STUDIES ON THE BIOSYSTEMATICS AND BIOLOGY OF STRIGEIDS (DIGENEA) PARASITIC IN FRESHWATER FISH

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To Ali, whose love makes everything possible.

DECLARATION

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

ABSTRACT

This study is concerned with two strigeid genera which utilise fish as their second intermediate host and piscivorous birds as a definitive host, i.e. *Apatemon* (*Apatemon*) Sudarikov, 1959 and *Ichthyocotylurus* Odening, 1969. Although the life-cycle has been ascertained for most *Ichthyocotylurus* spp., confusion and disagreement still exist as to the constituent species, while all of the life-stages have been described for only a single member of the subgenus *Apatemon* (*Apatemon*). In order to clarify species membership to these taxa and indeed the taxonomic position of the subgenus *Apatemon* (*Apatemon*) further information was required on the life-cycles and life-stages of these strigeids. Although, metacercariae from this family have been recorded from a variety of British fishes, confirmed records, i.e. those supported with life-cycle data, are limited to a single species. It was this lack of confidence in identifying metacercariae recovered from fishes and the lack of known good criteria for distinguishing the adults that prompted the present study.

Collections of metacercariae from a variety of hosts and locations were made, from which all subsequent life-cycle stages were obtained. The project aims were to establish the identity of the forms occurring in British fishes, by applying discriminatory techniques to the experimentally reared life-stages. In addition to traditional methods, techniques with little previous application to these genera were used and included, scanning electron microscopy (SEM), chaetotaxy, principal components analysis (PCA), and karyology. Furthermore, behavioural aspects such as the release patterns of cercariae from their molluscan hosts were studied to investigate whether they would prove to be of diagnostic value.

Metacercariae obtained from the sampling survey were tentatively identified, using all currently employed methods for their determination, i.e. morphology, nature of cyst, host and site specificities, as *Ichthyocotylurus erraticus* (Rudolphi, 1809), *I. variegatus* (Creplin, 1825), *Apatemon gracilis* (Rudolphi, 1819) and *A. annuligerum* (Nordmann, 1832). Material collected from Finland was considered to contain both *Ichthyocotylurus* spp. recovered in the U.K., as well as *I. platycephalus* (Creplin, 1825) and *I. pileatus* (Rudolphi, 1802). The *Ichthyocotylurus* spp. were found to be more host

specific than *A. gracilis*, although *A. annuligerum* was considered oioxenic to perch *Perca fluiatilis* L. Records of *I. erraticus* from gwyniad *Coregonus lavaretus* (L.) and grayling *Thymallus thymallus* (L.), and *A. gracilis* from arctic charr *Salvelinus alpinus* (L.) constitute first listings from Britain. The large number of sensilla present on the body surface of these metacercariae, observed by SEM and chaetotaxy, precluded their diagnostic use. PCA was, however, found to be of value for distinguishing between species and determining morphological variation within a species.

I. erraticus, *I. variegatus* and *A. gracilis* adults were successfully reared in experimental hosts using metacercariae from a variety of fish hosts, sites within a single fish host and geographical sites. The adults obtained enabled clarification of the identities assigned to the metacercariae. Those metacercariae believed to represent *I. pileatus* and *A. annuligerum* failed to establish in experimental hosts. Herring gulls and lesser black-backed gulls proved to be extremely good experimental hosts for both *Ichthyocotylurus* spp., with the vast majority of infections establishing and providing high yields of eggs and adults. These infections yielded information on the establishment, development, fecundity, site specificity, longevity and morphological variability of the adults. Aspects of the morphology and biology of *I. variegatus* adults recorded were found to support its validity as a species discrete from *I. platycephalus* which was in some doubt. The experimental hosts used for *A. gracilis* infections, domestic and mallard ducklings, were found to be less satisfactory. Challenges were performed with *A. gracilis* metacercariae from three sources, rainbow trout, salmon parr and stone loach. The latter source was the only one to result in egg producing adults, with specimens exhibiting normal morphology and demonstrating an increased longevity over adults raised from salmonid metacercariae. These findings suggest that the metacercarial host may affect the successful completion of the life-cycle.

Eggs of known origin were collected for all three cultured strigeid species, enabling further life-cycle studies, these were incubated and miracidia successfully raised. Developmental periods were found to be temperature dependent and differed for the three species at 20°C: *A. gracilis* < *I. erraticus* < *I. variegatus*. Light microscopy revealed the morphology of all three species to be identical, as were the epidermal plate formulae and chaetotaxy, indicated by silver-staining. The nomenclature for the

distribution of miracidial sensilla derived by Dimitrov *et al.* (1989) was amended to enable a full description of these species. Osmotic shock resulted in an improved deciliation of the miracidia compared to sonication and subsequent SEM observation confirmed the arrangement of body surface structures, while revealing sensilla forms. Behavioural aspects of *I. variegatus* miracidia were examined, with a maximum longevity (<11 hours) recorded at the lowest temperature studied (10°C), and host finding demonstrated to occur by an increased turning response in the presence of substances emitted from the susceptible snail host, following an initial unresponsive dispersal phase.

Ichthyocotylurus cercariae were found in naturally infected *Valvata piscinalis* which constitutes the first record in Britain of cercariae of this genus. Cercariae of *I. erraticus* and *I. variegatus* were successfully raised experimentally from miracidia of known identity and origin within naïve, experimentally raised *V. piscinalis* hosts, while *A. gracilis* cercariae were obtained from laboratory reared *Lymnaea peregra*. Cercarial developmental periods within the molluscan host were found to be temperature dependent and markedly different for the strigeid genera investigated, as were their behaviour and morphology. The *Ichthyocotylurus* spp. exhibit a distinct diurnal emergence rhythm from their molluscan host, being shed during the hours of daylight, while *A. gracilis* cercariae demonstrate a reciprocal pattern, emerging during the hours of darkness. Behavioural contrasts were also observed in longevities, emergence strategies (route of exit) and swimming behaviour. The two *Ichthyocotylurus* spp. were extremely similar, the only cercarial features found to be of diagnostic use were: the presence or absence of eye-spots; their differing developmental periods from miracidium to cercaria; the number and distribution of sensilla when compared by PCA; and their differing longevities at 20°C. Characters considered to be of value in differentiating between strigeid cercariae at the species level, including the armature, chaetotaxy pattern and resting posture, did not differ between these two species. SEM observations enabled descriptions of the variety and structure of sensilla present on different life-stages, while transmission electron microscopy revealed the internal structure of cercarial sensory structures.

Experimentally raised cercariae were found to be infective and the life-cycle was

completed for the three strigeid species. Host specificities were observed for *I. erraticus* and *A. gracilis*, being particularly stringent for the latter species, while site specificities recorded were as observed in natural infections. Metacercarial maturation periods (for encystment) were highly temperature dependent, being comparable for the two *Ichthyocotylurus* spp. and more rapid than for *A. gracilis* specimens.

Karyological studies were performed on the cercariae-releasing parthenitae of *I. erraticus*, *I. variegatus* and *A. gracilis*, the identity of which was confirmed through life-cycle studies. The chromosome number and morphology for the three species was described and enabled the ready discrimination of *I. erraticus* and *I. variegatus* cercariae which had proved to be problematical using comparisons of gross morphology. The strigeids described here and from all other sources have been found to possess 20 chromosomes in diploid sets.

Behavioural and developmental characteristics were found to be of taxonomic value for the separation of morphologically similar species at all life-stages. The fine surface structures of the strigeids investigated using SEM and chaetotaxy revealed many similarities at the species level, but provided evidence for their use in the separation of higher taxa. All comparisons of morphology were enhanced by the ability of PCA to discern subtle variation while karyological investigations provided precise species determinations. The two latter techniques in particular may greatly aid systematicians investigating taxonomic problems within other digenean families.

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1: GENERAL INTRODUCTION

INTRODUCTION

Members of the superfamily Strigeoidea Railliet, 1919 are digenetic trematodes with a life-cycle involving three or four hosts. Adults may be located within the digestive tract of birds, mammals or reptiles, parasite eggs being passed into the aquatic environment in the definitive host's faeces. There are many variations on the life-cycle pattern, although in all cases the egg hatches to release a miracidium which develops through two parthenogenetic generations and subsequently via, cercarial and metacercarial stages before completing the life-cycle in the form of the sexual adults. Typically, the miracidium and cercaria are free-living, while the parthenitae are confined to the first intermediate host (a mollusc) and the metacercaria to the second intermediate host (a mollusc, other invertebrate or fish).

Historically, a lack of life-cycle data necessitated the identification of strigeoid species using morphological features of the adult stage and their host specificities (see taxonomic monographs of Dubois, 1938, 1953, 1968, 1970a; Sudarikov, 1971). This proved rather problematical, with adult morphology tending to be highly conserved within families and providing few discriminating features; while the value of adult host specificity in strigeoid classification has been increasingly questioned (see the review in Blair, 1974; Hendrickson, 1986; Shoop, 1989). Indeed, Shoop (1989) points out that "Dubois classified the species according to the host's they inhabit even when he recognises such assemblages to be polyphyletic". Blair (1974) also indicated how identification was hindered by non-uniform material preparation and the influence of definitive host species, particularly experimental hosts, on the development of worms and their subsequent dimensions. Unfortunately, this situation has resulted in much confusion in the strigeoid literature.

Clearly, life-cycle data are vital for the accurate classification of strigeoids. Differences observed in the life-cycles of several species of *Apatemon* Szidat, 1928 prompted Sudarikov (1959) to create two genera: *Apatemon*, with cercariae possessing

10 flame-cells in the protonephridial system and encysting in fishes; and *Australapatemon*, with cercariae possessing 14 flame-cells and encysting in leeches. However, in their paper on Australian members of the Strigeida, Dubois & Pearson (1965) reduced Sudarikov's (1959) proposed divisions to the level of subgenera. The accumulation of such information for the genus *Cotylurus* Szidat, 1928 led Odening (1969) to recommend two subgenera: *Cotylurus*, with cercariae exhibiting pre-acetabular penetration glands and encysting in molluscs or leeches; and *Ichthyocotylurus*, with cercariae exhibiting post-acetabular penetration glands and encysting in fishes. Niewiadomska (1971a) subsequently argued that the latter two subgenera differed from each other at least as much as other recognised genera within the family Strigeidae Railliet, 1919 and should be raised to the full generic level (see full definitions in Appendix 1). She stated that a digenean genus should incorporate a set of species displaying similar morphological features at all corresponding stages of ontogeny. In the light of this if is difficult to see why Niewiadomska (1971a) did not also propose the elevation of *Apatemon* and *Australapatemon* back to the generic level.

This study is concerned with strigeids which utilise fish as their second intermediate host and piscivorous birds as definitive host, i.e. *Apatemon* (*Apatemon*) and *Ichthyocotylurus* Odening, 1969. Although, the life-cycle has been ascertained for most *Ichthyocotylurus* spp., confusion and disagreement still exists as to the constituent species, in particular the validity of *I. variegatus* (Creplin, 1825), while all of the life-stages have been described for only a single member of the subgenus *Apatemon* (*Apatemon*). In order to clarify species membership to these genera, and indeed the taxonomic position of the subgenera *Apatemon* and *Australapatemon*, further information is required on the life-cycles and life-stages of these strigeids.

Diagnoses of the family Strigeidae according to Shoop (1989) and the genera, *Apatemon*, *Ichthyocotylurus* and *Cotylurus* (based on Dubois, 1968; Odening, 1969; Niewiadomska, 1971a) are provided in Appendix 1. The general form of the life-cycle displayed by both *Ichthyocotylurus* and *Apatemon* (*Apatemon*) spp. is shown in Fig. 1.

Strigeoids employing fish as a second intermediate host may impart no discernable detrimental effects or, in some circumstances, may cause serious damage and even death. Cercarial penetration of the fish body, when occurring in sufficiently large numbers, can effectively disrupt the osmoregulatory function of the skin with potentially fatal consequences, particularly in fry. Subsequent migration of the tail-less cercariae to their 'chosen site' within the fish host invariably causes a degree of tissue damage and haemorrhaging, but this is typically minor and not life-threatening (Johnson, 1971; Hoffmann, Scheinert & Bibelriether, 1991). Once at this destination development proceeds to the infective metacercarial stage, where complications to the host arise primarily from the number of parasites present on, or in, the organs/cavity invaded.

Members of the genus *Diplostomum* Nordmann, 1832 infect many species of freshwater fish and are commonly specific to the eye. *Diplostomum* metacercariae infecting the lens of the eye induce cataractous changes which can result in lens rupture and exophthalmia, with partial or complete blindness (see *inter alia* Shariff, Richards & Sommerville, 1980). Impaired visual acuity causes abnormal feeding behaviour and ultimately loss of condition (Hendrickson, 1978). Consequently, this genus has received much attention in the literature (for a review see Brady, 1989), especially since the expansion of the fish farming industry where optimal growth rates are essential for economic success.

Strigeids with cercariae that penetrate and develop to metacercariae in fish have received far less attention than *Diplostomum*, particularly in Britain. Pathology resulting from infections with these strigeid metacercariae is most commonly associated with members of the genus *Ichthyocotylurus*. Although pathology was not investigated in the present study, it is pertinent to review it here because no previous account exists and what information is available, is largely confined to the eastern european literature. Markevich (1951) observed mortalities in ruffe *Gymnocephalus cernuus* (L.) which he attributed to heavy infections of *I. platycephalus* (Creplin, 1825) metacercariae; several hundreds to thousands of cysts were located in the organs of the body cavity

(swimbladder, kidneys, liver, and gonads), within the pericardial cavity, in the eye membranes, on the optic nerve and associated with the meninges and peritoneum. Weight loss in whitefish *Coregonus lavaretus* (L.) was considered by Petrushevskii & Shulman (1961) to result from *I. erraticus* (Rudolphi, 1809) metacercariae located within the pericardial cavities of hosts. Later, Bykhovskaya-Pavlovskaya & Petrushevskii (1963) recorded heavy burdens of these cysts, adhered to the hearts of lake whitefish, which were found to constitute up to 20% of the organ's weight. Such high intensities of infection in whitefish can result in flaccidity of the cardiac musculature and deformature of the heart (Sudarikov, 1971), which would account for the poor condition observed by Petrushevskii & Shulman (1961).

A single report exists of the detrimental effect of *Ichthyocotylurus* infection in British fishes. This observation was made by Campbell (1973), who noted cysts attached to the hearts of Loch Leven brown trout. These cysts were reported to be *I. erraticus* metacercariae, which, when occurring in numbers exceeding approximately 200, encased the heart and caused adhesion of the organ to the pericardium. The most recent and detailed examination of the pathology attributed to *I. erraticus* metacercariae was performed by Orecka-Grabda (1991) for infected vendace *Coregonus albula* (L.). She recorded cysts attached to the heart and the pericardium, as well as on the surface of the kidney under the external coat of connective tissue. Intensities of infection were not extreme, ranging from a single to 50 metacercariae on the heart, with approximately 20 cysts in superficial kidney layers. Even at these levels, histological evaluation of the heart condition revealed "myocarditis with biotic changes and proliferation of endothelial elements towards fibrination and scaring". Retrogressive changes were noted in the kidney, producing acute inflammation and disappearance of the nephron elements, with "progressing damage and liquefaction of glomeruli and canaliculi". Necrobiotic changes recorded in the liver were thought to result from the production of endotoxins, while destruction of blood cells caused hypersplenism. Even more serious were the mortalities reported by Tell (1980) in whitefish, smelt *Osmerus eperlanus* (L.), perch

Perca fluviatilis L. and ruffe resulting from a variety of *Ichthyocotylurus* infections in Lake Peipus-Pskov.

Reports of pathology in fish resulting from *Apatemon* metacercarial infections are scarce. Kozicka (1958) recorded changes in the brains of young cyprinids infected with an *Apatemon* sp. (referred to as *Tetracotyle* sp. 1) and in the eyes of perch containing *A. annuligerum* (v. Nordmann, 1832) (referred to as *Tetracotyle* sp. 2); both from Drużno Lake, Poland. The migration and encystment of the majority of *A. gracilis* (Rudolphi, 1819) metacercariae in heavily infected bullheads *Cottus gobio* (L.) were observed by Hoffmann, Scheinert & Bibelriether (1991) to cause little damage. This is in contrast to the earlier work of Zandt (1924), who noted haemorrhages and peritonitis in *A. gracilis* infected bullheads. Hoffmann *et al.* (1991) did, however, observe damage to the retina and consequent blindness when metacercariae were located within the eye. Detrimental effects of naturally acquired *A. gracilis* infections in rainbow trout were quantified by Tort, Watson & Priede (1987). Many fish examined contained over 50 metacercariae within the pericardial cavity and collagenous adhesions were observed between the heart and pericardium. These authors compared *in vitro* heart performance between infected (following removal of cysts and adhesions) and uninfected fish, noting that cardiac output in the former was only 20-40% of controls. Although there was no obvious mechanical damage attributable to the parasites, visible lesions were seen in the ventricular myocardium which they considered associated with the inflammatory response of the host to the parasite.

In contrast to *Diplostomum* spp. and most other intestine inhabiting adult digeneans, certain *Ichthyocotylurus* adults have been identified as being responsible for mortalities in definitive avian hosts. These deaths have resulted from intestinal blockages in both naturally and experimentally infected birds, caused by high intensities of infection with species which normally attain a large size; *I. platycephalus* and *I. variegatus* (see *inter alia* La Rue, 1927; Szidat, 1936; Odening, Mattheis & Bockhardt, 1970; Swennen, Heesen & Höcker, 1979). There are no British reports of mortalities in

birds attributed to strigeids, although adults have been recorded from three *Larus* spp. at Loch Leven, Scotland (Fraser, 1974).

Current views on the control of strigeoid infections remain the same as those reviewed by Brady (1989), which involved the discontinuation of the life-cycle. The most successful methods to this end are considered to be the removal of infected snail populations, preventing fish coming into contact with the infectious cercariae and denying birds access to the environs of the snail population, thereby preventing eggs entering the water body. A novel biological control method was proposed for *D. spathaceum* Rudolphi, 1819 infections, which was effected by the hyperparasitism of its sporocysts with the protozoon *Nosema strigeoidea* (see Palmieri, Heckman & Cali, 1976), but no further information has appeared subsequently. More recently, Whyte, Allan, Secombes & Chappell (1987) investigated the potential for vaccination of rainbow trout *Oncorhynchus mykiss* (Walbaum) against this *Diplostomum* sp. Neither approach has subsequently been implemented.

Although strigeid metacercariae have been recorded from a variety of British fishes, confirmed records, i.e. those supported with life-cycle data, are limited to one *Apatemon (Apatemon)* sp., *A. gracilis* (see Crocombe, 1959; Blair, 1974, 1976; Watson & Pike, 1993); the two latter authors successfully completing the life-cycle experimentally. Identifications of *Ichthyocotylurus* metacercariae which are based on morphology alone give rise to doubt. Odening's (1979) key which incorporated cyst structure improved the confidence in species discrimination. However, other life-cycle data are required for an accurate determination of species. *Ichthyocotylurus* adults were raised experimentally from Scottish perch metacercariae by Blair (1974). He proposed, on the basis of site specificities within the fish and definitive hosts, that these represented *I. variegatus*, but was unable to confirm the identification from adult morphology (young worms obtained were not fully developed), while metacercarial

morphology was not examined. A single natural host record of *I. erraticus* adults, which are well characterised from the remaining species of this genus, exists from British gulls (Fraser, 1974); while no records, even unsubstantiated, exist for *Ichthyocotylurus* cercariae in the United Kingdom.

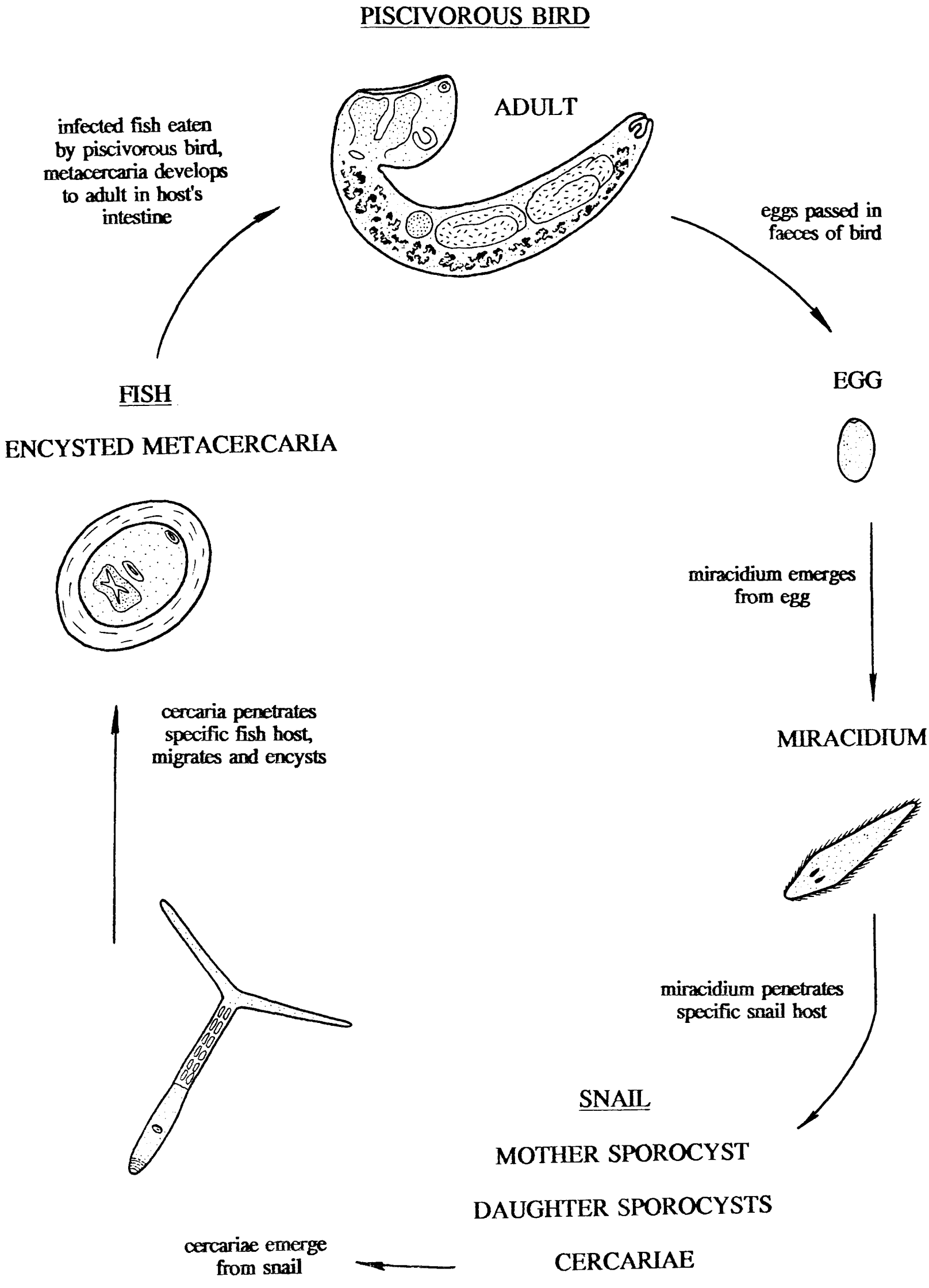
This lack of confidence in species records together with the potential pathology associated with *Ichthyocotylurus* spp. initiated the present study. Collections of metacercariae from a variety of hosts and locations were made, from which all subsequent life-cycle stages were experimentally obtained. The project aims were to establish the identity of the forms occurring in British fishes by applying a variety of traditional and modern taxonomic/identification techniques to all the experimentally reared life-stages. The importance of cercarial features, both morphological and behavioural, for the separation of both taxa and species is well established (see *inter alia* Niewiadomska 1970a, 1971b; Blair, 1974, 1977). Metacercarial morphology is also advocated by some authors to be of value for the discrimination of strigeid species (Niewiadomska 1971a, 1973; Odening, 1979). While, Shoop (1989) employed metacercarial, as well as adult, characters for the delineation of families within the Strigeoidea. Dubois (1970b, 1978), however, disagreed with the use of tetracotyle morphology in taxonomy, believing their features to be of "weak" diagnostic utility. The detailed examination of strigeid life-stages will, hopefully, also enable an evaluation of features presently considered of discriminatory value for the different ontogenetic stages and elucidate new criteria for their separation.

At present, the fine surface structures of strigeids have yet to be investigated with the scanning electron microscope, while the known chaetotaxy of this family is limited to a single species of miracidium (*Strigea falconispalumbi* Viborg, 1795; see Dimitrov, McCarthy & Kanev, 1991) and five species of cercaria (see Shigin, 1974; Blair, 1974; Richard, 1982; Zazornova, 1987), only one of which is known to utilise fish as the second intermediate host. No chaetotaxy studies have been performed on any life-stage of an *Ichthyocotylurus* sp. and it might prove to be a useful tool for their

discrimination, or act to support other evidence. The morphology, of similar strigeid species can now be compared with increased precision using multivariate analysis of morphometric data, which provides the facility to look at organisms in the form of a multidimensional image, thereby enabling the parasites to be examined using many measurements simultaneously. To date no such multivariate technique has been applied to strigeids and this might be valuable in distinguishing morphologically similar species. Karyological techniques have not been widely used for digenean problems and such knowledge on strigeids is scarce. The application of this technique to experimentally reared parthenitae (identity confirmed by life-cycle data) could reveal differences in the number and morphology of their chromosomes providing a more confident separation of species. Further, there are also behavioural aspects, such as the release patterns of cercariae from their molluscan hosts, which remain uninvestigated and may prove to be discriminatory.

No detailed study has been performed on the taxonomy of strigeids parasitic in fish since the 1970's (see *inter alia* Blair, 1974; all studies listed for Odening and his co-workers; Swennen *et al.*, 1979). This work sets out to explore the usefulness of such investigative techniques which have not previously been applied to strigeid systematics. The results may provide information beneficial to the understanding of these parasites and indicate possibilities for the broader application of the approaches used.

Fig. 1. General life-cycle of a strigeid utilising fish as its second intermediate host (not to scale).



CHAPTER 2: THE METACERCARIAE

INTRODUCTION.

Keys for the identification of *Ichthyocotylurus* metacercariae were provided by Markevich (1951) and Bykhovskaya-Pavlovskaya (1964) both of which are incomplete, Odening (1979) and Bauer (1987). The most reliable of the latter two is considered to be that of Odening (1979) and is shown below. Species separation in this key was based on cyst structure and comparative dimensions of internal organs. In the present study this key was primarily used, along with data on host and site specificities, to identify *Ichthyocotylurus* spp. prior to confirmation of identity by the experimental raising of adults. No such key exists for the metacercariae of *Apatemon (Apatemon)* spp., of which several members are still undescribed. Metacercariae of this subgenus were identified according to the descriptions and works of Kozicka (1961, 1972), Dubois (1968, 1974, 1980), Odening (1970) and Blair (1974, 1976). Host and site specificity is of great importance in the identification of *Apatemon (Apatemon)* spp. due to their morphological similarities.

Key for the identification of *Ichthyocotylurus* metacercariae, translated from Odening (1979).

1. Capsule and cyst tear easily; thin hyaline envelope, envelope of cyst forms on host and may be thin or thick.....**2.**

\- Capsule and cyst difficult to tear; envelope of cyst and capsule generally thick.....**3.**

2. Width of the ventral sucker greater than about 150μm; adhesive organ (93-161μm) of shorter than or of similar maximum length (rarely as long) to ventral sucker (109-257μm); pseudosuckers (52-155μm) similar length to oral sucker (93-150μm).....***I. platycephalus***

\- Width of ventral sucker less than 115μm; adhesive organ (110-276 x 132-345μm) distinctly larger than ventral sucker (109-257 in length); pseudosuckers (88-171μm) longer than oral sucker (65-90μm).....***I. variegatus***

3. Ventral sucker (50-90μm) a little wider than oral sucker (40-70μm); pseudosuckers longer than oral sucker.....***I. erraticus***

\- Ventral sucker (35-66μm) of similar width to oral sucker; pseudosuckers shorter than oral suckers .....***I. pileatus***

2.1. HISTORICAL PERSPECTIVE.

Strigeid species which utilise fish as their second intermediate hosts vary from oioxenic to euryxenic in their specificities. In addition, many demonstrate site preferences within a particular fish species. The known host records for members of the genus *Ichthyocotylurus* and subgenus *Apatemon* are listed by species below.

I. erraticus.

The tetracotyle or metacercarial stage of *I. erraticus* has frequently been recorded from fish in both Europe and North America, often under the synonym of *Tetracotyle intermedia* Hughes, 1928. The range of fish families were given by Odening (1979) as the Salmonidae and Osmeridae, plus rarely the Dallidae, Cottidae and Cyprinidae. Cysts are generally located in the pericardial cavity; although additional sites may include the kidney, and occasionally the body cavity, gonads and eyes (Odening, 1979).

Previous records of this species from the British Isles are limited to the Salmonidae. Copland (1957) and Roberts, Leckie & Slack (1970) recorded *I. erraticus* metacercariae in powan *Coregonus lavaretus* (L.), from Loch Lomond. Later, Wootten (1973a, b) found similar cysts in brown trout *Salmo trutta* L. and rainbow trout from Hanningfield reservoir; Campbell (1974) in brown trout from Loch Leven; and Mitchell, Halton & Smyth (1978) in rainbow trout from a County Antrim fish farm. In each case the metacercariae were located within the pericardial cavity of the fish host.

I. variegatus

Reflecting its original name "*Tetracotyle de la perche fluviatile*" (Moulinié, 1856), *I. variegatus* metacercariae are most commonly recorded in perch. Indeed, this species appears to be quite restricted in its range of fish hosts, to the Percidae (see Odening, 1979; Bauer, 1987). However, Rautskis (1988) listed additional Lithuanian hosts which included roach *Rutilus rutilus* (L.), and zander *Stizostedion lucioperca* (L.), while the zander was also identified as bearing *I. variegatus* metacercariae by

Rumyantsev, Permyakov & Alekseeva (1984). Whether some of these additional host records may actually represent *I. platycephalus* is unknown. The location of cysts was given by Odening (1979) as the wall of the swimbladder, the peritoneum and the pericardial cavity.

In the British Isles, Wootten (1973b) observed an unidentified *Ichthyocotylurus* sp. within the abdominal cavity of perch from Hanningfield reservoir; Blair (1974) and McGuigan & Sommerville (1985) recorded *I. variegatus* cysts in perch from two Scottish Lochs, Loch Lomond and Loch Fad, respectively; and similar infections were reported by Faulkner, Halton & Montgomery (1989) in perch from Loch Neagh, Northern Ireland.

I. platycephalus

A wide and diverse range of fish groups acts as host for metacercariae of this species, including the Percidae, Percopsidae, Cyprinidae, Salmonidae, Osmeridae, Escocidae, Gadidae and the Gasterosteidae (Odening, 1979); with the most frequent records from the Percidae (particularly perch, zander and ruffe) and Cyprinidae (Matthesis & Odening, 1980; Pugachev, 1983; Sudarikov, 1984; Rautskis, 1988). Many of the early reports are under the synonyms *T. ovata* v. Linstow, 1877 and *T. communis* Hughes, 1928. The metacercariae are usually located within the pericardial cavity, although cysts may also be found in the body cavity, on the wall of the swimbladder or within the orbit.

T. ovata (= *I. platycephalus*) was recorded from ruffe by Nicoll (1924) in his "Reference list of the trematode parasites of British freshwater fishes", although the origin of this record is not stated. Wootten (1973b) observed an *Ichthyocotylurus* sp. in the abdominal cavity of ruffe examined from Hanningfield reservoir; these he tentatively identified as *I. platycephalus*. Encysted metacercariae on the surface of the swimbladder in perch from Loch Leven were believed by Campbell (1974) to represent *Cotylurus cucculus* Thoss, 1897 or *T. communis* (= *I. platycephalus*). However, none of these

records were confirmed. Specimens excised from brown trout in Galway, Eire were identified as *I. platycephalus* by staff at The Natural History Museum, London, and were re-examined in the present study. At present this material remains the only credible record of any life-stage of this species from Britain and Ireland.

I. pileatus

Based on the works of Hughes (1928) and Razmaskin (1963, 1966, 1974, 1976), Odening (1979) listed the hosts of *I. pileatus* (Rudolphi, 1802) Odening, 1969 metacercariae as the Percidae (swimbladder), Salmonidae (*Coregonus* spp. - kidney) and Percopsidae (heart). Shigin (1983) also included the Cyprinidae as second intermediate hosts. The most commonly observed synonym for this metacercarial species is *T. diminuta* Hughes, 1928.

There are no British records of any *I. pileatus* life-stages.

A. gracilis

Many early records of *A. gracilis* were made under the synonyms *A. cobitidis* (Von Linstow, 1890) Vojtek, 1964, *A. pellucidus* (Yamaguti, 1933) and *A. gracilis pellucidus* (Yamaguti, 1933) Dubois, 1953. Blair (1976) stated that fish hosts of this species of metacercaria include members of six families (Salmonidae, Eleotridae, Cobitidae, Cottidae, Gasterosteidae and Gobiidae), with their scattered records suggesting at least a Holartic circumpolar distribution. The location of cysts within the fish host appears to vary according to the host family; the Salmonidae harbouring metacercariae predominantly within the pericardial cavity, the Cottidae within the body cavity and the Gasterosteidae within the eyes.

A. gracilis metacercarial cysts were recorded in the bullhead, *Cottus gobio* L. from South Wales by Crocombe (1959). The same metacercariae were recovered from rainbow trout, three-spined-sticklebacks *Gasterosteus aculeatus* L., and stone loach *Barbatula barbatulus* (L.), at the site of a fish farm on the River Almond, Scotland by

Blair (1974, 1976). Also in Scotland, Wootten & Smith (1980) recorded *A. gracilis* cysts in salmon *Salmo salar* L. parr from the River Almond, while McGuigan & Sommerville (1985) found infected feral and farmed rainbow trout in Loch Fad on the Isle of Bute.

Due to the similar location of *A. gracilis* and *I. erraticus* metacercariae in salmonids, there are several records of the latter which might actually have been *A. gracilis*. For example, Campbell suggested in a pers. comm. to Blair (1976) that this might have been the case with the cysts observed on the heart of brown trout from Loch Leven.

A. annuligerum

Nordmann (1832) described an encysted larva, which he called *Distomum annuligerum*, from the eyes of perch in Germany. These larvae were later identified by Kozicka (1961) as being of the tetracotyle type and renamed *Tetracotyle annuligerum*. In a series of experimental infections, Odening (1970) discovered that the metacercariae were those of a strigeid which he called *Apatemon (Apatemon) annuligerum*. At present the perch is the only known host for this species, which appears site specific for the humour of the eyes.

The first British record of this metacercariae in perch eyes was made by Nicoll (1924), although conspicuous by its absence was the fact he did not state that the larvae were encysted. More recently, Blair (1974) and McGuigan & Sommerville (1985) discovered perch from Loch Lomond and Loch Fad, respectively, to be infected with *A. annuligerum* cysts.

Apatemon (Apatemon) spp.

There are far fewer host records for the metacercariae of other species considered to belong to this subgenus (see Chapter 3.2; Introduction). The metacercaria of *A. fuligulae* Yamaguti, 1933 have been recorded from the musculature of *Parasilurus* (see Yamaguti, 1933; Markevich, 1951), *Pseudobagrus* (see Yamaguti, 1933), *Rutilus* and

Leuciscus spp. (see Osmanov, 1971, 1976) while *A. graciliformis* Szidat, 1928 metacercariae were recovered by Combes & Nassi (1977) from naturally and experimentally infected guppies *Poecilia reticulata* Peters. Furcocercariae of *A. graciliformis* were only found to develop within female fish of this ovoviviparous host, migrating to the ovary and penetrating the vitelline vesicles of an embryo, if present. The embryo develops normally if intensities of infection are low, with the metacercariae encysting a short time before parturition. Swimming of the young infected guppies is often impaired, possibly increasing the chance of predation and transmission to the definitive host. The authors state that in non-gravid female fish, cercariae penetrate the oocytes and exist as intra-cellular parasites. The subsequent fate of these larvae was not revealed.

2.2. TAXONOMIC STUDIES.

Strigeid metacercariae possess a cyst of parasite origin and commonly a capsule of host origin. The cyst wall is often thick and can be difficult to remove mechanically. Voge & Jeong (1971) demonstrated that *Cotylurus lutzi* Basch, 1969 metacercariae could be excysted in a non-enzymatic medium (Earle's balanced salt solution) using only a temperature stimulus (40-41°C), while Basch, DiConza & Johnson (1973) were able to induce excystment of the same parasite species by teasing in 0.35% saline at 40°C. However, experiments by Fried & Butler (1977) and Mitchell, Halton & Smyth (1978) showed that excystment of *C. strigeoides* Dubois, 1958 and *Ichthyocotylurus erraticus*, respectively, could not be induced by temperature alone. These authors found that an alkaline bile salt-trypsin medium at 39-41°C was necessary for the emergence of these metacercariae. Blair (1974, 1976) employed successive acid-pepsin and trypsin-tauroglycholate solutions at 40°C to achieve the excystment of *Apatemon gracilis* metacercariae. Such an acid pre-treatment (acid-pepsin or acid-saline) was found to be essential for the excystment of *C. cornutus* (Rudolphi, 1808) Szidat, 1928 metacercariae by Graczyk & Shiff (1993).

Several of the strigeid metacercarial species excised from naturally infected fish in this study were found to possess thick, tough cyst walls; while others were bound by thinner more fragile cysts. It was considered that undamaged metacercariae would be most efficiently liberated using artificial digests regardless of their cyst's nature, while digestion times might be characteristic for a species.

Thorough descriptions have been provided for all four *Ichthyocotylurus* spp. recognised by Odening (1979): *I. erraticus* (see Hughes, 1928, as *Tetracotyle intermedia*; Olson, 1970; Niewiadomska & Kozicka, 1970); *I. variegatus* (see Odening & Bockhardt, 1971); *I. platycephalus* (see Hughes, 1928, as *Cotylurus communis*; LaRue, 1932, as *C. communis*; Odening, Mattheis & Bockhardt, 1970); and *I. pileatus* (see Hughes, 1928, as *T. diminuta*). The metacercaria of *A. gracilis* have been described by Yamaguti (1933) (as *A. pellucidus*), Hoffman (1959), Crocombe (1959), Vojtek (1964a) (as *A. cobitidis*) and Blair (1974, 1976), while details of *A. annuligerum* metacercariae were provided by Kozicka (1961, 1972) and Odening (1970). Kozicka's (1972) description of the *A. annuligerum* metacercaria was based on a single excysted specimen, while that of Odening (1970) for this species (number of specimens not indicated) only included measurements of a few structures. Of the remaining metacercariae of the subgenus *Apatemon*, *A. fuligulae* is the only other described (Yamaguti, 1933).

The existing morphological knowledge of strigeid metacercariae is limited to light microscopical examinations. This study applied a number of other techniques to metacercariae excised from naturally infected fish, in order to provide additional information on these morphologically similar larvae. The surface structures of the metacercariae were examined using the scanning electron microscope (SEM) and the patterns of their surface sensilla (chaetotaxy) investigated using silver-stained specimens. Chaetotaxy has been employed extensively to discriminate morphologically similar cercariae (see Chapter 5.2; Introduction) and to a lesser extent, miracidia of different

species (see Chapter 4.2; Introduction). The technique has, however, rarely been applied to the metacercarial stage, with previous studies limited to *Diplostomum* spp. by Shigin, Chupilko & Klochkova (1985) and Brady (1989).

Variations in the morphology of metacercariae were also studied using principal components analyses (PCA). PCA expresses the relationship between measured parameters or variables. Each axis of the PCA plot is ordered by the amount of variation it explains; the x-axis shows the component which is responsible for the most variation within the sample and the y-axis the second most variable parameter. The third most variable parameter is placed at a right angle to the resultant of the first two, and so on. This form of analysis expresses the variability between specimens in the data set, as accurately as possible, using a small number of (principal) components. The technique has, in certain cases, proven to be a powerful method of discriminating taxonomically close species. It was successfully employed in the discrimination of *Diplostomum* metacercariae (Brady, 1989), as well as adult digenean species (Bray & des Clers, 1992; Gibson, Taskinen & Valtonen, 1992) and morphologically similar monogeneans (Silan & Maillard, 1989; Shinn, 1993; Shinn, des Clers, Gibson & Sommerville (1996).

None of these techniques have previously been applied to strigeid metacercariae. It was hoped that chaetotaxy and SEM studies would isolate species specific features, while PCA would support species separations made using Odening's (1979) key and identify any variations in morphology arising from the location within a host, the utilisation of different host species or the geographical isolation of populations of a species.

MATERIALS AND METHODS

2.1. NATURAL INFECTIONS.

Collection of fish for examination.

A wide range of fish species were obtained from a number of sources (listed in Table 1), and examined for the presence of strigeid metacercariae. Wild fish were collected with rod and line, fyke nets, seine nets, gill nets or using electro-fishing techniques; those obtained from fish farms were hand-netted out of the ponds and cages. Live fish were transported back to the laboratory in local aerated water.

Collection of Metacercariae.

All fish were examined immediately after killing. The body surface, body cavity and all organs contained therein, the heart and pericardium, eyes, brain and cranial cavities were examined for the presence of metacercarial cysts. Excised tissues were dissected in 0.85% physiological saline and metacercarial cysts removed using a fine pipette. Cysts removed from Finnish fish were transported to Scotland in vials containing physiological saline which were packed on ice. It was found that cysts could be stored in fresh saline at 4°C for several days without compromising viability. However, with the exception of Finnish material, only newly excised cysts were used for experimental infections (see Chapter 3.1.2; Materials and Methods).

Records were kept of the prevalence and intensity of infections, as well as the location of cysts/pre-encysted metacercariae within the fish host. The size, weight and when obvious, the sex of the fish host were also noted. Metacercarial identifications were made according to the works listed in Chapter 2; Introduction.

2.2. TAXONOMIC STUDIES.

2.2.1. Artificial digestion of cysts.

Newly excised encysted metacercariae were freed by subjecting them to pepsin

and trypsin-tauroglycholate solutions, as described by Blair (1974, 1976). Cysts were placed in a 0.8% solution of pepsin in Hanks' saline (buffered at pH 1.7-2.0) for 10 minutes at 40°C. They were then washed in several changes of saline, and incubated at 40°C in a solution of 0.5% trypsin and 0.3% sodium tauroglycholate, adjusted to pH 7.8. Unless otherwise stated, times recorded for excystment were made from in excess of 50 specimens and generally from several hundred.

2.2.2. Light microscopical observations of metacercariae.

Once the metacercariae had been freed from their limiting cysts they were fixed in Berland's fluid (19 parts glacial acetic acid and 1 part formalin), then stored in 80% alcohol until mounting. Before mounting, metacercariae were placed in a solution of 1 part glycerine and 9 parts 80% alcohol and maintained on a hot plate to allow the alcohol to evaporate. When this was completed the specimens were mounted unstained in glycerine jelly. Measurements were taken, when possible, from more than 15 specimens, on an Olympus BH2 microscope using interference phase contrast.

2.2.3. Chaetotaxy and scanning electron microscopical observations of metacercariae.

Chaetotaxy

The most satisfactory method of staining metacercarial sensilla was a modification of Brady's (1989) technique:

1. Metacercariae were digested from their limiting cysts.
2. The worms were placed in an embryo dish filled with distilled water.
3. Supernatant water was removed and an ice-cold solution of 0.5% silver nitrate pipetted in. Hot silver nitrate (60-65°C) was found to cause excessive contraction of the metacercariae. The embryo dish was then placed in the dark for 5 minutes.
4. After this period of incubation the worms were washed vigorously 5-10 times in distilled water.
5. The metacercariae were placed under an ultra-violet lamp (325nm) for 3 minutes

and then washed 5-10 times in distilled water. The efficiency of UV lamps varies greatly, and with some equipment a far shorter period of exposure may be necessary.

6. The worms were transferred into an embryo dish containing a solution of 1 part glycerine and 9 parts 80% alcohol and placed on a hot plate to allow the alcohol to evaporate.
7. Once the alcohol had completely evaporated the parasites could then be mounted in glycerine or glycerine jelly. These mountants allowed the specimens to be manipulated under the coverslip during examination with the light microscope.
8. Sensilla were best seen using a x100 objective and bright field illumination. Illustrations were drafted with the aid of a drawing tube.

This silver nitrate staining technique was performed on both *I. erraticus* and *I. variegatus* metacercariae; conclusions on the distribution of sensilla were drawn from 15 optimally stained specimens of each species. The deep ventral body concavity of *Apatemon* specimens, giving four overlaid body surfaces, precluded the accurate mapping of their body sensilla by this method.

Scanning electron microscopy (SEM)

Excysted metacercariae were placed in cacodylate buffer and washed several times by flushing with a pipette. They were then fixed in cacodylate buffered 3% glutaraldahyde for 1 hour at 4°C, washed again in cacodylate buffer, post-fixed in osmium tetroxide for one hour at room temperature and rewashed in cacodylate buffer. Before processing can proceed further the specimens must be cleaned to remove any remnants of debris acquired within their cysts. Debris was removed by placing the metacercariae in a 16% solution of glycerine and leaving in a rotator over night. The glycerine itself was then removed with three 2-hourly changes of 20% ethanol. Alternatively, surface material was displaced by mild sonication of the metacercariae

in a dilute detergent solution after the final cacodylate buffer wash. Care was required with the latter technique as over-sonication can damage specimens. The clean metacercariae were then dehydrated through an acetone series, critical point dried, mounted on stubs and coated with gold. Specimens were examined using an Hitachi Field Emission 800 scanning electron microscope.

Metacercariae initially fixed in Berland's fluid and stored in 80% alcohol were also processed for SEM analysis by rehydrating the specimens, post-fixing them in osmium tetroxide and then proceeding as described above.

2.2.4. Discrimination of metacercariae by Principal Components Analysis of metrical data.

The specimens to be included in the analyses were collected and prepared for light microscopical studies according to the techniques listed in 2.1, 2.2.1 and 2.2.2; Materials and Methods. Provisional identifications were made according to the works indicated in Chapter 2; Introduction.

Principal components analysis (PCA)

PCA expresses the relationship between measured parameters. The variables are related to one another either by their correlation or covariance matrix. In this study principal components (weighted linear components of the observed variables) were derived from the correlation matrix of "standardised" variables. The morphometric measurements required "standardising" to remove any geometric growth effect before they were incorporated into the PCA. This was achieved by Log (ln) transforming the data. Several PCAs were performed after the removal of specimens that were not relevant to a subsequent analysis. Although not performed here, it is also legitimate to remove small clusters of specimens (outliers) that are clearly isolated and prove on re-examination to be atypical. These multiple PCAs were necessary as the score given to, and graphical position of, each specimen is determined by all other specimens in the

analysis. The first component summarises as much of the joint variation as is possible, the second as much of the remainder as possible and is plotted at a right angle to the first. Successive components describe ever smaller proportions of the total variation and are plotted perpendicularly to the resultant of the previous components. Consequently, only the first three or four components ever need be considered, as these describe the vast majority of the overall variation. Plots of component pairs provide a two-dimensional view of the three-dimensional image (of the relationship between specimens) produced.

Eigen values indicate the amount of variation described by individual components, while component loadings or coefficients reveal how much each variable contributes to a particular component. Ellipses may be drawn upon the PCA plots to encompass a specific proportion of designated points or, to indicate confidence limits on the ellipse's centroid (the mean of the designated sample).

The morphometric measurements used in the present analyses were:

Ichthyocotylurus spp.

1. Body length (BL)
2. Body breadth (BB)
3. Oral sucker length (OSL)
4. Oral sucker breadth (OSB)
5. Pharynx length (PL)
6. Pharynx breadth (PB)
7. Ventral sucker length (VSL)
8. Ventral sucker breadth (VSB)
9. Distance from centre of ventral sucker to anterior extremity of body (VSA)
10. Lateral lappet fissure length (FL)*
11. Lateral lappet length (LL)*
12. Distance between anterior margins of lateral lappet fissures (DL)
13. Tribocytic organ length (TCOL)**
14. Tribocytic organ breadth (TCOB)**
15. Distance from posterior margin of tribocytic organ to posterior extremity of body (TCOP)

Odening's (1979) key refers to * as pseudosuckers and ** as adhesive organ.

Dimensions of the tribocytic organ and lappet length were omitted for metacercariae of the subgenus *Apatemon* due to the variation recorded in extension of the former and difficulty in the accurate measurement of the latter (see Chapter 2.2.2; Results). The following metrical features not observed in *Ichthyocotylurus* spp. were included for *Apatemon* spp.:

13. Forebody length (FBL)
14. Proteolytic gland length (PGL)
15. Proteolytic gland breadth (PGB)

Body length [1] was replaced by total body length (sum of fore- and hindbody lengths). All measurements are indicated in Figs 2, 3.

Analyses were performed on the groups of specimens listed below; numbers in parentheses represent the number of specimens incorporated into each analysis.

1. All *Ichthyocotylurus* spp.: *I. erraticus* (128); *I. variegatus* (162); *I. platycephalus* (1); *I. pileatus* (38). Total number of specimens = 329.

2. *I. erraticus* metacercariae excised from: Scottish powan (43); Scottish rainbow trout (15); Finnish whitefish (47); Finnish vendace (23). Total number of specimens = 128.

3. *I. variegatus* metacercariae excised from: Scottish perch (20); Scottish ruffe, swimbladder (47); Scottish ruffe, pericardial cavity (20); Scottish ruffe, orbit (10); Scottish ruffe, ovary (16); Finnish perch (32); Finnish ruffe (17). Total number of specimens = 162. All Scottish ruffe specimens were obtained from a single fish.

4. *A. gracilis* metacercariae excised from Scottish rainbow trout (43), Scottish salmon parr (33), Scottish arctic charr (3), Scottish stone loach (22), Welsh stone loach (18), Welsh bullheads (41) and *A. annuligerum* from Scottish perch (33). Total number of specimens = 193.

Abbreviations used in Figs 2, 3: (BB) Body breadth; (BL) Body length; (DL) Distance between lateral lappets; (FBL) Forebody length; (FL) Lateral lappet fissure length; (HBL) Hindbody length; (LL) Lateral lappet length; (OSB) Oral sucker breadth; (OSL) Oral sucker length; (PB) Pharynx breadth; (PL) Pharynx length; (PGB) Proteolytic gland breadth; (PGL) Proteolytic gland length; (TCOB) Tribocytic organ breadth; (TCOL) Tribocytic organ length; (TCOP) Distance from posterior margin of tribocytic organ to posterior extremity of body; (VSA) Distance from the mid-point of ventral sucker to anterior extremity of the body; (VSB) Ventral sucker breadth; (VSL) Ventral sucker length.

Fig. 2. Morphometric measurements recorded for *Ichthyocotylurus* metacercariae.

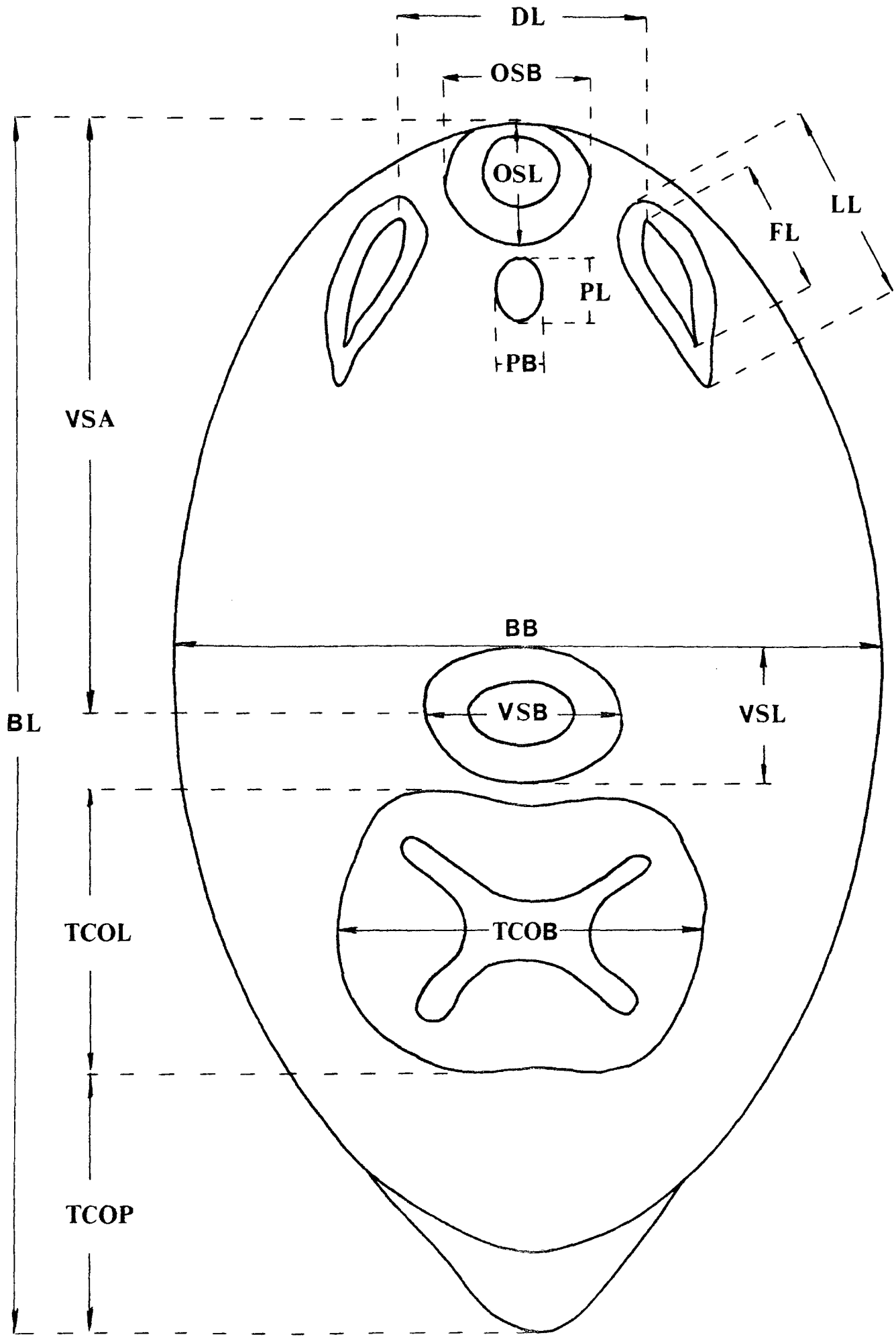
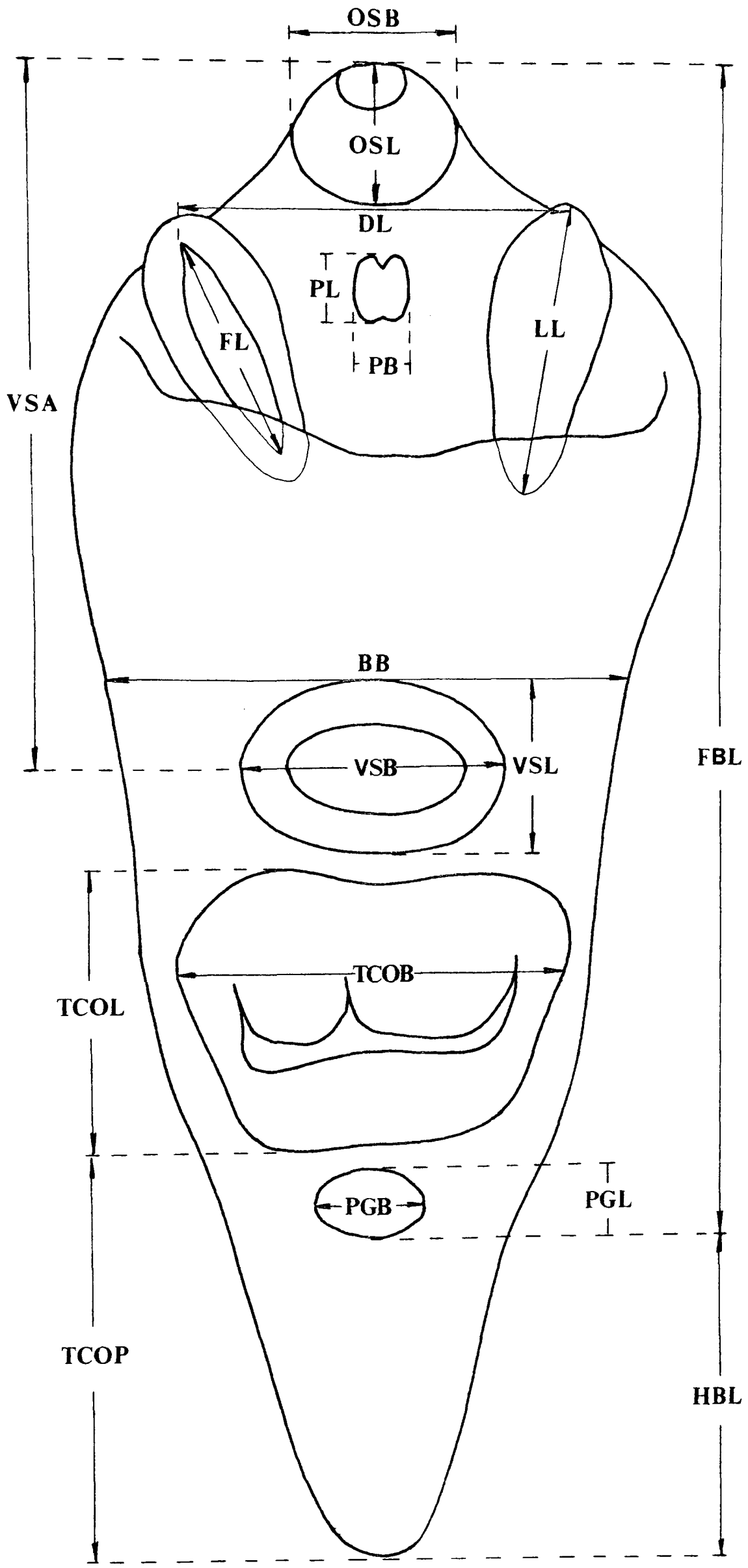


Fig. 3. Morphometric measurements recorded for *Apatemon* (*Apatemon*) metacercariae.



RESULTS

2.1. NATURAL INFECTIONS.

A list of the host records made in the present study are given in Table 1. More detailed analyses of these observations, including details of fish size and weight, prevalences, intensities and locations of infections are provided in Tables 2-5.

2.2. TAXONOMIC STUDIES.

2.2.1. Artificial digestion of cysts.

The metacercarial identifications were made on excysted mounted worms according to the works indicated in Chapter 2; Introduction. Excystation was never seen to occur without trypsin treatment and Table 6 gives the times required for emergence following incubation in this medium.

Table 6. Time required for excystation of strigeid metacercarial species in trypsin solution.

Metacercarial species	Nature of cyst wall	Time (minutes)
I. erraticus	thick (38-54μm)	40-120 (typically c. 50)
I. variegatus	thin (<15μm)	5-30 (typically c. 10)
I. pileatus	thick	30-120 (typically c. 40)
I. platycephalus	thin	6 and 8 minutes (two specimens)
A. gracilis	thick (54-63μm)	< 5 (typically less than 1)
A. annuligerum	thick (58-77μm)	< 5 (typically less than 1)

2.2.2. Light microscopical observations of metacercariae.

As stated in the introduction to this chapter, detailed morphological descriptions of the metacercariae of the four *Ichthyocotylurus* spp., *A. gracilis* and *A. annuligerum* have been provided by other authors and no additional information can be added here. Morphological measurements recorded for *I. erraticus*, *I. variegatus*, *I. pileatus*, *I. platycephalus*, *A. gracilis* and *A. annuligerum* metacercariae are provided in Tables 7,

8, 9, 10 and 11, respectively. These tables also include the most reliable taxonomic information compiled by other authors for these metacercariae. Figs 4 and 5 show photomicrographs of the 6 metacercarial species.

Table 1. Strigeid metacercarial host records observed in present study.

Host species, number of specimens examined in parentheses	Source	Date	Metacercarial identity
Powan (21)	Loch Lomond	Oct '91, June '92, July '93	*I. erraticus*
Gwyniad (5)	Llyn Tegid, (W)	Sep '93	*I. erraticus*
Whitefish (?)	Lake Kitka, (F)	Jul '93	*I. erraticus*
Vendace (?)	Lake Kitka, (F)	Jul '93	*I. erraticus*
Grayling (1)	Llyn Tegid, (W)	Sep '93	*I. erraticus*
Arctic char (7)	Isle of Skye (fish farm)	Jan '93	*A. gracilis*
Rainbow trout (155)	Loch Awe (fish farm I) Loch Awe (fish farm II) River Earn (fish farm) River Almond (fish farm) River Doon (fish farm)	Jan '92 Jan '92, Jul '92 Jan '92 Mar '92, Aug '92, Sep '92, Jan '93, May '93 Sep '92	- *I. erraticus/A. gracilis* *A. gracilis* *A. gracilis* *A. gracilis*
Brown trout (40)	Airthrey Loch burn Dumfrieshire fishery Stirlingshire fishery River Almond	Mar '92 Mar '92 Aug '93 Sep '93	- - - -
Brook trout (15)	Dumfrieshire fishery River Doon (fish farm)	Mar '92 Aug '92	- -
Salmon parr (62)	River Almond fishery	Aug '93	*A. gracilis*
Perch (186)	Humberside area, (E) Castle Semple Loch Due Loch Loch lomond Lake Kitka, (F) Llyn Tegid, (W) Stirlingshire loch	Jan '92 Feb '92, Aug '92 Jul '92 Aug '92, May '93, Jun '93, Jul '94 Jul '93 Sep '93 Jun '94	- *I. variegatus/A. annuligerum* - *I. variegatus/A. annuligerum* *I. variegatus* *I. variegatus* *I. variegatus/A. annuligerum*
Ruffe (32)	Loch Lomond Lake Kitka, (F) Llyn Tegid (W)	Apr '92, May '93 Jul '93 Sep '93	*I. variegatus* *I. variegatus/I. platycephalus/I. pileatus* *I. variegatus*
Pike (7)	Castle Semple Loch Due Loch Llyn Tegid, (W)	Oct '91 May '93 Sep '93	- - -
Roach (22)	Castle Semple Loch Humberside area, (E) Llyn Tegid, (W)	Oct '91 Jan '92 Sep '93	-
Rudd (10)	Humberside area, (E)	Jan '92	-
Bream (10)	Humberside area, (E)	Jan '92	-
Stone loach (146)	River Almond (site of a fish farm) Llyn Tegid beck, (W)	Apr '93, May '93, Jul '93, Aug '93, Sep '93, Jul '94 Sep '93	*A. gracilis* *A. gracilis*
Bullhead (40)	Llyn Tegid beck, (W)	Sep '93	*A. gracilis*
Minnow (10)	River Almond (site of a fish farm)	Jul '93	-
3-spined-stickleback (10)	Airthrey Loch	May '93, Jul '93	-

Unless otherwise indicated all sources are from Scotland. Other sources: (W) = Wales, (E) = England, (F) = Finland.

Table 2. Natural *Ichthyocotylurus erraticus* metacercarial infections.

Host species	Source	Date	Number of fish examined	Weight (g). Mean in parentheses	Total length (cm)	Prevalence of infection %	Intensity of infection. Mean in parentheses	Cyst location within fish host
Powan	Loch Lomond	Oct '91 Jun '92 Jul '93	7 9 5	101-346 (267) 111-405 (311) 94-378 (234)	21.0-29.0 21.0-31.5 18.0-29.5	100 100 100	120-700+ (approx. 350) approx. 150-500+ approx. 150-800+	Vast majority in the pericardial cavity*; several on the liver and other abdominal cavity organs.
Gwyniad	Llyn Tegid	Sep '93	5	?	16.5-21.4	20	single cyst	Inner surface of the pericardium
Grayling	Llyn Tegid	Sep '93	1	?	21.0	-	single cyst	Attached to the ventricle
Rainbow trout	Loch Awe (fish farm II)	Jan '92 Jul '92	20 22	139-344 (234) 271-414 (350)	22.5-31.0 24.5-31.0	95 36	1-8 (4.5) 1-5 (2.1)	pericardial cavity** pericardial cavity***

* cysts aggregated in clusters on the ventricle, often several deep; occasionally the mass of cysts caused adhesion between the ventricle and pericardium.

** mixed infection: 89% *I. erraticus* and 11% *A. gracilis*. *** mixed infection: 84% *I. erraticus* and 16% *A. gracilis*.

Table 3. Natural *Ichthyocotylurus variegatus* metacercarial infections.

Host species	Source	Date	Number of fish examined	Weight (g). Mean in parentheses	Total length (cm)	Prevalence of infection %	Intensity of infection. Mean in parentheses	Cyst location within fish host (see below for abbreviations)
Perch	Castle Semple Loch	Oct '91	4	8.0-11.5 (9.8)	9.4-10.2	100	32-49 (43.0)	92.5% Sbl, 5% Mes, 2% Orb, 0.5% Liv
		Feb '92	1	4.7	6.5	-	32	
		Aug '92	18	7.5-81.5 (60.4)	6.5-16.0	100	14-95 (53.6)	
	Loch Lomond	Aug '92	21	8.0-385.0 (114.6)	11.0-24.0	100	9-132 (60.5)	
		May '93	53	26.7-65.2 (41.4)	11.0-14.5	100	24-140 (60.0)	Similar distribution for all other records. In low infections, cysts were often found exclusively on the inner surface of the swimbladder.
		Jun '93	22	40.0-249.0 (103.3)	13.0-22.0	100	25-152 (72.0)	
		Jul '94	7	128.0-360.0 (209.0)	18.0-24.0	100	all >100	
	Llyn Tegid	Sep '93	5	?	16.0-24.5	100	5-18 (10.2)	
	Stirlingshire fishery	Jun '94	10	11.3-79.3 (49.6)	11.0-15.0	100	24-98 (45.3)	
Ruffe	Loch Lomond	Apr '92	12	9.0-19.2 (14.1)	7.0-10.8	100	67-360 (208)	65% Sbl, 14% Pcc, 13% Mes, 3% Bcw, 3% Ov, 2% Orb
		May '93	15	6.0-22.3 (15.7)	6.2-10.0	100	97-500+ (>300)	
	Llyn Tegid	Sep '93	5	?	8.0-12.5	100	2-8 (5.6)	Similar distribution for all other records. No cysts were ever found within the testes of male fish.

Abbreviations.

Bcw: body cavity wall
Liv: liver
Mes: mesenteries
Orb: orbit
Ov: ovary
Pcc: pericardial cavity
Sbl: swimbladder

Table 4. Natural *Apatemon gracilis* metacercarial infections.

Host species	Source	Date	Number of fish examined	Weight (g). Mean in parentheses	Total length (cm)	Prevalence of infection.	Intensity of infection. Mean in parentheses	Cyst location within fish host (for abbreviations see below)
Rainbow trout*	Loch Awe (fish farm II)	Jan '92	20	139-344 (234)	22.5-31.0	95	1-8 (4.5)	Pcc**
		Jul '92	22	271-414 (350)	24.5-31.0	36	1-5 (2.1)	Pcc***
	River Earn (fish farm)	Jan '92	20	220-393 (307)	27.0-32.0	95	1-16 (4.5)	Pcc
	River Almond (fish farm)	Mar '92	6	66-160 (128)	17.5-24.0	83	1-5 (2.2)	Pcc
		Aug '92	13	171-391 (320)	23.5-29.5	92	1-76 (26)	Pcc
		Sep '92	24	258-430 (323)	26.0-31.0	96	8-107 (28.1)	Pcc
		Jan '93	20	202-389 (314)	26.0-30.0	100	7-55 (31)	97% Pcc, 3% Pyl
		May '93	5	503-656 (568)	32.0-34.0	80	6-29 (12.5)	Pcc
	River Doon (fish farm)	Sep '92	10	181-264 (206)	22.5-26.0	100	16-141 (75.4)	97% Pcc, 2% Pyl, 1% Hum
Salmon parr	River Almond (DAFS)	Aug '93	62	11.7-62.4 (20.7)	9.0-16.8	94	1-6 (3.0)	61% Bc, 39% Pcc
Arctic char	Isle of Skye (fish farm)	Jan '91	7	?	12.5-15.5	43	1-2 (1.7)	80% Pcc, 20% Pyl
Stone loach	River Almond (site of a fish farm)	Apr '93	1	0.86	4.3	-	(4.0)	Bc
		May '93	10	0.21-0.68 (0.42)	2.6-4.2	100	1-6 (3.3)	Bc
		Jul '93	8	0.53-2.01 (1.34)	3.6-5.4	100	1-33 (9.0)	77% Bc, 23% Cc
		Aug '93	23	0.25-3.80 (0.81)	1.6-7.7	38	1-6 (2.6)	86% Bc, 11% Cc, 3% Hum
		Aug '93	18	0.25-3.90 (0.76)	1.6-6.6	67	1-5 (2.8)	85% Bc, 15% Cc
		Sep '93	32	0.27-2.41 (0.79)	3.0-6.7	78	1-19 (2.9)	82% Bc, 17% Cc, 1% M
		Jul '94	42	0.28-3.80 (0.93)	3.1-6.4	21	1-18 (3.4)	86% Bc, 14% Cc
	Llyn Tegid beck	Sep '93	6	2.97-7.51 (4.01)	6.3-8.7	100	1-19 (6.5)	77% Bc, 13% Pcc, 10% Cc
Bullhead	Llyn Tegid beck	Sep '93	40	0.37-3.05 (1.48)	2.8-5.6	68	1-13 (5.6)	91% Bc, 6% Cc, 3% Pcc

* heavy infections typically found as a monolayer of cysts on the inner surface of the pericardium, covered by a communal host response with melanin deposits.

** mixed infection: 89% *Ichthyocoylurus erraticus* and 11% *A. gracilis*. *** mixed infection: 84% *I. erraticus* and 16% *A. gracilis*.

Abbreviations. Bc: body cavity, Cc: cranial cavity, Hum: humour of the eye, M: musculature, Pcc: pericardial cavity, Pyl: pyloric caeca.

Table 5. Natural *Apatemon annuligerum* metacercarial infections.

Host species	Source	Date	Number of fish examined	Weight (g). Mean in parentheses	Total length (cm)	Prevalence of infection %	Intensity of infection. Mean in parentheses	Cyst location within fish host (see below for abbreviations)
Perch	Castle Semple Loch	Aug '92	18	7.5-81.5 (60.4)	6.5-16.0	44	1-5 (1.9)	
	Loch Lomond	Aug '92	21	8.0-385.0 (114.6)	11.0-24.0	57.9	1-2 (1.4)	
		May '93	53	26.7-65.2 (41.4)	11.0-14.5	56.6	1-4 (1.6)	In all cases cysts present within
		Jun '93	22	40.0-249.0 (103.3)	13.0-22.0	80	1-4 (1.7)	the humour of the eyes
		Jul '94	7	128.0-360.0 (209.0)	18.0-24.0	28	1-5 (3.0)	
	Stirlingshire fishery	Jun '94	10	11.3-79.3 (49.6)	11.0-15.0	90	1-9 (3.2)	

Table 7. Morphometric measurements (μm) of *Ichthyocotylurus erraticus* metacercariae.

Author	Present study					Hughes (1928)	Olson (1970)	Odening (1979)
Fish host	Powan	Rainbow trout	Grayling	Whitefish	Vendace	-	Brook trout	From previous authors
Source	Loch Lomond, Scotland	Loch Awe, Scotland	Llyn Tegid, Wales	Lake Kitka, Finland		Lake Huron, North America	Georgetown Lake, Montana, North America	
Fixative	Berland's fluid					?	Hot AFA or 10% formalin	
Number of specimens	46	15	1	47	23	4-10	10	
Cyst dimensions*	488-677 x 441-520	580-680 x 410-500	-	-	-	550-750 x 400-560	600-700 x 425-475	480-1480 x 400-1110
Body length	294-494 (394)	296-441 (354)	301	256-347 (304)	231-462 (333)	300-420 (380)	350-430 (394)	232-590
Body breadth	217-442 (321)	221-347 (267)	231	215-281 (247)	182-396 (266)	250-330 (300)	250-340 (300)	160-600
Oral sucker length	46-65 (55)	44-57 (51)	45	43-62 (52)	39-54 (48)	43-63 (59)	50-60 (57)	43-75
Oral sucker breadth	43-72 (62)	42-63 (49)	48	41-68 (48)	41-56 (50)	54-60 (58)*	50-70 (58)	40-70
Pharynx length	18-30 (23)	16-22 (19)	19	17-25 (20)	16-22 (19)	24-30 (28)*	-	17-30
Pharynx breadth	16-24 (20)	14-19 (16)	15	14-20 (17)	16-20 (17)	15-24 (19)*	-	12-26
Ventral sucker length	53-81 (63)	44-63 (53)	47	39-66 (50)	43-60 (51)	52-66 (57)	50-60 (58)	40-76
Ventral sucker breadth	62-95 (80)	63-86 (71)	64	56-72 (65)	56-72 (63)	56-77 (66)	60-80 (70)	50-90
Tribocytic organ length	100-174 (132)	91-158 (128)	120	87-138 (112)	78-144 (112)	70-80 (74)	90-110 (99)	66-140
Tribocytic organ breadth	104-186 (150)	102-164 (140)	122	101-134 (115)	82-132 (117)	90-110 (105)	90-130 (111)	90-150
Lateral lappet length	54-89 (75)	52-85 (70)	63	52-90 (65)	53-92 (71)	-	55-62 (59)	52-90 x 22-42
Lateral lappet fissure length	37-70 (55)	48-72 (57)	48	33-70 (47)	41-68 (52)	35-52 (49)	-	-

* Measurements from unfixed specimens.

Table 8. Morphometric measurements (μm) of *Ichthyocotylurus variegatus* metacercariae.

Author	Present study				Odening & Bockhardt (1971)	Odening (1979)
Fish host	Perch	Ruffe	Perch	Ruffe		From previous authors
Source	Castle Semple Loch, Scotland	Loch Lomond, Scotland	Lake Kitka, Finland		Lake area of the Spree and Havel Rivers, Germany	
Fixative	Berland's fluid				?	
Number of specimens	20	93	32	17	?	
Cyst dimensions*	586-685 x 545-633	-	-	-	587-793 x 551-719	570-800 x 400-752
Body length	422-595 (517)	290-649 (467)	347-520 (430)	305-516 (416)	492-749	380-880
Body breadth	365-480 (428)	194-517 (338)	248-454 (341)	239-371 (317)	352-771	300-771
Oral sucker length	62-76 (67)	50-90 (63)	47-68 (60)	43-70 (58)	66-90	65-90
Oral sucker breadth	65-86 (77)	47-94 (65)	47-70 (61)	49-82 (61)	66-104	60-104
Pharynx length	21-32 (28)	17-33 (25)	19-25 (23)	23-27 (24)	26-35	22-35
Pharynx breadth	19-29 (25)	14-30 (20)	13-24 (19)	14-26 (20)	30-40	17-40
Ventral sucker length	72-113 (89)	50-124 (76)	45-91 (67)	56-107 (71)	64-117	62-110
Ventral sucker breadth	94-119 (108)	60-134 (96)	70-120 (87)	74-117 (84)	77-132	77-114
Tribocytic organ length	152-221 (183)	103-258 (150)	105-188 (134)	103-194 (139)	145-276	110-276
Tribocytic organ breadth	164-224 (187)	101-205 (147)	109-169 (132)	117-196 (144)	169-345	132-345
Lateral lappet length	86-132 (118)	90-177 (125)	93-145 (118)	87-145 (111)	86-163 x 44-73	88-171 x 30-66
Lateral lappet fissure length	65-105 (80)	51-120 (81)	62-109 (78)	64-97 (79)	-	-

* Measurements from unfixed specimens.

Table 9. Morphometric measurements (μm) of *Ichthyocotylurus pileatus* and *I. platycephalus* metacercariae.

Author	Present study		Hughes (1928)	Odening (1979)	Odening *et al.* (1970)	Identified by the Natural History Museum, London	Odening (1979)
Metacercarial species	*I. pileatus*	*I. platycephalus*	*I. pileatus*		*I. platycephalus*		
Fish host	Ruffe		Perch	From previous authors	?	Brown trout	From previous authors
Source	Lake Kitka, Finland		Wampler Lake, Michigan, U.S.A		Lake area of the Spree and Havel Rivers, Germany	Galway, Eire	
Fixative	Berland's fluid		?		?	?	
Number of specimens	37	1	7-10		?	5	
Cyst dimensions	-	-	330-450 x 280-420	285-565 x 266-528	-	-	536-1334 x 448-980
Body length	140-225 (191)	908	155-233 (180)	155-429	778-1064	734-914 (793)	510-1300
Body breadth	128-214 (151)	594	134-190 (164)	134-342	551-675	384-563 (503)	342-900
Oral sucker length	31-42 (38)	124	42-53 (49)	35-66	114-138	91-93 (92)	87-150
Oral sucker breadth	29-46 (36)	126		35-66	107-155	87-91 (90)	107-155
Pharynx length	12-20 (15)	49	20-23 (22)*	13-26	35-52	26	35-54
Pharynx breadth	9-17 (13)	41	15-20 (18)*	13-22	24-41	24	22-41
Ventral sucker length	28-44 (35)	177	42-53 (48)	35-60	206-257	112-140 (123)	109-257
Ventral sucker breadth	37-49 (42)	192		35-66	206-235	118-150 (130)	150-250
Tribocytic organ length	55-116 (73)	173	63-105 (75)	63-150	159-207	100-128 (107)	92-200
Tribocytic organ breadth	52-96 (79)	202	70-105 (85)	62-180	183-311	116-203 (163)	140-367
Lateral lappet length	26-61 (45)	142	-	30-57 x 22-44	-	-	52-155 x 39-150
Lateral lappet fissure length	19-42 (32)	76	33-36 (35)	-	-	-	-

* Measured from live specimens.

Table 10. Morphometric measurements (μm) of *Apatemon gracilis* metacercariae.

Author	Present study					Hoffman (1959)	Vojtek (1964a)	Blair (1974)
Fish host	Rainbow trout	Salmon parr	Arctic char	Stone loach	Bullhead	Brook stickleback	?	Stone loach
Source	River Almond fishery, Scotland	River Almond fishery, Scotland	Isle of Skye fishery, Scotland	River Almond at site of fishery, Scotland	Llyn Tegid beck, Wales	North Dakota, North America	?	River Almond at site of fishery, Scotland
Fixative	Berland's fluid					Hot Bouin's	Mecuric -acetic fixative	Hot formol saline
Number of specimens	31	33	3	22	45	7	?	5
Cyst dimensions*	505-694 x 364-462 (n=10)	-	-	564-715 x 367-513 (n=10)	-	1000 x 600	584-884 x 474-584	542-660 x 356-426** (n=10)
Total body length	651-915 (778)	512-765 (651)	723-896 (798)	578-833 (701)	569-883 (732)	452-739 (547)	-	-
Forebody length	488-814 (593)	388-674 (505)	560-652 (604)	464-644 (548)	460-689 (551)	307-403 (345)	303-442 (390)	395-581 (444)
Forebody breadth	224-356 (283)	189-263 (215)	244-326 (275)	223-330 (279)	215-355 (270)	-	234-347 (282)	248-294 (266)
Hindbody length	101-264 (173)	91-190 (147)	163-244 (193)	90-256 (153)	113-214 (156)	115-192 (154)	114-189 (144)	144-243 (200)
Oral sucker length	71-94 (85)	66-89 (78)	77-87 (84)	72-97 (84)	70-105 (86)	52-80 (67)	64-106 (84)	57-86 (76)
Oral sucker breadth	77-105 (88)	66-89 (75)	80-93 (85)	72-103 (86)	72-148 (95)		59-104 (81)	80-89 (83)
Pharynx length	31-41 (35)	27-35 (32)	31-39 (35)	33-47 (38)	25-43 (36)	37-50 (42)	28-55 (39)	39-38 (35)
Pharynx breadth	26-38 (29)	25-31 (28)	28-33 (30)	27-39 (33)	25-35 (29)	25-45 (35)	31-48 (38)	27-32 (30)
Ventral sucker length	79-148 (110)	82-122 (92)	108-116 (111)	72-152 (120)	72-148 (101)	75-87 (82)	84-117 (100)	86-114 (100)
Ventral sucker breadth	110-176 (122)	96-121 (112)	121-131 (127)	105-169 (140)	105-148 (123)	110-112 (110)	78-129 (103)	104-118 (112)
Tribocytic organ length	108-293 (209)	124-218 (159)	201-314 (261)	183-303 (284)	161-313 (214)	125-137 (135)	42-69 (55)	-
Tribocytic organ breadth	148-255 (190)	113-198 (145)	165-247 (202)	156-243 (197)	149-244 (173)	125-182 (152)	59-104 (81)	-
Lateral lappet length	-	-	-	-	-	75-87 (82)	115-173 (143)	-
Lateral lappet fissure length	79-128 (96)	64-105 (78)	82-90 (86)	89-124 (126)	74-117 (92)	-	-	-
Proteolytic gland	36-61 (50) x 49-107 (76)	37-60 (42) x 37-90 (61)	44-59 (50) x 59-75 (69)	31-87 (51) x 52-103 (82)	41-111 (53) x 52-117 (77)	-	-	-

* Measurements from unfixed specimens. ** Cysts obtained from experimentally infected rainbow trout.

Table 11. Morphometric measurements (μm) of *Apatemon annuligerum* metacercariae.

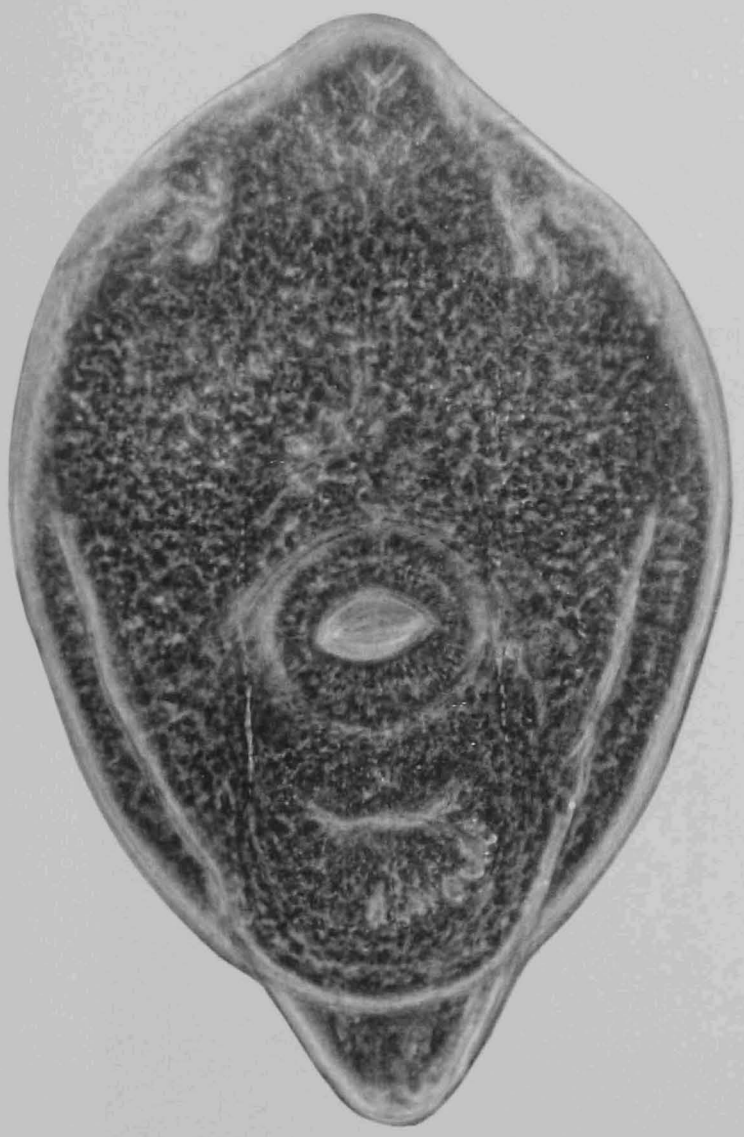
Author	Present study		Odening (1970)	Kozicka (1972)
Fish host	Perch			
Source	Loch Lomond, Scotland	Castle Semple Loch, Scotland	Lake area of the Spree and Havel Rivers, plus Berlin, Germany	Mazurian Lakes, Poland
Fixative	Berland's fluid		?	?
Number of specimens	19	14	?	1
Cyst dimensions*	690-750 x 445-543 (n=3)	-	1000 x 600-750	-
Total body length	533-924 (695)	750-1197 (982)	-	730
Forebody length	424-726 (530)	569-863 (745)	-	553
Forebody breadth	187-363 (264)	244-403 (327)	-	445
Hindbody length	109-299 (165)	181-315 (237)	-	177
Oral sucker length	73-104 (86)	86-116 (95)	104-114	118
Oral sucker breadth	70-107 (86)	83-116 (95)	107-114	110
Pharynx length	29-47 (36)	36-57 (40)	44-46	51
Pharynx breadth	25-37 (29)	27-34 (31)	46-48	44
Ventral sucker length	86-155 (116)	102-156 (119)	145-155	133
Ventral sucker breadth	105-167 (137)	123-172 (141)	155-159	184
Tribocytic organ length	143-347 (212)	141-363 (234)	-	-
Tribocytic organ breadth	137-261 (191)	132-312 (200)	-	-
Lateral lappet length	121-169 (138)	148-214 (172)	-	156 x 156
Lateral lappet fissure length	80-122 (99)	88-140 (103)	-	-
Proteolytic gland	38-66 (46) x 60-111 (80)	44-66 (54) x 61-78 (68)	-	51 x 118
Ovary primordium	-	28-39 (35) x 20-31 (26)		21 x 28
Anterior testis primordium	-	31-52 (42) x 23-39 (36)		38 x 73
Posterior testis primordium	-	27-62 (40) x 25-45 (40)		34 x 87

* Measurements from unfixed specimens.

Fig. 4. Photomicrographs of the four species of *Ichthyocotylurus* metacercariae.

I. erraticus

I. variegatus

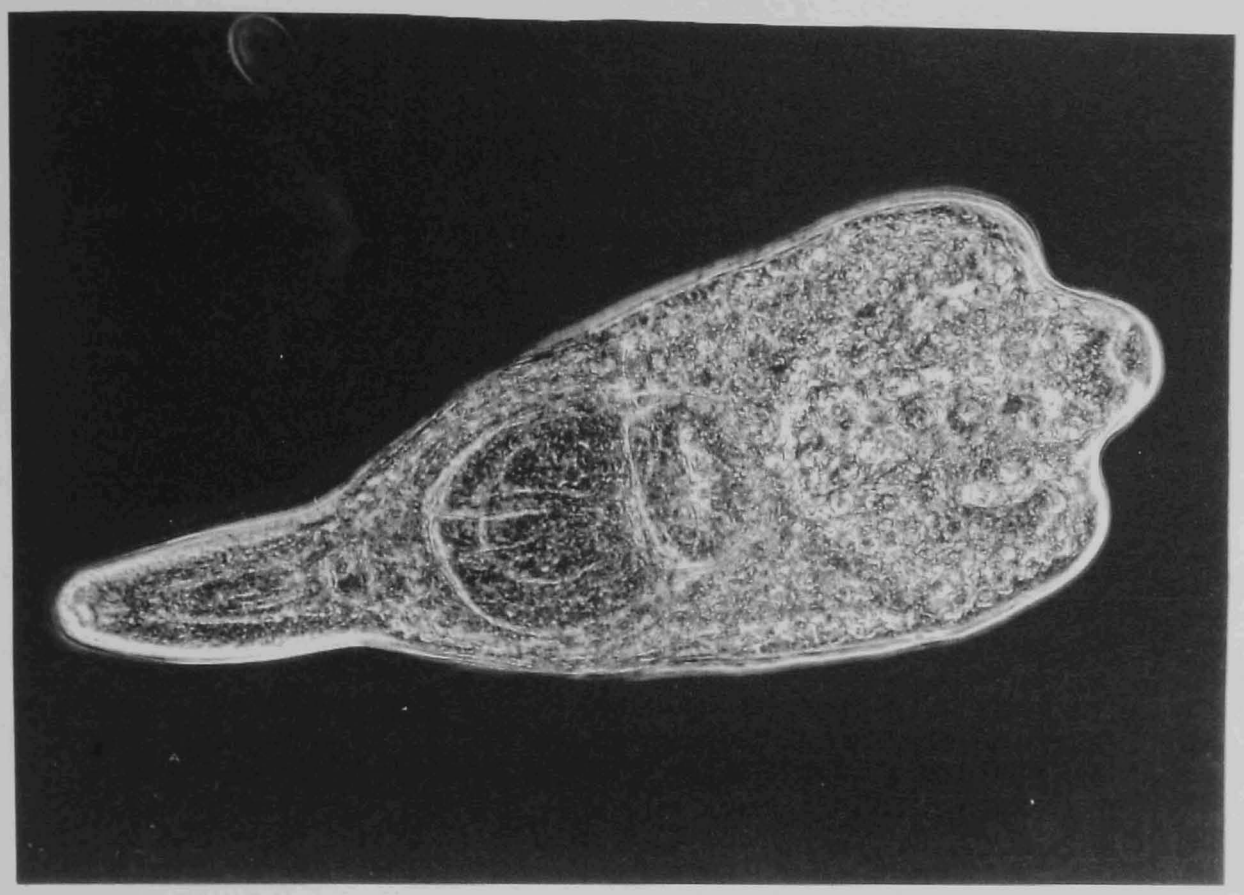


I. platycephalus

300µm

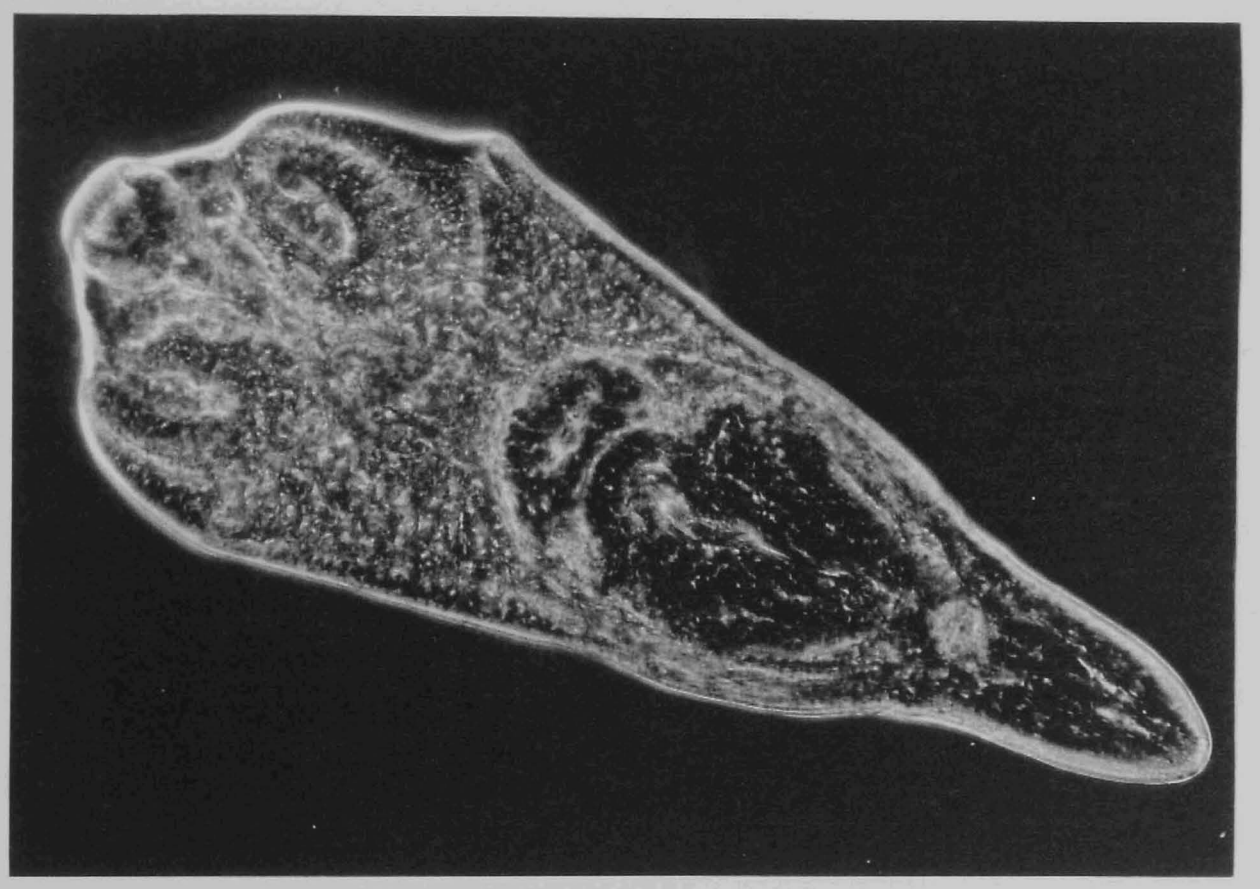
I. pileatus
Shown with tribocytic organ extended. Scale bar represents 600µm.

Fig. 5. Photomicrographs of two species of metacercariae belonging to the subgenus *Apatemon*.



A. gracilis

500µm



A. annuligerum

2.2.3. Chaetotaxy and scanning electron microscopical observations of metacercariae

Chaetotaxy

Clean, perfectly stained specimens were difficult to obtain due to intra-cyst debris remaining attached after excystment; also, some contraction of the metacercariae invariably occured during staining, regardless of the temperature of the silver nitrate solution employed. This contraction resulted in an increased depth and curvature of the specimens, making the mapping of peripheral body sensilla extremely problematic. Sensilla are numerous on the bodies of both *I. erraticus* and *I. variegatus* metacercariae, particularly anteriorly on the ventral surface and posteriorly on the dorsal surface (Fig. 6). Those on the ventral surface are arranged mainly in 2 concentric rings. The inner ring extends posteriorly from the posterior margin of the oral sucker to enclose the ventral sucker, while the outer ring is directed posteriorly from the anterior margin of the oral sucker, passes laterally to the lappets and descends to enclose the tribocytic organ. A high degree of variation was recorded in the number and distribution of these sensilla and no species specific patterns were observed. Indeed, no balanced symmetry in the sensillary pattern was seen for any of the specimens mapped. Similarly, no variation attributable to species was noted for the sensilla present on the oral or ventral suckers. The oral suckers of both species were found to bear a great number of sensilla located around the mouth (48-92), anterior to the mouth (22-51) and encircling the margin of the sucker (61-91). A typical distribution of these sensilla is shown in Fig. 7. Sensilla are less numerous on the ventral sucker, although variation in numbers were still observed, with between 14 and 24 sensory structures present. The most common sensillary arrangement recorded for the ventral suckers of both species, illustrated in Fig. 7, exhibited a ring of 16 sensilla surrounding the orifice, with a further ring of 6-8 peripherally.

Fig. 6. A typical sensillary distribution recorded for the ventral and dorsal body surfaces of *I. variegatus* and *I. erraticus* metacercariae, illustrating the apparent lack of symmetry.

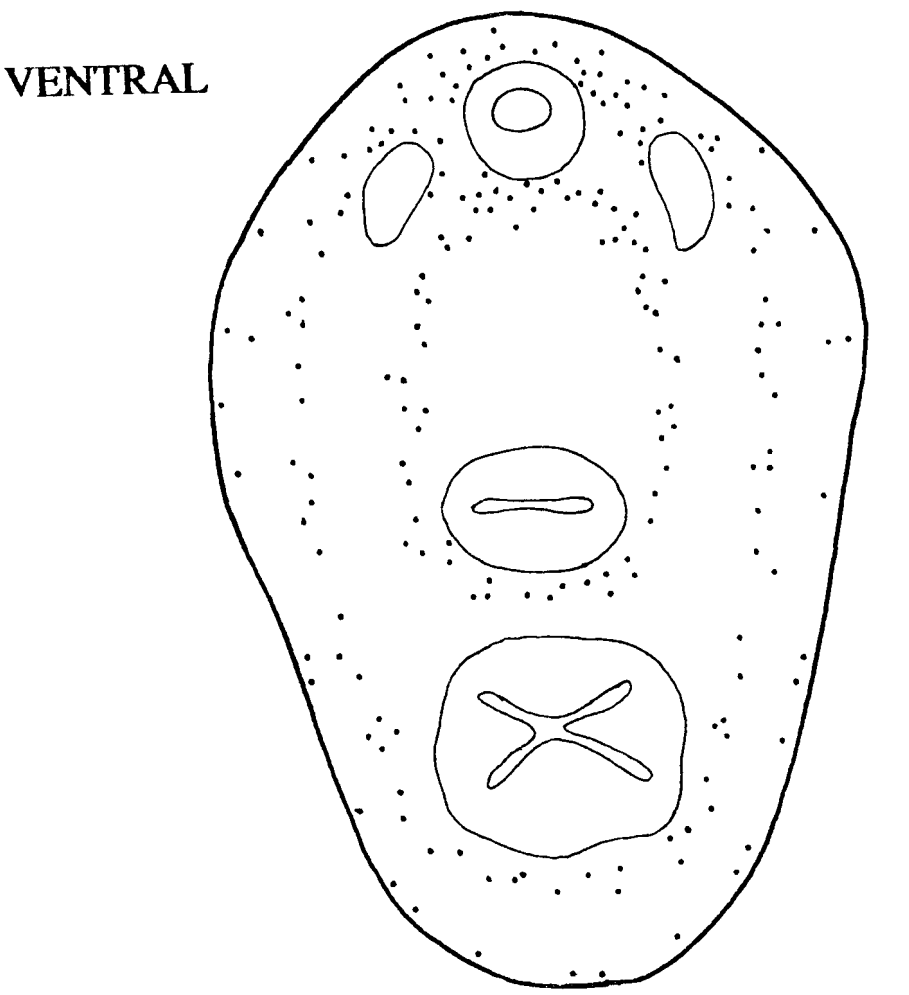


Fig. 7. Sensillary pattern recorded for the oral sucker of an *I. variegatus* metacercaria.

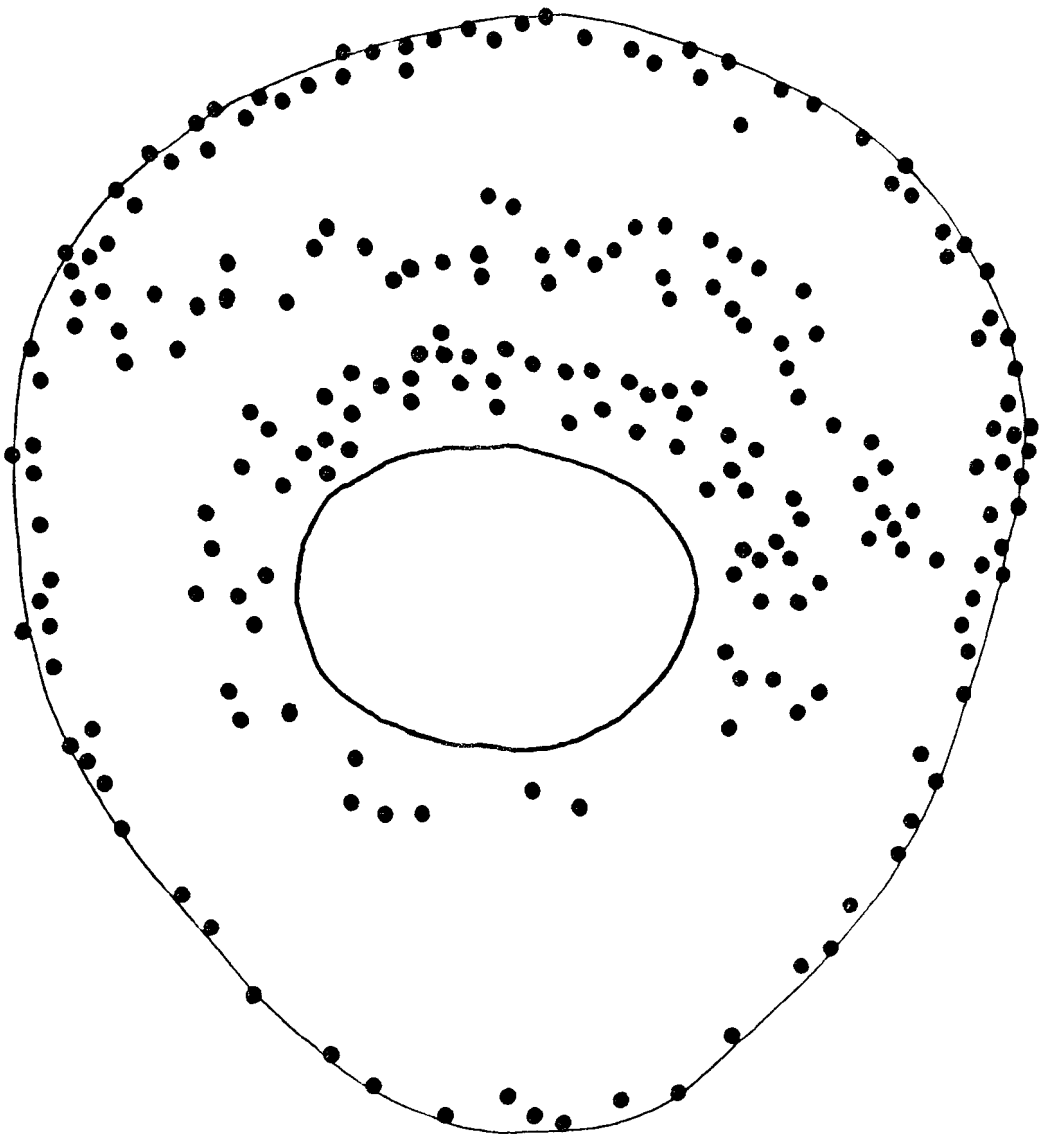
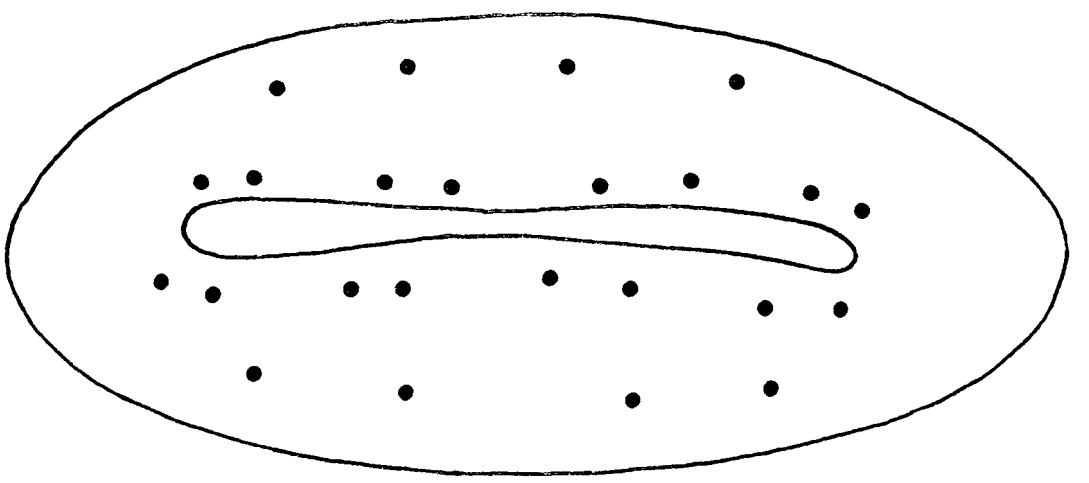


Fig. 8. A stylised representation of the most commonly recorded sensillary pattern for the ventral suckers of *I. erraticus* and *I. variegatus* metacercariae.



Scanning electron microscopy (SEM).

Of the two methods used to fix metacercariae for SEM examination, Berland's fluid provided more relaxed specimens than 3% cacodylate. The cleaning techniques applied to excysted metacercariae during SEM processing proved to be of limited success, with *Apatemon* spp. typically responding better than *Ichthyocotylurus* spp. The use of glycerine for the removal of debris followed by a brief sonication (5-10 seconds) in detergent solution provided the best results, although further modifications to the technique are still needed for optimum specimen preparation. Metacercariae appearing clean at lower magnifications were often found to have structural details obscured at higher magnifications, while particularly clean specimens were frequently damaged by the sonication stage of processing. Figs 9, 10, 11, 12, 13 show scanning electron micrographs of *I. erraticus*, *I. pileatus*, *I. variegatus, A. gracilis* and *A. annuligerum* metacercariae, respectively.

Ichthyocotylurus spp.

SEM examination of *Ichthyocotylurus* metacercariae revealed sensilla not observed by silver nitrate staining, including those present on the lateral lappets and tribocytic organs of all 3 species. The full complement of sensilla on these highly protrusible organs could not be determined, although 3 were consistently observed on the medial side of the lappet base (Fig. 14), 2 on the external distal margins of each tribocytic organ lip and several on the lateral sides of the tribocytic organ base (Fig. 15). The distribution of body sensilla was difficult to discern due to the numbers present, different body postures adopted and apparent intra-specific variation. The majority of *Ichthyocotylurus* metacercarial sensilla were found to comprise a low dome bearing a single short apical cilium (Figs 14, 15, 16, 20). The elevating domes appear to be absent for oral sucker sensilla, with the cilium emerging from within a pit (Figs 16, 17, 18, 19).

Only the peripheral oral sucker sensilla of *Ichthyocotylurus* metacercariae were

visible by SEM (Figs 16, 17, 18, 19). While the arrangement of these sensilla appeared more ordered than suggested by silver staining (indeed Fig. 17 shows bilateral symmetry), intra- and inter-specific variation in numbers was still recorded. The sensilla situated peripherally on the oral sucker of *I. pileatus* metacercariae (it is not known whether additional sensilla are present) were each found to possess a longer, broader cilium than the other *Ichthyocotylurus* spp., particularly when considered relative to the size of the oral sucker itself (Fig. 19).

The ventral suckers of both *I. erraticus* and *I. variegatus* metacercariae exhibited the same number and arrangement of sensilla, with 2 rings encircling the orifice at different levels, the deeper comprising 8 sensilla and the more superficial 16 sensilla (Fig. 20).

Apatemon (Apatemon) spp.

Although the SEM cleaning procedure provided relatively debris-free *Apatemon* specimens, the sensory cilia were occasionally lost. All visible sensilla of *A. gracilis* and *A. annuligerum* metacercariae were of the same type, resembling the domed form of the *Ichthyocotylurus* spp., but modified, with a finer, even shorter nipple-like cilium arising from a typically better defined dome (Fig. 21). SEM examination indicated that both species possess the same number and bilateral arrangement of oral sucker sensilla. These sensory structures are positioned: 4 on the posterior wall of the mouth (ventral wall not visible); 14, with poorly defined domes, spaced equally around the margin of the mouth; and 28, grouped in pairs, on the crest of the sucker (Figs 21, 22). A ring of 10 sensillum pairs, with particularly well defined domes, extends ventrally and laterally around the base of the oral sucker (Figs 13, 21). This border continues dorsally but the sensillary domes become less pronounced and more difficult to discern accurately. The sensilla on the visible region of the ventral body surface of *A. gracilis* and *A. annuligerum* are even more abundant than those on this region of the *Ichthyocotylurus* spp. These are particularly numerous on the rim of the ventral concavity and lateral to the lappets. The

lappets of both *Apatemon* spp., unlike those of the *Ichthyocotylurus* spp. were never seen to protrude; the fissures always maintaining their characteristic shape (Figs 12, 13). Few sensilla were found to encroach onto the lateral lappets of either *Apatemon* spp. No clear pattern was observed for the body sensilla in either *Apatemon* spp., and consequently no species-specific variations were recorded. In several specimens the most anterior portion of the ventral sucker could be seen, and was found to bear, several sensilla on its inner rim.

Fig. 9. Scanning electron micrograph of an *I. erraticus* metacercaria, with retracted lateral lappets (LL) and partly extended tribocytic organ (TCO).

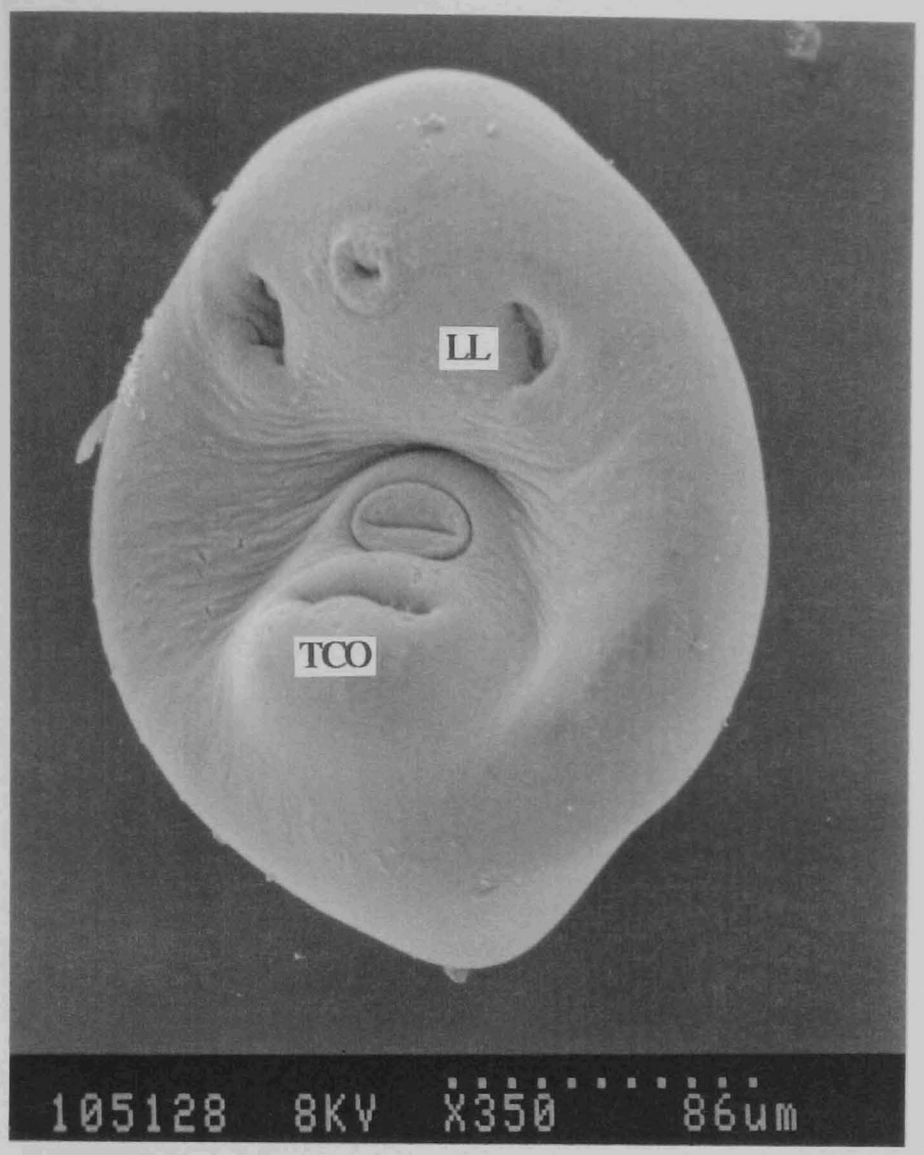


Fig. 10. Scanning electron micrograph of an *I. pileatus* metacercaria with sensilla (S) clearly visible on the oral sucker.

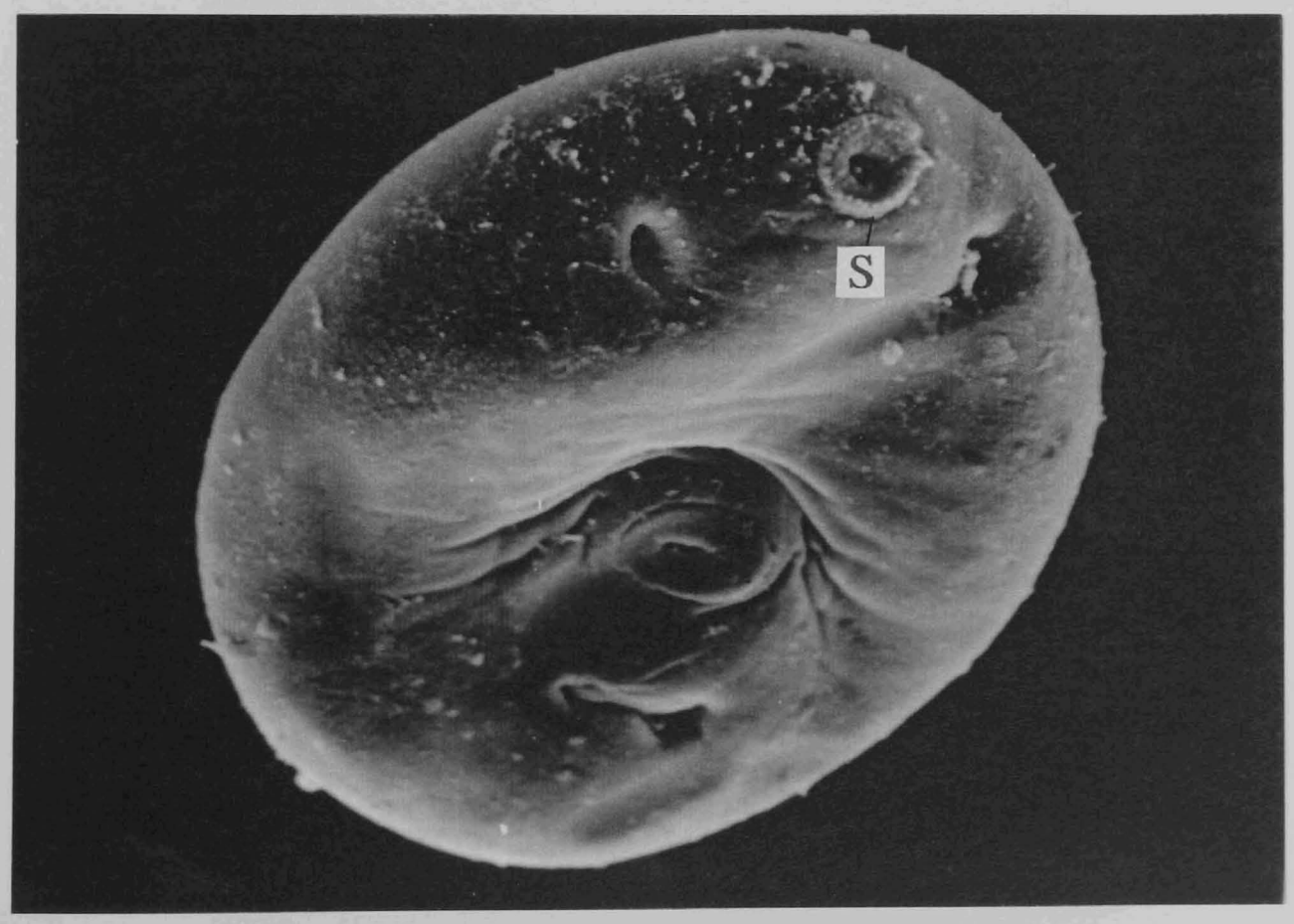
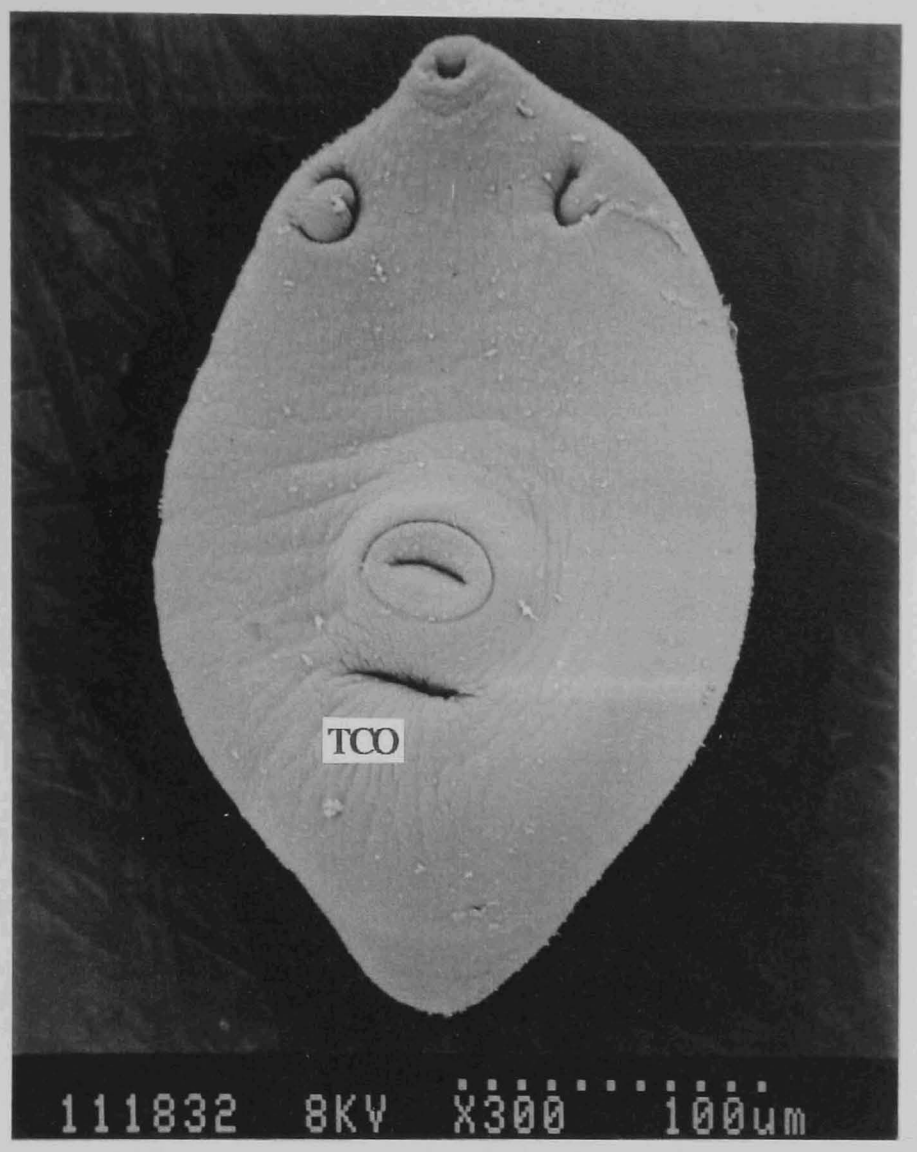


Fig. 11. Scanning electron micrographs of two *I. variegatus* metacercariae showing (top plate) tribocytic organ (TCO) retracted and (bottom plate) tribocytic organ extended.



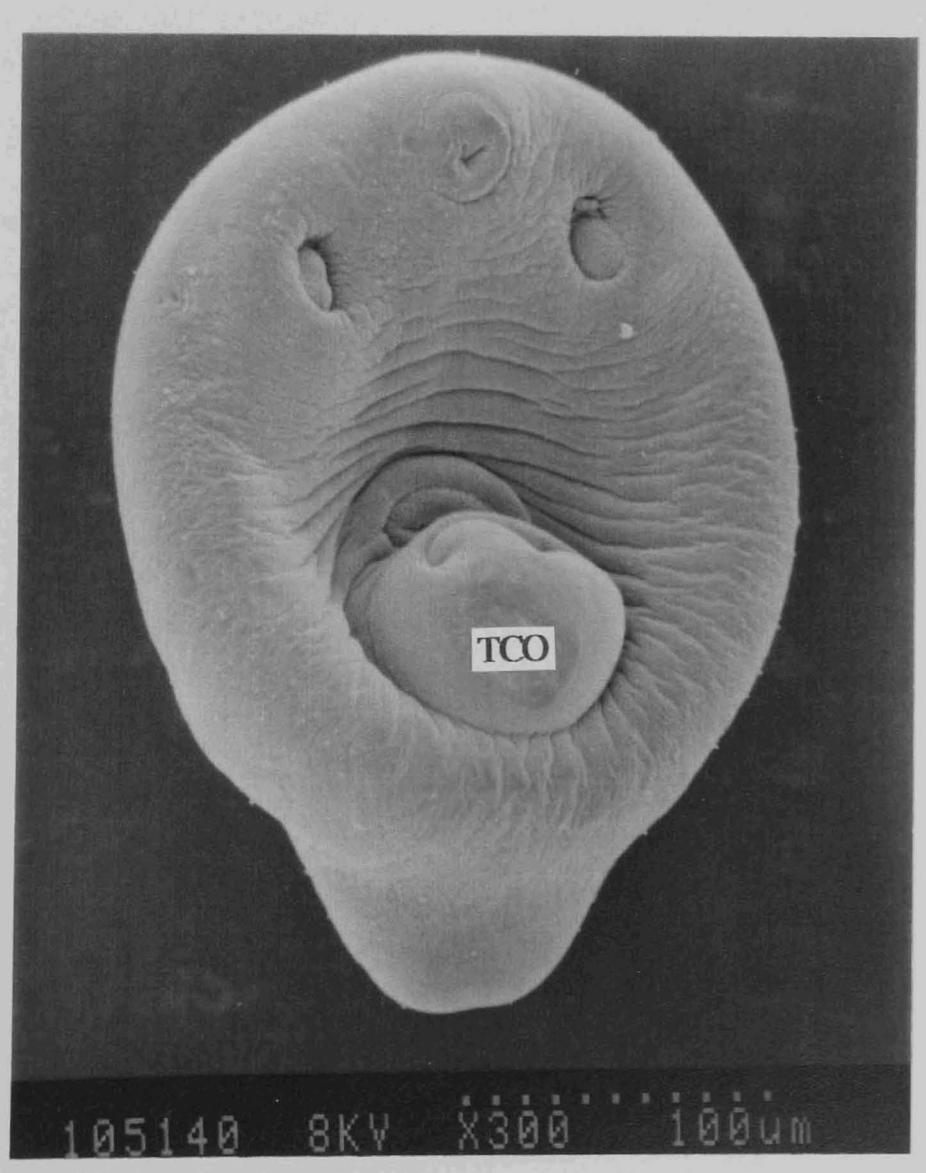


Fig. 12. Scanning electron micrograph of ventral surface of an *A. gracilis* metacercaria.

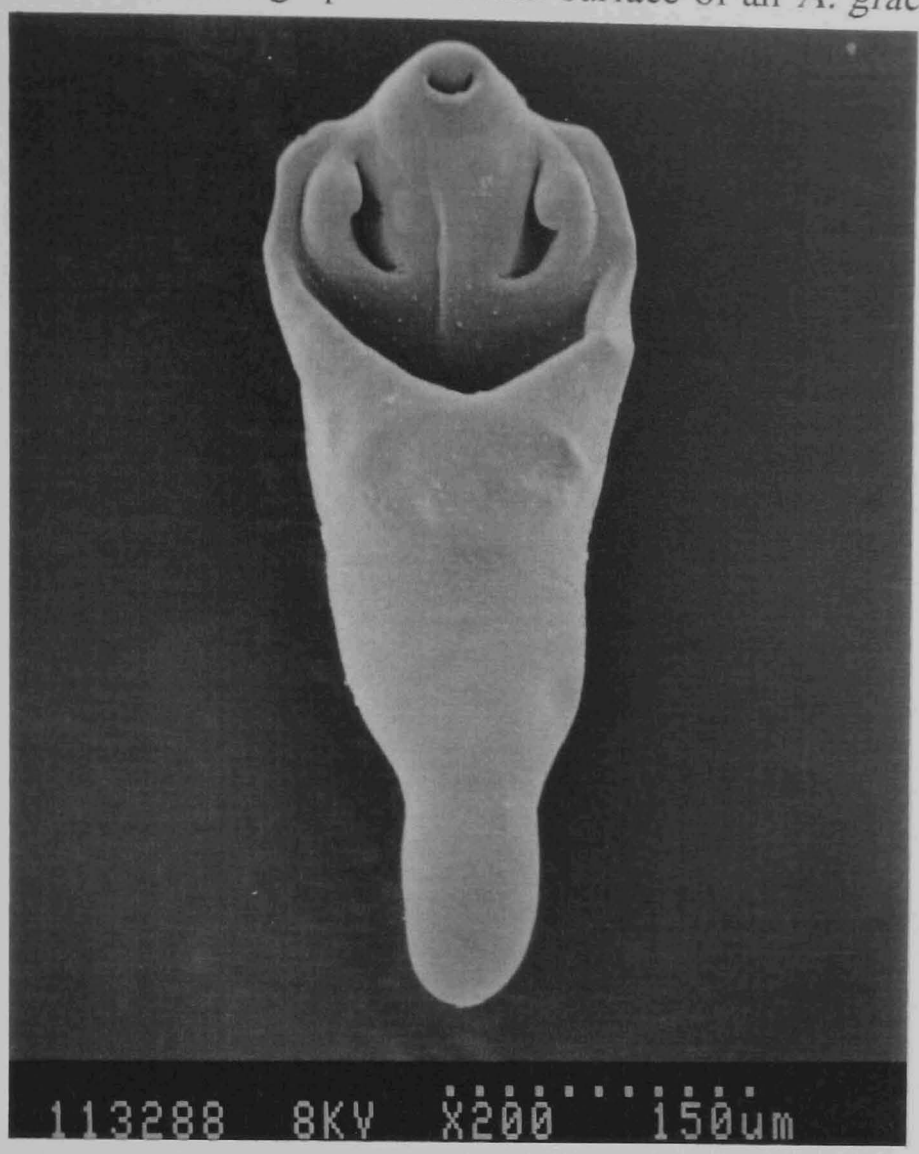


Fig. 13. Scanning electron micrograph of ventro-lateral surface of an *A. annuligerum* metacercaria.

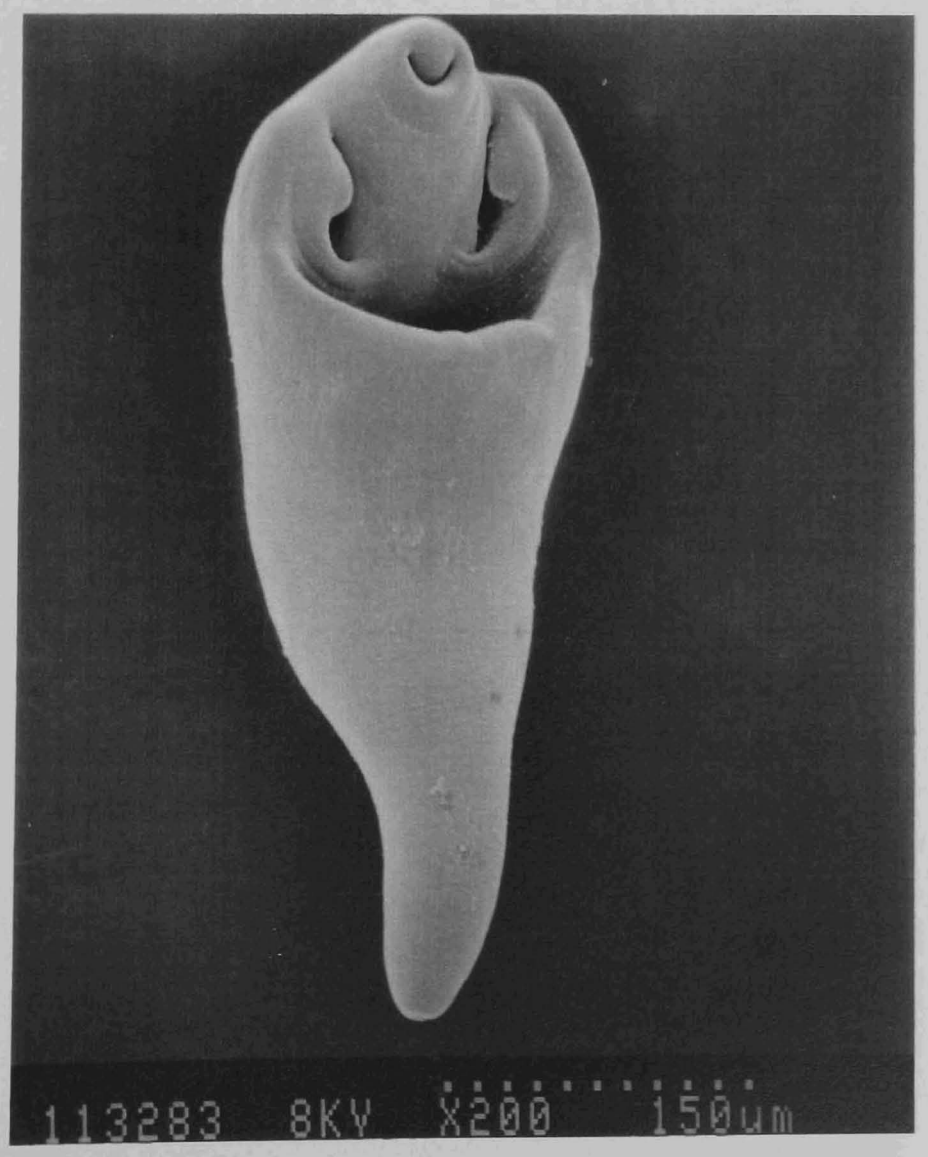


Fig. 14. Scanning electron micrograph of the extended lateral lappet of an *I. erraticus* metacercaria, with associated sensilla (arrowheads).

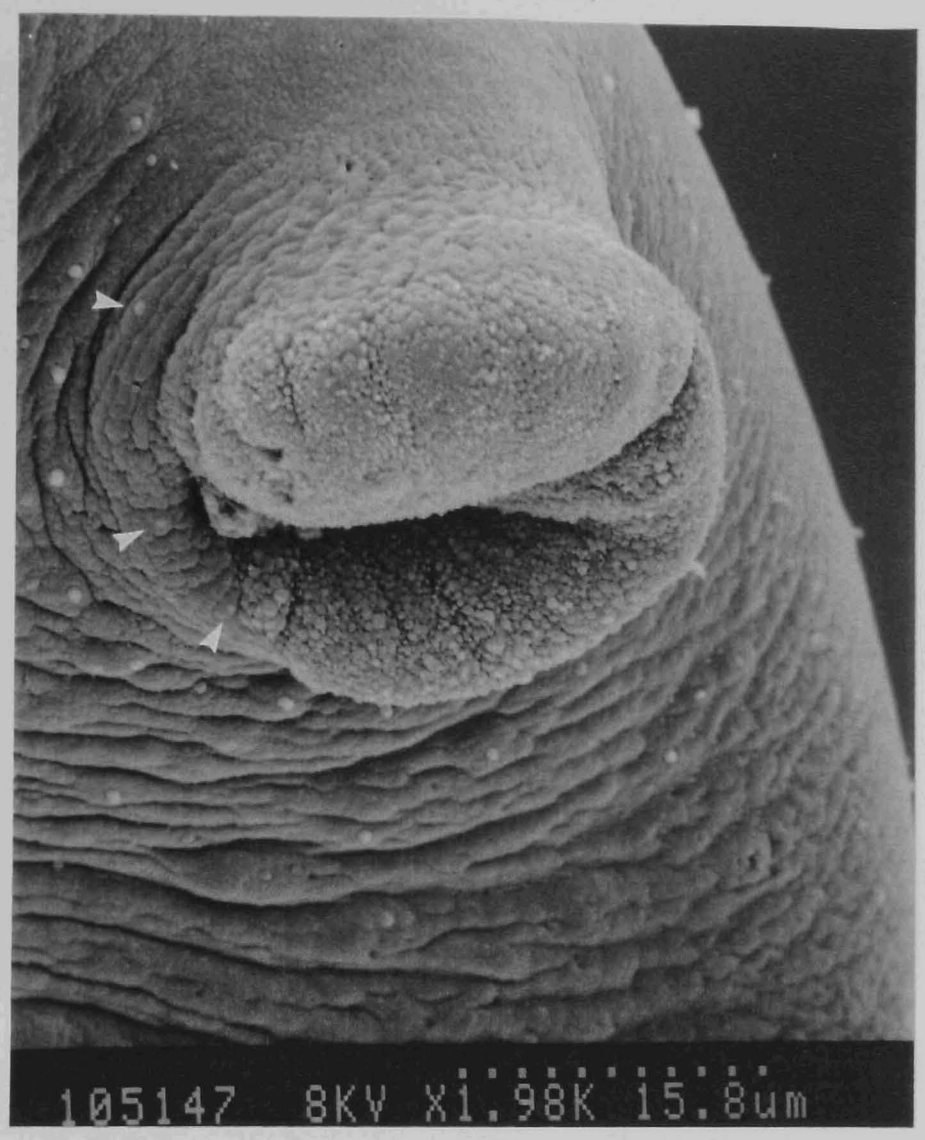


Fig. 15. Scanning electron micrograph of the ventral surface of an *I. variegatus* metacercaria showing sensilla (small arrowheads) on the tribocytic organ and damage resulting from the "cleaning" process (large arrowhead).

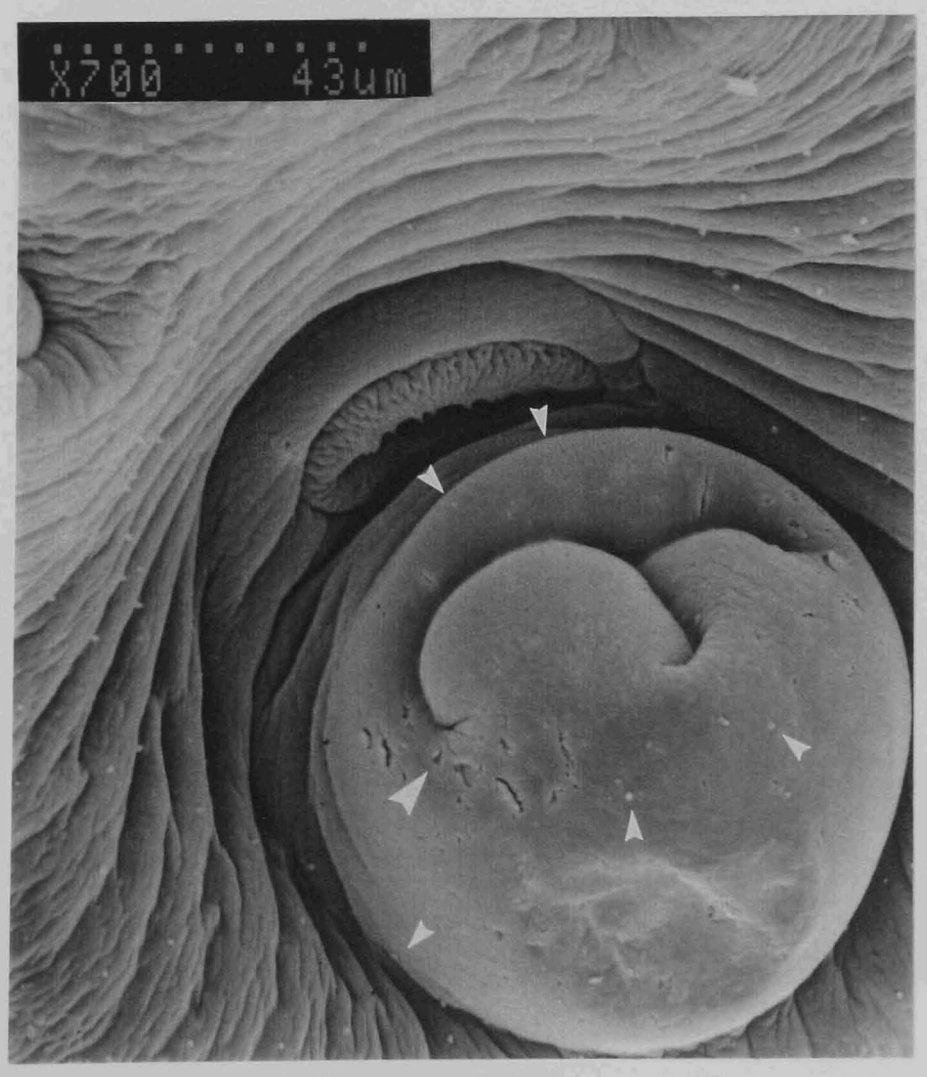


Fig. 16. Scanning electron micrograph of the anterior ventral surface of an *I. erraticus* metacercaria showing the body and oral sucker sensilla types.

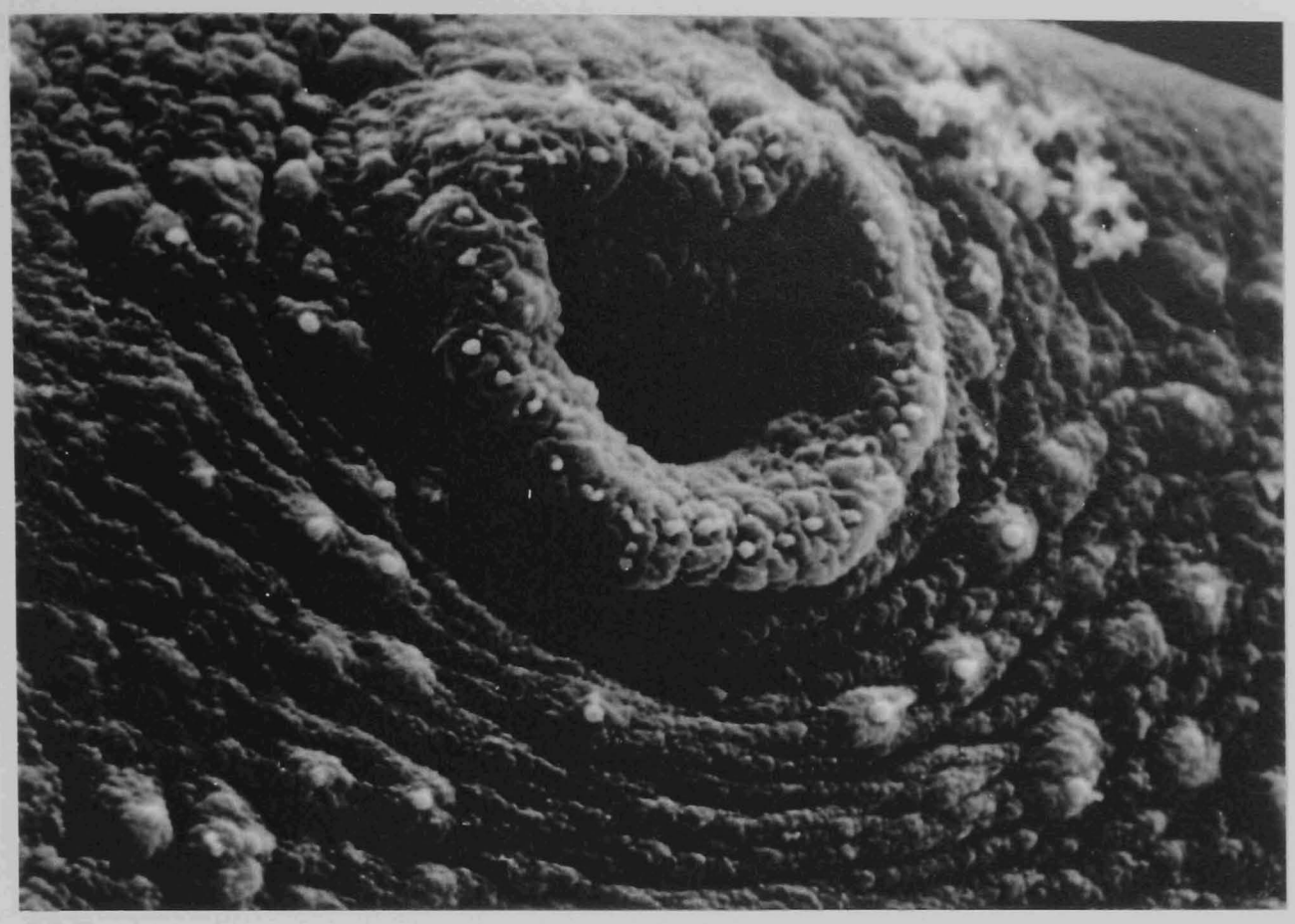


Fig. 17. Scanning electron micrograph of the ventral sucker of an *I. erraticus* metacercaria indicating the arrangement of peripheral sensilla.

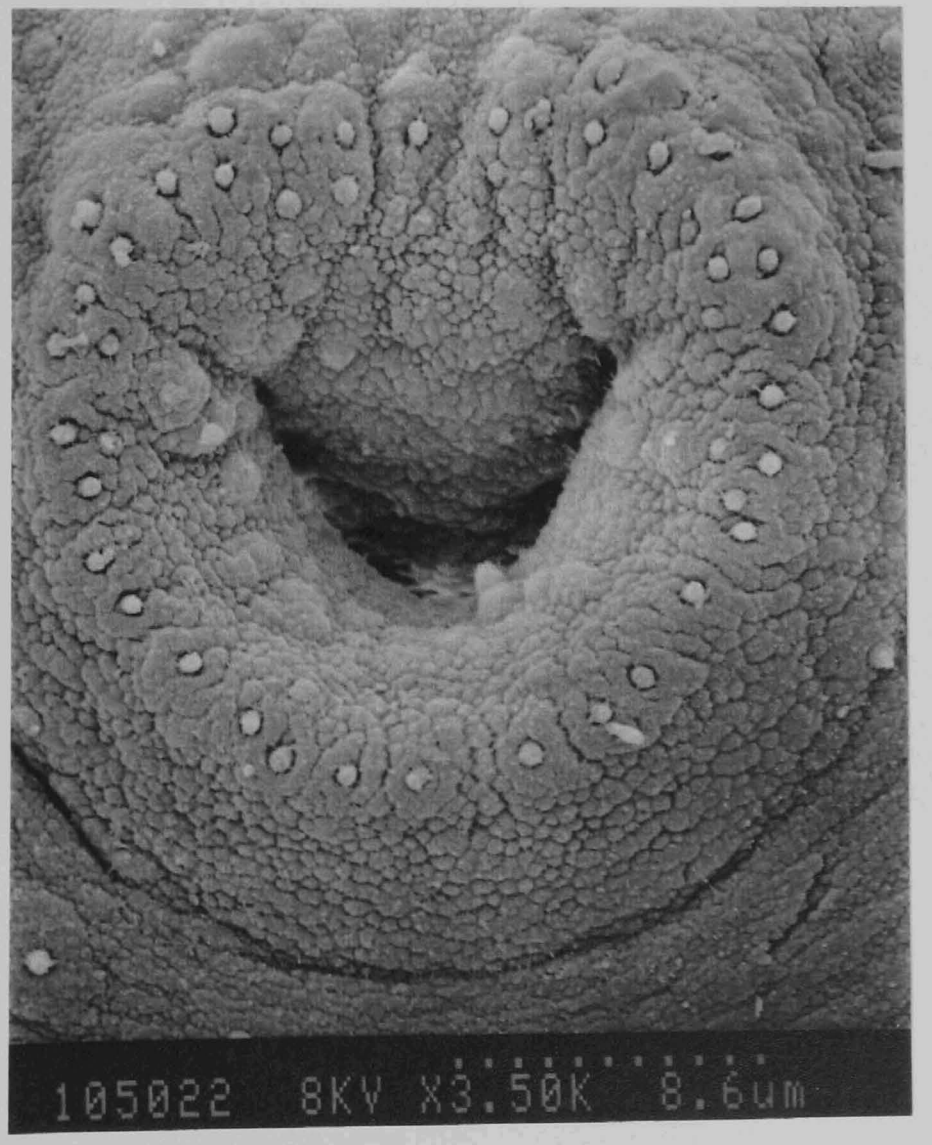
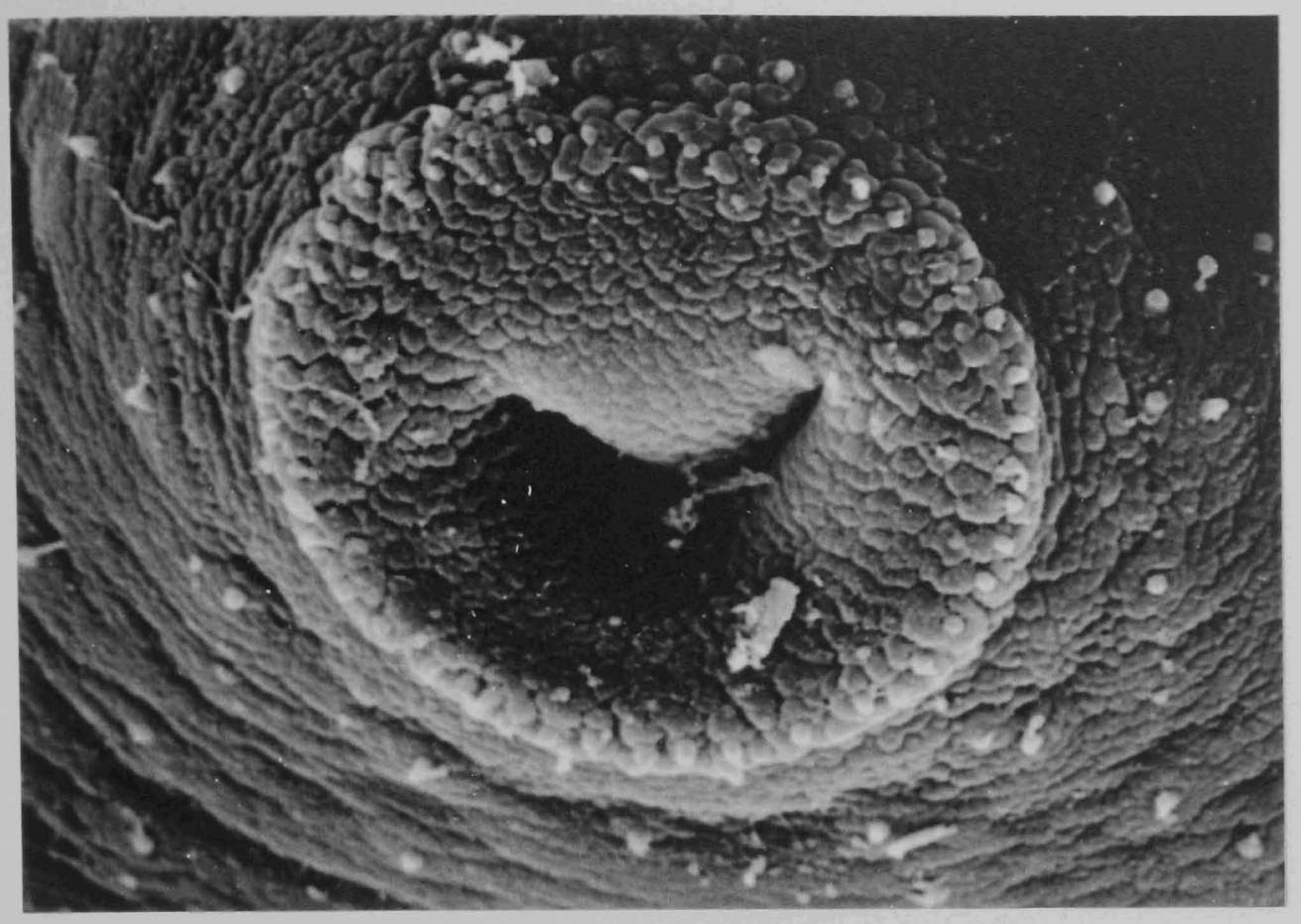


Fig. 18. Scanning electron micrographs of the oral suckers of an *I. erraticus* metacercaria (top) and an *I. variegatus* metacercaria (bottom).



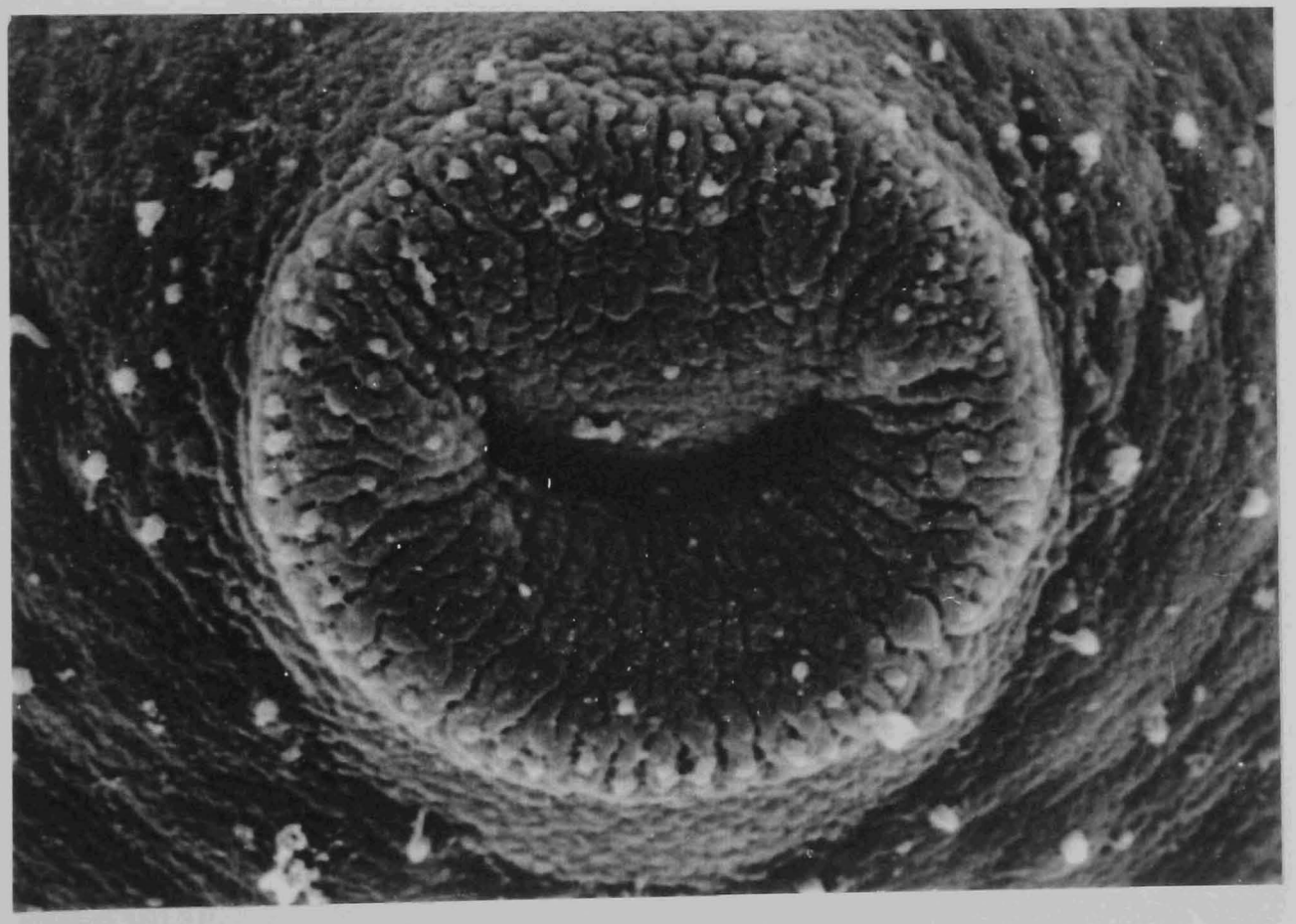


Fig. 19. Scanning electron micrograph of the oral sucker of an *I. pileatus* metacercaria showing the ring of large peripheral sensilla (arrowhead).

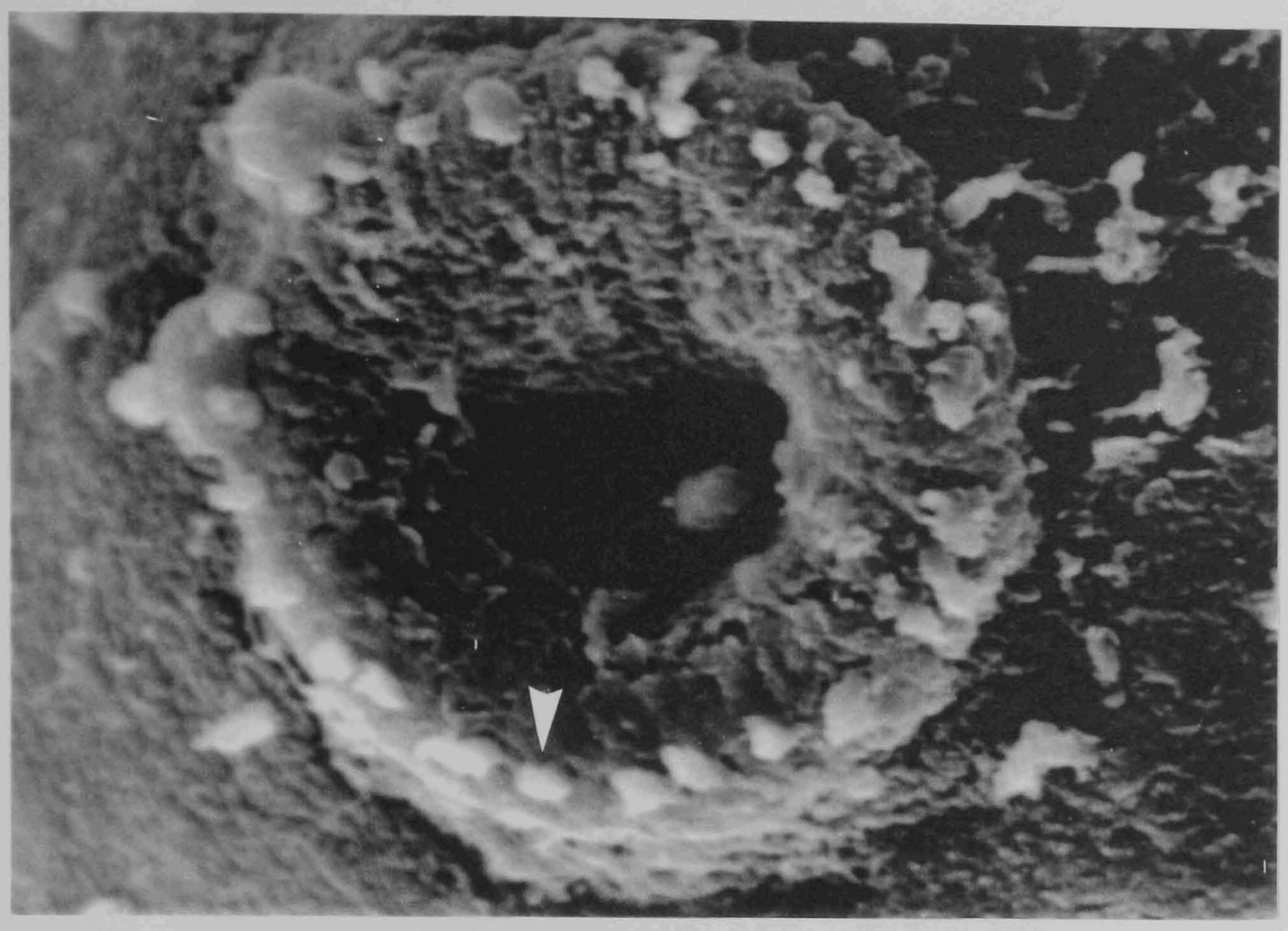


Fig. 20. Scanning electron micrograph of the ventral sucker of an *I. variegatus* metacercaria, arrowheads indicate the position of sensilla.

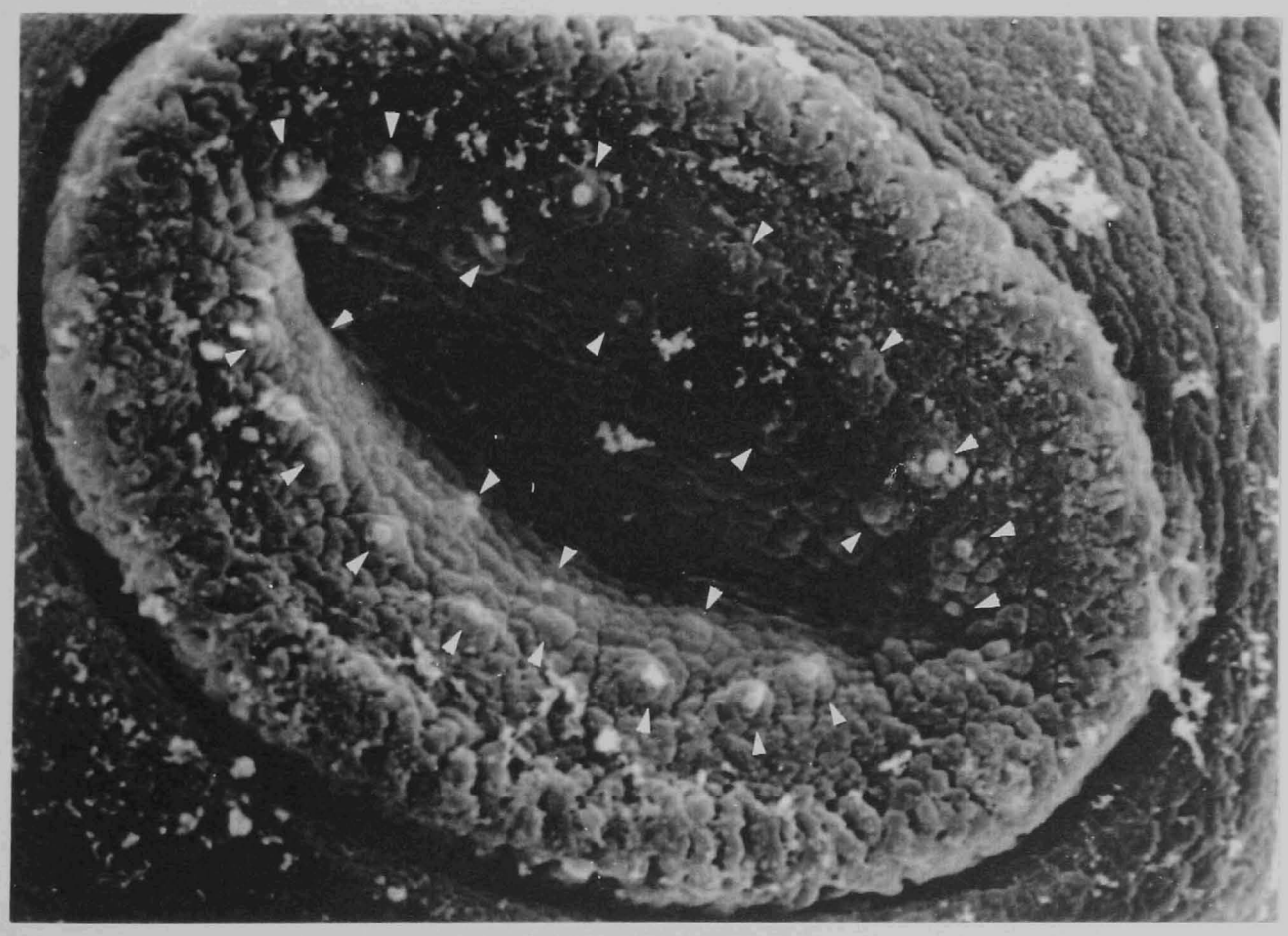


Fig. 21. Scanning electron micrograph of the oral sucker of an *A. gracilis* metacercaria.

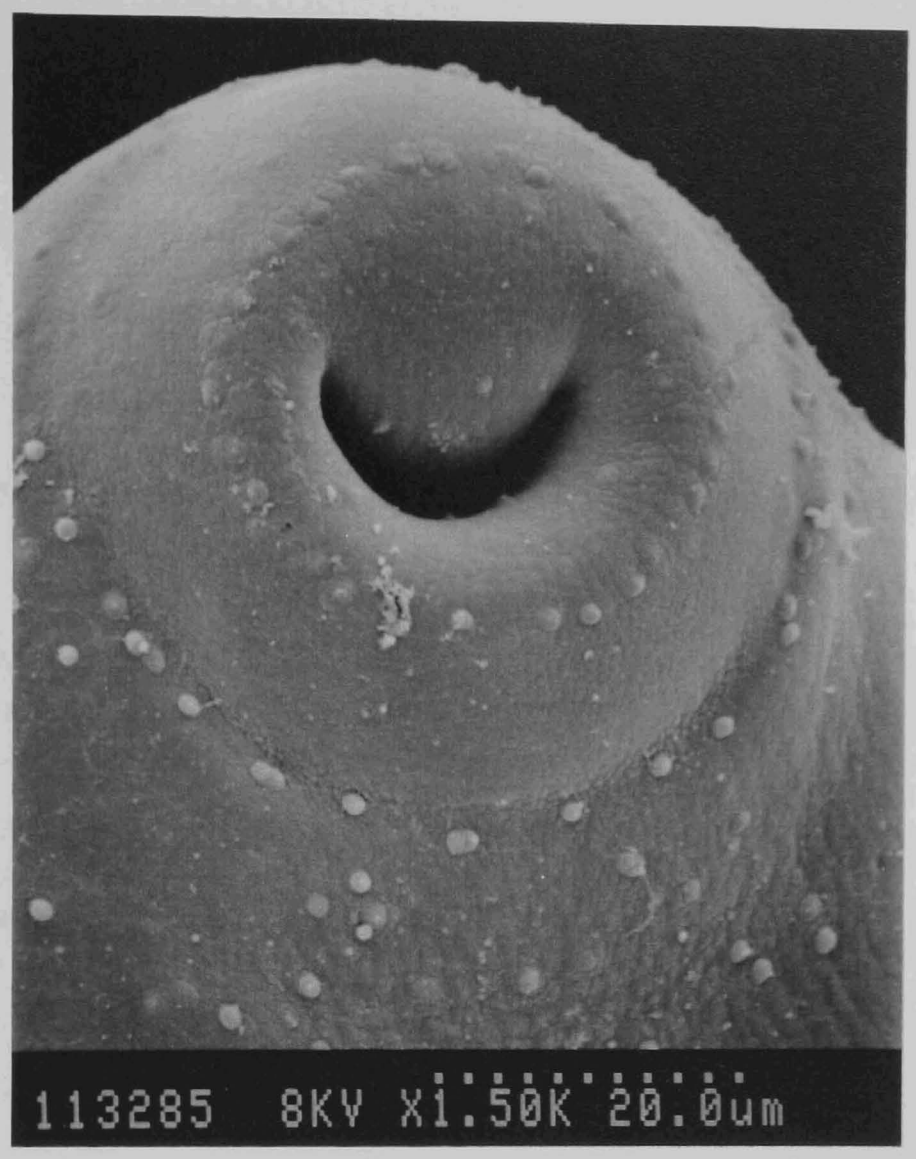
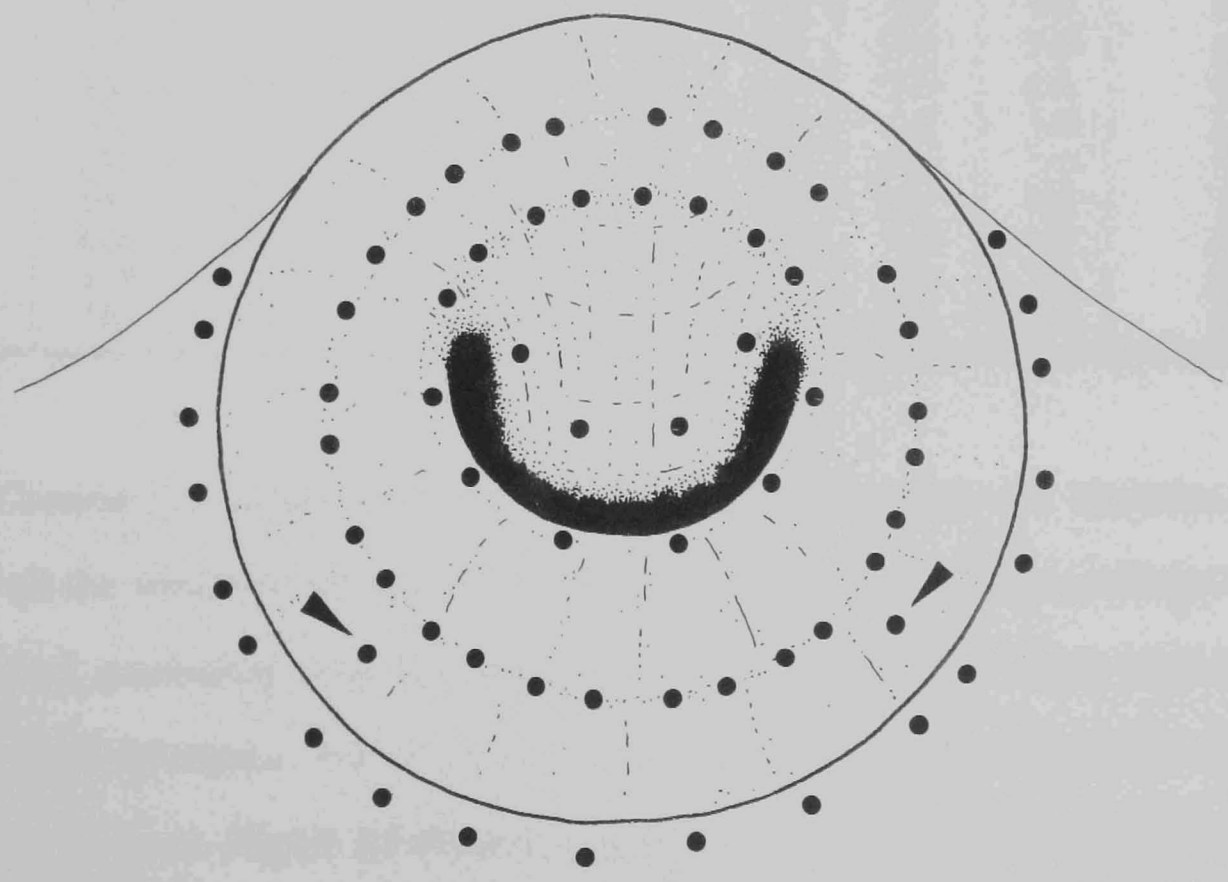


Fig. 22. Drawing indicating the position of *Apatemon* spp. oral sucker and associated sensilla. Arrowheads indicate sensilla not always observed.



2.2.4. Discrimination of metacercariae by principal components analysis of metrical features

1. Discrimination of *Ichthyocotylurus* spp.

Measurements from 327 metacercariae, representing all 4 *Ichthyocotylurus* spp., were incorporated into the analysis. The mean and coefficient of variation for each variable globally and for each species are given in Table 12. The coefficient of variation of each variable was found to be considerably larger globally than for each individual species, indicating substantial species differences in the size of each parameter.

Table 12. Mean and coefficient of variation for each variable (standardised data), globally and in each species (Coefficient of variation = 100 x standard deviation/mean). For an explanation of abbreviations see Fig. 2.

Variable	All *Ichthyocotylurus* spp. combined (n=327)		*I. erraticus* (n=128)		*I. variegatus* (n=162)		*I. pileatus* (n=37)		*I. platycephalus* (n=1)
	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Value
BL	5.92	5.47	5.83	2.69	6.13	3.15	5.26	1.92	6.81
BB	5.66	5.64	5.61	2.80	5.84	4.09	5.03	2.60	6.39
OSL	4.00	4.83	3.95	2.28	4.13	2.69	3.62	2.29	4.82
OSB	4.02	5.92	3.97	3.73	4.16	3.67	4.36	3.09	4.84
VSL	4.10	7.29	3.99	3.73	4.31	4.04	3.54	2.35	5.18
VSB	4.33	6.86	4.25	2.82	4.54	4.26	3.74	2.11	5.26
VSA	5.25	6.25	5.15	3.15	5.46	3.77	4.62	2.97	6.51
FL	4.10	8.61	3.95	4.10	4.38	4.04	3.45	5.54	4.33
LL	4.47	8.46	4.26	3.22	4.79	3.15	3.79	4.51	4.96
DL	4.68	6.11	4.64	3.90	4.83	4.08	4.13	3.29	5.43
PL	3.08	7.05	3.03	4.89	3.21	4.39	2.70	3.74	3.89
PB	2.91	10.21	2.92	10.45	3.02	7.65	2.52	5.59	3.71
TCOL	4.85	5.84	4.78	2.95	5.03	3.72	4.30	4.05	5.15
TCOB	4.33	5.28	4.87	3.39	4.99	3.65	4.36	2.82	4.97
TCOP	3.93	17.12	3.72	10.86	4.37	9.09	2.70	17.74	5.31

Correlations between the 15 variables, shown in Table 14, illustrate how the sizes of all the structures co-vary in the same direction, with a long specimen typically being broad, possessing long and broad internal structures and having large distances between these structures. The first 2 principal components account for 85.2% (Table 13) of the total variance. Figure 23 illustrates the plot of these components and demonstrates how Factor 1 (PCA1) readily discriminates between the 4 species. All variables,

particularly the body, oral and ventral sucker dimensions, and the length of the lateral lappets, contribute to PCA1, as revealed by their high component loadings in Table 13.

Table 13. Principal components analysis of the correlations between the 15 variables.

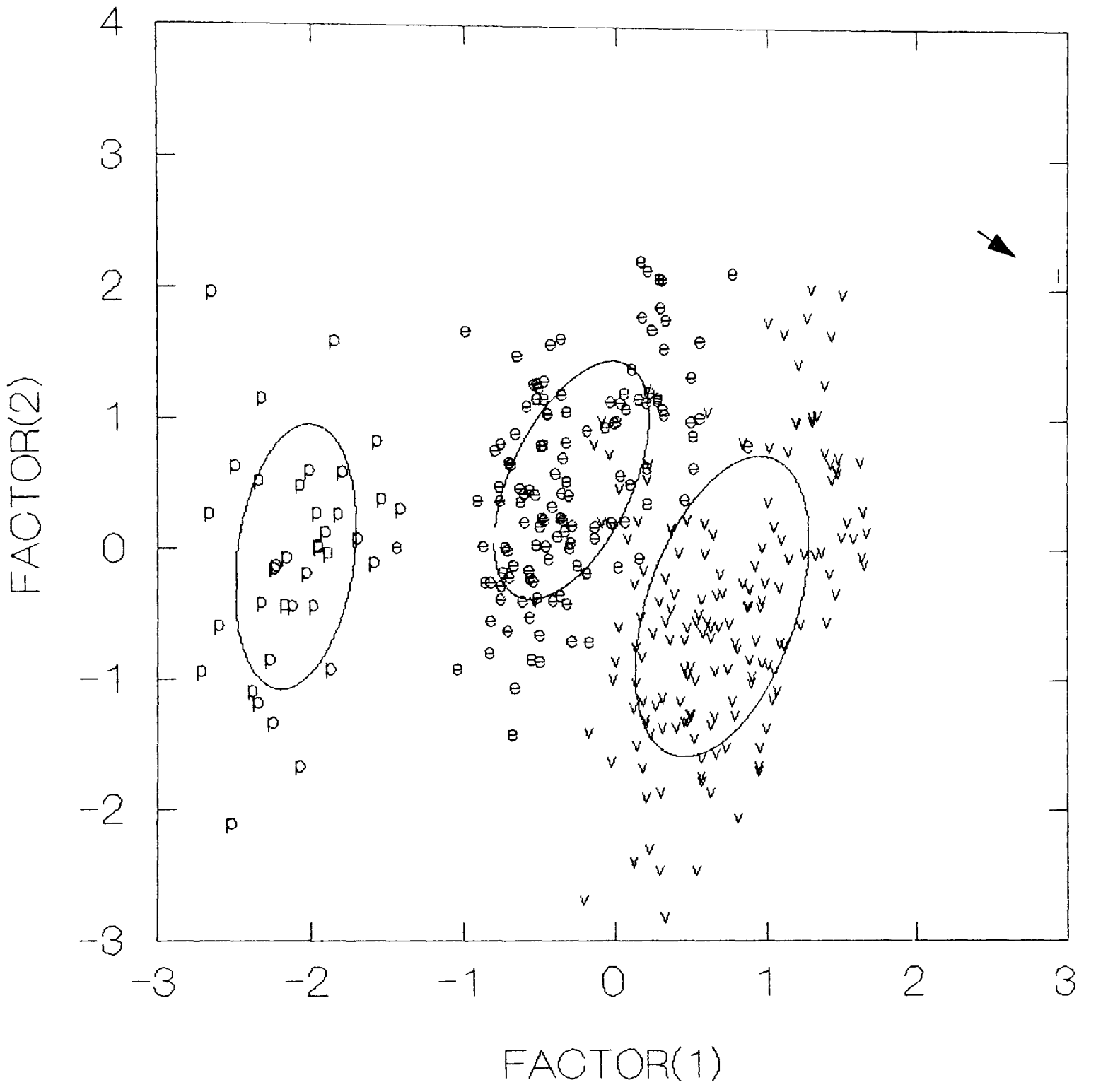
Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	12.24	0.54	0.49	0.38
Proportion (%)	81.61	3.61	3.25	2.53
Cumulative (%)	81.61	85.22	88.47	91.00

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
BL	0.951	-0.130
BB	0.933	0.083
OSL	0.901	-0.030
OSB	0.940	0.178
VSL	0.941	-0.015
VSB	0.952	0.025
VSA	0.894	-0.233
FL	0.897	-0.274
LL	0.903	-0.268
DL	0.894	0.217
PL	0.856	0.092
PB	0.859	0.330
TCOL	0.896	0.040
TCOB	0.897	0.231
TCOP	0.828	-0.251

Table 14. Correlation matrix of the 15 variables.

	BL	BB	OSL	OSB	VSL	VSB	VSA	FL	LL	DL	PL	PB	TCOL	TCOB	TCOP
BL	1.000														
BB	0.926	1.000													
OSL	0.835	0.773	1.000												
OSB	0.853	0.889	0.835	1.000											
VSL	0.886	0.853	0.867	0.881	1.000										
VSB	0.895	0.868	0.869	0.908	0.934	1.000									
VSA	0.953	0.855	0.809	0.774	0.833	0.817	1.000								
FL	0.845	0.805	0.786	0.811	0.823	0.844	0.793	1.000							
LL	0.839	0.796	0.813	0.811	0.844	0.860	0.794	0.932	1.000						
DL	0.827	0.878	0.744	0.872	0.829	0.842	0.756	0.734	0.757	1.000					
PL	0.765	0.721	0.845	0.816	0.793	0.817	0.723	0.743	0.753	0.721	1.000				
PB	0.737	0.784	0.770	0.865	0.774	0.811	0.652	0.716	0.735	0.794	0.781	1.000			
TCOL	0.863	0.838	0.772	0.795	0.858	0.855	0.805	0.789	0.794	0.792	0.727	0.736	1.000		
TCOB	0.848	0.894	0.765	0.858	0.812	0.821	0.779	0.746	0.715	0.835	0.727	0.797	0.846	1.000	
TCOP	0.793	0.737	0.714	0.756	0.749	0.744	0.756	0.776	0.794	0.717	0.674	0.692	0.652	0.702	1.000

Fig. 23. Map of the 327 *Ichthyocotylurus* specimens collected from Scottish, Welsh and Finnish fish as shown in the first plane of the principal components analysis. *I. erraticus* specimens are represented by (e), *I. variegatus* by (v), *I. pileatus* by (p) and *I. platycephalus* by (l). Ellipses surround 50% of points.



2. Discrimination of *I. erraticus* metacercariae from different hosts and geographically distant samples.

The initial analysis was performed on 128 metacercariae excised from Scottish powan, Scottish rainbow trout, Finnish whitefish and Finnish vendace. Table 15 shows the mean and coefficient of variation of each variable both globally and for each sample group.

Table 15. Mean and coefficient of variation for each variable (standardised data), globally and in each sample. For an explanation of abbreviations see Fig. 2.

Variable	All *I. erraticus* specimens (n=128)		Scottish powan (n=43)		Scottish rainbow trout (n=15)		Finnish whitefish (n=47)		Finnish vendace (n=23)	
	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V
BL	5.83	2.69	5.97	2.09	5.86	2.27	5.72	1.35	5.80	2.64
BB	5.61	2.80	5.76	2.21	5.59	2.09	5.51	1.25	5.57	2.68
OSL	3.95	2.28	4.00	2.18	3.93	1.63	3.95	1.98	3.87	1.76
OSB	3.97	3.73	4.13	2.35	3.88	2.55	3.87	2.43	3.91	2.00
VSL	3.99	3.73	4.13	2.54	3.97	2.67	3.90	3.03	3.92	2.40
VSB	4.25	2.82	4.37	2.06	4.26	2.04	4.18	1.36	4.15	1.59
VSA	5.15	3.15	5.25	3.22	5.18	2.59	5.06	1.86	5.13	3.37
FL	3.95	4.10	4.04	3.19	4.04	3.34	3.84	3.83	3.94	3.15
LL	4.26	3.22	4.32	3.43	4.24	3.40	4.20	2.45	4.26	3.01
DL	4.64	3.90	4.81	3.16	4.57	3.50	4.55	2.57	4.57	3.04
PL	3.03	4.89	3.15	4.98	2.93	3.86	2.99	2.84	2.94	3.23
PB	2.92	10.45	2.97	3.27	2.78	3.63	2.84	2.68	2.85	2.32
TCOL	4.78	2.95	4.87	2.51	4.84	2.87	4.71	2.36	4.71	2.72
TCOB	4.87	3.39	4.37	2.45	4.94	2.67	4.76	1.72	4.76	2.46
TCOP	3.72	10.86	3.89	9.05	3.63	13.77	3.56	11.57	3.81	7.03

As in the analysis involving all 4 *Ichthyocotylurus* spp. the variables were found to be positively correlated. The first 2 principal components account for 64.3% (Table 16) of the total variance. Sample separations described by these components according to geographical origin and host species are indicated in Figures 24 and 25, respectively. Fig. 24 suggests that there is morphological variation between *I. erraticus* metacercariae from Scotland and Finland. However, when these 2 populations are examined by host (Fig. 25), it is revealed that specimens from Scottish powan differ from Finnish *Coregonus* spp., while Scottish rainbow trout material share similarities with both groups. Plotting the relative positions of the variables acting on the first 2 principal components (Fig. 26) indicates visually their importance in these separations. The first

principal component (PCA1) discriminates using predominantly width-related variables (body breadth [BB], oral sucker breadth [OSB], ventral sucker breadth [VSB], tribocytic organ breadth [TCOB] and distance between the lateral lappets [DL]), with the second principal component (PCA2) opposing oral sucker length [OSL] at the bottom, to the distance from tribocytic organ to posterior body extremity [TCOP] at the top. Consequently, metacercariae excised from Scottish powan were isolated by PCA1 due to their broader bodies and internal organs (Table 15). A subsequent analysis between the 43 Scottish powan and 15 Scottish rainbow trout specimens indicated a similar separation to that shown in Fig. 25, with discrimination still determined by the larger width dimensions of the former. When the 70 *I. erraticus* specimens excised from the 2 Finnish *Coregonus* spp. were considered separately no clear separation was noted. Nevertheless, a proportion of the whitefish metacercariae were observed to be shorter than the vendace and remaining whitefish specimens, with a more anteriorly placed ventral sucker and tribocytic organ.

Table 16. Principal components analysis of the correlations between the 15 variables.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	8.32	1.32	1.18	0.88
Proportion (%)	55.45	8.78	7.84	5.84
Cumulative (%)	55.45	64.24	72.08	77.92

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
BL	0.858	0.153
BB	0.913	0.069
OSL	0.591	-0.530
OSB	0.876	0.013
VSL	0.775	-0.245
VSB	0.865	-0.094
VSA	0.707	0.073
FL	0.590	0.325
LL	0.549	0.325
DL	0.831	0.086
PL	0.708	-0.243
PB	0.713	0.058
TCOL	0.693	-0.319
TCOB	0.877	-0.087
TCOP	0.426	0.738

Fig. 24. Map of the 128 *I. erraticus* specimens in the first plane of the principal components analysis. Scottish specimens are represented by (S) and Finnish by (F). Ellipses surround 50% of points.

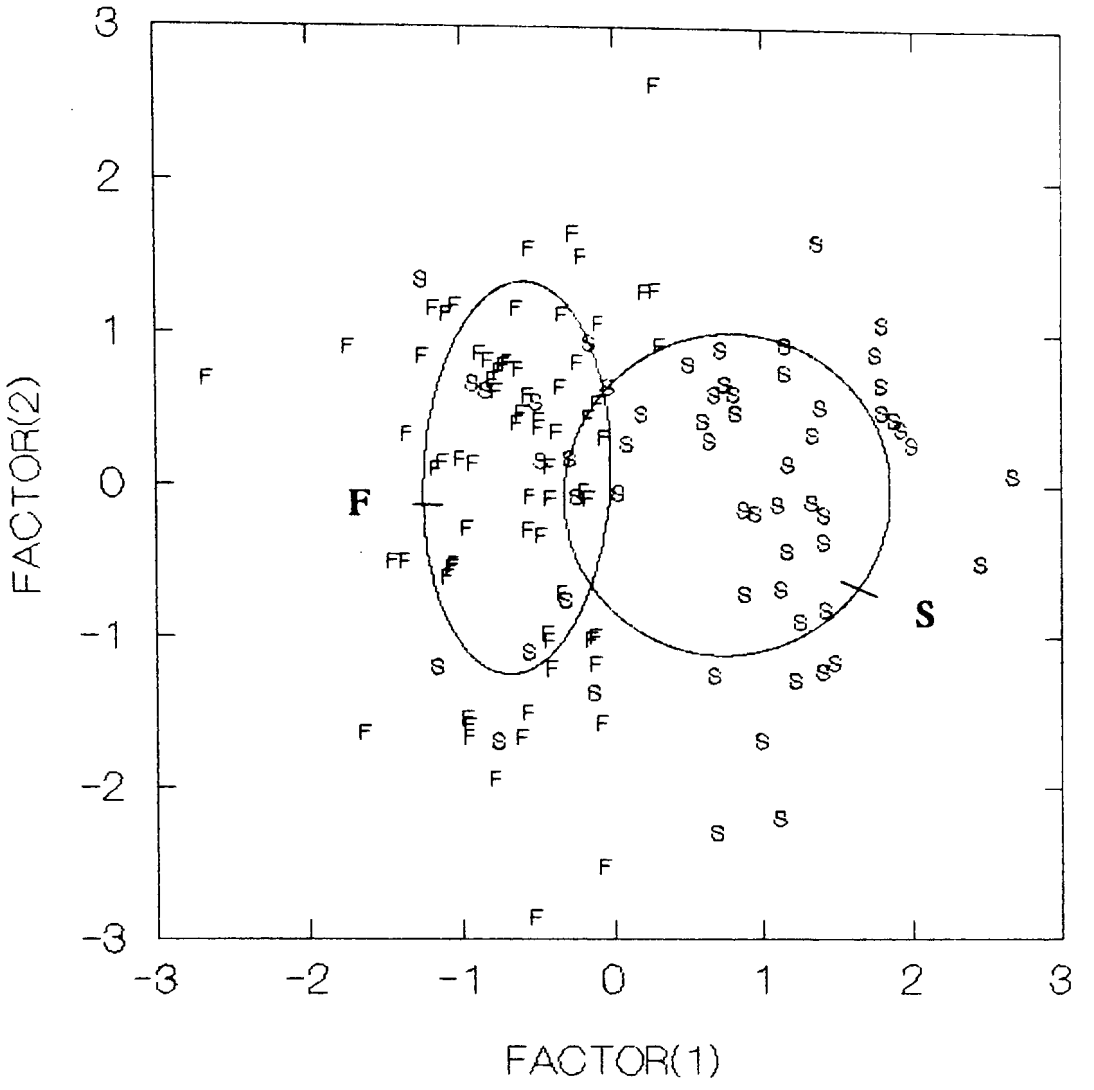


Fig. 25. Map of the 128 *I. erraticus* specimens in the first plane of the principal components analysis. Scottish powan specimens are represented by (p), Scottish rainbow trout by (r), Finnish whitefish by (w) and Finnish vendace by (v). Ellipses surround 50% of points.

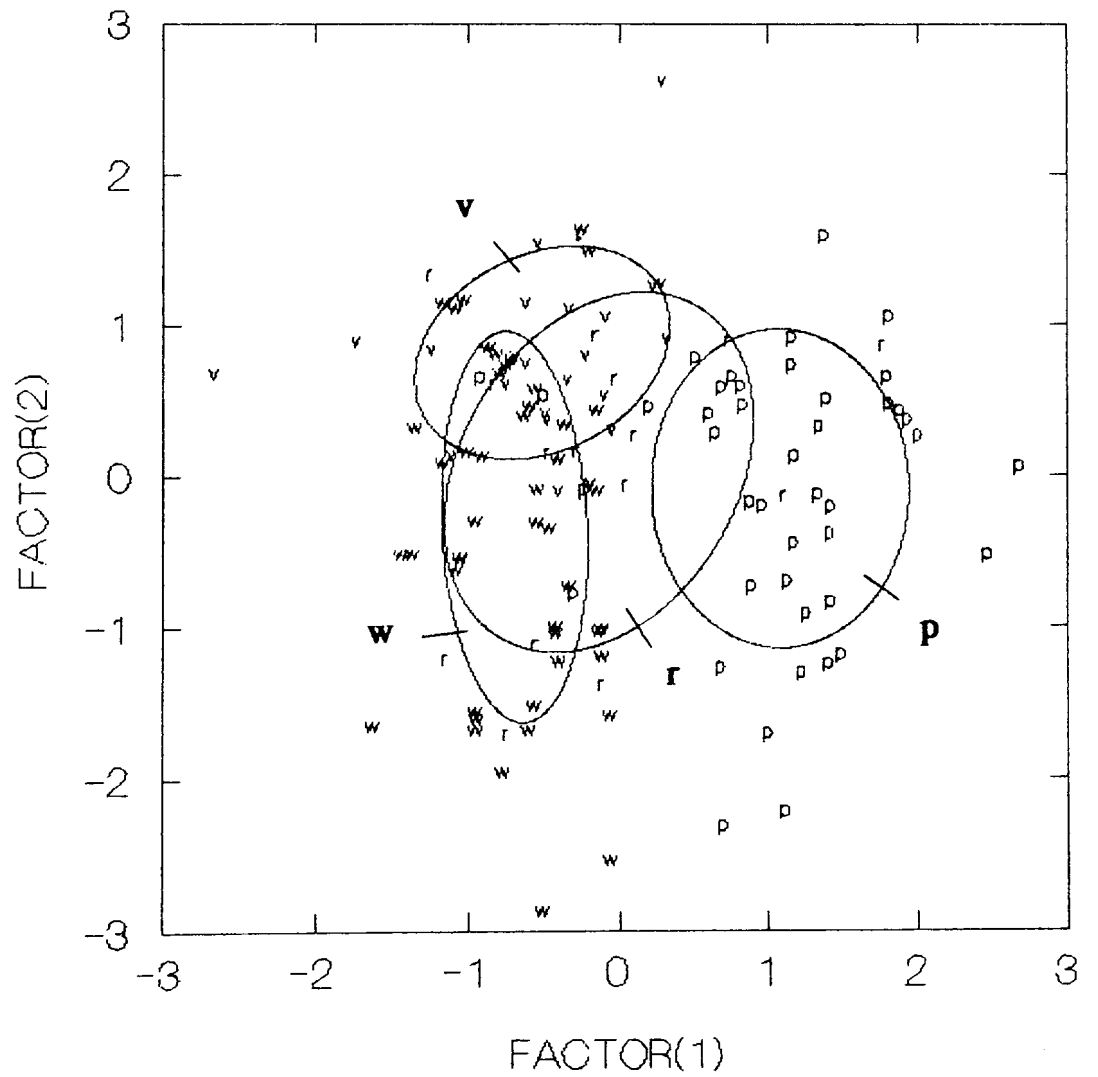
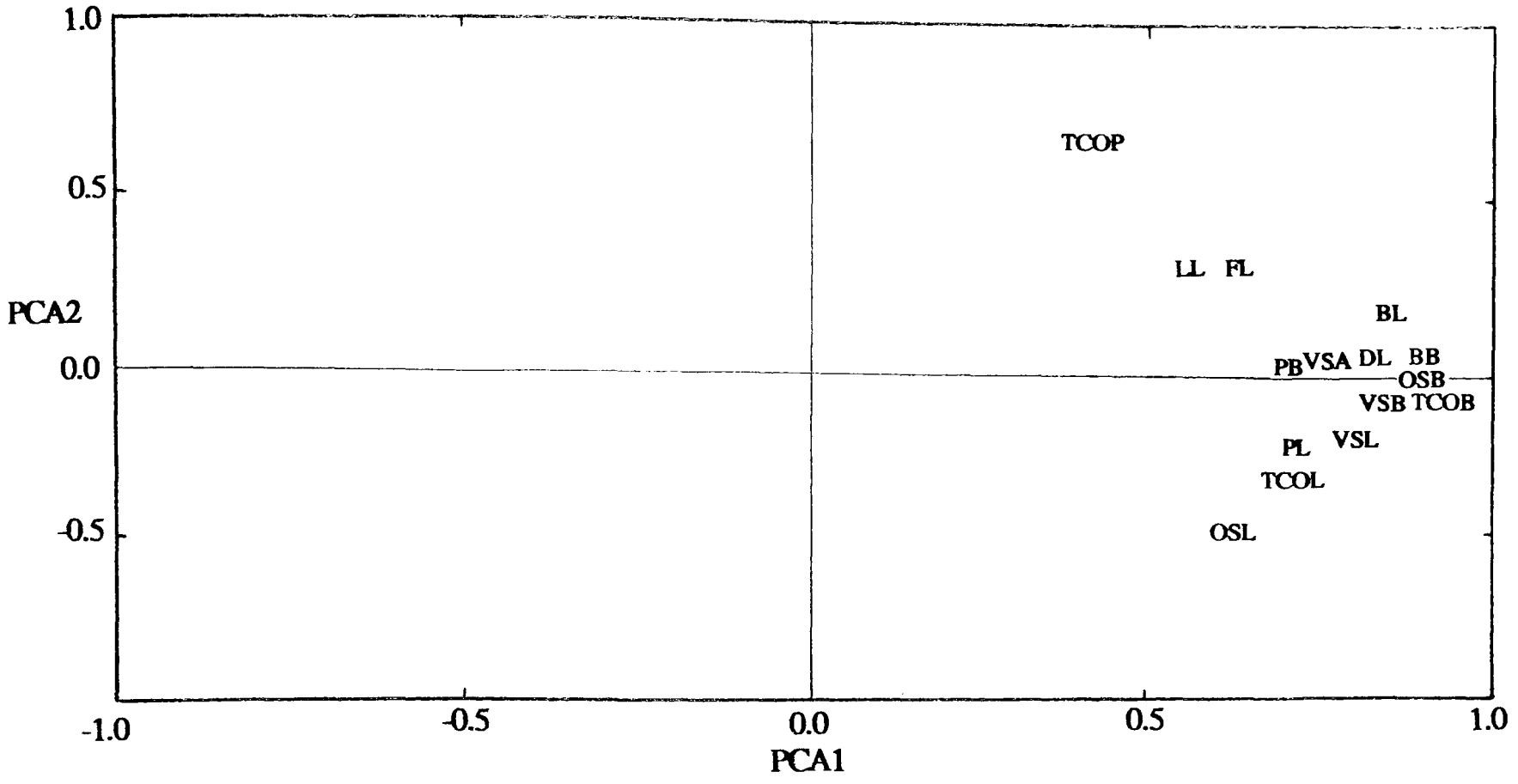


Fig. 26. Map of the 15 variables in the first plane of the principal components analysis on 128 *I. erraticus* specimens.



3. Discrimination of *I. variegatus* metacercariae from different hosts, different sites within a single host and geographically distant sources.

The initial PCA was performed on 162 specimens excised from Scottish perch, Scottish ruffe, Finnish perch and Finnish ruffe. The mean and coefficient of variation of each variable, both globally and for each sample, are provided in Table 17. Again, as with the 2 previous PCA analyses, all variables were found to be positively correlated. Table 18 shows that the first 2 principal components account for 61% of the total variance. The spatial plots described by these components are given in Figs 28, 29, 30. Clear separations, indicated by the ellipses surrounding 50% of designated points, were not obtained according to metacercarial source (Scotland and Finland, Fig. 28) or host (perch and ruffe, Fig. 29). However, when source and host of the metacercariae were considered together, as shown in Fig. 30, several discrete groupings were recorded, with PCA1 isolating Scottish perch metacercariae from those of both Finnish percids. This separation is emphasised by the imposition of 95% confidence limits on the centroids (Fig. 30). Fig. 27 demonstrated that oral sucker breadth [OSB], ventral sucker dimensions [VSB and VSL], tribocytic organ breadth [TCOB] and body dimensions [BB and BL] contributed most to PCA1, and reference to the raw data (summarised in Table 17) revealed that the discrimination was due to these dimensions being slightly larger for Scottish perch specimens.

Table 17. Mean and coefficient of variation for each variable (standardised data), globally and in each sample. For an explanation of abbreviations see Fig. 2.

Variable	All *I. variegatus* specimens (n=162)		Scottish perch specimens (n=20)		Scottish ruffe specimens (n=93)		Finnish perch specimens (n=32)		Finnish ruffe specimens (n=17)	
	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V
BL	6.13	3.15	6.39	1.59	6.13	3.64	6.09	1.72	6.01	2.40
BB	5.84	4.09	6.06	1.07	5.81	4.75	5.83	2.35	5.74	2.20
OSL	4.13	2.69	4.20	1.45	4.14	2.78	4.09	1.93	4.04	3.00
OSB	4.16	3.67	4.34	1.75	4.16	3.65	4.11	2.80	4.09	3.25
VSL	4.31	4.04	4.48	2.81	4.31	3.97	4.22	3.10	4.25	4.19
VSB	4.54	4.26	4.68	1.47	4.55	4.42	4.46	2.78	4.42	3.64
VSA	5.46	3.77	5.51	1.96	5.47	4.24	5.46	2.82	5.32	3.48
FL	4.38	4.04	4.37	3.73	4.39	4.47	4.35	3.20	4.36	3.26
LL	4.79	3.15	4.77	1.95	4.81	3.45	4.77	2.39	4.70	3.28
DL	4.83	4.08	5.01	2.60	4.84	3.97	4.75	3.89	4.72	3.60
PL	3.21	4.39	3.32	3.13	3.22	4.91	3.13	2.75	3.18	2.39
PB	3.01	7.65	3.21	2.71	3.00	5.00	2.93	4.06	2.97	5.35
TCOL	5.03	3.72	5.24	1.93	5.03	3.74	4.95	2.75	4.93	3.08
TCOB	4.99	3.65	5.23	1.41	4.98	3.62	4.90	2.35	4.96	3.07
TCOP	4.37	9.09	4.62	5.22	4.33	10.05	4.42	5.34	4.23	10.90

Table 18. Principal components analysis of the correlations between the 15 variables.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	7.45	1.70	1.20	0.97
Proportion (%)	49.64	11.32	8.01	6.49
Cumulative (%)	49.64	60.96	68.97	75.46

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
BL	0.811	-0.465
BB	0.853	-0.324
OSL	0.618	0.423
OSB	0.863	0.367
VSL	0.819	0.013
VSB	0.808	0.163
VSA	0.596	-0.616
FL	0.611	-0.912
LL	0.655	0.060
DL	0.697	-0.026
PL	0.502	0.592
PB	0.633	0.535
TCOL	0.688	-0.255
TCOB	0.802	-0.117
TCOP	0.446	0.014

Fig. 27. Map of the 15 variables in the first plane of the principal components analysis on 162 *I. variegatus* specimens.

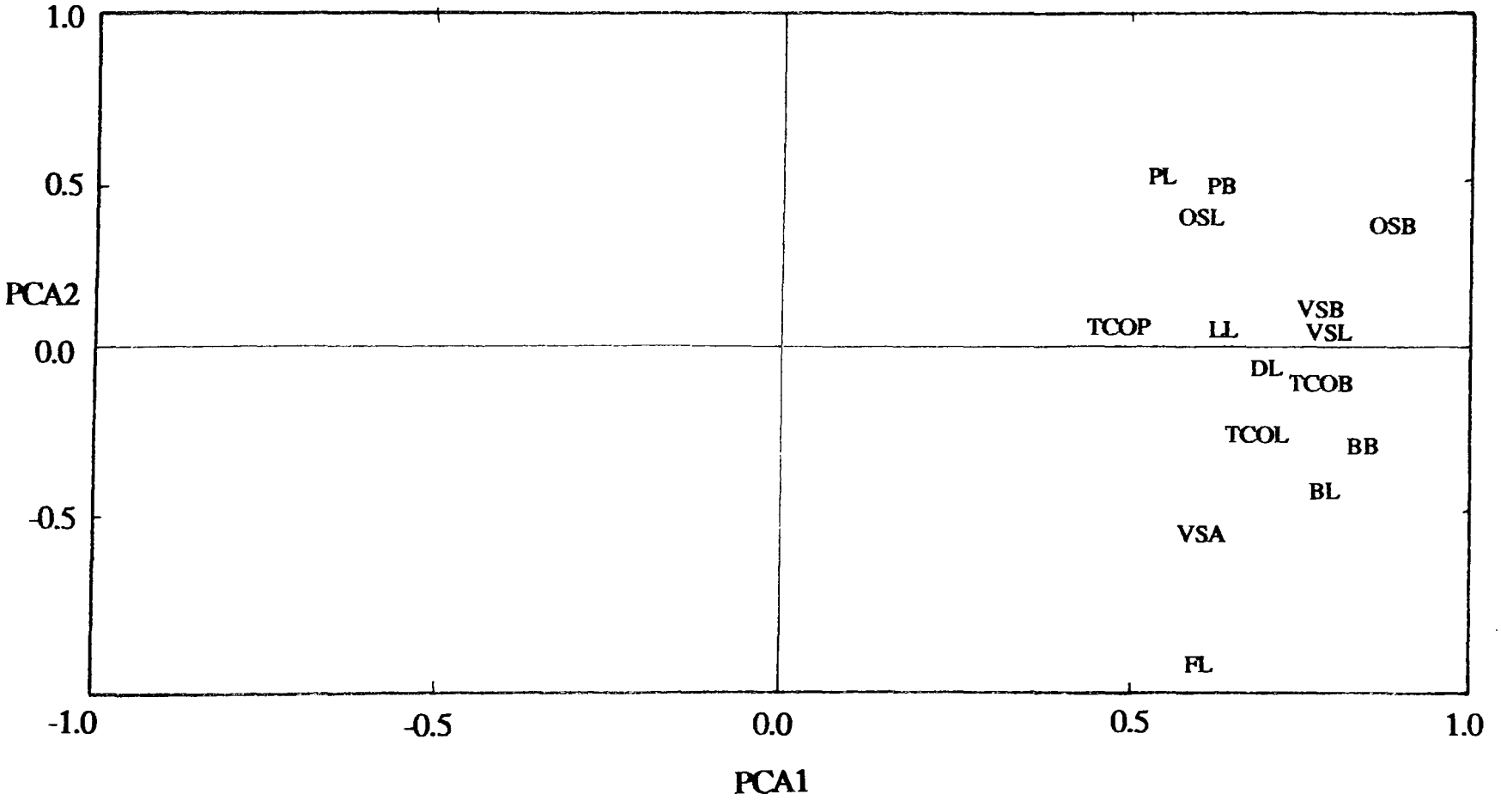


Fig. 28. Map of the 162 *I. variegatus* specimens in the first plane of the principal components analysis. Scottish specimens are represented by (S) and Finnish by (F). Ellipses surround 50% of points.

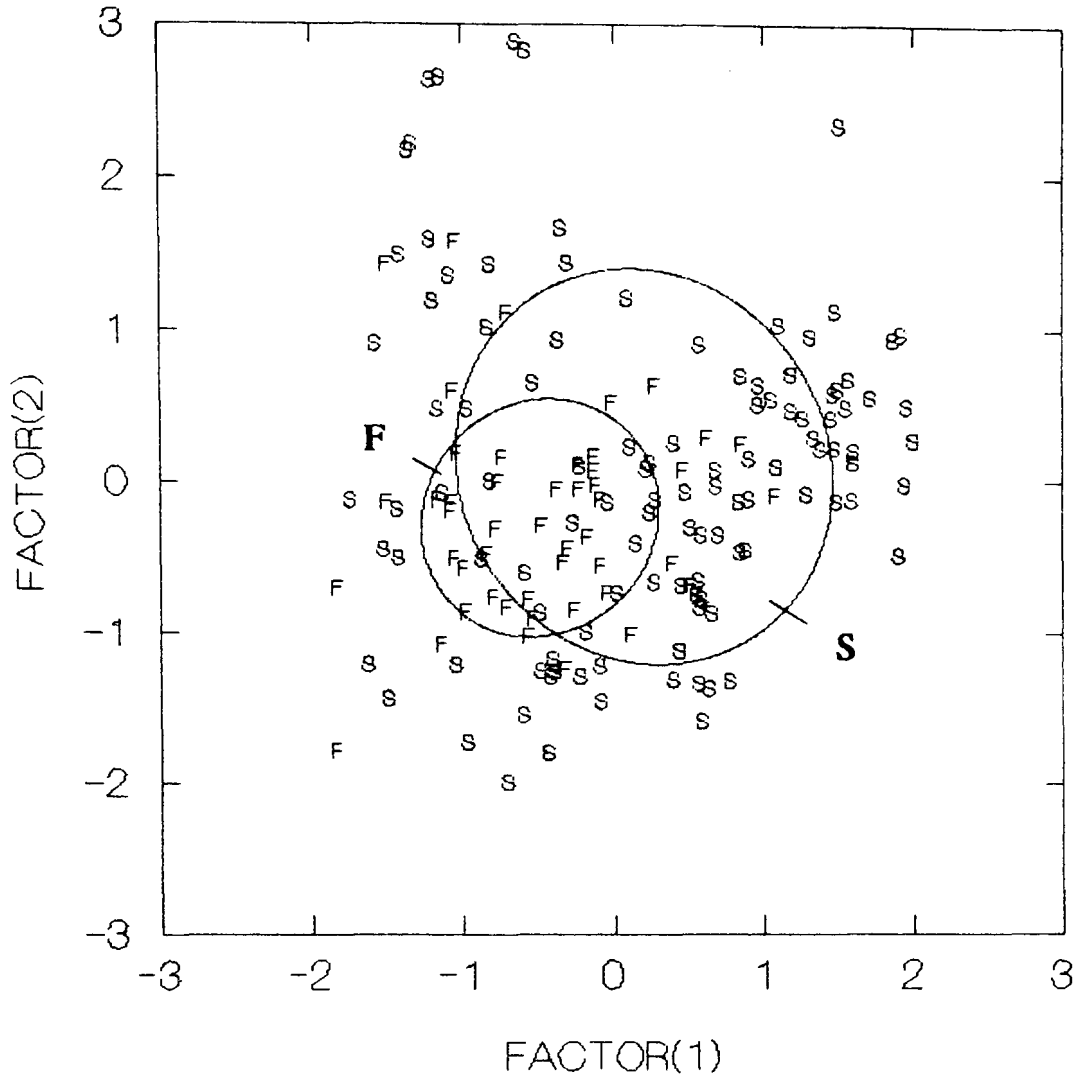


Fig. 29. Map of the 162 *I. variegatus* specimens in the first plane of the principal components analysis. Specimens excised from perch are represented by (P) and ruffe by (R). Ellipses surround 50% of points.

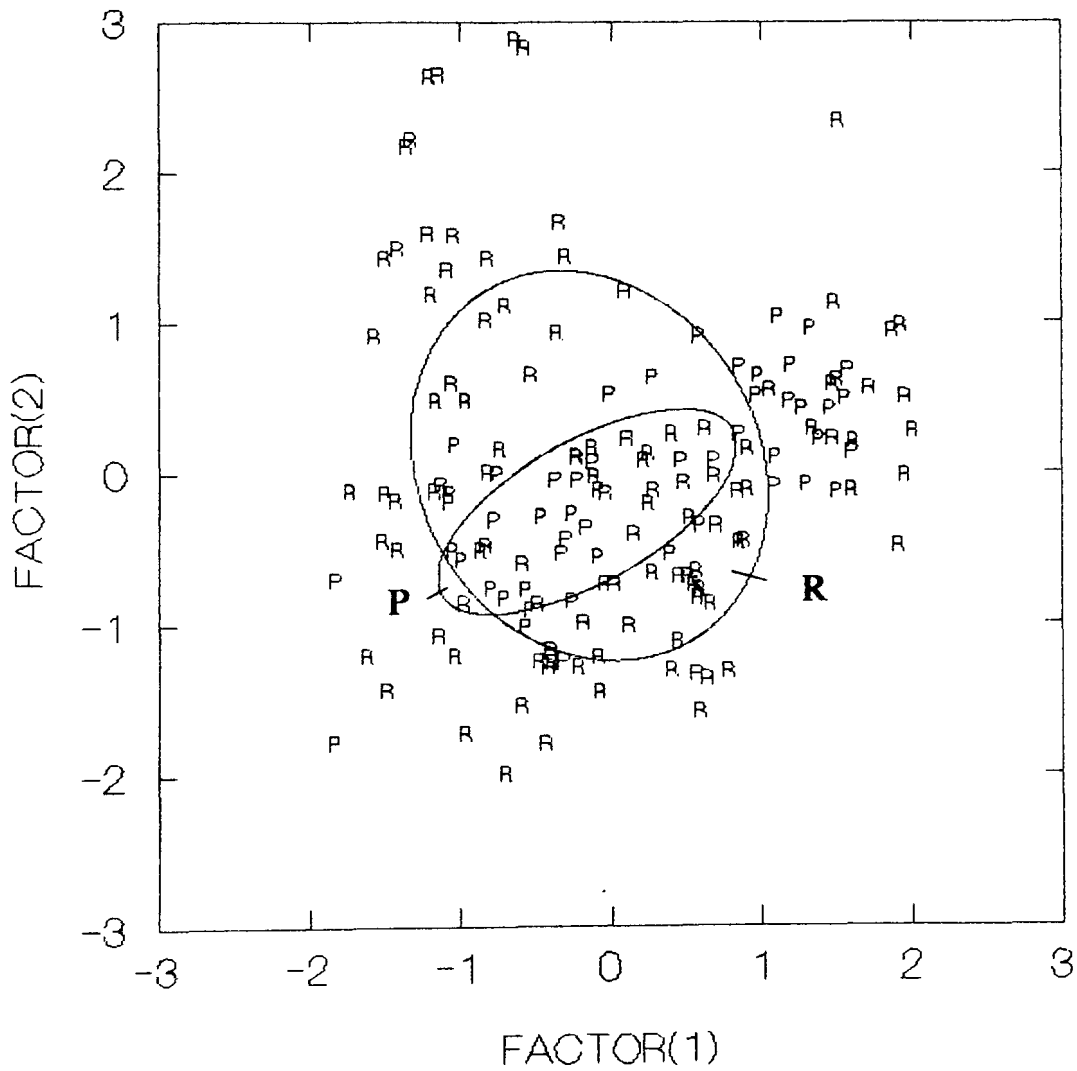
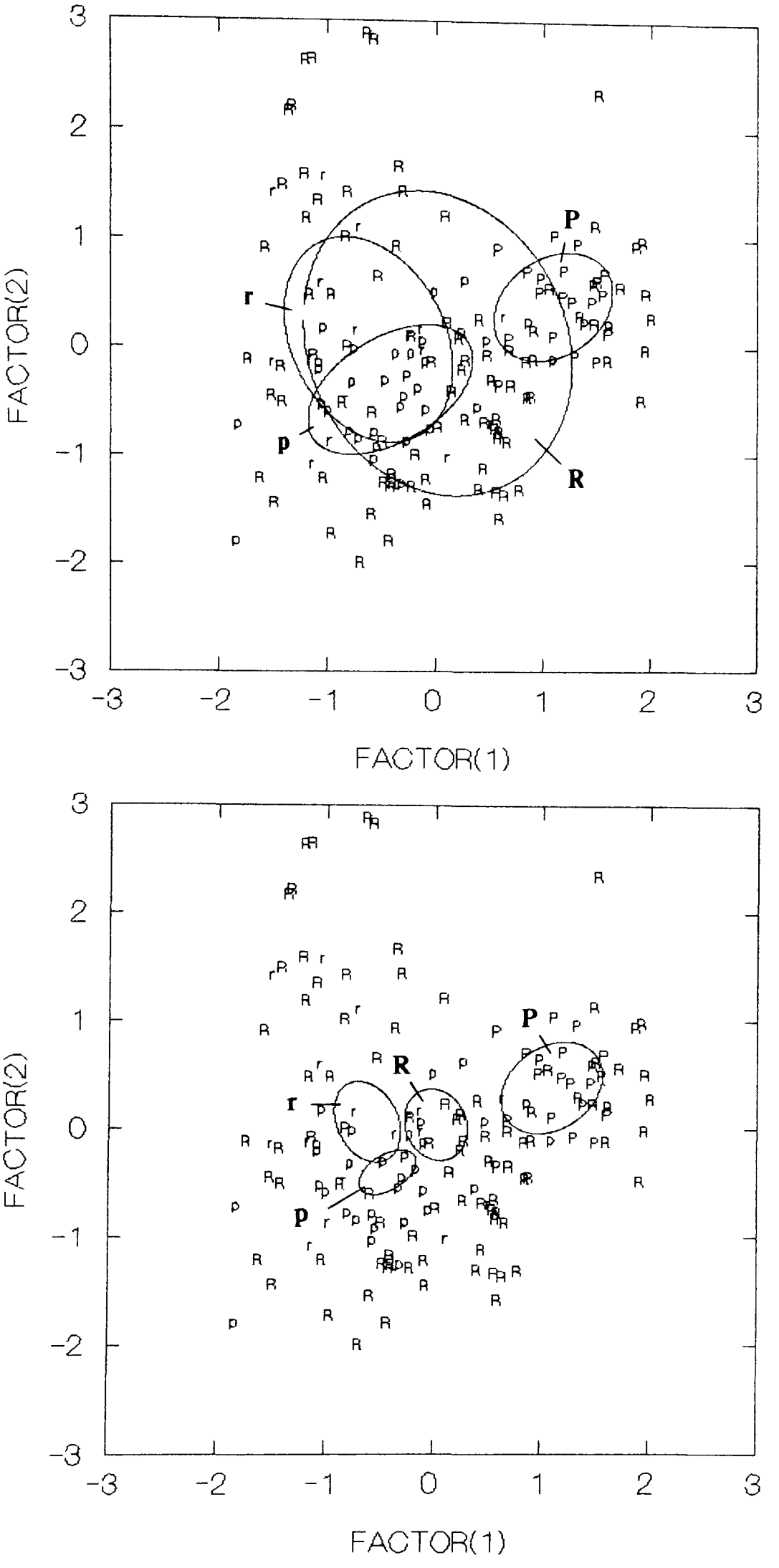


Fig. 30. Map of the 162 *I. variegatus* specimens in the first plane of the principal components analysis. Scottish perch specimens are represented by (P), Scottish ruffe specimens by (R), Finnish perch specimens (p) and Finnish ruffe specimens (r). Ellipses surround 50% of points (top). Ellipses indicate 95% confidence limits (bottom).



A further analysis was performed on the 93 *I. variegatus* specimens collected from 4 different locations (swimbladder, orbit, pericardial cavity and ovary) within a single ruffe. The mean and coefficient of variation of each variable globally and for each site are shown in Table 19.

Table 19. Mean and coefficient of variation for each variable (standardised data), globally and in each sample. For an explanation of abbreviations see Fig. 2.

Variable	All *I. variegatus* specimens (n=93)		Swimbladder (n=47)		Orbit (n=10)		Pericardial cavity (n=20)		Ovary (n=16)	
	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V
BL	6.13	3.64	6.17	3.29	6.20	2.10	6.05	9.12	6.08	4.18
BB	5.81	4.75	5.83	4.31	5.93	2.80	5.76	5.47	5.73	5.83
OSL	4.14	2.78	4.12	2.50	4.10	2.17	4.14	3.09	4.19	3.15
OSB	4.16	3.65	4.15	3.69	4.18	3.37	4.14	2.75	4.19	4.82
VSL	4.31	3.97	4.33	3.44	4.30	4.14	4.23	4.47	4.36	4.29
VSB	4.55	4.42	4.56	2.90	4.51	3.97	4.47	3.98	4.66	7.21
VSA	5.47	4.24	5.52	4.00	5.52	2.34	5.39	4.86	5.36	4.44
FL	4.39	4.47	4.38	4.31	4.43	4.06	4.36	4.91	4.43	3.61
LL	4.81	3.45	4.81	3.20	4.81	3.08	4.80	4.19	4.84	3.62
DL	4.84	3.97	4.84	4.11	4.91	3.16	4.81	4.41	4.84	3.53
PL	3.22	4.88	3.20	4.63	3.16	3.32	3.24	5.62	3.28	5.03
PB	3.00	5.00	3.00	5.00	2.94	6.46	3.04	4.31	3.01	4.95
TCOL	5.03	3.74	5.06	3.00	5.10	3.65	4.92	4.51	5.02	4.02
TCOB	4.55	3.62	5.00	3.44	4.98	2.37	4.93	3.55	4.96	4.76
TCOP	4.33	10.06	4.33	7.83	4.29	13.40	5.39	5.88	4.31	16.84

Here, the first 2 principal components described 64% of the total variance, as shown in Table 20. The plot of these components (Fig. 32) indicates that PCA2 is responsible for the separation of specimens, particularly those removed from the orbit and ovary. The variables contributing mainly to this separation (Fig. 31) are the length of the oral sucker and pharynx dimensions, which are larger in metacercariae from the ovary, position of the ventral sucker, which is situated more posteriorly in specimens from the orbit and whose metacercariae also have a greater distance between their lateral lappets.

Table 20. Principal components analysis of the correlations between the 15 variables.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	7.42	2.17	1.24	0.90
Proportion (%)	49.45	14.48	8.28	6.03
Cumulative (%)	49.45	63.93	71.21	77.24

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
BL	0.841	-0.424
BB	0.858	-0.332
OSL	0.555	0.541
OSB	0.830	0.372
VSL	0.809	-0.041
VSB	0.759	0.207
VSA	0.694	-0.510
FL	0.697	-0.131
LL	0.719	0.094
DL	0.414	0.679
PL	0.560	0.560
PB	0.616	-0.483
TCOL	0.756	-0.250
TCOB	0.379	0.178
TCOP	0.826	0.134

Fig. 31. Map of the 15 variables in the first plane of the principal components analysis on 93 *I. variegatus* specimens excised from a single ruffe.

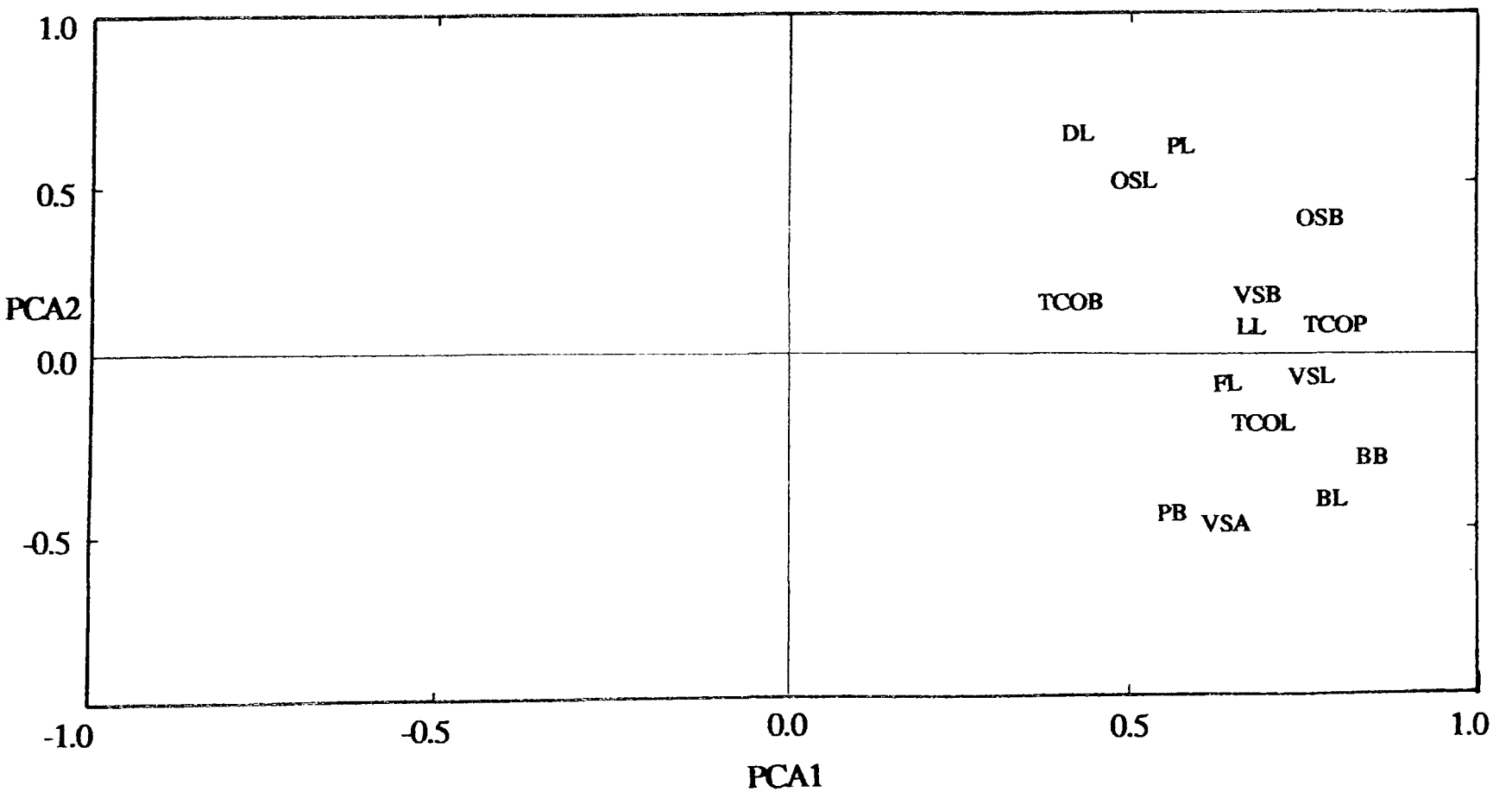
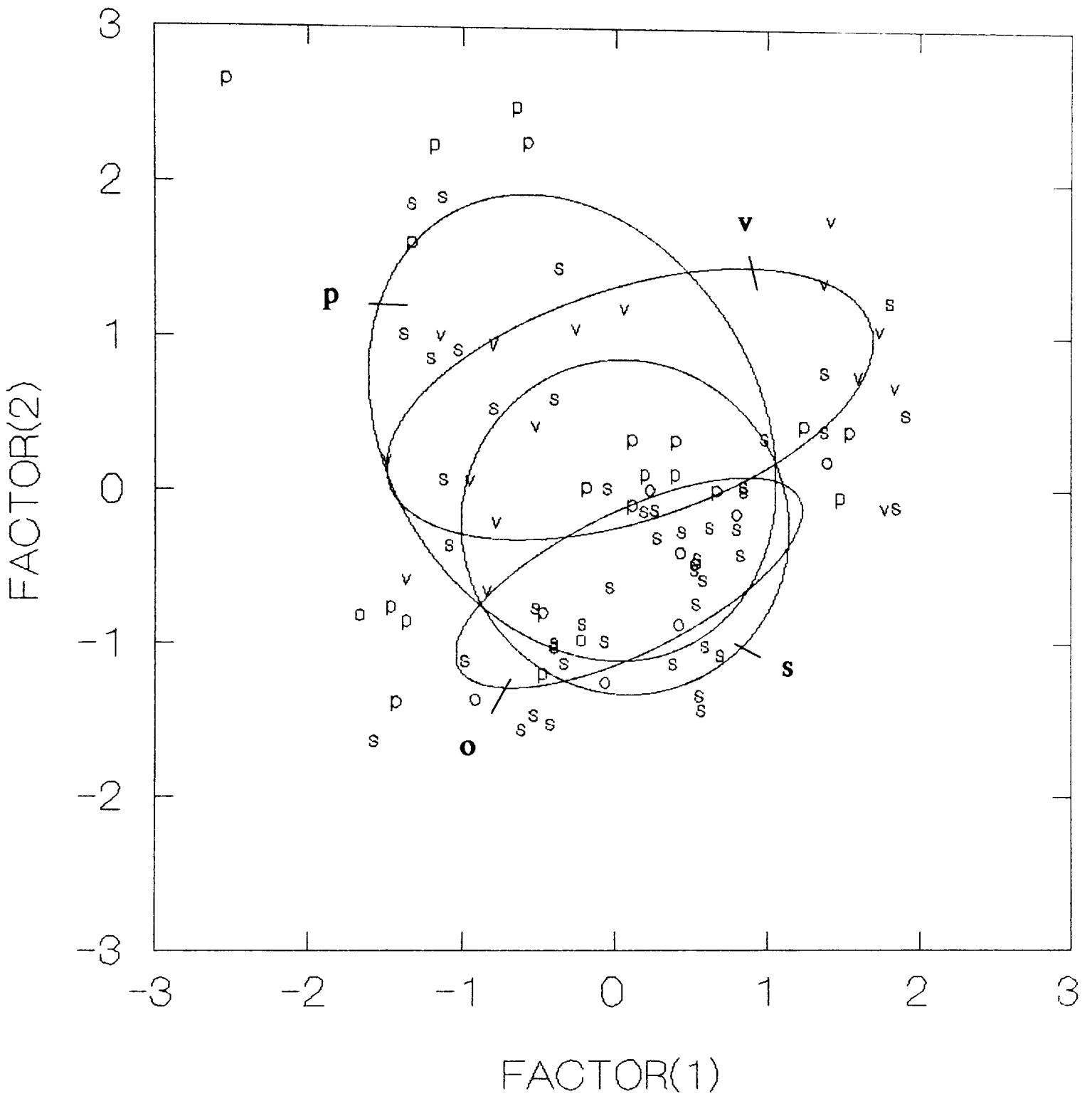


Fig. 32. Map of the 93 *I. variegatus* ruffe specimens in the first plane of the principal components analysis. Swimbladder specimens are represented by (s), orbit specimens by (o), pericardial cavity specimens (p) and ovary specimens (v). Ellipses surround 50% of points.



4. Discrimination of *Apatemon (Apatemon)* metacercariae.

The initial PCA was performed on 160 *A. gracilis* and 33 *A. annuligerum* metacercariae. The *A. gracilis* material was excised from 5 different fish hosts, 1 host being obtained from 2 geographically distant sites; these specimens were either considered as a whole (species) or according to host species and geographical origin (6 individual samples). The mean and coefficient of variation for each variable globally and for each sample are shown in Table 22.

When present, correlations between the variables were found to be positive and are given in Table 23. The first 2 principal components account for 57.4% (Table 21) of the total variation.

Table 21. Principal components analysis of the correlations between the 15 variables.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	6.69	1.91	1.06	0.87
Proportion (%)	44.61	12.76	7.08	5.82
Cumulative (%)	44.61	57.37	64.45	70.27

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
BL	0.793	-0.541
FBL	0.804	-0.453
BB	0.810	-0.037
OSL	0.703	0.010
OSB	0.637	0.083
VSL	0.616	0.432
VSB	0.720	0.349
VSA	0.757	-0.336
FL	0.656	0.350
DL	0.735	-0.260
PL	0.746	0.299
PB	0.481	0.399
PGL	0.494	-0.081
PGB	0.534	0.490
TCOP	0.318	-0.549

Fig. 33 illustrates the plot of these components, with ellipses surrounding 50% of the *A. gracilis* (all sources) and *A. annuligerum* metacercariae. Some separation of the 2

species was observed in this figure, resulting from variables acting in the first principal component. The imposing of 95% confidence limits on the centroids (Fig. 34) made the discrimination more readily visible. Both length and breadth related variables contribute to the separation, in particular, forebody length [FBL], body breadth [BB], total body length [TBL], the distance from ventral sucker to anterior body extremity [VSA], pharynx length [PL] and distance between the lappets [DL], as indicated in Fig 35. *A. gracilis* metacercariae are typically smaller in all of these measurements than *A. annuligerum* (see Table 22).

The same plot is shown in Fig. 36, with an ellipse still incorporating 50% of *A. annuligerum* specimens, but now with 50% of members from each individual *A. gracilis* sample also ellipsed. *A. gracilis* metacercariae excised from salmon parr were the only members of the species completely discrete from *A. annuligerum* specimens and represent specimens with particularly small features relevant to PCA1. Again, applying 95% confidence limits provided a better separation (Fig. 37), with all *A. gracilis* material other than Scottish stone loach specimens discriminated from *A. annuligerum* specimens by PCA1 variables. Scottish stone loach *A. gracilis* were separated from all other samples by possessing small measurements for variables acting on PCA2, including total body length [TBL], ventral sucker and proteolytic gland lengths [VSL and PGL], pharynx breadth [PB] and distance from the tribocytic organ to the posterior extremity of the body [TCOP] (see Fig. 35, Table 22).

The last consideration applied to the plot of these first 2 principal components was the placing of ellipses around 50% of *A. gracilis* specimens excised from salmonid hosts, *A. gracilis* specimens from non-salmonid hosts and *A. annuligerum* specimens. Fig. 38 suggests that *A. gracilis* specimens from non-salmonid hosts have more morphological similarities with *A. annuligerum* than those from salmonid hosts. This was emphasised by running separate PCA's between pairs of the 3 designated groupings and comparing their separation by 95% confidence limits on the centroids. These plots are given in Fig. 39 and show a clear separation for salmonid *A. gracilis* and *A. annuligerum* specimens, a slight separation for salmonid and non-salmonid *A. gracilis* specimens, and an overlap in groupings for non-salmonid *A. gracilis* and *A. annuligerum* specimens.

Table 22. Mean and coefficient of variation for each variable (standardised data), globally and in each sample group. For an explanation of abbreviations see Fig. 2

Variable	Both species specimens (n=193)		*A. gracilis* specimens														*A. annuligerum* specimens (n=33)	
			All *A. gracilis* specimens (n=160)		Rainbow trout specimens (n=43)		Salmon parr specimens (n=33)		Arctic char specimens (n=3)		Scottish stone loach specimens (n=22)		Welsh stone loach specimens (n=18)		Bullhead specimens (n=41)			
	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V
TBL	6.58	2.30	6.56	1.83	6.61	1.88	6.48	1.42	6.68	1.65	6.55	1.68	6.56	2.00	6.59	1.65	6.68	3.47
FBL	6.32	2.41	6.30	2.02	6.34	2.38	6.22	1.69	6.40	1.19	6.31	1.46	6.28	1.58	6.33	1.79	6.41	3.48
BB	5.57	3.07	5.55	2.88	5.62	2.03	5.37	1.73	5.61	2.78	5.62	1.96	5.50	2.96	5.61	2.51	5.65	3.56
OSL	4.43	2.05	4.41	1.86	4.42	1.86	4.36	1.31	4.43	1.63	4.42	1.86	4.40	0.77	4.45	2.20	4.94	2.18
OSB	4.45	2.90	4.44	2.91	4.44	2.07	4.34	1.64	4.44	1.78	4.45	2.49	4.39	2.19	4.55	3.30	4.49	2.67
VSL	4.66	3.69	4.65	3.61	4.70	2.96	4.52	1.88	4.71	0.74	4.77	3.82	4.61	2.39	4.64	4.18	4.75	3.47
VSB	4.84	2.62	4.82	2.53	4.87	2.16	4.72	1.34	4.84	0.89	4.94	2.41	4.77	2.91	4.81	1.87	4.93	2.31
VSA	5.80	2.95	5.78	2.63	5.82	3.35	5.68	1.92	5.84	1.70	5.84	2.28	5.79	1.57	5.80	2.22	5.90	3.70
FL	4.51	3.81	4.49	3.81	4.52	4.56	4.34	2.60	4.46	1.03	4.66	1.91	4.43	2.12	4.51	2.93	4.60	3.15
DL	4.96	3.99	4.94	3.50	5.03	3.06	4.79	2.46	4.90	4.98	4.89	4.03	4.96	2.84	4.97	3.10	5.09	4.95
PL	3.56	3.40	3.54	3.14	3.54	2.66	3.45	1.71	3.56	3.26	3.64	2.91	3.49	2.58	3.59	2.90	3.62	4.12
PB	3.37	3.09	3.36	3.07	3.33	2.52	3.34	1.92	3.40	2.82	3.50	2.74	3.30	3.27	3.37	2.61	3.40	3.12
PGL	3.87	4.96	3.87	4.94	4.28	4.40	3.79	3.54	3.90	4.05	3.90	6.15	3.77	3.34	3.97	5.11	3.88	5.05
PGB	4.27	4.82	4.26	5.02	4.42	4.56	4.10	4.20	4.24	3.51	4.38	5.55	4.18	3.80	4.35	4.28	4.31	3.83
TCOP	5.32	4.02	5.33	3.83	5.40	3.06	5.24	3.26	5.56	4.03	5.28	4.58	5.28	3.88	5.36	3.88	5.27	4.88

Table 23. Correlation matrix of the 15 variables. High positive correlations are shown in bold.

	TBL	BB	FBL	OSL	OSB	VSL	VSB	VSA	FL	DL	PL	PB	PGL	PGB	TCOP
TBL	1.000														
BB	**0.944**	1.000													
FBL	**0.613**	**0.606**	1.000												
OSL	0.520	0.511	0.495	1.000											
OSB	0.438	0.451	0.466	0.412	1.000										
VSL	0.260	0.299	0.466	0.374	0.332	1.000									
VSB	0.410	0.422	**0.626**	0.400	0.397	**0.623**	1.000								
VSA	**0.809**	**0.836**	0.456	0.502	0.380	0.377	0.371	1.000							
FL	0.323	0.366	0.497	0.448	0.369	0.492	0.503	0.411	1.000						
DL	**0.682**	**0.676**	**0.671**	0.452	0.502	0.323	0.452	0.569	0.391	1.000					
PL	0.414	0.412	0.565	0.580	0.472	0.467	0.557	0.451	0.528	0.431	1.000				
PB	0.198	0.248	0.306	0.314	0.279	0.390	0.423	0.263	0.374	0.133	0.511	1.000			
PGL	0.371	0.352	0.418	0.331	0.285	0.231	0.225	0.316	0.324	0.184	0.364	0.300	1.000		
PGB	0.175	0.235	0.391	0.337	0.384	0.466	0.552	0.261	0.440	0.286	0.485	0.226	0.149	1.000	
TCOP	0.480	0.326	0.338	0.188	0.074	0.048	0.131	0.257	0.002	0.243	0.126	-0.010	0.299	-0.036	1.000

Fig. 33. Map of the 193 *Apatemon* specimens in the first plane of the principal components analysis. *A. gracilis* specimens are represented by (g) and *A. annuligerum* by (a). Ellipses surround 50% of points.

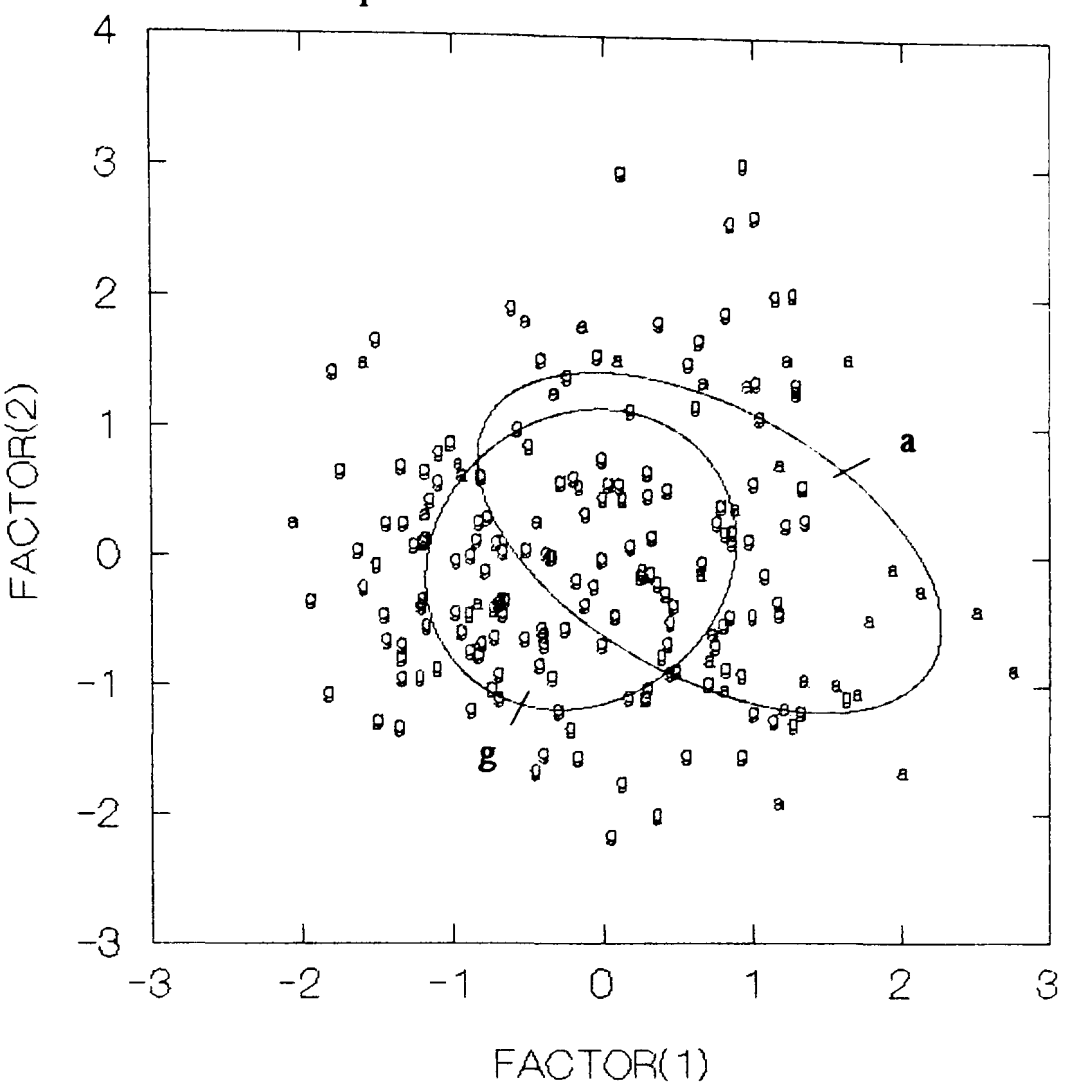


Fig. 34. Map of the 193 *Apatemon* specimens in the first plane of the principal components analysis. *A. gracilis* specimens are represented by (g) and *A. annuligerum* by (a). Ellipses indicate 95% confidence limits.

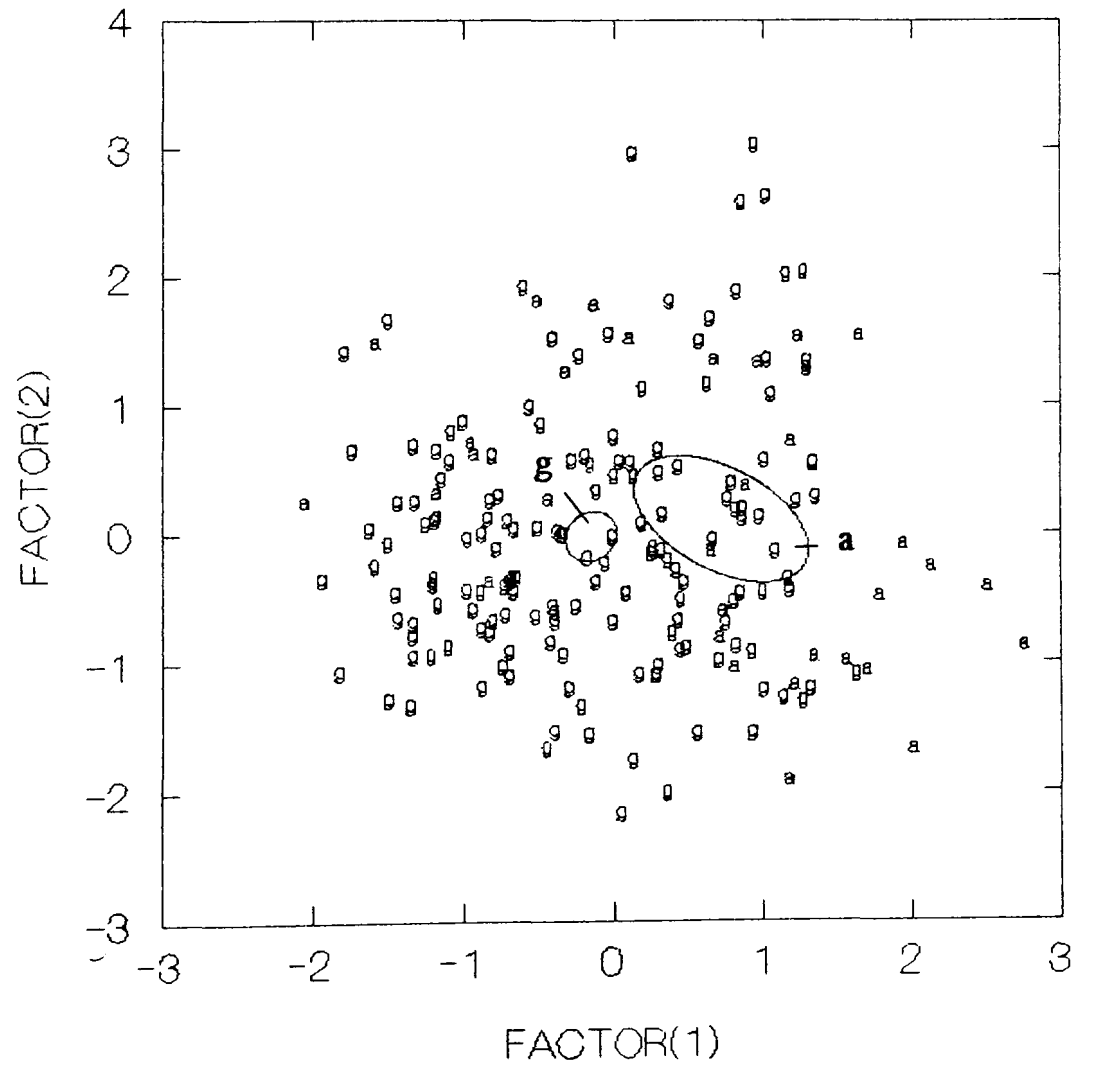


Fig. 35. Map of the 15 variables in the first plane of the principal components analysis on 193 *Apatemon (Apatemon)* specimens.

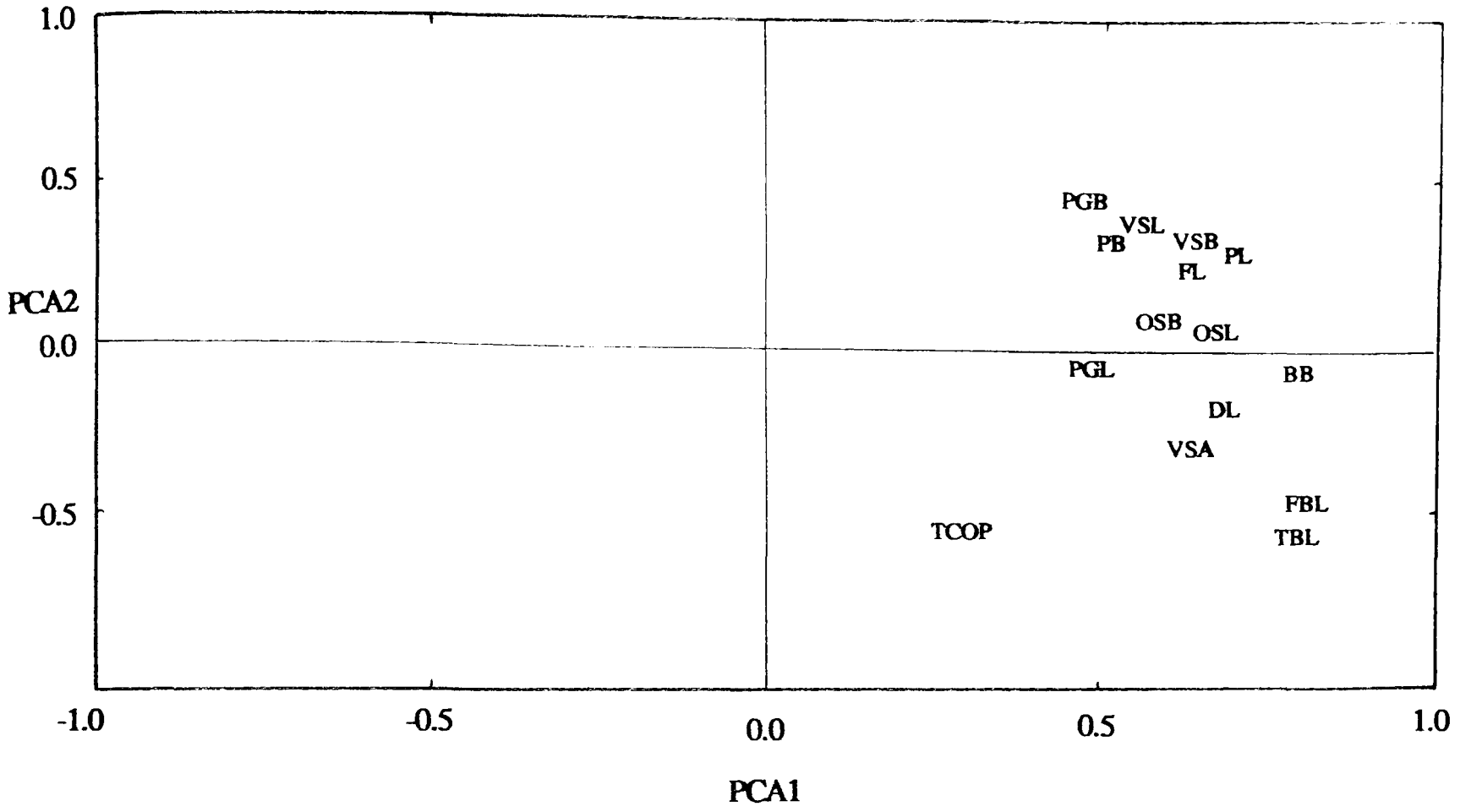


Fig. 36. Map of the 193 *Apatemon (Apatemon)* metacercariae in the first plane of the principal components analysis. *A. gracilis* specimens excised from rainbow trout are represented by (r), those from salmon parr (s), Scottish stone loach (l), Welsh stone loach (w), bullheads (b), charr (c) and *A. annuligerum* specimens by (a). Ellipses surround 50% of specimens.

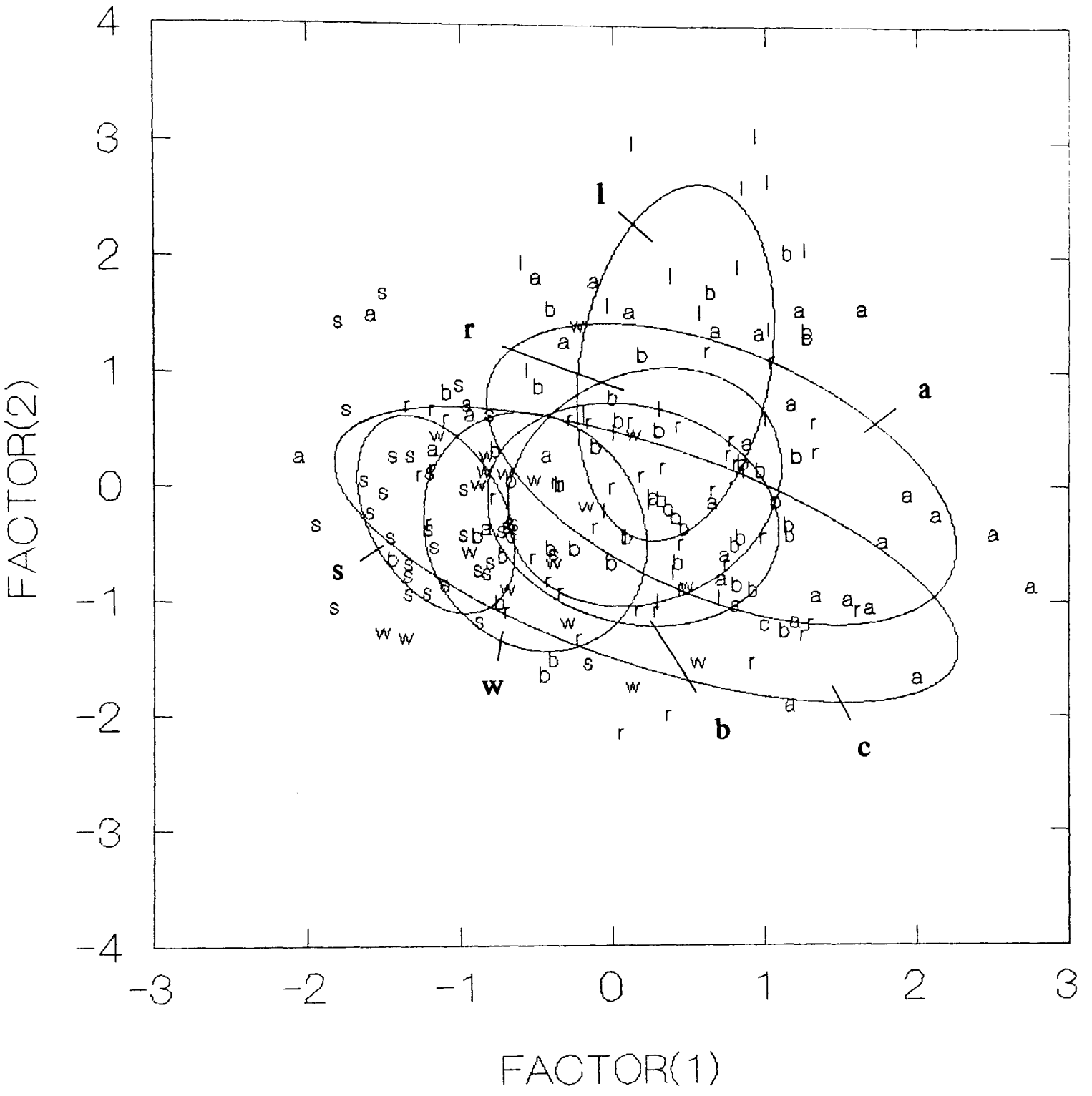


Fig. 37. Map of the 193 *Apatemon (Apatemon)* metacercariae in the first plane of the principal components analysis. *A. gracilis* specimens excised from rainbow trout are represented by (r), those from salmon parr (s), Scottish stone loach (l), Welsh stone loach (w), bullheads (b) and *A. annuligerum* specimens by (a). Ellipses indicate 95% confidence limits.

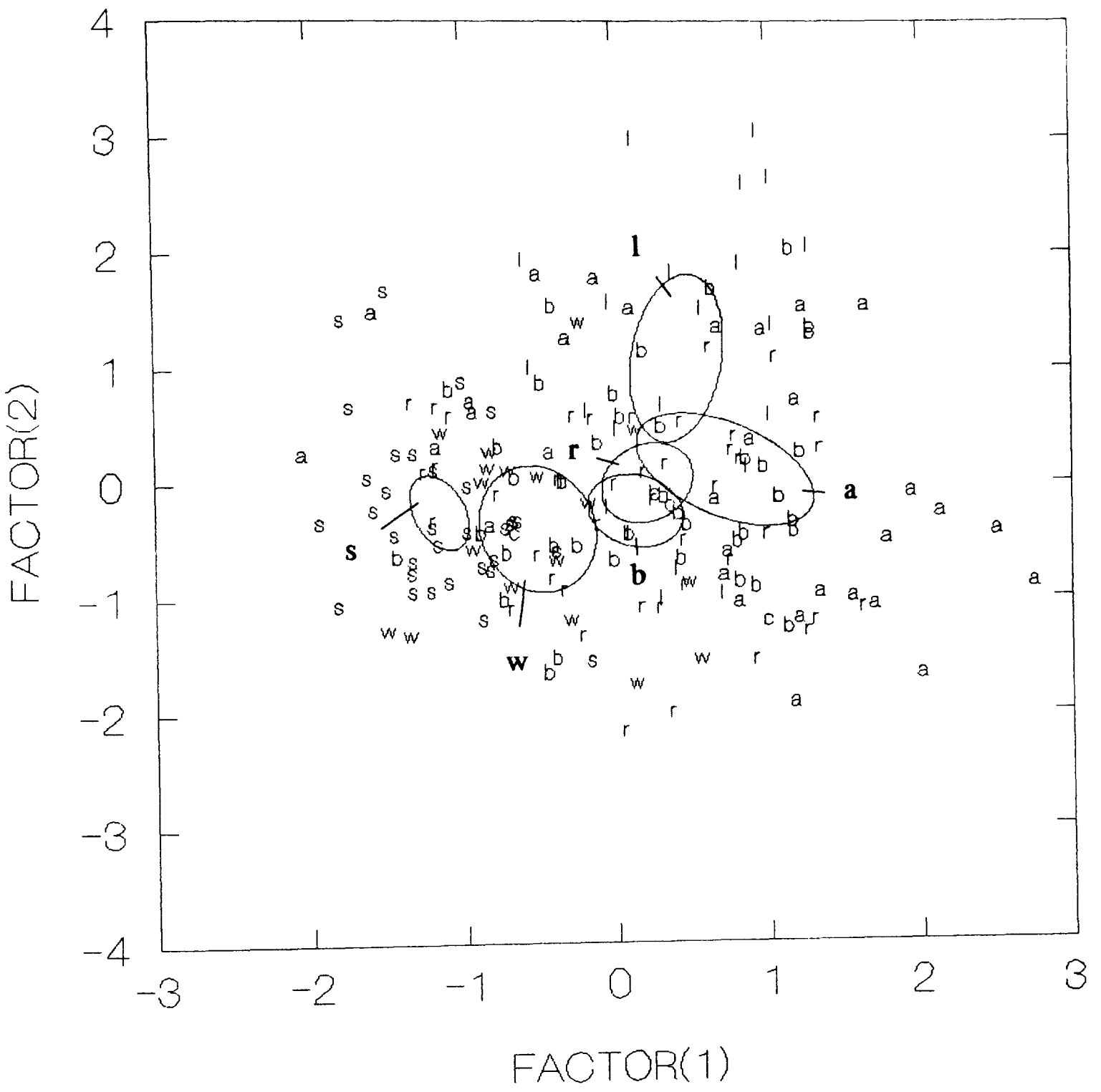


Fig. 38. Map of the 193 *Apatemon (Apatemon)* metacercariae in the first plane of the principal components analysis. Specimens excised from salmonids are represented by (s), those from non-salmonids (n) and *A. annuligerum* specimens by (a). Ellipses surround 50% of points.

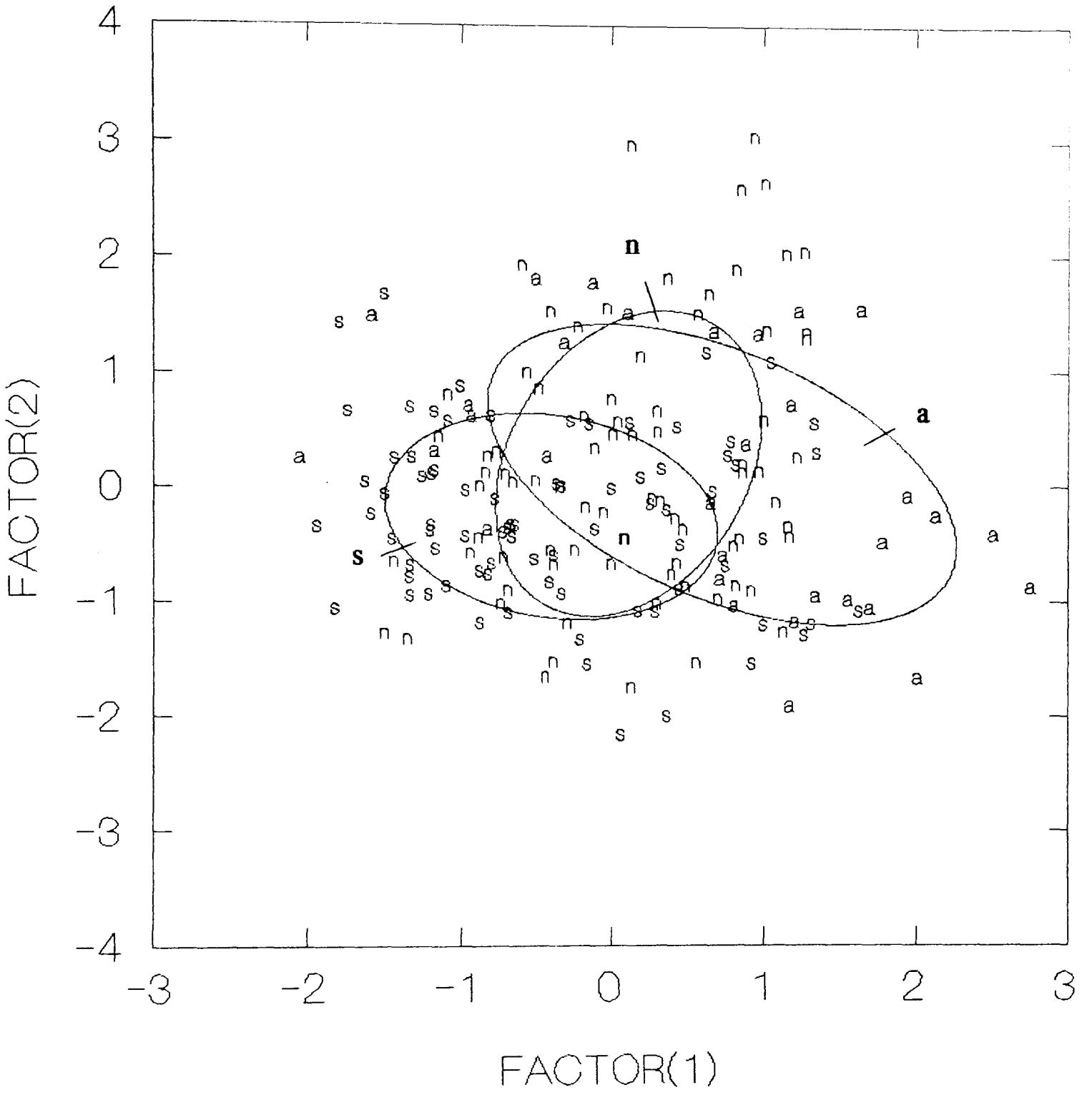
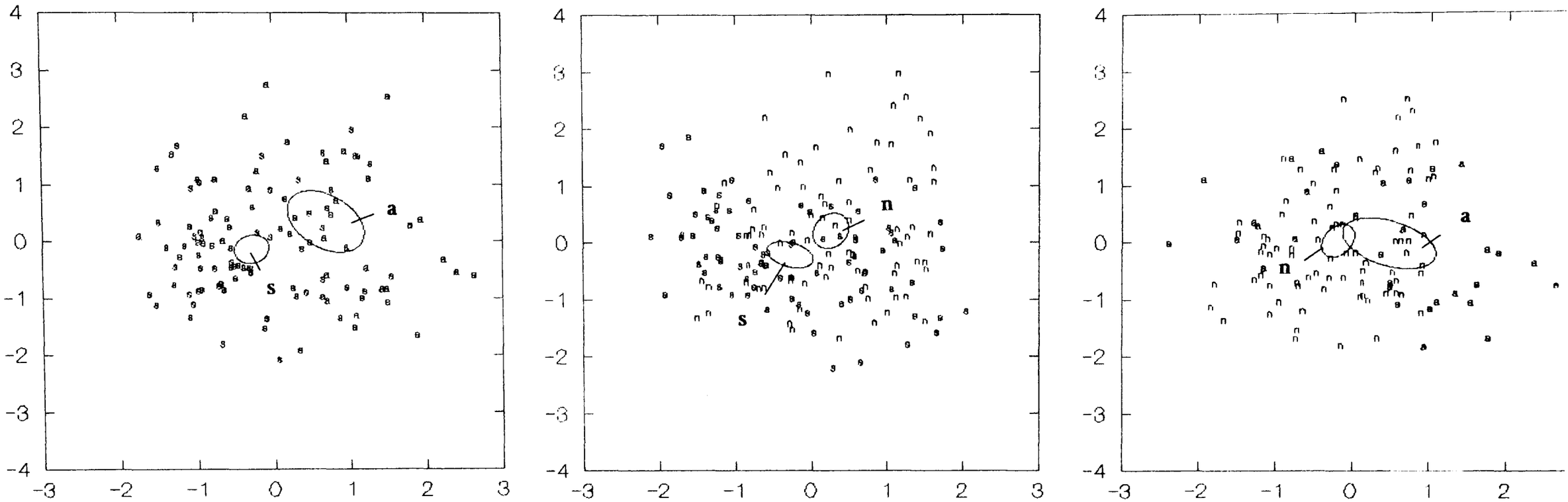


Fig. 39. Maps indicating the separation of *Apatemon (Apatemon)* metacercariae in the first plane of the principal components analysis. Map 1, 79 *A. gracilis* specimens excised from salmonid hosts (s) and 33 *A. annuligerum* specimens (a). Map 2, 79 *A. gracilis* specimens excised from salmonid hosts (s) and 81 *A. gracilis* specimens excised from non-salmonid hosts (n). Map 3, 81 *A. gracilis* specimens excised from non-salmonid hosts (n) and 33 *A. annuligerum* specimens (a). PCA1 acts along the x-axis and PCA2 along the y-axis. Ellipses indicate 95% confidence limits on the centroids.



Individual comparisons of morphological variation between *A. gracilis* metacercariae excised from different hosts were performed against *A. annuligerum* specimens by running further PCAs; similar comparisons were made between individual groups of *A. gracilis* metacercariae. Details of the 11 analyses would require a large amount of space, consequently only summaries are provided of the separating features for each, Tables 24 and 25, respectively.

Table 24. Summary of variables contributing most to the separation of *A. gracilis* specimens of different hosts from *A. annuligerum* specimens. Information compiled from Eigenvector value plots for individual PCA analyses between members of the 2 species.

Variables (in order of importance)	*A. gracilis* hosts								
	Salmonids				Non-salmonids				
	All salmonid specimens (separation by 2 PCA axes)		Rainbow trout	Salmon parr	All non-salmonid specimens	Scottish stone loach (separation by 2 PCA axes)		Welsh stone loach	Bullheads
1	TBL	PGB	TCOP	TBL	FBL	PGL	PGL	TBL	PGL
2	OSB	VSB	OSL	DL	BB	VSL	PB	FBL	TCOP
3	FBL	FBL	PGL	BB	TBL	TCOP	TCOP	VSA	DL
4	DL	TBL	PB	FBL	VSA	VSB	DL	OSL	PL
5	BB	VSL	PGB	OSB	PL	TBL	FL	DL	VSB

Table 25. Summary of variables contributing most to the separation of *A. gracilis* specimens excised from different hosts. Information compiled from Eigenvector value plots for individual PCA analyses between sample members.

A. gracilis hosts	All salmonid and non-salmonid specimens	Salmon parr and rainbow trout specimens	Salmon parr and Scottish stone loach specimens	Rainbow trout and Scottish stone loach specimens
Variables*				
1	BB	OSB	VSB	TBL
2	FBL	TBL	BB	TCOP
3	TBL	BB	PL	PL
4	PL	FBL	PGL	PB
5	VSA	DL	OSB	VSL

*variables: in order of importance.

DISCUSSION

2.1. NATURAL INFECTIONS.

I. erraticus (Tables 1 and 2).

I. erraticus infections were recorded from Scottish and Welsh *Coregonus* spp.: Loch Lomond powan and Llyn Tegid gwyniad *C. lavaretus* (L.). Prevalence of infection was 100% for powan and burdens extremely heavy, with more than 120 metacercariae being recovered from each fish. Cysts were clustered together on the ventricle, mainly at its apex and often several deep. Lower numbers were adhered in groups to the inner surface of the pericardium and a few, particularly in the heaviest infections, were present on the organs of the abdominal cavity. Intensities of infection were similar from each sample obtained and fish size and sex appeared to have little influence. A similar site specificity and intensity of infection was recorded for the cysts identified as *I. erraticus* by Copland (1957) from Loch Lomond powan. Only a single Llyn Tegid gwyniad, of five examined, was infected, and then only with a single metacercaria which was adhered to the inner surface of the pericardium. A grayling *Thymallus thymallus* (L.), seine netted from Llyn Tegid also bore a single *I. erraticus* metacercaria, but attached to the ventricle. Nevertheless, this specimen represents the first record of such an infection in a British grayling. Farmed Loch Awe rainbow trout were the only trout found to be infected with *I. erraticus* metacercariae. Intensities of infection were low (1-8) and mixed, with *A. gracilis* cysts also present. Both metacercarial species were located within the pericardial cavity, on the ventricle and inner surface of the pericardium and no segregation was noted. Wootten (1973a) observed the same site specificity of metacercariae thought to be *I. erraticus* in both rainbow and brown trout from Hanningfield reservoir, Essex, while also noting the clustering of cysts in heavier infections. However, Swennen *et al.* (1979, who completed the life-cycle experimentally) stated that these clusters of cysts in smelt and rainbow trout, from the Ijsselmeer, Netherlands, were typically found to be floating free, rather than attached,

inside the pericardial cavity. In studying the epidemiology of *I. erraticus* infections in rainbow and brown trout Wootten (1973a) regularly sampled fish from their introduction to Hanningfield reservoir in April 1968. Both trout species were uninfected in May, but metacercariae were found in June. In brown trout the prevalence of infection rose from 12.5% in June to 100% in November, with associated increases in intensities. He concluded that successive infections were superimposed as the fish aged. However, the pattern of infection in rainbow trout varied in an irregular manner throughout the year. An increase in the prevalence of infected brown trout, from Summer to Winter, was also observed by Campbell (1974, metacercarial identity not confirmed) for Loch Leven fish, while the same pattern was noted by Olson (1970) in a range of fish species from Georgetown Lake, Montana, U.S.A. Swennen *et al.* (1979) recorded increases in infection intensities in smelt of the same age class during the summer months. The prevalence of *I. erraticus* (and *A. gracilis* - mixed infections) metacercariae recorded from Loch Awe rainbow trout in the present study was lower in the July 1992 sample (36%) than the January 1992 (95%). This is surprising as the fish were of the same size class and recruitment of further metacercariae would have been expected during the Summer months. However, the stocking policy at this site is not known and unexposed fish might have been introduced into this sample group. Rainbow trout obtained from a second fish farm on Loch Awe were uninfected with either strigeid species, suggesting that the snail populations which harbour the parthenitae, and subsequently release the cercariae of these parasites, are localised. Regular samples of *I. erraticus* infected fish were not obtained and consequently further comments on a possible annual infection pattern cannot be made.

Cysts excised from Finnish whitefish and vendace, both from Lake Kitka also proved to be *I. erraticus*. Hearts were dissected from the fish by colleagues in Finland and sent to Scotland. Consequently, information regarding distribution of the metacercariae was not available, although intensities of infection were high.

I. variegatus (Tables 1 and 3).

I. variegatus infections were confined to the percids; perch and ruffe. Prevalences of infection were found to be 100% for all samples from sources yielding infected fish. Metacercarial cysts within perch were predominantly located on the inner surface of the swimbladder, although low numbers were often found on the mesenteries and other organs of the body cavity. A similar distribution of *I. variegatus* metacercariae was recorded by Blair (1974) in Loch Lomond perch. The majority of cysts within ruffe were similarly found on the inner surface of the swimbladder, but, in this host, metacercariae were also located in the pericardial cavity, ovaries and orbits. Metacercarial burdens in Loch Lomond ruffe (67-500+) were far higher than in the loch's perch (9-100+), and these higher burdens might be responsible for the wider distribution of cysts within these fish. A rather different location within ruffe was observed by Odening & Bockhardt (1971) who found cysts mainly around the intestines. Samples of Loch Lomond perch obtained in this study during the months of May, June and July 1993 showed increasing mean intensities of *I. variegatus* infection, 60, 72 and 140, respectively. However, these samples consisted of successively larger size classes. Consequently, the higher intensities could have resulted from burdens carried over from previous years rather than from new challenges between the sample dates. Ruffe and perch from Llyn Tegid both showed low intensities of infection, with mean recoveries of 5.6 and 10.2 cysts, respectively. These low intensities of infection with *Ichthyocotylurus* spp. in susceptible Llyn Tegid fish suggest either smaller populations of *Valvata* spp. in this lake than Loch Lomond or a lower prevalence of infection in the snail population.

Hearts excised from Finnish ruffe were infected with three *Ichthyocotylurus* spp., *I. variegatus*, *I. platycephalus* and *I. pileatus*. Such multiple infections (*I. variegatus* and *I. platycephalus*) in ruffe were also recorded by Swennen *et al.* (1979). No *I. platycephalus* or *I. pileatus* infections were recorded from the British fish examined. Given the cyst locations observed in the tentative mainland British records of *I.*

platycephalus by Wootten (1973b, abdominal cavity) and Campbell (1974, inner surface of the swimbladder), it is likely that these metacercariae were actually *I. variegatus*. Metacercariae excised from brown trout in Galway, Eire were identified as *I. platycephalus* by staff at The Natural History Museum, London. These specimens were re-examined here and, although poorly fixed, were found to conform to Odening's (1979) key for this species and closely resembled the specimens identified as *I. platycephalus* from Finish material. However, the quality of these preparations did not allow a full complement of measurements to be recorded and hence considered against the *I. variegatus* material using PCA.

All *Ichthyocotylurus* infections were recorded from still water sources, lochs and lakes (Table 1). The absence of *I. erraticus* infections in salmonid species from the rivers sampled suggests that the snail hosts (*Valvata* spp., see Chapters 4, 5) for this genus were not present, although potential definitive hosts were. This does not represent a typical habitat distribution for these snails; Cleland (1954) stated that they are found in rivers to quite small brooks, canals, ditches and lakes.

A. gracilis (Tables 1, 4)

A. gracilis metacercariae were recovered from a range of salmonid and non-salmonid hosts: rainbow trout; salmon parr; arctic charr, *Salvelinus alpinus* (L.); stone loach, *Barbatula barbatulus* (L.); and bullheads, *Cottus gobio* L.

The site specificity of this metacercarial species was found to vary according to the host species. Infections in rainbow trout were largely confined to the pericardial cavity, with cysts adhered to the inner surface of the pericardium, ventricle, bulbus arteriosus or free in the cavity itself. Often cysts were grouped together, but always in a single layer, and covered by a common fibrous host tissue reaction with melanin pigment spots. McGuigan & Sommerville (1985) found the pericardial cavity to be the sole location of *A. gracilis* metacercariae in Loch Fad rainbow trout, recording the ventricle as the most common site of encystment, but with no cysts recovered from the

inner surface of the pericardium. A wider distribution of these metacercariae was observed by Blair (1974, 1976) in four experimentally infected rainbow trout. He found 87% of cysts within the pericardial cavity, 10% in the body cavity, 2% in the cranium and orbits, and 1% in the humour of the eyes. The range of sites described by Blair may have resulted from the intensity of the infections induced (almost 500 cysts per fish), causing more atypical localisations. Such alternative encystment sites were also noted in the present study for rainbow trout with high intensities of infection (see Table 4). Prevalences of infection in rainbow trout were consistently high at the three fish farms (on different river systems) sampled, ranging between 80-100%, although intensities varied between sites. At the River Almond fish farm, prevalences and intensities of infection were seen to increase from August 1992 to January 1993 (Table 4).

A. gracilis metacercariae in the remaining species of infected salmonids appeared to be less site-specific for the pericardial cavity. Isle of Skye arctic charr showed a 43% prevalence of infection with light metacercarial burdens (1.7), 20% of cysts being associated with the pyloric caeca. The majority (61%) of *A. gracilis* metacercariae in salmon parr were recovered from the body cavity, typically associated with the pyloric caeca; lower numbers (39%) being present in the pericardial cavity. Although the prevalence of infection in the single sample of salmon parr was high (94%), intensities of infection were low (3). Salmon parr obtained from the same source during the years 1973 and 1974 by Wootten & Smith (1980) also exhibited similar distributions of what were almost certainly *A. gracilis* metacercariae, with 63% and 34% in the body and pericardial cavities, respectively. These authors recorded prevalences of infection of 96% in 1973 and 48% in 1974, with low intensities of infection for both years (2.4 and 1.1).

The distribution of *A. gracilis* cysts within stone loach from the River Almond was found to differ markedly to that observed in the Salmonidae, being located (in 140 fish examined) within the body cavity (82-100%), cranial cavity (0-23%), and (rarely) in the humour of the eye (0-3%) and the musculature (2 cases of a single cyst) rather

than in the pericardial cavity. Blair (1974) did, however, record a low proportion (9%) of pericardial cysts in stone loach from the River Almond and 13% of the metacercariae in stone loach from the Llyn Tegid beck were located in this site. In the present study small stone loach, less than 2.0cm true length, were never seen to harbour metacercariae, and Fig. 40 indicates that intensities of infection increased with increasing host size (age). The relationship between stone loach size, intensity of *A. gracilis* infection and month of capture for River Almond specimens is shown in Table 26.

Fig. 40. Relationship between stone loach length and intensity of infection.

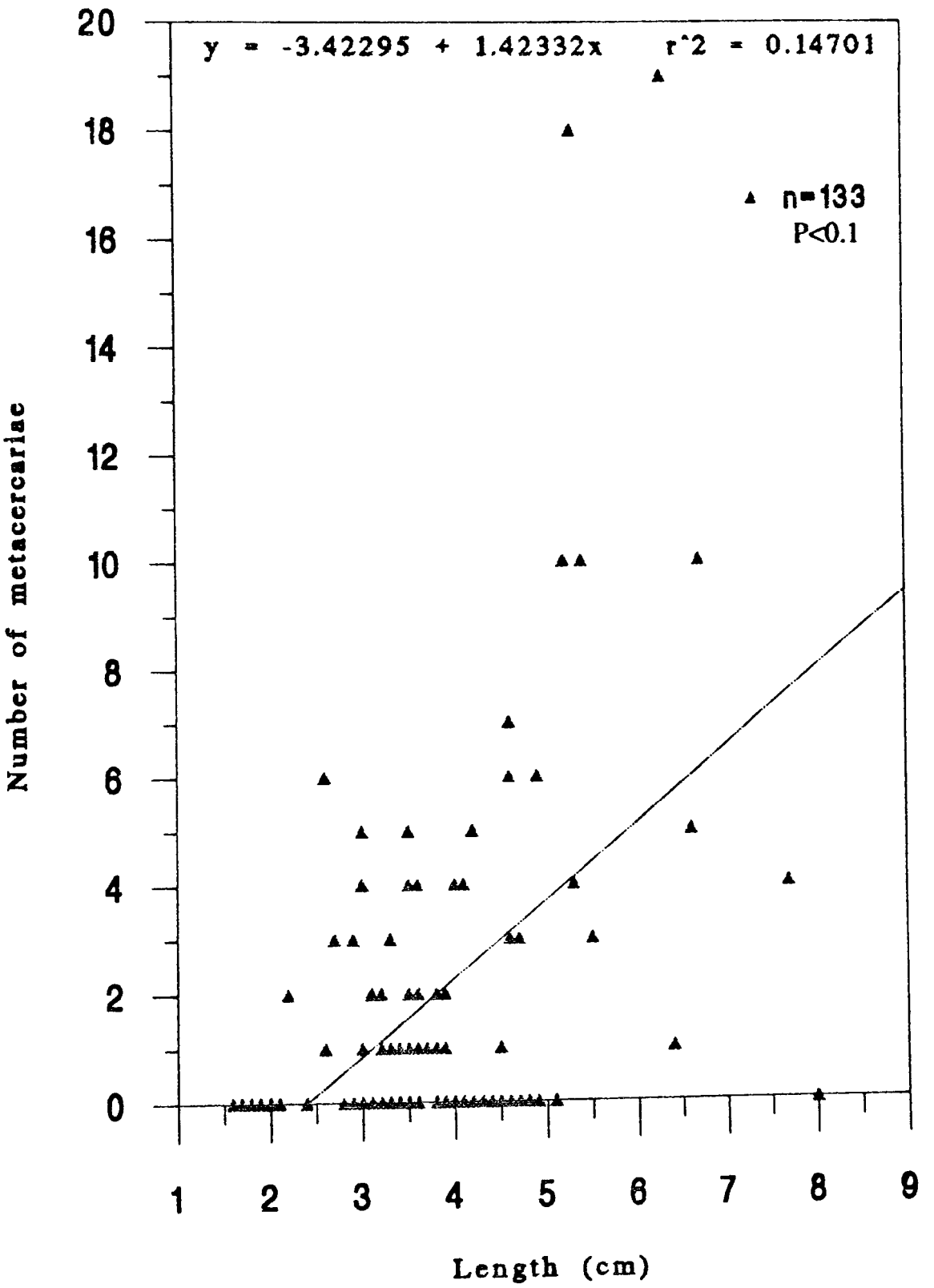


Table 26. Relationship between stone loach size, intensity of infection and month of capture.

Date	Stone loach size (cm)					
	<2.0	2.0-2.9	3.0-3.9	4.0-4.9	5.0-5.9	6+
	Mean intensity of infection. Number of stone loach examined in parentheses.					
May '93	0 (4)	3.3 (3)	3.3 (4)	4.5 (2)	-	-
July '93	-	-	2.5 (2)	6.5 (2)	14 (4)	-
August '93	0 (5)	0.5 (10)	1.4 (21)	4.7 (4)	-	-
September '93	0 (6)	0 (2)	1.0 (27)	0 (2)	3 (1)	14.5 (2)
July '94	-	0 (1)	0.1 (17)	0.5 (19)	9 (2)	1 (1)

These results suggest that *A. gracilis* burdens in River Almond stone loach of a particular size class are likely to be greater in May than in September. This is particularly puzzling when the percentage of unencysted, newly acquired metacercariae within these fish is considered. In May only encysted metacercariae were present, the cysts having been attained earlier in the year from over-wintered infected snails or from infections the previous Summer. In July 24% of metacercariae were pre-encysted, in August 47% and September 19%. Given the time required for cercarial migration through the host and for encystment (4+ weeks, see Johnson, 1971; Vojtek, 1972; Blair, 1974; present study, Chapter 6), this suggests that the cercarial challenge began some time after May, peaked at around July and dropped towards the end of the Summer. Consequently, if recruitment of infections occur, overall intensities would have been expected to increase from May to September, not the reverse. Many of the samples obtained were small which might account for these results; alternatively, heavily infected fish might have died or have been predated during the Summer months. In their monitoring of the *A. gracilis* infections in cultured River Almond salmon parr Wootten & Smith (1980) also observed a peak in metacercarial recruitment during the month of July.

Crocombe (1959) recorded *A. gracilis* cysts attached to the coelomic mesenteries

in naturally infected bullheads from Glamorgan in Wales. Here, the metacercariae in bullheads from the Llyn Tegid beck were not found to be confined solely to this site with the cranial (6%) and pericardial (3%) cavities also bearing cysts. Further differences in site specificities of this metacercaria were observed by Hoffman (1959) for the brook stickleback from North Dakota, U.S.A. (musculature) and Blair (1973, 1974, 1976) for the 3-spined-stickleback from the River Almond and Heidarvatn, Southern Iceland (humour of the eyes).

A. annuligerum (Tables 1, 5)

A. annuligerum cysts were found floating free in the humour of perch eyes from three sites in Central Scotland. Prevalences of infection were found to range between 28 and 90%, with consistently low intensities of infection (1-9) regardless of sample date or fish size. Perch obtained from Loch Lomond by Blair (1974) yielded far higher intensities of infection, with 70 cysts from just four fish, while McGuigan & Sommerville (1985) recorded up to 100 metacercariae in Loch Fad perch. The latter authors observed cysts within the retina and humour, and noted the presence of a thick (120μm) fibrous capsule surrounding the cyst. Such a host response was never observed in the present study and might result from burden size.

A. annuligerum were always recovered from perch which were also infected with *I. variegatus* metacercariae; the samples of perch from Humberside, England and the Due Loch, Central Scotland being uninfected with either species. The molluscan host of *A. annuligerum* is at present unknown (See Chapters 4, 5) and there is no particular reason to believe it to be specific to the same genus of snails (*Valvata*) as *Ichthyocotylurus* spp., *A. gracilis* being predominantly recorded from lymnaeids, making this a curious observation. However, *Apatemon* cercariae were recovered from naturally infected *V. piscinalis*, but these were believed to belong to the subgenus *Australapatemon* (see Chapter 5.1.1; Results).

No strigeid metacercariae were recorded from cyprinids in the present study (although only three species were examined and of these only roach from more than a single site), nor are there any previous confirmed records from British cyprinids. Nicoll (1924), in his reference list of trematode parasites from British freshwater fishes, recorded *I. platycephalus* (as *T. ovata*) from the peritoneum of bream *Abramis brama* (L.) and silver bream *Blicca bjoerkna* (L.), but gave no indication of the origin of these records. This location within the fish hosts does not represent that most typical for *I. platycephalus* (pericardial cavity), and the subsequent reference list of Kennedy (1974) did not include these reports. The Cyprinidae and Percidae are the most commonly recorded hosts for *I. platycephalus* metacercariae, and as both fish families have been widely studied, the presence of this species in Britain is unlikely.

2.2. TAXONOMIC STUDIES.

2.2.1. Artificial digestion of cysts.

The behaviour of *I. erraticus* metacercariae during excystment was described by Mitchell *et al.* (1978). Similar rotation, elongation and contraction by the metacercaria within the cyst was noted for all *Ichthyocotylurus* spp. in the present study. As the cyst wall progressively thinned the metacercarial movement resulted in increasing distortions of the cyst wall. Often a nipple-like protrusion was seen at the point of contact between the oral sucker and cyst wall, and it was at this point that the wall invariably ruptured, causing the worm to emerge anterior-end first. Mitchell *et al.* (1978) found that *I. erraticus* excystment usually took 1-2 hours to complete in an alkaline-trypsin solution, but could be reduced to 10-15 minutes if 0.2% cysteine hydrochloride was added to the medium. However, the effect of this reducing agent upon the metacercariae was unknown and consequently not routinely used by the authors. The average emergence time recorded here for this species was 50 minutes, which indicates the effectiveness of the pepsin pretreatment not employed by Mitchell *et al.* (1978). Cyst wall thickness of the *Ichthyocotylurus* spp. reflected the times required for excystation, with the thin

walled *I. variegatus* requiring less than 30 minutes in trypsin solution before emergence. The metacercaria of *Cotylurus cornutus* possesses a thick cyst which is even more tightly investing than those of *I. erraticus* and *I. pileatus*. Excystment of this species was observed by Graczyk & Shiff (1993) to require an acidic pretreatment. They found that, although the addition of pepsin to this solution provided optimum results, it was not an essential component. However, unlike *Ichthyocotylurus* spp., both Blair (1974) and Graczyk & Shiff (1993) noted that excystation of *C. cornutus* metacercariae was a largely passive event with the cyst wall appearing to adhere to the worm and simply thinning until the tegument became exposed to the medium. This difference may reflect structural differences observed in the cysts of the two genera; *C. cornutus* (see Blair, 1974), *C. flabelliformis* (Faust 1917) Van Haitsma, 1931 (see Hughes, 1929) and *C. brevis* Dubois & Rausch, 1950 (see Nasir, 1960) all bearing perforations in their cyst wall, unlike *Ichthyocotylurus* spp.

The method of excystation of the two *Apatemon* spp. was very different to that observed for the *Ichthyocotylurus* spp. This process was described in detail by Blair (1974, 1976) for the metacercaria of *A. gracilis* and was found here to apply equally to *A. annuligerum*. With these species the thinning of the cyst wall in trypsin solution occurs predominantly at one pole; the metacercaria exhibits little activity and the cyst retains its shape. Excystation is explosive, with the metacercaria being rapidly expelled under pressure from the thinnest pole along with the refractile cyst contents. The cyst itself contracts with the expulsion of the worm, leaving a smaller, more rectangular central cavity and thickened walls. As reported by Blair (1974, 1976), excystment occurs even if the metacercaria is dead, demonstrating that the process is the result of the medium upon the pressurised cyst and unlike, *Ichthyocotylurus* spp, not dependent upon metacercarial activity.

2.2.2. Light microscopical observations of metacercariae

The tetracotyle key of Markevich (1951) provided accurate information on the

two large *Ichthyocotylurus* spp., *I. variegatus* (as *T. perca fluviatilis*) and *I. platycephalus* (as *T. variegata*), but would not have always enabled their accurate separation. Bykhovskaya-Pavlovskaya's (1964) key misidentified the metacercaria of *I. platycephalus*, describing it as *I. pileatus*. This key also contained several errors, such as the ventral sucker of *I. erraticus* (as *T. intermedia*) metacercariae being smaller than the oral sucker. Later Hoffman (1967), based on Hughes's (1928) description, stated that the lappets of *I. erraticus* were markedly smaller than the oral sucker; this is not the case, even if fissure length is considered rather than actual lappet length. Prior to confirmation through life-cycle studies Odening's (1979) key enabled the confident identification of three of the four *Ichthyocotylurus* spp. However, the statement that the lappets of *I. pileatus* are always shorter than the oral sucker was not found to be correct. In this study the lateral lappets were typically of a similar or greater length than the oral sucker, although they were occasionally shorter. Odening (1979) also indicated that the maximum width of the *I. variegatus* ventral sucker was 115µm; here this dimension was exceeded in several specimens from all four sources investigated. The inclusion of this feature is peculiar, as he recorded ventral sucker widths of up to 132µm for this species in an earlier work (Odening & Bockhardt, 1971). The failings of the most recent *Ichthyocotylurus* key, that provided in Bauer (1987), were the rather narrow size ranges given for body and organ dimensions of certain species and its dependence on the ratio of the tribocytic organ length to body length for the identification of *I. erraticus*, a feature which was not always accurate. It is considered that a more accurate key to this genus would amend Odening's (1979) key by: replacing, for *I. variegatus,* "width of ventral sucker less than 115µm" with "width of ventral sucker less than 150µm" - the criterion which *I. platycephalus* exceeds; changing, for *I. pileatus*, "pseudo-suckers [i.e. lappets] shorter than oral suckers" to "pseudo-suckers of a similar length to oral sucker"; and adding, for *I. pileatus*, "length of tribocytic organ at least 1/3rd of body length", as proposed by Bauer (1987).

The morphological measurements recorded for *I. erraticus* metacercariae in the

present study (see Table 6) generally fell within the limits given by Odening (1979) for this species, although some of the tribocytic organ dimensions observed were found to exceed the upper limits of his range, particularly in specimens excised from powan and rainbow trout. This was probably due to the state of the organ on fixation; all of the larger dimensions being recorded from specimens with this structure partially extended, possibly as a result of the relaxant fixative, Berland's fluid.

Dimensions obtained for *I. variegatus* metacercariae from Scottish perch corresponded closely with those recorded in Odening & Bockhardt's (1971) description of this species. However, the dimensions of metacercariae from Scottish and Finnish ruffe and Finnish perch were less similar to Odening & Bockhardt's (1971) results. Most of the mean body and organ measurements for these specimens were within the range of Odening's (1979) key, although the smaller measurements were not. Cyst structure, comparative body dimensions and location within the host all indicated, however, that these specimens were smaller *I. variegatus* metacercariae than had previously been recorded. This conclusion was later confirmed by the morphology of cultured adults; see Chapter 3. Such *I. variegatus* metacercariae, with dimensions below the range indicated by Odening (1979), were also described by Pugachev (1983).

The body dimensions of *I. pileatus* from Finnish ruffe closely matched those of the original description by Hughes (1928), but the internal organs were very much towards the lower end of the range indicated by both Hughes (1928) and Odening (1979). All measurements taken from the single *I. platycephalus* specimen were within the limits described by Odening, Matthesis & Bockhardt (1970) and Odening (1979). This specimen also met the major criterion for its discrimination from *I. variegatus* metacercariae, i.e. a ventral sucker of greater (or equal) length to that of the tribocytic organ.

A proportion of *A. gracilis* and *A. annuligerum* metacercariae were found to contract in the artificial digest on excystment, with the oral sucker and lappets drawn towards the ventral concavity. These specimens were far less active than those which

did not contract. Measurements in the present study were taken from specimens that were active and extended prior to fixation. The use of Berland's fixative also ensured the most extended specimens possible. These factors may have been responsible for the dimensions in this study (from all sources) being larger than those previously recorded by other authors. However, if partial contraction of specimens was responsible for the shorter body dimensions recorded by other authors, a concomitant increase in forebody breadth might have been expected. Although the *A. annuligerum* specimen described by Kozicka (1972) was shorter and broader than those in the present study, this was not the case with *A. gracilis* metacercariae described by other authors, which were shorter and thinner (see Table 10).

The body dimensions obtained here for *A. gracilis* metacercariae corresponded most closely to those given by Blair (1974, 1976), but even his results indicated a significantly shorter forebody. Blair's measurements, shown in Table 10, can be compared directly to one set of dimensions recorded here for this species, as both were taken from River Almond stone loach. The greatest difference between these two data sets was in the ventral sucker dimensions, which were particularly large in the present study. Indeed, with the exception of salmon parr specimens, this structure was consistently larger in metacercariae from all present sources than observed by previous authors. The tribocytic organ of *A. gracilis* and *A. annuligerum* is situated at the base of the ventral concavity of the forebody. This position means that it does not contact the substratum on excystment, and its degree of extension when fixed is extremely variable. Consequently, measurements of its length and breadth are also highly variable, making it a poor diagnostic feature. No differences in morphological measurements of *A. gracilis* metacercariae were readily attributable to host differences, i.e. metacercariae recovered from members of the Salmonidae were not obviously larger than those from the Cottidae or Cobitidae, or vice-versa.

The description by Kozicka (1972) of the metacercaria of *A. annuligerum* was found to be completely satisfactory, although based on a single specimen. As Odening

(1970) only provided details of certain internal organs, the measurements of Kozicka's (1972) specimen remain the only previous complete morphological data set. Here, total body length was seen to differ greatly for specimens excised from perch of two Central Scottish lochs. Specimens from one site averaged almost 1mm in length, while metacercariae from the other were never seen to attain such a size. Surprisingly, the mean dimensions of the pharynx, oral and ventral suckers (Table 11) were smaller, from both present sources, than the smallest measurements recorded by Odening (1970) and Kozicka (1972). Nevertheless, comparative sizes of the ventral sucker and oral sucker were similar for all records. At present *A. gracilis* and *A. annuligerum* metacercariae are distinguished morphologically by the latter species possessing a ventral sucker which is much larger than the oral sucker (Dubois in a pers. comm. to Blair, 1974, but not published in Dubois, 1974, as indicated to Blair). Although this statement is correct, a comparison of the results obtained in the present study suggests that the same is also true, to a lesser extent, for *A. gracilis*. Consequently, this feature does not provide an adequate criterion for the discrimination of the two species.

A. fuligulae is the only other member of the subgenus *Apatemon* for which the metacercaria has been described. The details of this species' morphology, provided by Yamaguti (1933), differ markedly to those observed in the present study and by other authors for *A. gracilis* and *A. annuligerum*. The nature of the *A. fuligulae* cyst was not discussed, but Yamaguti (1933) stated that the metacercaria may be liberated without difficulty. He observed a forebody that was "flat", an oral sucker of larger dimensions than the ventral sucker and lappets that existed as "shallow suctorial depressions".

2.2.3. Chaetotaxy and scanning electron microscopical observations of metacercariae.

Silver nitrate staining and SEM examination of metacercariae indicated that sensilla were abundant on the surface of both *Ichthyocotylurus* and *Apatemon (Apatemon)* spp. Indeed, sensilla were far more numerous on the metacercaria than cercaria of these species (see Chapter 5.2.3; Results). This finding is in contrast to those

of Shigin *et al.* (1985) and Brady (1989), who, using silver staining techniques, observed a decrease in the number of sensilla from the cercarial to metacercarial stage of *Diplostomum* spp. Shigin *et al.* (1985) recorded low numbers of sensilla on the metacercaria of *D. parviventosum* Dubois, 1932. These sensilla were, with the exception of the ventral sucker, found to be consistent in their number and distribution. The later work of Brady (1989) also recorded low numbers of sensilla for the metacercariae of four *Diplostomum* spp. She produced chaetotaxy maps for each of these species and noted species-specific variations between them. The only intra-specific variation observed by Brady (1989) was in the arrangement of oral sucker sensilla, which she suggested might result from contraction of the organ. The failure to elucidate chaetotaxy patterns for the species of strigeid metacercariae in the present study resulted from the extremely large number of sensilla present and the subsequent difficulty in comparing individual maps. The use of silver staining and SEM to compile such data was further hindered by debris acquired from within the cyst or damage incurred removing this material. Consequently, chaetotaxy is not considered a viable method for aiding the identification of, or attempting to discriminate between, these metacercariae.

SEM examination emphasised the three-dimensional shape of metacercariae belonging to the two strigeid genera studied, as well as the variation in shape and extension that may be recorded in the organs of attachment. The greater ease in obtaining clean specimens of the subgenus *Apatemon* may reflect their non-active method of escape from the cyst. *Ichthyocotylurus* spp. are required to use their bodies to stretch the thinning cyst wall and rasp against it with their suckers to achieve freedom, whereas *Apatemon (Apatemon)* spp. are ejected under pressure through a ruptured pole of the cyst.

The number of sensilla most commonly recorded by silver staining for the ventral suckers of *I. erraticus* and *I. variegatus* was confirmed by SEM observation. However, the actual arrangement of the sensilla, as indicated by SEM, differed somewhat from that suggested by silver staining. Due to the contraction of the sucker

during silver staining the more superficial ring was drawn centrally, giving the impression that this was the inner of two concentric rings, see Fig. 8. SEM failed to show the presence of the large number of argentophilic structures (revealed by silver staining, Fig. 7) situated anterior to, and surrounding, the mouth of these *Ichthyocotylurus* spp. The apparent absence of these structures could be due to contraction of the oral sucker, causing the cilia to be obscured between the surface folds, although Fig. 18 indicates that contraction was often minimal. Alternatively, the argentophilic structures may represent non-ciliate sensilla or glandular papillae and may simply not be visible on the surface of the organ. A transmission electron microscope (TEM) study of the oral suckers would answer this question.

Sensilla were only observed on the tribocytic organ of *Ichthyocotylurus* spp. using SEM. The failure of silver staining to indicate these sensilla is explained by the retraction of the organ when using this technique and might account for their absence from the descriptions of Shigin *et al.* (1985) and Brady (1989) for *Diplostomum* spp.

Two types of sensilla were distinguished for *Ichthyocotylurus* metacercariae, those restricted to the oral sucker, with the cilium appearing to emerge from a pit, and those consisting of a dome bearing an apical cilium. *Apatemon (Apatemon)* spp. were only seen to possess a modified form of the latter. Similar domed ciliate sensilla were identified for newly excysted *Fasciola hepatica* metacercariae by Bennett (1975) and for the adults of *Philophthalmus megalurus* (Cort 1914) and *Echinostoma hortense* Asada, 1926 by Edwards, Nollen & Nadakavukaren (1977) and Torii, Tsuboi, Hirai & Nishida (1989), respectively. Bennett (1975) and Edwards *et al.* (1977) attributed tangoreceptor functions to the external domed sensilla they described. However, both of these authors had supporting TEM information on which to base their suppositions. Such an investigation is required to answer the questions posed for the sensilla of strigeid metacercariae. What are the respective functions of the oral sucker and body sensilla in *Ichthyocotylurus* spp.? Does the external structural variation in domed sensilla observed between *Ichthyocotylurus* and *Apatemon (Apatemon)* spp. reflect internal and

consequently functional differences? Do the larger cilia of the *I. pileatus* oral sucker sensilla reflect a difference in function to the equivalent sensilla in the other *Ichthyocotylurus* spp.?

2.2.4. Discrimination of metacercariae by Principal Components Analysis of metrical features.

Principal components analysis of the 327 *Ichthyocotylurus* specimens indicated four discrete groups which corresponded to the four species identifications made. All 15 variables incorporated into the analysis were found to contribute to the separation achieved, with dimensions of the body, oral and ventral suckers and lappet length of particular influence (as indicated by the variable coefficients, Table 13). As well as cyst structure, the features indicated here, i.e. relative sizes of both oral and ventral and pseudosuckers (lappets), are central to Odening's (1979) key for the identification of this genus. Unfortunately, only a single specimen of *I. platycephalus* was available for inclusion in this analysis. A separate examination of members of this species with *I. variegatus* metacercariae might have revealed further evidence for their taxonomic uniqueness.

Principal components analysis was also applied to the metacercariae of *A. gracilis* and *A. annuligerum* in an attempt to isolate discriminating morphological features. Although a separation of the two species was indicated with the imposition of 95% confidence limits on the centroids, a broad overlap was present when ellipses surrounded 50% of each species' specimens. This appeared to be due to the large intra-specific variation in many of the measurements recorded for *A. annuligerum*, coefficients of variation often exceeding those for *Apatemon* spp. globally (Table 22). When groupings according to *A. gracilis* host species was applied to this separation, it became apparent that salmon parr specimens possess the greatest morphological differences to *A. annuligerum* metacercariae (Figs 36, 37), with smaller body

dimensions, smaller pharynx length and less distance between the lappets being the features of principal significance (Fig. 35, Table 22). Imposing more general host groupings (salmonid and non-salmonid) on *A. gracilis* metacercariae suggested that non-salmonid specimens exhibit morphology intermediate to salmonid specimens and *A. annuligerum* (Figs 38, 39). When considered independently all individual samples of *A. gracilis* metacercariae demonstrated morphological variation from *A. annuligerum* specimens. However, variation was also present between individual *A. gracilis* samples.

Interestingly and contrary to Dubois's pers. com. to Blair (1974), oral and ventral sucker dimensions were not identified as major contributors to the discrimination of the two *Apatemon* spp., when all *A. gracilis* material was included in the analysis. Nevertheless, all individual analyses of *A. gracilis* samples against *A. annuligerum* specimens did include a dimension of either the oral or ventral sucker in the top five discriminating features, although never both (Table 24). Thus, if separation of these metacercarial species is to be attempted by morphology alone, it appears that a broader view is required.

Identifiable variations in morphology were observed for *I. erraticus* metacercariae from different hosts and geographically distant populations (Figs 24, 25). Analyses examining the combined effects of host and origin on *I. erraticus* morphology indicated: no obvious variation for specimens from the two Finnish *Coregonus* spp, Lake Kitka whitefish and Lake Kitka vendace; variation between Scottish powan and Scottish rainbow trout specimens; variation between Scottish powan and Finnish whitefish (both *Coregonus lavaretus*) specimens; but surprisingly little variation between Scottish rainbow trout specimens and those from both Finnish *Coregonus* spp. (Fig. 25). Morphological variation when present was predominantly seen in body and internal organ breadths. These findings show that *I. erraticus* metacercariae from Scottish powan tend to have broader dimensions than Scottish rainbow trout specimens (see Fig. 26), and suggest that conditions influencing Finnish material cause narrower dimensions in

specimens than observed in Scotland (comparison of Scottish and Finnish *C. lavaretus* specimens). Thus, if the limits of these influencing factors are not met by Finnish *Coregonus* specimens, Finnish rainbow trout *I. erraticus* metacercariae might be expected to demonstrate even narrower dimensions.

When considered purely on the basis of geographical separation or host species, morphological variation in *I. variegatus* metacercariae was seen to be minimal. However, when examined as separate samples, i.e. Scottish ruffe, Scottish perch, Finnish perch and Finnish ruffe material, a parallel situation was observed to that recorded for *I. erraticus* metacercariae. Separation was achieved between Scottish perch specimens and all other material on the basis of longer, broader bodies, larger ventral suckers, broader oral suckers and tribocytic organs (Fig. 27, Table 18). Specimens from both Finnish ruffe and perch demonstrated little morphological variation, while Scottish ruffe bore more similarities to the Finnish material than to Scottish perch metacercariae (Fig. 29).

The location within a host was also found to result in morphological variation, as seen for *I. variegatus* metacercariae from a single ruffe. Specimens excised from the orbit and ovary displayed the greatest variation, with individual metacercariae from the pericardial cavity and swimbladder sharing similarities with those from both locations. Ovarian specimens showed larger body and organ dimensions than orbit specimens, perhaps reflecting developmental differences pre-encystment due to the respective nutritional status of the locations.

Niewiadomska & Szymański (1991) recorded host-induced variability in experimentally reared *D. paracaudum* (Iles, 1959) metacercariae. The variability of *D. spathaceum* (Rudolphi, 1819) metacercariae in relation to host species, host size, age and density of the parasites was investigated by Graczyk (1991). The results obtained indicated that all four parameters contribute to variability, but it is the host species in which the metacercariae develop which is of decisive importance. In the present study

the geographical origin of specimens was sometimes seen to be of equal importance to the fish host in causing morphological variability, e.g. *I, erraticus* metacercariae from Scottish and Finnish *C. lavaretus*. It must be remembered that the present study used metacercariae excised from naturally infected fish, and consequently the results obtained may be compromised by the influence of the other parameters identified by Graczyk (1991), although the employment of standardisation removed the potential effect of age related growth.

In summary; it appears that *Ichthyocotylurus* metacercariae can be discriminated by details of their morphology and cyst structure, although further life-cycle data are required for confirmation of the identities assigned. *Apatemon gracilis* and *A. annuligerum* are extremely similar morphologically and differ greatly from the other known member of this subgenus, *A. fuligulae*. The large number of sensilla present on the body surface of these strigeid species, as observed by silver-staining and SEM, precluded their diagnostic use. Principal components analysis was, however, found to be of value in distinguishing between species and determining morphological variation within a species.

CHAPTER 3: THE ADULTS

INTRODUCTION

The aims of the work reported in this chapter were three-fold: to obtain adults to confirm the identifications made in Chapter 2; to examine the effect of metacercarial origin (fish host/geographical location) upon adult establishment, development and morphology; and to harvest eggs for further life-cycle work.

3.1. THE CULTURE OF METACERCARIAE TO ADULTS

Strigeids whose metacercariae utilise fish as their second intermediate host develop naturally to the adult stage in piscivorous birds. In the laboratory, the use of the parasite's natural host is not always possible, even when known. Other methods enabling the rearing of the digenean adult from its metacercaria include the use of *in vitro* culture, chicken embryo infections or alternative experimental hosts.

An early review of *in vitro* culture techniques for raising digenean adults was provided by Erasmus (1972); these procedures typically met with only limited success and parasite eggs, when obtained, were not found to be viable. Later, Mitchell, Halton & Smyth (1978) managed to raise *Ichthyocotylurus erraticus* adults in tissue culture media supplemented with chicken serum, although parasite eggs obtained from these adults also failed to develop. The transplantation of metacercariae onto the live medium of chick embryo chorioallantois has provided more promising, although still mixed results. Leno & Holloway (1986) successfully obtained gravid adults of *Diplostomum spathaceum* (Rudolphi, 1819), the eggs of which produced viable miracidia. Experiments by Irwin, Saville & Chubb (1989) yielded gravid *D. spathaceum* adults, but of a smaller size than worms from natural infections, while Brady (1989) failed to culture adults from a series of challenges with *Diplostomum* spp. Most recently, Strain & Irwin (1995) used this technique to rear the adults of three microphallid species, which they found to be indistinguishable from their definitive descriptions. Although the latter authors were satisfied with the development of their worms and considered them to be

morphologically 'normal', there must always be some concern regarding comparisons made between specimens raised in natural and artificial systems.

In taxonomic studies it is most important that adults obtained experimentally retain normal morphology. For this reason, it was decided that the culture of metacercariae to adults would be best attempted using recognised natural hosts, whenever possible, or at least the most favourable experimental hosts available.

Natural and experimental hosts.

Ichthyocotylurus erraticus

Odening (1979) listed natural definitive hosts of *I. erraticus* as the Laridae, Gaviidae and Podicipediformidae, the first two being the most widely recorded. *I. erraticus* adults were found in a collection examined at Kaliningrad from the common gull *Larus canus,* and lesser black-backed gull *L. fuscus*, by Dubois (1938). More recently, Niewiadomska & Kozicka (1970) discovered four adults in two specimens of the black-headed gull *L. ridibundus*, from the Mazurian lakes, Poland, and, having examined 326 birds all considered to be definitive hosts by Dubois (1968), concluded both prevalence and intensity of infection to be very low in the definitive host. They performed two experimental infections; one upon a naïve laboratory reared black-headed gull and the second on an *L. canus/L. fuscus* hybrid obtained from Warsaw zoo. In both cases a proportion of the *I. erraticus* metacercariae excised from *Coregonus* spp. successfully established.

Massive infections of *I. erraticus* adults in two California gulls *L. californicus*, one of which died, were observed by Olson (1970), with 900 and 2500 specimens being present. He experimentally infected four immature California gulls, which were judged to be free from trematode burdens, with *I. erraticus* cysts (referred to as *Tetracotyle intermedia* Hughes, 1928) obtained from the pericardial cavities of salmonid fish. After four days the gulls began passing large numbers of trematode eggs and were found on autopsy to harbour *I. erraticus* adults.

Fraser (1974) examined 70 gulls of five species for trematodes at Loch Leven, Scotland. She discovered *I. erraticus* in lesser black-backed gulls, herring gulls *L. argentatus* and greater black-backed gulls *L. marinus*, during the months of June and July. The prevalence of infection ranged between 11% for herring gulls and 14% for lesser black-backed gulls to 36% for greater black-backed gulls, with intensities of between 5 and 52 adults. This parasite was also found in unspecified gull species in the Ijsselmeer, Netherlands by Swennen, Heessen & Hocker (1979), with heavy infections being normally mixed with other species of the genus.

The gaviids (loons/divers), *Gavia stellatus* and *G. articus*, were reported as hosts of *I. erraticus* by Dubois and Rausch (1950), Dubois (1968), Odening (1978; *G. stellatus*) and Sudarikov (1984), although details of infections were not provided.

I. variegatus

Definitive host records for *I. variegatus* adults were summarised by Odening (1979) as being predominantly from the Laridae, but also included the Podicipedidae and rarely the Anatidae. Odening & Bockhardt (1971) and Blair (1974) successfully raised this species experimentally in black-headed gulls and Halton & Faulkner (1988) in both black-headed and herring gulls. Surprisingly, given the prevalence of *I. variegatus* metacercariae recorded in this and other studies (see Chapter 2), natural infections of adults have not been observed in British gulls (Pemberton, 1963; Fraser, 1974).

I. platycephalus

The range of natural hosts recorded for *I. platycephalus* adults embraces all of the families listed for *I. erraticus* and *I. variegatus*, plus the Pelecanidae and Acciptridae (Odening, 1979). Other authors, such as McDonald (1981) and Bauer (1987), have simply stated that the hosts are "normally gulls". Many of the host species listed for *I. platycephalus* include records for the synonymous *Cotylurus cucullus* (Thoss, 1897) and

C. communis (Hughes, 1928) La Rue, 1932 (see *inter alia* La Rue, 1932; Razmashkin, 1966; Shigin, 1983).

Odening, Mattheis & Bockhardt (1970) obtained *I. platycephalus* adults from experimental infections in black-headed gulls, and Swennen *et al.* (1979) achieved similar results in black-headed, herring and lesser black-backed gulls.

It is worth noting that there is some disagreement regarding the validity of *I. variegatus* as a species distinct from *I. platycephalus*. This matter will be discussed later in 3.2; Taxonomy of adults. However, it is the present author's view that the two species are distinct and that some natural host records for *I. platycephalus* may in fact represent *I. variegatus* adults.

I. pileatus

Natural host records of *I. pileatus* adults are mainly confined to the Laridae, *Larus*, *Sterna* and *Chlidonias* spp. (Dubois, 1968; McDonald, 1981; Shigin, 1983), although Sudarikov (1984) also included the Gaviidae and Anatidae. Razmashkin (1966) described the development of *I. pileatus* metacercariae to adults based on material obtained experimentally from common gulls.

***Apatemon (Apatemon)* spp.**

Records of natural infections of birds by *Apatemon* spp. must be treated with some caution, as historically there has been much confusion regarding the identification of members of this subgenus (see 3.2; Taxonomy of adults). Host records from non-piscivorous waterfowl should be viewed with suspicion, although the consumption of infected dead/moribund fish during foraging cannot be discounted.

A. gracilis

The most widely reported natural hosts of *A. gracilis* adults are members of the

genus *Mergus*, e.g. Yamaguti (1933), Dubois (1968), Odening (1978), McDonald (1981), Shigin (1983), Sudarikov (1984) and Watson & Pike (1993). Indeed, Dubois (1968) limited natural host records to members of this genus: the goosander *Mergus merganser*; smew *M. albellus*; red breasted merganser *M. serrator*; hooded merganser *M. cucullatus*; and scaly sided merganser *M. squamatus*. More recently, McDonald (1981) stated that *A. gracilis* adults are "now believed to be restricted to mergansers".

Many of the early host records for this species were from other anatids; mainly ducks (listed in *inter alia* Vojtek, 1971; Shigin, 1983; Sudarikov, 1984). Of these, most are likely to involve misidentifications, arising from the historical confusion with *A. minor* Yamaguti, 1933 (reviews in Vojtek, 1964b; Blair, 1974), for which ducks are the most common definitive hosts (Dubois, 1968). Nevertheless, some of these records are more probable than others, with the scaup *Aythya marila*, (see Odening, 1978) and other occasionally piscivorous ducks representing potential alternative definitive hosts.

Experimental infections with *Apatemon gracilis* metacercariae have, through necessity, typically been restricted to readily accessible duck species or gulls. Domestic ducklings *Anas platyrhynchos* var. domestica, were successfully used to obtain *Apatemon gracilis* adults by Yamaguti (1933, referred to as *A. gracilis pellucidus*), Vojtek (1964a, referred to as *A. cobitidis cobitidis*), Crocombe (1969, referred to as *A. gracilis pellucidus*) and Blair (1974). However, Watson & Pike (1993) were unable to experimentally infect domestic ducklings, but found eider ducklings *Somateria mollisima*, to be excellent experimental hosts. Challenges of black-headed gulls with *A. gracilis* metacercariae failed to establish (Blair, 1974).

A. annuligerum

The known natural hosts of *A. annuligerum* are limited to a single species, the buzzard *Buteo buteo*. Oltenau, Lungu & Popescu (1968) discovered *Apatemon* adults in the intestine of a buzzard from the Danube Delta. They identified these worms as *A. gracilis*, but Dubois (1980) suggested that the specimens were probably misidentified

and actually represented *A. annuligerum*. In a series of experiments Odening (1970) attempted to infect five species of bird with *Distomum annuligerum* v. Nordmann, 1832 cysts excised from the eyes of perch. Adult worms of the genus *Apatemon* were recovered from the small intestine of a buzzard; all other infections failing. Odening (1970), therefore, transferred the species to this genus as *Apatemon* (*Apatemon*) *annuligerum*. The only experimental infections with *A. annuligerum* metacercariae attempted in Britain were performed by Blair (1974). He fed cysts excised from the eyes of Loch Lomond perch to three black-headed gulls and a domestic duckling, but no adults were recovered.

Definitive host records of the remaining species considered to belong to the subgenus *Apatemon* (see 3.2) are largely confined to the Anatidae, e.g. *A. graciliformis* (experimental infection, see Dubois & Nassi, 1977), *A. somateriae* Dubois, 1948 (eiders and scoters, see Dubois, 1968) and *A. fuligulae* (diving ducks, see Yamaguti, 1933); although *A. buteonis* (Yamaguti, 1933) is only recorded from birds of prey (see Yamaguti, 1933; Dubois, 1968).

3.2. TAXONOMY OF ADULTS.

As noted in Chapter 1, much confusion exists in the literature on strigeid systematics due to the reliance on often poorly differentiated adult morphology and the lack of life-cycle details. Such a situation was, and still is to a certain extent, true for species belonging to the genus *Ichthyocotylurus* and subgenus *Apatemon (Apatemon)*. Indeed, Sudarikov (1984) declared the definitions of Bykhovskaya-Pavlovskaya (1962) and his own monographs of (1959, 1971) as out of date.

I. erraticus was initially described as *Amphistoma erraticum* Rudolphi, 1809 and has remained historically unchallenged. The validity of the other small (<4.5mm) species *I. pileatus* has similarly not been questioned, although it was referred to as *C. variegatus* by Szidat (1929b) and, in re-establishing its identity, Dubois (1938) attributed to it the

erroneous synonym *C. variegatus*. The remaining species of the genus are large, being greater than 5mm in length. Two such species were originally identified: *Amphistoma platycephalus* Creplin, 1825, located within the cloaca of the definitive host; and *A. variegatus* Creplin, 1825 within the host's intestine. Subsequently, a number of large forms were described, some of which are now confidently synonymised, while others are still considered discrete species by certain authors (for reviews, see Odening, Mattheis & Bockhardt, 1969, 1970; Odening & Bockhardt, 1971; Mattheis & Odening, 1980). At present the most widely accepted composition of the genus is that proposed by Odening (1979), who ascribed four species: *I. platycephalus* [syns: *C. cucullus* (Thoss, 1897); *C. communis* (Hughes, 1928)]; *I. variegatus* [syn. *C. cumultitestis* (Dubois, 1962)]; *I. erraticus*; and *I. pileatus*. Notable variations on the structure of the genus are those of Dubois (1978) and McDonald (1981) who both maintained that *I. variegatus* is a synonym of *I. platycephalus,* and Shigin (1983), who retained *C. cucullus* as a valid species.

Detailed descriptions for *I. erraticus* adults were provided by Dubois (1968), Niewiadomska & Kozicka (1970), Fraser (1974); for *I. variegatus* by Dubois (1968, as *Cotylurus cumultitestis* Dubois, 1962), Odening & Bockhardt (1971); for *I. platycephalus* by Dubois [1968, as *C. platycephalus communis* (Hughes, 1928) La Rue, 1932 and as *C. platycephalus platycephalus* (Creplin, 1925) Szidat, 1928]; and for *I. pileatus* by Dubois (1968).

The number of species comprising the subgenus *Apatemon* is unclear due to the absence of life-cycle data available (all life-stages are known only for *A. gracilis*) and the presence of only a single adult morphological criterion enabling subgeneric allocation (poorly developed nature of the genital cone, according to Dubois, 1968). McDonald (1981) listed eight species from waterfowl, based on the descriptions of Dubois (1968), Dubois & Angel (1972) and Dubois & Nassi (1977). However, three of these species are given as *species inquirendae* by Dubois (1968), and of the remaining

five, only *A. gracilis*, *A. graciliformis* and *A. fuligulae* are known to have piscine second intermediate hosts. Two additional species, *A. annuligerum* and *A. buteonis*, with the adults in birds of prey, are also considered members of this subgenus (*inter alia* Dubois, 1968; Odening, 1970; Dubois, 1974), although only the former has been found to have metacercariae in fish.

Detailed descriptions of *A. gracilis* were provided by Hoffman (1959), Vojtek (1964a), Dubois (1968), Blair (1974); *A. annuligerum* by Dubois (1974); *A. graciliformis* by Dubois & Nassi (1977); *A. fuligulae* by Yamaguti (1933), Dubois (1968); and *A. somateriae* (2 subspecies) and *A. buteonis* by Dubois (1968).

MATERIALS AND METHODS

3.1. CULTURE OF METACERCARIAE TO ADULTS.

3.1.1. Collection and maintenance of birds.

Herring gulls and lesser black-backed gulls were selected as experimental hosts for *Ichthyocotylurus* adults, as both are widely reported as natural hosts and have previously been successfully utilised experimentally for this genus of parasite (see Chapter 2.1; Introduction). The availability of other piscivorous birds for experimental purposes, which may be considered more effective definitive hosts for particular *Ichthyocotylurus* species, was limited by wildlife protection acts. Due to the unavailability of mergansers for use as experimental hosts, it was decided that infections with *A. gracilis* metacercariae would be attempted in mallard *Anas platyrhynchos* var platyrhynchos, domestic and eider ducklings. No birds of prey were available for experimental work with *A. annuligerum* and infections were attempted using the same avian hosts as those employed for *A. gracilis* and *Ichthyocotylurus* spp.

Gulls.

During the summers of 1992 and 1993 a mixture of herring gull and lesser black-backed gull eggs were collected from the island of Inchmickery in the Firth of Forth. The collections were carried out under a Scottish Natural Heritage (SNH) licence and assisted by SNH staff.

The gull eggs were transported as quickly as possible to Stirling within an insulated box, where they were placed in an incubator at 39±1°C with a relative humidity of 70%. Eggs were manually rotated three or four times per day. After hatching, the chicks were maintained in the incubator until their feathers had dried. Young birds were then placed under a heater lamp in an artificial nest made from pressed paper in a plastic tank. Chicks which hatched out at the same time were kept together. Later, the artificial nests and young birds were moved into separate indoor

cages with solid floors. Chicks were maintained on a diet of cooked white fish (hokey or coley) mixed with cooked sardines and a small amount of bran. Young chicks were hand fed four times per day, whilst older birds were able to feed themselves. Water was provided *ad libitum* and once per day it was supplemented with multivitamins.

Ducklings

Day-old domestic (Aylesbury) ducklings were obtained from a small hatchery in Ayrshire and day-old mallards from a wildlife centre in Stirlingshire. The ducklings were maintained in small indoor pens and were fed *ad libitum* on a soaked commercial chick meal supplemented with cod liver oil.

Mallard eggs were also obtained from the wildlife centre in Stirlingshire. These eggs were incubated under the same conditions as the gull eggs and on hatching were treated as the day-old ducklings.

Eider duck eggs were collected from the Sands of Forvie Nature Reserve, Aberdeenshire under a SNH licence and assisted by SNH staff. Eider eggs were incubated at a temperature of 41±1°C.

3.1.2. Administration of metacercarial cysts.

The sources of metacercariae were the same as those detailed in Chapter 2 and are listed below.

I. erraticus: Loch Lomond powan *Coregonus lavaretus*, River Almond rainbow trout *Onchorhynchus mykiss* and Lake Kitka whitefish *C. lavaretus.*

I. variegatus: Loch Winnoch, Lake Kitka and Bothnian Bay perch *Perca fluviatilis* and Loch Lomond and Lake Kitka ruffe *Gymnocephalus cernuus* (Loch Lomond ruffe material from 3 different sites within the host).

I. pileatus: Lake Kitka ruffe.

A. gracilis: River Almond rainbow trout (introduced to all experimental hosts), salmon parr *Salmo salar* (introduced to mallards) and stone loach *Barbatula barbatulus* (introduced to mallards)

A. annuligerum: Loch Lomond perch.

Unfortunately, insufficient numbers of *I. platycephalus* metacercariae were obtained to enable experimental infections. Details of the infections performed are indicated in Tables 27-32; chicks/ducklings were given an identification number to enable a direct comparison of egg production, percentage recovery of adults and their distribution within the host.

Excised metacercarial cysts were administered to the experimental avian hosts either by admixture with food or by gavage. The gulls fed readily from the hand and a known quantity of metacercariae were added to food and given to the chicks. Often whole tissues (heart, pericardium or swimbladder) with adhered cysts were offered, but occasionally loose cysts were pipetted onto and then wrapped in the food material. The ducklings were infected by placing the parasites into the oesophagus using a piece of plastic tube connected to a 5ml syringe bearing metacercarial cysts in physiological saline. In all cases the birds were observed following infection to ensure that the metacercariae were not regurgitated.

3.1.3. Examination of faeces for eggs and egg counts.

Birds were removed individually from their enclosures daily at 1pm and faecal samples collected from the substrate using a spatula or pipette as appropriate. The volume of material was measured, enabling the later calculation of egg number per ml of faeces, and then suspended in distilled water. The faeces were gently homogenised for approximately one minute before being sieved through a series of micro-pore meshes. Large faecal material was removed when the sample was washed through 150µm and then 100µm meshes. The eggs in the remaining suspension were then

retained using a 45μm mesh and flushed free of any residual faecal matter using fresh distilled water. The eggs were washed off the mesh into a large petri dish, where they were isolated and placed onto a Sedgewick Rrafter grid for counting. When numbers of eggs recovered were too large to count accurately, a proportion of the evenly distributed monolayer covering the grid was removed, counted separately and the total calculated.

Infections in several mallard ducklings which failed to produce eggs before day 7 p.i. were considered to have failed and these birds were subsequently reinfected using other sources of metacercariae, as gravid worms were not found to be present in similar cases. The re-infections were all performed in excess of 21 days after their initial exposure and after treatment with the anthelminthic, Panacur (Fenbendazole).

3.1.4. Recovery of adults.

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

After killing the bird, the digestive tract (from duodenum to cloaca) was removed and placed in a large petri dish containing a small amount of physiological saline. The intestine was divided into 5cm sections, placed into individual small petri dishes containing saline and examined in sequence. Each section was opened longitudinally under the dissecting microscope and faecal matter flushed free of the surface with a pipette. It was then possible to observe whether the adults were free in the lumen or attached to the villi of the gut. Unattached adults were removed using a pipette and loosely attached individuals were displaced with a brush. Most worms were still firmly adhered and it was often necessary to nip off the villus with fine forceps to prevent damage to the parasite. All worms were briefly washed in saline to remove debris and then rapidly fixed in either Berland's fluid or cacodylate buffered 3%

glutaraldehyde while still alive.

The number and percentage of worms recovered, their position in the host's intestine and whether or not they were attached were all recorded. Regression analyses were applied to indicate if significant relationships existed between percentage recovery of worms and the variables of: chick age on infection; number of metacercariae administered; and duration of infection.

3.1.5. Development of the adult hindbody.

The hindbody was considered to start at the posterior margin of the proteolytic gland and extend to the posterior tip of the worm, as indicated in Fig. 41. This dimension was measured from light microscope preparations of adults excised at different periods p.i. and used as an indicator for the time required to attain full-maturity (maximum length achieved). The measurement was recorded with the aid of Kontron image analysis equipment linked to an Olympus BH2 microscope. This apparatus enabled a line to be drawn along the long axis of a projected image of the structure, using a light pen. The line was compared to a known standard distance by the computer and subsequently converted into the actual length.

3.2. TAXONOMY OF ADULTS.

3.2.1. Light microscopical observations of adults.

Adults were recovered from the intestines of experimental hosts, as described above in 3.1.4. Live specimens were fixed in Berland's fluid for about 2 minutes before being transferred to 80% alcohol for storage. Worms for light microscope preparations were selected at random, stained in Mayer's paracarmine, cleared in Beechwood creosote and mounted in Canada Balsam. Due to the size of *I. variegatus* adults, it was necessary to support the coverslip in order to cause the minimum amount of specimen distortion. Morphometric measurements recorded for the strigeid adults are shown in Fig. 41. The measurements were taken on an Olympus BH2 microscope from specimens

mounted in lateral view, although dorso-ventral mounts were also made which aided the observation of certain structures. Hindbody length and the distance from the ovary to the forebody were measured as described in 3.1.4, hindbody breadth was recorded at the anterior margin of the ovary, and sucker dimensions were taken along their own axes. In addition to the measurements indicated in Fig. 41, the distances from the anterior margins of the oral and ventral suckers to the lip of the forebody were recorded for *I. variegatus* adults.

3.2.2. Scanning electron microscopical observations of *Ichthyocotylurus* adults.

Experimentally raised *I. erraticus* and *I. variegatus* specimens were washed in saline to remove debris from the host's intestine and fixed in cacodylate buffered 3% glutaraldehyde while still alive. The material was then processed, mounted and observed as described for metacercariae in Chapter 2.2.3; Materials and Methods. Paucity of *A. gracilis* adults precluded SEM examination of their surface structures.

3.2.3. Discrimination of adults by Principal Components Analysis of metrical features.

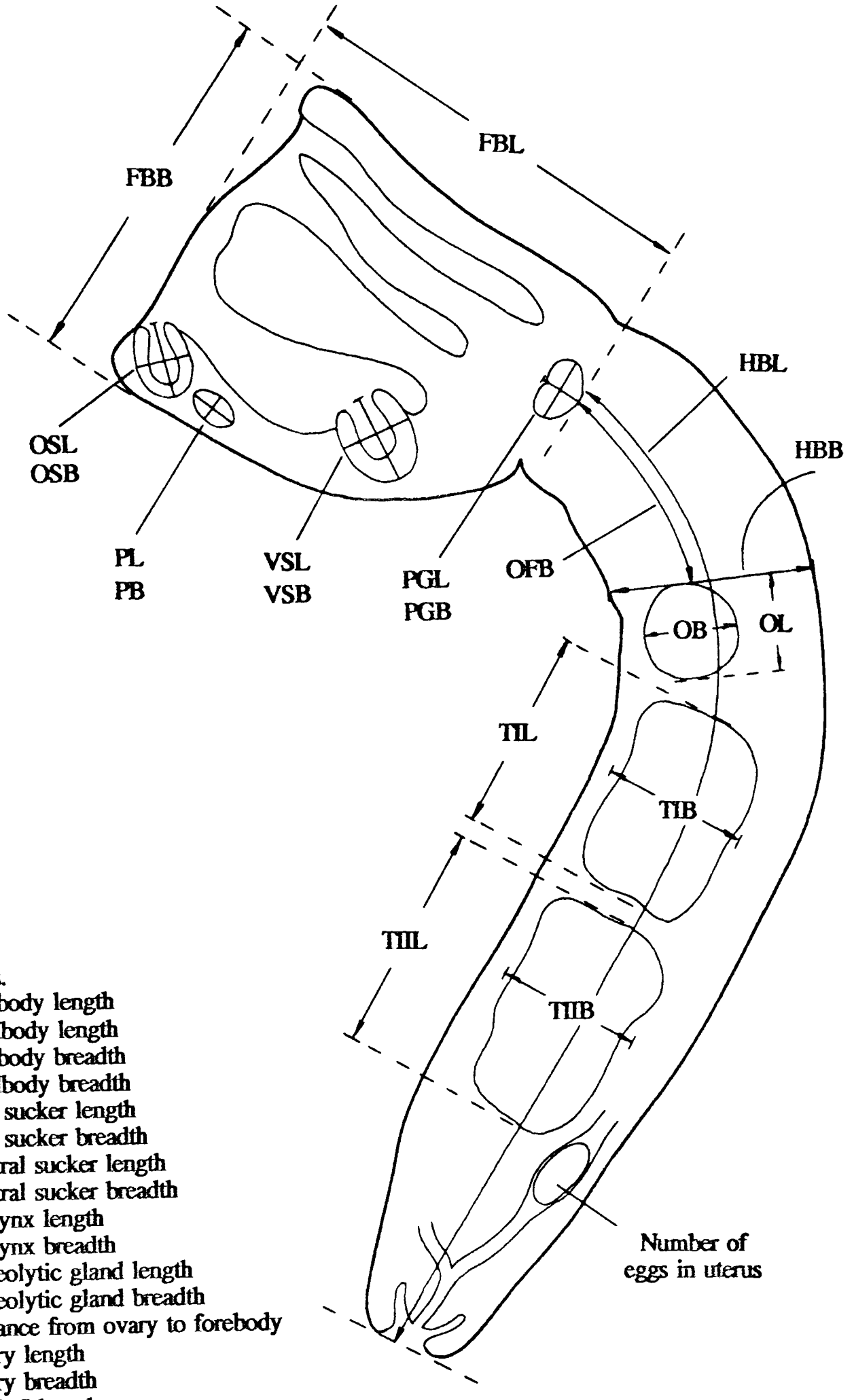
Morphometric analyses were performed as described in Chapter 2.2.4; Materials and Methods. Measurements were taken from light microscope preparations of 36 *I. erraticus*, 33 *I. variegatus* and 15 *A. gracilis* adults. The metrical features (variables) used in the analyses are shown in Fig. 41. Dimensions of the oral sucker and pharynx of *I. variegatus* specimens were not included in the analysis of this species, as a full data set of these features was not attainable (structures were often obscured by a host villus within the forebody). The proteolytic gland dimensions were not measured for either *Ichthyocotylurus* spp.

Analysis 1. *I. erraticus* adults. 20 specimens raised from Loch Lomond powan metacercariae and 16 specimens raised from Lake Kitka whitefish metacercariae. Both groups of adults were excised from their experimental hosts at 14 days p.i.

Analysis 2. *I. variegatus* adults. 10 specimens raised from Loch Lomond ruffe metacercariae (14 days p.i.), 14 specimens raised from Loch Winnoch perch metacercariae (14 days p.i.) and 9 specimens raised from Lake Kitka perch metacercariae (15 days p.i.).

Analysis 3. *A. gracilis* adults. 6 specimens raised from rainbow trout metacercariae (5 days p.i.), 4 specimens raised from salmon parr metacercariae (4 days p.i.) and 5 specimens raised from stone loach metacercariae (5 days p.i.). All metacercarial hosts (fish) were obtained from the River Almond, Scotland.

Fig. 41. Morphometric measurements recorded for strigeid adults.



Abbreviations.

FBL.	Forebody length
HBL.	Hindbody length
FBB.	Forebody breadth
HBB.	Hindbody breadth
OSL.	Oral sucker length
OSB.	Oral sucker breadth
VSL.	Ventral sucker length
VSB.	Ventral sucker breadth
PL.	Pharynx length
PB.	Pharynx breadth
PGL.	Proteolytic gland length
PGB.	Proteolytic gland breadth
OFB.	Distance from ovary to forebody
OL.	Ovary length
OB.	Ovary breadth
TIL.	Testis I length
TIB.	Testis I breadth
TIIL.	Testis II length
TIIB.	Testis II breadth

RESULTS

3.1. CULTURE OF METACERCARIAE TO ADULTS.

3.1.1. Analysis of faecal egg counts.

Faecal egg counts were monitored throughout the infection in gull chicks (Tables 27 and 28) and ducklings. Egg counts were used as an assessment of the establishment of the infection in the birds, the maturation age of the adults, fecundity, peak egg production and longevity. Due to maintenance conditions of the birds, it was not possible to determine the total egg production.

***Ichthyocotylurus* spp.**

Attempted infections of ducklings with *Ichthyocotylurus* metacercariae failed to yield any eggs in the faeces of experimental hosts (Table 31).

I. erraticus

All experimental infections with *I. erraticus* metacercariae in gull chicks resulted in the establishment of adults. Table 27 shows the number of eggs recovered daily per 1 ml sample of faeces. Egg production typically began at 4 days post-infection (p.i.). The number of eggs recovered then increased rapidly, attaining a peak at around 8 days p.i. before dropping steadily. By day 20 p.i. egg release was minimal and was observed to last until day 45 p.i. in one individual (Fig. 42).

The number of eggs recovered from birds infected with metacercariae excised from Loch Lomond powan initially appeared to be proportional to the number of cysts administered. This relationship can be seen in Table 27, with the higher intensity challenges (400 cysts) averaging approximately 3 times the egg production of the lower intensity challenge (150 cysts). However, as the course of the infection progressed (from about 2 weeks p.i.), this relationship was lost. Table 27 also indicates that the single infection using metacercariae obtained from rainbow trout yielded higher numbers of

eggs per cyst introduced than the powan material, while Finnish whitefish cysts yielded lower numbers of eggs per cyst administered.

I. variegatus

Experimental single infections in gull chicks with *I. variegatus* metacercariae were all successful. However, in 3 mixed infections of *I. variegatus* and *I. pileatus*, metacercariae of both species failed to establish. The results of faecal egg counts for gull chicks infected with *I. variegatus* metacercariae are given in Table 28. Egg production began after a prepatent period of 1-4 days. The number of eggs released increased to a maximum at around day 12 p.i. (Fig. 42). In a single monitored bird, eggs stopped appearing in the faeces at 31 days p.i. Table 28 indicates that the number of eggs recovered from infected birds per cyst administered was comparable, regardless of fish host (ruffe/perch) and geographical origin (Scotland/Finland).

The number of eggs produced per introduced metacercaria was markedly higher for *I. variegatus* than *I. erraticus*, as can be seen in Fig. 42. Although a larger proportion of *I. variegatus* adults were recovered, (see below, 3.1.2; Recovery of adult worms) this increase in egg recovery was predominantly due to their greater fecundity (see 3.2.1; Light microscopical observations of adults).

Apatemon spp.

A. gracilis

It was hoped that experimental infections would be performed on eider ducklings, as well as on domestic ducklings and mallards, but all eggs obtained proved to be non-viable.

Only a small number of eggs were ever recovered from ducklings infected with cysts removed from salmon parr or rainbow trout and, typically, these infections produced no eggs (Tables 31 and 32).

All infections with *A. gracilis* cysts removed from stone loach established in

mallard ducklings. The onset of egg release was on day 4 p.i. and peak production was rapidly attained; the duration of egg release was short, stopping at about day 15 p.i. in a single monitored duckling (Table 32).

A. annuligerum

A. annuligerum cysts from the eyes of Loch Lomond perch were administered to a gull chick and a mallard duckling (Table 29). No eggs were recovered from either infection.

3.1.2. Recovery of adult worms.

Ichthyocotylurus species

No adult worms were recovered from experimental infections in ducklings (Table 31). Single infections in gull chicks were largely successful, adults generally being recovered unless the infection was allowed to continue to take its full course (i.e. until egg production ceased). However, only a single worm was obtained from 3 mixed infections of *I. pileatus* and *I. variegatus* metacercariae (Table 30).

I. erraticus

Details of the recovery of *I. erraticus* adults from experimentally infected gull chicks are provided in Table 29. The mean recovery of worms was 26% (range: 0-62%).

Regression analyses were performed on the percentage recovery of adults compared to the age of the gull chick at infection (Fig. 43) and the number of metacercariae administered (Fig. 44). These graphs show that there is no linear relationship between these variables ($P>0.1$). However, due to the limited number of experimental hosts available, it was impossible to compare each variable totally in isolation and consequently pertinent trends may have been obscured.

The greatest return of worms was found when an infection was terminated at the

onset of egg production (day 5 p.i.: 68% of worms recovered) and numbers of adults obtained after 24 days p.i. was minimal. The gull chick sacrificed when egg production had ceased (day 45 p.i.) was found to be free from infection. However, no significant correlation ($P>0.1$) was demonstrable between duration of infection and percentage recovery of adults (Fig. 45).

The percentage recovery of adults developing from Finnish whitefish metacercariae (23%) was slightly lower than that recorded for Scottish powan metacercariae (32%) in a comparible infection. The high return of eggs (per metacercaria administered) from rainbow trout derived adults is explained by their large percentage recovery (41%).

I. variegatus

Table 30 provides details of the recovery of *I. variegatus* adults from experimentally infected gull chicks. The mean recovery of worms was 40% (range 0-91%). No significant correlations were recorded between the percentage recovery of adults and the age of the host at infection, the number of metacercariae introduced or the duration of the infection (Figs 43-45).

A slightly higher recovery rate was recorded for adults originating from cysts excised from perch (45%) than from ruffe (35%). Metacercariae from Scottish and Finnish perch were equally successful in establishing, with 44% and 46% recovered as adults, respectively. Infections were also performed using cysts excised from 3 locations within a single ruffe (swimbladder, pericardial cavity and orbit). All 3 infections established, but metacercariae originating from the pericardial cavity appeared twice as successful as those from the other sites.

The single infection allowed to continue to its conclusion (end of egg production) was halted at 31 days p.i.; no *I. variegatus* adults were recovered.

Apatemon species

A. gracilis

No adults were obtained from a single gull chick infected with 313 *A. gracilis* metacercariae removed from rainbow trout, and very few adults were recovered from experimental infections utilising Aylesbury or mallard ducklings as definitive hosts. The age of the experimental bird at infection (1 to 77 days) was not found to affect the establishment or recovery of adults. Adults were only ever retrieved from infections that were terminated at or before day 5 p.i.

Of the 3 fish hosts bearing *A. gracilis* metacercariae, infections using cysts excised from salmon parr were the least successful, with adults recovered from 17% of birds. Ducklings infected with metacercariae from stone loach (mallards) and rainbow trout (Aylesburys) yielded adults in 40% of attempted infections. The recovery of adults recorded from infections with material of the latter 2 sources may be artificially low as several infections were allowed to continue past 5 days p.i. in order to harvest, or attempt to harvest, eggs and no adults were ultimately obtained. In all successful *A. gracilis* infections the percentage recovery of adults was low (7-24%).

The results suggest that in these experimental avian hosts *A. gracilis* metacercariae may begin development but often fail to achieve maturity. The highest returns of adults were obtained when infections were stopped on days 3 (19%) and 4 (24%) p.i. In both these cases the adults were still immature (see 3.1.4; Results).

A. annuligerum

No adults were recovered from the infections attempted using *A. annuligerum* metacercariae (Tables 29 and 31).

3.1.3. Site specificity of adults within experimental definitive hosts.

The vast majority of all worms recovered from experimental infections were alive and firmly attached to a villus within the host's gut. Only worms attached in such

a manner were included in the analyses of adult distribution shown in Figs 46, 47. Bird identification numbers in Tables 29-32 correspond to those given in Fig. 46, enabling cross-referencing of infection regimes and distribution of worms within the experimental definitive host.

Both *Ichthyocotylurus* spp. were absent from the duodenum of infected birds (Figs 46, 47). Fig. 46 demonstrates that *I. variegatus* adults were recovered from the distal half of the small intestine and, in 9 out of the 11 infections, from the rectum. The distribution of *I. erraticus* adults was wider than that recorded for *I. variegatus*, specimens being found in the distal 80% of the small intestine, often in the rectum and in a single case the cloaca (Fig. 46). However, of 551 *I. erraticus* adults recovered, 507 (92%) were located within the last 40% of the small intestine and 82% of these within the terminal 20% (Fig. 47). Although 4 of 7 *I. erraticus* infections extended into the rectum (Fig. 46), only 16 worms (0.3%) were recovered from this region (Fig. 47). The origin of the *I. erraticus* metacercariae (species of fish host/ geographical sample site) was not seen to affect the distribution of worms within the definitive host (Table 29 and Fig. 46). *I. variegatus* adults were also most abundant in the distal 20% of the small intestine (74% of 797 worms), but this species was also commonly present in the host's rectum with 11% of the total number of worms recovered from this region (Fig. 47). The distribution of adult *I. variegatus* worms did not appear to be affected by metacercarial location within the fish host (Table 30 and Fig. 46; gull infections 4, 5 and 6: metacercariae removed from different sites within a single ruffe). The species of fish host (ruffe/gull infections 1-6, or perch/gull infections 7-10 and 14-15; Table 30 and Fig. 46) from which *I. variegatus* metacercariae were obtained also had little influence upon the attachment site of the adult. Nevertheless, the two infections not extending into the host's rectum both involved metacercariae excised from perch and in these cases adults were present more proximally in the small intestine (Table 30, Fig. 46; gull infections 8 and 15).

Unlike *Ichthyocotylurus* spp., Fig. 47 shows that *A. gracilis* adults were located

proximally within the digestive tract of their experimental hosts, with 26% of adults recovered from the duodenum and 63% from the first 5th of the small intestine. No worms were found beyond the first 40% of the host's small intestine. Adults developing from metacercariae excised from rainbow trout were found almost exclusively within the small intestine (24 of 25 specimens), salmon parr derived adults were located within the duodenum, while adults obtained from stone loach metacercariae were situated in both regions (Tables 31, 32 and Fig. 46).

3.1.4. Development of the hindbody of the worms.

Adults developing from Scottish powan (41 specimens) and Scottish perch (31 specimens) metacercariae were used as a guide to the hindbody development of *I. erraticus* and *I. variegatus*, respectively. Fig. 48 shows the hindbody length of worms at different periods p.i. The hindbody of *I. erraticus* adults develops rapidly, attaining 87% of its maximum size by day 5 p.i. and its full length by day 14 p.i. In the mature worm the hindbody length was found to correlate strongly with the total body length ($p< 0.001$), constituting 86% of overall length (Fig. 49). It is interesting to note that the position of the *I. erraticus* adult within the small intestine of its host appears to affect the development of its hindbody (Fig. 50). Worms recovered from progressively posterior regions of the intestine possessed proportionally larger hindbodies ($P< 0.1$). However, the number of eggs present in the uterus of adults was not found to correlate significantly with intestinal location (Fig. 51) or hindbody length (Fig. 52).

Fig. 48 indicates that hindbody development in *I. variegatus* adults is initially less rapid, with only 55% of the maximum length attained at day 5 p.i. However, by day 8 development appears to be complete, having reached 97% of the maximum length recorded in day 19 p.i. adults. The hindbody of mature *I. variegatuus* adults was also found to correlate with total body length ($p< 0.01$), comprising 76% of overall length (Fig. 49).

A. gracilis adults were only recovered when infections were terminated at or

before day 5 p.i., and hence it was not possible to monitor hindbody growth or assess whether full development was achieved. However, with rainbow trout derived adults in domestic ducks, it was observed that worms obtained at 3 days p.i. were not mature, being non-ovigerous, smaller than 5-day-old adults (78% of total body length) and with their hindbodies accounting for 50.4% of total body length compared to 62.6% at day 5 p.i.

Adults obtained from rainbow trout metacercariae only ever attained 53% of the total body length of those derived from stone loach metacercariae. The proportion of hindbody to total body length was also greater for adults developing from stone loach metacercariae (68.7% at day 5 p.i.). However, these apparent developmental differences may have resulted from the different experimental hosts from which the adults were collected (domestic ducklings for rainbow trout and mallard ducklings for stone loach). Nevertheless, if egg production mirrors developmental state, then this would not be so, as only negligible numbers of eggs were ever recovered from rainbow trout metacercarial infections in either host.

Table 33 shows the mean body lengths and number of eggs *in utero* of the strigeid adults raised. The number of eggs within the uterus of each *I. variegatus* specimen could not be discerned, even at 5 days p.i., due to the large numbers present. *I. erraticus* specimens developing from Finnish whitefish metacercariae attained a greater mean length (4.06mm) and contained larger numbers of eggs (52) than equivalent specimens developing from Scottish powan metacercariae, 3.46mm and 30.4 eggs. Morphological differences between these two adult populations are examined in 3.2; Taxonomy of adults. Although, as stated above, negligible numbers of eggs were collected from certain infections with *A. gracilis* metacercariae, Table 33 shows that adults collected after 3 days p.i. included gravid specimens, regardless of metacercarial origin.

Table 27. Results of faecal egg counts from gull chicks infected with *I. erraticus* metacercariae.

Chick I.D. number	Fish host	Source of fish	Number of cysts administered	Number of eggs collected/ml of faeces at different number of days post-infection (p.i.)											
				1	2	3	4	5	6	7	8	9	10	11	12
1	Powan	Loch Lomond	400	0	0	0	248	1000*							
2	"	"	400	0	0	0	127	363	2584	3120	4380	3450	1280	-	1160
3	"	"	400	0	0	0	395	1700	2700	2400	4300	500	2120*		
4	"	"	400	0	0	0	60	522	2900	5600	3600	4000	3400	-	2400
5	"	"	150	0	0	0	430	365	700	1600	1900	1900	1400	-	700
6	Rainbow trout	Loch Awe	17	0	0	2	36	159	78	298	900	130	-	-	660*
7	Whitefish	Lake Kitka - Finland	200	0	0	1	26	825	912	242	180	290	690	1600	560
8	"	"	200	0	0	0	0	111	420	744	890	190	257	420	550
9	"	"	100	0	0	0	128	155	*						

* day of sacrifice.
- no count taken

Table. 27 (continued). Results of faecal egg counts from gull chicks infected with *I. erraticus* metacercariae.

Chick I.D. Number	Number of eggs collected/ml of faeces at different number of days post-infection (p.i.)											
	13	14	15	17	20	22	24	26	30	35	40	45
1												
2	950	600*										
3												
4	1820	730	1200	540	85	560	95	295	31	39	21	0*
5	860	2400	1140	780	35	10	6*					
6												
7	-	*										
8	550	390	40*									
9												

* day of sacrifice.

Table 28. Results of faecal egg counts from gull chicks infected with *I. variegatus* metacercariae.

Chick I.D. number	Fish host	Metacercarial species	Sample site	Number of cysts administered	Number of eggs collected/ml of faeces at different number of days post-infection (p.i.).							
					1	2	3	4	5	6	7	8
1	Ruffe	*I. variegatus*	Loch Lomond	350	0	0	0	0	600	4900	16700*	
2	"	"	"	200	0	0	8	2	550*			
3	"	"	"	200	0	7	41	320	-	1760	1100	5000
4	"	"	"	100	0	0	4	1	94	-	980	2720
5	"	"	"	100	0	0	2	0	250	-	1850	4100
6	"	"	"	150	0	16	127	820	-	-	150	30
7	Perch	"	Loch Winnoch	200	0	0	1	0	55	840	2400	7600*
8	"	"	"	200	0	9	152	322	730	3400	5650	3800
9	"	"	"	100	0	4	15	37	742	3000	3700	5000
10	"	"	"	200	0	0	12	76	438	590	740	2850
11	Ruffe	*I. variegatus/ I. pileatus*	Lake Kitka, Finland	200	0	0	0	2	2	0*		
12	"	"	"	150	0	0	*					
13	"	"	"	100	0	1	0	*				
14	Perch	*I. variegatus*	"	60	0	0	0	2	750	730	1250	1900
15	"	"	BB**	100	0	0	0	1	456	3300	2400*	

* day of sacrifice. **Bothnian bay, Finland.

Table 28 (continued). Results of faecal egg counts from gull chicks experimentally infected with *I. variegatus* metacercariae.

Chick I.D. number	Number of eggs collected/ml of faeces at different number of days post-infection (p.i.)															
	9	10	11	12	13	14	15	17	19	20	22	24	26	28	30	31
1																
2																
3	-	24000	38000	28000	-	48000	42000	-	5200	-	6400	5000	1100	10	0	*
4	1420	1140	450	-	790	1650*										
5	2380	3650	4400	-	2450	6200	5550*									
6	2250	-	776	3440	2800	1890	4080*									
7																
8	-	-	2830	-	-	3000	4100	2600	3150*							
9	-	8600	9650	-	-	8600	9200*									
10	-	4700	4600*													
11																
12																
13																
14	2600	3900	4300	2500	2100	1750	900*									
15																

* denotes date of sacrifice. - counts not taken.

Table 29. Recovery of adult worms from gull chicks experimentally infected with *I. erraticus*, *A. gracilis* and *A. annuligerum* metacercariae.

Chick I.D. number	1	2	3	4	5	6	7	8	9	10	11
Age at infection (days)	4		3		2	11	16	16	19	26	28
Fish host	Powan					R. trout	Whitefish			R. trout	Perch
Sample site	Loch Lomond					Loch Awe	Lake Kitka, Finland			Perthshire fish farm	Loch Lomond
Metacercarial species	*I. erraticus*									*A. gracilis*	*A. annuligerum*
Metacercarial location within fish host	Ventricle and pericardial cavity										Humour of eyes
Number of cysts administered	400	400	400	400	150	17	200	200	150	313	14
Days post-infection at sacrifice	5	14	10	45	24	12	14	15	6	5	5
State of egg release at sacrifice	onset	down	peak	end	down	peak	down	down	peak	no release	
Number of adults recovered	249	102	104	0	7	7	59	0	23	0	0
Percentage recovery	62	26	26	0	5	41	30	0	15	0	0

Table 30. Recovery of adult worms from gull chicks experimentally infected with *I. variegatus* metacercariae.

Chick I.D. number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Age on infection (days)	2	14	4	4	13	7	2	8	8	9	13	12	12	9	7
Fish host	Ruffe						Perch				Ruffe			Perch	
Sample site	Loch Lomond. Scotland.						Loch Winnoch. Scotland				Lake Kitka, Finland				Bothnian Bay, Finland
Metacercarial species	*I. variegatus*										*I. variegatus/ I. pileatus*			*I. variegatus*	
Metacercarial location in fish host	Sbl	Sbl	Sbl	Sbl	Pcc	Orb	Sbl	Sbl	Sbl	Sbl	Pcc	Pcc	Pcc	Sbl	Sbl
Number of cysts administered	350	200	200	100	100	150	200	200	100	200	200	150	100	60	100
Days post-infection at sacrifice	7	5	31	14	15	15	8	19	15	11	6	4	5	15	7
State of egg release at sacrifice	peak	onset	end	down	peak		up	down	peak		no eggs released			down	peak
Number of adults recovered	317	54	0	12	30	19	63	83	66	70	0	1	0	14	68
Percentage recovery	91	27	0	12	30	13	32	43	66	35	0	0.7	0	23	68

Abbreviations: Swimbladder (Sbl); Pericardial cavity (Pcc); Orbit (Orb).

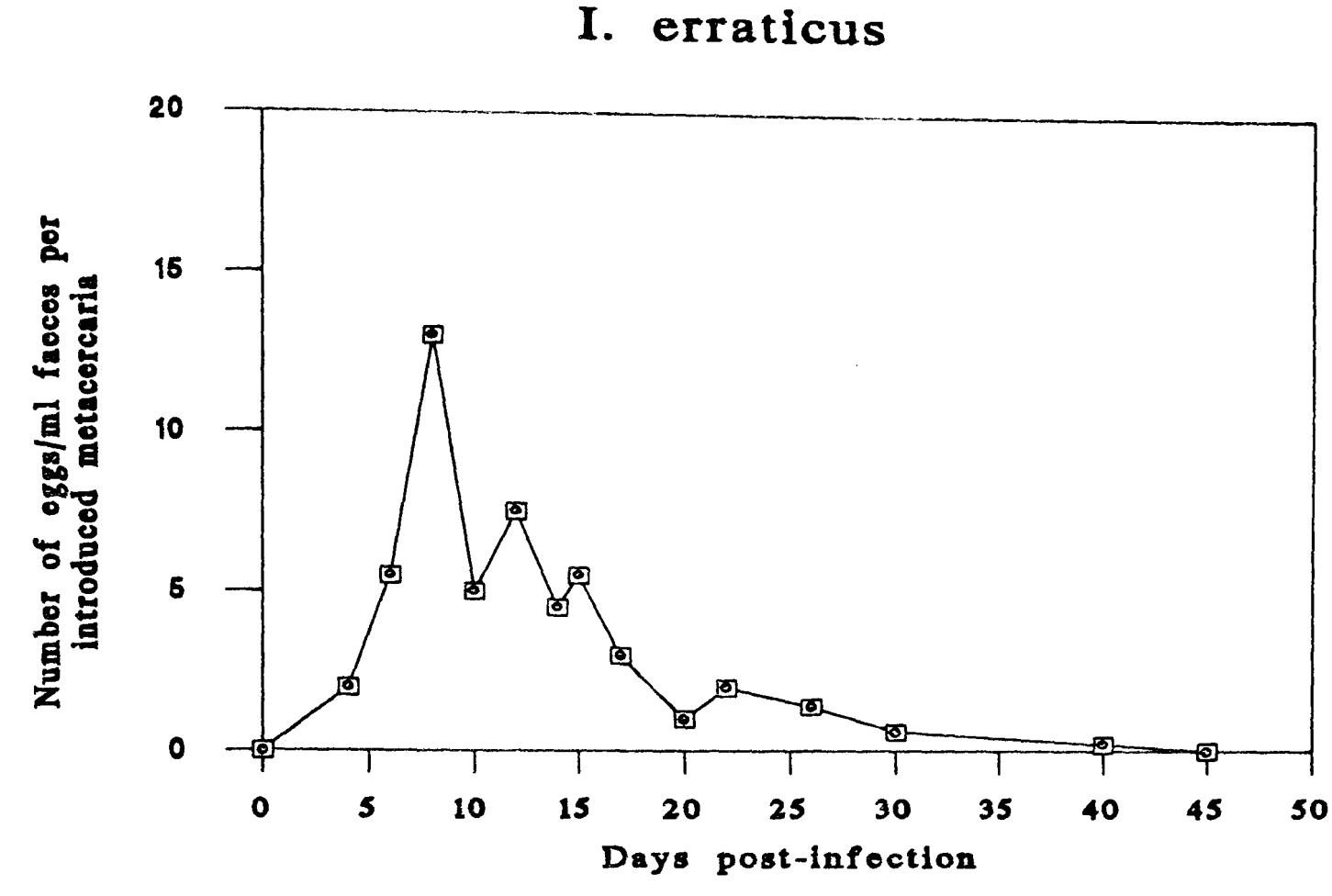
Table 31. Recovery of adult worms from ducklings experimentally infected with *A. gracilis* metacercariae excised from rainbow trout, *I. erraticus* from powan, *I. variegatus* from ruffe and *A. annuligerum* from perch.

Duckling I.D. number	1	2	3	4	5	6	7	8	9	10	10	11	12	9
Bird species	Aylesbury ducklings								Mallard ducklings					
Age on infection (days)	6	9	9	18	27	27	27	43	5	6	23	32	35	44
Metacercarial species	*A. gracilis*					*I. variegatus*	*I. erraticus*	*A. annuligerum*	*A. gracilis*					
Fish host	Rainbow trout					Ruffe	Powan	Perch	Rainbow trout					
Sample site	Perthshire fish farm					Loch Lomond			Perthshire fish farm					
Metacercarial location in fish host	Pericardial cavity					Body cavity	Pericardial cavity	Humour of eyes	Pericardial cavity					
Number of cysts administered	95	95	85	100	90	200	100	12	50	120	35	35	100	100
Days post-infection at sacrifice	12	8	3	7	5	5	5	5	Reinfected					
Number of eggs released	0	0	3	0	0	0	0	0	0	0	0	0	0	0
Number of adults recovered	0	0	16	0	9	0	0	0	-	-	-	-	-	-
Percentage recovery	0	0	19	0	10	0	0	0	-	-	-	-	-	-

Table 32. Recovery of adult worms from mallard ducklings experimentally infected with *A. gracilis* metacercariae excised from stone loach and salmon parr.

Duckling I.D. number	10	9	12	11	13	14	15	16	17	18	19
Age on infection (days)	63	77	56	77	1	3	3	11	13	24	28
Condition of bird on infection	Previously challenged				Naïve						
Fish host	Stone loach		Salmon parr						Stone loach		
Sample site	Perthshire fish farm										
Metacercarial location in fish host	Body cavity and cranial cavity		Body cavity and pericardial cavity						Body cavity and cranial cavity		
Number of cysts administered	14	30	60	60	30	49	30	17	33	4	40
Days post-infection at sacrifice	8	5	5	5	5	5	5	4	5	5	16
Number of eggs released	10	70	0	0	0	0	0	1	234	57	191
Number of adults recovered	0	2	0	0	0	0	0	4	5	0	0
Percentage recovery	0	7	0	0	0	0	0	24	15	0	0

Fig. 42. Effect of duration of infection upon egg recovery from gulls infected with *Ichthyocotylurus* spp.



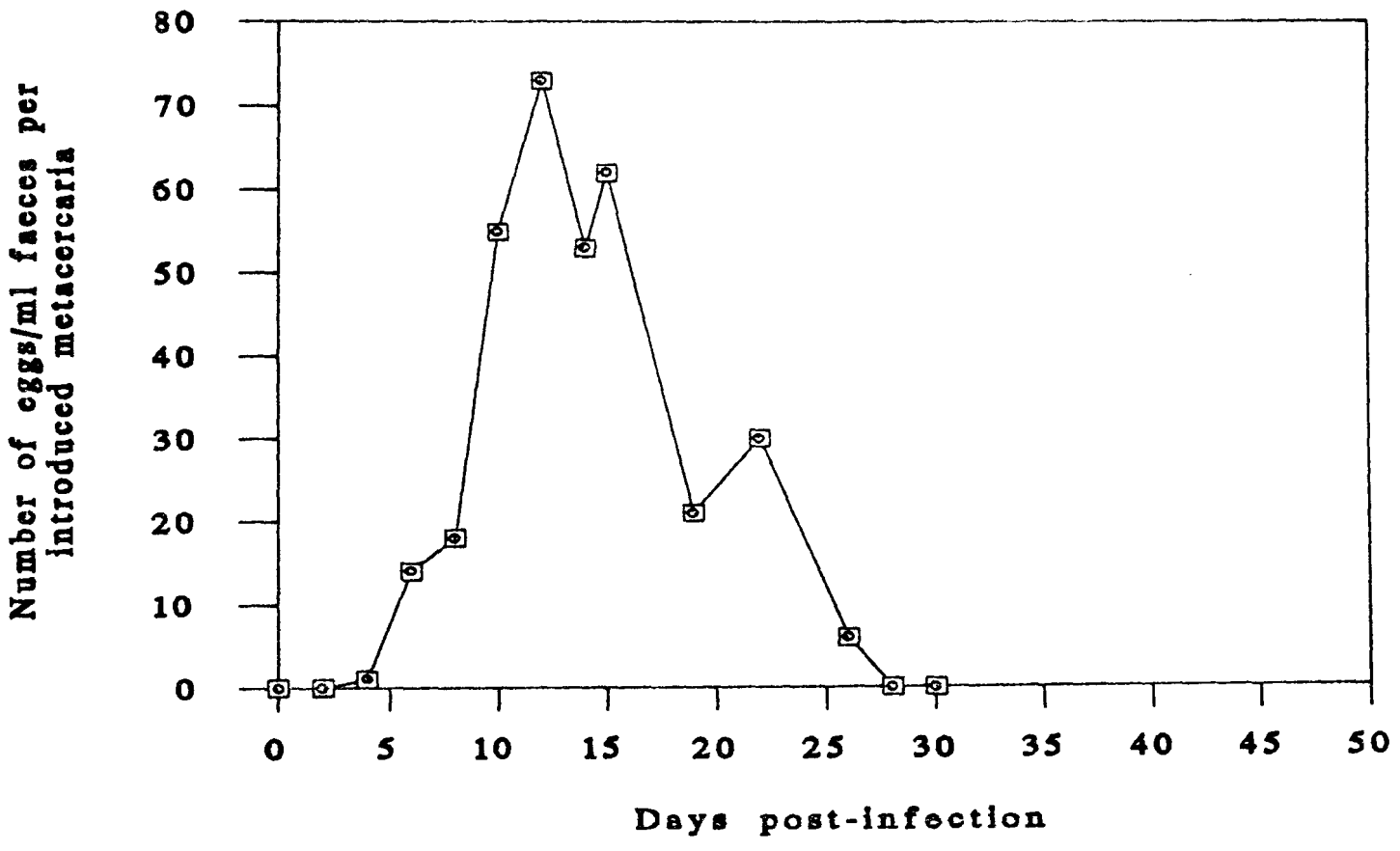


Fig. 43. Effect of gull age at infection on the percentage recovery of adults.

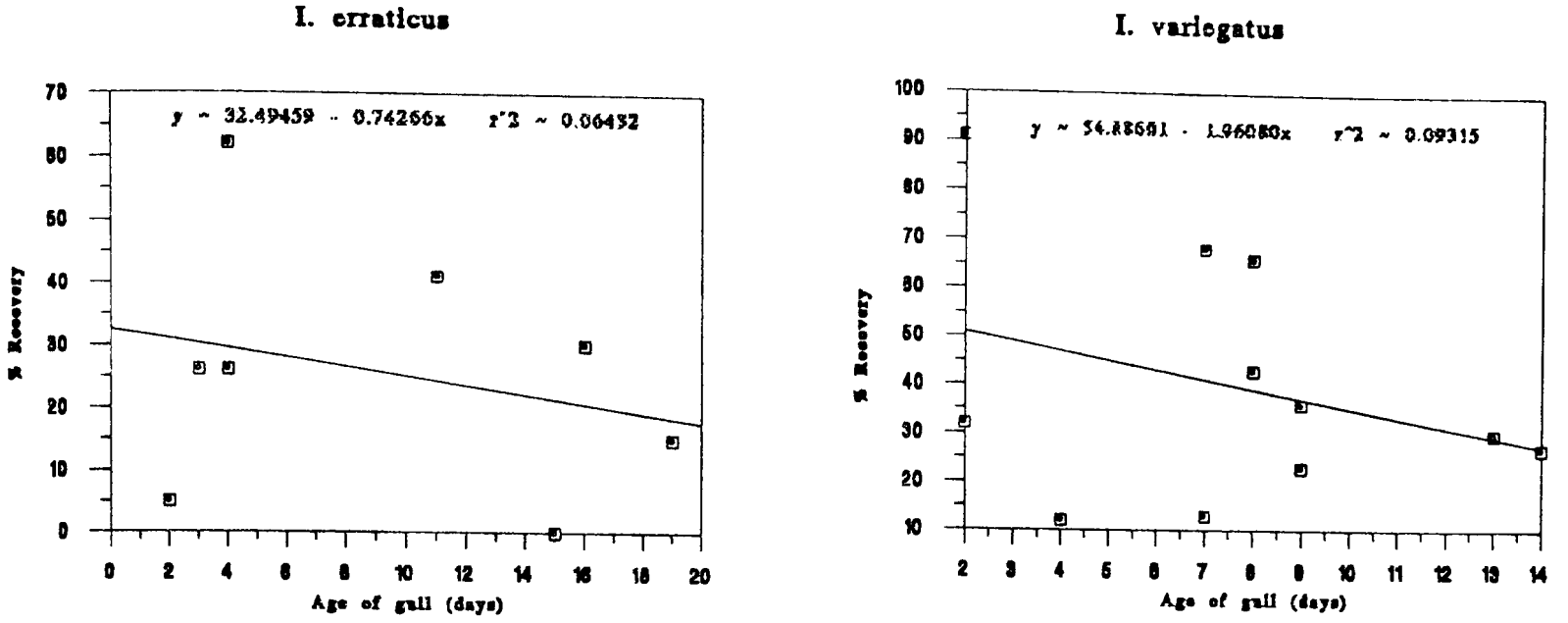


Fig. 44. Effect of the number of metacercariae administered on the recovery of adults.

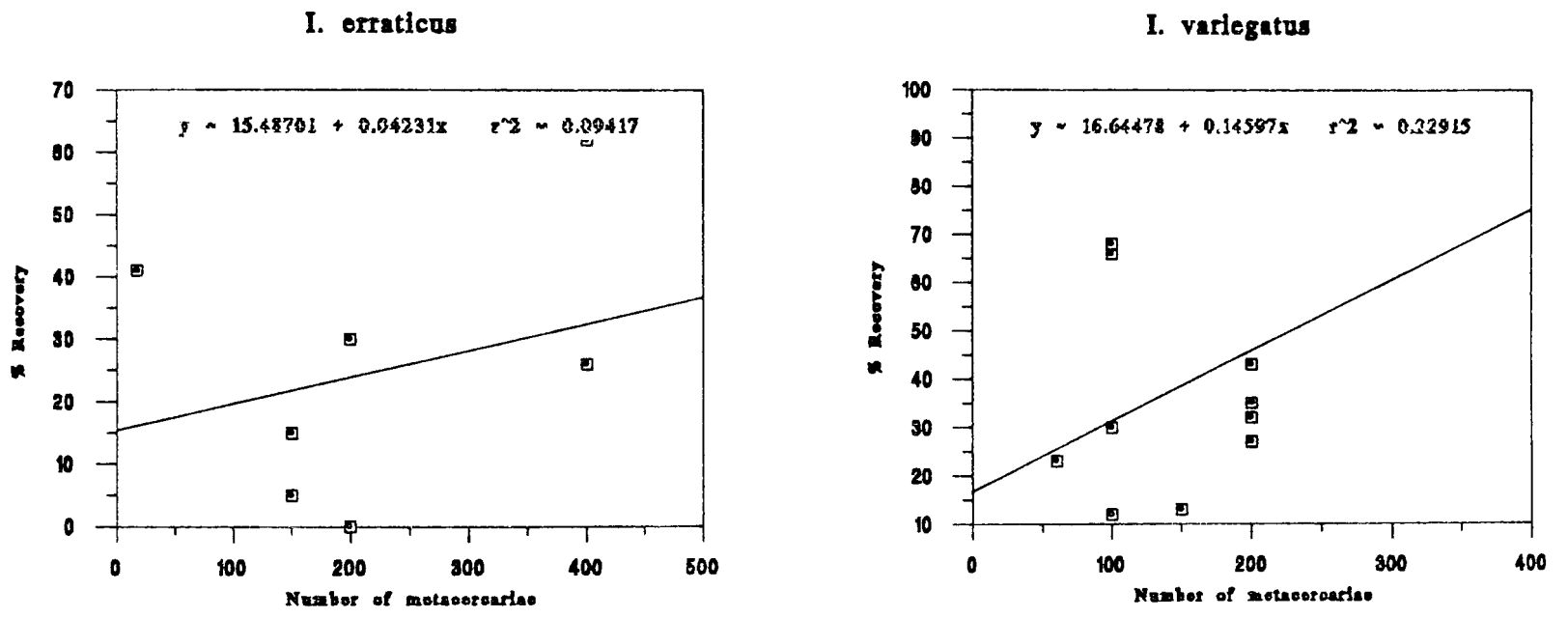


Fig. 45. Effect of the duration of infection on the percentage recovery of adults.

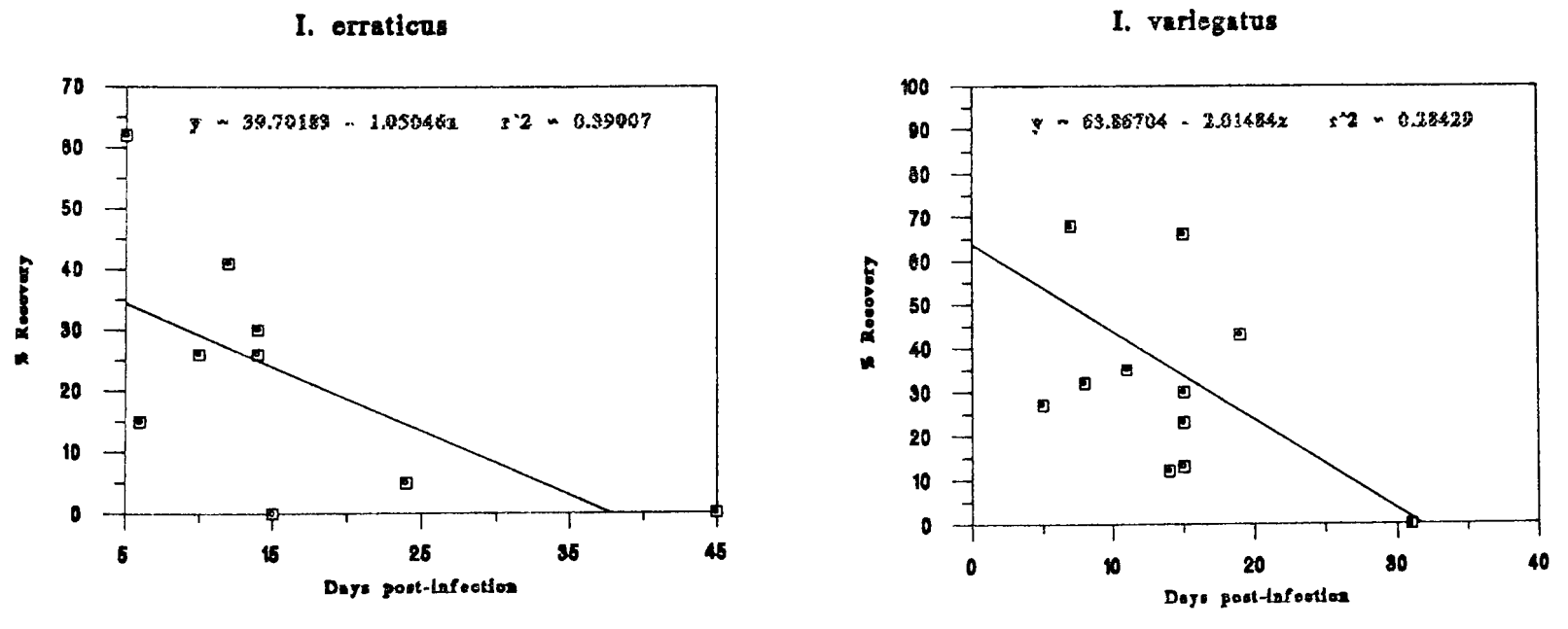
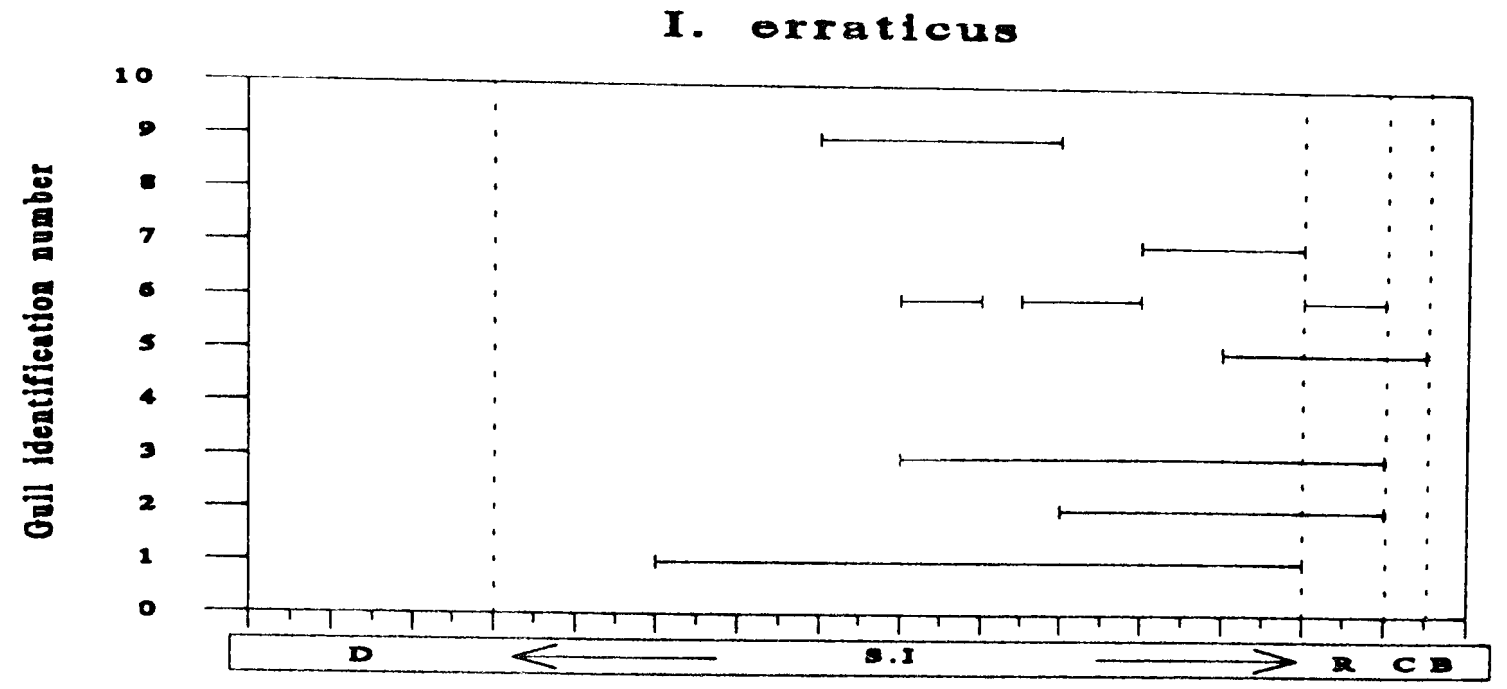
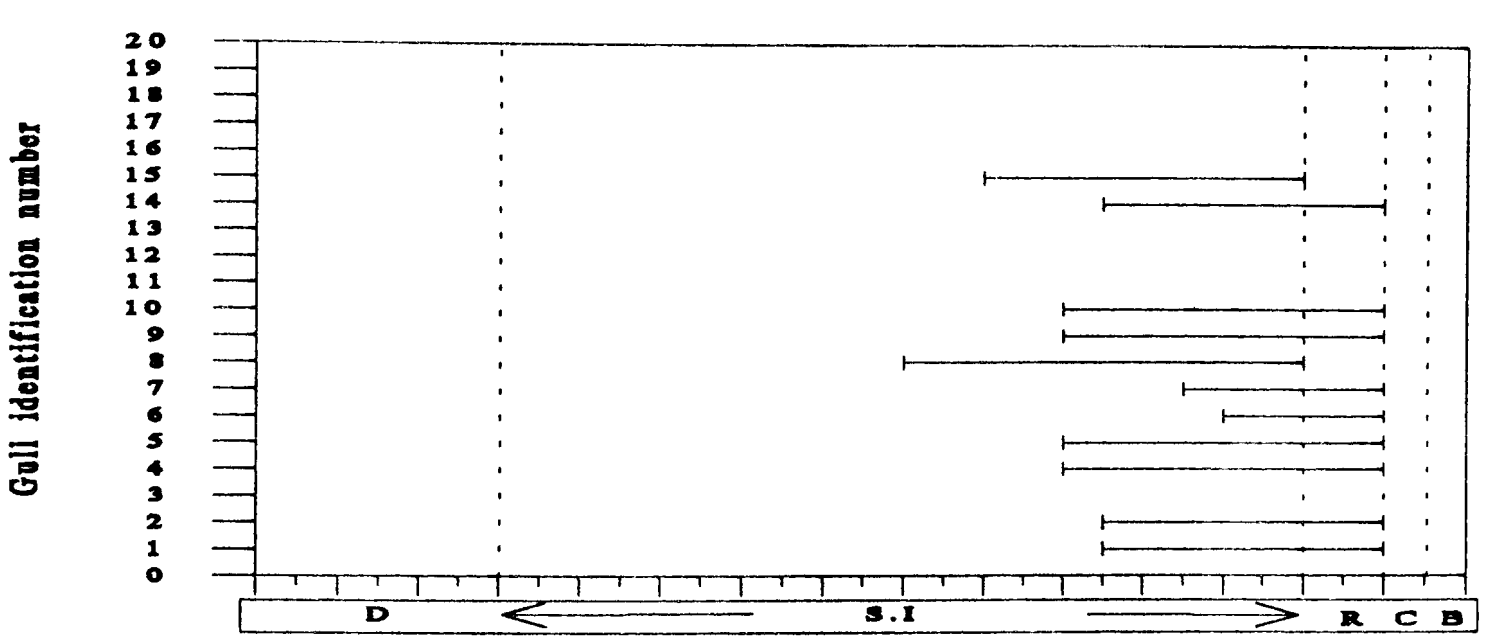
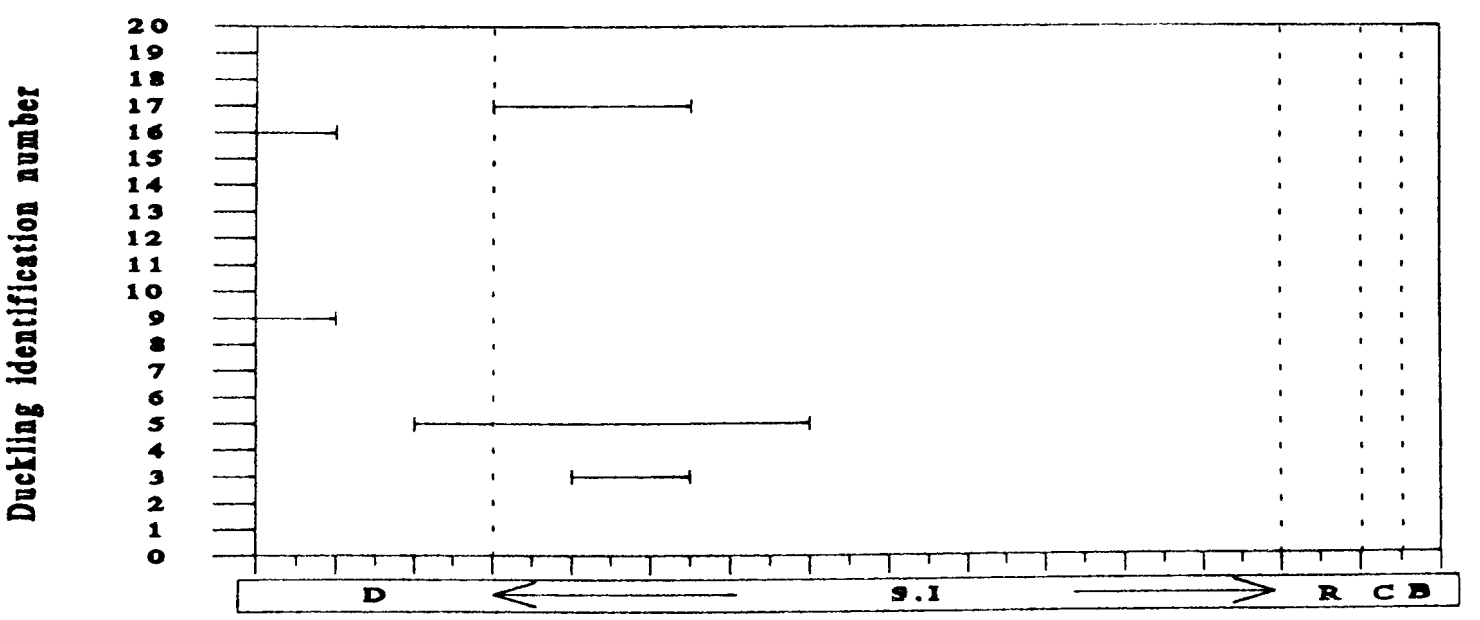


Fig. 46. Distribution of adults within the digestive tracts of experimental hosts.

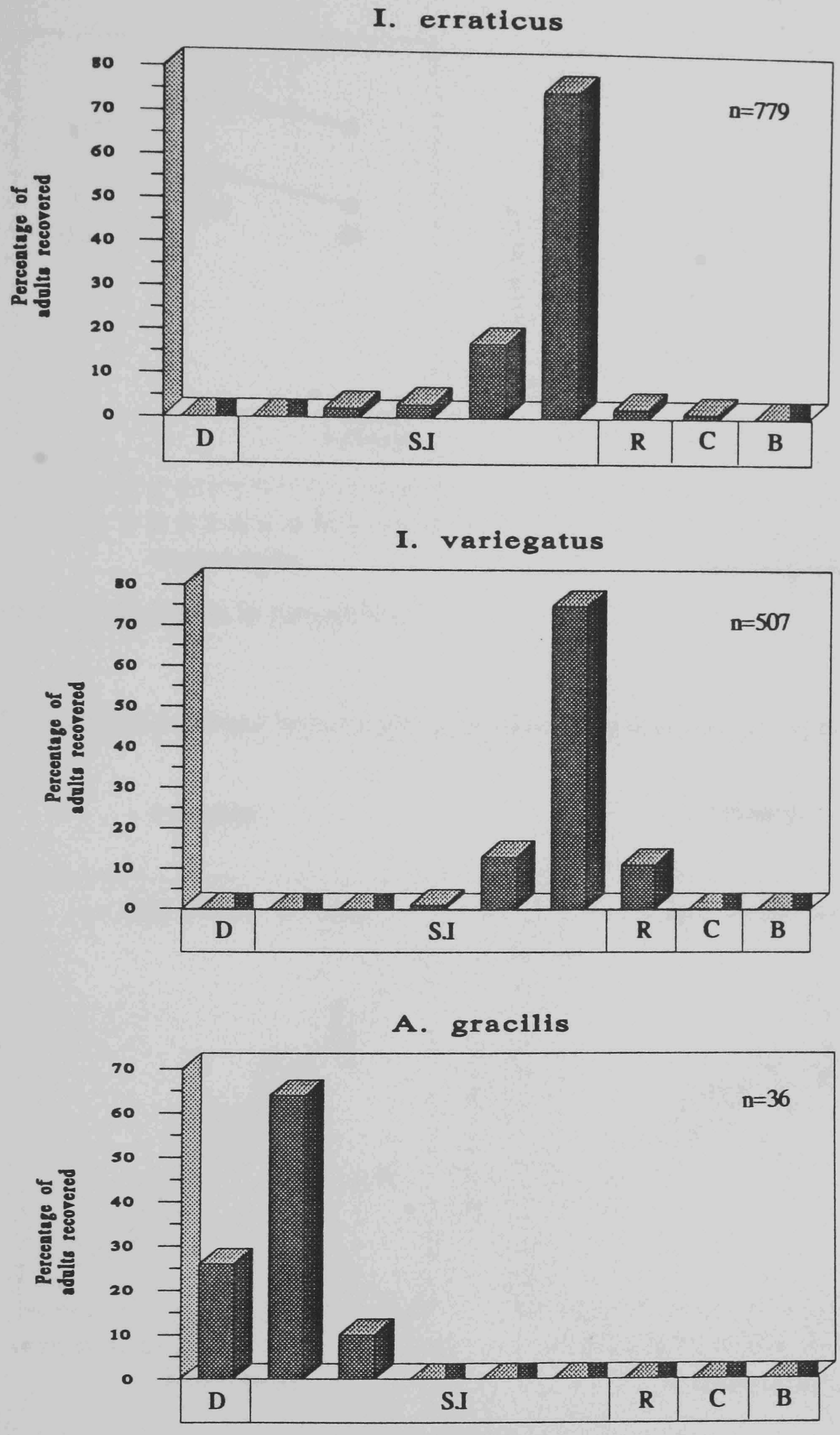






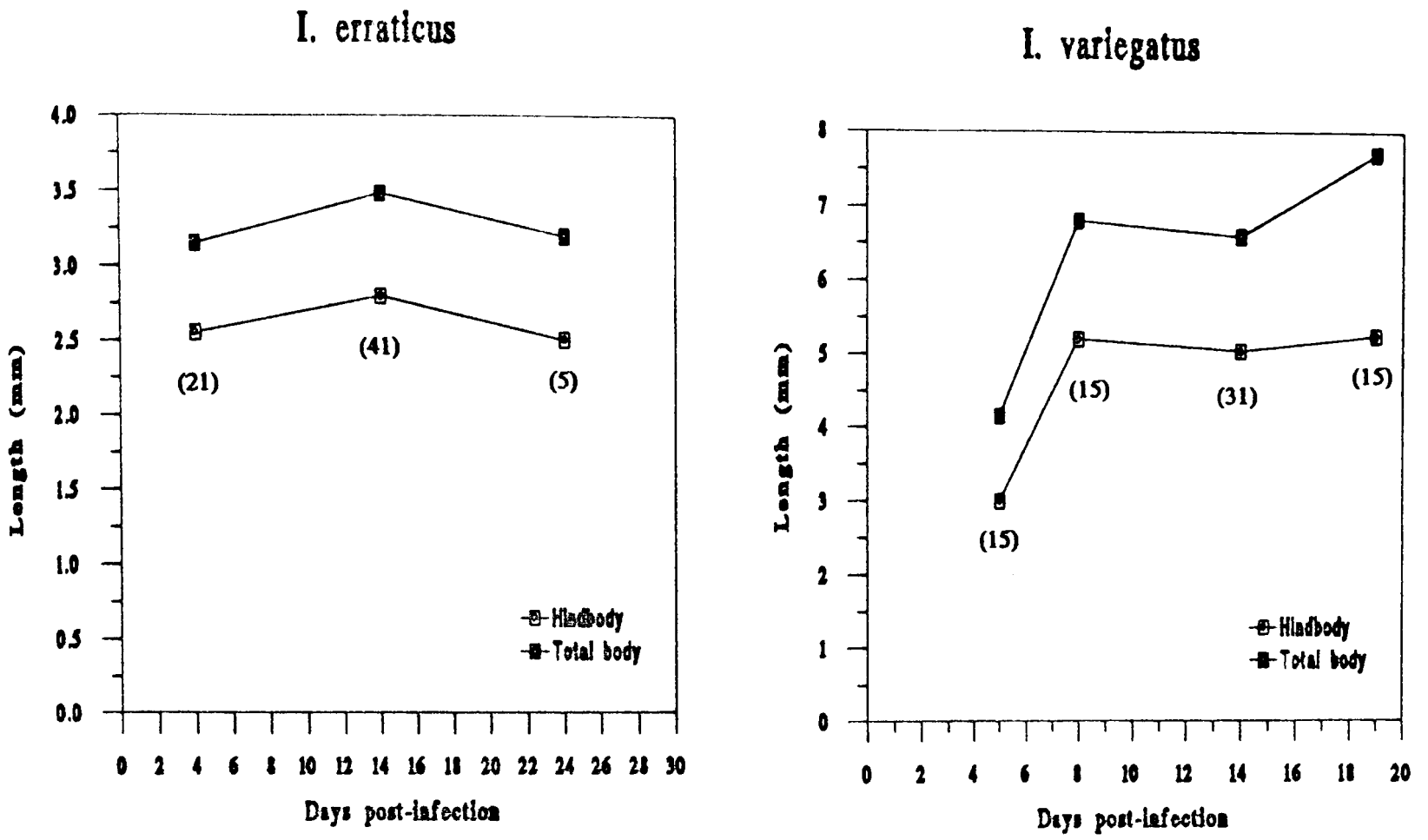
Abbreviations: Duodenum (D); Small intestine (S.I); Rectum (R); Cloaca (C); Bursa Fabricius (B).

Fig. 47. Percentage recovery of adults from different regions of the host's digestive tract.



Abbreviations: Duodenum (D); Small intestine (S.I); Rectum (R); Cloaca (C); Bursa Fabricius (B).

Fig. 48. Development of *Ichthyocotylurus* adults.



Number of specimens in parentheses.

Fig. 49. Relationship between body length and hindbody length in *Ichthyocotylurus* spp.

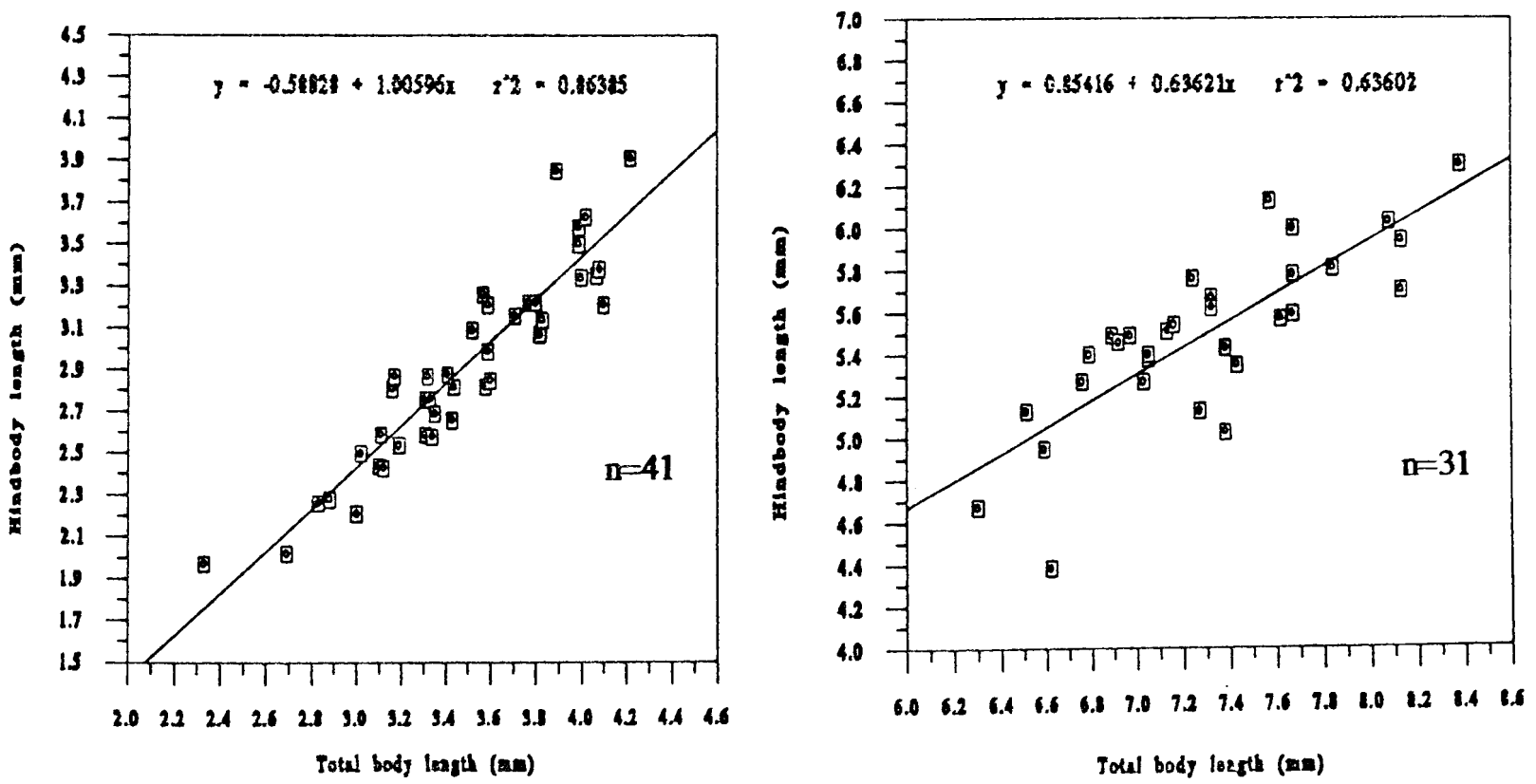


Fig. 50. Relationship between the location of *I. erraticus* adults in the host and relative length of the hindbody.

n=41; P<0.1

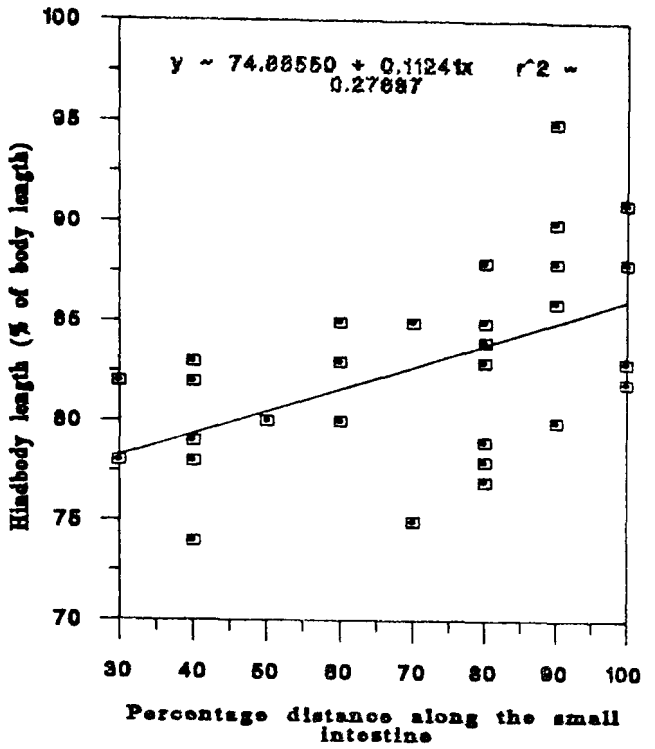


Fig. 51. Relationship between the location of *I. erraticus* adults in the host's intestine and the number of eggs *in utero*.

n=41; P>0.1

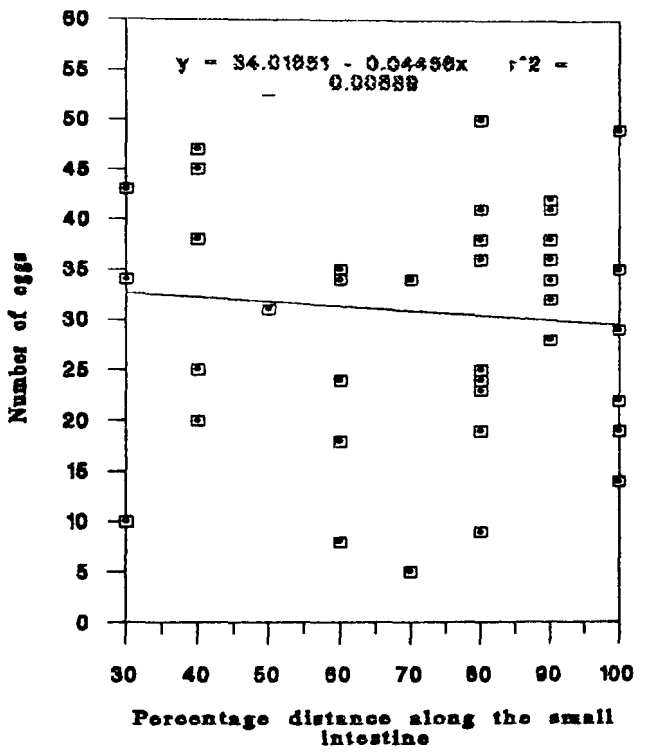


Fig. 52. Relationship between the relative hindbody length of *I. erraticus* adults and the number of eggs *in utero*.

n=41; P>0.1

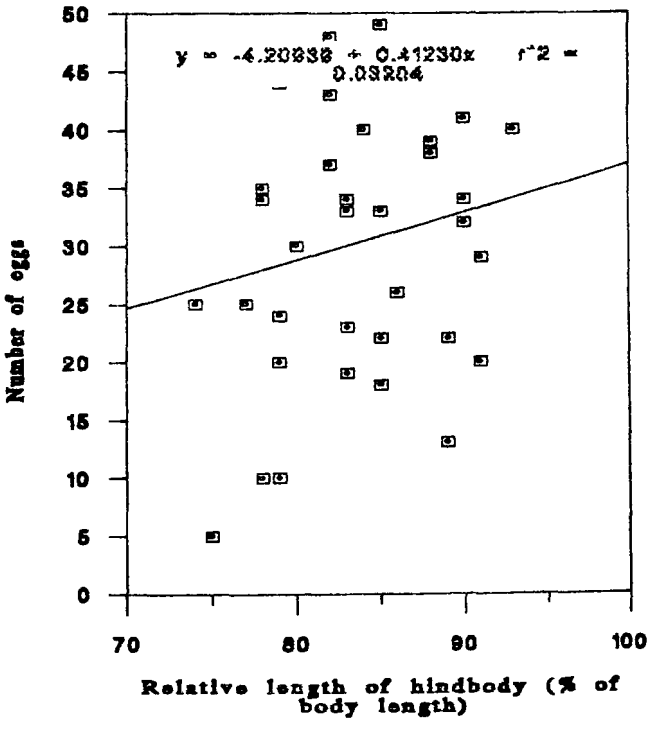


Table 33. Mean lengths (mm) of adult worms at various days post infection.

Species	Source	Age (days p.i.)	No. of specimens	Mean body length	Range (S.D.).	Mean number of eggs
I.v	1	5	15	4.25	3.59-4.92 (0.35)	*
I.v	1	14	12	6.19	4.65-7.41 (0.84)	*
I.v	2	8	15	6.79	4.35-7.57 (0.73)	*
I.v	2	14	31	6.72	5.23-7.95 (0.82)	*
I.v	2	19	15	7.61	6.62-8.38 (0.43)	*
I.v	3	15	10	7.82	6.97-8.69 (0.53)	*
I.e	4	5	21	3.16	2.52-3.87 (0.33)	22.2
I.e	4	14	41	3.46	2.33-4.22 (0.42)	30.4
I.e	4	24	5	3.16	2.56-3.56 (0.35)	34.2
I.e	5	14	20	4.06	3.32-4.77 (0.43)	52
A.g	6	5	5	2.14	1.93-2.38 (0.15)	5
A.g	7	4	4	1.29	1.15-1.47 (0.12)	4
A.g	8	3	16	0.89	0.51-1.26 (0.18)	0
A.g	8	5	9	1.14	0.89-1.47 (0.18)	2.4

Abbreviations: *I. variegatus* (I.v); *I. erraticus* (I.e); *A. gracilis* (A.g).

* Average number of eggs too numerous to count.

Sources are:
1. Ruffe: Loch Lomond, Scotland.
2. Perch: Loch Winnoch, Scotland.
3. Perch: Lake Kitka, Finland.
4. Powan: Loch Lomond, Scotland.
5. Whitefish: Lake Kitka, Finland.
6. Stone loach: River Almond, Scotland.
7. Salmon parr: River Almond, Scotland.
8. Rainbow trout: River Almond, Scotland.

3.2. TAXONOMY OF ADULTS.

3.2.1. Light microscopical observations of adults.

Light microscopical observations of the experimentally raised *I. erraticus* and *A. gracilis* adults complied with the descriptions provided by the authors listed in 3.2; Introduction, and confirmed the identifications made at the metacercarial stage. As stated in 3.2; Introduction, the status of *I. variegatus* as a valid species is doubted by certain authors. However, morphological data acquired for the adults raised from metacercariae attributed to *I. variegatus* corresponded to the previous descriptions for this species, while exhibiting differences to *I. platycephalus* descriptions, and supported the identifications made. The paucity of *I. platycephalus* metacercariae obtained (2 specimens from Finnish ruffe) precluded the culture of adults; consequently no direct comparison of the morphology of these 2 species could be made. Experimental infections with metacercariae believed to be *I. pileatus* and *A. annuligerum* were unsuccessful, as explained previously in 3.1, and consequently their identifications could not be confirmed.

Morphological measurements recorded in the present study and by other authors for the adults of *I. erraticus*, *I. variegatus* and *A. gracilis* are provided in Tables 34-36, respectively. Photomicrographs of the 3 species are given in Figs 53-56.

3.2.2. Scanning electron microscopical observations of *Ichthyocotylurus* adults.

Few sensilla were present on the hindbodies or exposed regions of the forebodies of either *Ichthyocotylurus* spp. Those observed all terminated in a short cilium surrounded by a low collar. Scanning electron micrographs of the 2 species are provided in Figs 53, 54.

Table 34. Morphometric measurements (μm) of *Ichthyocotylurus erraticus* adults. Mean measurements in parentheses.

Author	Present study		Dubois (1968)	Niewiadomska & Kozicka (1970)	Fraser (1974)
Avian host	Herring or lesser black-backed gulls		?	Common gull x lesser black-backed gull hybrid	Greater black-backer gull
Metacercarial host	Loch Lomond powan	Lake Kitka whitefish	?	Mazurian lakes vendace	?
Age of adults (days p.i.)	14	15	?	4-10 staggered infection	?
Fixative	Berland's fluid		?	Heat killed, 75% alcohol	?
Number of specimens	20	16	?	20	15
Total body length	2830-4220 (3505)	3320-4770 (4033)	up to 4200	1580-3140	3100-5080 (4170)
Forebody length	306-750 (478)	685-1030 (863)	450-1000	390-630	670-1400 (920)
Forebody breadth	563-1424 (862)	706-1038 (868)	540-1130	-	680-880 (770)
Hindbody length	1969-3906 (3018)	2490-3840 (3180)	1400-3200	1180-2590	2430-3790 (3240)
Hindbody breadth	188-625 (395)	437-759 (578)	360-900	-	360-690 (530)
Oral sucker length	95-145 (109)	120-155 (139)	100-215	69-104	91-113 (101)
Oral sucker breadth	91-143 (118)	113-157 (136)	90-180	76-122	94-133 (118)
Pharynx length	73-125 (100)	89-128 (114)	65-155	-	72-107 (84)
Pharynx breadth	61-95 (75)	70-115 (91)	55-135	-	59-87 (72)
Ventral sucker length	153-256 (182)	173-264 (222)	143-270	104-157	120-204 (164)
Ventral sucker breadth	150-228 (182)	149-231 (204)	125-235	97-191	125-178 (156)
Ovary length	134-306 (180)	117-212 (172)	90-200	76-146	122-182 (148)
Ovary breadth	117-206 (156)	140-264 (178)	125-250	-	109-209 (162)
Testis I length	275-498 (404)	355-602 (477)	145-675	139-282	255-501 (386)
Testis I breadth	244-413 (317)	264-536 (387)	200-600	-	220-419 (322)
Testis II length	331-732 (511)	528-825 (609)	225-720	198-383	321-523 (415)
Testis II breadth	200-417 (284)	264-462 (347)	215-680	-	218-417 (324)
Egg dimensions	94-113 (103) x 51-66 (59)	110-125 (115) x 55-70 (63)*	80-125 x 50-74	87-108 x 45-69	98-108 x 57-70

* Egg dimensions measured *in utero*

Table 35. Morphometric measurements (μm) of *Ichthyocotylurus variegatus* adults. Mean measurements are in parentheses.

Author	Present study			Dubois (1968, as *Cotylurus cumultitestis*)	Odening & Bockhardt (1971)
Avian host	Herring or lesser black-backed gulls			*Spheniscus humbodti*	Black-headed gulls
Metacercarial host	Loch Lomond ruffe	Loch Winnoch perch	Lake Kitka perch	?	?
Age of adults (days p.i.)	15	15	14	?	?
Fixative	Berland's fluid			?	?
Number of specimens	10	14	9	?	?
Total body length	4650-7410 (6145)	5230-7950 (6717)	6970-8690 (7858)	up to 6000	up to 1.3cm
Forebody length	903-2120 (1608)	1250-2240 (1546)	1700-2300 (1942)	1120-1780	-
Forebody breadth	1160-2320 (1819)	1430-2240 (1715)	1760-2490 (2087)	1150-1710	-
Hindbody length	3220-5500 (4526)	3900-5750 (5105)	5150-6640 (5914)	3000-4380	-
Hindbody breadth	975-2140 (1709)	1160-2140 (1485)	1390-1790 (1660)	910-1370	-
Oral sucker length	250-270 (260) *4	150-250 (198) *9	230-260 (252) *6	160-210	242-345
Oral sucker breadth	170-260 (223) *4	150-210 (179) *9	190-260 (223) *6		207-307
OS-AFB	40-80 (60)	20-160 (73)	30-240 (150)	-	-
Pharynx length	110-190 (167) *6	150-180 (165) *4	190-220 (210) *6	150-180	183-242
Pharynx breadth	80-160 (130) *6	110-120 (115) *4	130-190 (155) *6	105-160	152-197
Ventral sucker length	180-320 (265)	160-280 (231)	210-330 (280)	220-300	221-314
Ventral sucker breadth	250-320 (276)	210-320 (269)	280-330 (299)		324-383
VS-AFB	400-750 (596)	400-950 (562)	410-730 (584)	-	-
Ovary length	281-454 (370)	248-619 (425)	396-512 (487)	210-350	-
Ovary breadth	264-743 (500)	413-875 (518)	454-644 (525)	380-490	-
Testis I length	623-1307 (953)	643-1370 (1134)	934-1536 (1221)	600-890	-
Testis II breadth	789-1390 (1083)	685-1720 (1197)	955-1515 (1227)	640-960	-
Testis II length	830-1370 (1133)	809-1760 (1387)	1162-2179 (1520)	700-1100	-
Testis II breadth	643-1307 (974)	747-1580 (1129)	996-1349 (1155)	640-1000	-
Egg dimensions	90-120 (109) x 60-80 (67)**	90-115 (105) x 55-65 (61)**	105-130 (108) x 60-80 (69)**	90-120 x 57-71	95-132 x 44-69

*x indicates number of specimens for which measurement was recorded. ** Egg dimensions measured *in utero*. Abbreviations: Distance from oral sucker to anterior margin of forebody (OS-AFB); distance from ventral sucker to anterior of forebody (VS-AFB).

Table 36. Morphometric measurements (µm) of *Apatemon gracilis* adults. Mean measurements in parentheses.

Author	Present study			Dubois (1968)	Blair (1974a)		Watson & Pike (1993)
Avian host	Domestic ducklings	Mallard ducklings		*Mergus* sp.?	Domestic ducklings		Eider duckling
Metacercarial host	Rainbow trout	Salmon parr	Stone loach	?	Rainbow trout	Stone loach	Rainbow trout
Age of adults (days p.i.)	5	4	5	?	?	?	36
Fixative	Berland's fluid			?	Hot 4% formol saline		Hot 70% ethanol
Number of specimens	6	4	5	?	4	9	16
Total body length	1050-1470 (1222)	1150-1470 (1290)	1930-2380 (2140)	up to 2500	1031-1567 (1200)	1275-1449 (1386)	1560-2580 (1630)
Forebody length	385-566 (466)	493-617 (553)	608-723 (667)	400-720	395-697 (479)	480-612 (522)	420-700 (452)
Forebody breadth	344-443 (390)	304-411 (350)	452-501 (477)	340-540	308-365 (341)	325-372 (346)	-
Hindbody length	623-804 (740)	641-962 (757)	1283-1656 (1470)	930-1800	620-870 (722)	782-930 (864)	1100-1880 (1178)
Hindbody breadth	271-402 (353)	206-337 (276)	247-321 (285)	430-600	266-356 (292)	287-395 (327)	-
Oral sucker length	84-109 (100)	92-115 (107)	98-119 (111)	110-180	84-97 (93)	97-105 (102)	-
Oral sucker breadth	92-119 (107)	76-94 (89)	92-121 (103)	80-140	68-89 (81)	76-89 (83)	-
Pharynx length	51-76 (67)	57-68 (63)	66-80 (73)	60-80	48-58 (54)	49-61 (56)	-
Pharynx breadth	39-66 (50)	47-60 (52)	55-62 (58)	52-80	40-49 (45)	44-65 (56)	-
Ventral sucker length	66-164 (135)	144-154 (148)	150-183 (163)	180-255	103-120 (112)	124-143 (137)	-
Ventral sucker breadth	92-174 (128)	125-162 (136)	117-176 (163)	110-245	89-133 (107)	105-148 (120)	-
Proteolytic gland length	57-135 (93)	70-125 (86)	82-119 (95)	-	-	-	-
Proteolytic gland breadth	107-185 (112)	76-123 (97)	105-148 (126)	-	-	-	-
Ovary length	84-115 (103)	72-119 (90)	113-160 (142)	110-130	72-95 (86)	80-120 (97)	-
Ovary breadth	119-172 (151)	103-127 (112)	113-123 (120)	150-180	-	-	-
Ovary position**	(18%)	(15%)	(20%)	22-40%	-	-	-
Testis I length	156-238 (209)	148-181 (158)	296-362 (339)	210-380	171-190 (180)	190-228 (207)	-
Testis I breadth	172-262 (221)	148-197 (179)	189-214 (202)	270-360	-	-	-
Testis II length	189-353 (267)	173-238 (208)	345-411 (381)	270-435	193-262 (223)	205-310 (260)	-
Testis II breadth	172-271 (227)	156-206 (183)	181-222 (204)	250-340	-	-	-
Egg dimensions	81-112 (92) x 48-64 (55)*	84-100 (94) x (48-64 (57)*	87-119 (103) x 58-71 (65)*	90-115 x 60-80	-	92-102 (97) x 65-74 (70)	-

* eggs measured *in utero*. ** distance from ovary to anterior margin of hindbody as a percentage of hindbody length.

Fig. 53. Photomicrograph and scanning electron micrographs of *I. erraticus* adults raised from powan metacercariae within experimental gull hosts. A). Photomicrograph of lateral view of adult. B). Scanning electron micrograph of the ventral body surface. C). Scanning electron micrograph of the ventral view of the forebody showing the oral sucker (OS) and lappets (L), with arrowheads indicating the lips of the tribocytic organ.

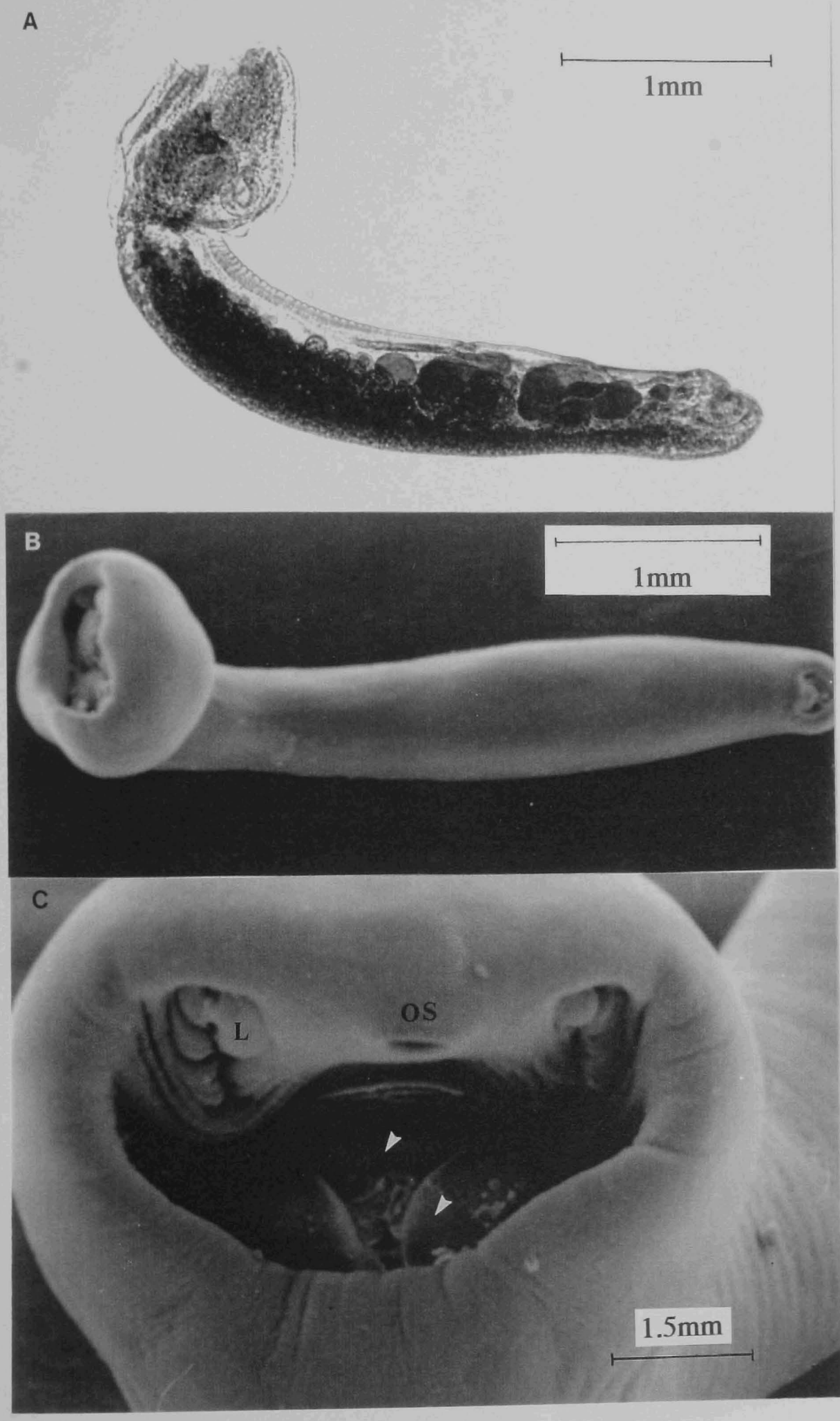
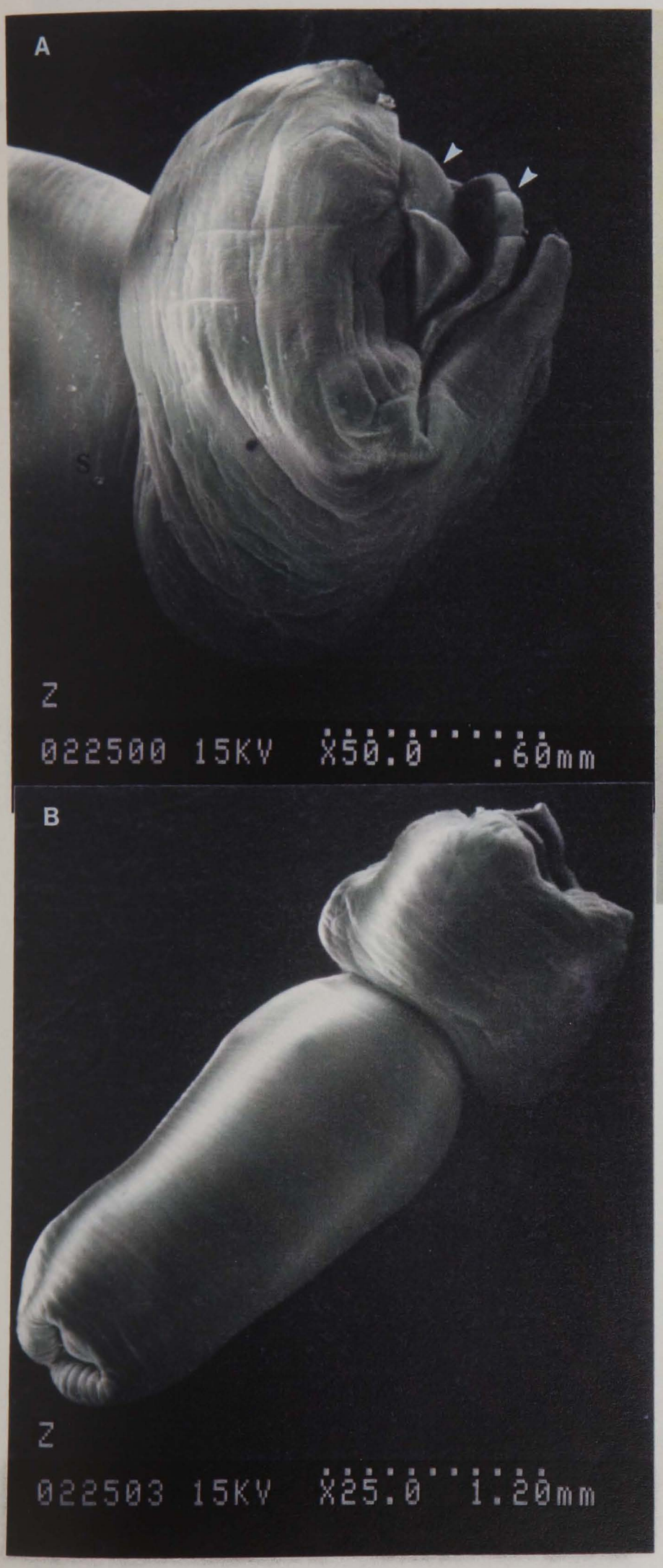
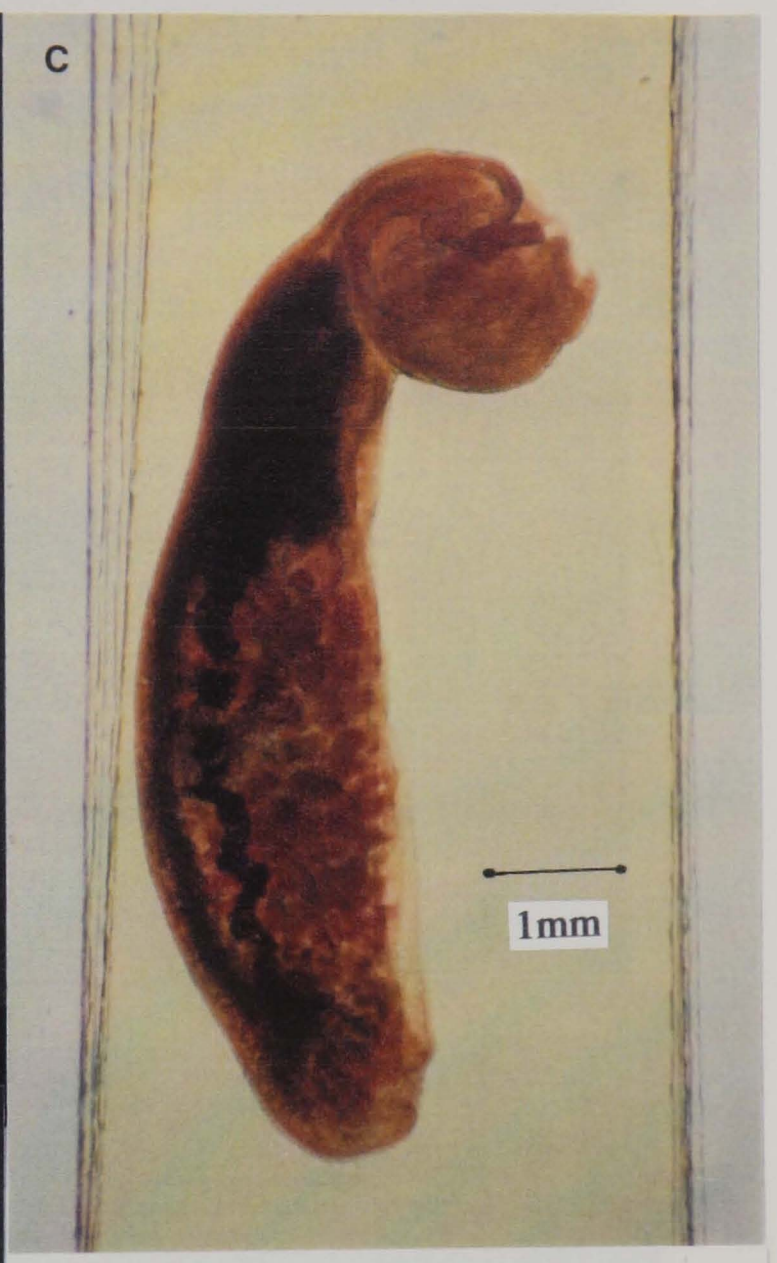


Fig. 54. A). and B). Scanning electron micrographs of a 5 day p.i. *I. variegatus* adult raised from a Loch Lomond ruffe metacercaria within an experimental gull host. A). Ventro-lateral view of forebody showing tribocytic organ lips (arrowheads) and sensillum (S). B). Ventral body surface. C). and D). Photomicrographs of the lateral view of 14 day p.i. *I. variegatus* adults, raised from Loch Lomond ruffe metacercariae and recovered from the same experimental gull host, demonstrating the variation in body form.





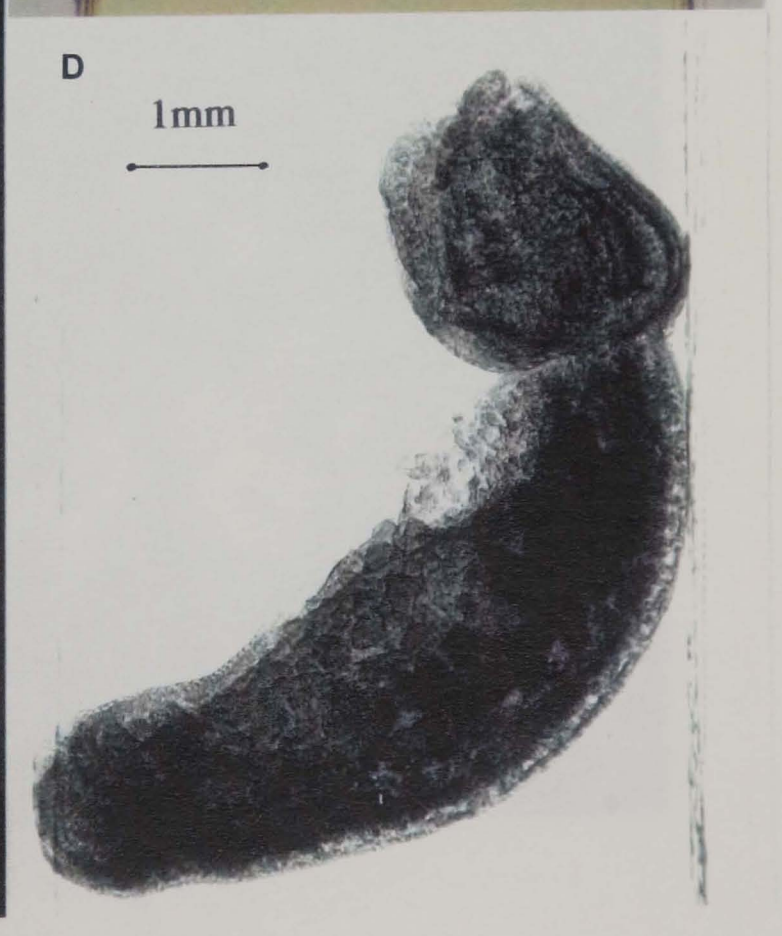


Fig. 55. Photomicrographs of *I. variegatus* adults *in situ* within the small intestine of an experimental gull host. Arrowhead in B) indicates localised region of hypertrophied villi surrounding one particular worm.

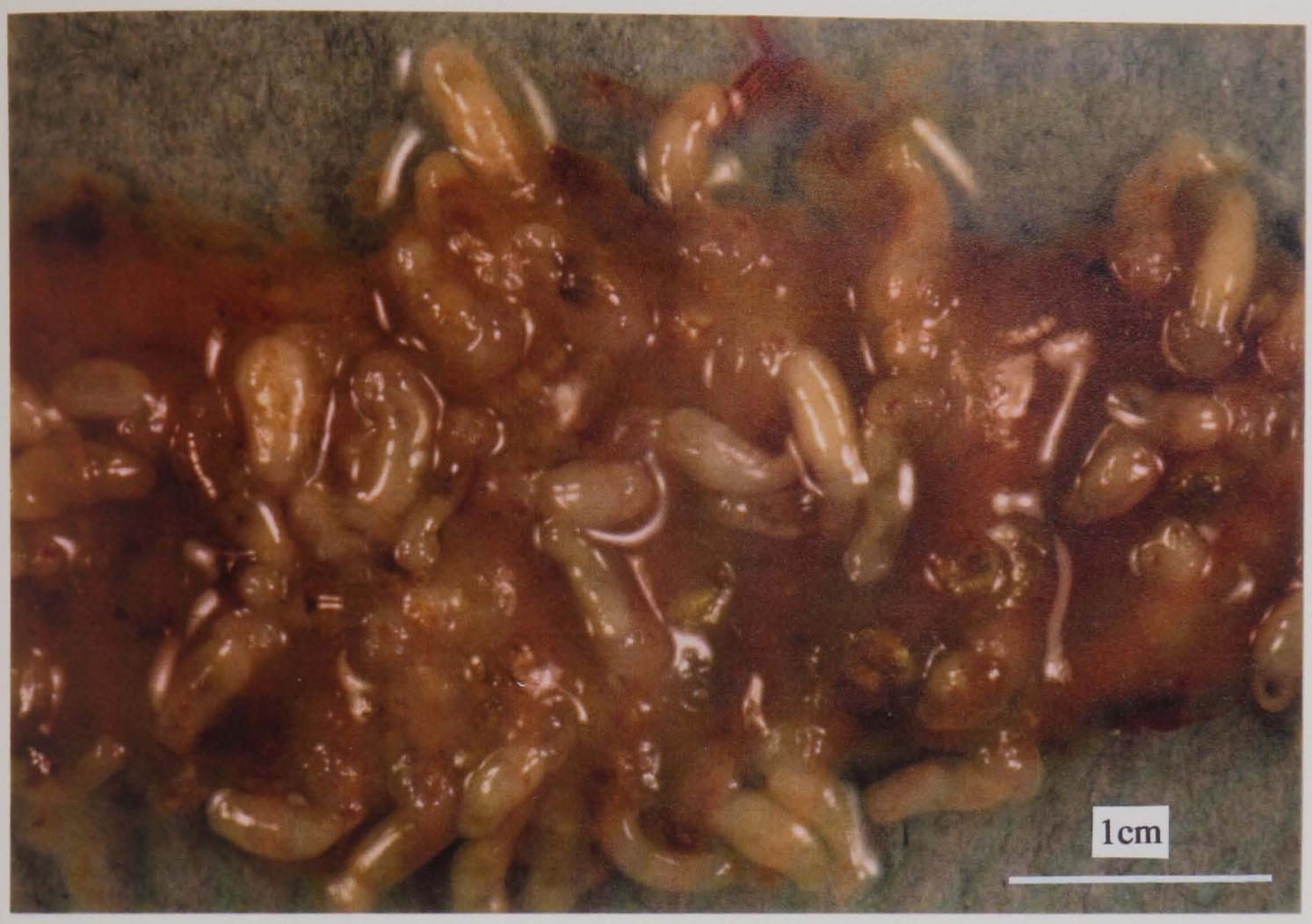
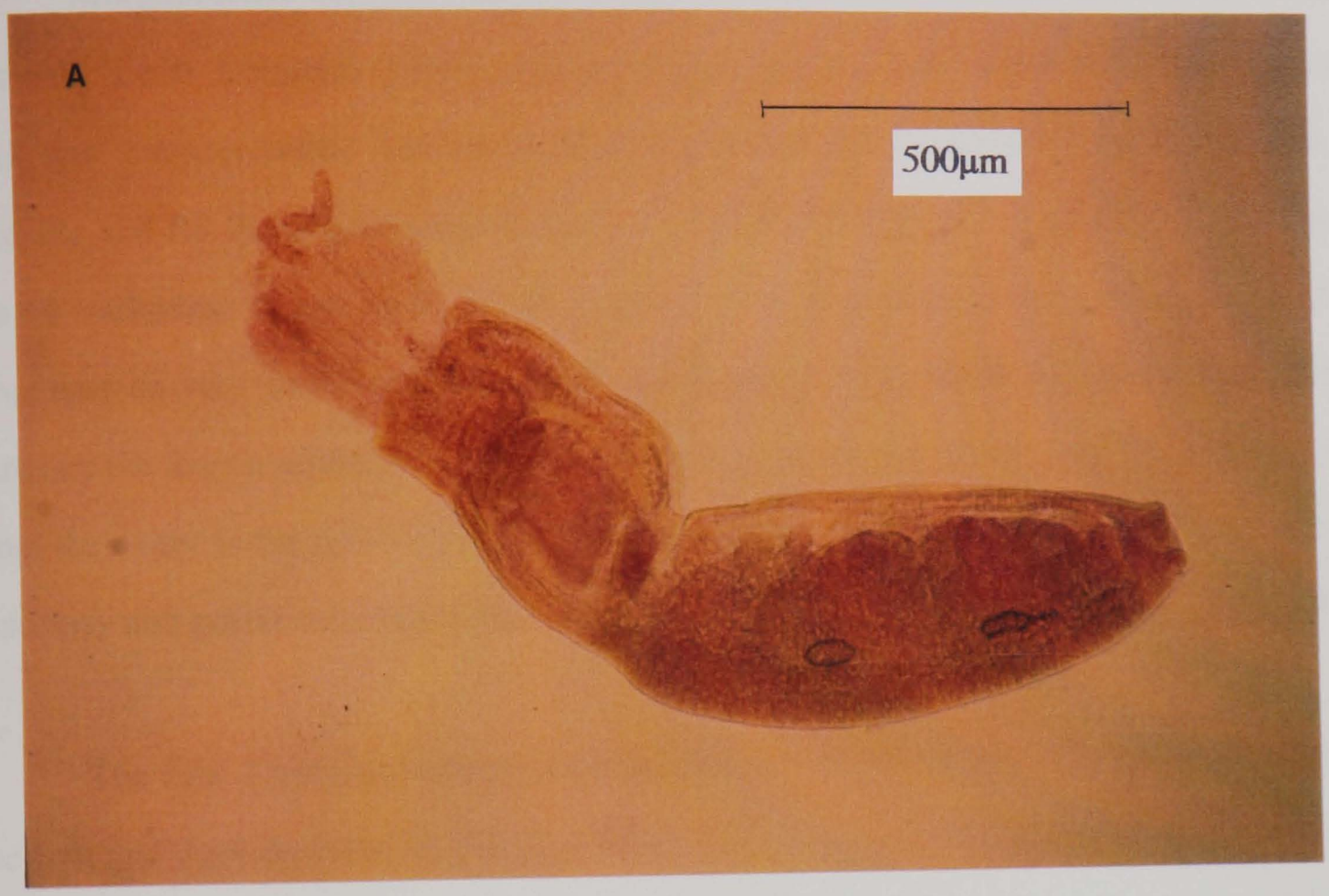
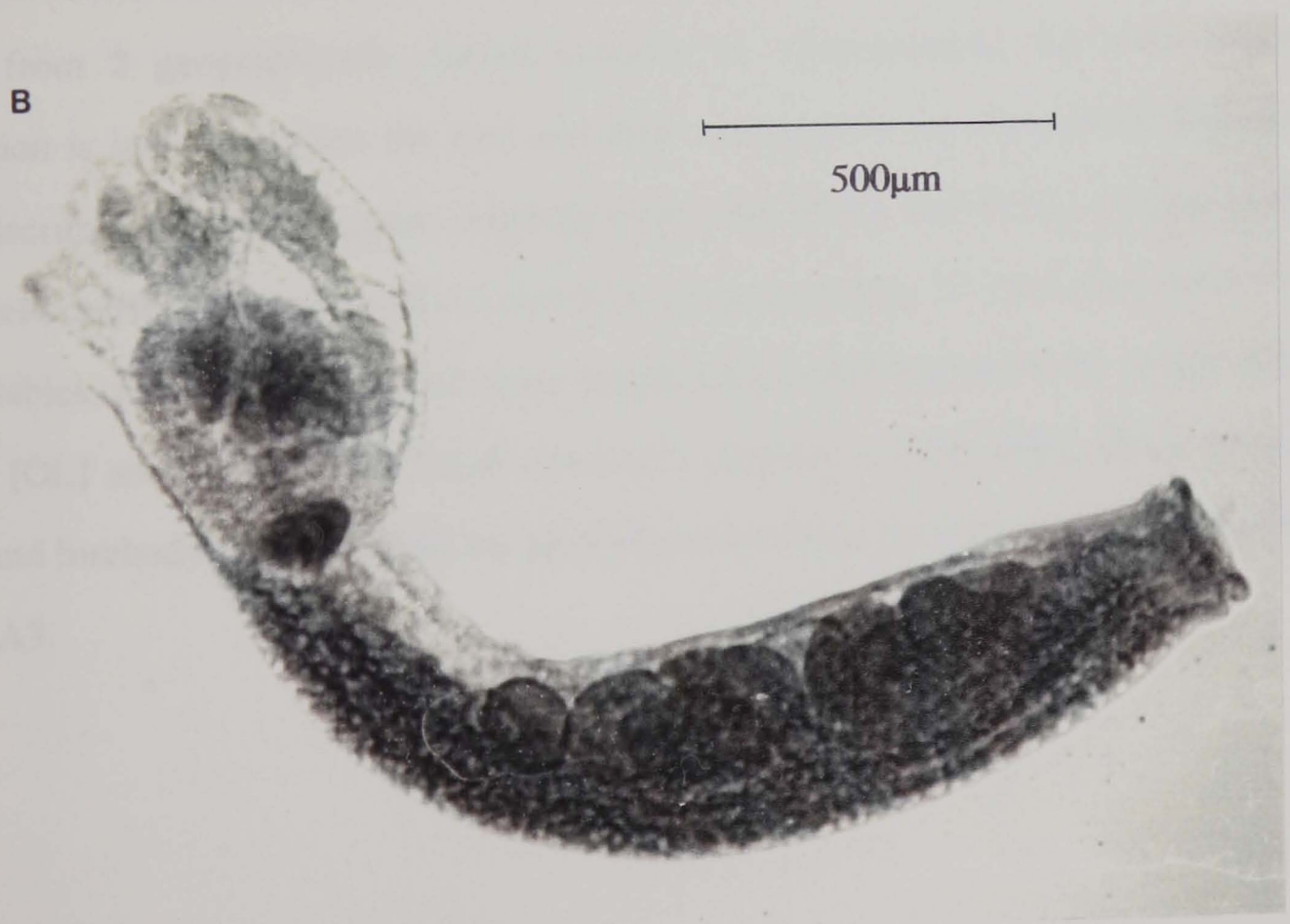


Fig. 56. Photomicrographs of *A. gracilis* adults. A). Adult raised from a rainbow trout metacercaria within an experimental domestic duck host. B). Adult raised from a stone loach metacercaria within an experimental mallard host.





3.2.3. Discrimination of adults by Principal Components Analysis of metrical features.

I. erraticus

Morphometric analysis was performed on 36 *I. erraticus* adults, 20 raised from Scottish (Loch Lomond) powan metacercariae and 16 from Finnish (Lake Kitka) whitefish metacercariae. The mean and coefficient of variation for each variable, globally and for the 2 individual samples, are given in Table 37. Correlations between the 18 variables, shown in Table 39, illustrate how the sizes of the vast majority of structures co-vary in the same direction. Particularly high correlations were recorded between the length related variables of total body length, hindbody length and distance from the ovary to the forebody; illustrating that large specimens tend to possess a large hindbody and posteriorly displaced ovary. No large negative correlations were observed.

The first 2 principal components account for 58.5% of the total variance, while the first and third describe 55.5% (Table 38). Fig. 57 illustrates the plot of the first two components and demonstrates how Factor 1 (PCA1) discriminates between the adults raised from 2 geographically distant samples of metacercariae. An even clearer separation is indicated when the first and third components are considered (Fig. 58), with discrimination in this plane resulting from both PCA1 and PCA3. A plot of the variables contributing most to PCA1 and PCA3 is given in Fig. 59. This map shows that all variables, with the exception of ovary length [OL], contribute to PCA1, while ovary length [OL] and the breadth-related characters: pharynx breadth [PB], ovary breadth [OB] and forebody breadth [FBB] are primarily responsible for the separation described by PCA3.

Table 37. Mean and coefficient of variation for each variable (standardised data), globally and in each sample of *I. erraticus* adults (Coefficient of variation = 100 x standard deviation/mean). For an explanation of abbreviations see Fig. 41.

Variable	All *I. erraticus* adults combined (n=36)		Scottish powan specimens (n=20)		Finnish whitefish specimens (n=16)	
	Mean	C.V	Mean	C.V	Mean	C.V
TBL	8.21	1.77	8.15	1.60	8.30	1.43
FBL	6.42	5.73	6.15	4.21	6.76	1.55
HBL	8.02	1.82	7.99	1.89	8.06	1.69
FBB	6.75	2.27	6.74	2.75	6.75	1.60
HBB	6.12	5.59	5.94	5.77	6.35	2.93
OSL	4.81	3.33	4.71	2.99	4.93	1.64
OSB	4.83	2.77	4.76	2.86	4.91	1.59
VSL	5.29	3.12	5.20	2.60	5.41	2.24
VSB	5.25	3.01	5.19	3.10	5.31	2.47
PL	4.66	2.92	4.59	2.79	4.73	2.22
PB	4.39	3.53	4.31	2.76	4.50	2.84
OPG	7.05	4.51	6.91	5.02	7.22	2.29
OL	5.16	3.41	5.18	3.46	5.13	3.39
OB	5.09	3.46	5.04	2.70	5.15	3.98
TIL	6.06	2.87	5.99	2.40	6.14	2.88
TIB	5.83	3.64	5.74	3.22	5.94	3.27
TIIL	6.30	2.89	6.22	3.01	6.41	1.75
TIIB	5.74	3.45	5.67	3.51	5.84	2.60

Table 38. Principal Components Analysis of the correlations between the 18 variables used for *I. erraticus* adults.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	8.37	2.17	1.62	1.27
Proportion (%)	46.48	12.04	9.02	7.07
Cumulative (%)	46.48	58.52	67.54	74.61

Coefficient of each variable on the first two principal components.			
Variable	PCA1	PCA2	PCA3
TBL	0.901	0.314	-0.089
FBL	0.669	-0.442	0.278
HBL	0.735	0.560	-0.264
FBB	0.541	0.149	-0.437
HBB	0.499	-0.783	0.031
OSL	0.789	-0.003	0.324
OSB	0.590	-0.030	0.079
VSL	0.636	-0.192	-0.174
VSB	0.781	-0.111	0.047
PL	0.725	0.153	0.237
PB	0.620	0.058	0.585
OFB	0.773	0.441	0.083
OL	0.151	0.075	-0.634
OB	0.553	-0.451	-0.479
TIL	0.762	0.245	-0.200
TIB	0.717	-0.408	-0.141
TIIL	0.744	0.329	0.184
TIIB	0.745	-0.332	-0.055

Table 39. Correlation matrix of the 18 variables used for *I. erraticus* adults.

	TBL	FBL	HBL	FBB	HBB	OSL	OSB	VSL	VSB	PL	PB	OFB	OL	OB	TIL	TIB	TIIL	TIIB
TBL	1.000																	
FBL	0.529	1.000																
HBL	**0.920**	0.160	1.000															
FBB	0.527	0.147	0.546	1.000														
HBB	0.227	**0.765**	-0.078	0.239	1.000													
OSL	0.663	0.572	0.475	0.374	0.385	1.000												
OSB	0.493	0.435	0.357	0.204	0.233	0.371	1.000											
VSL	0.512	0.489	0.379	0.304	0.457	0.453	0.308	1.000										
VSB	**0.705**	0.620	0.351	0.368	0.506	0.658	0.506	0.359	1.000									
PL	0.684	0.585	0.525	0.318	0.296	0.619	0.331	0.500	0.391	1.000								
PB	0.498	0.522	0.321	0.121	0.269	0.632	0.452	0.311	0.371	0.613	1.000							
OFB	**0.834**	0.293	**0.818**	0.321	-0.028	0.611	0.461	0.301	0.635	0.554	0.482	1.000						
OL	0.221	0.094	0.240	0.246	0.057	-0.066	0.117	0.334	0.029	0.151	-0.131	-0.018	1.000					
OB	0.419	0.384	0.329	0.341	0.500	0.292	0.283	0.412	0.447	0.197	0.061	0.267	0.187	1.000				
TIL	**0.744**	0.364	**0.705**	0.485	0.216	0.447	0.446	0.405	0.508	0.524	0.524	0.652	0.207	0.422	1.000			
TIB	0.486	0.418	0.366	0.321	0.601	0.492	0.464	0.493	0.569	0.274	0.274	0.472	-0.022	0.651	0.479	1.000		
TIIL	0.697	0.372	0.632	0.432	0.168	0.643	0.335	0.483	0.515	0.599	0.599	0.643	0.030	0.074	0.606	0.405	1.000	
TIIB	0.494	0.425	0.373	0.394	0.533	0.580	0.361	0.425	0.514	0.456	0.456	0.516	-0.030	0.667	0.469	**0.835**	0.464	1.000

Fig. 57. Map of the 36 *I. erraticus* adult specimens in the first plane of the Principal Components Analysis. Specimens raised from Scottish powan metacercariae are represented by (s) and those from Finnish whitefish metacercariae by (f). Ellipses surround 50% of points.

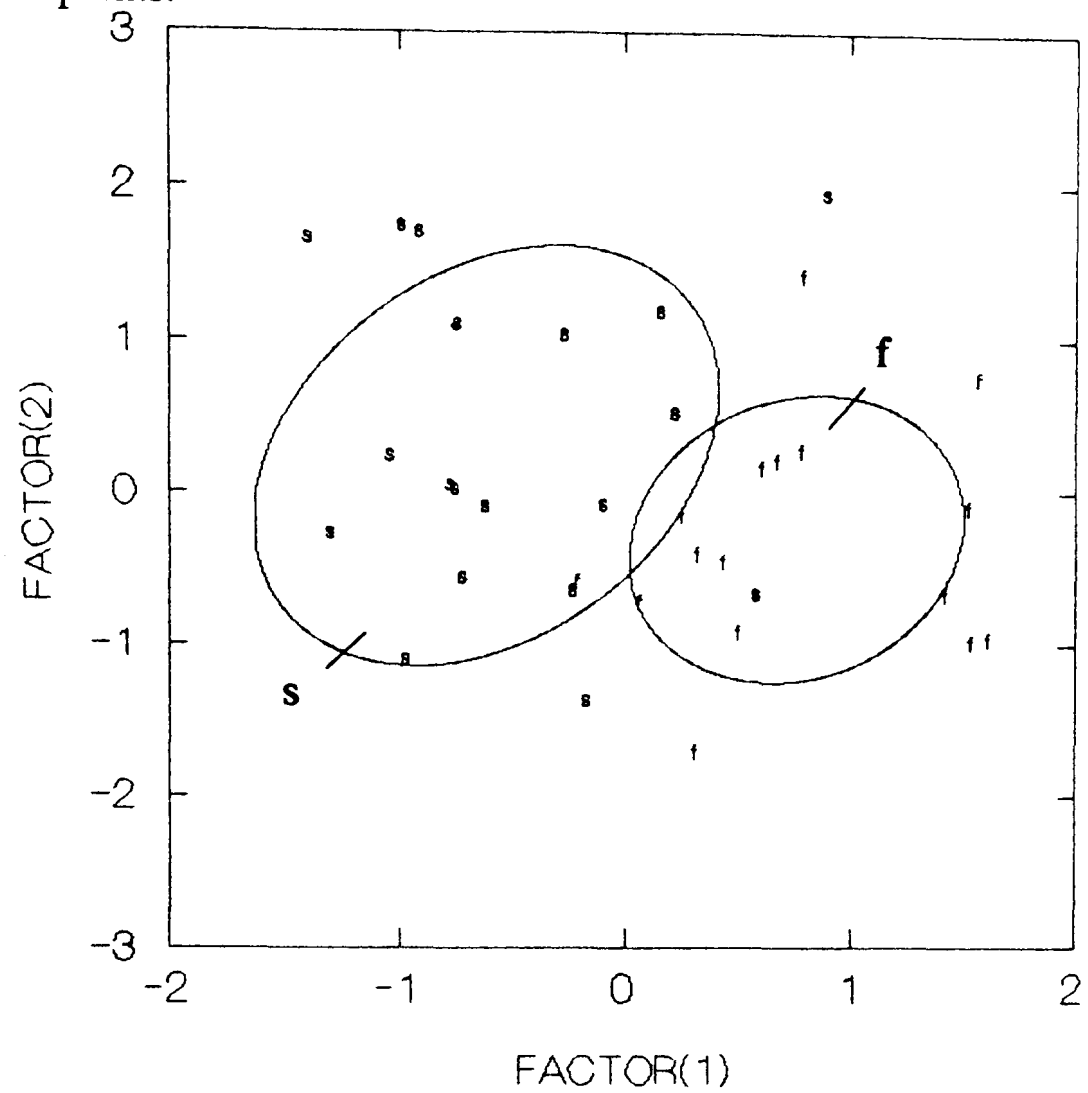


Fig. 58. Map of the 36 *I. erraticus* adult specimens in the second plane of the Principal Components Analysis. Specimens raised from Scottish powan metacercariae are represented by (s) and those from Finnish whitefish metacercariae by (f). Ellipses surround 50% of points.

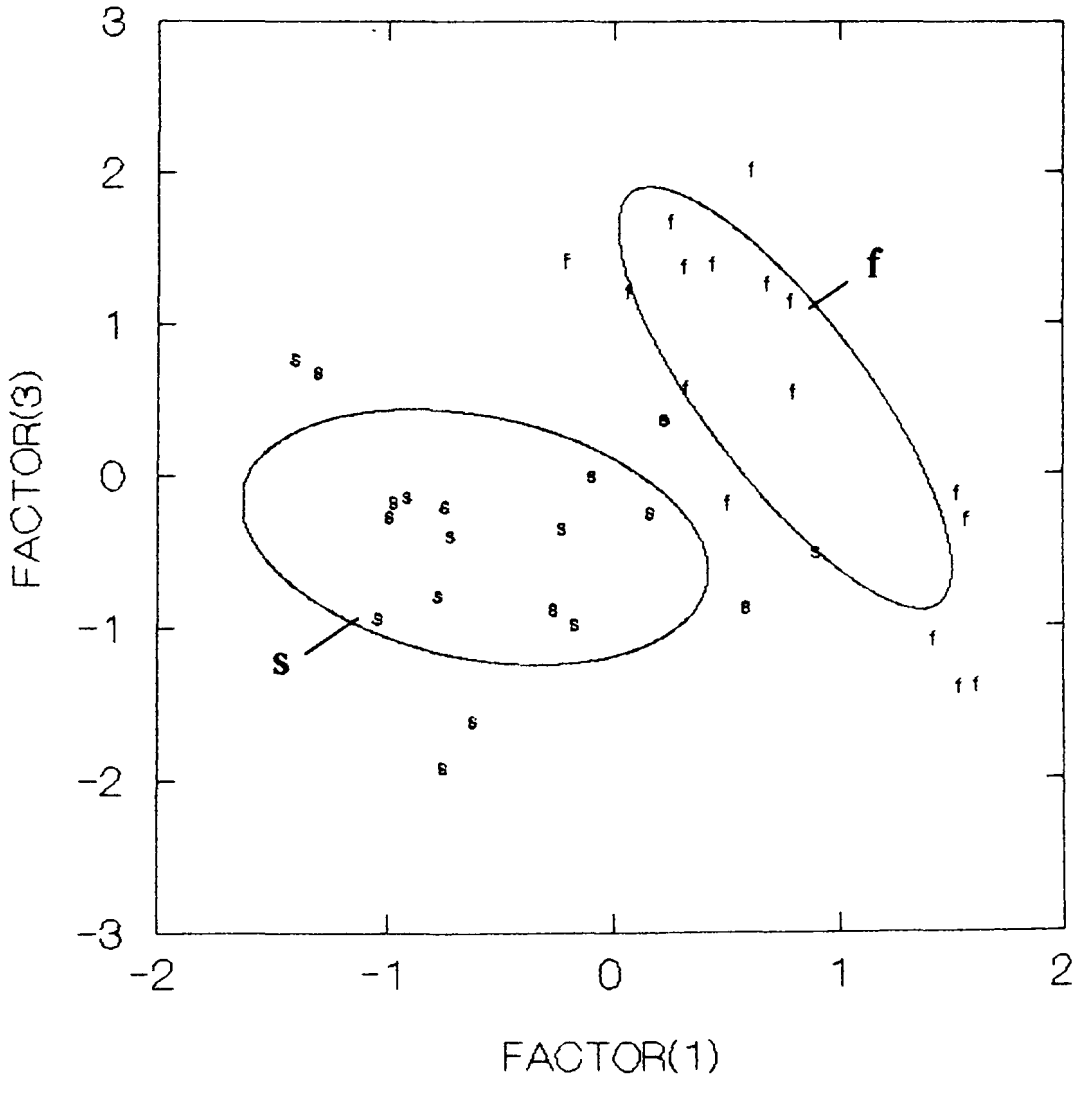
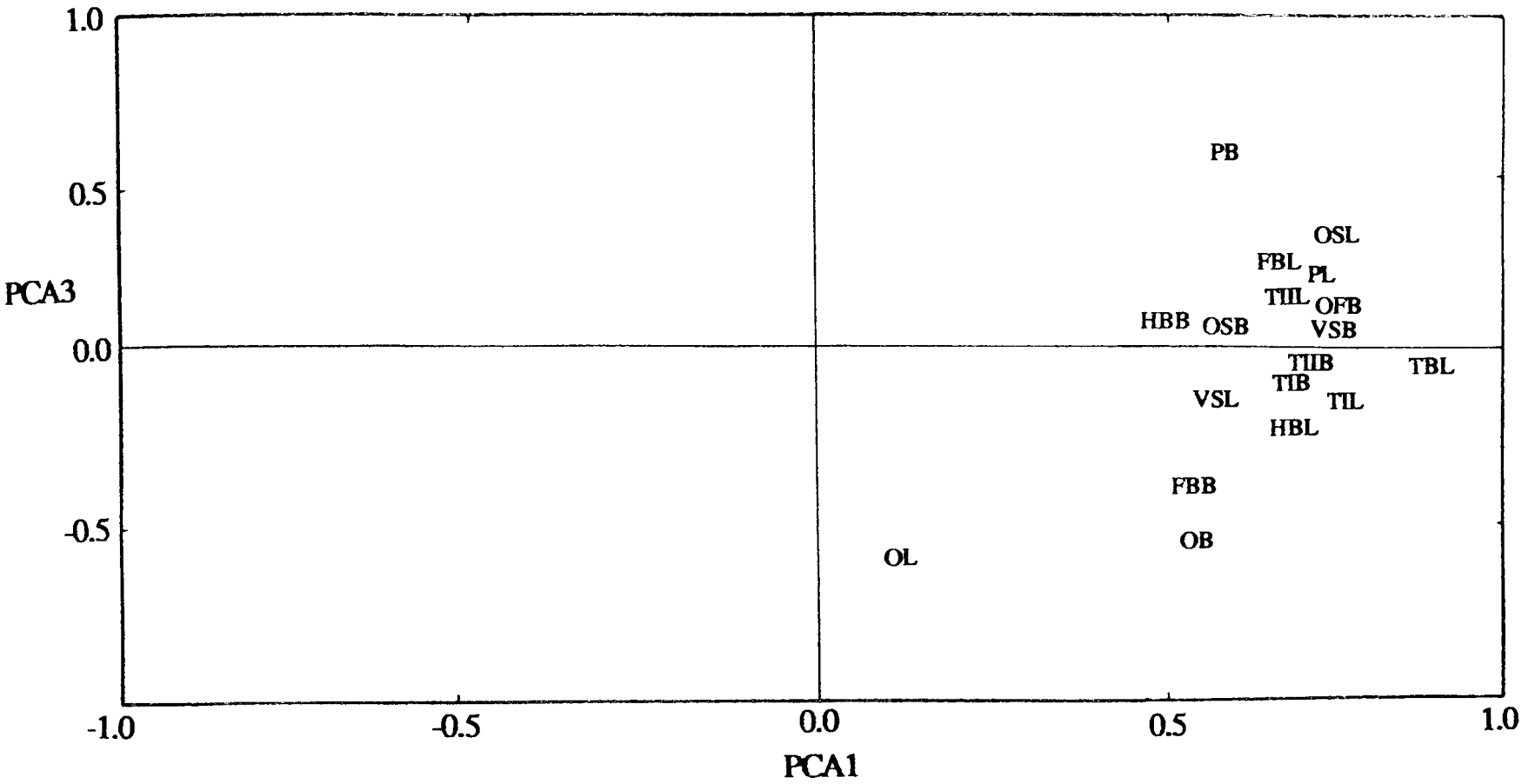


Fig. 59. Map of the 18 variables used for *I. erraticus* adults in the second plane of the Principal Components Analysis.



I. variegatus

Morphometric analysis was applied to 33 *I. variegatus* adults, 10 raised from Scottish (Loch Lomond) ruffe metacercariae, 14 from Scottish (Loch Winnoch) perch metacercariae and 9 from Finnish (Lake Kitka) perch metacercariae. The mean and coefficient of variation of each variable, globally and for the 3 individual samples, are given in Table 40. Correlations between the 14 variables, shown in Table 41, illustrate how the sizes of all the structures co-vary in the same direction. The high correlations were predominently between like-related variables, e.g. total body length [TBL] and forebody length [FBL], hindbody length [HBL] and testis I length [TIL] or, testis I breadth [TIB] and testis II breadth [TIIB]; although the length and breadth of testis II were also highly correlated.

The first 2 principal components account for 67.2% of the total variance (Table 42). Fig. 61 illustrates the plot of the first two components, with ellipses surrounding 50% of each sample's points. Some separation is indicated between the Scottish and Finnish adults along PCA1, while a further division is shown between adults derived from Scottish perch metacercariae and the remaining specimens along PCA2. The discrimination between the samples is emphasised when 95% confidence limits are imposed on the centroid (Fig. 62). A map of the variables contributing most to PCA1 and PCA2 is given in Fig. 60. This map shows that the length related variables, total body length [TBL], hindbody length [HBL] and testes dimensions [TIL, TIB, TIIL, TIIB] contribute primarily to PCA1, while ventral sucker length [VSL] is mainly responsible for the separation according to PCA2.

The directions of the ellipses generated demonstrates the variation present within each sample. Adults raised from Scottish ruffe metacercariae vary mainly according to PCA1 variables (horizontal ellipse), this is also shown by the high coefficients of variation seen for this sample's variables in Table 40. Both samples of perch metacercariae derived adults are surrounded by approximately vertical ellipses, demonstrating variation in their variables acting along PCA2, particularly in the lengths of their ventral suckers.

Table 40. Mean and coefficient of variation for each variable (standardised data), globally and in each sample of *I. variegatus* adults (Coefficient of variation = 100 x standard deviation/mean). For an explanation of abbreviations see Fig. 41.

Variable	All *I. variegatus* adults combined (n=33)		Scottish ruffe specimens (n=10)		Scottish perch specimens (n=14)		Finnish perch specimens (n=37)	
	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V
TBL	8.82	1.79	8.71	2.29	8.81	1.49	8.97	0.80
FBL	7.42	2.60	7.36	3.17	7.37	2.20	7.57	1.48
HBL	8.54	1.90	8.41	1.81	8.53	1.56	8.68	0.91
FBB	7.51	2.48	7.48	3.41	7.44	1.69	7.64	1.36
HBB	7.36	2.70	7.42	3.42	7.29	2.65	7.41	1.23
OFB	7.09	3.58	6.98	3.02	7.06	3.61	7.28	3.01
VSL	5.53	3.06	5.57	3.21	5.45	2.77	5.63	2.31
VSB	5.62	1.82	5.60	1.46	5.59	2.09	5.70	0.81
OL	6.02	4.19	5.90	3.14	6.01	5.08	6.18	2.06
OB	6.21	4.09	6.17	5.04	6.21	4.51	6.26	2.17
TIL	6.98	3.32	6.84	3.35	7.01	3.30	7.10	2.25
TIB	7.03	3.04	7.02	2.82	7.21	3.30	7.31	2.91
TIIL	7.18	3.37	6.96	3.29	7.06	3.48	7.06	1.91
TIIB	6.97	3.13	6.85	3.97	7.01	2.94	7.05	1.48

Table 41. Correlation matrix of the 14 variables used for *I. variegatus* adults.

	TBL	FBL	HBL	FBB	HBB	OFB	VSL	VSB	OL	OB	TIL	TIB	TIIL	TIIB
TBL	1.000													
FBL	**0.826**	1.000												
HBL	**0.972**	0.675	1.000											
FBB	0.703	0.662	0.644	1.000										
HBB	0.584	0.620	0.503	0.738	1.000									
OFB	0.700	0.435	0.742	0.429	0.438	1.000								
VSL	0.452	0.583	0.360	0.579	0.532	0.258	1.000							
VSB	0.534	0.559	0.491	0.601	0.497	0.447	0.510	1.000						
OL	0.619	0.398	0.656	0.343	0.278	0.252	0.175	0.167	1.000					
OB	0.589	0.489	0.559	0.492	0.546	0.252	0.294	0.054	0.682	1.000				
TIL	0.792	0.533	**0.821**	0.467	0.444	0.566	0.265	0.360	0.668	0.544	1.000			
TIB	0.613	0.485	0.606	0.480	0.572	0.538	0.178	0.451	0.402	0.529	0.620	1.000		
TIIL	**0.820**	0.527	**0.858**	0.523	0.437	0.673	0.161	0.474	0.558	0.508	0.689	0.750	1.000	
TIIB	0.673	0.435	0.702	0.522	0.518	0.619	0.120	0.502	0.389	0.418	0.612	**0.850**	**0.819**	1.000

Table 42. Principal Components Analysis of the correlations between the 14 variables used for *I. variegatus* adults.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	8.04	1.65	1.26	0.83
Proportion (%)	57.44	11.77	8.99	5.92
Cumulative (%)	57.44	67.21	76.20	82.12

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
TBL	0.949	0.022
FBL	0.778	-0.340
HBL	0.925	0.163
FBB	0.770	-0.408
HBB	0.720	-0.379
OFB	0.710	0.090
VSL	0.487	-0.706
VSB	0.620	-0.457
OL	0.628	0.384
OB	0.657	0.190
TIL	0.809	0.284
TIB	0.776	0.218
TIIL	0.855	0.322
TIIB	0.793	0.255

Fig. 60. Map of the 14 variables used for *I. variegatus* adults in the first plane of the Principal Components Analysis.

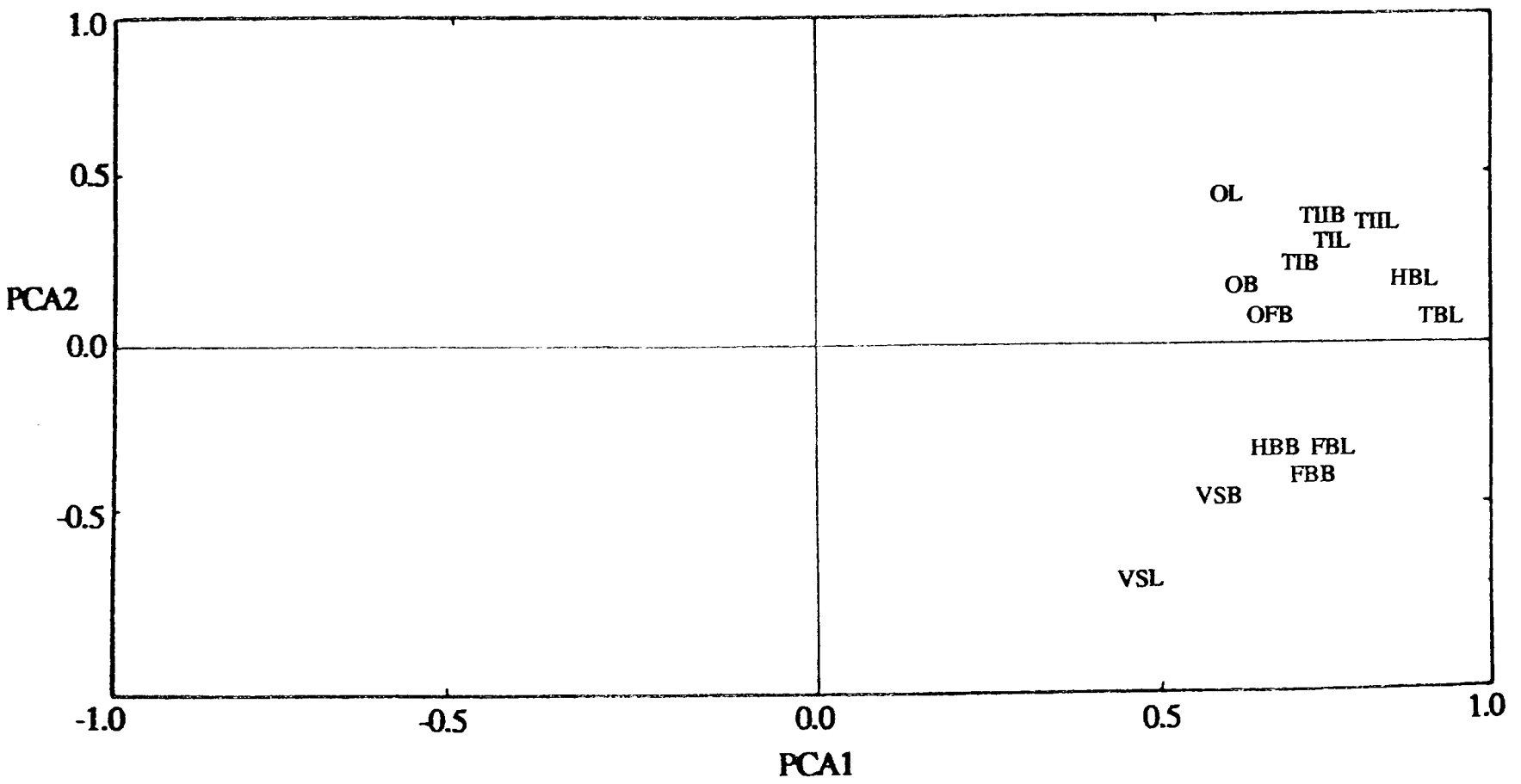


Fig. 61. Map of the 33 *I. variegatus* adult specimens in the first plane of the Principal Components Analysis. Specimens raised from Scottish ruffe metacercariae are represented by (r), from Scottish perch metacercariae by (p) and Finnish perch metacercariae by (f). Ellipses surround 50% of points.

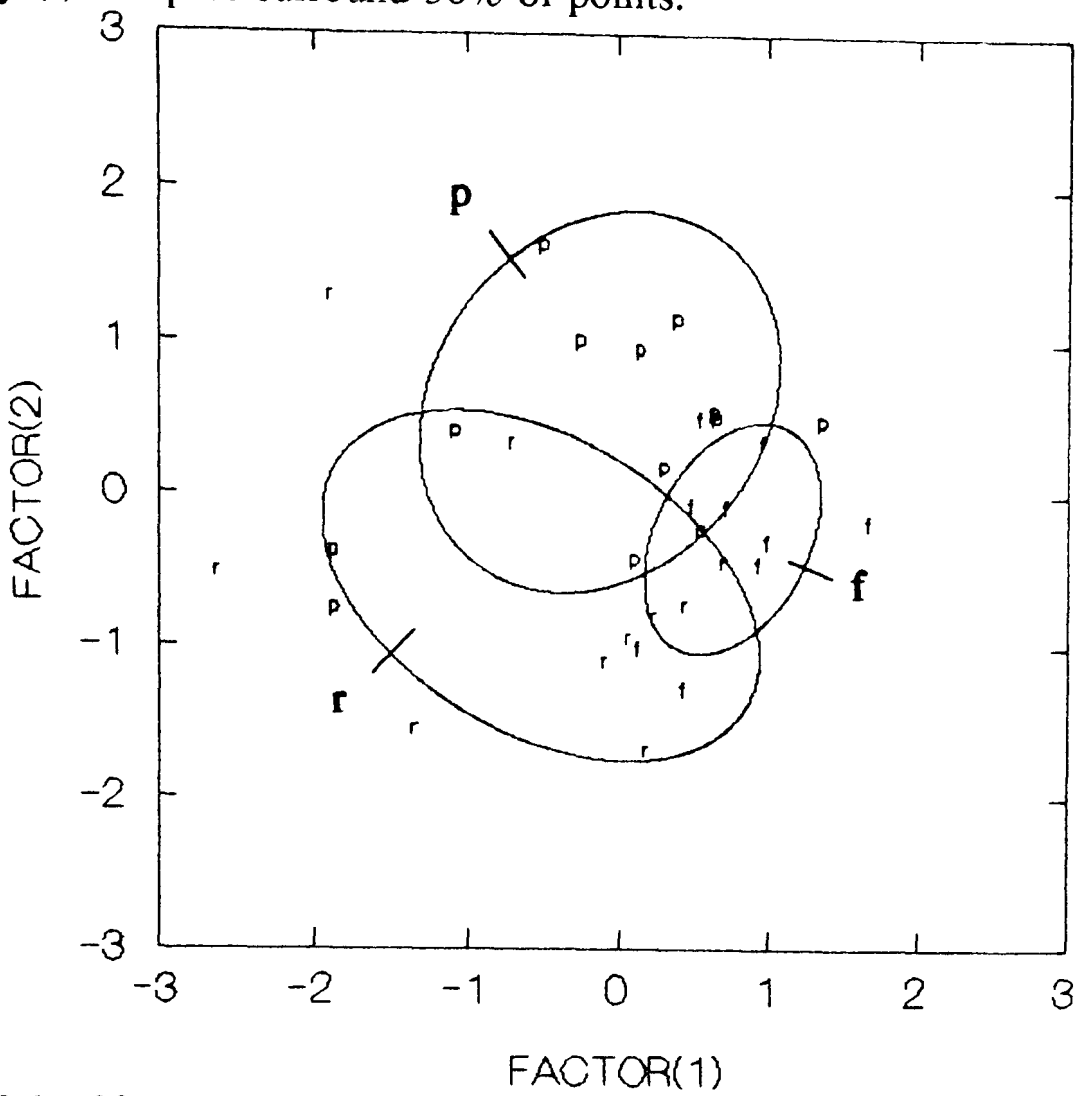
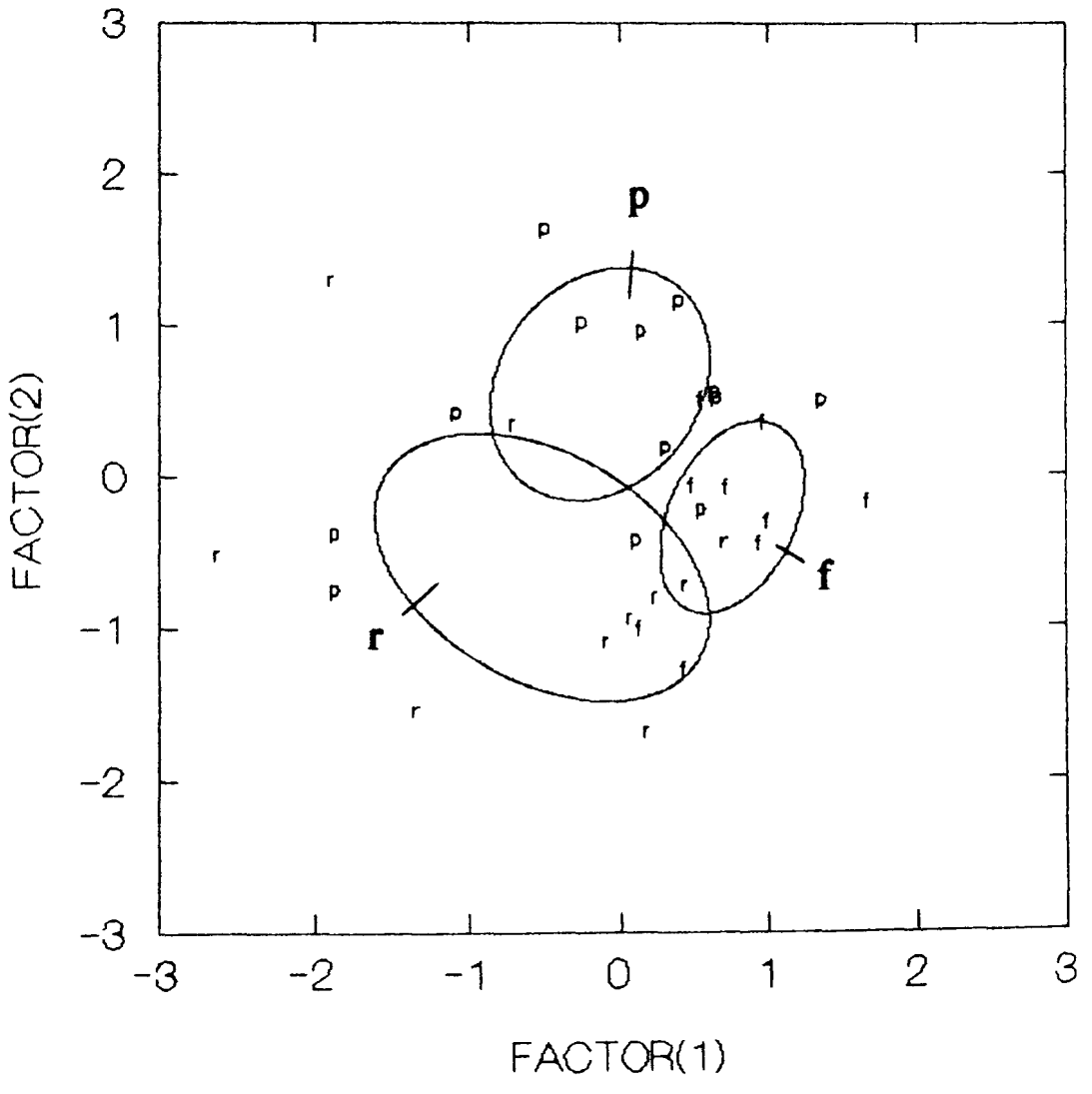


Fig. 62. Map of the 33 *I. variegatus* adult specimens in the first plane of the Principal Components Analysis. Specimens raised from Scottish ruffe metacercariae are represented by (r), from Scottish perch metacercariae by (p) and Finnish perch metacercariae by (f). Ellipses indicate 95% confidence limits.



A. gracilis

Morphometric analysis was performed on 15 *A. gracilis* adults raised from the metacercariae of 3 River Almond fish hosts, rainbow trout (6), salmon parr (4) and stone loach (5). The mean and coefficient of variation for each variable, globally and for the 3 individual samples, are given in Table 43. Correlations between the 18 variables, shown in Table 45, illustrate how the sizes of the vast majority of structures co-vary in the same direction. Particularly high correlations were typically recorded between like-related variables, e.g. hindbody length [HBL] and the distance from the ovary to the forebody [OFB], hindbody breadth [HBB] and ovary breadth [OB], although they were also recorded between length and breadth related variables, e.g. hindbody length [HBL] and forebody breadth [FBB].

The first 2 principal components account for 63.9% of the total variance (Table 44). Fig. 63 illustrates the plot of the first two components, with ellipses surrounding 50% of each sample's points. This plot shows a distinct separation of the 3 samples; adults raised from stone loach metacercariae are discriminated from the 2 salmonid samples by variables acting on PCA1, while the 2 salmonid derived samples are distinguished from each other by variables acting on PCA2. A map of the coefficients of these variables (Fig. 64) indicates that length related features contribute most to PCA1; total body length [TBL], hindbody length [HBL] and both testes lengths [TIL and TIIL]. PCA2 opposes length and breadth related features, with hindbody breadth [HBB], testes breadths [TIB and TIIB], ovary breadth [OB], oral sucker length [OSL] and forebody length [FBL] all of importance.

Table 43. Mean and coefficient of variation for each variable (standardised data), globally and in each sample of *A. gracilis* adults (Coefficient of variation = 100 x standard deviation/mean). For an explanation of abbreviations see Fig. 41.

Variable	All *A. gracilis* adults combined (n=15)		Stone loach specimens (n=5)		Rainbow trout specimens (n=4)		Salmon parr specimens (n=6)	
	Mean	C.V	Mean	C.V	Mean	C.V	Mean	C.V
TBL	7.30	3.89	7.66	1.02	7.16	1.41	7.10	1.87
FBL	6.28	3.07	6.47	1.53	6.27	1.63	6.13	2.71
HBL	6.83	5.20	7.29	1.28	6.62	2.81	6.60	1.74
FBB	6.00	2.60	6.17	0.68	5.85	2.29	5.96	1.51
HBB	5.72	3.50	5.65	1.81	5.60	4.46	5.86	2.75
OSL	4.66	2.06	4.71	1.68	4.67	2.27	4.61	2.04
OSB	4.60	2.78	4.63	2.48	4.48	2.23	4.67	2.27
VSL	4.98	4.60	5.09	1.43	5.00	0.58	4.87	6.98
VSB	4.88	3.59	4.92	3.05	4.91	2.46	4.83	4.80
PGL	4.50	4.80	4.55	3.19	4.43	6.12	4.50	6.80
PGB	4.81	4.74	4.83	2.65	4.56	4.36	4.95	3.88
OFB	5.07	10.93	5.67	3.58	4.89	7.32	4.68	8.93
OL	4.69	5.18	4.95	2.77	4.48	5.07	4.63	2.53
OB	4.84	3.06	4.79	0.73	4.72	2.14	4.98	2.49
TIL	5.43	6.00	5.82	1.41	5.06	1.88	5.34	2.21
TIB	5.31	2.69	5.31	0.90	5.18	2.63	5.39	2.82
TIIL	5.63	5.12	5.94	1.09	5.33	2.50	5.57	3.77
TIIB	5.33	2.83	5.32	1.39	5.20	2.42	5.41	3.12

Table 44. Principal Components Analysis of the correlations between the 18 variables used for *A. gracilis* adults.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	7.35	4.16	1.98	1.45
Proportion (%)	40.82	23.12	11.01	8.04
Cumulative (%)	40.82	63.94	74.95	82.99

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
TBL	0.941	-0.270
FBL	0.722	-0.445
HBL	0.943	-0.191
FBB	0.892	0.138
HBB	0.085	0.786
OSL	0.330	-0.465
OSB	0.395	0.501
VSL	0.405	-0.236
VSB	0.420	-0.195
PGL	0.010	0.046
PGB	0.298	0.592
OFB	0.900	-0.267
OL	0.915	0.140
OB	0.018	0.842
TIL	0.943	0.047
TIB	0.296	0.868
TIIL	0.943	0.154
TIIB	0.203	0.851

Table 45. Correlation matrix of the 18 variables used for *A. gracilis* adults.

	TBL	FBL	HBL	FBB	HBB	OSL	OSB	VSL	VSB	PL	PB	OFB	OL	OB	TIL	TIB	TIIL	TIIB
TBL	1.000																	
FBL	**0.835**	1.000																
HBL	**0.978**	0.715	1.000															
FBB	0.784	0.567	**0.806**	1.000														
HBB	-0.153	-0.412	-0.071	0.157	1.000													
OSL	0.304	0.243	0.307	0.413	-0.232	1.000												
OSB	0.187	0.263	0.133	0.404	0.154	-0.155	1.000											
VSL	0.465	0.366	0.460	0.198	0.144	0.051	-0.151	1.000										
VSB	0.329	0.417	0.260	0.306	0.018	0.492	0.323	0.071	1.000									
PL	0.052	-0.141	0.144	-0.008	0.024	-0.145	-0.255	-0.352	-0.187	1.000								
PB	0.041	0.033	0.031	0.528	0.318	0.075	0.813	-0.256	0.263	-0.279	1.000							
OFB	**0.922**	0.744	**0.913**	0.670	-0.022	0.384	0.133	0.510	0.485	0.101	-0.080	1.000						
OL	**0.854**	0.528	**0.902**	**0.817**	0.222	0.131	0.268	**0.807**	0.196	0.106	0.181	**0.807**	1.000					
OB	-0.211	0.391	-0.143	0.102	**0.800**	-0.449	0.316	0.092	-0.271	-0.058	0.376	-0.187	0.257	1.000				
TIL	**0.880**	0.593	**0.906**	**0.874**	0.035	0.307	0.385	0.303	0.239	0.078	0.353	0.770	**0.897**	0.070	1.000			
TIB	0.052	-0.191	0.145	0.354	0.715	-0.262	0.443	-0.097	-0.004	0.111	0.517	0.077	0.357	0.612	0.283	1.000		
TIIL	**0.847**	0.558	**0.865**	0.791	0.209	0.227	0.455	0.311	0.358	0.111	0.301	**0.846**	0.877	0.133	**0.923**	0.411	1.000	
TIIB	-0.002	-0.186	0.080	0.263	0.614	-0.359	0.408	-0.241	-0.170	0.185	0.396	0.021	0.296	0.590	0.194	**0.927**	0.333	1.000

Fig. 63. Map of the 15 *A. gracilis* adult specimens in the first plane of the Principal Components Analysis. Specimens raised from rainbow trout metacercariae are represented by (r), from salmon parr metacercariae by (s) and stone loach metacercariae by (l). Ellipses surround 50% of points.

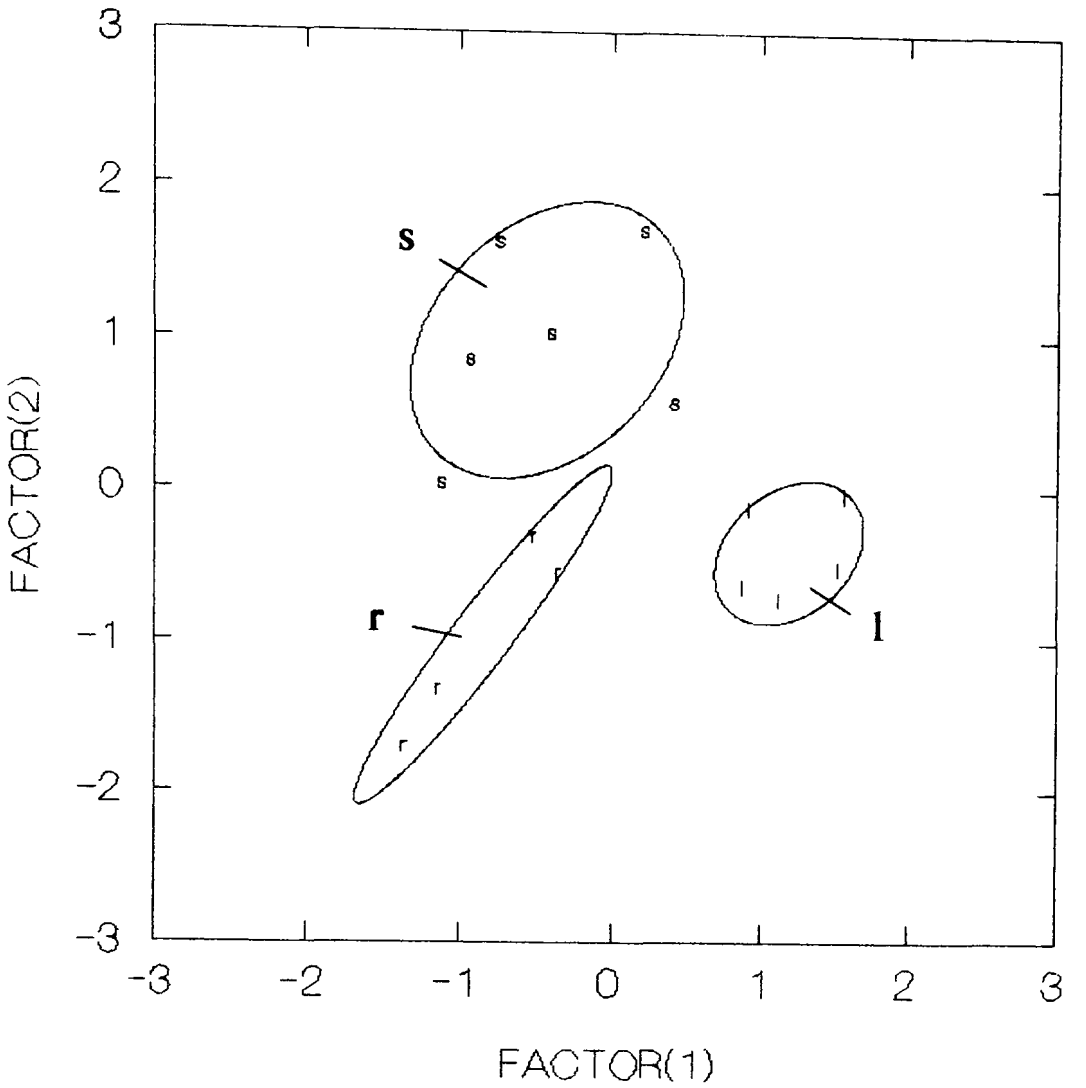
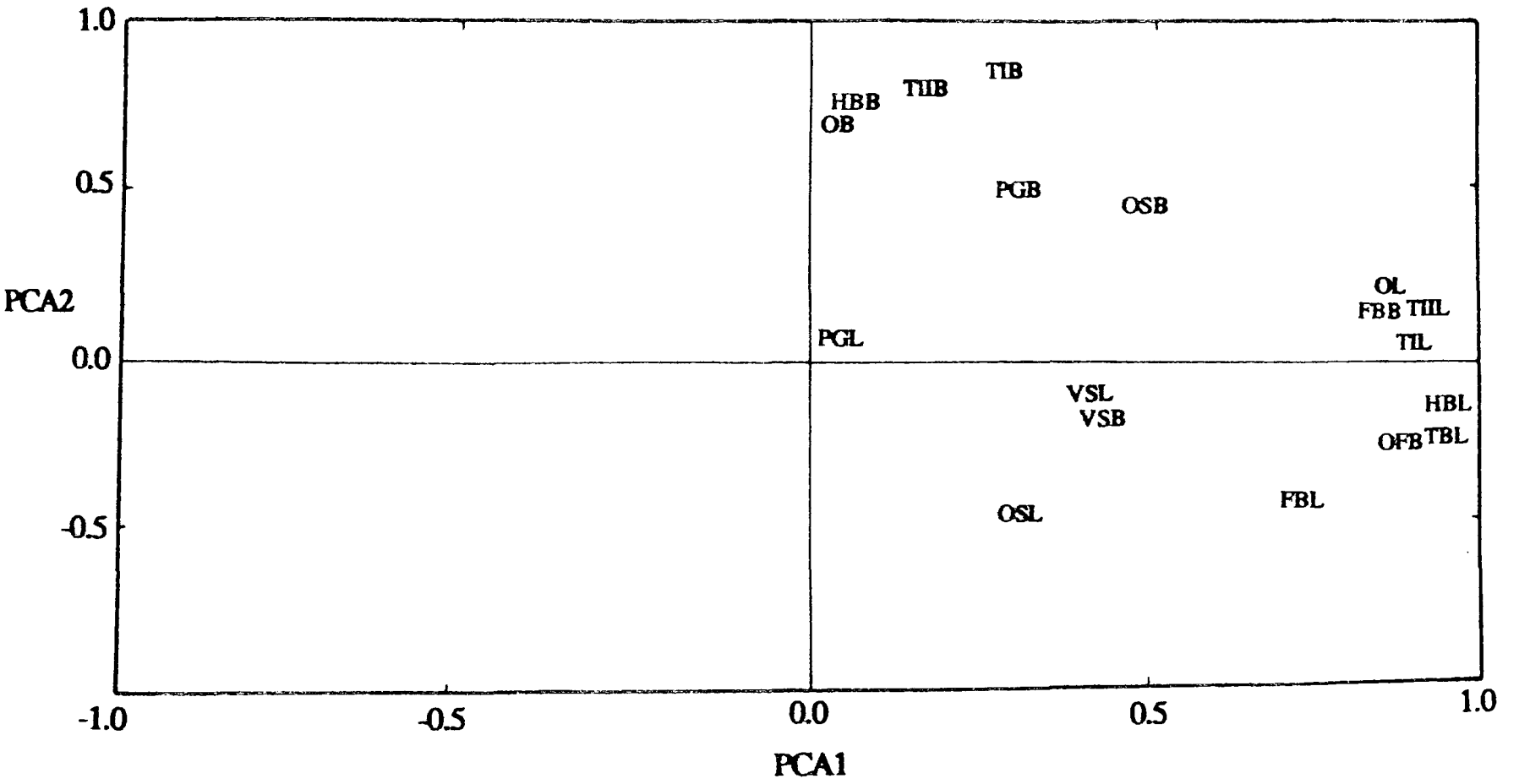


Fig. 64. Map of the 18 variables used for *A. gracilis* adults in the first plane of the Principal Component Analysis.



DISCUSSION

3.1. CULTURE OF METACERCARIAE TO ADULTS.

Adult worms of the genus *Ichthyocotylurus* (*I. erraticus* and *I. variegatus*) were readily obtained from gull chicks (herring gulls and lesser black-backed gulls) infected with metacercariae excised from British fishes. These species were also reared from Finnish metacercariae. Unfortunately, until full adult plumage is acquired, it is extremely difficult to distinguish between the two very closely related gull species used in this study. Consequently, all conclusions made regarding adult development presume equal success in both gull species.

I. pileatus and *I. variegatus* metacercariae from joint infections of Finnish ruffe failed to establish in experimental gull chicks. Both gull species employed are listed as natural hosts for *I. pileatus* (see Dubois, 1968) and single infections of *I. variegatus* metacercariae from Finland were successful. Competition between the two species might be expected, as both develop primarily in the bird's small intestine. However, this should not result in the exclusion of both species and suggests that compromised metacercarial viability, and not host unsuitability or competition, was responsible for the failure of these infections.

The development of *Ichthyocotylurus* adults within the gull host was rapid. The onset of egg production by *I. erraticus* adults was typically on day 4 p.i., as previously recorded by Olson (1970) for experimental infections in California gulls and Swennen *et al.* (1979) in herring gulls. Niewiadomska & Kozicka (1970) obtained young (immature) adults 26 hours p.i. and Razmashkin (1966) mature adults after 4 days p.i. Adults recovered in this study were found to have attained 87% of their total body length and to be gravid by day 5 p.i. However, the number of eggs present in the uterus of adults at day 5 p.i. (mean 20.2, n=21) was significantly lower ($p<0.001$) than those present in adults at day 14 p.i. (mean 30.4, n=41) (Table 33), suggesting that full

maturity is not achieved by day 5 p.i.

Once egg release has started, the number of *I. erraticus* eggs present in the hosts faeces was found to rise rapidly and attain a peak at around day 8 p.i. before dropping steadily. Swennen *et al.* (1979) also observed that a peak in egg production was achieved at about day 10 p.i. and that recovery decreased after this time. They recorded similar release patterns for *I. variegatus* and *I. platycephalus* adults.

No correlation was found between the recovery of *I. erraticus* adults and duration of infection. This finding was probably due to the low sample size involved (9 infections). It appears that there is a progressive loss of established adults from around day 10 p.i., as the egg numbers recovered decrease after this time, while the number of eggs *in utero* was not found to drop once maturity was reached. Lower percentage recoveries of *I. erraticus* adults were obtained from Finnish whitefish metacercariae than from Scottish powan material. This decrease was probably due to a reduction in the number of viable cysts administered (the cysts were excised in Finland and transported to Scotland over a period of days) and also explains the lower return of eggs (per metacercaria introduced) from gulls infected with this material.

Olson (1970) noted that an initial *I. erraticus* infection lasted for 14 days. The results recorded here agree with those of Swennen *et al.* (1979), who found *I. erraticus* eggs in infected gulls faeces up to 40 days p.i.

All previous records of *I. erraticus* adults, from both natural and experimental infections, have reported the worms as being primarily located in the small intestine. Indeed, Dubois (1968), Niewiadomska & Kozicka (1970), Olson (1970), Odening (1979) and McDonald (1981) all simply stated that the worms are present in the small intestine. A wider distribution of *I. erraticus* adults in young naturally infected herring, lesser black-backed and greater black-backed gulls was recorded by Fraser (1974), with worms recovered from the cloaca and bursa Fabricius, as well as the posterior region of the small intestine. Swennen *et al.* (1979) recorded *I. erraticus* adults within the digestive

tract of a herring gull at 6 days p.i.; all adults being located within the small intestine. No worms were found in the proximal 30%, 94% of worms were situated between 40 and 70% down the intestine and only 6% in the terminal 30%. Comparable data from this study i.e. experiments where gulls were sacrificed at days 5 and 6 p.i. also showed that adults were solely located in the small intestine. The distribution of adults in the bird sacrificed at day 6 p.i. closely resembled that of Swennen *et al.* (1979), with all worms removed from the 40-70% region of the small intestine. Whereas, in a bird autopsied 5 days p.i. adults were found throughout the terminal 80% of the small intestine, with almost 90% of worms in the most distal 20%.

In these experimental infections, gulls sacrificed after 10 days p.i. yielded no adults from the proximal half of the small intestine and in four of five birds, several adults were present in the rectum. Indeed, the proximal limits of distribution decreased as the duration of the infection increased: < 6 days p.i. - distal 80%; 10-12 days p.i. - distal 50%; 14 days p.i. - distal 30%; 24 days p.i. - distal 10%. Whether a migration down the intestine occurs, or whether proximally situated adults are 'lost' first is difficult to determine. It may be that the more proximal regions of the small intestine are not as favourable for sustaining *I. erraticus* adults as the more distal positions. The fact that distally situated worms possess proportionally larger hindbodies supports this theory, but these adults are no larger (total body length remains constant regardless of location) and they do not contain greater numbers of eggs in their uterus. Alternatively, these data can be interpreted in respect to forebody size, which is proportionally smaller in distally located worms. Perhaps the smaller forebody reflects the nature of the niche (smaller villi), with this feature enhancing the worm's ability to remain attached distally.

The development of *I. variegatus* adults was also rapid, with the first worms maturing and releasing eggs after only 3 to 4 days p.i. However, although all specimens were found to be gravid by day 5 p.i., at this age their hindbody only comprised 55%

of total body length and maximum size was not attained until day 8 p.i. Odening & Bockhardt (1971) recorded a slightly longer prepatent period of 4 to 7 days (typically 6) for *I. variegatus* adults in blackheaded gulls, but failed to comment upon levels of egg production during the course of their experimental infections. They considered the life span of *I. variegatus* adults to be between 10 and 30 days (typically 10 to 15).

Odening, Mattheis & Bockhardt (1970) completed the life-cycle of *I. platycephalus* experimentally, raising adults in blackheaded gulls. They found that eggs were first released in the faeces of experimental birds 5 to 6 days p.i. and that the duration of infection was typically 15 to 23 days; slightly longer than that of *I. variegatus*. In the present study and that of Swennen *et al.* (1979) eggs were recovered from the faeces of birds infected with *I. variegatus* adults until 28 and 35 days p.i., respectively. However, it must be remembered that the termination of egg production indicates the absence of any releasing adults and hence the limit of the population's longevity, not that of the average individual. A number of residual eggs may also be passed in the faeces after the death and loss of the last adults. The pattern of egg production recorded here (presuming individual egg production does not decrease with time - the numbers are too large to determine this) suggests that *I. variegatus* adults, like *I. erraticus*, are progressively lost. In this case from about day 12 to 14 p.i.

Brady (1989) experimentally infected herring gulls, a known natural host, with *Diplostomum* spp. She noted that the onset of egg production began before day 7 p.i., that a slight peak occurred at 21 days p.i. and that numbers decreased after 49 days. She continued monitoring infected birds for 61 days and egg release was continuous throughout this period. The number of eggs recovered per ml of faeces was far lower than for either of the *Ichthyocotylurus* species. Judging from the data of Brady (1989) and the present study, it appears that strigeoid adults adopt at least two egg release strategies. Egg production may be high and adult longevity low (<40 days: *Ichthyocotylurus* spp.), or fewer eggs may be produced but over a longer period (>60 days: *Diplostomum* spp.).

Niewiadomska (1973) considered the longevity of other adult members of the Strigeoidea to be relatively short. Certain species do, however, have a far greater life span. Odening (1965) demonstrated that two species of *Neodiplostomum* Railliet, 1919 adults live for about two years, and similar data were reported by Pearson (1961) for a third species. The longevities of *Strigea* spp. were recorded as 7-18 months by Odening (1967). Unfortunately, quantitative data are not given by these authors regarding egg production.

The distribution of *I. variegatus* adults was found to be more localised than that of *I. erraticus*. In all infections worms were confined to the distal half of the small intestine (typically the terminal 30%) and rectum. Of 507 worms recovered, not a single specimen was ever observed within the cloaca or bursa Fabricius. The duration of infection was not found to affect the distribution. Odening & Bockhardt (1971) recovered *I. variegatus* adults from the posterior third of the small intestine and rectum in experimentally infected blackheaded gulls. The development of *I. variegatus* adults in herring and blackheaded gulls was monitored by Halton & Faulkner (1988), who noted that excystment of metacercariae occurred within 3 hours of ingestion and that worms attached to the mucosa half way down the small intestine. The rapid development of the worms between days 2 and 6 p.i. was associated with their migration down the small intestine towards the rectum.

A multiple infection experiment was performed by Swennen *et al.* (1979), feeding herring gulls with fish known to contain metacercarial cysts of *I. erraticus*, *I. variegatus* and *I. platycephalus*. Examination of the birds' digestive tracts revealed adults of all three species: *I. erraticus* was located in the posterior region of the small intestine; *I. variegatus* also occupied this region of the intestine but extended into the large intestine (rectum); and *I. platycephalus* was exclusively situated within the cloaca and bursa Fabricius. No overlap in habitat was seen for the two latter species.

The spatial separation of *I. variegatus* and *I. platycephalus* adults within the

definitive host was considered by Razmashkin (1966), Swennen *et al.* (1979) and Odening (1979) to be species specific. Dubois (1968, 1978) and McDonald (1981) disputed the existence of *I. variegatus* as a valid species, considering it a misidentification of *I. platycephalus*. However, these authors list the location of *I. platycephalus* within the avian host as the cloaca and bursa Fabricius (Dubois, also included the rectum), never the small intestine.

Fish host and location of the metacercariae within a single fish host was not found to influence the distribution of *Ichthyocotylurus* adults within the experimental definitive host. However, location within the fish host did appear to affect success of establishment; an aspect of infection which has not been examined previously. In two comparable *I. variegatus* infections terminated after 15 days p.i., one with metacercariae excised from the pericardial cavity and the other with cysts from the orbits of the same fish, the former yielded a 30% recovery of adults and the latter only 13%. This apparent difference in viability may result from the nature of the host response which is known to differ depending on the tissue invaded.

Initial infections attempted with *A. gracilis* metacercariae involved cysts excised from naturally infected rainbow trout and fed to Aylesbury ducklings. Negligible numbers of eggs were obtained from infected ducklings, although ovigerous adults were collected when the infection was terminated at 5 days p.i. The utility of domestic ducks as experimental hosts for *A. gracilis* has met with mixed success by other authors. Yamaguti (1933) performed three experiments in which tetracotyle larvae from the body cavity of the fish *Mogurndu obscura* were fed to domestic ducklings. The resulting adults he described as *Apatemon pellucidus* (a synonym of *A. gracilis*, according to Dubois, 1968). In these experiments he found that worms matured within 10 days. The first eggs appeared in the faeces 3 days p.i. and increased in number to day 8. Egg production continued for 94 days p.i. and the eggs were found to be viable. Two male

khaki campbell ducklings were infected by Crocombe (1959) with 150 and 120 cysts respectively from the mesenteries of the bullhead *Cottus gobio*. He obtained 73 adults from the first bird at 4 days and 14 adults at 9 days p.i. from the second. These adults, he concluded, were identical to those described by Yamaguti (1933). Later, Vojtek (1964a) infected several species of duck (including domestic ducks) with metacercariae from bullheads, stone loach and the mottled black sea goby *Proterorhinus marmoratus*. The resulting adults he called *Apatemon cobitidis cobitidis* (developing from bullhead and stone loach metacercariae) and *A. cobitidis proterorhini* (from the goby metacercariae). Both of these forms are now considered synonymous with *A. gracilis* (see, Dubois, 1968). Successful infections in domestic ducks were also obtained by Blair (1974), who fed the hosts metacercariae from rainbow trout, sticklebacks and stone loach (all fish were from the same source used in the present study: the River Almond, Perthshire). His experimental infections established for all challenges with stone loach metacercariae, 9 of 10 challenges with rainbow trout material and 2 of 3 challenges with stickleback metacercariae. He stated that the adult worm matures in about four days, but did not provide details of adult age (days p.i.) on recovery, percentage recovery or longevity of worms. More recently, Watson & Pike (1993) experienced a similar lack of success to that recorded in the present study, and were unable to obtain any *A. gracilis* adults from domestic ducklings infected with rainbow trout metacercariae. It is interesting that the majority of adults raised developed from bullhead and stone loach material, while only a single author (Blair, 1974) achieved success with salmonid metacercariae.

Due to the lack of success with domestic ducks in the present study, an alternative experimental host was used for subsequent *A. gracilis* infections, all be it, its close relative the mallard. No eggs were found in the faeces of birds infected with metacercariae from rainbow trout, or from salmon parr, but all infections using stone loach metacercariae produced eggs.

Watson & Pike (1993) investigated the development and morphology of *A. gracilis* adults raised in four different avian hosts: herring gulls; domestic ducklings (see before); domestic chicks *Gallus gallus*; and eider ducklings. All metacercariae used in the infections were excised from rainbow trout. They recovered viable eggs from two infected eider ducklings* for 52 and 35 days respectively and upon autopsy collected adults from one individual at 64 days p.i.

Eider ducks are reported as natural hosts for two forms of *Apatemon* (*Apatemon*) adults: *A. somateriae somateriae* Dubois, 1948 and *A. s. fischerie* Dubois, 1968, which may explain why they appear to be such effective experimental hosts for the closely related *A. gracilis*. However, why *A. somateriae* forms are found particularly in the eider duck, which feeds primarily on mussels and not fish, is puzzling.

Hoffman (1959) infected chicks (species not specified) with *A. gracilis* cysts obtained from the brook stickleback *Eucalia inconstans*. Eighteen percent of experimental birds yielded ovigerous adults in 3-12 days and eggs recovered proved to be viable. Two of three *A. gracilis* adults obtained by Watson & Pike (1993) from a domestic chick were ovigerous at day 6 p.i. and eggs also proved to be viable. They found that infections were short-lived in this host (maximum of 14 days) and that the number of eggs produced was low. The durations of infection which they observed resemble the population longevity recorded in the present study for stone loach derived *A. gracilis* adults in mallard experimental hosts. Adults obtained here from stone loach metacercariae differ from Watson & Pike's chick adults in that all present worms were ovigerous and egg production tended to be greater.

* As explained in Chapter 3; Materials and Methods, attempted incubations of eider eggs failed and a comparison of *A. gracilis* infections with those performed in other avian hosts could not be performed. The eider eggs were collected from the same source (Sands of Forvie Nature Reserve) as those used by Watson & Pike (1993) in 1986. Unfortunately, there now appears to be a major problem at this site, as only a single duckling fledged from some several hundred breeding pairs in the Summer of 1994.

No adults were retrieved from the herring gull examined at day 5 p.i. in the present study, while those obtained by Watson & Pike (1993) from this host at 3 days p.i. were stunted and non-ovigerous.

It is interesting to note that when *A. gracilis* adults were obtained from infections in the present study of a longer duration than 3 days p.i., gravid individuals were present, regardless of metacercarial origin. Why then, were so few eggs ever recovered from the faeces of birds infected with rainbow trout (3 eggs from 11 infections) and salmon parr (1 egg from 6 infections) metacercariae? The smallest dimension (width) of all eggs (see 3.2; Table 36) was larger than the 45µm collecting sieve and faecal sampling methods were constant for all infections. Possibly, the stunted size of the adults recovered from rainbow trout and salmon parr material resulted in physiological conditions within the worms which enabled initial egg production, but restricted their release and further production?

The results obtained in this study, and by other authors, appear to indicate that the success of *A. gracilis* adults in an experimental host depends not only upon the definitive host employed but also on the source of metacercariae, i.e. the fish host. It would be useful to determine whether *A. gracilis* metacercariae excised from stone loach, bullheads, salmon parr and rainbow trout develop equally well in eider ducklings or, indeed, whether they would all establish optimally in the recognised natural host, mergansers?

A. annuligerum adults were not recovered from the experimental infections performed using a mallard duckling and a gull chick, and the lack of metacercarial material prevented further attempts to culture these worms. Consequently, the identity of these metacercariae was not confirmed, although it is likely based on the present knowledge of members of the subgenus *Apatemon*. Records in the literature indicate that this species and *A. buteonis* are specific to birds of prey. *A. annuligerum* adults were

raised experimentally in a buzzard by Odening (1970), but no further life-cycle studies were performed and the miracidium and cercaria (its molluscan host unknown) remain undescribed. The only natural host record for *A. annuligerum* is also from a buzzard (Oltenau *et al.*, 1968). However, it is unlikely that this bird represents the sole definitive host for this species, particularly as it is not a specialist fish feeder. Consequently, the recognised host list will probably broaden with further host records, as hypothesised for strigeids in general by Niewiadomska (1973).

All adults of the subgenus *Apatemon* (*Apatemon*) are located proximally within the intestine of their definitive hosts, but records are not always clear as to whether the duodenum is included in this distribution. Dubois (1968) and Sudarikov (1984) gave the location of *A. gracilis* adults from natural infections as the duodenum and proximal region of the small intestine. In experimental infections, Yamaguti (1933, using domestic ducklings) stated that *A. gracilis* adults were located in the intestine; Crocombe (1959, using khaki Campbell ducklings) and Blair (1974, using domestic ducklings), indicated the small intestine; Hoffman (1959, using chicks) and Watson & Pike (1993, using domestic chicks and eider ducklings) gave the site as the upper third of the small intestine. In this study *A. gracilis* adults were recovered from the duodenum and the proximal 40% of the small intestine. Within this range there appeared to be some spatial separation of adults according to metacercarial origin. However, due to the low number of successful infections, no firm conclusions could be made. As experimental infections were terminated at 3-5 days p.i. in order to recover adults, no study of the relationship between distribution and the duration of infection was possible.

3.2. TAXONOMY OF ADULTS.

With the exception of the forebody lengths recorded for *I. erraticus* adults raised from Scottish metacercariae, which were rather short, the morphology of all specimens corresponded closely to that described by previous authors (Table 34). Principal

Components Analysis on the metrical features of Scottish and Finnish *I. erraticus* specimens indicated that morphological variation occured primarily in the generally smaller dimensions, the longer, thinner ovaries and the narrower pharynx of the former. The typically shorter forebody of Scottish worms was not shown to have particular importance in the discrimination of the two worm populations (see Fig. 59).

The total body lengths of *I. variegatus* adults attained at 14-15 days p.i. ranged between 4.65-8.69mm (pooled data from adults raised from all metacercarial sources), often exceeding the maximum size (6mm) recorded by Dubois (1968, for *Cotylurus cumultitestis*) but falling short of the limit indicated by Odening & Bockhardt (1971) (13mm). Body dimensions were found to vary widely for *I. variegatus* adults, even when of the same age, metacercarial source and recovered from the same host. Nevertheless, worms raised from Finnish perch metacercariae tended to be larger than those from Scottish perch metacercariae, with Scottish ruffe material providing the smallest specimens. PCA indicated that greater hindbody length and testes dimensions were also important in differentiating Finnish adults from Scottish, while adults derived from Scottish ruffe metacercariae possessed larger ventral suckers than those raised from Scottish perch material.

All *I. variegatus* adults were seen to possess the same body shape, with a 'closed' sub-globular or sub-hemispherical forebody and cylindrical hindbody (see Figs 54, 55). Such a body form was also recorded by Dubois (1968, as *C. cumultitestis*), Odening & Bockhardt (1971) and Swennen *et al.* (1979) for this species. The descriptions of Dubois (1968, as *C. platycephalus communis*), Odening *et al.* (1970), Niewiadomska (1970b, as *C. platycephalus* and *C. cucullus*) and Swennen *et al.* (1979) for *I. platycephalus* adults all indicate an 'open' bowl-shaped forebody and, with the exception of Swennen *et al.* (1979), also provide illustrations which show a more ovoid hindbody for this species, than occurs in *I. variegatus*. The body dimensions, particularly those of the hindbody, recorded for *I. platycephalus* by Dubois (1968, as *C.*

platycephalus communis), are somewhat broader than recorded here and by himself (1968, as *C. cumultitestis*) for *I. variegatus*. These measurements do, however, exhibit a large overlap in ranges and do not provide a basis for discrimination of the two species. Ratios of hindbody and forebody lengths were calculated for these species by Dubois (1968) and similarly showed an overlap of ranges. Niewiadomska (1970a) used the ratio of forebody and hindbody breadths to distinguish *C. cucullus* and *C. platycephalus* adults (the former now known to be a synonym of the latter). Her findings were later found to result from differences in the timing of fixation, post-excision (see Odening, 1971; Swennen *et al.,* 1979), and demonstrate the precarious nature of basing discrimination on body ratios. Internal organ dimensions are extremely similar in both species and offer no species specific characteristics. Unfortunately, the paucity of *I. platycephalus* metacercariae recovered from Finnish material and their absence from the British fishes examined precluded the culture of adults. Consequently, morphometric analyses, which might have enabled discrimination of the two species by the collective comparison of subtle morphological differences, could not be applied.

Previous authors have all described the position of the oral sucker in *I. variegatus* adults as subterminal (Dubois, 1968 as *C. cumultitestis*; Odening & Bockhardt, 1971; Swennen, Heessen & Hocker, 1979) and this was also recorded in the present study. Indeed, Swennen *et al.* (1979) considered the position of the oral and ventral suckers to be of diagnostic merit in the discrimination of this species from *I. platycephalus*, observing that both suckers were "more deeply imbedded" in the latter species. These measurements were recorded for *I. variegatus* specimens (Table 35) in the present study to enable their future quantitative comparison with *I. platycephalus* adults. Sudarikov (1984) provided a key for the identification of *Ichthyocotylurus* adults in which *I. variegatus* and *I. platycephalus* were discriminated by their location within the gut of the definitive host, ratio of fore- and hindbody lengths and distribution of vitelline follicles. No reference was made to body shape or the position of suckers, while examination of figures in the literature suggests that the criterion of a more

extensive vitellarium in *I. variegatus* is tenuous.

The low percentage recovery of *A. gracilis* adults and their short longevity observed in 3.1 indicated the unsuitability of the experimental definitive hosts employed. Worms recovered in the present study and by Blair (1974) from infections in domestic ducklings all exhibited a similar morphology; adults raised from rainbow trout metacercariae attained an average body length of about 1.2mm (both studies, Table 36) and those developing from stone loach metacercariae 1.4mm (Blair, 1974). These body lengths are only approximately half the size of those recorded for *A. gracilis* adults recovered from the natural host (Dubois, 1968). Although many of the worms in the present study were poorly developed, most internal organ dimensions were within the range indicated by Dubois (1968). The only species characteristic of these adults that did not conform was the position of the ovary, which was situated in the anterior 15-20% of the hindbody (Table 36) and which fell closer to that recorded for other members of the subgenus *Apatemon.* Nevertheless, it is not clear where Dubois (1968) recorded this measurement from, i.e. the centre or anterior margin of the ovary, and if it is the former, then all worms do comply with the range he gave for *A. gracilis* (22-40%).

Watson & Pike (1993) demonstrated that the eider duck is a far more suitable experimental host for *A. gracilis* (excised from rainbow trout) than the domestic duckling, with worms of up to 2.6mm recovered. However, although the mallard and domestic duck are varieties of the same species, *A. gracilis* infections performed here with stone loach metacercariae in the mallard produced adults with an average length of 2.1mm, far larger than attained by Blair's (1974) adults derived from the same metacercarial source in the domestic duck. The most noticeable morphological differences between the latter two groups of adults were the increase in hindbody and testes lengths.

Further infections in mallards revealed that metacercarial origin may influence

the morphology of the adults to almost the same extent as the experimental host employed. *A. gracilis* metacercariae excised from salmon parr and fed to mallards developed into adults that only ever attained a size comparable to that recorded for specimens reared in domestic ducklings (1.3mm). In an attempt to obtain eggs from adults derived from rainbow trout metacercariae, infections in mallards were allowed to continue for extended periods and were never terminated at, or before, 5 days p.i. Consequently, no adults developing from this material were ever recovered from mallards. Unfortunately, this prevented a comparison of their morphology with those adults reared from the other two metacercarial sources in this host; stone loach (adults 2.3mm in length) and salmon parr (adults 1.3mm in length).

PCA identified the most significant morphological differences between the adults obtained from rainbow trout metacercariae (in domestic ducks) and salmon parr metacercariae (in mallards) as; larger forebody and oral sucker lengths in the former, and larger hindbody breadths and breadths of organs contained therein, in the latter. Whether these differences reflect avian host-induced or metacercarial-induced variation is unknown.

In summary, *I. erraticus*, *I. variegatus* and *A. gracilis* adults were successfully reared in experimental hosts using metacercariae from a variety of origins (fish hosts, sites within a single fish host and geographical sites); the adults obtained enabled clarification of the identities assigned to metacercariae. Infections attempted using metacercariae believed to represent *I. pileatus* and *A. annuligerum* were unsuccessful; the former was thought to be due to compromised metacercarial viability and the latter to the use of unsuitable experimental hosts.

Herring gulls and lesser black-backed gulls proved to be extremely good experimental hosts for both *Ichthyocotylurus* spp., with the vast majority of infections establishing and providing high yields of eggs and adults. These infections allowed information on the establishment, development, fecundity, site specificity, longevity and

morphological variability of the adults to be gathered, enhancing the data acquired by previous authors. Aspects of the morphology and biology of *I. variegatus* adults recorded were found to support its validity as a species discrete from *I. platycephalus*.

The experimental hosts used for *A. gracilis* infections, domestic and mallard ducklings, were not found to be satisfactory, with few eggs and adults recovered and poor development often attained. However, the development of *A. gracilis* metacercariae of this species excised from stone loach appeared less influenced by these hosts than those from two salmonid species. All challenges with stone loach metacercariae established, resulted in adults exhibiting normal morphology, produced collectable quantities of eggs and demonstrated an increased longevity. These findings suggest that metacercarial host may affect future development.

Eggs of known origin were collected for all three cultured strigeid species, enabling further life-cycle studies.

CHAPTER 4: THE MIRACIDIA

INTRODUCTION

4.1. DEVELOPMENTAL STUDIES.

The eggs of many trematodes, including strigeids, are operculate and embryonate in water. Accounts of the development of such eggs were provided in the reviews of Smyth (1966) and Erasmus (1972), and also by Olson (1970), while experimental studies of environmental factors influencing the development and hatching of operculate digenean eggs have been described for *Fasciola hepatica* (see Rowcliffe & Ollerenshaw, 1960), *Diplostomum spathaceum* (see Blair, 1974) and *Diplostomum* spp. (see Brady, 1989). Blair's (1974) study concluded that the development and hatching of *D. spathaceum* eggs were largely controlled by temperature, that they were capable of normal development and hatching under all likely conditions of pH, salinity and oxygen concentrations, that hatching could occur in the dark, and that they were able to survive long periods at low temperatures.

Here, experimentally obtained eggs of *Ichthyocotylurus erraticus*, *I. variegatus* and *Apatemon gracilis* were incubated in an attempt to raise the miracidial stages. The development of the miracidia was monitored and the effect of temperature on this development investigated.

4.2. TAXONOMIC STUDIES.

Light microscopical investigations on the morphology of miracidia are numerous (*inter alia* Lynch, 1933; Pearson, 1961; review of Erasmus, 1972). Such studies on miracidia of the genus *Ichthyocotylurus* were performed by Olson (1970; *I. erraticus*), Odening *et al.* (1970; *I. platycephalus*) and Odening & Bockhardt (1971; *I. variegatus*), with the miracidium of *I. pileatus* remaining undescribed. The only miracidium described for members of the subgenus *Apatemon* is that of *A. gracilis* (Vojtek, 1964a, as *A. cobitidis*). In this study the morphology of the miracidia of the three strigeid species raised from experimentally obtained eggs was investigated and compared with

existing knowledge.

Silver nitrate staining of miracidia reveals the periphery of the epidermal plates, the opening(s) of the apical gland, the number and distribution of sensilla as well as the location of the excretory pores. Many authors have described the number and arrangement of miracidial epidermal plates using this technique (*inter alia* Pearson, 1961; Vojtek, 1964a; Peters, 1966), but few have also recorded the chaetotaxy. An early exception to this was the work of Pearson (1961) on *Neodiplostomum intermedium* Pearson, 1959, prior to the descriptions by Albaret (1984) on the argentophillic surface structures of *Schistosoma* spp. Later, Dimitrov, Samnaliev & Sey (1989) provided nomenclature for the description of miracidial chaetotaxy and subsequently applied this system to the miracidium of *Paramphistomum ichikawai* Fukui, 1922. The only existing description of argentophillic structures of a strigeid miracidium is that of Dimitrov, McCarthy & Kanev (1991) for *Strigea falconispalumbi* Viborg, 1795 (= *S. falconis*). In the present study, this technique was applied to *I. erraticus*, *I. variegatus* and *A. gracilis* miracidia.

Scanning electron microscopy (SEM) of miracidia has enabled descriptions of structures not seen using light microscopy and in certain cases has isolated species specific features. For example, Eklu-Natey, Wüest, Swiderski, Striebel & Huggel (1985) were able to record that *Schistosoma* spp. exhibited different patterns of anastomosing membrane foldings on the terebratorium. In this study SEM was used, for the first time, to examine the surface structures of strigeid miracidia.

4.3. BEHAVIOURAL STUDIES.

Studies on the longevity and infectivity of miracidia have concentrated mainly on *Schistosoma* spp., particularly *S. mansoni* (see Oliver & Short, 1956; Farley, 1962; Prah & James, 1977; Anderson, 1978; Anderson, Mercer, Wilson & Carter, 1982), and no information appears to be available regarding strigeids. Temperature was considered by Anderson *et al.* (1982) to be one of, if not the, most limiting factors influencing the

survival of *S. mansoni* miracidia, and it would seem most likely that this parameter is of similar importance to temperate species. A series of infectivity experiments could not be performed with *Ichthyocotylurus* miracidia in this study due to the short supply of naïve snail hosts (*Valvata piscinalis*). Similarly, such an examination of the senility of *A. gracilis* miracidia was precluded due to the paucity of available parasite eggs. However, an investigation of the longevity of *I. variegatus* was undertaken to determine age groupings relevant for the behavioural (chemotactic) experiments performed later in the study. Longevity data on this miracidial species would also provide an estimation of senility rate, and hence, the degree of rapidity required for experimental infections in snails.

The behavioural patterns of miracidia, particularly their photo-, thermo-, geo- and chemotactic responses, have been extensively studied (see review of Erasmus, 1972). However, the majority of recent behavioural investigations have, as with other studies on miracidia, concentrated on *Schistosoma* spp. and *Fasciola hepatica* (see *inter alia* Christensen, 1980; Samuelson, Quinn & Caulfield, 1984; Haas, Gui, Haberi & Ströbel, 1991; Howe & Nollen, 1992). The results obtained, even for a single miracidial species, have often provided differing or contradictory views (see review of Haas, 1988), which reflects the difficulties inherent in the experimental techniques employed to study such phenomena. Observations on tactic responses of strigeid miracidia have so far been unquantified and remained largely incidental (Olson, 1970; Swennen *et al.*, 1979). In their study on the host finding behaviour of *Schistosoma japonicum* Katsurada, 1904 miracidia, Haas *et al.* (1991) advocated the use of specially constructed choice chambers which enabled the quantification of results. These authors also reported that snail conditioned water could be satisfactorily employed for chemotactic experiments. In this study the accumulation and turning behaviour of *I. variegatus* miracidia was investigated using choice chambers modelled on those of Haas *et al.* (1991), while the use of snail conditioned water allowed many trials to be performed with a limited supply of snails.

MATERIALS AND METHODS.

4.1. DEVELOPMENTAL STUDIES.

Collection, storage and incubation of eggs.

Eggs were collected in distilled water from the faeces of experimentally infected birds, as described in Chapter 3.1.3; Materials and Methods. The eggs (50-400, depending upon availability) were transferred to small, 3ml petri-dishes, the distilled water being replaced with artificial spring water (A.S.W) (Maccinis & Voge, 1970), and incubated in a light:dark regime of L16:D8. *I. erraticus* eggs obtained from gulls experimentally infected with metacercariae from powan and *I. variegatus* eggs originating from perch and ruffe metacercarial infections were maintained at 18, 20 or 24°C. Due to the paucity of material, *A. gracilis* eggs from ducks infected with stone loach metacercariae were kept at a single incubation temperature of 20°C. The A.S.W was replaced daily with water heated to the appropriate temperature, to reduce bacterial growth.

Often it was necessary to store eggs until other limiting conditions for experimental infections in snails were met; this was achieved by placing the unembryonated eggs in distilled water at 4°C. Typically, several thousand eggs were stored in such a manner within a 5ml plastic Bijoux bottle. Egg viability was maximised by changing the distilled water with water of the same temperature several times per week. Hatching data for eggs stored in this way were considered separately from fresh eggs.

Monitoring miracidial release.

Eggs were examined within the petri-dish under a dissecting microscope twice weekly for the development of miracidial eye-spots. Thereafter, eggs were monitored periodically each day for miracidial release. The free-swimming miracidia were best observed under the dissecting microscope using an incident light source. The period

over which hatching took place for any given batch, the "duration of hatching", was recorded. Hatching success of egg batches was determined by calculating the percentage of empty shells at the end of incubation. Incubation was terminated when no miracidial hatching had been observed for several days and microscopical examination of unhatched egg subsamples revealed them to be non-viable. A more informative method of calculating hatching success would have been to remove empty egg cases on a daily basis, as performed by Blair (1974), enabling 50% hatch rates (which represent the populations' average developmental period) to be calculated. However, the number of eggs typically incubated within each 3cm petri-dish in the present study made this impractical.

Occasionally, practical requirements necessitated the delaying of miracidial release; this was attempted by denying light to the developed miracidia.

4.2. TAXONOMIC STUDIES.

4.2.1. Light microscopical observations of miracidia.

Newly emerged (less than 1 hour post-hatch) *I. erraticus*, *I. variegatus* and *A. gracilis* miracidia of known life-history were removed from their petri-dishes using a drawn out pipette and either fixed in hot (60-65°C) 5% formalin or placed in dilute methyl cellulose to minimise movement and allow the observation of live specimens. Miracidia were measured unstained in these media, coverslip pressure on the specimens being avoided. Mean measurements were calculated from a minimum of 10 individuals.

4.2.2. Miracidial chaetotaxy.

Miracidial sensilla were stained using the technique described for metacercariae in Chapter 2.2.3; Materials and Methods. Several modifications to this method were necessary for optimal results with miracidia. A solution of hot (60-65°C) rather than cold (4°C) 0.5% silver nitrate was used in order to reduce specimen contraction, and the period of dark incubation was reduced to 2.5 minutes to prevent over staining. The

necessary exposure time to UV light (approximately 10 seconds at a wavelength of 325nm) was found to be very short and best results were often obtained using natural light. Drawings were made with the aid of a drawing tube and the chaetotaxy pattern compiled from a minimum of 50 specimens. Descriptions of the epidermal plate and sensillary arrangements were made according to the nomenclature proposed by Dimitrov, Samnaliev & Sey (1989). However, modifications to this system were necessary to account for sensilla located between plates of a particular tier, while a new system was proposed for terebratorial sensilla. Consequently, Dimitrov *et al.* (1989) were referred to in the results when changes from their nomenclature were applied.

4.2.3. Scanning electron microscopical observations of *I. erraticus* and *I. variegatus* miracidia.

Newly emerged *I. erraticus* and *I. variegatus* miracidia of known life-history were fixed in cacodylate buffered 3% glutaraldehyde or hot 5% formalin. The samples were then processed as described for metacercariae in Chapter 2.2.3; Materials and Methods. The free swimming miracidia, unlike metacercariae, did not require 'cleaning' before processing. However, in order to study the surface of the miracidia, the removal of their locomotory cilia was necessary. This deciliation was attempted in two ways:

1. Sonication. Miracidia were fixed in absolute ethanol and deciliated in the same medium with an ultrasonic cleaner at 50kHz for 9-12 minutes. Such a technique was successfully employed for human and avian schistostomatid miracidia by Eklu-Natey *et al.* (1985) and Maejima, Yazaki, Fukumoto & Kamo (1989).

2. Osmotic shock was used by Rosenbaum & Carlson (1969) to achieve the cilia amputation of the ciliate *Tetrahymena pyriformis*. The miracidia in artificial spring water (A.S.W) were cooled to 4°C to minimise movement and excess water removed. At zero time, 2.5 ml of the concentrated miracidia were added to 5 ml of medium A (10mM EDTA.2Na; 50mM sodium acetate, pH 6.0) in a 50 ml conical flask and mixed by

swirling. At 30 seconds, 2.5 ml of cold distilled water was added, followed 0.25 ml of 0.2M $CaCl_2$ at 90 seconds. The suspension was then mixed by inverting the flask several times. At 3 minutes and 30 seconds, the suspension of cells was subjected to mechanical shearing (4-5 times) by sucking in and out with a fine pipette. Immediately after deciliation (at about 4 minutes) the miracidia were fixed in 3% glutaraldehyde.

After both deciliation techniques the specimens were post-fixed in 1% osmium tetroxide and processed in the same way as ciliated miracidia.

4.3. BEHAVIOURAL STUDIES.

4.3.1. Longevity of the *I. variegatus* miracidium.

The longevity of *I. variegatus* miracidia was investigated at 4 temperatures; 10, 15, 20 and 25°C. Fifty newly emerged (0-1 hour post-hatching) miracidia were carefully pipetted into a small petri-dish containing A.S.W of the desired temperature. The petri-dish was then placed in an illuminated incubator. Miracidia were observed hourly under a dissecting microscope and any dead specimens removed. Stationary miracidia were presumed to be dead. Care was taken to minimise temperature changes within the A.S.W during examinations. Each experiment was replicated 3 times.

4.3.2. Chemotactic behaviour of the *I. variegatus* miracidium.

The chemotactic behaviour of *I. variegatus* miracidia was investigated at room temperature (20±2°C) using two pieces of equipment; a two-arm-chamber and a T-chamber, based upon those utilised by Haas *et al.* (1991).

The two-arm-chamber (Fig. 65) was used to quantify the accumulation of the miracidia in response to differing stimuli. The chamber was illuminated from the directions of the side arms with cold light sources and the system filled with artificial spring water (A.S.W). Ten to 30 miracidia were then placed in the isolated central chamber. Following a 10 minute acclimation period the closure ring was turned to the open position, thereby connecting the main chamber with the side arms. After 1 minute

the closure ring was turned back to the closed position, enabling the number of miracidia in the test and control arms to be recorded. Any possible bias of the chamber was tested by performing the trials without the introduction of any stimuli (10 replicates). Two experiments were subsequently performed with the test and control arms alternated and the apparatus thoroughly cleaned for each trial:

1. 10 minutes before miracidial release a living naïve *Valvata piscinalis* specimen (natural molluscan host) was placed at the end of the test arm and secured with silicone rubber holders. The snail was removed just prior to the opening of the closure ring, after the establishment of a chemical gradient. Miracidia of 4 age classes were employed: 0-1 hours; 1-2 hours; 2+ hours; and a mixed class of any active specimens. (Minimum of 10 replicates each).

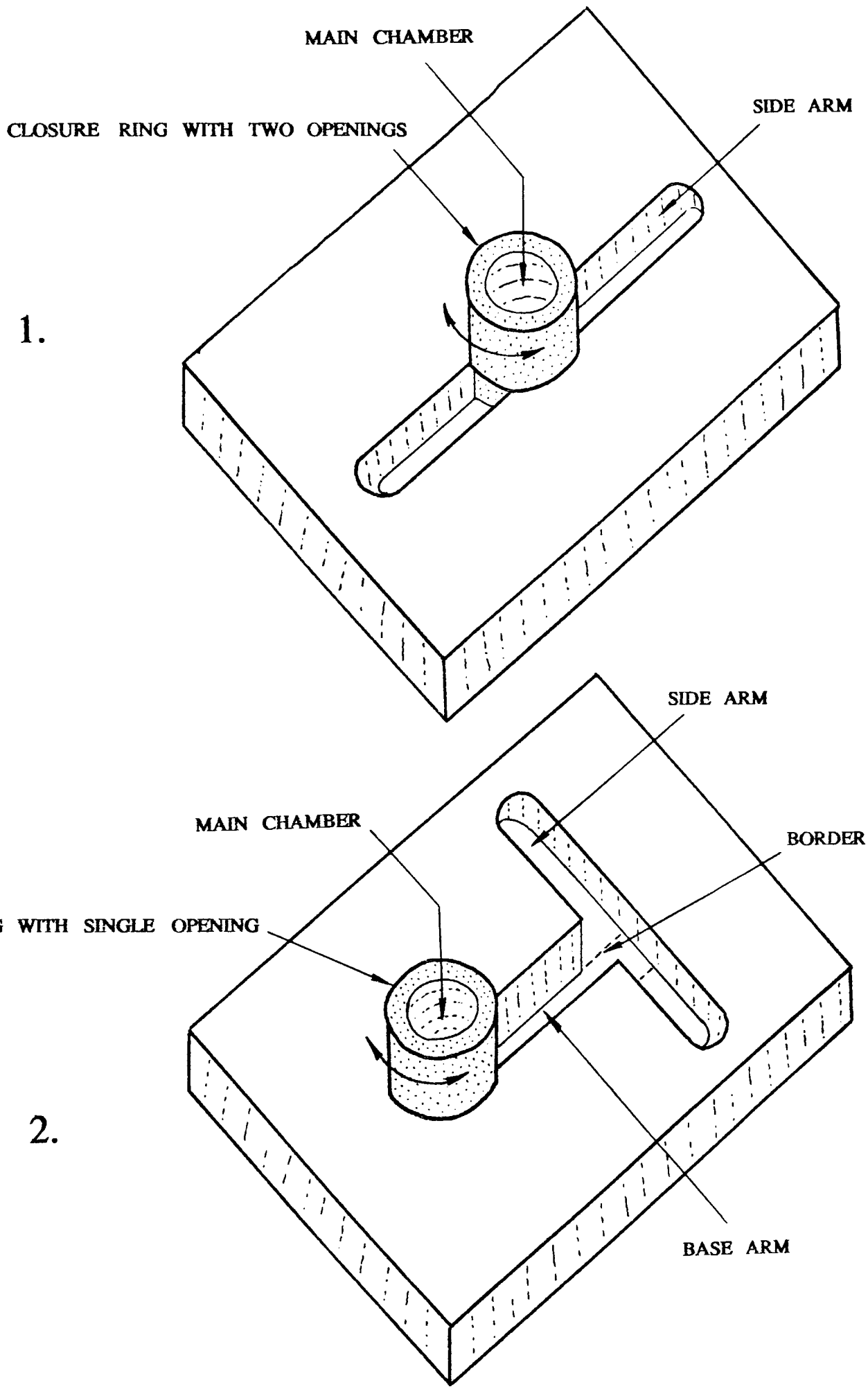
2. Experiment 1 was repeated using miracidia of a mixed age class but with *Lymnaea peregra* specimens (an unsuitable molluscan host) rather than *V. piscinalis* providing the stimulus. (10 replicates)

Miracidial chemotactic swimming behaviour was also quantified in a T-chamber (Fig. 65). The chamber was illuminated from the directions of the test and control arm with cold light sources and the system being filled with A.S.W. A batch of 20-50 mixed-age miracidia were placed in the main chamber and a naïve *V. piscinalis* specimen secured using silicone rubber holders at the end of the test arm. After an 8 minute acclimation period (the time required for the spreading chemical gradient to reach the base arm, as previously estimated by A.S.W. stained with phenol red), the snail was removed and the closure ring opened, allowing the miracidia access to the base arm. Miracidial swimming paths were observed for 5 minutes using a dissecting microscope. Crossings of the borderlines toward test and control arms were recorded only for those miracidia that approached along the base arm. The experiment was replicated 20 times. As with the two-arm chamber, the test arm was alternated and the apparatus thoroughly cleaned for each

experiment. Tests for bias in the apparatus were carried out in the absence of any external stimuli.

The results obtained from both pieces of equipment were subjected to statistical analysis. This was performed using the χ^2 test which evaluates the observed and the expected results if the selection was random. Findings were considered significant at values of $P<0.05$.

Fig. 65. Perspex choice chambers for the quantification of miracidial host-finding behaviour. 1. Two-arm-chamber. 2. T-chamber. Both chambers shown at approximately actual size.



RESULTS

4.1. DEVELOPMENTAL STUDIES.

Hatching, for all 3 species studied, was typically observed to occur towards the end of the light phase of the L:D cycle, after more than 10 hours of illumination. It was found that, when practical purposes necessitated, the timing of emergence could be delayed for several days if eggs were denied light at the point of hatching. A larger proportion of miracidia than usual would then hatch when illumination was resumed. However, for all 3 species, hatching was found to occur in the dark as well as in the light, if illumination was denied for prolonged periods.

The developmental periods of strigeid eggs at different incubation temperatures recorded in this study are provided in Table 46 together with those reported by other authors for comparison. All eggs for which developmental rates are shown in the table were hatched under a L16:D8 regime.

I. erraticus

In the present study *I. erraticus* eggs required, on average, 13 days before miracidial hatching commenced at 24°C. This period was extended by some 11 days at a temperature of 18°C. For each temperature regime, peak hatching typically occurred 2-3 days after its onset. The duration of hatching showed the same relationship as onset of hatching to temperature, being reduced in warmer conditions (Table 46). Temperature also appeared to affect the proportion of eggs successfully producing miracidia with a maximum average return of 87% at 20°C, slightly less at 18°C (81%) and a minimum average (66%) at 24°C, the highest temperature studied.

The storage of eggs at 4°C for 6-16 weeks prior to incubation at 20°C had little effect on the onset of hatching (on average delayed by 1 day). Similarly, the percentage hatch of these eggs was only reduced marginally to a mean of 80%, although a large standard deviation was recorded. However, with eggs stored in this manner for 8

months, the onset of hatching was delayed until 21 days and only 13% were found to hatch (single batch).

I. variegatus

No variation was recorded in the developmental periods or percentage hatch rates for eggs of *I. variegatus* derived from gulls infected with ruffe or perch metacercariae, and these results were pooled in Table 46. *I. variegatus* eggs were even more susceptible to incubation temperatures than *I. erraticus*, with a mean difference of 19 days between miracidial emergence at 18°C and 24°C. Although, similar temperature relationships were displayed, developmental periods of *I. variegatus* eggs were longer than those recorded for *I. erraticus* and the percentage hatch rate was markedly smaller (39-45%).

The effect of storing eggs at 4°C prior to incubation at 20°C was minimal for 6-16 weeks, but it seriously reduced percentage hatching (8%) after 8 months.

A. gracilis

Due to their smaller number, *A. gracilis* eggs were only incubated at 20°C, the temperature found to yield the highest returns for the other strigeid miracidia. Their developmental period at this temperature was the most rapid of all the species studied, with the first miracidia hatching after 13 days. The viability of these eggs was found to be particularly high, with in excess of 88% producing miracidia.

Table 46. Developmental periods (days) and percentage hatch rates of strigeid miracidia.

Species	Author	Temp (°C)	Start of hatching	Peak hatching	Duration of hatching	Percentage hatch
			Range with mean±S.D in parentheses			
I. erraticus	Olson (1970)	24	(12)	15-16	(9)	-
	Swennen *et al.* (1979)	15	(44)	-	-	-
		23	(13)	13-14	(11)	-
	Present study	18	21-26 (24±2.2)	(27±2.2)	(22±5.1)	74-88 (81±5.7)
		20	**16-19 (17±1.2)**	**(19±0.7)**	**(20±5.6)**	**83-94 (87±4.9)**
		24	11-14 (13±1.4)	(14±1.4)	(8±2.9)	59-75 (66±7.4)
		20*	**16-20 (18±1.8)**	**(21±2.5)**	**(15±4.9)**	**57-92 (80±13.9)**
		20**	**21**	**24**	**-**	**13**
I. variegatus	Odening & Bockhardt (1971)	20	18-23	-	-	-
	Swennen *et al.* (1979)	20	21-23	-	-	-
	Present study (pooled ruffe and perch material, see text)	18	34-40 (38±2.8)	(40±2.1)	(20±5.0)	37-41 (39±1.6)
		20	**19-26 (23±2.9)**	**(25±2.5)**	**(20±4.6)**	**26-57 (40±12.0)**
		24	16-20 (19±1.6)	(20±2.5)	(6±3.7)	15-90 (45±32.4)
		20*	**18-28 (25±4.3)**	**(27±4.2)**	**-**	**26-36 (33±1.6)**
		20**	**24**	**-**	**-**	**8**
I. platycephalus	Odening *et al.* (1970)	20	(22)	-	-	-
	Swennen *et al.* (1979)	20	21-23	-	-	-
A. gracilis	Vojtek (1964a)	22	14-15	-	-	-
		29-30	(11)	-	-	-
	Present study	20	13-17 (15±1.6)	(16±1.6)	(7±5.0)	88-95 (91±2.5)

* Eggs stored at 4°C for 6-16 weeks prior to incubation. ** Eggs stored at 4°C for 8 months prior to incubation, only a single sample monitored. All samples with standard deviations indicated are from in excess of two replicates.

Figures in bold to aid comparison of eggs incubated at the same temperature in the present study.

4.2. TAXONOMIC STUDIES.

4.2.1. Light microscopical observations of miracidia.

A table of the body dimensions obtained in the present study and by previous authors for strigeid miracidia is presented in Table 47. The miracidia of all 3 strigeid species studied possess the same body shape and arrangement of internal organs. The ciliated cylindrical body tapers anteriorly and posteriorly from the level of the lateral papillae to end as rounded cones. Anteriorly the body ends in a non-ciliated, protrudable terebratorium, which is also commonly referred to in the literature as the apical papilla. This typically takes the form of an angular cap, although it appears as a smooth extension of the body when fully extended or is unseen when fully retracted, giving the anterior end a more rounded appearance. The miracidium of each species possess 4 tiers of ciliated epidermal plates, the number and arrangement of which are described in 4.2.2; Miracidial chaetotaxy. A large lateral papilla is clearly visible on each side of the miracidium situated between the first and second plate tiers. Cilia anterior to the lateral papillae decrease progressively in length towards the terebratorium. Morphological measurements recorded for the miracidia fixed in hot 5% formalin are provided in Table 48. The internal organisation of the *I. erraticus* miracidium was discussed in some detail by Olson (1970) and this description applies equally to the miracidia of *I. variegatus* and *A. gracilis*. In all cases 2 kidney-shaped eye-spots are located dorsally on either side of the mid-sagittal plane at the level of the lateral papillae. The excretory systems which consist of 2 pairs of flame cells and their collecting tubules are arranged as described by Olson (1970), although the orientation of the flame cells was found to be more varied. The flask-shaped apical gland opens on the terebratorium via what appears to be a single collecting duct. No cephalic glands, which are commonly recorded for other miracidia positioned laterally to the apical gland(s) (see review of Erasmus, 1972), were observed, however, a glandular mass is located posteriorly.

Table 47 indicates that live specimens tend to be longer and thinner than their fixed equivalents. The dimensions recorded for the three species in the present study were similar, although *I. erraticus* miracidia appear somewhat longer than those of *I. variegatus*.

Table 47. Dimensions of strigeid miracidia. All measurements are in micrometres, with mean values in parentheses.

Species	Author	Condition of specimens	Number of specimens	Body length	Body breadth
				Range with mean in parentheses	
I. erraticus	Olson (1970)	Fixed: hot AFA	?	150-175 (161)	37-50 (43)
	Present study	Live	10	134-188 (163)	31-59 (43)
		Fixed: hot 5% formalin	20	106-157 (132)	36-63 (48)
I. variegatus	Odening & Bockhardt (1971)	Live	?	132-160	22-48
	Present study	Live	10	122-185 (153)	26-58 (36)
		Fixed: hot 5% formalin	15	101-143 (118)	39-79 (49)
I. platycephalus	Odening *et al.* (1970)	Live	?	111-170	22-38
		Fixed in ?	?	123-147	58-74
I. erraticus *I. variegatus* *I. platycephalus*	Swennen *et al.* (1979)	Live ?	?	c. 150	c. 35
A. gracilis	Vojtek (1964a)	?	?	135-196	31-42
	Present study	Fixed: hot 5% formalin	10	99-140 (124)	33-46 (43)

Table 48. Morphological measurements of strigeid miracidia fixed in hot 5% formalin. All measurements are in micrometres, with mean values in parentheses.

Morphological feature	Miracidial species		
	I. erraticus	*I. variegatus*	*A. gracilis*
	n=20	n=15	n=10
Body length	96-157 (132)	91-143 (118)	99-140 (124)
Body breadth	36-63 (48)	39-79 (49)	33-46 (43)
Terebratorium length	4-8 (5)	4-27 (7)	2-11 (6)
Terebratorium breadth	10-22 (16)	11-29 (16)	9-18 (13)
Eye-spot length	11-14 (12)	9-13 (11)	9-11 (10)
Eye-spot breadth	6-9 (8)	6-8 (7)	6-8 (7)
Distance between eye-spots	1-14 (8)	0-13 (4)	-
Distance from eye-spots to anterior	14-36 (24)	13-44 (21)	14-25 (20)
Length of lateral papilla	(5)	(5)	-
Distance from lateral papilla to anterior	23-44 (30)	20-40 (27)	22-31 (27)
Length of cilia (tier II)	(12)	(11)	-
Length of cilia (tier III)	(10)	(10)	(11)
Length of cilia (tier IV)	(10)	(10)	-

4.2.2. Miracidial chaetotaxy.

The epidermal plate formula and sensillary pattern were found to be the same for the miracidia of all 3 strigeid spp. studied; representative diagrams of the 3 species are shown in Figs 66, 67. Photomicrographs of these structures are given for an *I. variegatus* miracidium in Figs 68, 69.

Epidermal plate formula (Figs 66, 69).

$E_I + E_{II} + E_{III} + E_{IV} = 6 + 9 + 4 + 3 = 22$

$E_I = 1 + 1\ E_IV + 1 + 1\ E_IL + 1 + 1\ E_ID$

$E_{II} = 1\ E_{II}MV + 1 + 1\ E_{II}V + 1 + 1\ E_{II}VL + 1 + 1\ E_{II}DL + 1 + 1\ E_{II}D$

$E_{III} = 1 + 1\ E_{III}VL + 1 + 1\ E_{III}DL$

$E_{IV} = 1 + 1\ E_{IV}VL + 1\ E_{IV}MD$

The first tier of epidermal plates (EI) comprised 6 trapezoid plates, 2 dorsal, 2 lateral and 2 ventral; the second tier (EII) had 9 rectangular plates, 2 dorsal, 2 dorso-lateral, 2 ventro-lateral, 2 ventral and 1 medio-ventral; the third (EIII) 4 broadly rectangular plates, 2 dorso-lateral and 2 ventro-lateral; and the fourth (EIV) 3 triangular plates, 1 medio-dorsal and 2 ventro-lateral (see Figs 66, 69).

Sensillary pattern.

1. Terebratorium (Figs 67, 68).

Unfortunately, sensilla could not be discriminated from the opening(s) of the apical gland by silver nitrate staining of specimens. The distribution of these argentophilic structures did not fit well with the nomenclature proposed by Dimitrov *et al.* (1989), which is based upon groupings situated on 3 axes which run through the centres of the first tier plates. In this study the bilateral symmetry of arrangement of the structures recorded enabled a simpler nomenclature to be employed based upon their position in relation to the first tier plates themselves. The trapezoid shape of these plates can be

extrapolated to the centre of the terebratorium, creating sectors pertaining to the plates (see Fig. 67). Thus, structures located in the dorsal epidermal plate sectors are placed together as the dorsal terebratorial group (TD). Such a system is less subjective than the arbitrary groupings used by Dimitrov *et al.* (1989).

T = TV + TL + TD = 20
TV = 4 + 4 = 8
TL = 3 + 3 = 6
TD = 3 + 3 = 6

The ventral group consisted of 1 large (2.5µm diameter) and a triangle of 3 small (1-2µm diameter) structures, with the large body positioned dorso-medially to the triangle. The lateral group contained an intermediate sized (2µm diameter) and 2 large structures in line with the long axis of the lateral plate, the most medial being the smaller body. The ventral group also comprised a triangle of 3 small structures which were associated with the medial body of the lateral group. The arrangement of these structures is shown in Figs 67, 68.

2. Body surface (Figs 66, 69).

The nomenclature of Dimitrov *et al.* (1989) for sensory papillae on the body surface of miracidia presumed that the structures are positioned posteriorly to the epidermal plates and did not account for sensilla situated within a tier of plates. Consequently, the letter 'm' was adopted for sensilla located in a mid-plate position.

$SE_I + SE_{II} + SE_{III} = 14$
$SE_I = 1 + 1\ SE_Im^*D\text{-}E_Im^*L + 1 + 1\ SE_ID + 2 + 2\ SE_IL + 1 + 1\ SE_IV$
$SE_{II} = 1 + 1\ SE_{II}V\text{-}E_{II}VL$
$SE_{III} = 1 + 1\ SE_{III}m^*DL\text{-}E_{III}m^*VL$
m^* = mid-plate position

One intermediate sized sensillum (2µm diameter) was found between the dorsal and

lateral epidermal plates of the first tier, situated mid-way down their length, and a single small (1μm diameter), sensillum was located at the base of each epidermal plate of the first tier. Additionally, at the base of the 2 lateral plates of this tier was a large sensillum (2-3μm diameter) which is often referred to as the lateral papilla. One sensillum was positioned between the bases of the ventral and ventro-lateral plates of the second tier and a further sensillum between the ventro-lateral and dorso-lateral plates of the third tier, mid-way down their length. The arrangement of these sensilla is indicted in Figs 66, 69. Fig. 66 shows the position of the excretory pores which were found at the base of each of the ventro-lateral plates of the third tier.

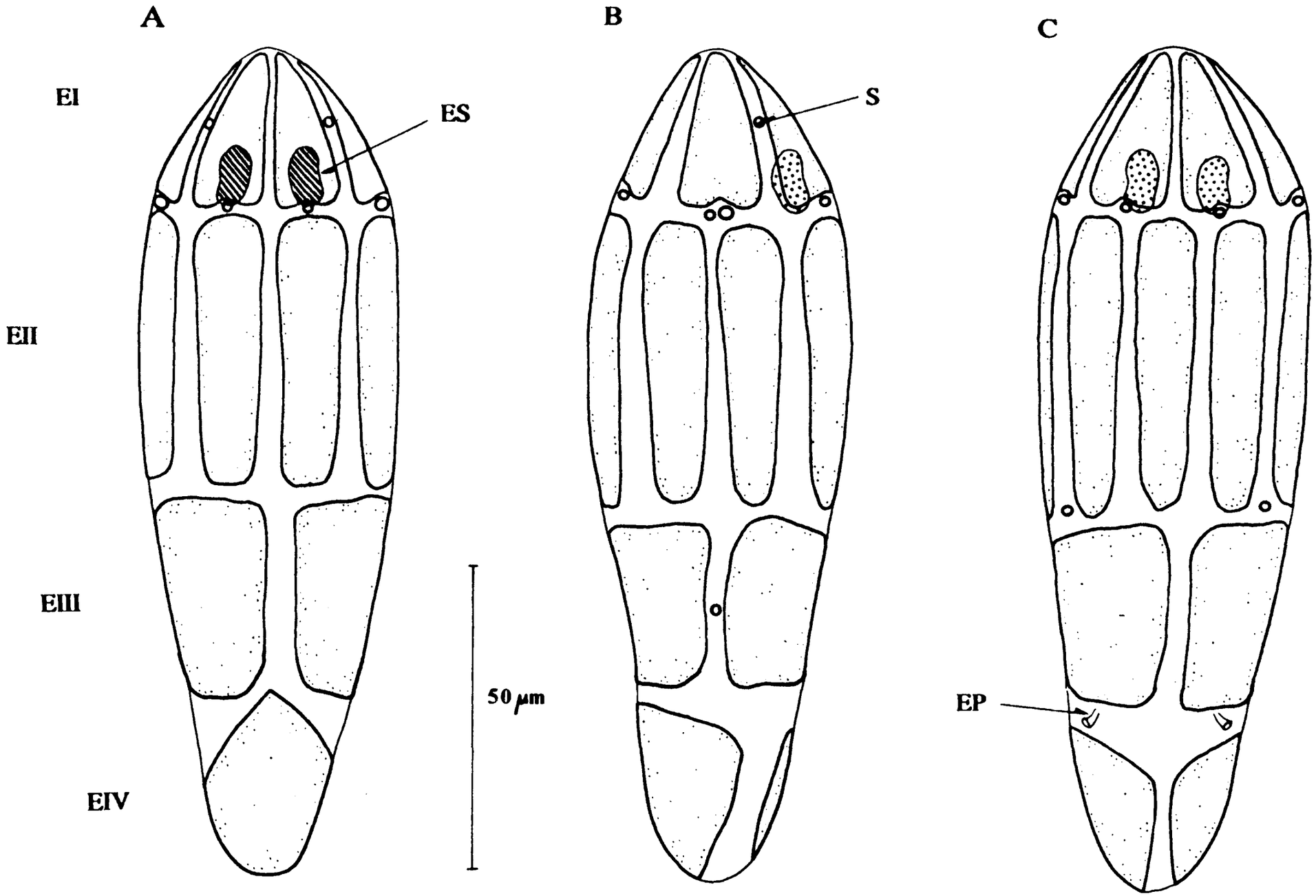


Fig. 66. The arrangement of epidermal plates and sensilla common to *I. erraticus*, *I. variegatus* and *A. gracilis* miracidia. A). Dorsal view. (EI-EIV = epidermal plate tiers; ES = eye-spots). B). Lateral view. (S = sensillum). C). Ventral view (EP = excretory pores).

Fig. 67. Arrangement of argentophilic structures on the terebratorium of *I. erraticus*, *I. variegatus* and *A. gracilis* miracidia (EP = epidermal plates; ES = eye-spots; S = sensilla). The interrupted lines indicate sectors which enable a description of sensilla position to be made. Eye-spots are situated dorsally.

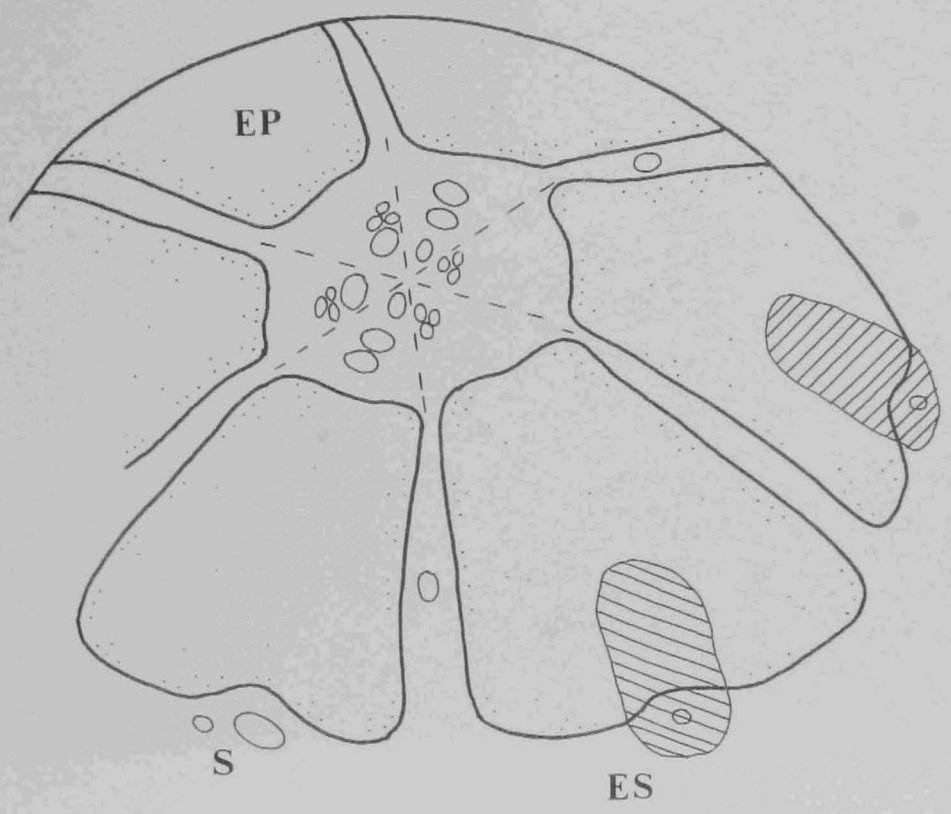


Fig. 68. Photomicrograph of the terebratorium of a silver stained *I. variegatus* miracidium.

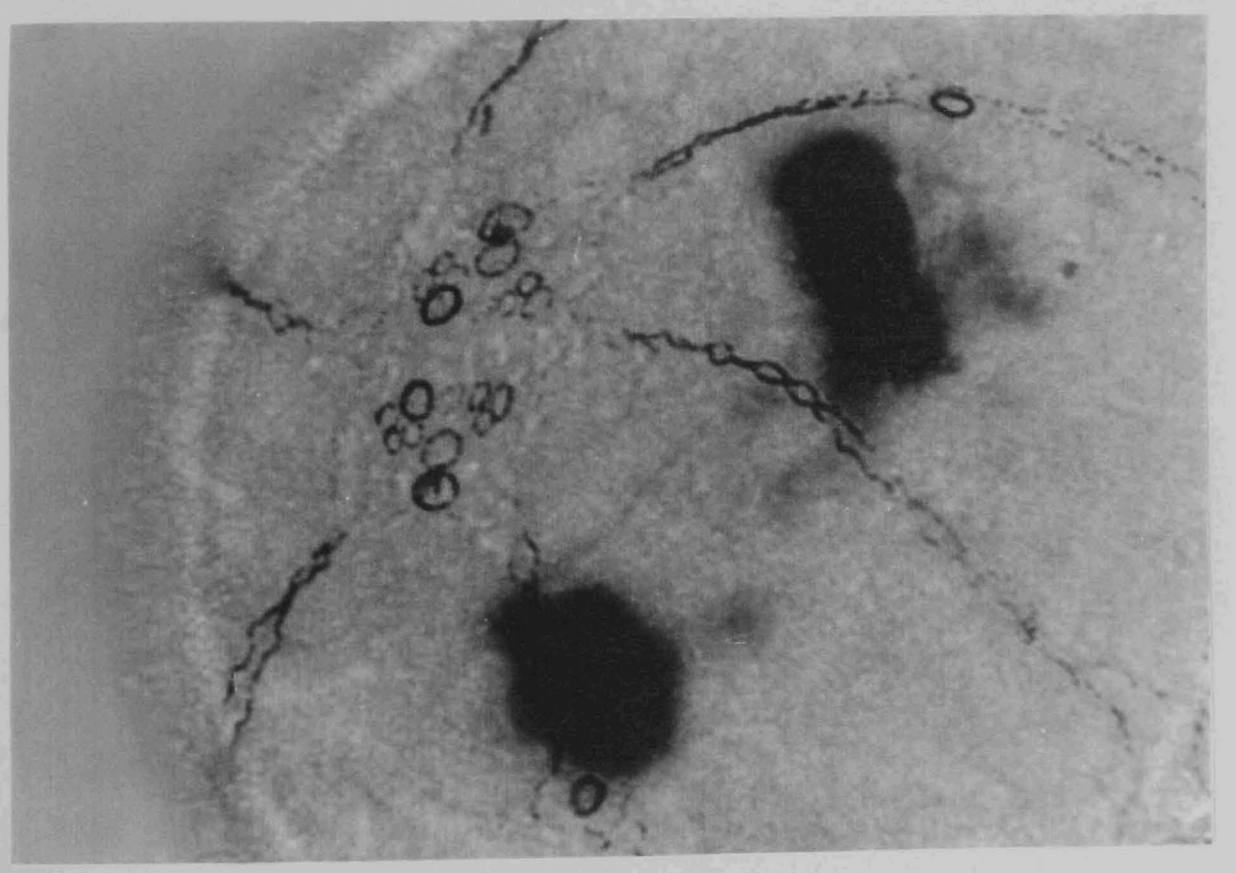
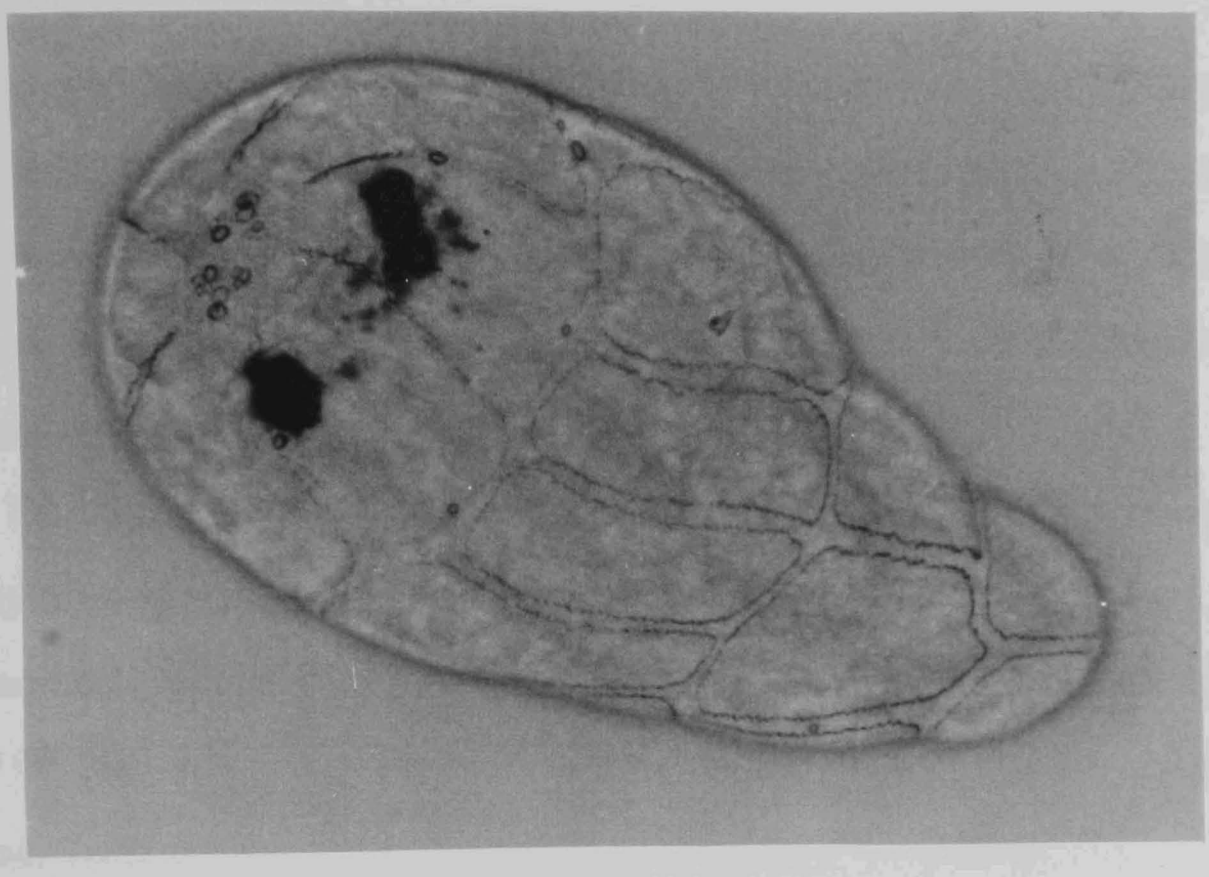
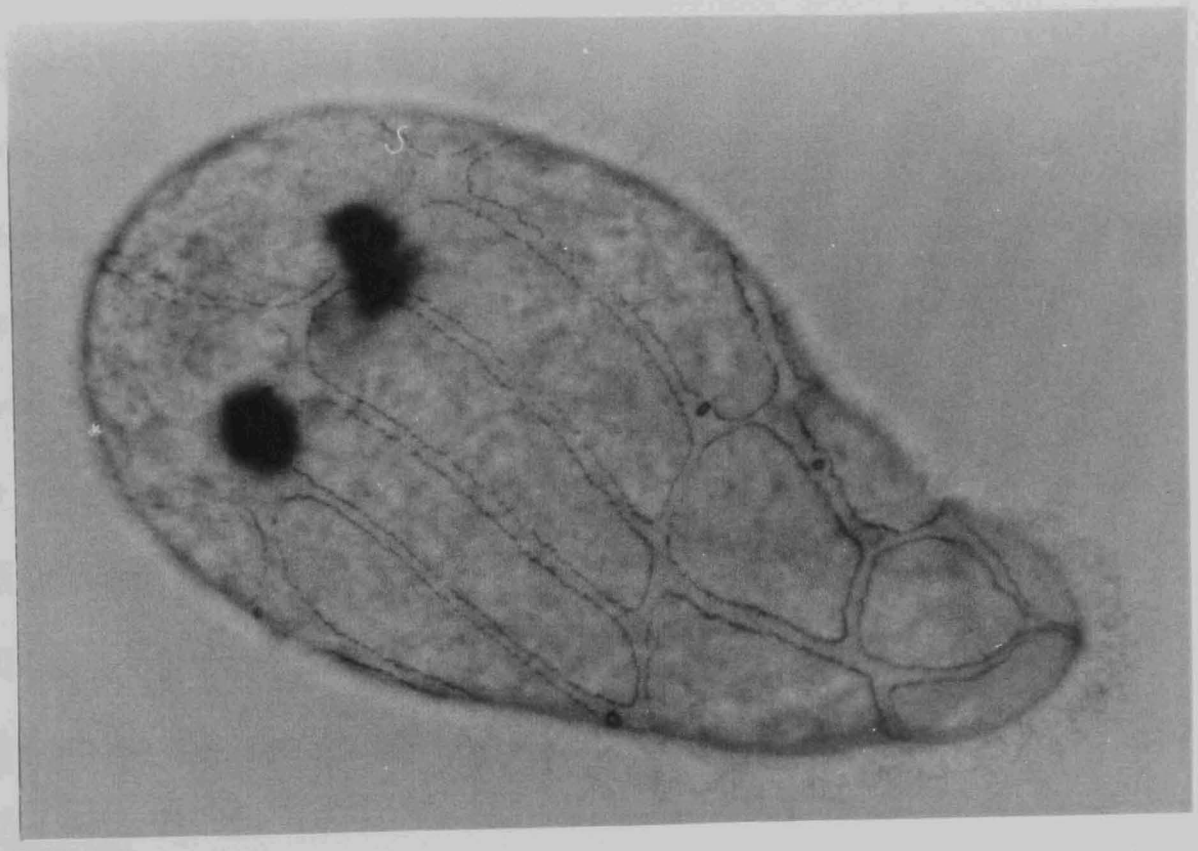


Fig. 69. Photomicrographs of a silver stained *I. variegatus* miracidium showing the arrangement of epidermal plates and sensilla.



Dorsal view



Ventral view

4.2.3. Scanning electron microscopical observations of *I. erraticus* and *I. variegatus* miracidia.

The dense covering of cilia on these species of miracidia is shown in Fig. 70. Unfortunately, neither technique employed to remove miracidial locomotory cilia was completely successful. Sonication of miracidia resulted in the shearing of cilia, not at their base but mid-way down their length and this remaining 'stubble' still obscured the surface of specimens. Continued sonication beyond this point resulted in the destruction of the miracidia themselves.

Deciliation by osmotic shock proved more effective, with the removal of the majority of body cilia being achieved, leaving the epidermal plates exposed. However, cilia located anteriorly on the epidermal plates of the first tier were still not removed (Fig. 71). The presence of these cilia, plus the tendency of the terebratorium to be retracted, made the surface of this region very difficult to examine.

SEM observations of miracidia following osmotic shock provided confirmation of the number and location of body sensilla but failed to yield new information regarding terebratorial structures. No differences were recorded in the surface structure of *I. erraticus* or *I. variegatus* miracidia. Figs 70, 71 show the anterior ends of 2 *I. variegatus* miracidia, the first with its cilia in place and the second following deciliation by osmotic shock. The terebratorium is devoid of ciliated epithelial plates and appears to be covered with corrugated membranous folds (Fig. 71). At the base of each of the first tier epidermal plates, located in a notch, is a sensillum which terminates in a flattened crescent-shaped structure. Situated posteriorly to this sensillum at the base of the lateral first tier plates is the lateral papilla. This consists of a bulbous projection, the surface of which was thrown into many folds. The surface detail of these structures can be seen in Figs 72, 73. All other body sensilla of the 2 *Ichthyocotylurus* spp. studied were of a short, uniciliate form (Fig. 71). The excretory pores, which measure about 1μm in diameter, are surrounded by a cytoplasmic fold. This fold forms a collar bearing fine microvilli (Fig. 74).

Fig. 70. The anterior end of an *I. variegatus* miracidium showing the dense arrangement of locomotory cilia.

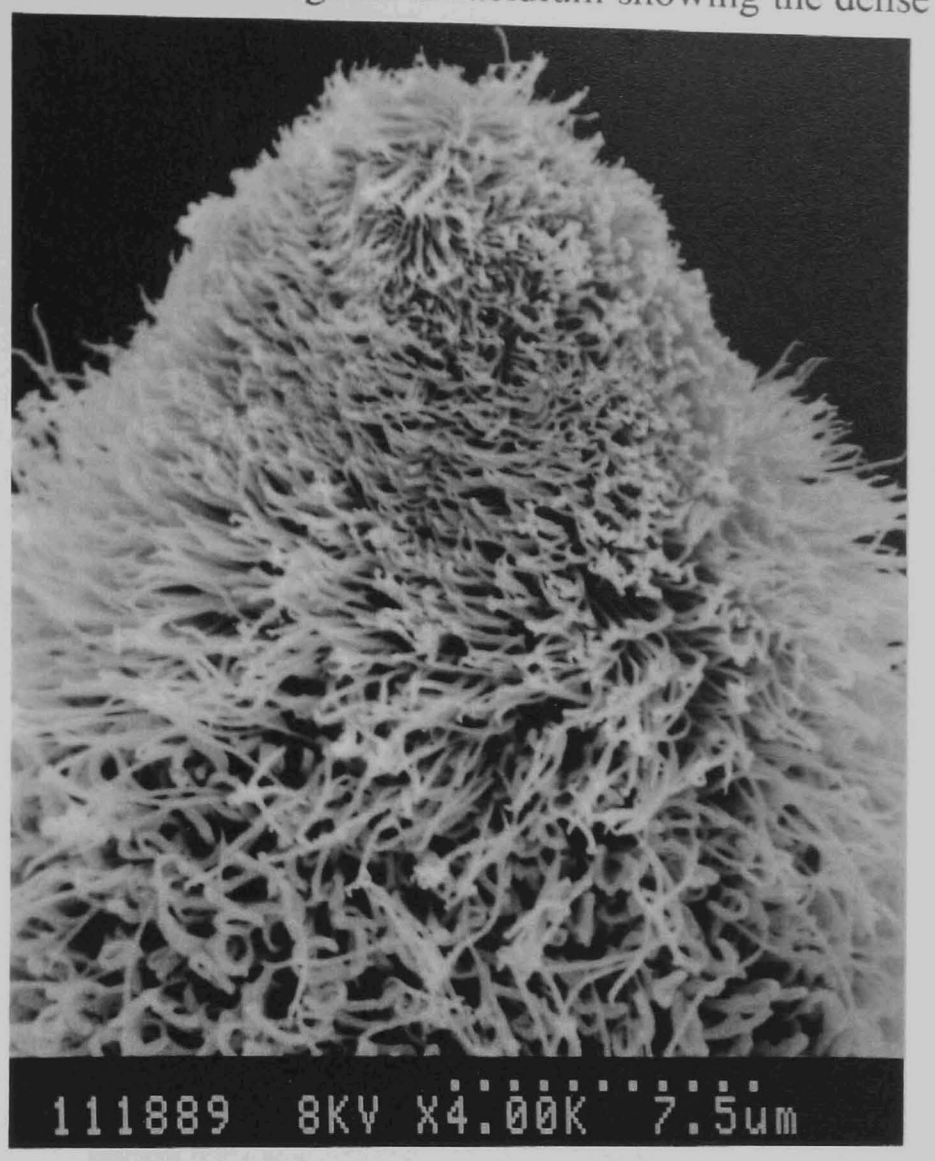


Fig. 71. The anterior end of an *I. variegatus* miracidium which has been deciliated by osmotic shock (T = terebratorium, S = sensillum, LP = lateral papilla, EP = epidermal plate).

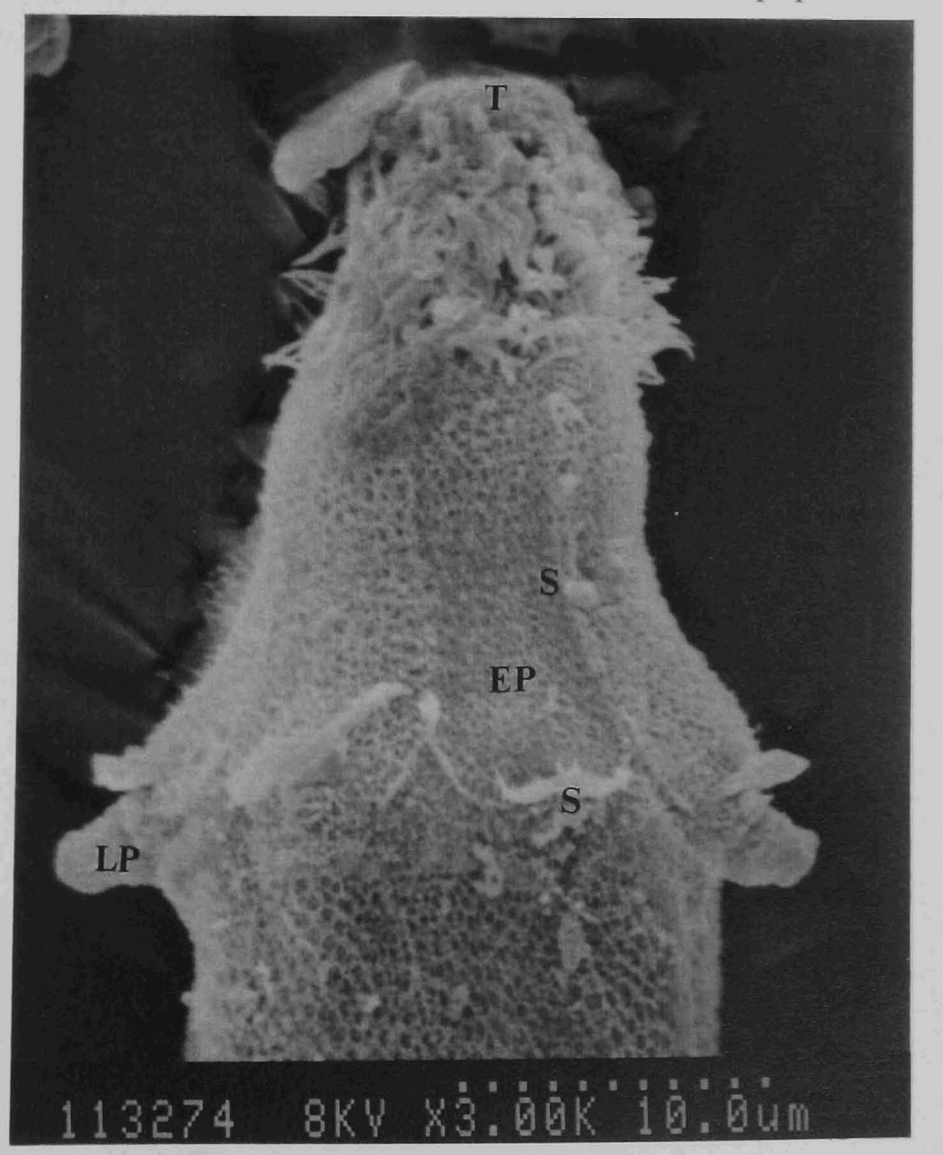


Fig. 72. The body surface of an osmotically shocked *I. erraticus* miracidium showing the position of the sensilla (S) and lateral papilla (LP) at the base of the first tier epidermal plates (EP). One of the second tier plates has been lost.

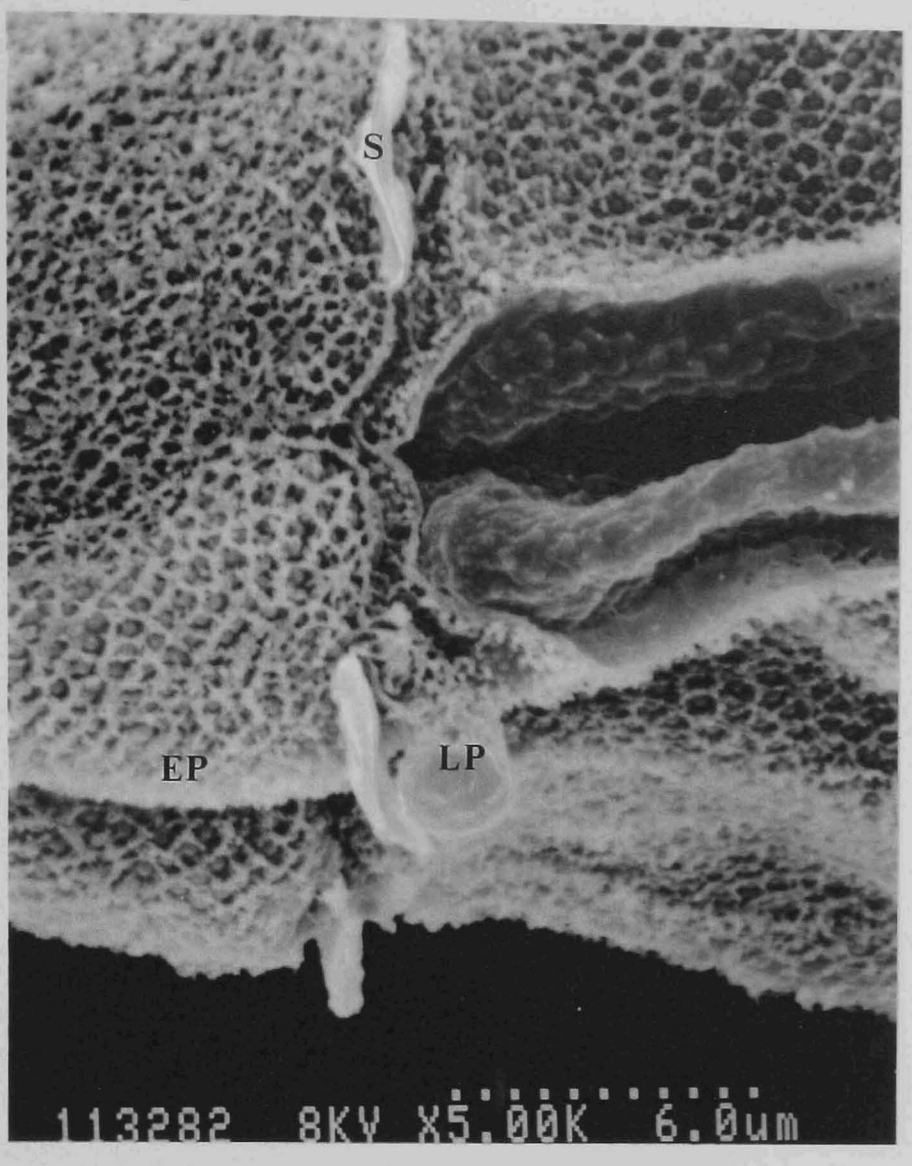


Fig. 73. The lateral papilla (LP) and associated sensillum (S) at the base of the lateral first tier epidermal plate.

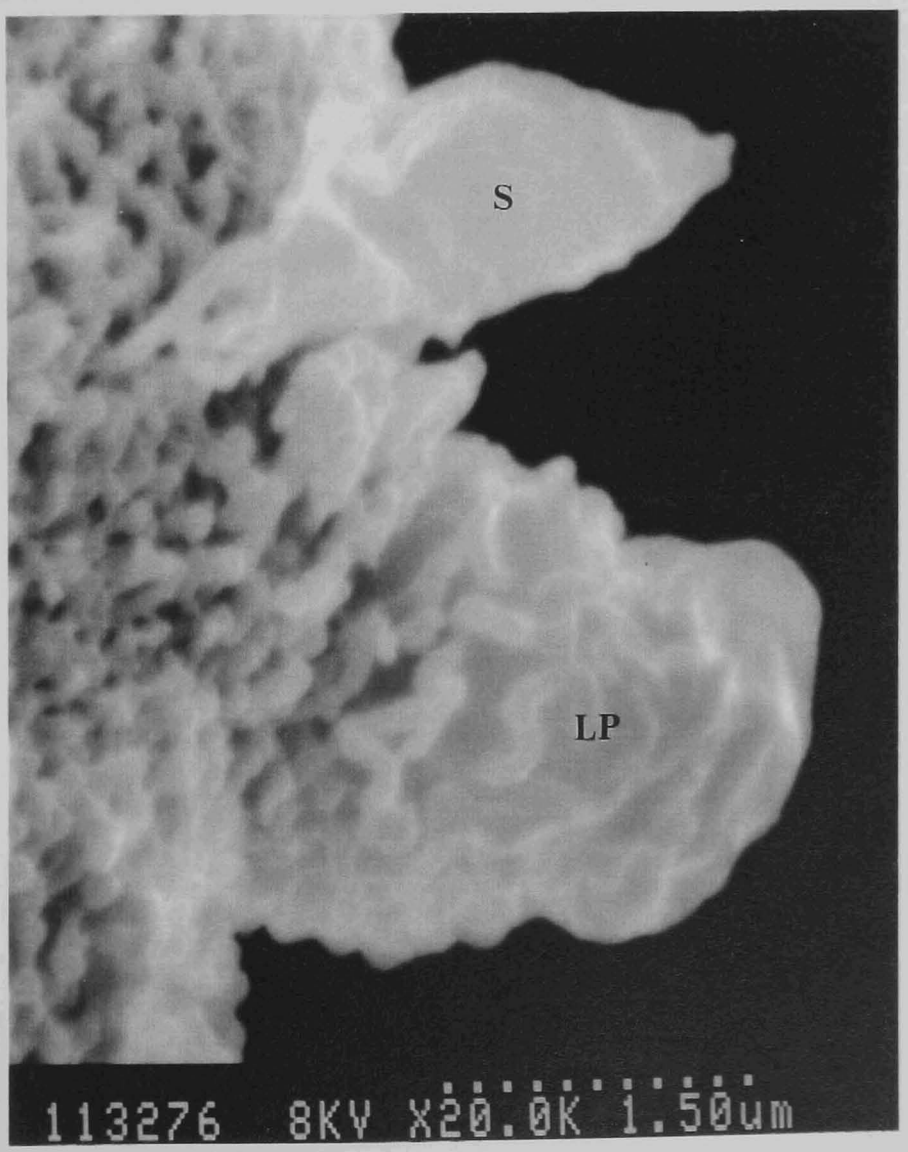
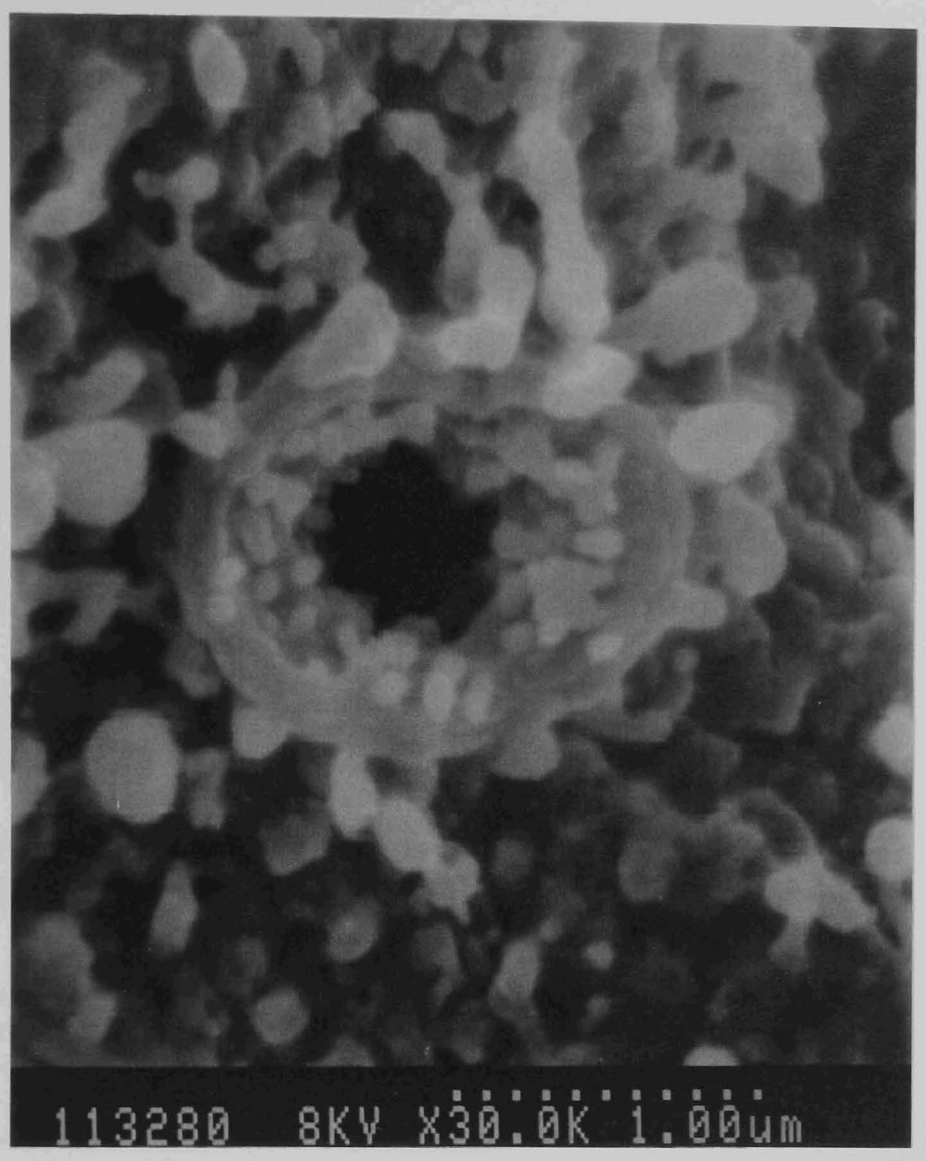


Fig. 74. Excretory pore of an *I. variegatus* miracidium.



4.3. BEHAVIOURAL STUDIES.

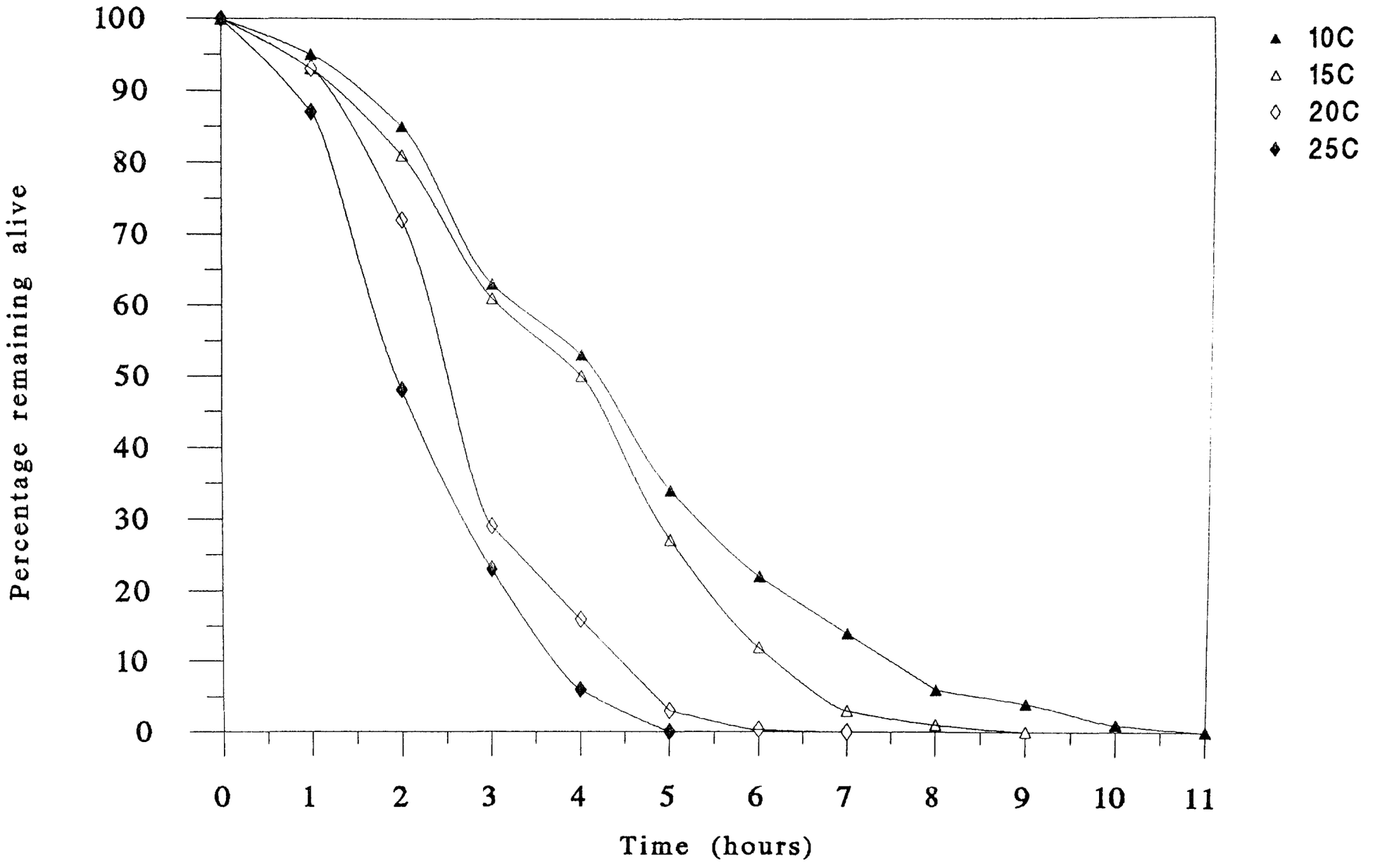
4.3.1. Longevity of the *I. variegatus* miracidium.

The longevity of *I. variegatus* miracidia at different temperatures are indicated in Table 49, which presents minimum and maximum survival times and the point at which 50% are dead. Fig. 75 illustrates the cumulative survival of miracidia at the different temperatures employed. The results obtained revealed 50% mortalities after 2-3, 3-4, 4-5 and 5-6 hours at temperatures of 25, 20, 15 and 10°C, respectively. The maximum longevity of these miracidia was observed as 10-11 hours at 10°C, but only 4-5 hours at 25°C.

Table 49. Longevities of *Ichthyocotylurus variegatus* miracidia at different temperature regimes, derived from mean values recorded for 3 replicates.

Temperature °C	First mortalities	50% dead	Maximum survival
	hours post-release		
10	1-2	5-6	10-11
15	1-2	4-5	9-10
20	1-2	3-4	7-8
25	1-2	2-3	4-5

Fig. 75. Cumulative survival of *I. variegatus* miracidia at different temperature regimes. Each point represents the mean of three replicates (n=50).



4.3.2. Chemotactic behaviour of the *I. variegatus* miracidium.

The results of the tests for bias in the apparatus used to investigate chemotactic behaviour are given in Tables 50 and 51. These tables show that in the absence of differential stimuli, miracidial movement was random in the 2-arm-chamber (P>0.9) and T-chamber (P>0.1).

Table 50. Test for bias in the 2-arm-chamber using *Ichthyocotylurus variegatus* miracidia with the only external stimulus bi-directional light.

Miracidial age (hours)	Number of miracidia (pooled from replicates)				Number of replicates	Value of χ^2	Probability*
	Left arm	Central reservoir	Right arm	Total			
Mixed	131 (31.2%)	162 (38.6%)	127 (30.2%)	420	15	0.06	P>0.9

Table 51. Test for bias in the T-chamber using *I. variegatus* miracidia in the absence of an external stimulus.

Miracidial age	Number of miracidia (pooled from replicates) crossing the boundary in the direction of the		Number of replicates	Value of χ^2	Probability*
	left arm	right arm			
Mixed	158 (51.6%)	148 (48.4%)	10	0.327	P>0.1

* Considered to be significant at a value of P<0.05.

The results obtained in the present study for chemotactic behaviour by *I. variegatus* miracidia are given in Tables 52, 53.

Table 52. Accumulation of *Ichthyocotylurus variegatus* miracidia in snail-conditioned water (SCW) in the 2-arm-chamber.

Snail species	Conditions	Miracidial age (hours)	Number of miracidia (pooled from replicates)				Number of replicates	Value of χ^2	Probability*
			Test arm	Central reservoir	Control arm	Total			
Valvata piscinalis	Illumination	Mixed	177 (50.6%)	132 (37.7%)	41 (11.7%)	350	15	84.8	P<0.001
		<1	25 (14.7%)	118 (69.4%)	27 (15.9%)	170	10	0.08	P>0.9
		1-2	83 (63.9%)	22 (16.9%)	25 (19.2%)	130	10	31.2	P<0.001
		2+	50 (22.2%)	137 (60.9%)	38 (16.9%)	225	10	1.64	P>0.05
	No illumination	Mixed	130 (50.6%)	56 (21.8%)	71 (27.6%)	257	10	16.5	P<0.001
		<1	71 (47.3%)	25 (16.7%)	54 (36.0%)	150	10	2.31	P>0.1
Lymnaea peregra	Illumination	Mixed	60 (28.3%)	104 (49.1%)	48 (22.6%)	212	10	1.33	P>0.1

* Considered to be significant at a value of P<0.05.

Table 53. Chemotactic orientation of *I. variegatus* miracidia in the T-chamber.

Miracidial age (hours)	Number of miracidia (pooled from replicates) crossing the boundary in the direction of the		Number of replicates	Value of χ^2	Probability*
	test arm	control arm			
Mixed	257	255	20	0.008	P>0.9

* Considered to be significant at a value of P<0.05.

DISCUSSION

4.1. DEVELOPMENTAL STUDIES.

The results as shown in Table 46 indicate that temperature has a considerable effect on the rate of strigeid miracidial development, with increasing temperatures reducing the amount of time required for hatching. A difference of 11 days was recorded between the first *I. erraticus* eggs to hatch at 18°C (24 days) and 24°C (13 days), while a 19 day difference was observed for *I. variegatus* eggs at these temperatures, 38 and 19 days, respectively. Swennen *et al.* (1979) recorded a developmental period of 13 days for the emergence of the first *I. erraticus* miracidia at 25°C, with a much delayed development at 15°C (44 days). Considering their results (at 15 and 25°C) together with those of the present study (18, 20 and 24°C) suggests an exponential relationship between development and temperature for this species of miracidium. It appears that the maximum maturation rate occurs at temperatures not greatly exceeding 25°C, while decreasing temperatures have a progressively larger arresting effect on development.

Such a relationship between temperature and developmental times were noted by Dubois (1929) for the eggs of *Strigea tarda* (=*Cotylurus cornutus*), by Rowcliffe & Ollerenshaw (1960) for *Fasciola hepatica* and by Blair (1974) for *D. spathaceum*. A more linear effect was recorded by Brady (1989) for the eggs of *Diplostomum* spp. incubated at 15, 20 and 25°C, with average developmental differences of eight and six days observed between the two lower and two higher temperatures, respectively. The development of *F. hepatica* (see Rowcliffe & Ollerenshaw, 1960) and *D. spathaceum* (see Blair, 1974) eggs was not thought to occur at below 10°C, nor to be further accelerated at temperatures exceeding 30°C.

In his detailed study of the effects of environmental factors on the development of *D. spathaceum* eggs, Blair (1974) also noted that temperature could affect viability. He observed that viability was compromised at temperatures of 40°C and at or below

15°C. However, differences at 20, 25 and 30°C were found to be negligible. This finding differs from the present study where a decreased viability was observed for *I. erraticus* eggs incubated at 24°C (average of 66%) compared to those at 20°C (average of 87%). The viability of *I. variegatus* eggs was not found to be affected by the maintenance temperatures used in the present study, but all average percentage hatches were lower than those recorded for *I. erraticus* eggs; maximum of 45%. The reason for the decreased viabilities of *I. variegatus* eggs may be due to the higher egg densities employed during incubation.

Many of the batches of eggs used for experimental infections in this study had previously been stored at 4°C, arresting their development. The effect of cold storage (4°C) on the subsequent developmental times and viability of *D. spathaceum* eggs at 25°C was investigated by Blair (1974). He was unable to quantify the results obtained for unembryonated eggs due to contamination of watchglasses, but noted that an undeterminable proportion still hatched following six months at 4°C. Results gained for embryonated eggs (maintained at 25°C for 7 days prior to cold storage) after five months storage showed developmental periods and viabilities comparable to controls, and only slightly lengthened developmental periods were observed after 11 months. Here, extended storage (longer than about 6 months) of both *I. erraticus* and *I. variegatus* unembryonated eggs was found to cause developmental periods to be only moderately lengthened, but viabilities to be drastically reduced (below 15%). It is possible that the addition of antibiotics to the storage medium (as used by Blair, 1974) would have increased the viabilities presently recorded, while the isolation of eggs into smaller groups prior to storage would have enabled a more complete removal of associated contaminants, particularly uric acid crystals. Nevertheless, the results obtained, from storage conditions far less suitable than would be expected to occur naturally, indicated the extended viability of strigeid eggs and their likely ability to overwinter. Whether embryonation of eggs prior to cold storage is beneficial to their future development is unclear.

In the present study it was observed that miracidia tended to hatch towards the end of the light phase of the L:D cycle, but if light was denied for extended periods the mature miracidia would eventually (after several days) emerge in the dark. Thus, for these three strigeid miracidia light was found to have a stimulatory effect on hatching but was not a prerequisite. No such phenomenon was noted by Blair (1974) for *D. spathaceum* miracidia, who found the developmental period unaffected by the absence of light.

4.2. TAXONOMIC STUDIES.

4.2.1. Light microscopic observations of miracidia.

As indicated by the results (Table 47), live specimens tend to be longer and thinner than their fixed equivalents. In this study *I. erraticus* body dimensions were found to be longer than *I. variegatus*; this is supported by Odening & Bockhardt's (1971) dimensions of live *I. variegatus* miracidia, which were shorter than the dimensions recorded for fixed *I. erraticus* miracidia by Olson (1970). A comparison of the measurements for fixed *I. erraticus* miracidia obtained during the present study and those of Olson (1970) suggests that hot AFA provides less contracted specimens than hot 5% formalin and may be a preferable fixative. The only directly comparable dimensions given by different authors were for live *I. variegatus* specimens taken by Odening & Bockhardt (1971) and the present study. These measurements were very similar, although a narrower size range was recorded by Odening & Bockhardt (1971). Vojtek (1964a) recorded rather longer *A. gracilis* miracidia than in the present study but did not indicate whether the dimensions were for live or fixed specimens.

The morphology of the three species of strigeid miracidia investigated was found to be identical. The arrangement of internal organs corresponded closely to those described by Olson (1970) for the miracidium of *I. erraticus*. However, in his description, Olson (1970) failed to observe the posteriorly located glandular mass recorded here for all three strigeid miracidia. Odening *et al.* (1970) also recorded such

a gland in a figure of the *I. platycephalus* miracidium. A gland opening at the posterior extremity of the body was also noted for strigeoid miracidia of the genera *Alaria*, *Diplostomum* and *Neodiplostomum* (see review of Erasmus, 1972). The function of this gland is at present unknown. Histochemical or TEM studies, such as those performed by Wilson (1970) on *F. hepatica* and by Kinoti (1971) and Wikel & Bogitsh (1974) on *Schistosoma* spp., would provide more information on the organisation of these glands, including details of their collecting ducts.

4.2.2. Miracidial chaetotaxy.

A review of the known epidermal plate patterns in miracidia was produced by Peters (1966). In this list strigeoids possessed either 21 or 22 epidermal plates. The review of Erasmus (1972) stated that the epidermal plate formula for many strigeoid trematodes is 6+8+4+3=21. Such a pattern was recorded by Vojtek (1964a) for *A. gracilis* (as *A. cobiditis*), Mathias (1925) for *Cotylurus brevis* (as *Strigea tarda*) and Dimitrov *et al.* (1991) for *Strigea falconispalumbi*. However, in contrast to Vojtek (1964a), the present study recorded an extra mid-ventral plate for the miracidium of *A. gracilis,* giving nine in the second tier and a total of 22. The same number and arrangement of epidermal plates were also recorded here for *I. erraticus* and *I. variegatus*, i.e. 6+9+4+3=22. It was suggested by Pearson (1961) that the likely formula throughout the Suborder Strigeata was in fact 6+9+4+3=22. He proposed that errors in ascertaining the epidermal plate formula may arise from the assumption that the plates in the second tier are arranged symmetrically in pairs about the mid-sagittal plane, when there is actually a mid-ventral unpaired plate. This theory is supported by the present data for the three strigeid species, especially when that of *A. gracilis* had previously been incorrectly described. The latter formula was also recorded for the strigeid miracidia of *C. lutzi* (see Basch, 1969), *I. platycephalus* (see Odening *et al.*, 1970) and *I. variegatus* (see Odening & Bockhardt, 1971), as well as for many schistostomatid and diplostomid miracidia (Pearson, 1961; Dönges, 1964; Southgate & Knowles, 1977;

Albaret, 1984; Eklu-Natey *et al.* 1985; Maejima, Yazaki, Fukumoto & Kamo, 1989). Although no variation was observed in the epidermal plate numbers in this study, other authors, including Eklu-Natey *et al.* (1985) and Dimitrov *et al.* (1991), have reported intraspecific variation. The presence of four tiers of plates appears to be constant for most families throughout the Strigeata and, generally, the arrangement within the tiers is highly conserved, particularly for tiers one, three and four. However, a notable exception is the Bucephalidae, which has only anterior plates and these bear appendages (Peters, 1965). Miracidia from other orders may possess four tiers of plates but with an alternative number of plates per tier; for example, several *Echinostoma* spp. have 6+6+4+2=18 (Peters, 1965) and the amphistome *Gigantocotyle explanatum* (Creplin, 1847) has 6+8+4+2=20 (Dunn, Hanna & Nizami, 1987). While, members of the Fasciolidae have five rather than four tiers of plates (Peters, 1965; Køie, Christensen & Nansen, 1976) and miracidia of the Gorgoderidae possess three tiers (Peters, 1965).

The only previous detailed investigation on the distribution of strigeid miracidial sensilla was that of Dimitrov *et al.* (1991) for *Strigea falconispalumbi*. Their description corresponded closely to that recorded here for *I. erraticus*, *I. variegatus* and *A. gracilis*. They were also unable to differentiate between sensilla and gland openings on the terebratorium and recorded the same 20 argentophillic structures in this region. The only difference in the distribution they recorded was the more ventral and lateral location of the large body which formed part of the ventral group (TV) described here.

Dimitrov *et al.* (1991) emphasised the problems associated with the mapping of terebratorial argentophillic structures, i.e. that small papillae (sensilla or gland openings) are not readily visible in weakly stained preparations, but, when intensely stained, tend to merge, obscuring their true number.

The body sensilla recorded for the *Ichthyocotylurus* spp. and *A. gracilis* differed from *S. falconispalumbi* only in the presence of an additional receptor located between the dorso-lateral and ventro-lateral plates of the third tier. Twenty argentophillic structures were also described by Pearson (1961) on the terebratorium of the

diplostomid, *Neodiplostomum intermedium*. However, two of these structures were located adjacent to the anterior margins of the lateral first tier plates and were visible in live specimens. He concluded that these were the openings of the cephalic glands (not present in the strigeid miracidia so far described). The remaining 18 argentophillic structures were organised in an identical pattern to that described for the strigeid species in the present study, with just the two large ventral members absent. Pearson (1961) was also unable to determine which of these structures represented the opening(s) of the apical gland. His description of the remaining chaetotaxy of the *N. intermedium* miracidium also closely resembled that recorded here and by Dimitrov *et al.* (1991) for strigeid species. Like *S. falconispalumbi*, *N. intermedium* was found to differ from the *Ichthyocotylurus* spp. and *A. gracilis* in the absence of a sensillum between the dorso-lateral and ventro-lateral plates of the third tier. In contrast to the strigeid species, Pearson (1961) believed that the lateral papillae of *N. intermedium* were not argentophillic. He still recorded two silver stained structures in this region, and if correct regarding the lateral papillae, then the second of these argentophillic structures indicates an additional sensillum; a further difference to the strigeid species. Nevertheless, the chaetotaxy of these members of the families Strigeidae and Diplostomidae are extremely similar, particularly on the terebratorium. The position of the two excretory pores of *N. intermedium* were also found to be the same as recorded for strigeid species.

Chaetotaxy descriptions by Albaret (1984) for several *Schistosoma* spp. indicated more body sensilla than recorded for strigeoid miracidia, with several species having a large number between the second and third plate tiers. The position of the excretory pores was also found to differ for *Schistosoma* spp., opening between the lateral plates of the third tier (Albaret, 1984).

If a shorthand nomenclature is to be followed for miracidial chaetotaxy it needs to be specific and relate to particular landmarks. The new terebratorial nomenclature proposed here divides the terebratorium into defined sectors and removes the need for

arbitrary groupings. Previously there was no symbol to indicate the mid-plate location of a structure. Here it was proposed that the letter 'm' precedes the epidermal plate position to describe such a location.

4.2.3. Scanning electron microscopical observations of *I. erraticus* and *I. variegatus* miracidia.

The application of sonication for the deciliation of these species of miracidia was not successful, with a persistent 'stubble' obscuring the surface structures. Other authors have managed to remove the cilia from all but the first tier of epidermal plates for schistostomatid (Køie & Frandsen, 1976; Eklu-Natey *et al.*, 1985; Dunn, Hanna & Nizami, 1987; Maejima *et al.*, 1989) and *Fasciola hepatica* miracidia (Køie, Christensen & Nansen, 1976) using this method. They sonicated their miracidia in either absolute alcohol or benzene, with results being achieved after 2-30 minutes. The inability to shear the most anterior cilia was believed to be due to their dense arrangement rather than differences in structure (Køie *et al.*, 1976). Fortunately for these authors, the terebratorium of the studied miracidia tended to remain proud of these cilia, enabling examination.

The present study represented the first application of osmotic shock for the deciliation of miracidia. This technique proved far more successful than sonication for the two *Ichthyocoylurus* spp., removing the cilia from all but the first tier of epidermal plates. SEM observations of miracidia following deciliation provided confirmation of the number and location of body sensilla but failed to yield new information regarding terebratorial structures. The types of sensilla which have been recorded on the terebratorium of digenean miracidia by other authors include those with uniciliate nerve endings and multiciliate pits (*inter alia* Wilson, 1970; Brooker, 1972; Køie & Frandsen, 1976; Køie *et al.*, 1976; Maejima *et al.*, 1989). Due to the tendency of the *Ichthyocotylurus* miracidia examined to retract their terebratorium on fixation, some modification to the deciliation techniques (to achieve the removal of the most apical cilia) or to the fixation method (to obtain specimens with an extended terebratorium) is

required to enable SEM observation of this structure. TEM studies would reveal the types of sensilla present on the terebratorium, but a full series of serial sections would be required to indicate their arrangement.

The type and arrangement of sensilla between the first two plate tiers of the *Ichthyocotylurus* spp. were identical to those recorded for *F. hepatica* by Wilson (1970) and Køie *et al.* (1976). However, unlike the *Ichthyocotylurus* spp. which possess other, more posteriorly placed, uniciliate sensilla, these authors observed no further sensory structures on the body of *F. hepatica*. A more extensive range of sensilla types was recorded by Eklu-Natey *et al.* (1985), who observed uni- and multiciliate forms between the first and second tier plates of four species of human *Schistosoma* miracidia. They also found multiciliate receptors between the second and third plate tiers for three of these species. No multiciliate sensilla were present on the bodies of either species of *Ichthyocotylurus* miracidium.

Each of the excretory pores of the *Ichthyocotylurus* spp. were found to be bordered by a microvilli lined collar. Maejima *et al.* (1989) recorded a similar excretory opening, for the miracidium of *Gigantobilharzia sturniae* (Tanabe, 1951). However, the pore of *G. sturniae* was larger, some 2-3µm in diameter, and was bordered by two such collars.

4.3. BEHAVIOURAL STUDIES.

4.3.1. Longevity of the *I. variegatus* miracidium.

I. variegatus miracidia were found to have a survival period of up to 11 hours at 10°C and shorter longevities at more elevated temperatures. From the figure presented by Mattheis & Odening (1980) it appears that the free-living life span of *I. platycephalus* miracidia was one to two days, but they did not reveal at what temperature this observation was made. Nevertheless, a survival period approaching two days is far greater than the maximum of 11 hours obtained here for *I. variegatus*. A longevity somewhat closer to the present results, though still prolonged, was described

by Olson (1970) for the miracidium of *I. erraticus*. He found that miracidial swimming became slower and more erratic after six hours, although most remained active for 12 hours and several for as long as 17 hours. Olson also failed, however, to indicate the temperature at which his observations were taken. The differences observed by the present study, Olson (1970) and Mattheis & Odening (1980) for *Ichthyocotylurus* miracidial longevities is marked, even considering the latter authors' lack of quantitative data. This seems difficult to explain, given their common molluscan host and close morphological similarities. However, neither Olson (1970) or Mattheis & Odening (1980) stated the media in which the miracidia were maintained, a factor which may have affected the results obtained. Maximum longevities of a similar magnitude to the present study were recorded for the miracidia of *Fasciola hepatica*, which survived for 4-12 hours at 22°C (Bryant & Williams, 1962), *Neodiplostomum intermedium* three hours at 25°C (Dönges, 1964), *Isthmiophora melis* (Schrank, 1788) 11 hours at 22°C (Dönges, 1974) and *Transversotrema patialense* (Soparkar, 1924) eight hours at 25°C (Bundy, 1981). It must be remembered that the survival parameter of maximum longevity is only a property of a miracidial population in that it defines the age at death of the oldest member. The times presented in Table 49 at which 50% of the population remains alive provides more information on normal miracidial life spans. Mathematical models can also be used to estimate the mean expected longevity; such modelling provided Bundy (1981) with an estimation of a 2.6 hour mean life span for the miracidium of *T. patialense*, some 5.4 hours short of the recorded maximum longevity.

Miracidial mortality is believed to be due to the progressive utilization of their finite energy reserves of glycogen and lipid granules (Anderson, Mercer, Wilson & Carter, 1982). The effect of temperature upon mortality is likely, therefore, to result from changes in the rate of resource utilization. This was reflected in the present study by enhanced miracidial activity and decreased longevity at higher temperatures. Several authors have noted that miracidial longevity can be extended if their environment is nutrient enriched (Bennett & Jenkins, 1950; Bryant & Williams, 1962). Thus, it appears

that in some cases at least, uptake of external nutrients is possible by miracidia. Halton & Smyth (1978) noted that maximum survival of the miracidia of *Schistosoma mansoni* Sambon, 1907 and *S. haematobium* (Bilharz, 1852) occurred at moderate (these are tropical parasites) temperatures (18-30°C), rather than at low (5-10°C) or high temperatures (>35°C). Similar effects of temperature upon longevity were recorded by Anderson *et al.* (1982) for *S. mansoni* miracidia, but their observed optimal temperature for survival was somewhat lower, at 15°C. In contrast to larval survival, Anderson *et al.* (1982) found that the rate of snail infection was at a maximum at 25°C. Although not investigated in the present study, such a relationship may also exist for the miracidium of *I. variegatus* with its higher levels of activity at this temperature.

The effect of miracidial age and life span on the infectivity of *Diplostomum spathaceum* miracidia was investigated by Waadu (1991). In this study the author maintained miracidia at 20±2°C for varying periods before exposing them to a potential snail host. As one of the periods was 24 hours, at least a small proportion of this species of miracidium must survive in excess of a day at this temperature. However, infections performed with miracidia of different age classes indicated that infectivity drops rapidly after two hours, and after 10 hours successful infections were in single figures. In a similar series of experiments Waadu & Chappell (1991) studied the effect of temperature on miracidial infectivity. Their results suggested that infection was possible at temperatures as low as 6°C, with 3.5% of challenged *Lymnaea peregra* subsequently found to release cercariae. However, following exposure to the miracidia the snails were then maintained at 20°C. Consequently, it is not known whether development would have proceeded at the low infection temperature. An increase in infection rates was observed at above 12°C, with optimal temperatures recorded as 14-20°C, although higher temperatures were not investigated. The fact that *D. spathaceum* miracidia may infect their snail host at 6°C would appear to have little significance anyway as miracidial development within the egg is generally considered to cease at temperatures of below 10°C, precluding release (*inter alia* Erasmus, 1972). Infectivity experiments

could not be performed here due to the reasons indicated in 4.3; Introduction, and such a study has yet to be undertaken for a strigeid species.

The half-life (50% dead) of *S. douthitti* miracidia was found by Farley (1962) to be greatly affected by miracidial hatching time, with late hatchers having a far shorter longevity. No similar studies appear to have been performed on strigeid miracidia, nor any other species whose eggs require a lengthy developmental period in the aquatic environment. The hatching date (developmental duration) was not noted for the miracidia used in the present studies on longevity and this might have had some bearing on the survival results obtained, which appear to be short compared to Olson's (1970) and Mattheis & Odening's (1970) incidental findings. Further experiments could be performed to ascertain whether incubation temperature, and hence developmental rate, has an effect upon subsequent longevity and infectivity. Another possibility for the apparently short longevity could result from the medium employed - artificial spring water, its composition resulting in physiological effects causing a rise in energy utilisation and premature death.

The relatively short longevity of *I. variegatus* miracidia and their susceptibility to variations in temperature emphasised the need for using newly hatched specimens and moderate temperature regimes in experimental infections. This apparently short longevity would necessitate the rapid location and subsequent penetration of a suitable molluscan host, suggesting that host finding might be an active process. As a consequence of these results and Waadu's (1991) findings, that the infectivity of the longer lived *D. spathaceum* miracidia decreases rapidly after two hours, it was decided that the age classes employed in studies on miracidial behavioural responses should concentrate on young specimens, i.e. 0-1, 1-2 hours post-hatching (see 4.3.2).

The maximum survival of these miracidia at a temperature of around 15°C would favour the infection of snails in the early Summer months, resulting in the release of cercariae towards the end of the Summer, as was found by Swennen *et al.* (1979) in the Ijesselmeer, Netherlands. (see Chapter 5.1.1; Discussion).

4.3.2. Chemotactic behaviour of the *I. variegatus* miracidium.

The phototactic and geotactic responses of *I. variegatus* miracidia were not studied quantitatively. However, when collecting miracidia from a petri-dish illuminated on one side, no obvious accumulation was observed in relation to the light source; miracidia tend to swim just off the bottom of the container, principally around its periphery. The lack of an obvious phototactic response by *I. variegatus* miracidia was in contrast to the findings of Olson (1970), who stated that *I. erraticus* miracidia were positively phototactic. Similar positive phototactic responses were noted by Swennen *et al.* (1979) for the miracidia of *I. erraticus*, *I. variegatus* and *I. platycephalus*. However, none of these observations were confirmed by quantitative experiments. Keshavarz-Valian & Nollen (1980) identified positive geotactic and phototactic behaviour with miracidia of the eyefluke *Philophthalmus grali*. They found that the strong positive geotaxis overrode the phototactic response when both were tested simultaneously, that both were somewhat diminished by extreme deviations from room temperature and that no significant phototaxis could be demonstrated in 'older' miracidia. These findings demonstrate the need for rigidly controlled investigations into miracidial behaviour.

A consideration of the life-cycle of the snail *Valvata piscinalis* provides some indication as to the most likely behaviour which would enhance *Ichthyocotylurus* miracidial host finding. Studies on the life-cycle and habits of this snail were made by Hunter (1961) and Cleland (1954). Russel-Hunter's findings are particularly relevant, as they were performed in Loch Lomond where *Ichthyocotylurus* infections in fish were abundant. Both authors agreed that *V. piscinalis* is a bottom dwelling annual with only a single breeding season in each year. It is during the breeding season that adults leave the bottom to feed and spawn on weed, although many individuals are still found on the mud. The young hatch in the Spring or Summer (according to Cleland, 1954), June to September (according to Hunter, 1961), as miniature adults, and, after a short period on the weed, descend to the mud. It is possible that the adults might become infected while on the weed, although specimens remaining on the bottom may be more prone to

infection. In both these cases cercarial release would occur towards the end of the Summer as recorded by Swennen *et al.* (1979). In the first instance, a positive phototactic response would aid host location, while the second would favour a negative response. However, overwintering infections in adults would not yield cercariae, as the molluscs do not survive to the end of the following Spring. Cercarial release occurring in the Spring, as observed in the present study (see Chapter 5.1.1; Results), would result from overwintering in juvenile snails that were infected in the previous Summer/Autumn. These snails would not have attained an infectable size until long after they had left the weed and proceeded onto the mud to feed. Consequently, a negative phototactic or dominant geotactic response would be required by the miracidia to achieve transmission on the mud bottom.

The present results (Table 52) showed that *I. variegatus* miracidia accumulated in *V. piscinalis* conditioned water ($P<0.001$). However, when the experiment was repeated with miracidia of separate age classes, a significant accumulation was recorded only for miracidia of between 1 and 2 hours old ($P<0.001$). Miracidia of less than 1 hour in age, or older than 2 hours, showed no significant movement towards the snail-conditioned water, $P>0.9$ and $P>0.05$, respectively. These results suggest a very short period in which the miracidia are receptive to stimuli emitted from the molluscan host. Newly hatched miracidia may be more responsive to other stimuli, such as light or gravity, which would aid dispersal and bring them into areas likely to be inhabited by *Valvata* spp. Consequently, these stimuli may override the chemotactic response. Experiments performed in the absence of illumination still failed to produce a significant movement towards the snail-conditioned water in young (<1 hour) miracidia, but, when compared to illuminated behaviour, did indicate the presence of a phototactic response. The central chamber provided some shade from the light sources directed towards the test and control arms, and the proportion of young miracidia remaining in the central chamber was far higher in illuminated than unilluminated trials, 69% and 17%, respectively. This difference was also observed, to a lesser extent, for mixed age

miracidia, with 38% remaining in the central chamber in illuminated conditions and 22% in unilluminated. Thus, as the miracidium ages, it is possible that the negative phototactic response diminishes or, alternatively, the chemotactic response is heightened. This does not, however, explain the large percentage of >2 hour-old miracidia remaining in the central chamber (61%). If *Ichthyocotylurus* miracidia exhibit a positive phototactic response, as proposed by Olson (1970) and Swennen *et al.* (1979), then, in the absence of any other stimulus, the majority would be expected to be found in the side arms rather than the central chamber. Table 50 shows that this was not the case with *I. variegatus* miracidia. The work of Takahashi, Mori & Shigeta (1961) demonstrated that age, temperature and light intensity were all important in the phototactic response of *Schistosoma japonicum*, with, for example, a positive phototaxis at low light intensities but a negative response at higher intensities. None of these parameters were indicated for the *Ichthyocotylurus* spp. studied by Olson (1970) or Swennen *et al.* (1979), while only a single light intensity was employed in the present study for *I. variegatus*. The lack of such information might explain our conflicting findings.

When a *Lymnaea peregra* specimen was placed into the test arm, the resulting snail-conditioned water did not cause the accumulation of *I. variegatus* miracidia (P>0.1, see Table 52). This suggests that the chemical stimuli (termed miraxones by Chernin, 1970), which attract *I. variegatus* miracidia to its natural host, *V. piscinalis*, are absent in this unsuitable host. Whether the stimulus/stimuli are present in other more closely related groups of snail would require further investigation. Chernin (1970) found that *Schistosoma mansoni, S. japonicum* and *Fasciola hepatica* miracidia were responsive to stimulants produced by both susceptible and insusceptible snails. However, Cable (1972) listed conflicting findings on this subject, while later work has suggested that no single miraxone is stimulatory to miracidia of all species, with a variety of organic and inorganic substances believed to be responsible (Keshavarz-Valian, Nollen & Maynard, 1981).

I. variegatus miracidia failed to orientate towards the test arm containing *V.*

piscinalis conditioned water in the T-chamber (probability of random orientation: $P>0.9$, see Table 53). Miracidia were seen to swim in straight paths along the base arm but rapidly adopt turning movements when reaching the T-junction and the front of the chemical gradient. This increase in random turning movements, or klinokinesis, was shown theoretically by Patlack (1953) to lead to the aggregation of animals, while Chernin (1970), Shiff & Kriel (1970), Mason & Fripp (1976), Mason (1977), Samuelson *et al.* (1984) and others have shown that such behaviour leads to the aggregation of miracidia in snail-conditioned water. Although *I. variegatus* miracidia were not observed to advance preferentially towards the arm containing snail-conditioned water, once present, they were far more likely to remain than those entering the control arm. This suggests that chemoklinokinesis (also termed chemokinesis) rather than chemotaxis is responsible for host location in this miracidial species. Once the initial contact has been made between miracidium and host surface the 'contact and return' response (MacInnis, 1965) is initiated, leading to penetration activity. The T-chamber results obtained by Hass *et al.* (1991) for *S. japonicum* miracidia indicated chemotactic driven orientation. This approach stimulus was not found to be host specific, with host selection not occurring until the attachment behavioural phase. Perhaps, the less rapid chemoklinokinetic stimuli are unique to a particular snail species, while chemotactic stimuli are less specific.

Collectively, the present results suggest that a chemosensitive response is involved in the location of the molluscan host by *I. variegatus* miracidia, but emphasise the need for knowledge of other tactic responses. Further more detailed and structured studies are required before any firm conclusions can be drawn regarding these attractants, but experiments performed with snail extracted compounds might enable the attractive stimulant/stimuli for this miracidial species to be identified.

In summary, eggs of *I. erraticus*, *I. variegatus* and *A. gracilis* obtained from experimental infections in gulls and ducks were incubated and miracidia successfully

raised. Developmental periods were found to be temperature dependent and differed for the three species at 20°C: *A. gracilis* < *I. erraticus* < *I. variegatus*. Light microscopy revealed the morphology of all three species to be identical, as were the epidermal plate formulae and chaetotaxy, indicated by silver-staining. The nomenclature of Dimitrov *et al.* (1989) for the mapping of body sensilla was modified to enable a full description of these miracidia and a new system proposed for terebratorial structures. Osmotic shock resulted in the partial deciliation of the miracidia and subsequent SEM observation confirmed the arrangement of body surface structures, while revealing sensilla forms. Behavioural aspects of *I. variegatus* miracidia were examined, with a maximum longevity (<11 hours) recorded at the lowest temperature studied (10°C), and host finding appearing to occur by an increased turning response in the presence of substances emitted from the snail host, following an unresponsive dispersal phase. Incidental observations suggested the miracidia of this species to be negatively phototaxic for at least part of its life span.

CHAPTER 5: THE PARTHENITAE AND CERCARIAE

INTRODUCTION

Strigeoid cercariae present many features which are of potential taxonomic use. Many morphological structures, such as spination, penetration glands, caudal bodies, eye-spots and the excretory system, are subsequently lost or greatly modified (generally becoming more complex) with the transition to the metacercarial stage. Variation in specificity to the molluscan hosts from which they emerge and to the subsequent hosts, as well as behavioural characteristics while in this free-living stage, may also be of taxonomic value. Reviews of the history of strigeoid cercarial classification were provided by Niewiadomska (1971b) and Blair (1974). It was proposed by Niewiadomska (1970a, 1971b) that these cercariae may be readily placed in appropriate adult genera or subgenera according to the structure of their excretory systems and the number and arrangement of their penetration glands, and this was applied by Blair (1977) in a key to British strigeoid cercariae with known life histories. However, effective criteria for separating species within a genus or subgenus using cercarial characteristics are still not established. Niewiadomska (1971b) considered body proportions, caudal body number and spination details to be the primary diagnostic features, with resting posture of less importance. Subsequently, Blair (1977) concurred with Niewiadomska's criteria with the exception of body proportions. These are, Blair considered, too prone to intra-specific variation, particularly when cercariae originate from different molluscan hosts or from geographically distant samples. He also added the specificity to the second intermediate host as of diagnostic importance.

A section from Blair's (1977) key to the genera and subgenera of British strigeoid cercariae is provided below. This has been amended to account for the elevation, by Niewiadomska (1971a), of the subgenera *Cotylurus* and *Ichthyocotylurus* to the full generic status.

4a Cercariae with flame cell formula 2[(2 + 2) + ((2 + 2) + (2))] = 20; transverse excretory commissure anterior to the ventral sucker; gut caeca terminate much closer to the bladder than to the ventral sucker.....**5**

4b Cercariae with 14 or fewer flame cells; complete or incomplete excretory commissure posterior to the ventral sucker, or anterior and posterior; gut caeca terminate slightly posterior to, or well anterior to, ventral sucker.

Genus *Apatemon*.....6

5a Two pairs of penetration gland cells anterior to the ventral sucker; no clusters of cilia in the recurved portion of the bladder arm.

Genus *Cotylurus*

5b Two pairs of penetration gland cells posterior to the ventral sucker (an additional pair anterior to the ventral sucker in *I. erraticus*); clusters of cilia in recurved portion of the bladder arm (not reported for *I. erraticus*).

Genus *Ichthyocotylurus*

6a Flame cell formula 2[(2) + ((2) + (1))] = 10; six pairs of penetration gland cells posterior to ventral sucker; complete transverse excretory commissure behind ventral sucker; gut caeca very short, terminating well anterior to ventral sucker.

Subgenus *Apatemon*

6b Flame cell formula 2[(2) + ((2 + 2) + (1))] = 14; four pairs of penetration gland cells posterior to ventral sucker; complete or incomplete transverse excretory commissures both posterior and anterior to ventral sucker; gut caeca terminate closer to ventral sucker than to bladder.

Subgenus *Australapatemon*

In the following study an attempt was made to raise the cercarial stage of *Ichthyocotylurus erraticus*, *I. variegatus* and *Apatemon gracilis* experimentally in order to investigate aspects of the biology and morphology of these cercariae relevant to the key of Blair (1977) and evaluate their value at the species level.

5.1 MOLLUSCAN STAGES.

In demonstrating the life-cycle of *I. erraticus* in North America, Olson (1970)

was unable to isolate the natural snail host. He monitored 10 species of snail (including *Valvata lewisi* and *Lymnea stagnalis*) for cercarial emergence and exposed rainbow trout, a known intermediate host, to the released cercariae, but no *I. erraticus* infections resulted. Determination of the snail host was attempted experimentally by exposing snails to *I. erraticus* miracidia. Successful infections occurred only with *V. lewisi* and the identity was confirmed by raising metacercariae from the resultant cercariae. Similar findings were made by Johnson (1971). *V. lewisi* is not native to Europe, although several other species of this genus are, including *V. piscinalis*, *V. cristata*, *V. macrostoma* and *V. helicoidea*. The natural molluscan host of both *I. variegatus* and *I. platycephalus* in the Müggleseе, Germany were identified by Odening & Bockhardt (1971) and Odening, Mattheis & Bockhardt (1970), respectively, as *V. piscinalis*. These cercariae were only found to emerge from *V. piscinalis*, although some 27 gastropod and 4 bivalve species were investigated. Swennen *et al.* (1979) found this snail species to be the molluscan host of *I. erraticus, I. variegatus* and *I. platycephalus* in the Ijsselmeer, Netherlands. All of these European authors recorded that experimental infections of *Ichthyocotylurus* spp. only established in *V. piscinalis*. They did not, however, attempt to infect the less abundant and easily obtained *Valvata* spp. from their regions. Another *Valvata* species, *V. helicoidea*, has been identified as the natural primary host to *I. erraticus* (see Barsiene, Petkevichute, Stanevichute & Orlovskaya, 1990) and *I. platycephalus* (see Grabda-Kazubskaya & Kiseliene, 1991) in eastern Europe. An alternative snail host was identified for *I. erraticus* by Orlovskaya (1979) as *Lymnaea stagnalis*.

The molluscan host of the fourth member of this genus, *I. pileatus*, was listed as unknown by Odening (1978), Pugachev (1983), Shigin (1983) and Sudarikov (1984). However, Anikieva (1983) recorded four snail species, *Radix ovata*, *L. stagnalis*, *Anisus vortex* and *Galba palustris*, as hosts. More recently, Barsiené, Petkevichute, Stanevichute & Orlovskaya, 1990 gave *L. zazurensis* from the Chukotka region of Siberia as the intermediate host, while Timoshenko (1990) listed three lymnaeid species, *L. stagnalis*,

L. auricularis and *L. palustris* from the River Selengi delta in Asian Russia. Thus it appears that *I. pileatus* is atypical in not being specific to *Valvata* spp. However, the identity of these cercariae must be in some doubt, as it is not clear how these authors have identified their cercariae and no descriptions are provided. Indeed, there does not appear to be any formal description of this species of cercaria.

No natural infections of molluscs yielding *Ichthyocotylurus* cercariae have been recorded from, and no such cercariae have been experimentally raised in Britain.

Less is known of the primary intermediate hosts of the species thought to belong to the subgenus *Apatemon*, where, with the exception of *A. fuligulae* (*Anisus acronicus*, see Barsiené *et al.*, 1990) and *A. graciliformis* (*Biomphalaria glabrata*, see Combes & Nassi, 1977) the only known molluscan hosts are those of *A. gracilis*. The most widely recorded host is the pulmonate snail *Lymnaea peregra* (see *inter alia* Vojtek, 1964a; Dubois, 1968; Blair, 1973, 1974, 1976, 1977; Odening 1978; Sten'ko, 1977, in Sudarikov, 1984). Odening (1978) also included *Radix balthica* and Dubois (1968) *L. palaustris*. Due to the similarities in cercarial morphology and lack of information on their life-cycles, many early descriptions of strigeid cercariae, particularly of *A. gracilis,* are either dubious (Crocombe, 1959) or erroneous (Stunkard, Willey & Rabinowitz, 1941). For example, the cercaria accredited to *A. gracilis* by Crocombe (1959) in South Wales emerged from *Ancylastrum fluviatilis*; this he named *Cercaria duodecaglandis.* His description differed from that provided by the other authors in Table 58 by the presence of an extra pair of post-acetabular flame-cells, giving a total of 12. Although numeric variation in structures such as flame-cells and penetration glands have been recorded (Niewiadomska, 1970b), this consistently high number (no intraspecific variation observed), plus its atypical molluscan host, raise doubts as to its identity.

The only certain record of natural infections of *A. gracilis* cercaria from Britain are those of Blair (1974, 1976, 1977), who obtained his material from Central Scotland. He also successfully raised the cercariae from experimentally raised miracidia in naïve

laboratory-reared *L. peregra*.

In addition to misidentifications of strigeid cercariae, there are also several descriptions of cercariae with unknown life-cycles which may represent members of this group. For example, three new cercariae described by Ginetsinskaya (1959) exhibit many similarities to those of the genus *Ichthyocotylurus*. *Cercaria valvatae* and *C. abyssalis* were both found to develop within *V. piscinalis*, while *C. spinulosa* developed within *L. stagnalis*. The position of these cercariae was discussed by Odening *et al.* (1970) and they considered *C. valvatae* in particular to relate to the genus *Ichthyocotylurus*.

5.2 TAXONOMIC STUDIES.

Conventional morphological studies were performed upon the experimentally raised cercariae and compared to the existing knowledge. In addition, multivariate analyses which were successfully employed in Chapter 2 to discriminate between morphologically similar metacercariae and demonstrated characters of diagnostic value, were applied to the cercariae of *I. erraticus* and *I. variegatus*.

Argentophilic papillae (sensilla) have been studied on the cercariae of various species of trematodes, including strigeids (Shigin, 1974; Blair, 1974; Richard, 1982; Zazornova, 1987), and several authors have shown that differences in sensillary patterns (i.e. chaetotaxy) can be important in differentiating species. Richard (1971) recorded distinct patterns for members of the superfamilies Strigeoidea (principally the Diplostomidae, Schistosomatoidea, Echinostomatoidea, Plagiorchioidea and Allocreadioidea); while sensillary patterns enabled Short & Kuntz (1976) to distinguish *Schistosoma rodhaini* Brumpt, 1931 and *S. mansoni* Sambon, 1907 cercariae. Brady (1989) also found this technique of value in discriminating morphologically similar *Diplostomum* spp. However, in describing features useful in the identification of

cercariae of seven *Diplostomum* spp., Niewiadomska & Kiseliene (1990) omitted chaetotaxy, as they found that there were several species with no clear differences.

Our knowledge of the armature and sensillary arrangement of *Ichthyocotylurus* spp. is restricted to light microscopical observations of *I. erraticus* cercariae presented by Swennen *et al.* (1979) and the surface structures of *I. variegatus* and *I. platycephalus* cercariae described by Odening & Bockhardt (1971) and Odening *et al.* (1970), respectively. However, all descriptions of the number and distribution of sensilla of *Ichthyocotylurus* spp. are limited to those bearing a cilium of a size which could be seen unstained under the light microscope, and thus the cercarial chaetotaxy of members of this genus has not been previously studied in detail. The armature and the distribution of sensilla of *Apatemon gracilis* cercariae were described by Blair (1974). His description of the armature was also limited to light microscopical observations, but the chaetotaxy pattern was compiled from silver-stained specimens.

In this chapter the chaetotaxy and armature of *I. erraticus*, *I. variegatus* and *A. gracilis* cercariae are re-examined using silver-stained preparations and scanning electron microscopy (SEM). Morphometric analyses were also applied to the distribution of these sensilla on the two *Ichthyocotylurus* spp. Multivariate analyses based on cercarial papillae (=sensilla) morphometric indices were used successfully by Bayssade-Dufour, Cabaret, Ngendahayo, Albaret, Carrat & Chabaud (1989), Cabaret, Bayssade-Dufour, Albaret, Ngendahayo & Chabaud (1990) and Albaret, Bayssade-Dufour & Ngendayayo (1993) for the identification of *Schistosoma* spp. However, the technique applied by these authors required multiple samples or isolates of cercariae which constituted some hundreds of specimens.

Ultrastructurally, the most widely studied cercarial sense organs are those of *Schistosoma mansoni* (see Robson & Erasmus, 1970; Nuttman, 1971; Morris, 1971; Hockley, 1973; Mclaren, 1980). These authors identified three basic types of sensilla on the surface of this cercaria, comprising two uni-ciliate forms and a multi-ciliate pit-like structure. In addition, Morris (1971) described a sub-tegumental aciliate sensory

organelle, Cousin & Dorsey (1987) a bi-ciliate receptor and Short & Gagné (1975) a lamellate structure, believed to be a photoreceptor. Similar uni-ciliate sensilla have been found on the cercariae of *Parorchis acanthus* Nicoll, 1906 (see Rees, 1971), *Neophasis lageniformis* (Lebour, 1910) (see Køie, 1973) and *Podocotyle staffordi* Miller, 1941 (see Gibson, 1974). More recently, Pariselle & Matricon-Gondran (1985) described several forms of uni- and multi-ciliated sensory receptors for the cercaria of *Nicolla gallica* (Dollfus, 1941). While, Zdárská (1992) identified three non-ciliate, four uni-ciliate and one multi-ciliated sensilla for the cercaria of *Echinostoma revolutum* (Frölich, 1802).

These findings have, therefore, demonstrated that cercariae exhibit a wide range of sensillary types incorporating the majority of forms described for other digenean life-cycle stages: miracidia (Wilson, 1970; Dunn, Hanna & Nizami 1987); redia (Køie, 1971; Czubaj & Niewiadomska, 1988); sporocysts (Czubaj & Niewiadomska, 1991); metacercariae (Hoole & Mitchell, 1981); and adults (Køie, 1973; Edwards, Nollen & Nadakavukaren, 1977; Ip & Desser, 1984; Torii, Tsuboi, Hirai & Nishida, 1989). This complexity in sensory receptors is present throughout the Platyhelminthes, as demonstrated by the work of Rohde (1990) and Rohde & Watson (1989, 1990a-c, 1992) on an aspidogastrean and studies on the Monogenea by, *inter alia*, Lyons (1969, 1972) and El-Naggar, Khidr & Kearn (1991).

Transmission electron microscopy (TEM) has not previously been used to study the fine structure of strigeid cercariae. In this section TEM is employed to examine the sensilla of *I. variegatus* cercaria in order to determine whether the differences observed in external structure using SEM correspond to variation in internal structure, and to see whether further sensillary forms can be identified.

5.3. BEHAVIOURAL STUDIES.

The cercariae of many digenean species must emerge from their molluscan host in order to begin the second free-living stage of their life-cycle. Although not

extensively studied there is a general consensus that cercariae utilise the circulatory system of the molluscan host in order to reach this site of emergence. Duke (1952) and Ritchards (1961) concluded that *Schistosoma mansoni* cercariae pass along the venous blood vessels to the pseudobranch and collar where emergence occurs. Similarly, for *Neodiplostomum intermedium* Pearson, 1959 (see Pearson, 1961) and *Cercaria X* of Taylor & Baylis, 1930 (see Probert & Erasmus, 1965), the main route of migration within the snail host was considered to be the vascular system, but with emergence occurring from the mantle sinus. Indeed, Ginetsinskaya (1988), considered the route described by Probert & Erasmus (1965) for *Cercaria X* through *Lymnaea stagnalis* to be typical for most cercarial species: the cercariae perforate the wall of the digestive gland and enter the haemocoel of the mollusc. From here they penetrate the venous vessels, passing through the rectal and hepatic sinuses, to the blood vessels of the lung (in pulmonates), and finally enter the heart. Contractions of the ventricle cause the cercariae to be expelled along with the blood into the pulmonary artery and subsequently into the mantle sinuses. On reaching a site where the sinus abuts with the mantle edge, the cercariae penetrate through its wall and then through the mantle tissues prior to emerging into the environment.

Pearson (1961) found that, for a number of strigeid species, the point of emergence established by an individual was utilised by all the subsequent cercariae released from the infected mollusc. Cercarial emergence from a common exit was also recorded for several species of diplostomid and such an aperture is generally referred to as an 'escape pore' (Ginetsinskaya, 1988). These pores are believed to close between emissions, with the edges of the aperture interlocking.

Once free from their molluscan host furcocercariae show a range of swimming behaviour, but the most typical and widely reported, particularly within the strigeoids, involves short periods of rapid tail-first swimming broken by longer periods of rest. During the swimming phase the cercaria tends to rise in the water. This movement then ceases, the furcae are spread, which aids floatation, and the cercaria sinks slowly,

anterior-end downwards (Erasmus, 1972). This resting posture of furcocercariae in the aquatic environment was considered by Niewiadomska (1971b) and Blair (1977) to be of systematic importance, although Blair conceded that its value is reduced by the lack of variation in some genera or subgenera. *Diplostomum* cercariae exhibit a particularly wide range of postures with variations in furcal spread, angle of tail-stem and angle of body (Blair, 1974, 1977; Brady, 1989).

The emergence strategies of the strigeid species, *A. gracilis*, *I. erraticus* and *I. variegatus* have not previously been studied and are considered here in relation to the 'typical' route. Swimming behaviour and resting posture have been discussed for these species by Crocombe (1959), Olson (1970), Odening & Bockhardt (1971), Blair (1974, 1976, 1977), Swennen *et al.* (1979) but are further studied here to supplement and contrast to the results obtained by the other authors.

Cercarial emergence from the molluscan host is directly influenced by environmental factors; of these light and temperature appear to play the most important roles. However, in the natural environment the influence of these is so interrelated that it is often difficult to ascertain which has the greater significance (Ginetsinskaya, 1988).

It was reported by Combes & Théron (1977) that the emergence of many digenean cercariae from their snail hosts exhibits a circadian rhythm. Such behaviour has been widely studied in schistosomes which display a range of release patterns. Faust & Hoffman (1934) found that *Schistosoma mansoni* cercariae emerged in greatest numbers during sunlight. This was confirmed by Kuntz (1947) and subsequently by many other authors. *S. douthitti* (Cort, 1914) cercariae emerge nocturnally (Olivier, 1950; Ginetsinskaya, 1988). In another variation, Raymond & Probert (1991) observed that *S. margrebowiei* (La Roux, 1933) cercariae exhibit an "ultradien" rhythm, with emergence following the onsets of light and darkness. These release strategies are often very well defined. Mouchet, Théron, Brémond, Sellin & Sellin (1992) were able to discriminate between the cercariae of four sympatric *Schistosoma* spp. on this basis

alone. Similarly, Mouahid, Monè, Chaib & Théron (1991) isolated *S. bovis* (Sonsino, 1876) and *S. haematobium* (Bilharz, 1852) cercariae from dual infected snails by their distinctive shedding patterns; and the chronobiology of the emergence patterns enabled Pagès & Théron (1990) to distinguish two geographical strains of *S. intercalatum* (Fisher, 1934). Théron (1989) observed that hybridisation between *S. mansoni* and *S. rodhaini*, with respective diurnal and nocturnal cercarial emergence rhythms, led to F1 and F2 generations of cercariae which were characterised by two unequal emergence peaks, one diurnal and the other nocturnal. The relative importance of these peaks depended upon which *S. mansoni* strain (early or late) was used for the hybridisation with *S. rodhaini*.

Marked differences in the cercarial emergence strategies of members of other digenean families, in response to light, have been demonstrated by Lewis, Welsford & Uglem (1989) for *Proterometra* spp. and by Taskinen, Valtonen & Gibson (1991) for two bucephalids infecting *Anodonta anatina*.

Experiments have shown that the emergence patterns of several species can be altered by maintaining snail hosts in varying light:dark (L:D) cycles. The ability of light to stimulate or inhibit cercarial emergence was first demonstrated by Giovannola (1936) on cercariae of *Diplostomum flexicaudum* Cort & Brooks, 1928. This author noted that changing the normal daily periodicity of lighting resulted in corresponding shifts in the intensity of cercarial emergence. The previous daily rhythm was then regained by restoring the normal cycle of lighting. Asch (1972) identified light as the sole environmental factor controlling the diurnal emergence of *S. mansoni* cercariae. He found that all visible portions of the light spectrum initiated this response and that intensities as low as 100-200 ergs/cm^2/sec were required. The numbers of *S. mansoni* cercariae emerging during this diurnal cycle was found to be enhanced by exposing snails to an extended dark period before exposure to light (Kuntz, 1947). Most authors have imposed L:D 12:12 cycles in experimental studies (*inter alia* Glaudel & Etges, 1973; Nojima & Sato, 1982; Krishna & Rao, 1986; Kitaguchi, Oshima, Saito &

Kanayama, 1992). In this study the pattern of cercarial release was observed using a L:D (16:8) photoperiod which corresponded approximately to the natural light conditions experienced during emission of strigeid cercariae in the field (late Spring/Summer months). Lewis, Welsford & Uglem (1989) monitored cercarial emergence under both L:D 12:12 and L:D 15:9 cycles. They found that percentage release during light and dark periods was extremely similar for both photoperiods (statistical comparison not provided). Nevertheless, total numbers emitted were reduced for the latter cycle.

It must be remembered, however, that the emergence of some digenean cercariae may not be influenced by changes in illumination (Wisniewski, 1937; Chernogovenko, 1962, in Ginetsinskaya, 1988).

MATERIALS AND METHODS.

5.1. MOLLUSCAN STAGES.

5.1.1. Natural cercarial infections.

Snails were collected from their natural habitat as parental stock to rear a population of naïve individuals with which to perform experimental infections. However, a survey of natural infections in wild snails was also carried out at the following sites where fish harbouring strigeid metacercariae were known to occur:

1. River Earn at the site of a fish farm, Comrie, Perthshire.
2. River Almond at the site of a fish farm, Perthshire.
3. Castle Semple Loch at Loch Winnoch Nature Reserve, Ayrshire.
4. Loch Lomond, Stirlingshire.

Lymnaea peregra were obtained from all four sources, whereas *Valvata piscinalis* were only recovered from the 2 loch habitats. The apparent absence of *V. piscinalis* from the two rivers does not reflect its general habitat preference, it being commonly recorded in flowing water (Cleland, 1954; Russel-Hunter, 1961). As mentioned in 5.1; Introduction, the genus *Valvata* is the sole confirmed molluscan host of *Ichthyocotylurus* spp. and its scarcity in these rivers would account for the absence of *Ichthyocotylurus* metacercariae in these fish populations (see Chapter 2).

Snails were collected from the first two sites using metal sieves attached to long wooden broom handles. At Loch Lomond, snails were harvested from the margins by hand and from deeper water using a fine meshed dredge net. Snails were transported back to the laboratory in water from the river or loch from which they were collected. They were maintained at room temperature in plastic tanks containing aerated artificial spring water (ASW) and washed vegetation from their natural habitats. *L. peregra* were fed *ad libitum* on cooked porridge oat flakes. The diet of *V. piscinalis*, which is a

known detritus feeder (Fretter & Graham, 1962), was fish faeces; but this proved to be unsuitable, resulting in many mortalities, and it was changed in December 1992 to a commercial tropical fish food with far greater success. The ASW was changed twice-weekly.

Wild snails were monitored daily for cercarial release by placing small groups in petri-dishes containing ASW between the hours of 9am and 6pm. If cercariae were found in the water the snails were further separated to enable the isolation of the shedding individual. Snails were monitored over a period of several months. Released cercariae were identified as accurately as possible using *inter alia* Yamaguti (1971), Combes (1980) and the key of Blair (1976).

5.1.2. Experimental infections: development from miracidia to cercaria.

Source of molluscs.

Valvata piscinalis from parasite-free colonies reared in the laboratory were infected with *I. erraticus* and *I. variegatus* miracidia when they attained a shell length of 1.5-2.5mm and naïve *Lymnea peregra* with *A. gracilis* miracidia when they reached a shell length of 4-6mm. These size ranges were selected as it was considered that smaller snails might be unable to survive the miracidial challenge, while large snails are generally believed to be less susceptible to infection (*inter alia* Anderson, Mercer, Wilson & Carter, 1982).

Source of miracidia.

The *I. erraticus* miracidia used had hatched from eggs shed by birds experimentally infected with Loch Lomond powan metacercariae, the *I. variegatus* miracidia from Loch Lomond ruffe metacercariae and *A. gracilis* miracidia from River Almond stone loach metacercariae.

Mode of infection.

The infections were performed in 2 ways:

(1) A batch of 10 eggs about to hatch and a single uninfected snail were placed together in each of the cells of a tissue culture multi-dish which were filled with ASW. The ASW was not replaced until at least 4 miracidia had hatched from each batch of eggs and were no longer visible in the water.

(2) 5 newly hatched miracidia were introduced with a Pasteur pipette into a cell containing an uninfected snail in ASW.

The latter method was felt to be necessary as snails (particularly *V. piscinalis*) were often found to consume the unhatched eggs and it was not known whether this route would result in an infection. The number of infections attempted with each snail species was limited by different factors: (1) the number of *V. piscinalis* attaining an infectable size enabled 41 specimens to be exposed to *I. erraticus* miracidia and 64 individuals to *I. variegatus* miracidia; and (2) the number of eggs and consequently miracidia obtained from experimental *A. gracilis* infections in birds allowed for just 16 *L. peregra* challenges.

Snail maintenance.

After infection, snails were kept separately in the cells of tissue culture multi-dishes containing ASW within an incubator set at 20±2°C, with a photoperiod of 16 hours of light per day (7am-11pm). Experimentally infected *L. peregra* were fed as before. *V. piscinalis* challenged before December 1992 were fed on fish faeces, while subsequently infected specimens were given tropical fish flakes. In all cases the ASW was changed daily.

Experimentally infected *L. peregra* were monitored daily for cercarial release after 3 weeks and *V. piscinalis* 4 weeks post-infection (p.i.). All the cercariae used in the following study were experimentally reared.

5.2. TAXONOMIC STUDIES.

5.2.1. Light microscopical observations of cercariae.

Specimens required for microscopical examination were collected from snails placed in fresh ASW with no food material present, ensuring clean preparations. All cercariae used were newly released (less than 2 hours post release). Cercariae were observed both alive and fixed.

Nasir & Erasmus (1964) recommended that cercariae should be fixed in hot 10% formalin and this proposal was adopted by Blair (1974). Here, cercariae were fixed in hot (60-65˚C) 5% or 10% formalin and measured in these media, coverslip pressure on the specimens being avoided. Mean measurements, in micrometres, were taken from 30 individuals and are indicated in Fig. 76.

Many structures were most easily observed in live specimens which were examined in ASW under a coverslip. Several internal features, particularly the excretory system, became more apparent as the water evaporated. The resting posture and swimming behaviour of cercariae were observed using a stereo dissecting microscope. Swimming behaviour was also recorded using video equipment (see video examination of cercarial release below).

5.2.2. Discrimination of *Ichthyocotylurus erraticus* and *I. vareigatus* cercariae by principal components analysis of metrical features.

Morphometric analyses were performed, as described in Chapter 2.2.4; Materials and Methods. Measurements were taken from light microscope preparations of 30 *I. erraticus* and 39 *I. variegatus* cercariae which had emerged from experimentally infected snails. All of the specimens were fixed in hot 5% formalin and were unstained. The metrical features (variables) used in the analyses are shown in Fig. 76.

Measurements were standardised to remove the effect of size. This was achieved by converting the data to a percentage of total body length (sum of body length, tail-stem length and furca length) or by logging (ln) the data. The latter method is

considered less susceptible to fixation effects. All measurements are in micrometres.

5.2.3. Chaetotaxy and scanning electron microscopical observations of cercariae.

Chaetotaxy.

Cercarial sensilla were stained using the method described for metacercariae in Chapter 2.2.3; Materials and Methods. The only modification to this protocol was the reduction of the dark incubation period to 2.5 minutes which prevented overstaining. Drawings were made with the aid of a drawing tube and the chaetotaxy pattern compiled from a minimum of 50 specimens. Descriptions were based on the nomenclature of Richard (1971) which was accepted by Bayssade-Dufour (1979). In this system the pattern of chaetotaxy reflects the central nervous system (CNS) of a hypothetical cercaria. In 1982, Niewiadomska & Moczoń determined the morphology and topography of the CNS of a cercaria subsequently considered to be *Diplostomum pseudospathaceum* Niewiadomska, 1984. Recently, Niewiadomska (1994) applied the chaetotaxy of *D. pseudospathaceum* to this known internal morphology causing this author to reinterpret the sensillary pattern of *Diplostomum* spp. She suggested that the proper relationship between the CNS and sensory apparatus should be analysed in other groups of furcocercariae. However, since the detailed organisation of the CNS of strigeid cercariae was not investigated in this study, the established nomenclature of Richard (1971) was used.

Scanning electron microscopy.

Cercariae were fixed in 3% cacodylate buffered glutaraldehyde or hot 5% formalin. They were then processed and viewed as described for metacercariae in Chapter 2.2.3; Materials and Methods. Statistical analyses using Student's t-tests for differences between the means of two small samples (population variances not assumed to be equal) were applied to meristic data when appropriate.

5.2.4. Discrimination of *I. erraticus* and *I. variegatus* cercariae by principal components analysis of sensilla number and distribution.

The silver nitrate staining of cercarial sensilla and their morphometric analysis were performed as described in 5.2.3 and 5.2.2; Materials and Methods, respectively. Morphometric measurements and meristic data (the total number of sensilla present on the dorsal and ventral surfaces of the tail-stem and on the furcae) were taken from 30 specimens of each species emerging from experimentally infected snails. The metrical (Fig. 77) and meristic features (variables) given below were used in the analyses.

Variables:

1-4.	Distance (horizontal) between dorsal surface sensilla (D_I - D_{IV}).
5 (meristic).	Number of furcal sensilla.
6-9.	Distance (vertical) between ventral surface sensilla (L_I - L_{IV}).
10-12.	Distance (vertical) between dorsal surface sensilla (S_I - S_{III}).
13 (meristic).	Number of tail-stem sensilla.
14-18.	Distance (horizontal) between ventral surface sensilla (V_I - V_V).

All morphometric measurements were in micrometres. The measurements were logged to remove the effect of size, while meristic data were untransformed.

5.2.5. Transmission electron microscopical observations of *I. variegatus* cercarial sensilla.

Cercariae were fixed in cacodylate buffered 3% glutaraldehyde at 4°C for 2-4 hours. The fixative was pipetted off and cacodylate rinse added for 12 hours. In order to improve handling of specimens and minimise loss during processing, the cacodylate rinse was then replaced with an agar solution. Once set, the agar was cut into pieces and the cercariae contained therein post-fixed in 1% osmium tetroxide for 1 hour. The specimens were then treated with a graded series of acetones and resin mix: 60% acetone (10 min.); 90% acetone (10 min.); 100% acetone (15 min., 2 changes); 100% acetone + resin mix (1:1, 30 min.); 100% acetone + resin mix (1:3, 30 min.); resin mix only (60 min.).

When specimens were in resin solutions the vials were placed on an angled rotator which allows streaming of the resin and facilitates impregnation. BEEM capsules

were warmed in the oven for 1 hour prior to embedding in order to remove any moisture which may have been present. One drop of resin was placed in the bottom of the capsule, the specimen then added and the capsule filled with resin before polymerisation in the oven at 60°C for 48 hours. Sections were cut at 80nm using a diamond knife on a Reichert Ultracut E microtome, floated onto copper grids, allowed to dry and stained with uranyl acetate and lead citrate. Sections were viewed using a Philips 301 transmission electron microscope.

5.3. BEHAVIOURAL STUDIES.

5.3.1. Cercarial emergence strategies and swimming behaviour.

Histological examination of snails.

Experimentally infected *V. piscinalis* used for histological examination were removed from their shells (to maximise absorbtion of fixative) and fixed in either Bouin's fluid (75ml picric acid, 25ml formalin, 5ml glacial acetic acid) or hot 10% formalin for 24 hours. After dehydration in an automatic tissue processor, specimens were placed in wax mounts and 5µm sections cut using a microtome. The sections were stained in Mayer's haematoxylin and eosin (H&E) or Cason's trichrome and mounted in Pertex. Serial sections of *V. piscinalis* specimens were examined to trace the migratory route of *I. erraticus* and *I. variegatus* cercariae through their molluscan host to the point of emergence.

Video examination of cercarial emergence and swimming behaviour.

The emergence and subsequent swimming behaviour of cercariae were recorded using a Hitachi monochrome video camera mounted on an Olympus BH2 trinocular microscope. The ASW-filled cell of a tissue culture multi-dish, containing a cercariae releasing snail, was positioned on the stage of the microscope under the camera. Illumination was provided from below by the microscope light source and images were grabbed at 40ms intervals by a Kontron 386 microcomputer with a 387 coprocessor chip

and graphics tablet.

Further observations on the swimming behaviour of cercariae newly released into tissue culture multi-dish cells were made under a dissecting microscope with a cold incident light source directed from the side.

5.3.2. Cercarial release patterns.

The patterns of cercarial release were only monitored from experimentally infected snails. All snails were fed and maintained as described in 5.1.2; Materials and Methods, with a photoperiod of light:dark 16:8. Illumination during the hours of light was provided by tungsten strip lights within the incubator. The intensity of illumination was not measured but was kept constant.

The number of cercariae produced and their emergence rhythm was studied in 12 *V. piscinalis* 5 known to be shedding *I. erraticus* and 7 *I. variegatus*. The experiment was repeated using 6 *L. peregra* releasing *A. gracilis* cercariae. The absence of light upon cercarial emission was investigated using a further 3 *V. piscinalis* specimens shedding *I. variegatus* cercariae which had been kept in conditions of total darkness from the time of infection.

Upon the onset of cercarial release the snail was placed into a vial (5ml plastic Bijoux for *V. piscinalis*; 20ml plastic Universal for *L. peregra*) 2/3 full of fresh, aerated ASW. Every 2 hours the snail was removed and placed into a similar vial. Any cercariae present in the previous vial were fixed by the addition of 10% formalin. This procedure was continued for a 24 hour period, enabling the total number of cercariae and their pattern of release to be recorded. The transfer of snails to different vials minimised their exposure to varying environmental conditions by enabling the cercarial counts to be performed at a later date. Individual snails were monitored periodically from the onset of cercarial release to its cessation (or the death of the snail). The following observation regime was employed, where possible, for each snail: onset (day 1), days 2, 5, 7, 10, 14 post-release and then every 7 days until cessation. Between these

days snails were returned to their normal culture tray cells and fed *ad libitum*. The data collected involved a total of 1,440 2-hourly counts.

The number of cercariae emerging during the light and dark, or the corresponding times for D:D treatment groups, was compared using χ^2 to that which would be expected if the timing of emergence were independent of L:D cycling. A probability value of $P<0.05$ was considered to represent a significant difference in all comparisons.

5.3.3. Longevity of cercariae.

The longevity of newly released, experimentally raised cercariae was investigated at different temperature regimes. *I. erraticus* and *I. variegatus* cercariae were studied at 15°C and 20°C (3 replicates of 100 cercariae each). *I. variegatus* cercariae were studied at 2 additional temperatures, 5°C and 10°C (single batch of 100 cercariae each); while the longevity of *A. gracilis* cercariae was recorded at 20°C (2 replicates of 100 cercariae). The techniques employed were the same as those described for miracidia in Chapter 4.3.1; Materials and Methods.

Key to morphometric measurements recorded from cercariae as indicated in Fig.76.

Abbreviations: (BL) body length; (TSL) tail-stem length; (FL) furca length (average); (BB) body breadth; (TSB) tail-stem breadth; (FB) furcae breadth (average); (AOL) anterior organ length; (VSL) ventral sucker length; (VSB) ventral sucker breadth; (VSA) distance between the anterior extremity of the body and the mid-point of the ventral sucker; (CVS) distance between division of oesophagus into caeca and the anterior margin of the ventral sucker; (CP) distance between the termination of the caeca and the posterior extremity of the body; (PL) pharynx length; (PB) pharynx breadth.

Fig. 76. Schematic illustration of a furcocercaria indicating the morphometric measurements recorded.

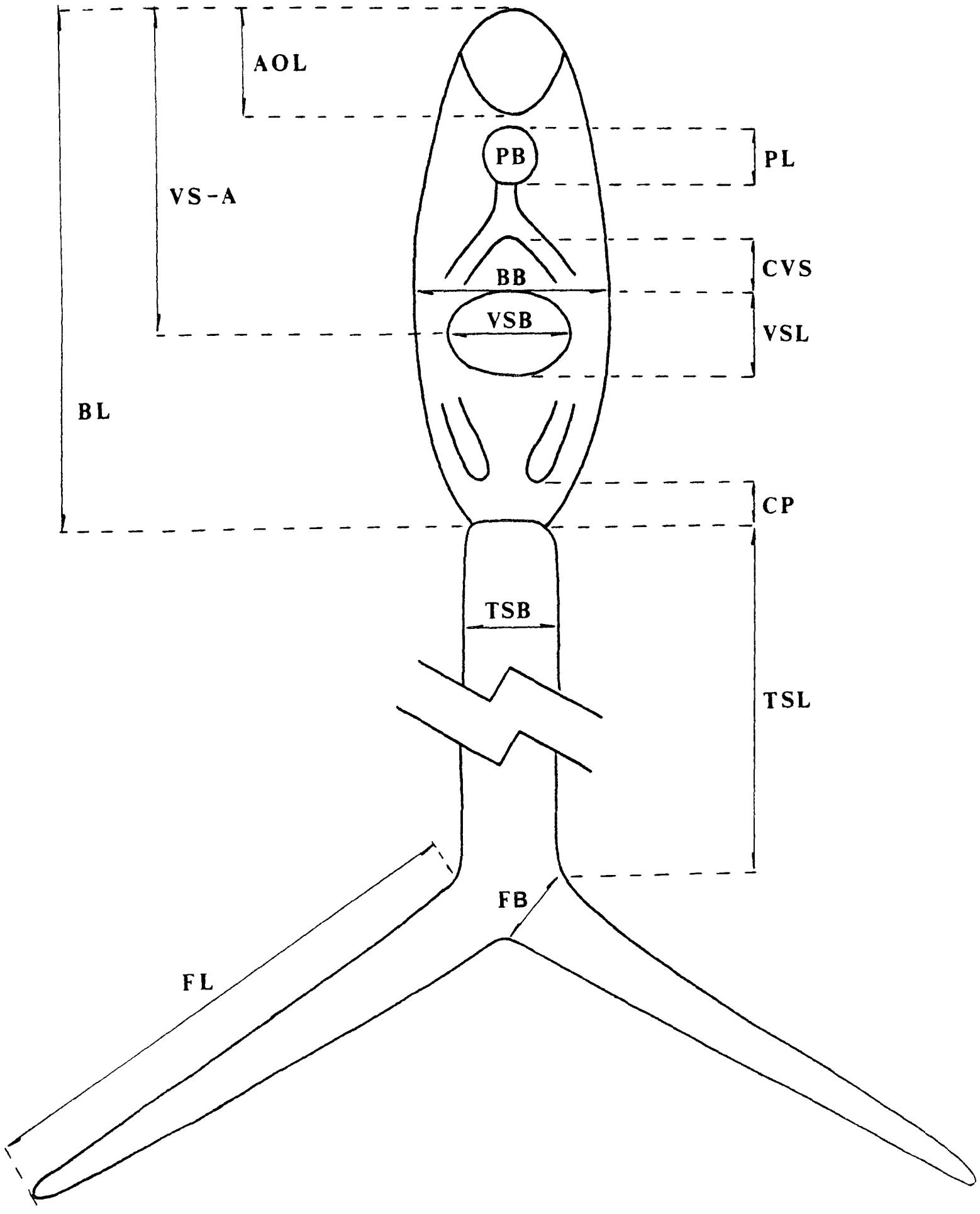
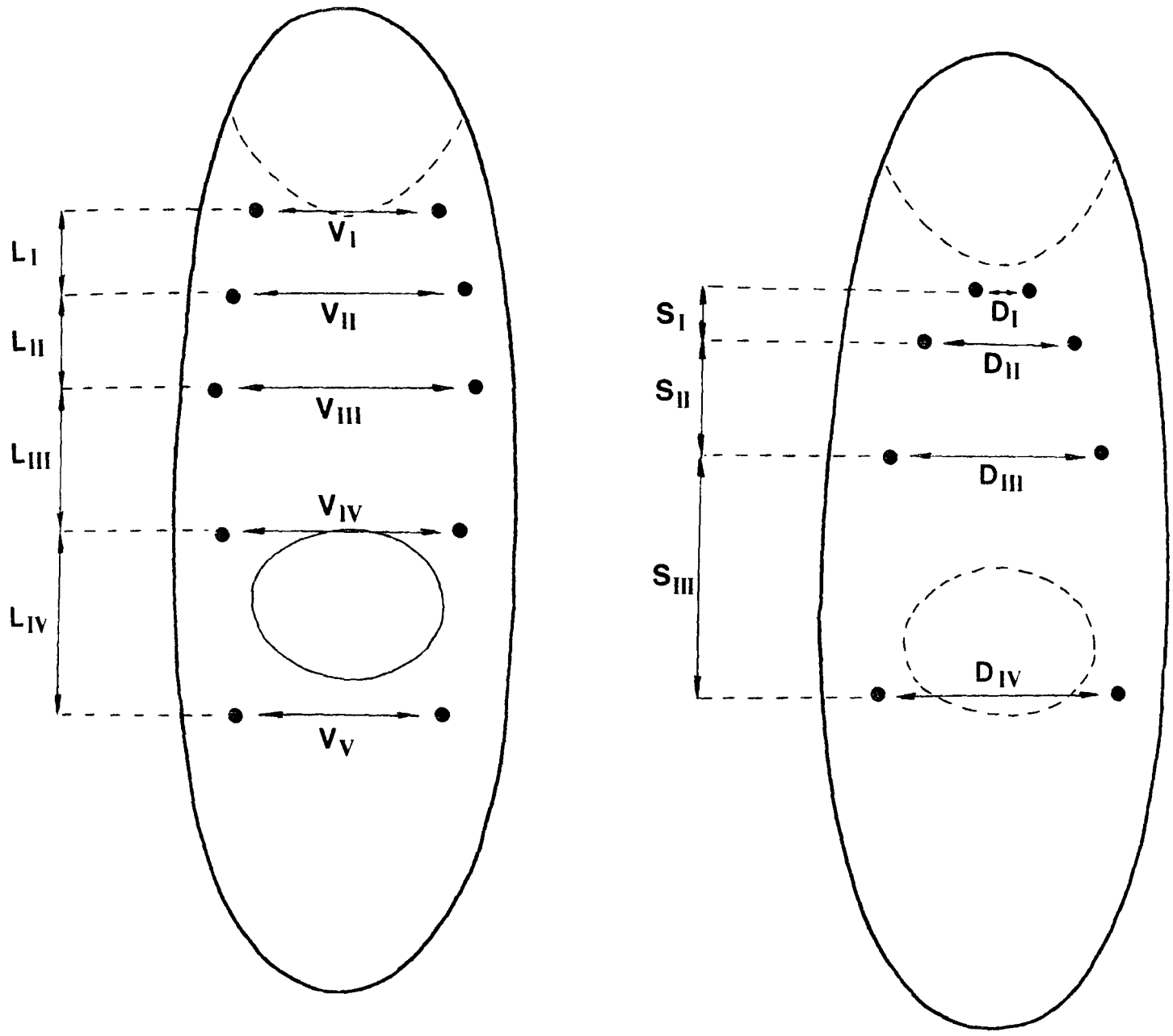


Fig. 77. Schematic illustration of the body of an *Ichthyocotylurus* cercaria indicating the measurements between sensilla used as variables for multivariate analyses.



VENTRAL SURFACE

DORSAL SURFACE

KEY:

L_I - L_{IV}: **Vertical distance between ventral surface sensilla**

V_I - V_V: **Horizontal distance between ventral surface sensilla**

S_I - S_{III}: **Vertical distance between dorsal surface sensilla**

D_I - D_{IV}: **Horizontal distance between dorsal surface sensilla**

RESULTS

5.1. MOLLUSCAN STAGES.

5.1.1. Natural infections.

The snails were found to have a low prevalence of infection with strigeid cercariae. Table 55 shows the number of *V. piscinalis* and *L. peregra* collected at each site and subsequently found to be releasing cercariae. Of 614 *L. peregra* and 795 *V. piscinalis* monitored, only 25 (4.1%) and 11 (1.4%) snails, respectively, were seen to release furcocercariae. Using the key of Blair (1977) and the works of Dubois (1968), Olson (1970), Odening & Bockhardt (1971), Blair (1974) and Swennen *et al.* (1979), 19 of these snails were infected with species identified as strigeids as follows: 5 (0.82%) *L. peregra* released a species of *Apatemon* (*Apatemon*) thought to represent *A. gracilis* and 1 (0.16%) an *Apatemon* (*Australapatemon*) sp., whereas, 10 (1.3%) *V. piscinalis* shed an *Apatemon (Australapatemon)* sp. and 1 (0.13%) a species of *Ichthyocotylurus,* believed to be *I. variegatus*.

5.1.2. Experimental infections: development from miracidium to cercaria.

Infections of *Valvata piscinalis*.

Initial experimental infections of *V. piscinalis* (snails maintained on a diet of fish faeces) with *I. erraticus* and *I. variegatus* miracidia all failed to produce cercariae, with the snails dying before cercarial release was achieved. Dissection of recent mortalities revealed that 51% of *I. erraticus* and 70% of *I. variegatus* challenges in *V. piscinalis* had been successful with either mother-sporocysts or occasionally, at 40 days plus post-infection (p.i.), daughter-sporocysts present. In several of these infections live daughter-sporocysts were found 70+ days after infection, far longer than the period recorded for cercarial emergence by other authors (see Table 54). The reason for the failure to obtain cercariae was believed to be due to the host's diet of fish faeces not reaching the required nutritional levels. These data are therefore not included in Table

54.

Subsequently, *V. piscinalis* were fed a diet of commercial tropical fish flakes and mortalities were greatly reduced. These later infections produced cercariae in 40% of *I. erraticus* and 36% of *I. variegatus* infections. The developmental periods from miracidial penetration to cercarial emergence recorded here and by other authors are shown in Table 54.

Table 54. Developmental period from miracidial penetration to cercarial release.

Species	Days post-infection before cercarial emergence (mean ± S.D).	Author	Temperature
I. erraticus	26-44 (35)	Olson (1970)	?
	21+	Swennen *et al.* (1979)	Room temp. ?
	35-52 (42 ± 6.8)	Present study	20°C
I. variegatus	Minimum of 54-61	Odening & Bockhardt (1971)	20°C
	c. 84	Swennen *et al.* (1979)	Room temp. ?
	45-62 (53 ± 7.0)	Present study	20°C
I. platycephalus	c. 84	Swennen *et al.* (1979)	Room temp. ?
	28-81	Mattheis & Odening (1980)	20-28°C

Table 55. Infections in *Lymnaea peregra* and *Valvata piscinalis* collected during 1992 and 1993.

SNAIL SPECIES	SITE	MONTH	NUMBER COLLECTED	NUMBER RELEASING FURCOCERCARIAE	NUMBER RELEASING OTHER CERCARIAE
Lymnaea peregra	River Earn at the site of a fish farm	May '92	45	5 (11.1%): *Diplostomum* spp. 1 (2.2%): *Apatemon (Apatemon)* sp.	6 (13.3%): *Notocotylus* sp. 1 (2.2%): Xiphidocercaria sp.
		August '92	250	1 (0.4%): *Diplostomum* sp.	0
	River Almond at the site of a fish farm	April '93	53	0	7 (13.2%): *Notocotylus* sp. 1 (1.9%): Xiphidocercaria sp.
		May '93	21	0	6 (29%): *Notocotylus* sp.
		August '93	13	3 (23%): *Diplostomum* spp.	2 (15%): *Notocotylus* sp.
	Loch Lomond	June '92	4	2 (50%): Unknown identity	1 (25%): *Notocotylus* sp.
		April '93	50	4 (8%) *Diplostomum* spp. 3 (6%) *Apatemon (Apatemon)* sp. 1 (2%) *Apatemon (Australapatemon)* sp.	3 (6%): Xiphidocercaria sp. 1 (2%): Gymnocephalus sp.
		June '93	2	2 (100%): *Diplostomum* spp.	0
	Castle Semple Loch	June '92	70	1 (1.4%): *Diplostomum* sp.	4 (5.7%): Gymnocephalus sp. 1 (1.4%): *Notocotylus* sp.
		August '92	1	0	0
		September '92	43	1 (2.3%) *Diplostomum* sp. 1 (2.3%) *Apatemon (Apatemon)* sp.	0
		February '93	55	0	1 (1.8%): Gymnocephalus sp.
		April '93	7	0	1 (14%): Xiphidocercaria sp.
Valvata piscinalis	Loch Lomond	April '93	613	1 (0.15%): *Ichthyocotylurus* sp.	1 (0.15%): Xiphidocercaria sp.
		June '93	96	0	0
	Castle Semple Loch	June '92	70	8 (11.4%) *Apatemon (Australapatemon)* sp.	1 (1.4%): Xiphidocercaria sp.
		August '92	9	2 (22.2%) *Apatemon (Australapatemon)* sp.	4 (44.4%): Unknown identity
		April '93	7	0	0

From examining snail mortalities it was noted that the mother-sporocysts of the later infections attained a greater size than those observed in the earlier unsuccessful infections, being similar for both species and measuring an average of 2.7mm x 160µm at 6 weeks p.i. Mother-sporocysts bearing germ-balls were observed as early as 13 days p.i. for *I. erraticus* and after 18 days for *I. variegatus*. Young mother-sporocysts were located in the region between the head and the foot, with older sporocysts bearing discernable daughters nearer to the digestive gland. Tangles of thread-like daughter-sporocysts were observed at the base of the digestive gland from around 5 weeks p.i. and, thereafter, more apically within this organ. The extent of the infiltration of daughter-sporocysts within the digestive gland can be seen in Fig. 98.

Infections of *Lymnaea peregra*.

Experimental infections of *L. peregra* with *A. gracilis* miracidia were markedly more successful than *Ichthyocotylurus* infections in *V. piscinalis*, with 63% of exposed snails releasing cercariae. The developmental period ranged between 28-39 days and averaged 4.5 weeks at 20°C.

5.2. TAXONOMIC STUDIES OF CERCARIA.

5.2.1. Light microscopical observations of cercaria.

Morphological measurements and taxonomic characteristics recorded for *Ichthyocotylurus erraticus*, *I. variegatus* and *Apatemon gracilis* cercariae are provided in Tables 56, 57 and 58, respectively. These tables also include the most recent and reliable taxonomic information compiled by other authors for these cercariae.

Table 56. Morphometric details recorded for the cercaria of *Ichthyocotylurus erraticus*.

Author	Olson (1970)	Swennen *et al.* (1979)	Present study
Source of cercaria	experimental infection		
Method of fixation	hot AFA	flame-fixed	hot 5% formalin
Body length	230-280	191-232	182-256 (216)
Body breadth	50-60	50-82	42-66 (50)
Tail-stem length	270-290	270-337	211-309 (252)
Tail-stem breadth	30-35	40-50	37-54 (44)
Furcae length	220-275	263-294	231-297 (261)
Furcae breadth	20-23	18-30	17-25 (21)
Anterior organ	43-52 x 25-30	40-55 x 25-32	40-59 (48)
Pharynx	11 (diameter)	-	11-14 (12) x 10-15 (12)
Ventral sucker length	23-30 (diameter)	25-32 (diameter)	22-30 (26)
Ventral sucker breadth			24-34 (27)
Caudal bodies	-	5 or 6 pairs	6 pairs
Penetration glands	3 pairs: 1 pre-, 2 post-acetabular	2 pairs: 1 level with, 1 post-acetabular	2 pairs: both post-acetabular
Flame-cell formula	2[(2 + 2) + (2 + 2 + (2))] = 20		
Excretory commissure	-	pre-acetabular	
"Treibwimperflammen"*	-	2 sets of 3	
Caeca	-	long, with 6 septa	
Eye-spots	-	none	

All measurements are in µm, with mean measurements in parentheses. Details of spination and sensilla are provided in 5.2.3; Results.

*"Treibwimperflammen": are thought to be flame-cell-like structures with rapidly beating cilia.

Table 57. Morphometric details recorded for *Ichthyocotylurus variegatus* and *I. platycephalus* cercariae.

Species	*I. variegatus*			*I. platycephalus*
Author	Odening & Bockhardt (1971)	Swennen *et al.* (1979)	Present study	Odening *et al.* (1970)
Source of cercaria	experimental infection			
Method of fixation	not stated	flame-fixed	hot 5% formalin	not stated
Body length	184-277	states dimensions and other characteristics agree with those given by Odening & Bockhardt (1971)	173-297 (226)	114-316
Body breadth	73-88		37-68 (52)	70-110
Tail-stem length	220-294		231-305 (269)	173-352
Tail-stem breadth	44-66		39-56 (45)	41-59
Furcae length	257-330		235-297 (267)	173-294
Furcae breadth	29-44		18-27 (23)	17-37
Anterior organ	37-47 x 20-37		42-68 (52)	30-37 x 19-32
Pharynx	9-14 x 10-14		10-15 (12) x 11-15 (13)	9-17 x 12-16
Ventral sucker length	24-31		22-32 (27)	23-32
Ventral sucker breadth	27-31		25-34 (29)	25-32
Caudal bodies	6 pairs		6 pairs	6 pairs
Penetration glands	2 pairs: 1 post-, 1 pre- or post-acetabular		2 pairs: both post-acetabular	2 pairs: both post-acetabular
Excretory commissure	pre-acetabular		pre-acetabular	pre-acetabular
"Treibwimper -flammen"*	2 sets of 3		2 sets of 3	2 sets of 3
Flame-cell formula	2[(2 + 2) + (2 + 2 + (2))] = 20			
Caeca	long, with 10-12 septa	long, with 7-8 septa	long, with 6-10 septa, typically 6 or 7	long, with 8-11 septa
Eye-spots	1 pair anterolateral to ventral sucker	as Odening & Bockhardt (1971)	1 pair anterolateral to ventral sucker - not always observed	1 pair anterolateral to ventral sucker

All measurements are in µm, with mean measurements in parentheses. Details of spination and sensilla are provided in 5.2.3; Results.

*"Treibwimperflammen": are thought to be flame-cell-like structures with rapidly beating cilia.

Table 58. Morphometric details recorded for the cercaria of *Apatemon gracilis*.

Author	Vojtek (1964a). (As *A. cobitidis. cobitidis)*	Blair (1974).	Sten'ko (1977, in Sudarikov, 1984)	Present study A	Present study B
Source	natural infection			experimental infection	
Method of fixation	unknown	hot 10% formalin	10% formalin	hot 5% formalin	hot 10% formalin
Body length	143-185 (163)	110-135 (123)	83-109 (91)	153-193 (170)	124-161 (134)
Body breadth	45-64 (53)	32-40 (35)	35-40 (40)	44-50 (47)	44-54 (47)
Tail-stem length	120-162 (135)	124-142 (137)	78-114 (91)	124-147 (133)	118-128 (123)
Tail-stem breadth	34-50 (42)	32-44 (38)	31-49 (40)	38-50 (44)	35-46 (37)
Furcae length	126-185 (165)	154-163 (159)	104-143 (129)	155-176 (169)	133-155 (145)
Furcae breadth	-	-	-	17-21 (19)	15-23 (18)
Anterior organ	42-50 (45) x 29-42 (32)	30-34 (31) x 21-25 (23)	22-31 (28) x 17-26 (21)	37-45 (41) -	34-40 (38) -
Pharynx	11x11	small	-	11x9	11x9
Ventral sucker length	21-22 (22)	15-17 (16)	16-19 diameter	19-22 (20)	16-20 (18)
Ventral sucker breadth	17-25 (22)	15-17 (16)		20-25 (22)	19-21 (20)
Flame-cell formula	2[(1 + 1) + (1 + 1 + (1))] = 10				
Caudal bodies	6 large pairs				
Penetration glands	4 post-acetabular linear pairs	6 post-acetabular pairs	4 post-acetabular pairs	4(?) post-acetabular pairs	
Excretory commissure	post-acetabular				
Caeca length	very short, well in front of ventral sucker				
Eye-spots	1 unpigmented pair anterolateral to ventral sucker				

All measurements are in μm, with mean measurements in parentheses. Details of spination and sensilla are provided in Chapter 5.2.3; Results.

5.2.2. Discrimination of *Ichthyocotylurus erraticus* and *I. vareigatus* cercariae by principal components analysis of metrical features.

Similar PCA results were obtained regardless of the method employed to standardise the data. The results given below were from data converted into a proportion of the total cercarial length.

The mean and coefficient of variation of each variable, globally and for each species, are given in Table 59. The low variation about the global mean for most variables indicates the similarities between the two species of cercariae.

Table 59. Mean and coefficient of variation for each standardised variable, globally and in each species (Coefficient of variation = 100 x standard deviation/mean). For an explanation of abbreviations see Fig. 76.

Variable	Both spp. combined (n=69)		*I. erraticus* (n=30)		*I. variegatus* (n=39)	
	Mean	Coefficient of variation (%)	Mean	Coefficient of variation (%)	Mean	Coefficient of variation (%)
(BL)	29.62	7.3	29.69	7.6	29.56	7.2
(TSL)	34.95	5.5	34.49	7.1	35.31	3.7
(FL)	35.43	4.4	35.82	4.4	35.12	4.3
(BB)	6.91	15.7	6.86	12.2	6.94	18.0
(TSB)	6.01	9.9	6.05	8.3	5.99	11.0
(FB)	2.93	10.1	2.89	7.0	2.99	11.6
(AOL)	6.70	11.2	6.61	8.2	6.77	12.9
(VSL)	3.53	12.1	3.51	9.0	3.54	14.1
(VSB)	3.81	10.0	3.74	6.9	3.87	11.7
(VSA)	19.03	11.6	19.63	11.5	18.57	11.3
(CVS)	4.53	24.4	5.28	17.1	3.95	22.3
(CP)	3.51	17.3	3.45	15.0	3.55	18.8
(PL)	1.62	11.4	1.66	10.6	1.60	11.9
(PB)	1.66	11.3	1.65	9.3	1.66	12.8

Correlations between the 14 variables, shown in Table 60, illustrate how the sizes of some structures may co-vary in the same direction (e.g. ventral sucker length [VSL] and ventral sucker breadth [VSB] or body length [BL] and distance from ventral sucker to the anterior body extremity [VSA]), vary inversely (e.g. body length [BL] and body breadth [BB]) or are completely independent (e.g. anterior organ length [AOL] and ventral sucker length [VSL]).

The first 2 principal components account for 53.95% (Table 61) of the total variance. The multi-dimensional aspect of the correlations between variables is indicated

by their relative positions on the first 2 components as shown in Fig. 78. The first principal component (PCA1) places predominantly width-related variables (body breadth [BB], tail-stem breadth [TSB], ventral sucker length [VSL] and ventral sucker breadth [VSB]) on the right hand side, as opposed to those describing elongation (body length [BL], distance from the ventral sucker to the anterior body extremity [VSA] and distance from the caeca to the posterior extremity of the body [CP]) on the left hand side. The second principal component (PCA2) opposes tail-stem length [TSL] at the bottom, to both length and breadth related variables including tail-stem breadth [TSB], distance from the ventral sucker to the anterior extremity [VSA] and pharynx length [PL]. Fig. 79 illustrates that the morphological variation between the 69 specimens shown by the first two components did not reflect species differences. In this figure the ellipses surround 50% of the points (specimens), a further plot with ellipses describing 95% confidence limits also failed to separate the two species. Plots showing the spatial separation of specimens in other planes (components 1 and 3, 2 and 3) similarly failed to separate *I. erraticus* and *I. variegatus* cercariae.

Table 60. Correlation matrix (high negative and positive coefficients in bold).

	BL	TSL	FL	BB	TSB	FB	AOL	VSL	VSB	VSA	CVS	CP	PL	PB
BL	1.000													
TSL	**-0.710**	1.000												
FL	-0.506	-0.248	1.000											
BB	**-0.727**	0.534	0.346	1.000										
TSB	-0.241	0.112	0.195	**0.629**	1.000									
FB	-0.250	0.199	0.099	0.396	0.514	1.000								
AOL	0.100	-0.161	0.059	0.031	0.186	0.181	1.000							
VSL	-0.503	0.287	0.341	**0.735**	0.494	0.441	-0.001	1.000						
VSB	-0.464	0.344	0.216	**0.724**	0.531	0.482	0.029	**0.752**	1.000					
VSA	**0.825**	**-0.668**	-0.317	**-0.616**	-0.071	-0.168	0.118	-0.364	-0.037	1.000				
CVS	0.187	-0.100	-0.135	-0.066	0.191	0.030	-0.179	0.073	0.112	0.102	1.000			
CP	0.530	-0.505	-0.111	-0.472	-0.196	-0.350	-0.071	-0.319	-0.452	0.604	-0.047	1.000		
PL	-0.222	0.027	0.339	0.406	0.443	0.261	0.133	0.549	0.414	-0.037	0.035	-0.045	1.000	
PB	-0.196	0.149	0.087	0.473	0.516	0.388	0.230	0.431	0.514	-0.135	-0.008	-0.176	0.314	1.000

Table 61. Principal components analysis of the correlations between the 14 variables.

Eigenvalues and proportion of the variance explained by the first four principal components.				
Eigenvalue	5.34	2.21	1.40	1.21
Proportion (%)	38.17	15.78	10.03	8.70
Cumulative (%)	38.17	53.95	63.98	72.68

Coefficient of each variable on the first two principal components.		
Variable	PCA1	PCA2
BL	-0.792	0.473
TSL	0.569	-0.617
FL	0.393	0.104
BB	0.916	-0.020
TSB	0.623	0.528
FB	0.566	0.306
AOL	0.064	0.397
VSL	0.803	0.230
VSB	0.819	0.187
VSA	-0.679	0.611
CVS	-0.017	0.227
CP	-0.613	0.352
PL	0.484	0.503
PB	0.549	0.417

Fig. 78. Map of the 14 variables in the first plane of the principal components analysis on 69 specimens.

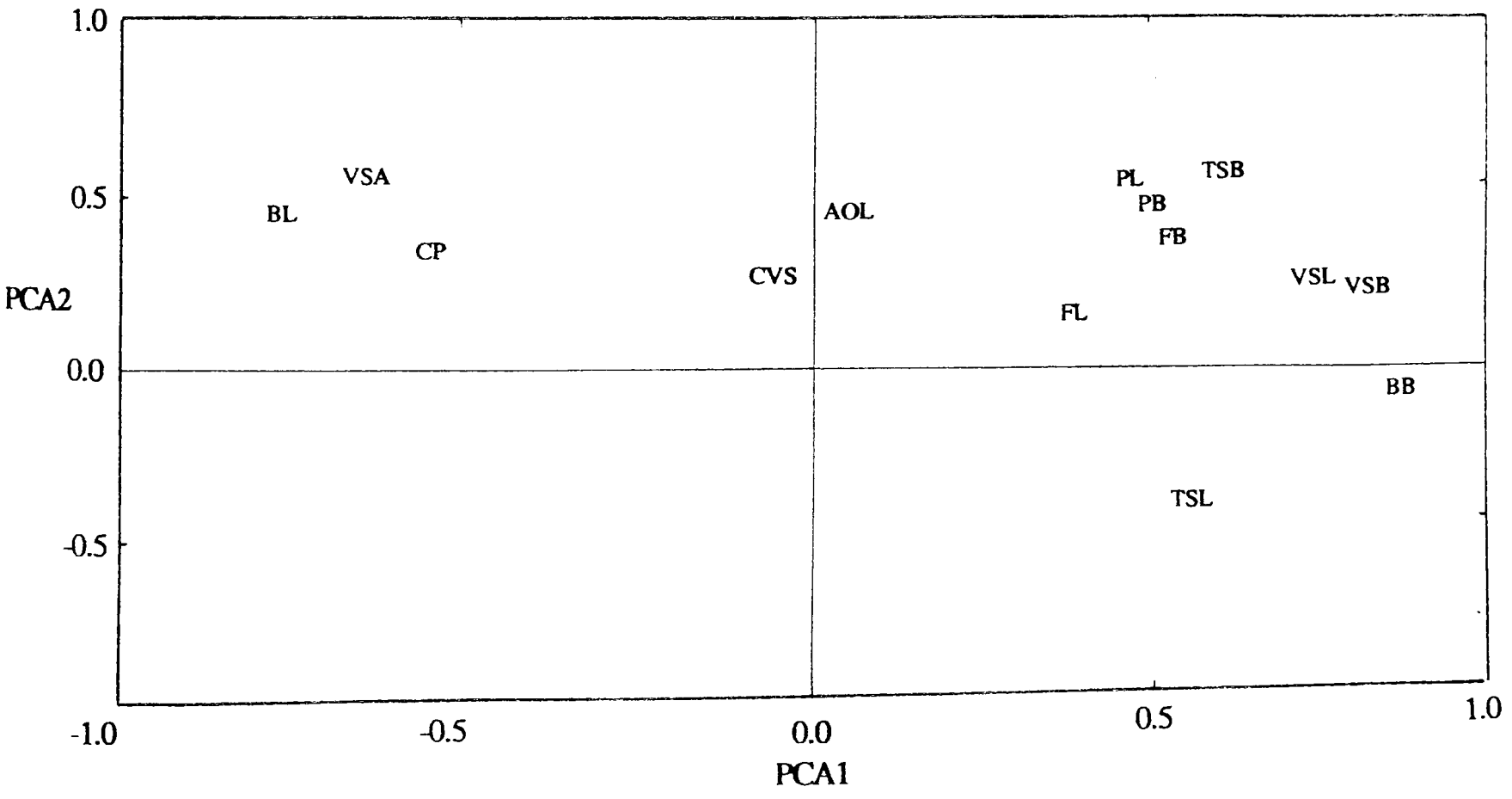
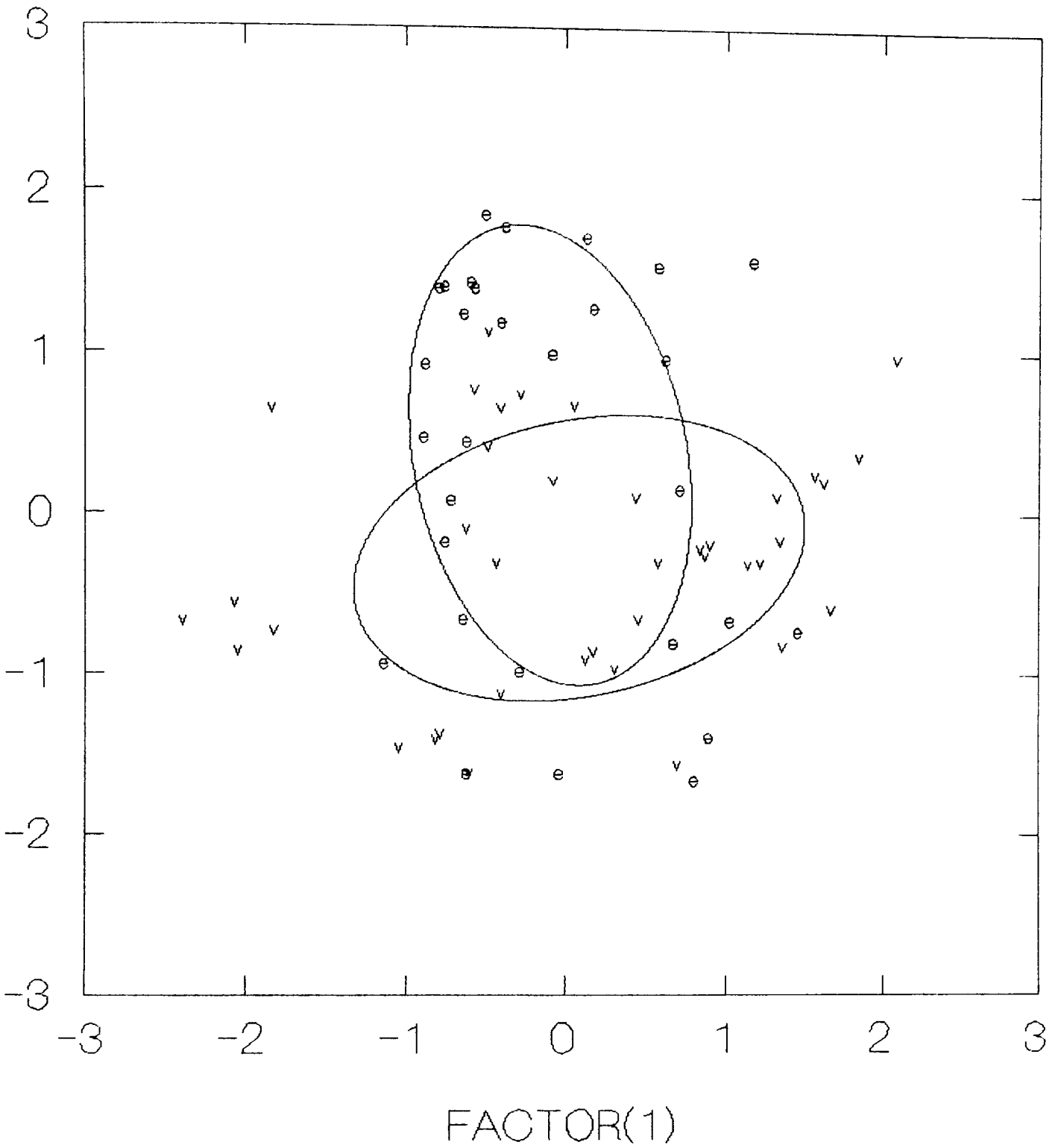


Fig. 79. Map of the 69 specimens in the first plane of the principal components analysis. *I. erraticus* specimens are represented by (e) and *I. variegatus* by (v). Ellipses surround 50% of points.



5.2.3. Chaetotaxy and scanning electron microscopical observations of cercariae.

Chaetotaxy of *I. erraticus* and *I. variegatus* cercariae based on silver-stained specimens.

Both species of *Ichthyocotylurus* studied appear to possess exactly the same number and distribution of sensilla (Fig. 80). Their chaetotaxy pattern was:

Cephalic region (Fig. 80A, B, C, D)

C_I: 1 C_IV in mouth + 1 + 8 C_IV + 5 C_IA* + 0 C_IL + 3$_I$D

C_{II}: 1 + 1 C_{II}V + 5 + 4 C_{II}L + 1 + 1 C_{II}D

C_{III}: 1 C_{III}V + 3 + 1 C_{III}L + 1 C_{III}D

(A* = Apical)

Body (Fig. 80A, B, C)

A_I: 1 A_IV + 2A_IL + 1 + 2 A_ID

A_{II}: 1 A_{II}V + 1 A_{II}L + 1 A_{II}D

A_{III}: 1 A_{III}V + 0 A_{III}L + 1 A_{III}D

M_I: 0 M_IV + 2 M_IL + O M_ID

P_I: 1 P_IV + 0 P_IL + 1 P_ID

P_{II}: 0 P_{II}V + 0 P_{II}L + 0 P_{II}D

P_{III}: 0 P_{III}V + 0 P_{III}L + 1 P_{III}D

Ventral sucker (Fig. 80A, B)

Ten sensilla were situated in 2 circles: $4S_I + 6S_{II}$

Tail-stem (Fig. 80E)

A total of 52-56 sensilla were located on the tail-stem. The two lateral groups of sensilla exhibit a constant number and arrangement, but some variation was recorded for dorsal and ventral groups (12-14 each surface):

Anterior lateral groups: 2 + 2.

Posterior lateral groups: 2 + 2 + 2 + 2 + 2.

Ventral and dorsal groups: 4 + 3(2) + 3(2) + 1(2) + 0(1) + 1.

Furcae (Fig. 80F)

The sensilla were situated on the dorsal and medial surfaces of each furca. Variation in number (21-26) and particularly position was often noted. The most commonly recorded distribution of these sensilla was:

Proximal group: 2 medial + 1 dorsal.

Poral group: 3 medial + 1 dorsal.

Predistal group: 3 medial + 1 dorsal.

Distal group: 3 + 2 + 2 + 2 + 2 + 2 (all medial).

Chaetotaxy of *A. gracilis* cercaria based on silver-stained specimens.

The chaetotaxy pattern recorded was:

Cephalic region (Fig. 81A, B, C, D)

C_I: $1C_IV$ in mouth + 1 + 4 C_IV + $2C_IA^*$ + $2C_IL$ + $5C_ID$

C_{II}: $1C_{II}V$ + 2 + 6 $C_{II}L$ + 1 + 1 $C_{II}D$

C_{III}: 1 + 3 $C_{III}V$ + 1 + 1 + 1 $C_{III}L$ + 1 + 1 $C_{III}D$

(A^* = Apical)

Body (Fig. 81A, B, C)

A_I: 1 A_IV + 0 A_IL + 1 A_ID

A_{II}: 1 $A_{II}V$ + 0 $A_{II}L$ + 0 $A_{II}D$

A_{III}: 0 $A_{III}V$ + 0 $A_{III}L$ + 0 $A_{III}D$

M_I: 1 M_IV + 0 M_IL + 0 M_ID

P_I: 1 P_IV + 0 P_IL + 0 P_ID

P_{II}: 0 $P_{II}V$ + 0 $P_{II}L$ + 0 $P_{II}D$

P_{III}: 0 $P_{III}V$ + 0 $P_{III}L$ + 1 $P_{III}D$

Ventral sucker (Fig. 81A, B)

Three sensilla were situated in a triangle, 2 lateral and 1 posterior: $3S_I + 0S_{II}$

Tail-stem (Fig. 81E)

A total of 34 sensilla were located on the tail-stem. The distribution of these sensilla was:

Posterior lateral groups: 2 + 2 + 2 + 2 + 2.

Ventral and dorsal groups: 1 + 1 + 1 + 3 + 1.

Furcae (Fig. 81F)

The sensilla were situated on the ventral, dorsal and medial surfaces, as well as the tip of each furca. The distribution of these sensilla was:

Proximal group: 1 ventral + 1 dorsal + 2 medial.

Poral group: none.

Predistal group: 2 + 2 (all medial).

Distal group: 2 medial + 2 (from a single terminal collar).

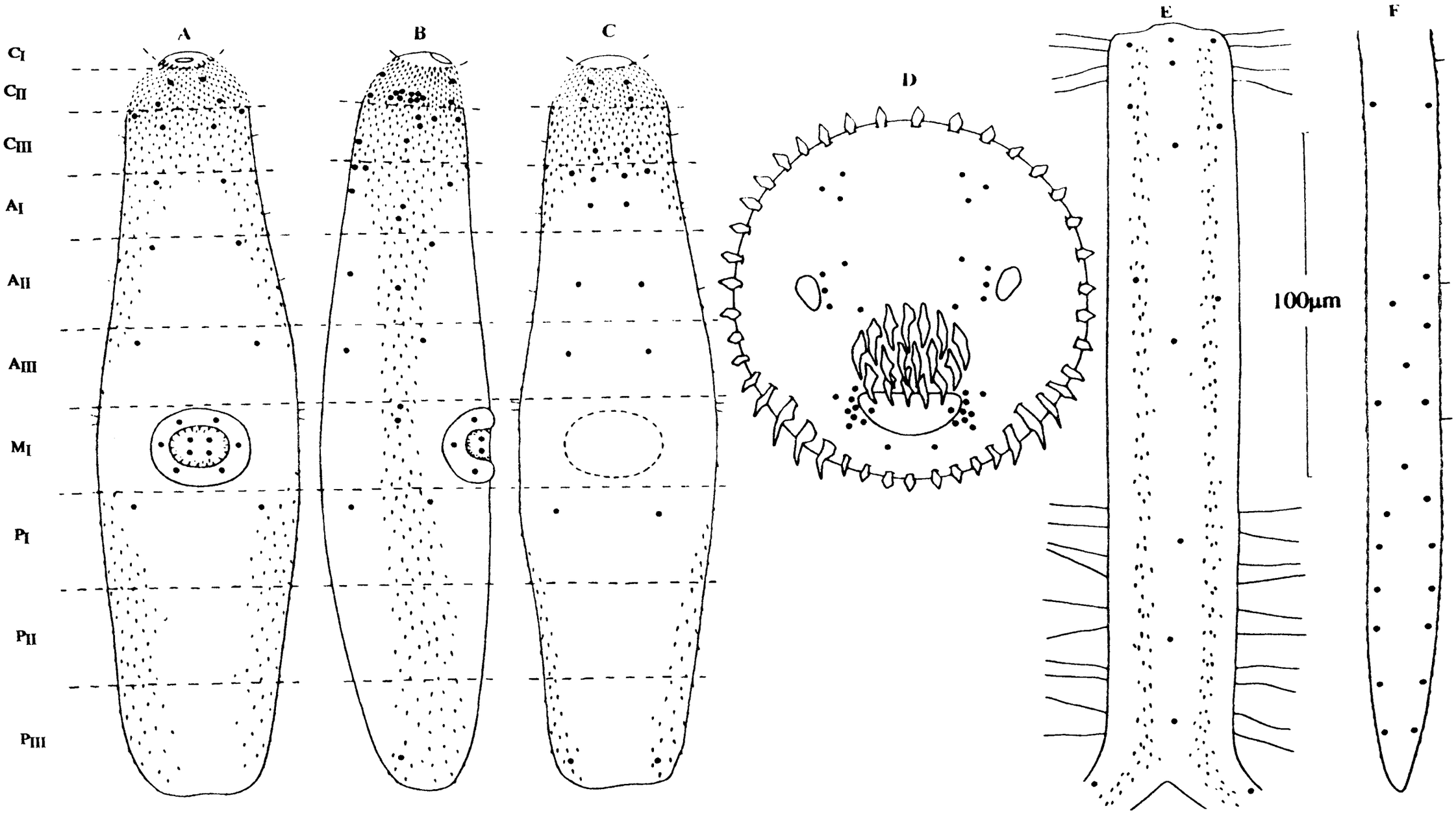


Fig. 80. *Ichthyocotylurus erraticus* and *I. variegatus* cercarial chaetotaxy. A). Body, ventral view; B). Body, lateral view; C). Body, dorsal view; D). *En face* view of anterior extremity bearing cephalic cycle I (not to scale); E). Tail-stem (dorsal and ventral surfaces are identical); F). Medial surface of furca (dorsal surface to the right). Scale bar: 100µm.

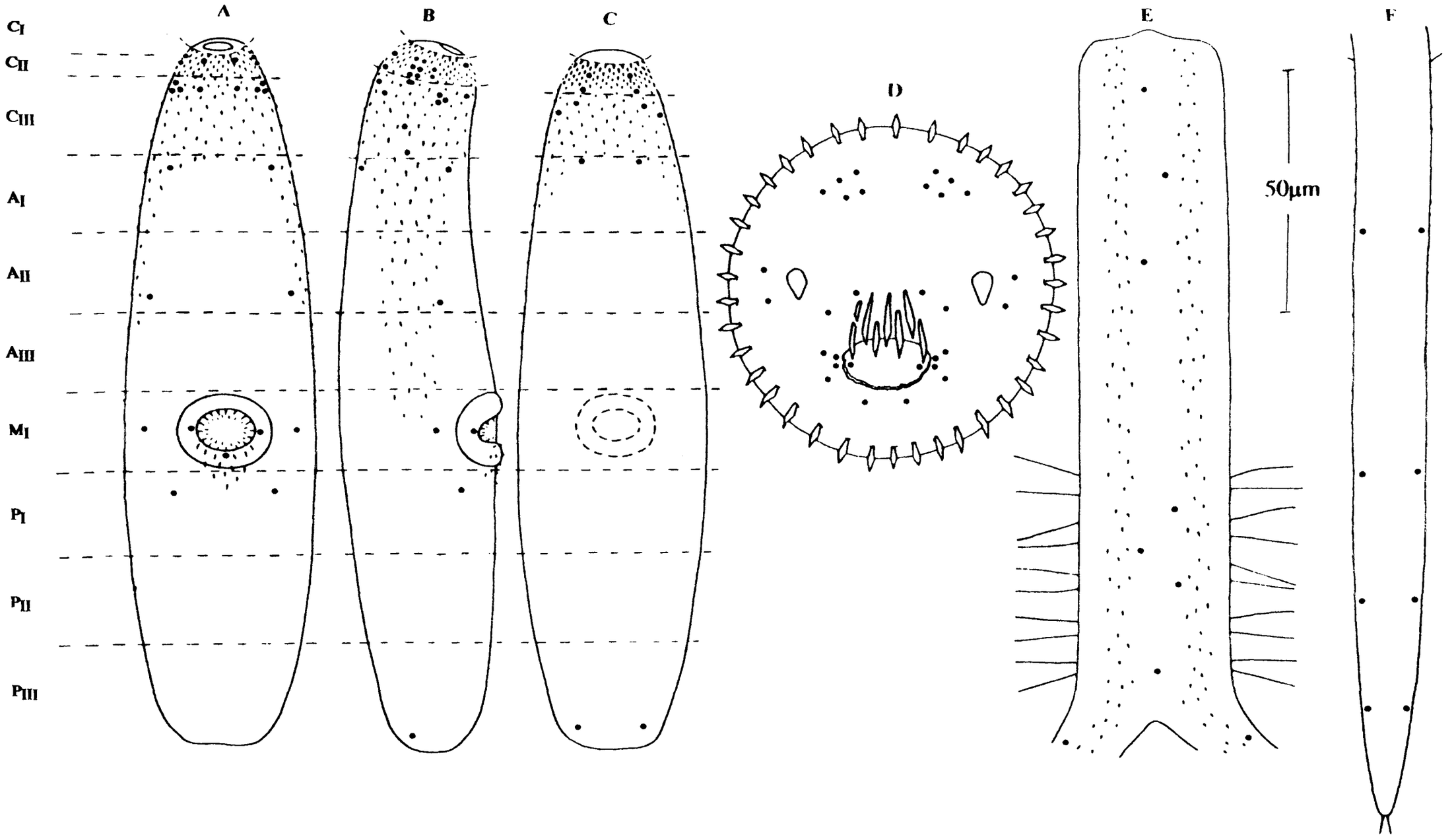


Fig. 81. *Apatemon gracilis* cercarial chaetotaxy. A). Body, ventral view; B). Body, lateral view; C). Body, dorsal view; D). *En face* view of anterior extremity bearing cephalic cycle I (not to scale); E). Tail-stem (dorsal and ventral surfaces are identical); F). Medial surface of furca (dorsal surface to the right). Scale bar: 100µm.

SEM: armature of *I. erraticus* cercariae

The unarmed crown bears the mouth ventrally and the pores of the penetration glands laterally (Fig. 82A, B). There is a central tuft of 17-22 large pre-oral spines arranged in 3-4 rows (Fig. 82A, B), and surrounding the unarmed anterior extremity is the post-oral collar of spines, comprising 10-15 alternating rows (Figs 82A, B, 83B). These spines are enlarged in the anterior rows, lateral to the mouth (Figs 82A, 83C). Beyond the posterior row of the collar there is an area of peg-like spines which extend as far as the anterior organ's distal margin both dorsally and ventrally; this area continues laterally along the entire body length and encroaches onto the dorsal and ventral surfaces posterior to the ventral sucker (Figs 82A, 83A, B, C). The ventral sucker bears two complete and one partial row of inwardly pointing spines, totalling approximately 80 (72-96), of the type found in the pre-oral tuft and post-oral collar (Fig. 83D). These spines comprise a broad base, are laterally flattened and hooked. Four bands of peg-like spines run along the tail-stem, one either side of the mid-line, both dorsally and ventrally, and continue along the margins of the furcae; each band consists of 2-4 rows (Figs 84A, B, 85A). All spines present on the body, tail-stem and furcae are directed posteriorly.

SEM: armature of *I. variegatus* cercariae

The distribution and type of spination was almost identical to that recorded for *I. erraticus* cercariae. The pre-oral tuft of *I. variegatus* was found to contain 12-19 spines in 3-4 rows and the post-oral collar possesses 8-14 rows of spines.

SEM: armature of *A. gracilis* cercariae

The general spination closely resembles that of the two *Ichthyocotylurus* spp., as does the form of the corresponding spines. On the unarmed body tip, the mouth is positioned ventrally and the two pores of the penetration glands open laterally (Figs 86A, B, 87A, C). The pre-oral tuft is not as well armed as the *Ichthyocotylurus* spp.,

consisting of 7-12 spines in 2-3 rows (Figs 86A, B, 87A) and the post-oral collar comprises 6-8 alternating rows of spines (Figs 87A, B, 88A, B). These spines were of a more uniform size than observed for *Ichthyocotylurus* spp. and were not enlarged lateral to the mouth. Beyond the posterior row of the collar, peg-like spines extend as far as the posterior margin of the anterior organ both dorsally and ventrally; this area is continued laterally to the level of the ventral sucker (Figs 86A, 87B, 88A, B). The ventral sucker bears 2-3 rings of inwardly pointing spines of the type found in the pre-oral tuft and post-oral collar. The exact number of rows and spines present on this organ is difficult to discern, as SEM preparation results in its retraction (Fig. 87D). Some spines are present on and beyond the posterior lip of the ventral sucker and form a small post-acetabular patch (Figs 86A, 87D, 88A). Tail-stem and furcal spination were as described for both *Ichthyocotylurus* spp. (Fig. 89A, B, C).

SEM sensilla observations

Scanning electron microscopy revealed no more sensilla than perfectly stained light microscope preparations, indeed there were several sensilla that were consistently not observed. Nevertheless, this technique did enable a variety of different sensilla types to be identified. The following forms were distinguished for the *Ichthyocotylurus* spp.: (a) those with a long cilium and loose, low collar (Figs 83C, 84A, B); (b) those with a cilium of intermediate length and a loose, low collar (Figs 83B, 84C, 85A); (c) those with a short cilium and tightly investing collar, the length of which may equal that of the exposed cilium (Figs 82A, B, 83A, B, D, 85B); (d) those with no visible cilium which may be unciliated or multiciliated pits (Fig. 82B). *Apatemon gracilis* cercariae also exhibit types (a) (Fig. 89A, B); (b) (Figs 87A, B, C, 89C, D); and (d) (Fig. 87B, C). However, the sensilla of this species which bear a short cilium invariably possess low collars; these may be tight or loose but were never seen to invest a significant proportion of the cilium. These sensilla are referred to as type (e) (Figs 86A, B, 87C, 88A, B).

Sensilla with a long cilium are confined to the tail-stem; those with an intermediate cilium are situated on the furcae and the dorsal body surface of *Ichthyocotylurus* spp. and the furcae and cephalic cycles of *A. gracilis*; those with a short cilium make up most of the cephalic and body cycles, and all those on the ventral sucker (ventral sucker sensilla were not observed for *A. gracilis*); sensilla with no visible cilium occur amongst those dorsally on the unarmed body tip of *Ichthyocotyluus* spp. and *A. gracilis*, plus laterally in the cephalic circles of *A. gracilis*.

Cilia were occasionally 'lost' during specimen preparation, being sheared at their base and leaving only the collar. These artifacts tended to be restricted to sensilla comprising the lateral groups of cephalic cycles I and II, and to those of the tail-stem (Figs 84A, 85B, 89A).

The distribution of different sensilla in the chaetotaxy patterns are described below. As the sensilla pattern of the body shows bilateral symmetry, only a single side is described.

I. erraticus and *I. variegatus*

Unless otherwise stated all sensilla of the cephalic cycles are of the short cilium and tight collar type. Cephalic cycle I (C_I) consists of 18 sensilla (on each body side). The mouth bears 1 sensillum within its inner margin. Its outer margin is bordered by 1 sensillum ventrally and 8 in line as far as the pre-oral spines (Fig. 82A). Of this group of 8 sensilla, 5 of the most medial consistently possess extremely short cilia with less tightly applied and distinct collars (Fig. 82A). The apical sensilla (5) are arranged in an arc between the antero-lateral margin of the pre-oral spines and the medial margin of the pore of the penetration gland (Figs 82A, B, 85B). One of the apical sensilla was never seen to possess a cilium, although a collar was always present (Fig. 82B). The 3 dorsal sensilla are arranged in a triangle, with the most dorsal member being either aciliate or a multiciliate pit with no visible cilium or collar present (Fig. 82B).

The C_{II} cycle comprises 13 sensilla lying within the spines of the post-oral collar

(Figs 82A, 83B, 85B).

The 6 sensilla of cycle C_{III} are located approximately between the post-oral collar and the posterior margin of the sparsely distributed spines (ventrally and dorsally) (Figs 82A, 83B). A dorsal pair possess intermediate length cilia with a loose, low collar (Fig. 83B).

The unarmed region anterior to the level of the ventral sucker is divided equally into A_I, A_{II}, A_{III} and M cycles. Some spines may encroach upon the A_I cycle. Cycles A_I and A_{II} each bear a pair of sensilla with intermediate length cilia. All other sensilla within these cycles possess a short cilium and tight collar (Fig. 83A, B, C).

All sensilla of cycles P_I and P_{III} are of the short cilium type. Cycle P_{II} is absent (Fig. 83C).

The ventral sucker possesses 4 sensilla on the inner margin (internal to the 3 rows of spines) and 6 on the outer margin. These are also of the short cilium type (Fig. 83D).

The sensilla present on the tail-stem all have a long cilium with loose, low collar (Fig. 84A, B).

The furcal sensilla possess intermediate length cilia and loose, low collars (Figs 84C, 85A).

A. gracilis

Unless otherwise stated all sensilla of the cephalic cycles are of the short cilium and low, non-investing collar type. Cephalic cycle I (C_I) consists of 15 sensilla (on each body side). The mouth bears 1 sensillum within its inner margin. Its outer margin is bordered by 1 sensillum ventrally and 4 laterally (Figs 86B, 87A). The 2 apical sensilla are located between the antero-lateral margin of the pre-oral spines and the medial margin of the pore of the penetration gland (Figs 86A, B, 87A). The 2 lateral sensilla lie lateral to the pore of the penetration gland (Figs 86A, B, 87A). Of the 5 dorsal sensilla, the 2 most dorsal members comprise 1 with a cilium of intermediate length and

1 which is aciliate (Fig. 87C).

The C_{II} cycle bears 11 sensilla; of the 8 lateral members 2 have cilia of an intermediate length (Fig. 87A, B).

Nine sensilla make up cycle C_{III}. There are 3 lateral sensilla within this cycle; the most anterior is aciliate, while the other 2 possess cilia of an intermediate length (Fig. 87B).

All sensilla comprising the A, M and P cycles are of the short cilium and low collar type (Figs 86A, 88A, B).

The tail-stem and furcal sensilla are of the same types as described for the *Ichthyocotylurus* spp. (Fig. 89A, B, D).

Previous observations on the armature and sensilla of *Ichthyocotylurus* spp. and *A. gracilis* cercariae are provided in Tables 62 and 63.

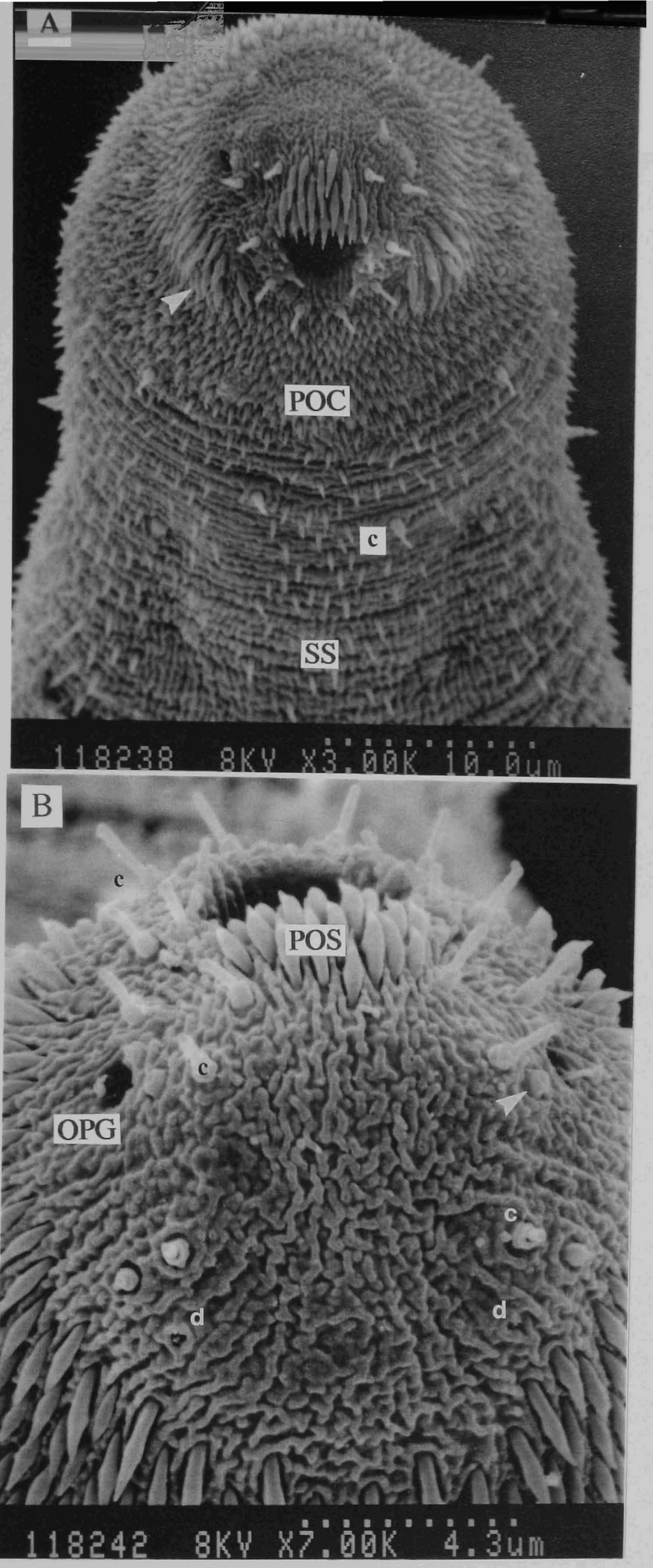


Fig. 82. Armature and sensilla of *I. erraticus* cercaria. A). Antero-ventral view of the cercaria showing the mouth surrounded by pre-oral spines and sensilla, the post-oral collar of spines (POC) and sparse, peg-like spines (SS). Arrow head indicates the region of enlarged spines within the post-oral collar. B). Antero-dorsal view of the unarmed body tip indicating the pre-oral tuft of spines (POS) and the outlets of the penetration glands (OPG). Arrow head indicates sensillum which was never seen to bear a cilium. Lower case letters refer to sensillary types - see text.

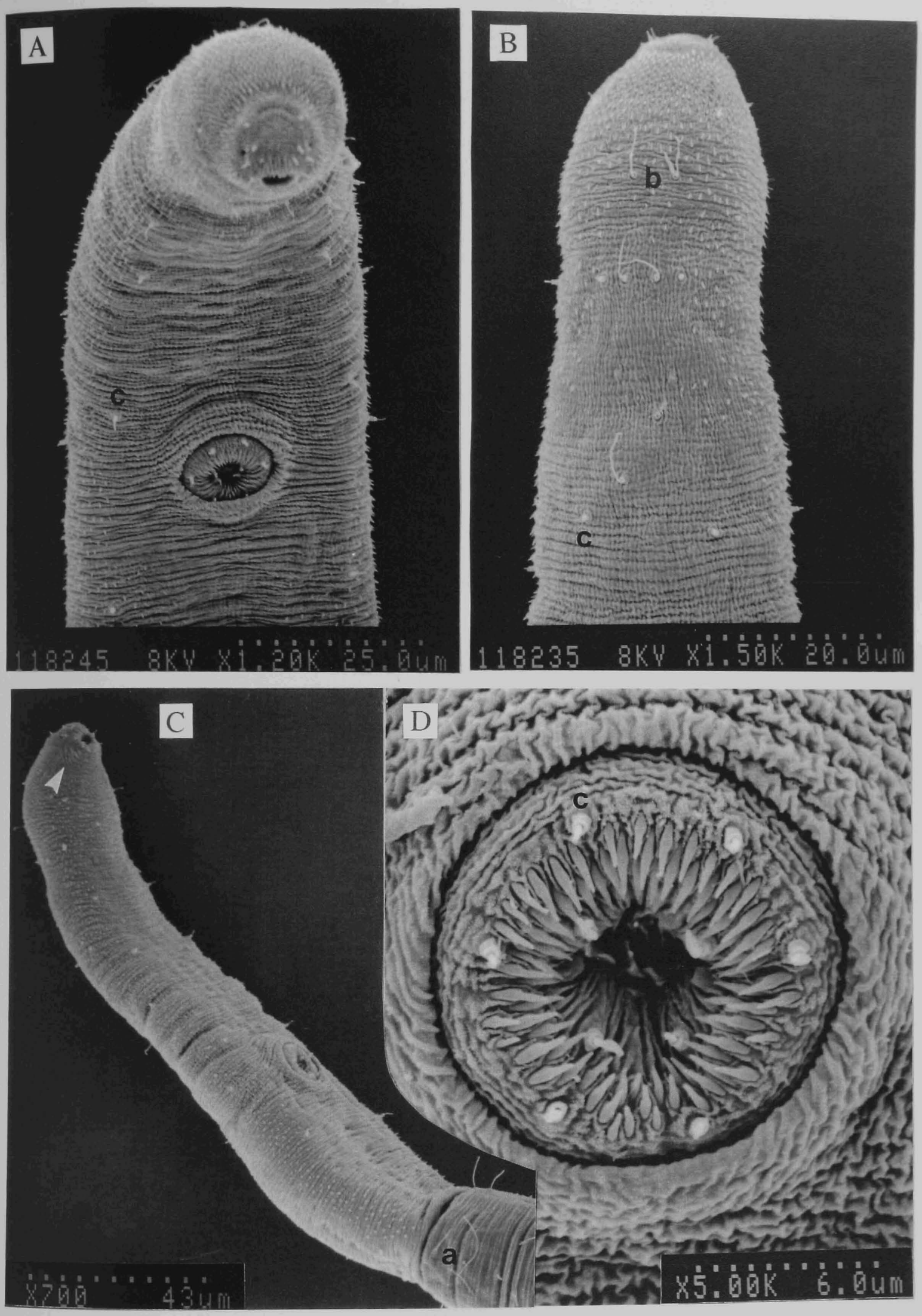
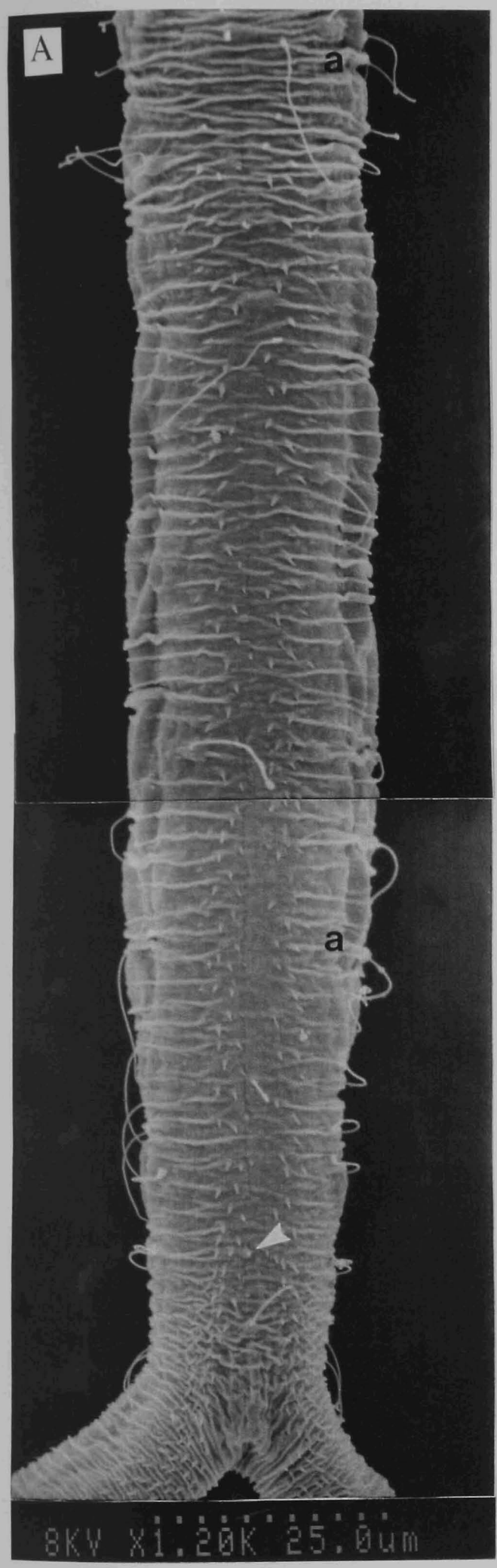
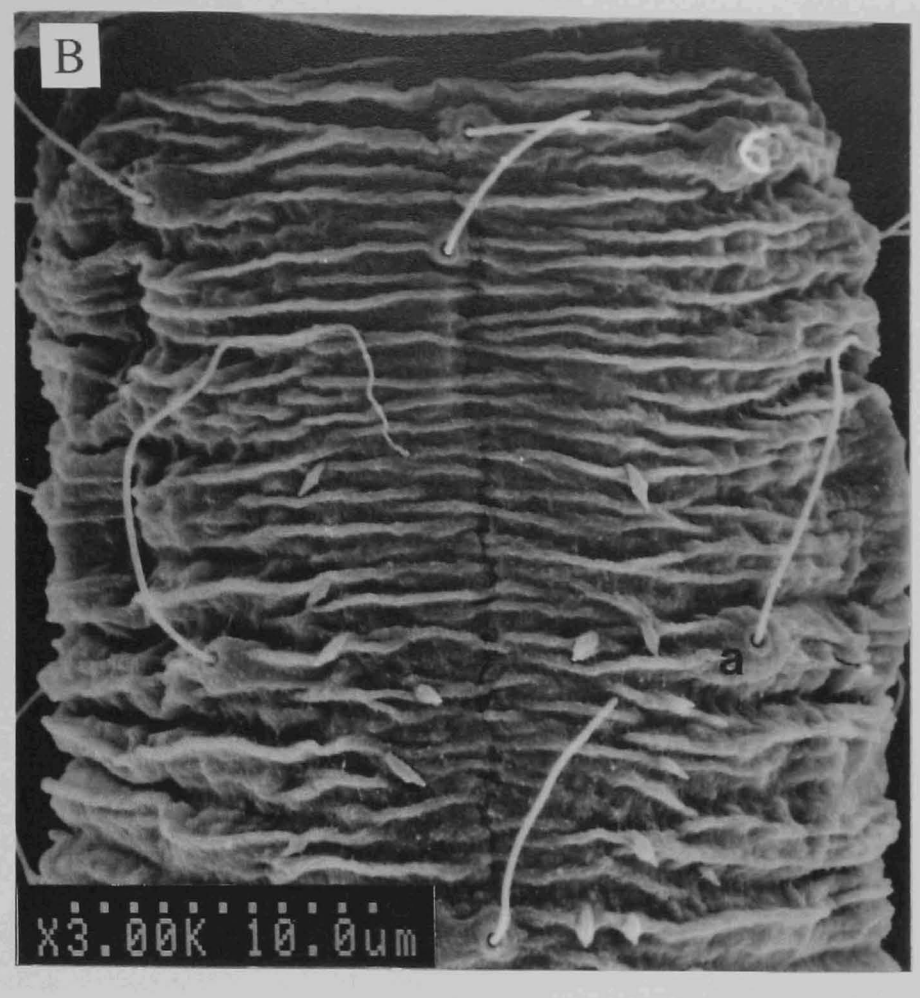


Fig. 83. Armature and sensilla of *I. erraticus* cercaria. A). Ventral body surface. B). Anterior half of dorsal body surface. C). Lateral body surface - arrow head as Fig. 84A. D). Ventral sucker. Lower case letters refer to sensillary types.





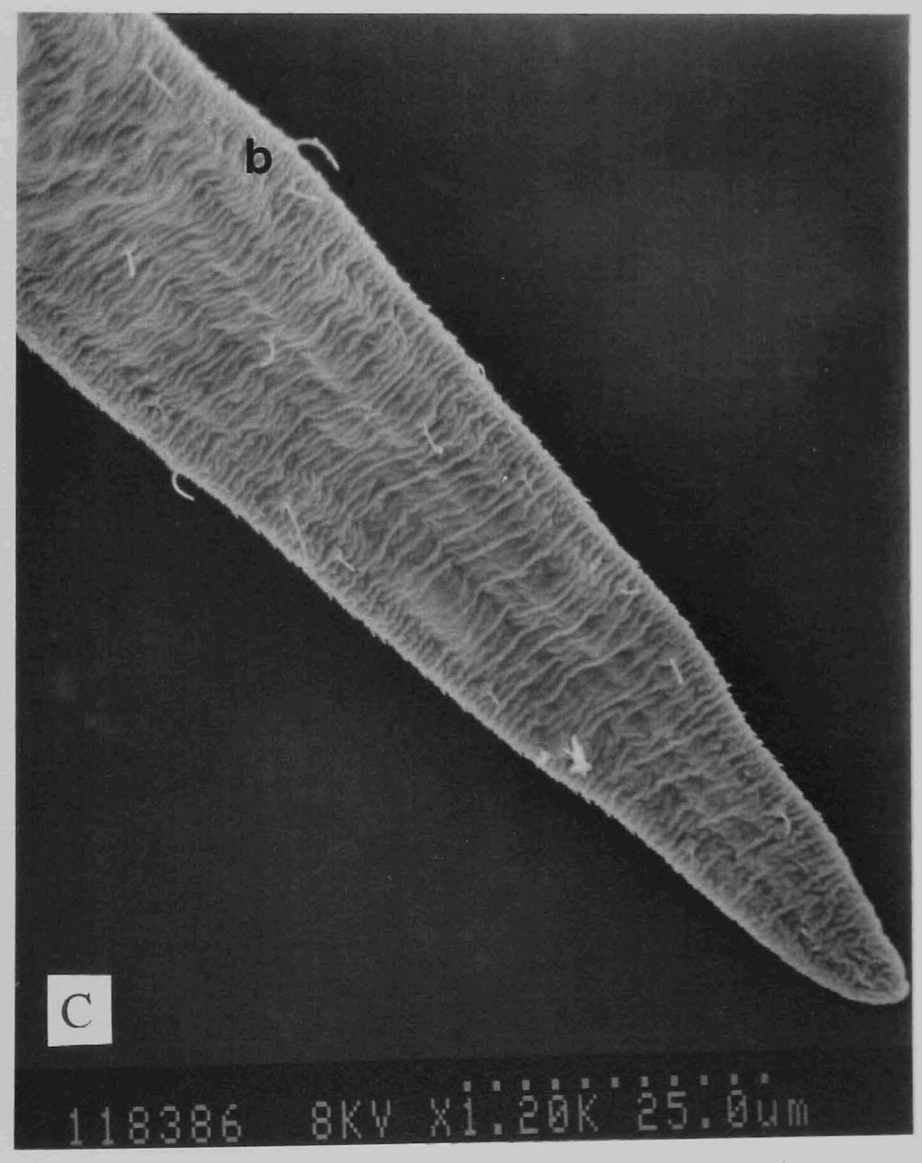


Fig. 84. Armature and sensilla of *I. erraticus* cercaria. A). Ventral surface of the tail-stem. Arrow head indicates a damaged cilium. B). Anterior end of the ventral surface of the tail-stem. C). Medial view of a furca. Lower case letters refer to sensillary types.

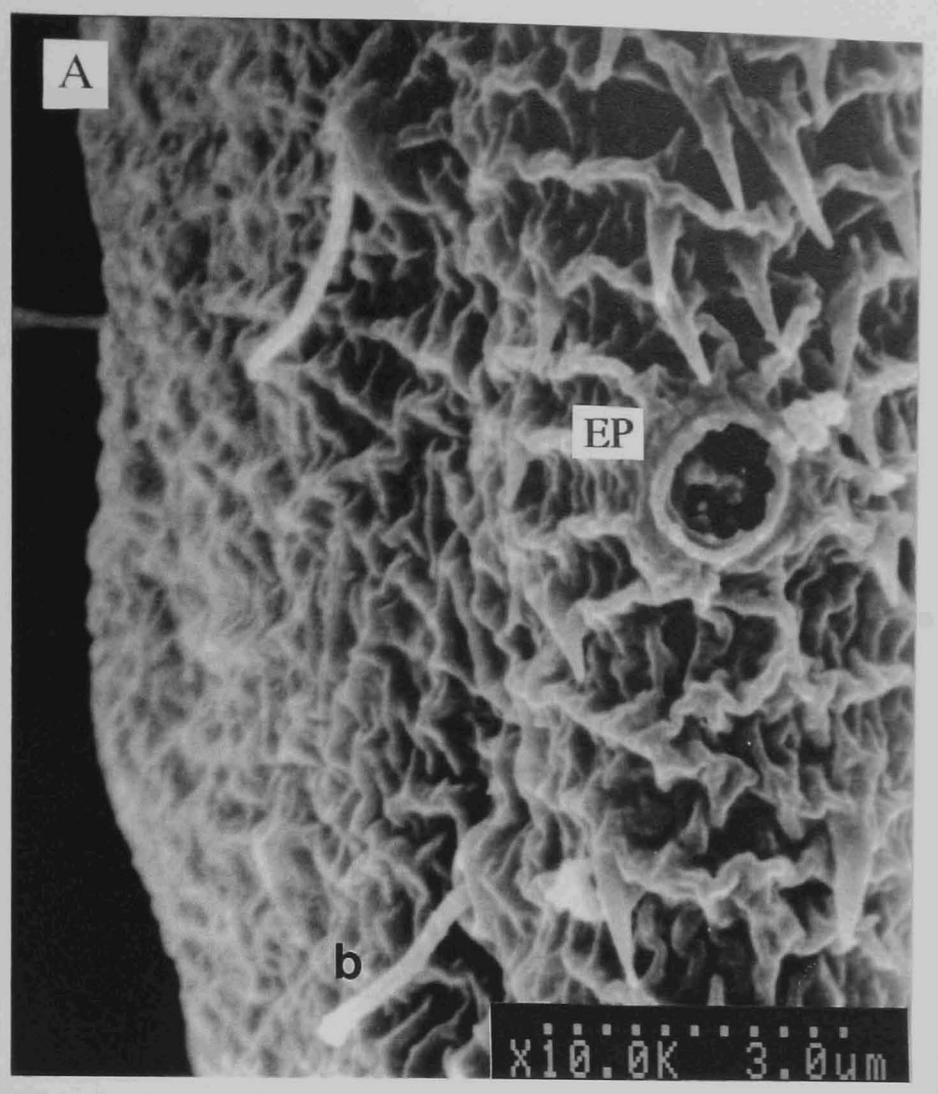
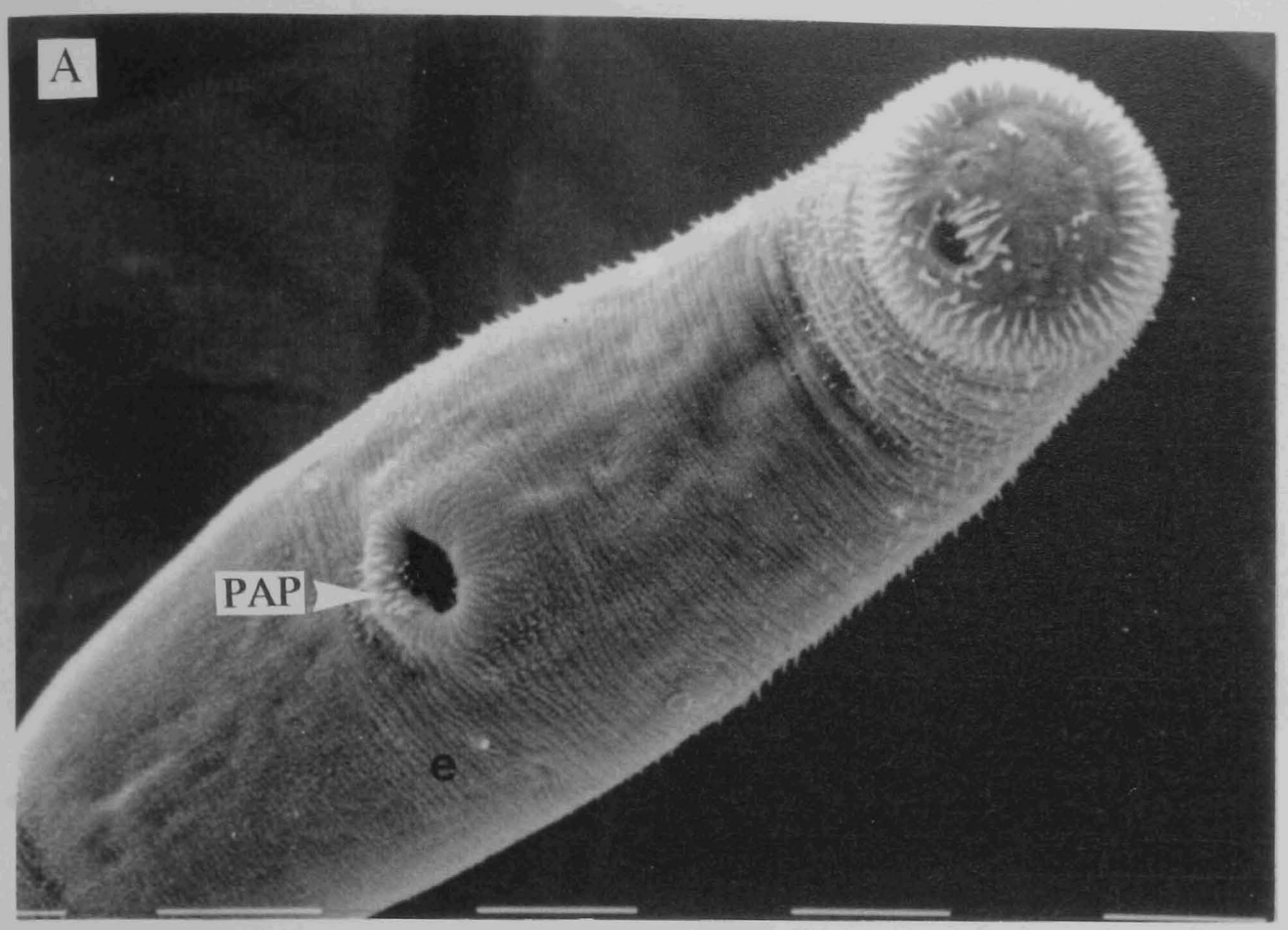


Fig. 85. Armature and sensilla of *I. erraticus* cercaria. A). Dorso-medial view of a furca showing the excretory pore on the dorsal ridge. B). Lateral view of the body with sensilla in the region of the post-oral collar - arrow head indicates damaged sensillum. All lower case letters refer to sensillary types.



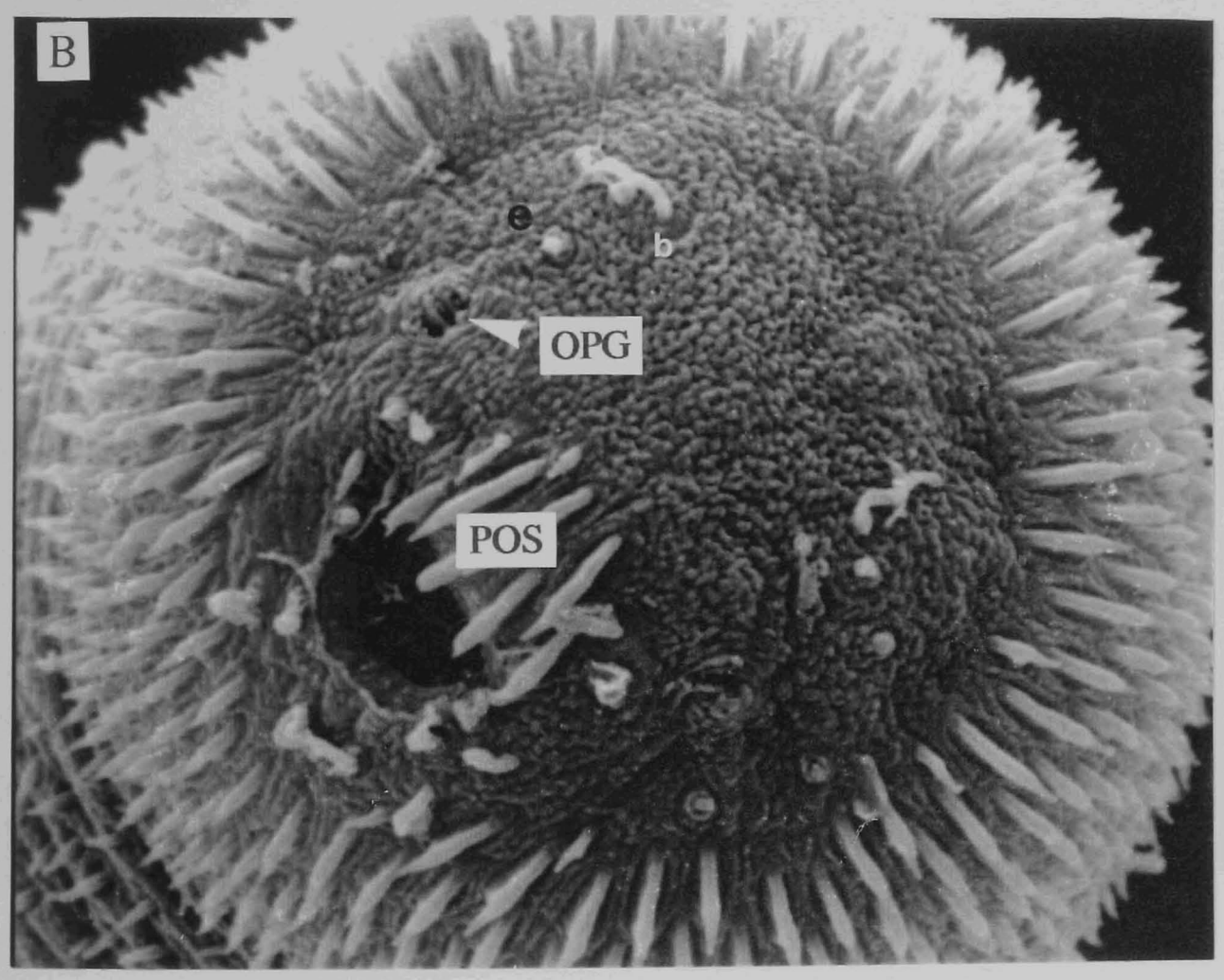


Fig. 86. Armature and sensilla of *A. gracilis* cercaria. A). Ventral body surface showing the post-acetabular patch of spines (PAP). B). Apical view of the unarmed anterior extremity indicating the pre-oral tuft of spines (POS) and the outlets of the penetration glands (OPG). Lower case letters refer to sensillary types.

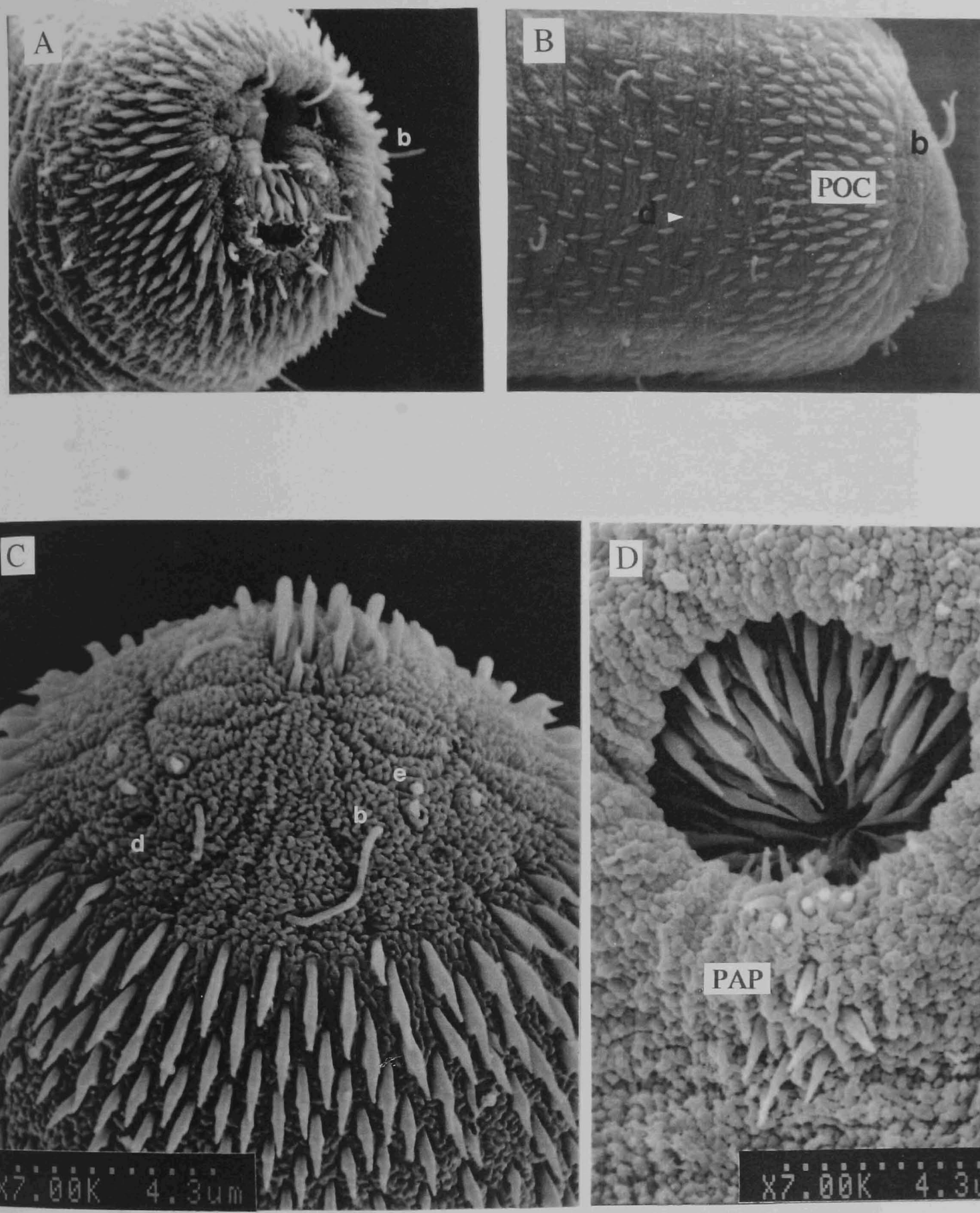
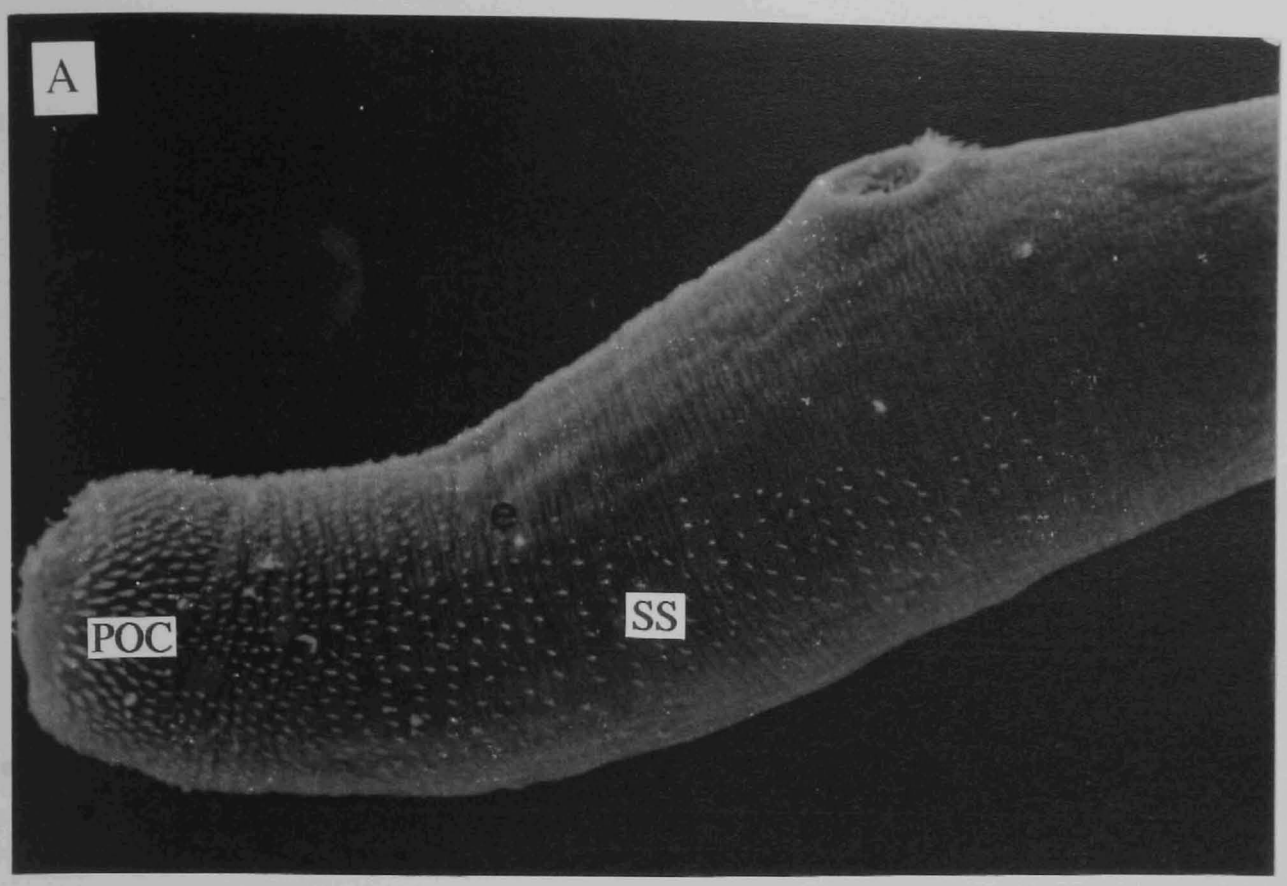


Fig. 87. Armature and sensilla of *A. gracilis* cercaria. A). Lateral view of the body surface showing the post-oral collar of spines (POC) and the sparsely distributed peg-like spines (SS). B). Dorsal view of the body surface showing the post-oral collar of spines (POC) and the sparsely distributed peg-like spines (SS). Lower case letters refer to sensillary types.



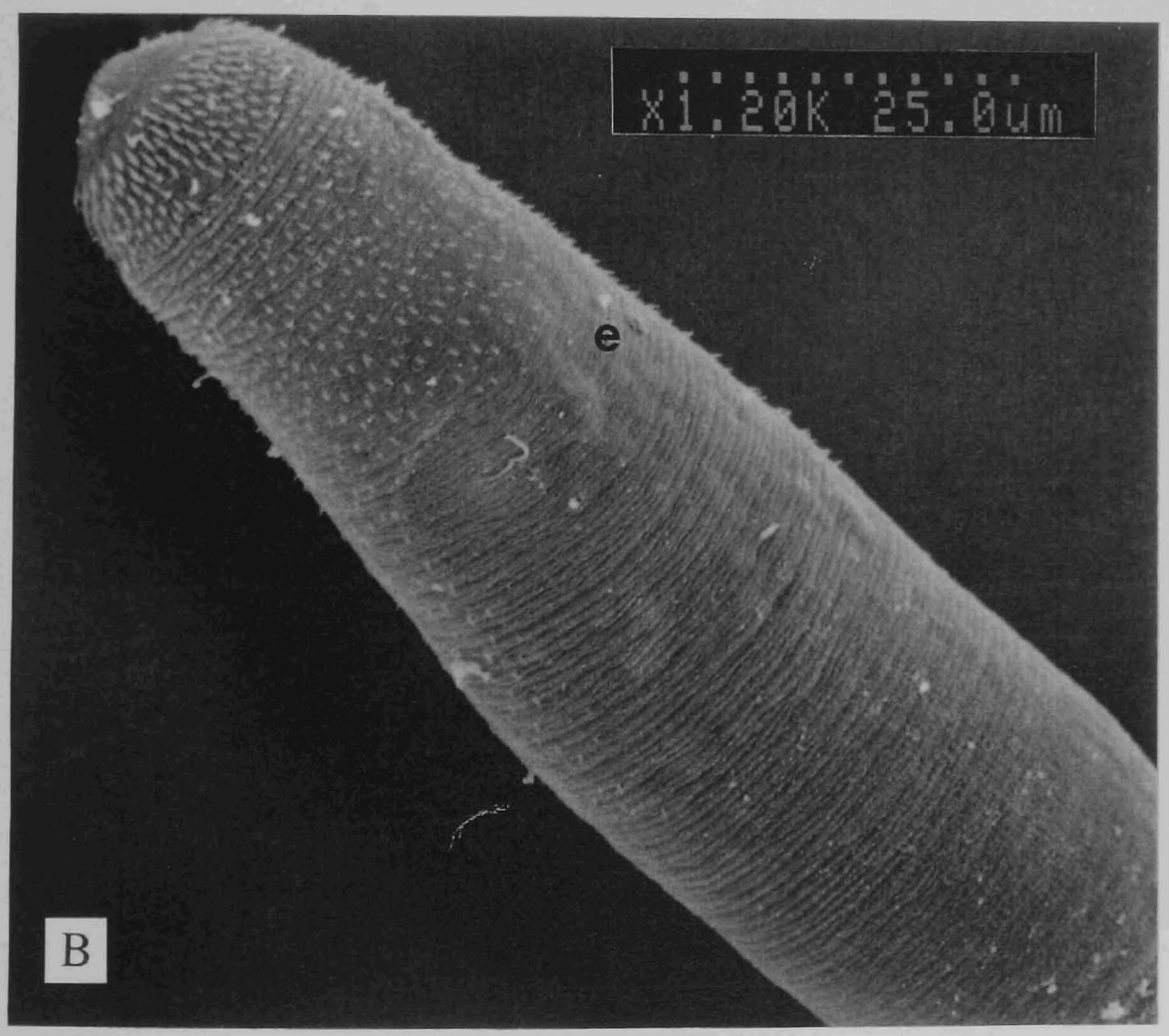
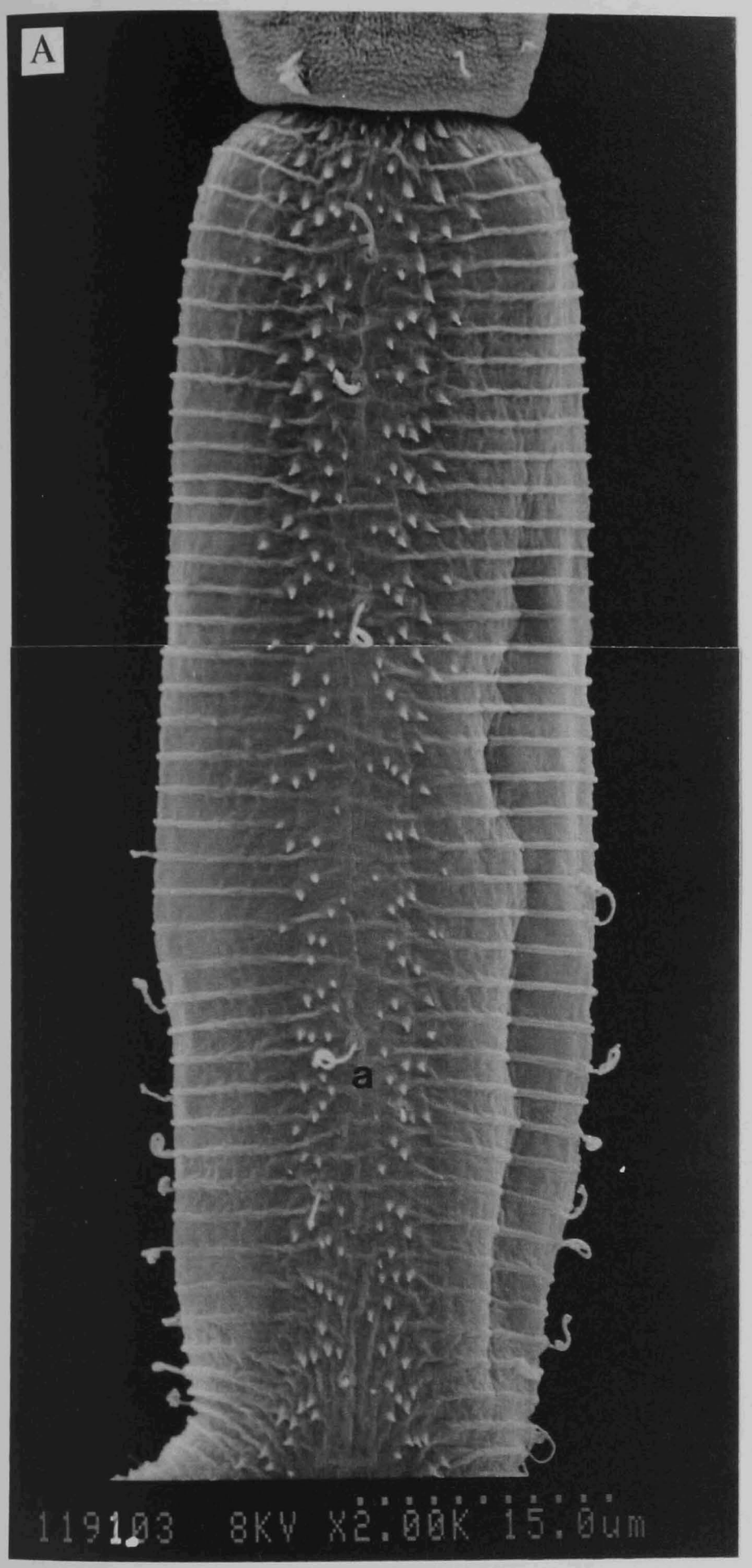
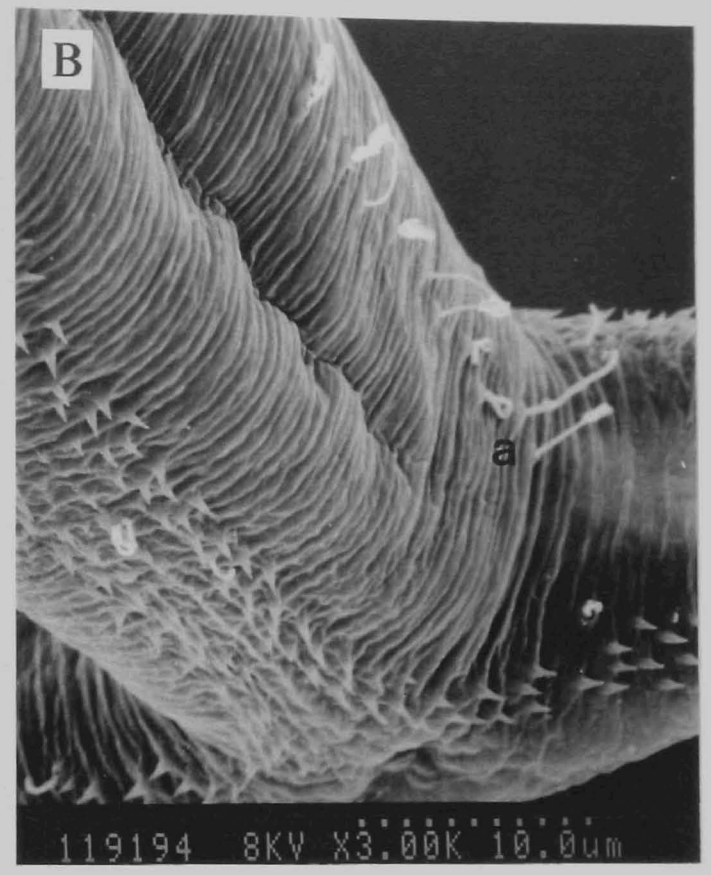


Fig. 88. Armature and sensilla of *A. gracilis* cercaria. A). Anterolateral view of the body surface. B). Lateral view of anterior region of the body surface showing the post-oral collar of spines (POC). C). Antero-dorsal view of the unarmed anterior extremity. D). Ventral sucker and post-acetabular patch of spines (PAP). Lower case letters refer to sensillary types.





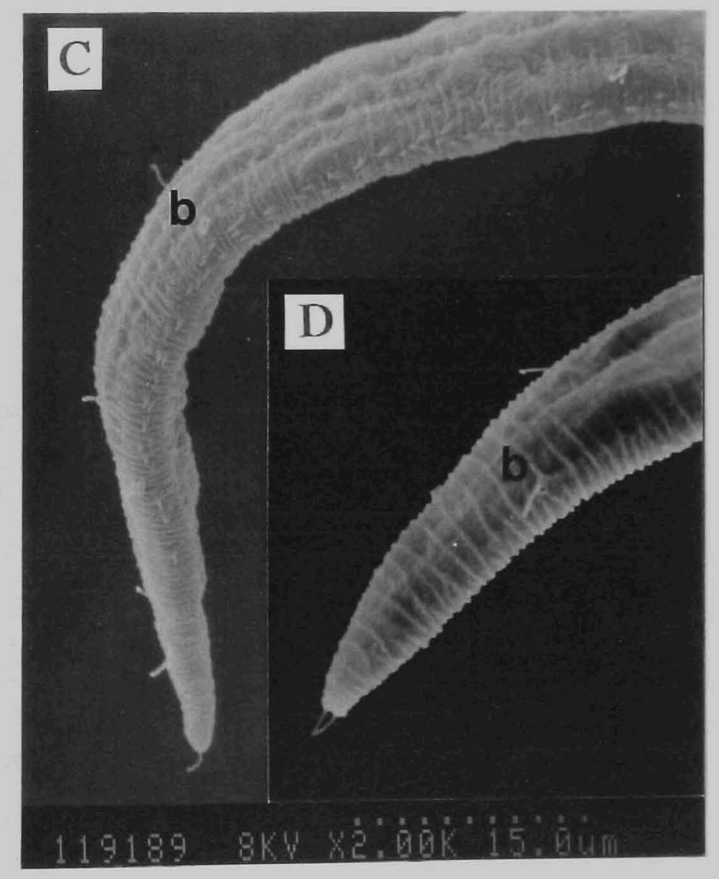


Fig. 89. Armature and sensilla of *A. gracilis* cercaria. A). Ventral surface of the tail-stem. B). Ventro-lateral view of the posterior end of the tail-stem. C). and D). Medio-lateral views of the furca.

Table 62. A comparison of the existing knowledge of the armature and chaetotaxy of three *Ichthyocotylurus* spp.

Species	*I. erraticus*	*I. variegatus*	*I. platycephalus*
Author	Swennen *et al.* (1979)	Odening & Bockhardt (1971)	Odening *et al.* (1970)
Armature:			
Pre-oral spines	presence noted	about 17 in 3 rows	not mentioned
Post-oral collar	presence noted	8 - 13 rows	5 rows
Body	covering of fine spines	as present study - figured	present, extent indistinct - figured
Ventral sucker	55 hooks in 2 rows	86 - 106 spines in 2 rings	62 - 64 spines in 2 rings
Sensilla:			
Cephalic	1 lateral pair	1 lateral pair	1 lateral pair
Body	3 lateral pairs	5 lateral pairs	4 lateral pairs
Tail-stem: Ventral surface Dorsal surface Antero-lateral Mid-lateral Postero-lateral	 - 4 proximal pairs 2 + 2 - 2 + 2 + 2 + 2 + 2	 - - 2 + 2 + 2 2 2 + 2 + 2 + 2 + 2	 - - 2 + 2 + 2 - 2 + 2 + 2 + 2 + 2
Furcae	8 medial, 3 lateral	origin of sensilla difficult to discern in figure - appears 3 medial, 2 lateral	5 ventral - figure

Table 63. A comparison of the existing knowledge of the armature of the cercaria of *Apatemon gracilis*.

Author	Crocombe (1959) As *Cercaria duodecaglandis*	Vojtek (1964a) As *Apatemon cobiditis cobiditis*	Blair (1974)	Sten'ko, 1977, in Sudarikov, (1984)	Present study
Armature:					
Pre-oral spines	11	8	7 - 10	9	7 - 12
Post-oral collar	9 - 11 rows	6 rows	6 - 7 rows	5 rows	6 - 8
Body	sparse spination to the level of the caeca - figure	sparse spination to the level of the caeca - figure	sparse spination to the level of the caeca and a patch of post-acetabular spines	sparse spination to the level of the caeca.	sparse spination to the posterior margin of the anterior organ, dorsally and ventrally but continued laterally to the ventral sucker. A patch of post-acetabular spines.
Ventral sucker	single ring of inwardly pointing spines	3 irregular rings (17 - 27 spines per ring)	3 irregular rings (18 - 20 spines per ring)	2 rings	2 - 3 rows
Sensilla:					
Cephalic	none described	none described	see text	indicated in chaetotaxy map, unpaired distribution	see text
Body					
Tail-stem: Ventral surface Dorsal surface Antero-lateral Mid-lateral Postero-lateral	 - - - - 9		 1 + 1 + 1 + 3 + 1 1 + 1 + 1 + 3 + 1 - - 2 + 2 + 2 + 2 + 2	 1 + 3 + 1 1 + 1 + 3 + 1 - - 2 + 2 + 2 + 2 or 2 + 2 + 2 (contradiction in figures)	as Blair (1974)
Furcae	none described		1 dorsal, 1 ventral, 8 medial, 2 terminal	13 medial	as Blair (1974)

5.2.4. Discrimination of *I. erraticus* and *I. variegatus* cercariae by principal components analysis of sensilla number and distribution.

The mean and coefficient of variation for each variable globally and for each species are given in Table 64. The relationship between the 18 variables are indicated in the correlation matrix (Table 65).

Table 64. Mean (μm) and coefficient of variation for each variable, globally and in each species (Coefficient of variation = 100 x standard deviation/mean). For an explanation of abbreviations see Fig. 77.

Variable	Both spp. combined (n=60)		*I. erraticus* (n=30)		*I. variegatus* (n=30)	
	Mean	Coefficient of variation (%)	Mean	Coefficient of variation (%)	Mean	Coefficient of variation (%)
D1	9.18	24.14	9.47	25.08	8.88	22.98
D2	28.32	18.58	31.23	16.49	25.40	13.74
D3	36.68	22.33	38.98	15.76	34.37	27.25
D4	55.74	13.94	54.58	15.67	56.90	12.04
F	23.48	6.00	23.40	5.33	23.57	6.66
L1	19.74	23.70	19.55	22.78	19.93	24.89
L2	30.25	16.03	29.53	15.45	30.97	16.46
L3	61.32	15.19	58.97	17.25	63.67	12.32
L4	48.15	18.31	45.03	15.50	51.27	18.43
S1	7.79	40.64	8.58	41.50	7.00	36.19
S2	45.32	15.80	45.10	15.06	45.53	16.72
S3	56.50	17.24	56.67	19.42	56.33	15.04
TS	29.77	10.04	28.63	9.90	30.90	8.85
V1	27.12	15.37	27.80	16.88	26.43	13.32
V2	39.34	14.17	40.58	15.97	38.10	11.14
V3	47.23	10.72	47.42	12.10	47.03	9.30
V4	61.93	12.56	63.97	12.44	59.90	11.95
V5	63.02	9.52	63.10	9.61	62.93	9.59

The PCA results of the first 2 components (Fig. 90), which account for 35.21% of the total variance (Table 66) failed to indicate any separation on either axis. However, Fig. 91 shows that components 1 and 3, explaining 34.09% of the total variance (Table 66), do demonstrate a degree of separation along the third axis (PCA3). Furthermore, when 95% confidence limits are imposed upon this map (Fig. 92) a total separation of the 2 species is achieved. The PCA of the variables analysed (Fig. 93) shows those which had the highest and lowest coordinates on the third axis and therefore contribute most to this dispersal. These variables are the vertical distances between the ventral surface sensilla [L1-L4] and the number of tail-stem sensilla [TS] (all with high positive values), and the horizontal distances between the dorsal surface sensilla [D2] and [D3] (with high negative values).

Table 65. Correlation matrix (high positive coefficients in bold).

	D1	D2	D3	D4	F	L1	L2	L3	L4	S1	S2	S3	TS	V1	V2	V3	V4	V5
D1	1.000																	
D2	0.172	1.000																
D3	0.092	**0.667**	1.000															
D4	0.179	0.173	0.173	1.000														
F	-0.019	-0.018	0.138	0.124	1.000													
L1	0.039	-0.116	-0.168	0.045	0.067	1.000												
L2	0.083	-0.203	-0.071	0.078	-0.018	0.429	1.000											
L3	-0.029	-0.142	-0.136	-0.029	0.004	0.184	0.357	1.000										
L4	-0.023	-0.131	0.070	0.118	0.109	0.148	0.378	0.176	1.000									
S1	0.270	0.345	0.330	0.230	-0.086	0.217	0.121	0.049	0.188	1.000								
S2	0.258	0.231	0.162	0.334	0.130	0.150	-0.002	0.141	0.171	0.272	1.000							
S3	0.158	0.207	0.025	0.131	-0.014	0.223	0.311	0.194	0.098	0.209	0.085	1.000						
TS	0.199	-0.139	-0.110	0.154	0.086	0.195	0.137	0.348	0.225	0.112	0.323	0.037	1.000					
V1	0.092	0.047	0.169	-0.132	0.037	0.217	0.186	0.101	-0.050	0.136	-0.086	-0.098	-0.090	1.000				
V2	0.154	0.099	0.136	-0.138	-0.104	0.329	0.225	0.160	0.018	0.238	0.061	0.134	0.051	**0.643**	1.000			
V3	0.104	-0.003	0.168	-0.300	0.033	0.022	0.235	0.208	-0.049	0.082	-0.052	0.081	0.091	0.550	**0.643**	1.000		
V4	-0.043	0.119	0.133	-0.133	0.104	0.316	0.351	0.189	0.054	0.283	0.073	0.070	0.044	0.480	0.509	**0.636**	1.000	
V5	-0.069	-0.070	0.177	-0.041	0.008	0.190	0.490	0.362	0.239	0.207	-0.071	0.141	-0.059	0.437	0.427	0.591	**0.684**	1.000
	D1	D2	D3	D4	F	L1	L2	L3	L4	S1	S2	S3	TS	V1	V2	V3	V4	V5

Table 66. Principal components analysis of the correlations between the 18 variables.

Eigenvalues and proportion of the variance explained by the first four principal components				
Eigenvalue	3.928	2.411	2.209	1.284
Proportion (%)	21.82	13.39	12.27	7.135
Cumulative (%)	21.82	35.21	47.48	54.62
Coefficient of each variable on the first three components				
Variable	PCA1	PCA2	PCA3	
D1	0.155	0.450	-0.021	
D2	0.085	0.608	-0.596	
D3	0.217	0.504	-0.585	
D4	-0.064	0.637	0.180	
F	0.040	0.133	0.071	
L1	0.453	0.063	0.412	
L2	0.577	-0.034	0.488	
L3	0.413	-0.047	0.479	
L4	0.236	0.215	0.466	
S1	0.405	0.571	-0.106	
S2	0.131	0.645	0.179	
S3	0.269	0.313	0.204	
TS	0.166	0.269	0.526	
V1	0.656	-0.230	-0.336	
V2	0.753	-0.097	-0.223	
V3	0.741	-0.248	-0.271	
V4	0.798	-0.120	-0.146	
V5	0.784	-0.192	-0.004	

Fig. 90. Map of the 60 specimens in the first plane of the principal components analysis. *I. erraticus* specimens are represented by (e) and *I. variegatus* by (v). Ellipses surround 50% of points.

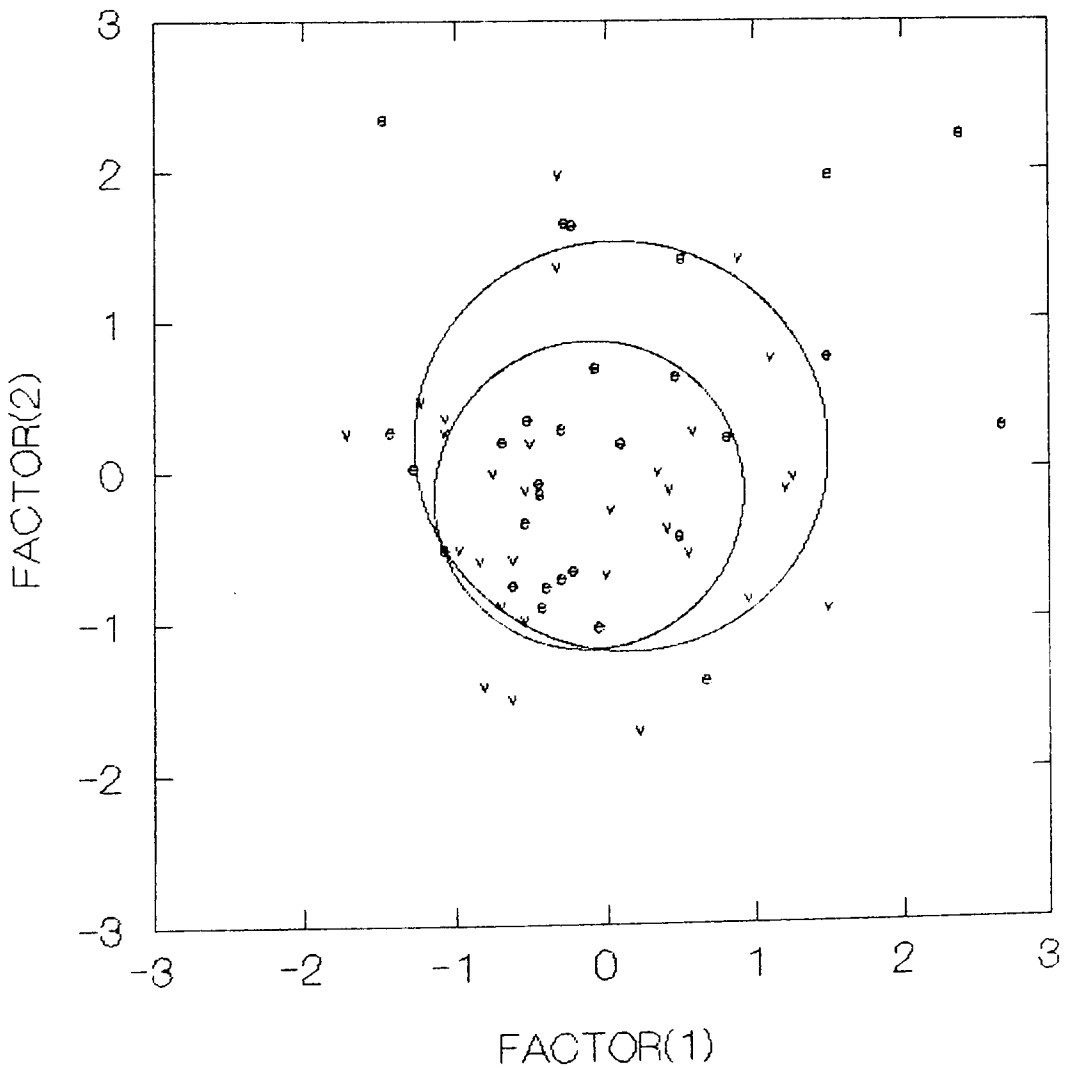


Fig. 91. Map of the 60 specimens in the second plane of the principal components analysis. *I. erraticus* specimens are represented by (e) and *I. variegatus* by (v). Ellipses surround 50% of points.

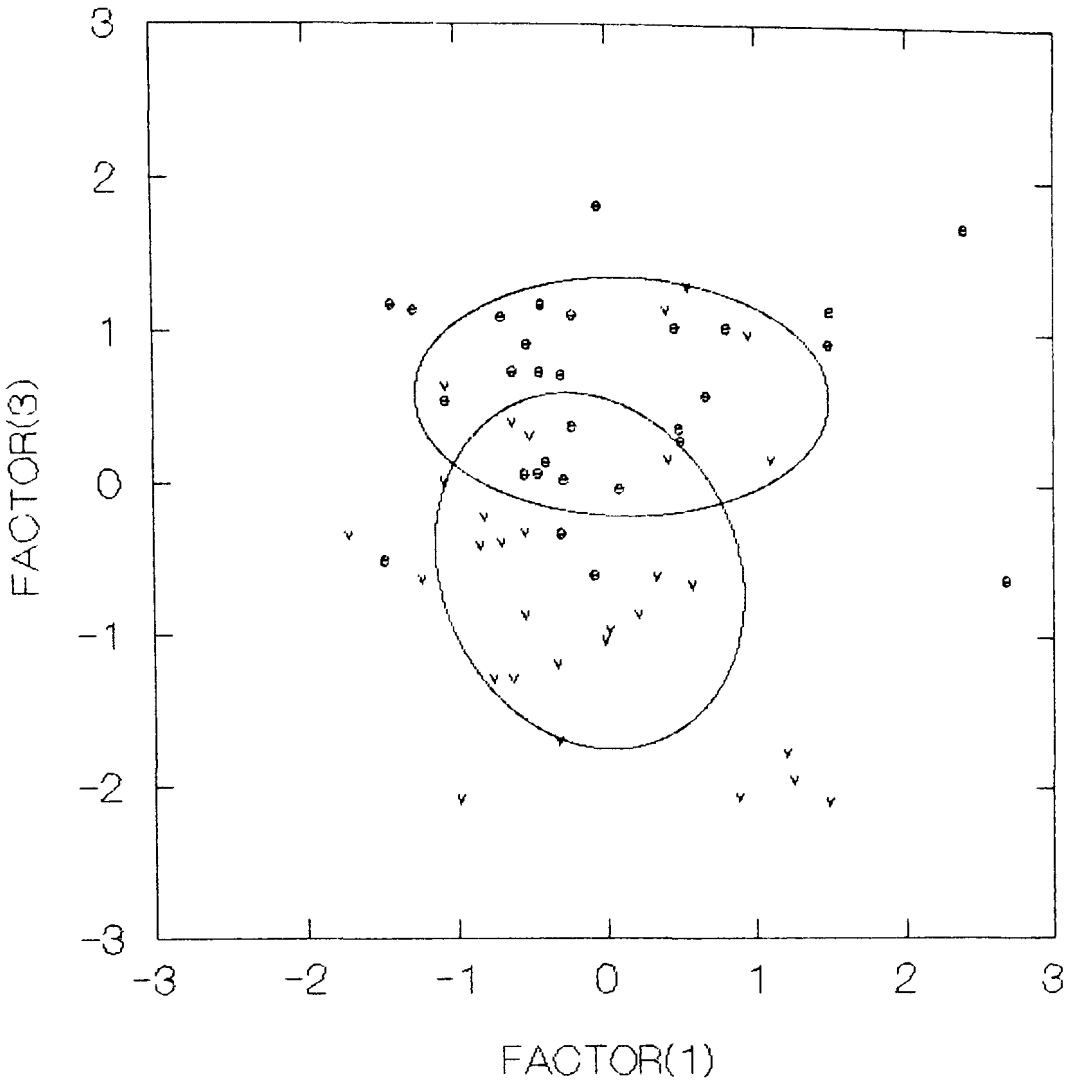


Fig. 92. Map of the 60 specimens in the second plane of the principal components analysis. *I. erraticus* specimens are represented by (e) and *I. variegatus* by (v). Ellipses represent 95% confidence limits.

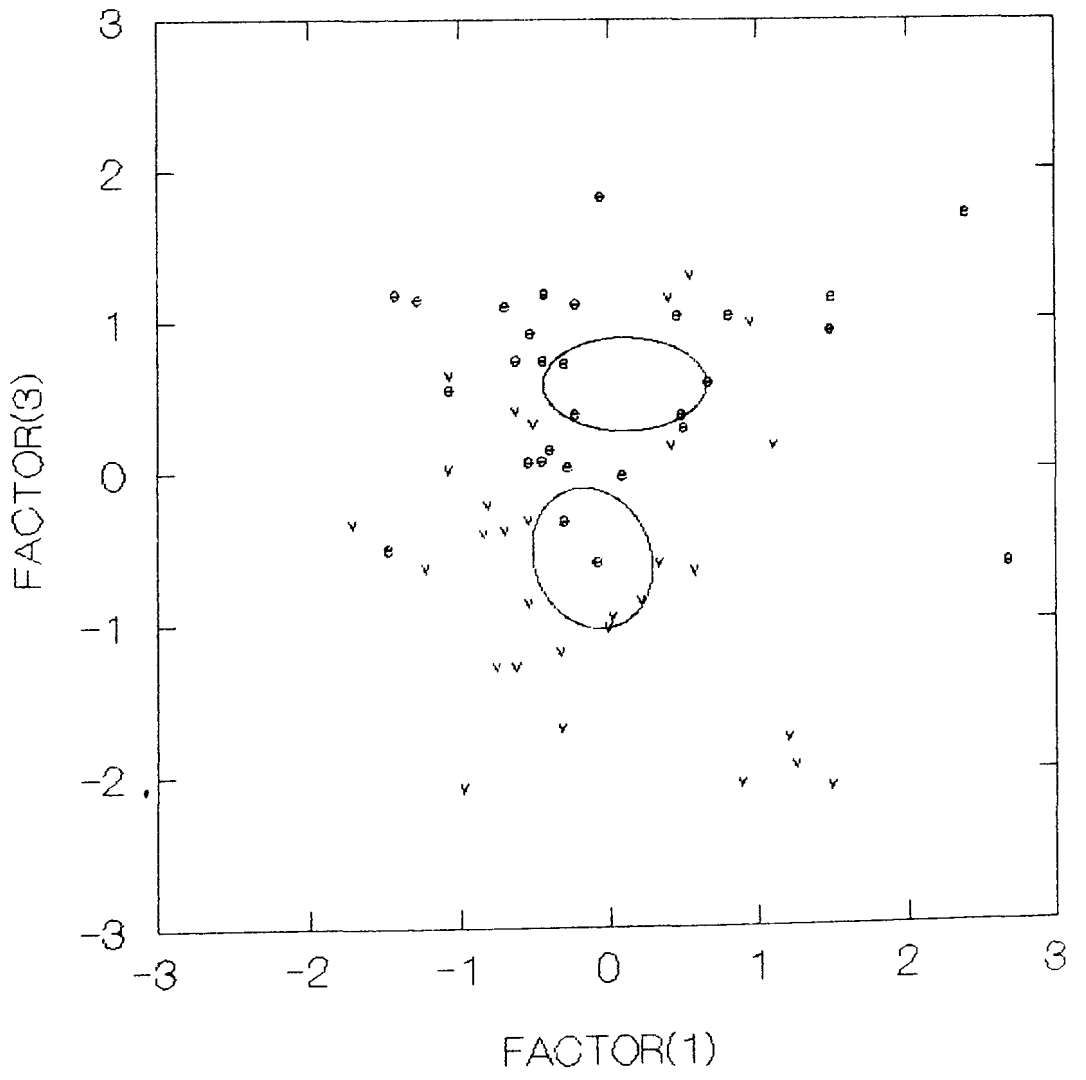
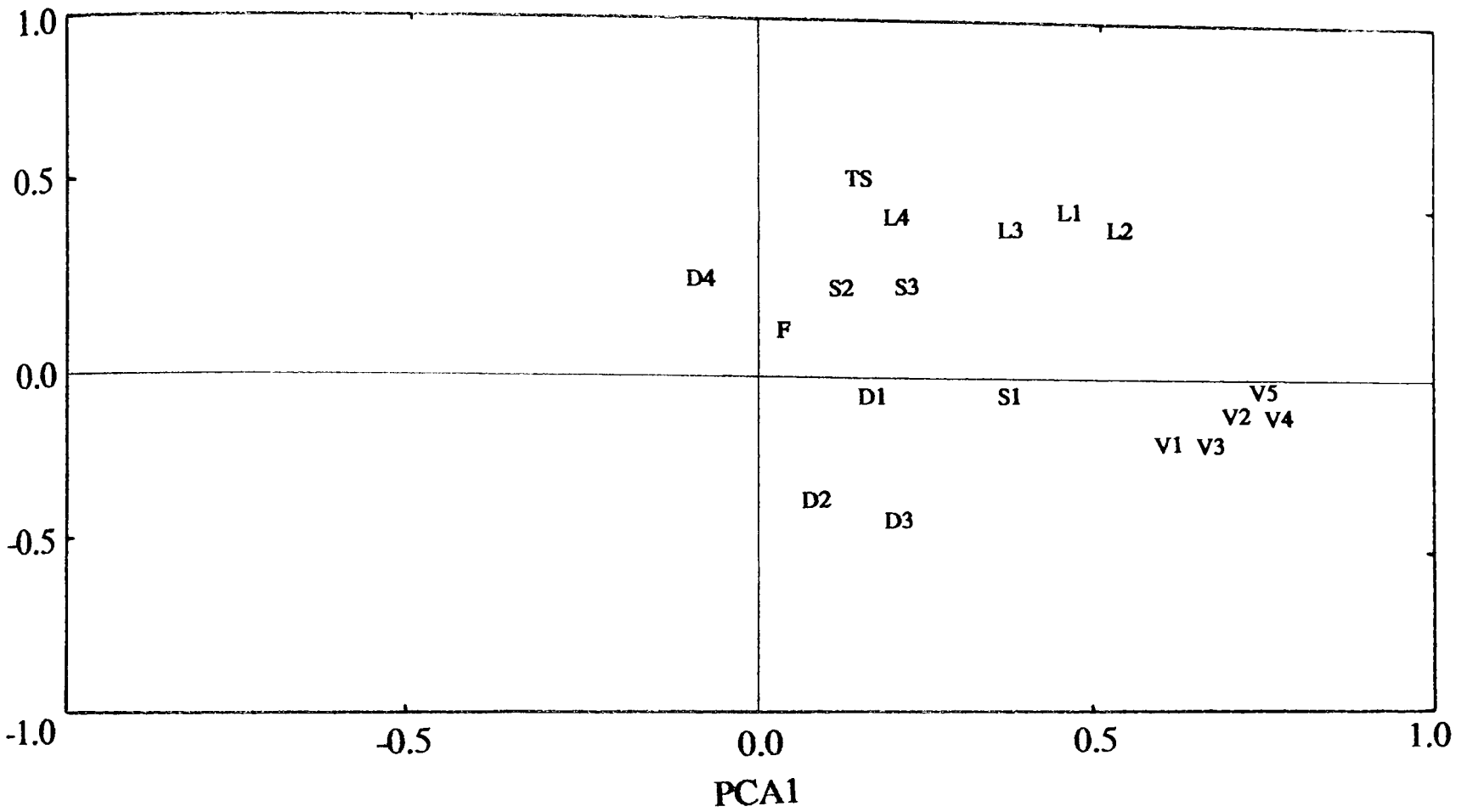


Fig. 93. Map of the 18 variables in the second plane of the principal components analysis on 60 specimens.



5.2.5. Transmission electron microscope observations of *I. variegatus* cercarial sensilla.

The 2 sensillary forms identified with the SEM as a and b, which possess a low loose collar but with cilia of differing lengths, appear using TEM to share the same internal structure. However, only rather thick TEM sections were obtained of these sensilla types and few additional details could be discerned. Fig. 94 indicates that no dilation of the sensory cell was observed, with the cilium appearing to send rootlets directly to the cup-shaped tegumental collar. The only other feature that could be discerned was a large electron-dense disc situated vertically below the cilium and its limiting collar.

Sensilla described as type c using SEM, possess a short cilium and tightly investing collar, being situated on the body and particularly in the cephalic region. This sensillary type can be seen in Fig. 96. TEM revealed that the cilium emerges from a bulbous thistle-shaped extension of a sensory process. The cilium, exhibiting the typical 9x2 + 2 axoneme arrangement, arises from a closed basal body without an attached rootlet. The thistle-shaped nerve bulb is attached to the tegument by a septate desmosome at the level of the basal body. Tightly applied to the inner surface of the bulb adjacent to the desmosome is an electron-dense ring. A further 2 such rings are present, 1 close to the basal body and the other, taller band, lateral to and extending below this. The cytoplasm of the nerve bulb contains numerous electron-lucent vesicles and microtubules. The distal part of the sensory cell from which the cilium protrudes is surrounded by a tall circular fold of tegument which forms the tightly investing collar.

Type d sensilla, located apically on the body of the cercaria, were thought to be aciliate when viewed with the SEM. However, TEM revealed that a short sensillum is present, being housed within a shallow hemispherical pit with a broad external opening (Fig. 95). No basal body was observed, although ciliary rootlets were seen to radiate towards the base of the sensory cell which was lined with an electron-dense region. This dense lining lies against the cap-like desmosome binding the cell to the tegument. An electron-dense region is also present around the pit, encapsulating it, and a discrete electron-dense disc is situated below the base of the pit. No associated dendron was observed with this type of sensillum.

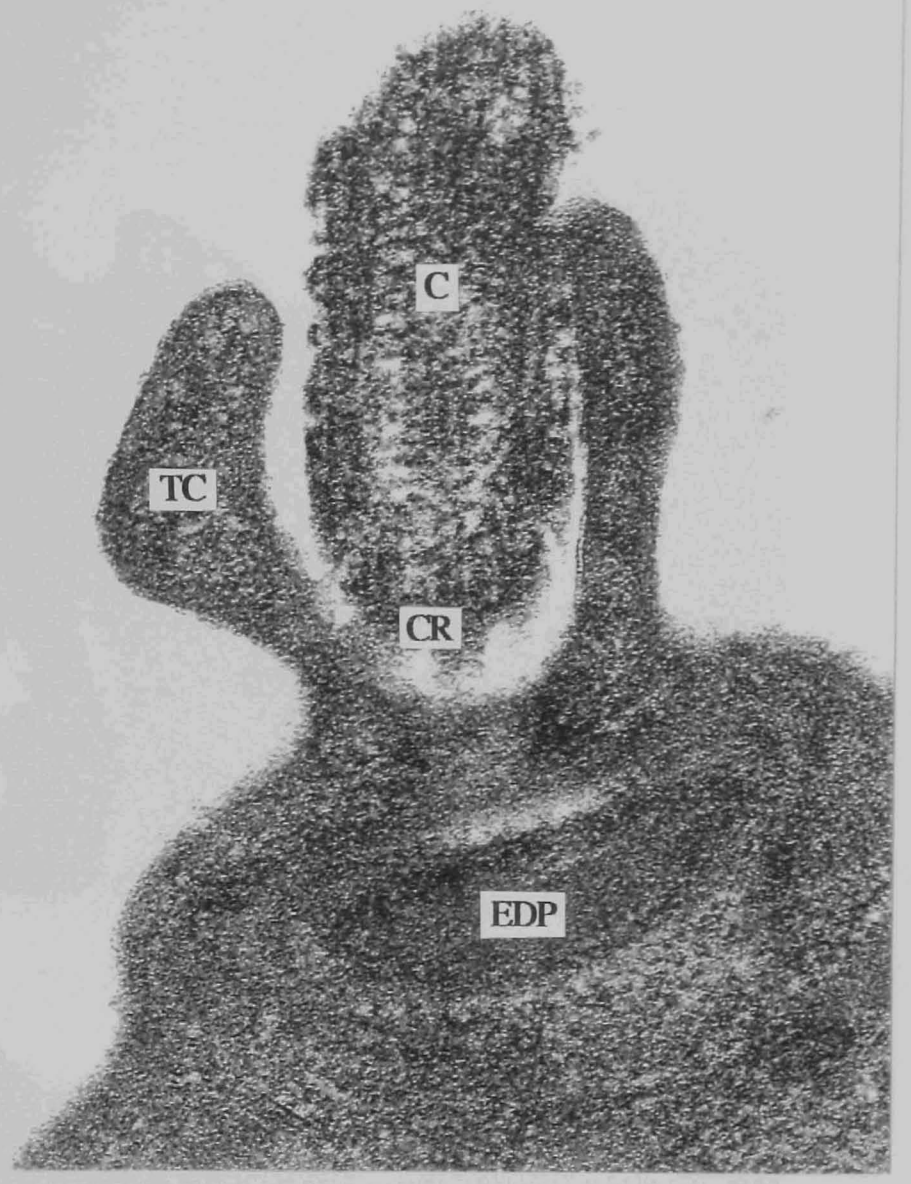


Fig. 94. TEM photomicrograph of sensillum type a. The cilium (C) sends ciliary rootlets (CR) to the tegumentary collar (TC). Posteriorly lies the discrete electron dense plate (EDP). (x 120,000).

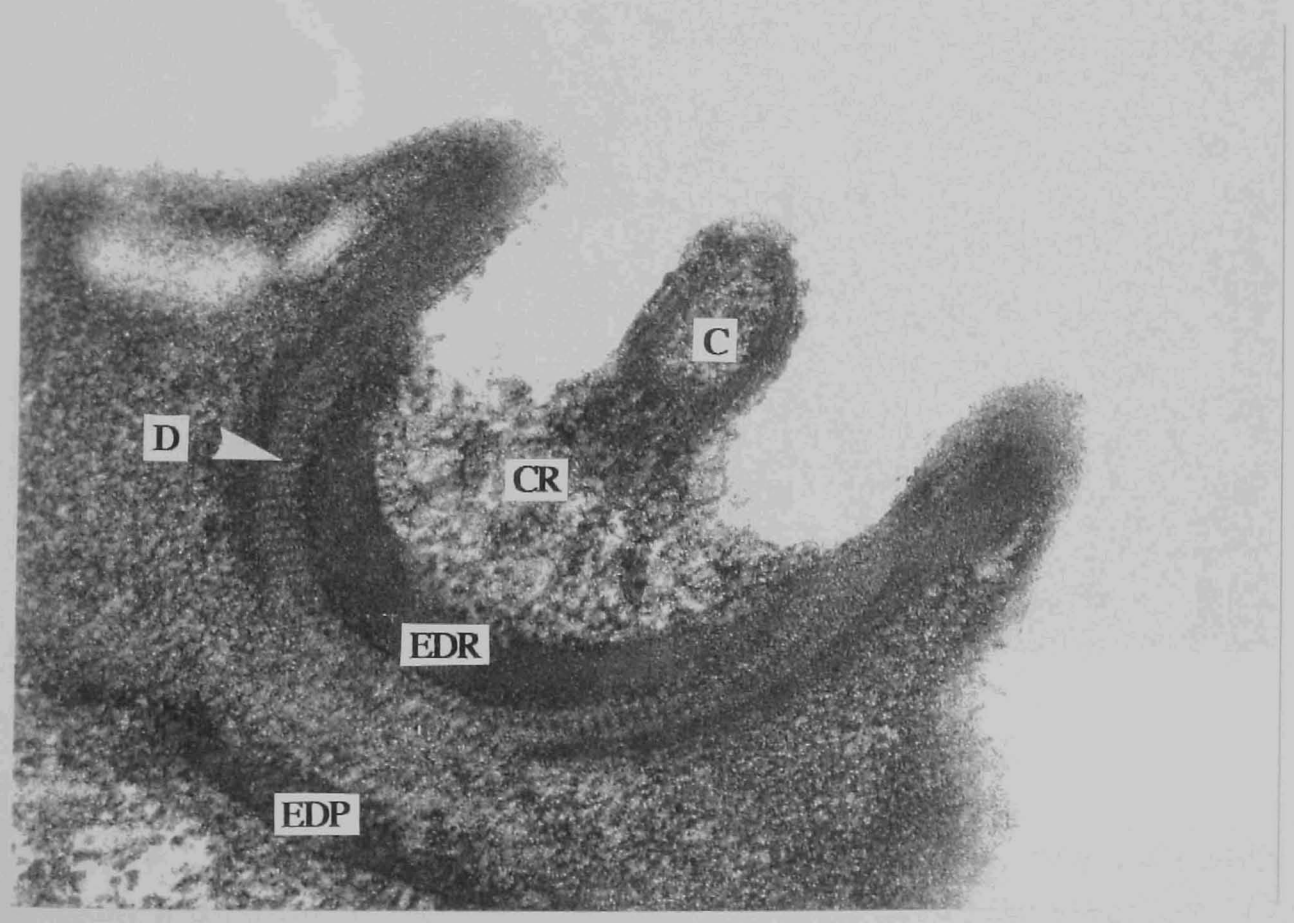
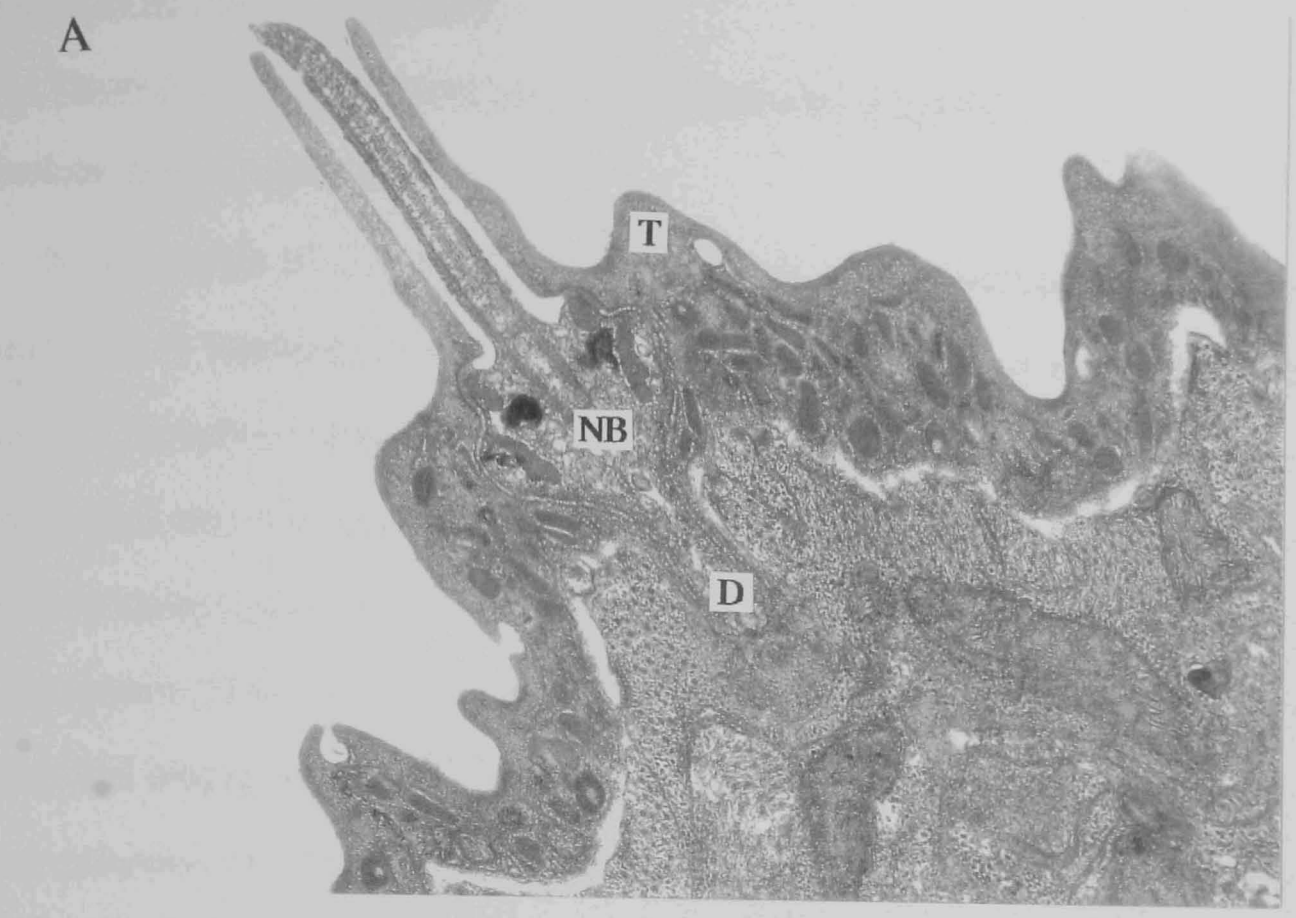


Fig. 95. TEM photomicrograph of sensillum type d. The short cilium (C) sends ciliary rootlets (CR) towards the base of the sensory cell which is lined with an electron-dense region (EDR). The base of the sensory cell is bound to the tegumentary pit by a cap-like desmosome (D). Below the pit lies an electron-dense plate (EDP). (x80,000).



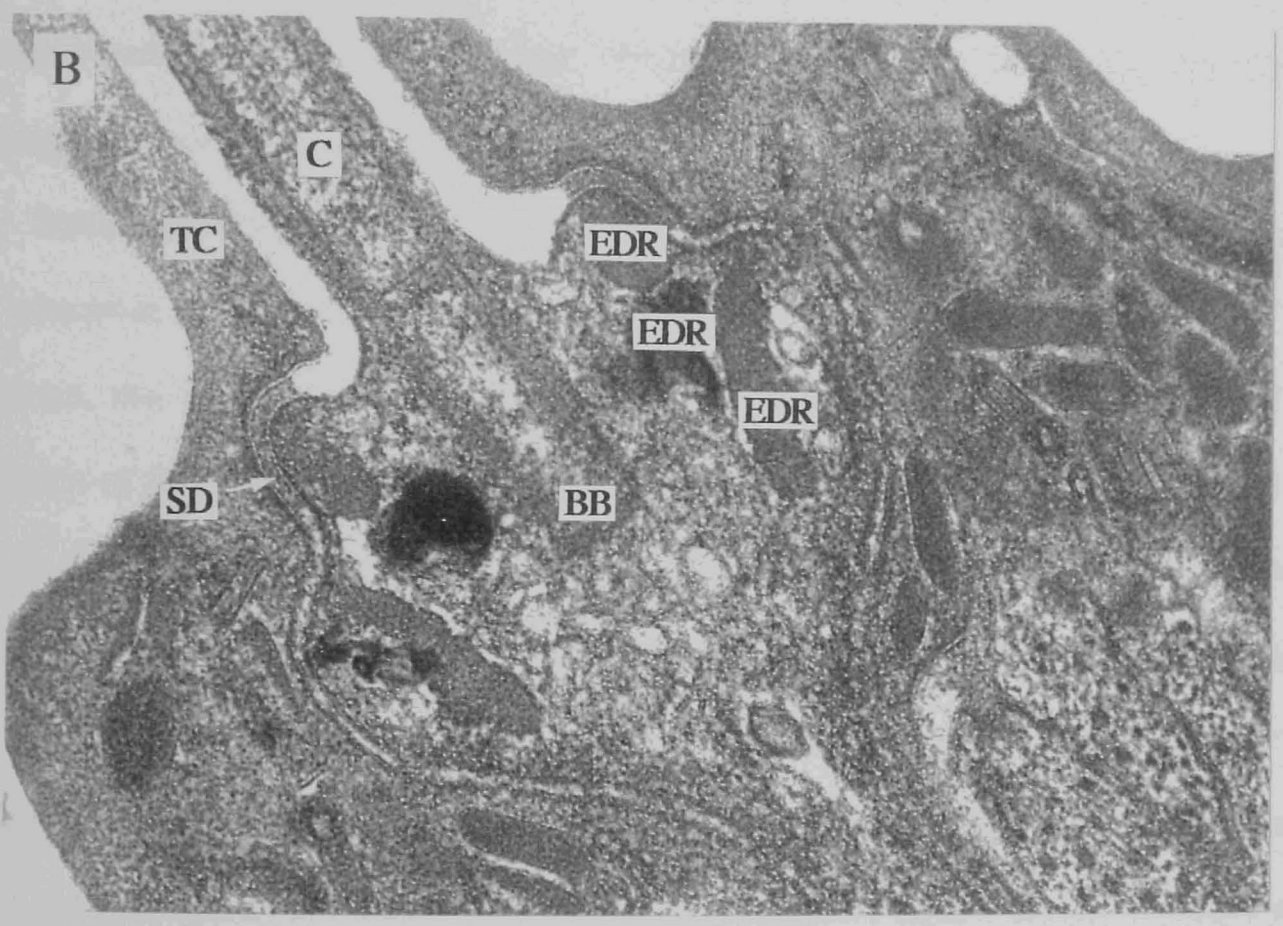


Fig. 96. A. and B. are TEM photomicrographs of sensillum type c. The nerve bulb (NB) is formed from a dilation of the dendron (D); it is attached to the tegument (T) via a septate desmosome (SD). The tegumentary collar (TC) invests the cilium (C) which arises from a closed basal body (BB). Three electron-dense rings (EDR) are present within the sensory bulb which also contains numerous electron-lucent vesicles. (A. x 30,000, B. x 60,000).

5.3. BEHAVIOURAL STUDIES.

5.3.1. Cercarial emergence strategies and swimming behaviour.

I. erraticus* and *I. variegatus

Both species of *Ichthyocotylurus* cercariae were observed emerging from the terminal 3 or 4 lamellae of the bipectinate ctenidium (gill) of infected *V. piscinalis* specimens. Cercariae could be seen crawling, anterior end first, along the axis of the ctenidium and into the most terminal lamellae. Generally, only a few filaments had an established escape pore, and these appeared to be sought rather than actively create a new emergence point. When in a suitable lamella, little activity was required to re-open the pore and emergence was largely passive, with the cercaria being extruded by the internal pressure. As the furcae passed through the pore it closed behind them and frequently the tips remained caught. At this point the cercaria began a violent undulating movement until both furcae were free. Sequential frames of an *I. erraticus* specimen emerging are shown in Fig. 97.

Serial histological sections of *Ichthyocotylurus* infected *V. piscinalis* showed that these cercariae do utilise the circulatory system of the host to reach their site of emergence, the ctenidium. Cercariae penetrate the haemocoelic channels lying among the lobules of the digestive gland and pass in these vessels to the region of the visceral ganglion where the vessels unite (also with the venous vessels draining other body regions) to form the visceral vein. This vein supplies the left kidney before emerging as the efferent renal vein and splitting into small branches which pass across the mantle skirt near the rectum and genital duct. These channels then pass to the ctenidial leaflets. Figs 98 to 102 show cercariae situated in the digestive gland (many within daughter sporocysts), within the haemocoelic channels, the venous vessels and the ctenidium. Unfortunately, cercariae were not observed actively entering the haemocoelic channels and it is not known whether they exit from a single or multiple sites.

The rapid bursts of tail first swimming activity exhibited by both *Ichthyocotylurus* spp. lasted for no more than 2 seconds. This motion was not captured

on film and, therefore, the finer details of this action could not be discerned. The cercariae were seen to hang in the water for up to several minutes, sinking very slowly. The resting posture was interrupted when the cercaria had traversed most of the water body or in response to a disturbance such as a shadow or vibration. Both *I. erraticus* and *I. variegatus* cercariae assumed the same resting posture, with the furcae spread at approximately 150° apart, the tail-stem and body hanging vertically down, but with the anterior half of the body bent ventrally in a hook-like manner. The resting positions of these cercariae recorded in the present study and by other authors are given in Table 67.

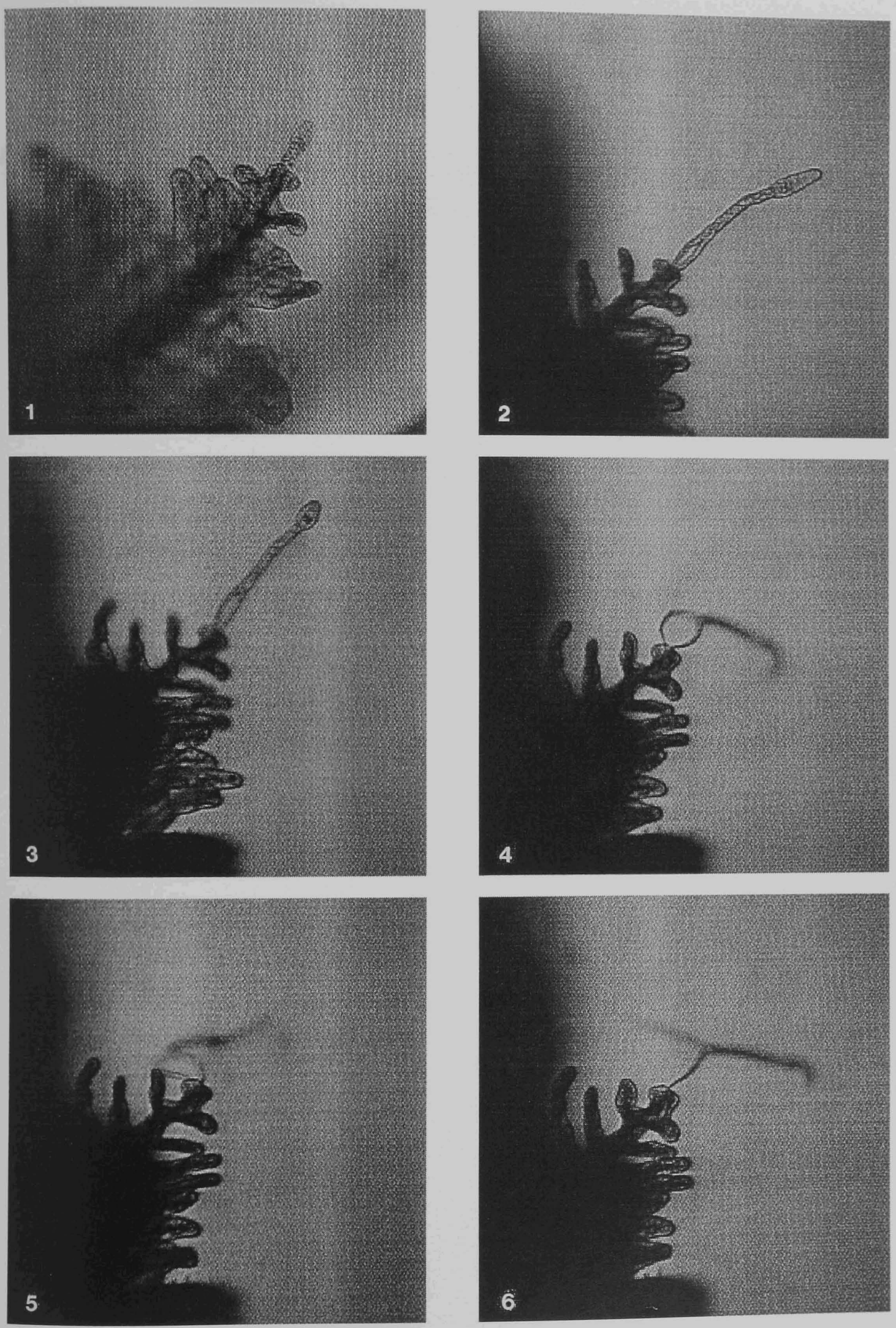
No strong phototaxis was observed for *I. erraticus* or *I. variegatus* cercariae, although, as already mentioned, a shadow response was present.

A. gracilis

The migratory route of *A. gracilis* cercariae within their *L. peregra* hosts, from the digestive gland to their point of exit, was not followed using serial sections, although cercariae were seen to emerge consistently from the same point on the inner surface of the mantle. Once free from the snail host, *A. gracilis* cercariae show a very active swimming behaviour, moving tail first, pausing rarely and then for less than a second. The swimming mechanism was captured in images taken at 160ms intervals and is shown in Fig. 103. While following a straight (not helical) path the cercaria rotates about its long axis with the furcae acting like a propeller and performing a spinning 'frontcrawl-like' stroke. Occasionally the cercaria does pause and Table 67 shows the resting posture of this species which was as described for the *Ichthyocotylurus* spp.

A. gracilis cercariae were found to be negatively phototactic with specimens accumulating away from the light source in an illuminated container.

Fig. 97. Series of images showing the emergence of an *Ichthyocotylurus erraticus* cercaria from the ctenidium of an experimentally infected *Valvata piscinalis*.



The series follows in chronological order.

Key for histological sections of *I. erraticus* infected *V. piscinalis* specimens (Figs 98-102).

Abbreviations: (bv) blood vessel; (c) cercaria; (ct) ctenidium; (dg) digestive gland; (ds) daughter sporocyst; (f) foot); (hc) haemocoelic channel; (h) heart; (k) kidney; (mc) mantle cavity; (ms) mantle skirt; (pt) palial tentacle; (vg) visceral ganglion.

Fig. 98. Longitudinal histological section of an *I. erraticus* infected *V. piscinalis* specimen showing daughter-sporocysts bearing cercariae within the digestive gland. (Cason's, x25 approx.)

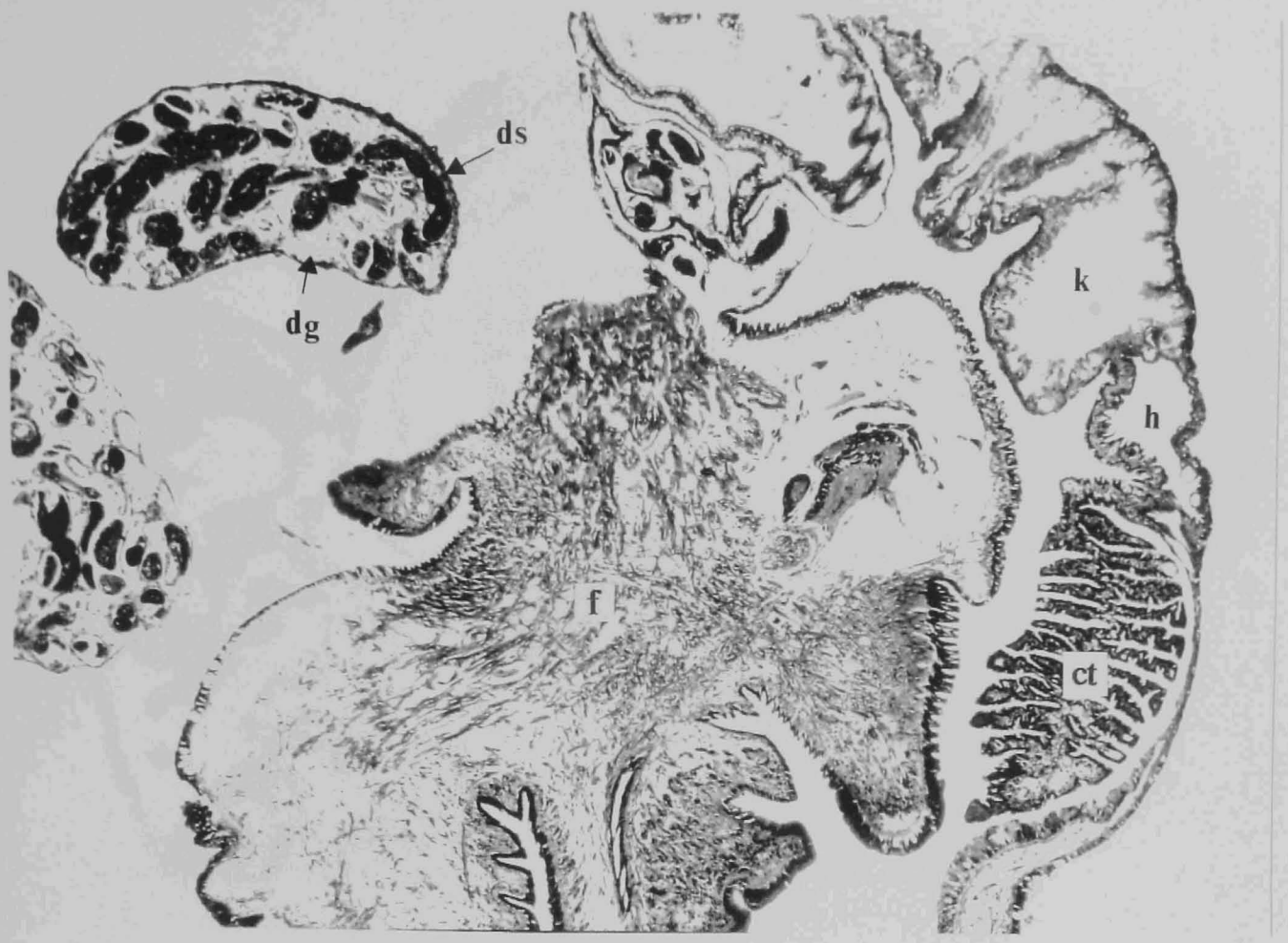


Fig. 99. Section showing *I. erraticus* cercariae in haemocoelic channels at the base of the digestive gland. (H&E, x160 approx.).

Fig. 100. Longitudinal section through an *I. erraticus* infected *V. piscinalis* specimen showing blood vessels (arrowed) bearing cercariae in the region of the visceral ganglion. (Cason's, x25 approx.).

Fig. 101. Detail of *I. erraticus* cercariae within venous vessels; enlarged from previous Fig. (Cason's, x160 approx.).

Fig. 102. Longitudinal section showing *I. erraticus* cercariae within the terminal lamellae of the ctenidium. (H&E, X160 approx.).

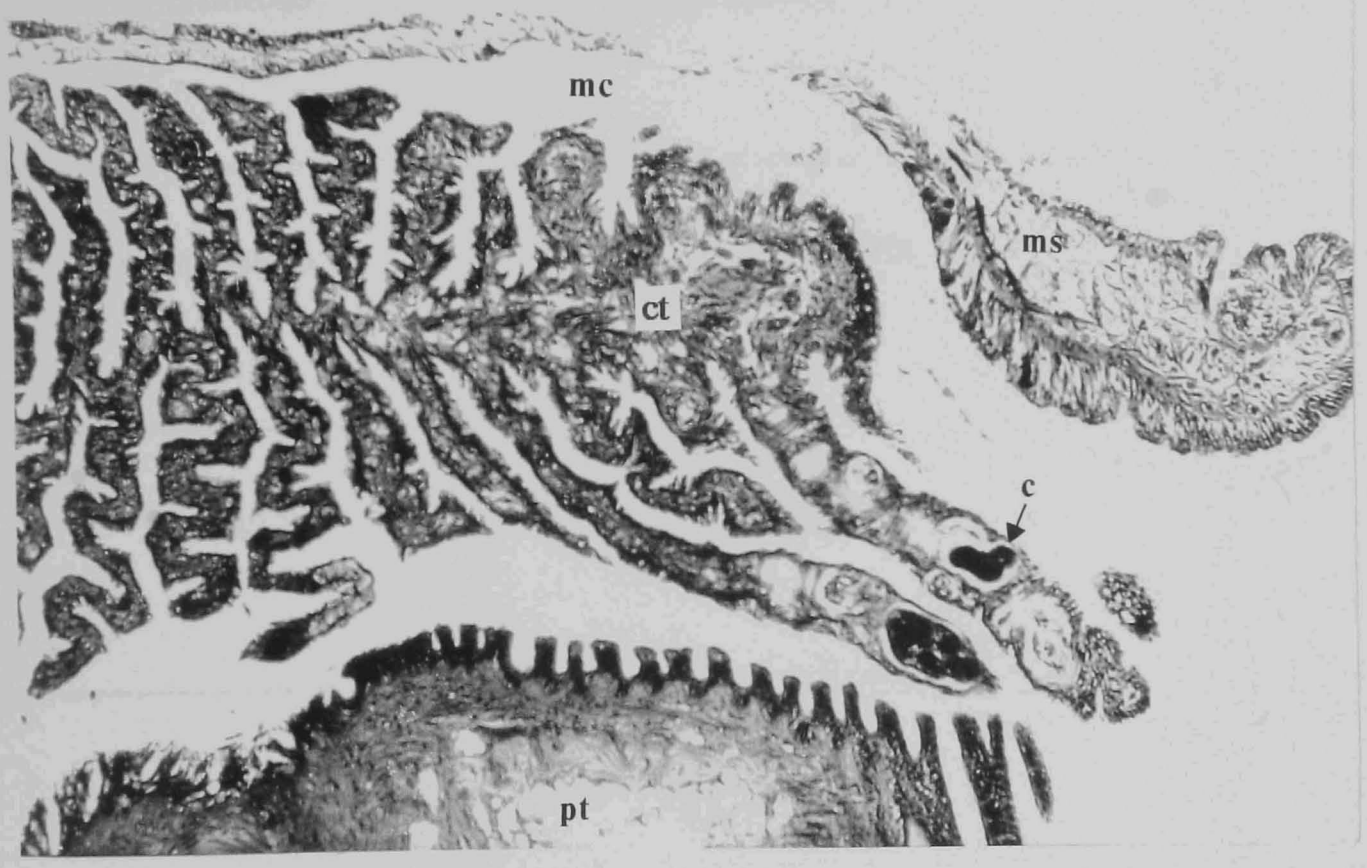


Table 67. The resting postures recorded for cercariae of *Ichthyocotylurus* spp. and *Apatemon gracilis*.

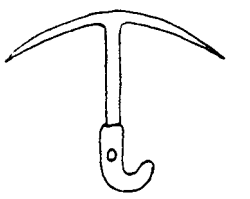
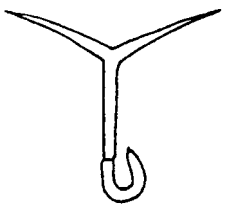
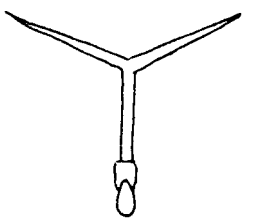
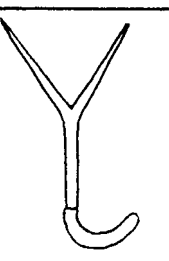
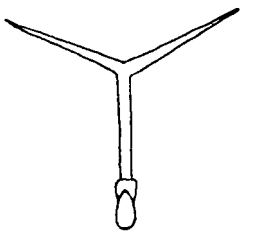
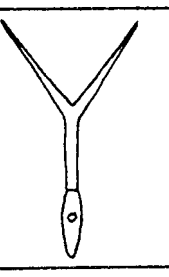
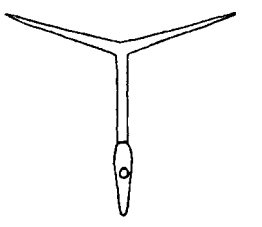
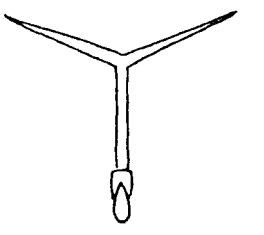
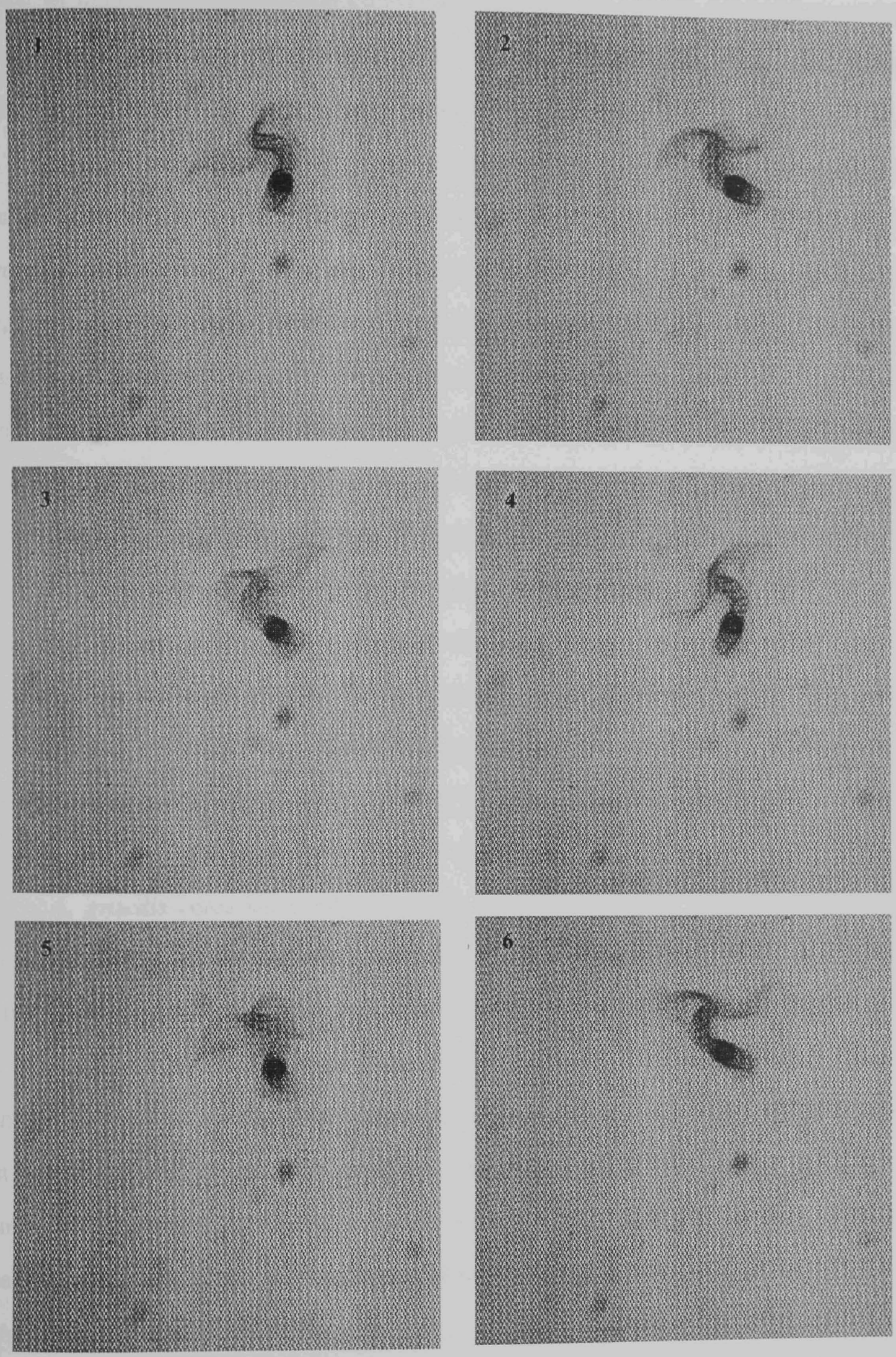
Species	Author	Posture
I. erraticus	Olsen (1970)	
I. erraticus	Swennen *et al.* (1979)	
I. erraticus	Present study	
I. variegatus	Odening & Bockhardt (1971)	
I. variegatus	Present study	
I. platycephalus	Odening, Matthesis & Bockhardt (1970)	
C. duodecaglandis (= *A. gracilis*)	Crocombe (1959)	
A. gracilis	Present study	

Fig. 103. Swimming behaviour of the cercaria of *Apatemon gracilis*.



Images follow chronologically at 160ms intervals.

5.3.2. Cercarial release patterns.

In light:dark (L:D) cycling conditions, all 3 strigeid species exhibited cercarial emergence patterns that were correlated with the L:D cycle (a significant deviation from pattern expected if emergence occurred randomly; $P<0.05$).

Fig. 104. shows that both *Ichthyocotylurus* spp. exhibit a diurnal pattern of release, with cercariae emerging predominantly during the light period; 93.3% of *I. erraticus* and 81.8% of *I. variegatus*. With the onset of the light period, the number of cercariae emerging immediately rose. There was, however, a lag of 2 hours before large numbers of cercariae were released. Numbers then increased to a peak at 1-3pm (6-8 hours after the onset of the light period), before decreasing for the remaining 6 hours of this period. The emission rates continued to decrease during the hours of darkness.

From the pooled data gathered throughout the duration of release, an average of 154 *I. erraticus* and 127 *I. variegatus* cercariae emerged daily.

The release pattern of *I. variegatus* cercariae, whose hosts were maintained in the dark from the time of infection (6-7 weeks earlier) is also shown in Fig. 104. Statistical tests indicated that no significant pattern was evident ($P>0.05$). Although the release pattern was disrupted in the absence of light, the average number of cercariae released during a 24 hour period (125) was unaffected.

A. gracilis cercariae exhibited a very different circadian rhythm to the other strigeids studied, with the majority (72.6%) emerging nocturnally (Fig. 104). In contrast to the *Ichthyocotylurus* spp., this species showed a marked and immediate response to the onset of darkness, with a rapid increase in the number of cercariae released. These numbers peaked 2-4 hours after the onset of darkness before decreasing steadily for the last 4 hours of this period. The numbers emerging actually increased for the first 2 hours of the light period before rapidly decreasing to a residual level by 3pm (8 hours after the onset of light). This level was maintained until the next dark period. The number of *A. gracilis* cercariae released in a 24 hour period was far greater than observed for the *Ichthyocotylurus* spp., with an average of 2778 specimens emerging

daily.

The duration of release from infected *V. piscinalis* was typically around 30 days for both *Ichthyocotylurus* spp., although occasionally it extended to 80 days. *A. gracilis* cercariae were generally released from infected *L. peregra* for longer periods, averaging in excess of 60 days. The number of cercariae released during the course of the infection was seen to peak at around day 10 for both *Ichthyocotylurus* spp. under L:D cycling conditions (Fig. 105). Numbers emerging during a 24 hour period were then seen to drop as the infection progressed. A peak production of *I. variegatus* cercariae, released from snails that were maintained in the dark, was achieved rather later, at around 20 days post-onset, before rapidly decreasing to lower levels (Fig. 105). Due to a break of 30 days in the monitoring of *A. gracilis* infected snails, the most productive period is unclear (Fig. 105). However, numbers of cercariae emitted were not seen to tail-off towards the latter stages of the infection, as occurred with the *Ichthyocotylurus* spp.

5.3.3. Longevity of cercariae.

Cercarial longevities at different temperatures are indicated in Table 68 and in Figs. 106-108.

Table 68. Longevity of cercariae at different temperatures.

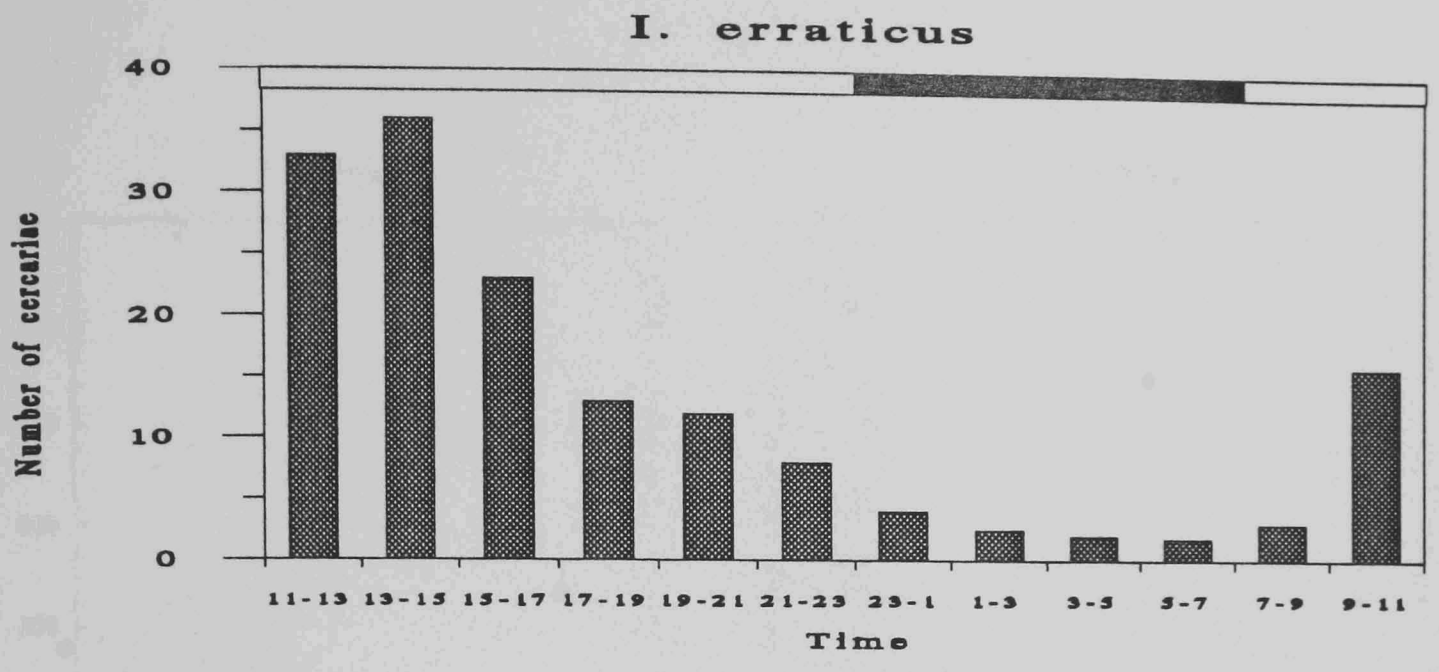
Species	Temperature (°C)	First mortalities	50% dead, extrapolated from the respective graphs (Figs 106-108)	Maximum survival
		hours post-release		
I. erraticus	15 20	2 2	20 6	49 13
I. variegatus	5 10 15 20	5 2 2 2	?* 5 23 10	24+* 14 55 23
A. gracilis	20	1	5	11

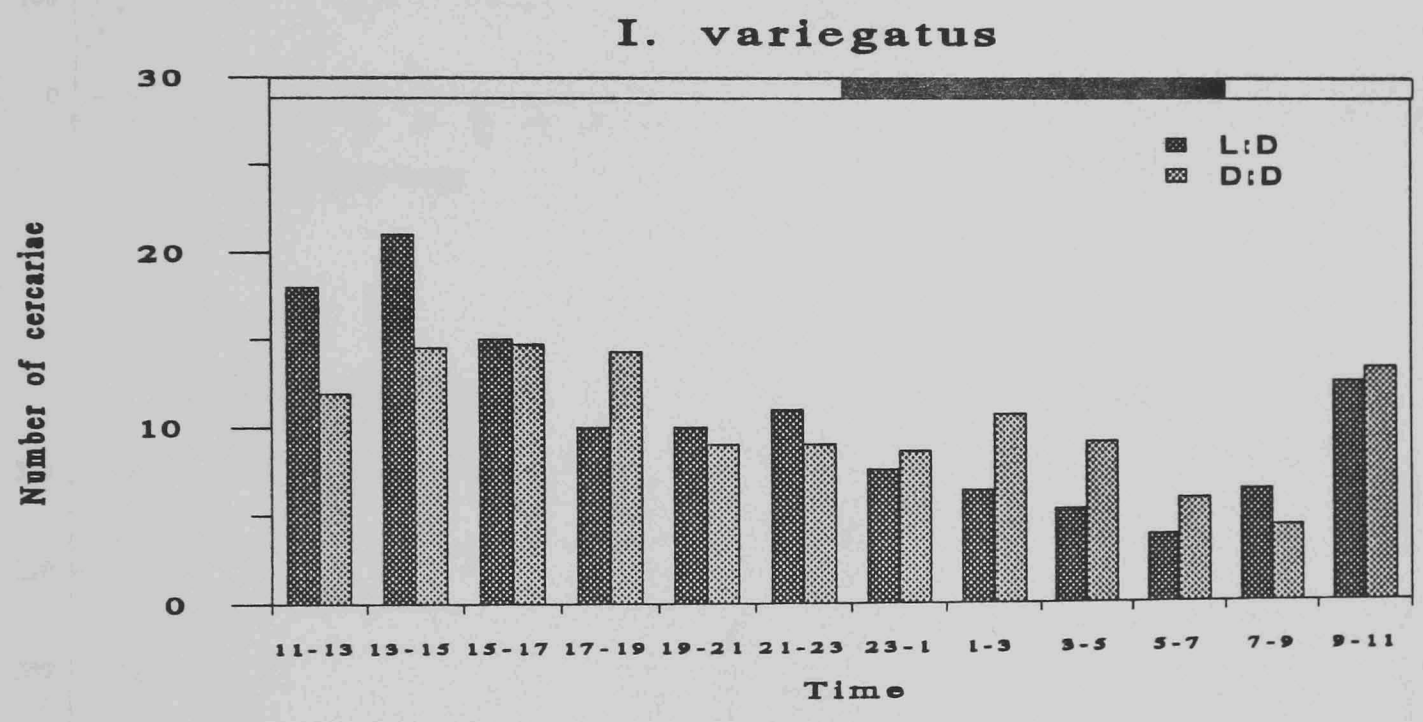
* Precise figures difficult to achieve as cercariae soon stopped swimming and dropped to the bottom where they remained alive but largely inactive. A proportion of these specimens were seen to regain activity with an increase in water temperature.

For both *Ichthyocotylurus* spp. there was a marked difference in survival at different temperatures. The longevity of the cercariae at 15°C was similar, with the first mortalities occurring after 2 hours, P50 at around 20 hours and maximal survival in excess of 2 days (Fig. 106). At a temperature of 20°C longevity was greatly reduced, by half for *I. variegatus* and by 2/3 for *I. erraticus* cercariae. Fig. 107 shows the differences for the two species at this temperature. The longevity of *I. variegatus* cercariae was also decreased at 10°C, with a maximal recorded survival of only 14 hours (Fig. 108).

A. gracilis cercariae had the shortest life-span at 20°C, with mortalities recorded within the first hour and maximal survival lasting no more than 11 hours.

Fig. 104. The emergence of strigeid cercariae from experimentally infected snails in relation to light:dark (L:D) cycling conditions or constant dark (D:D) conditions.





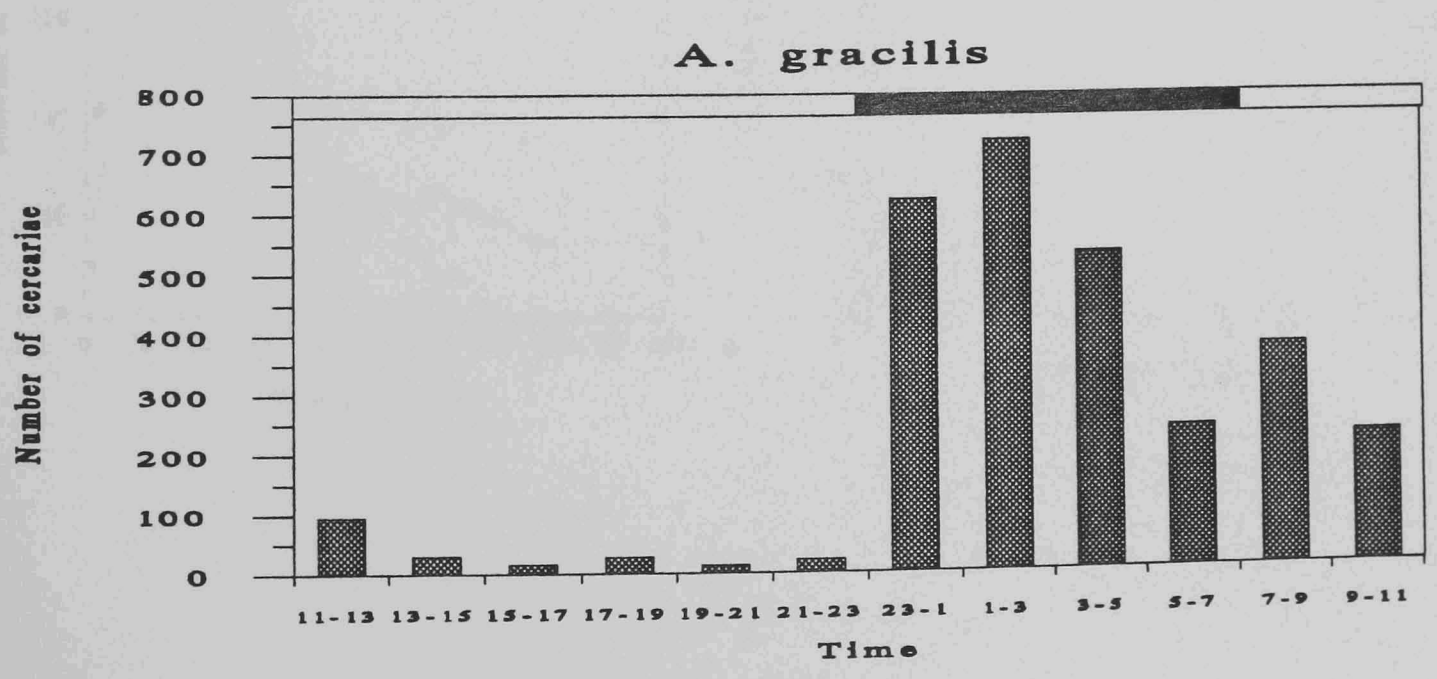
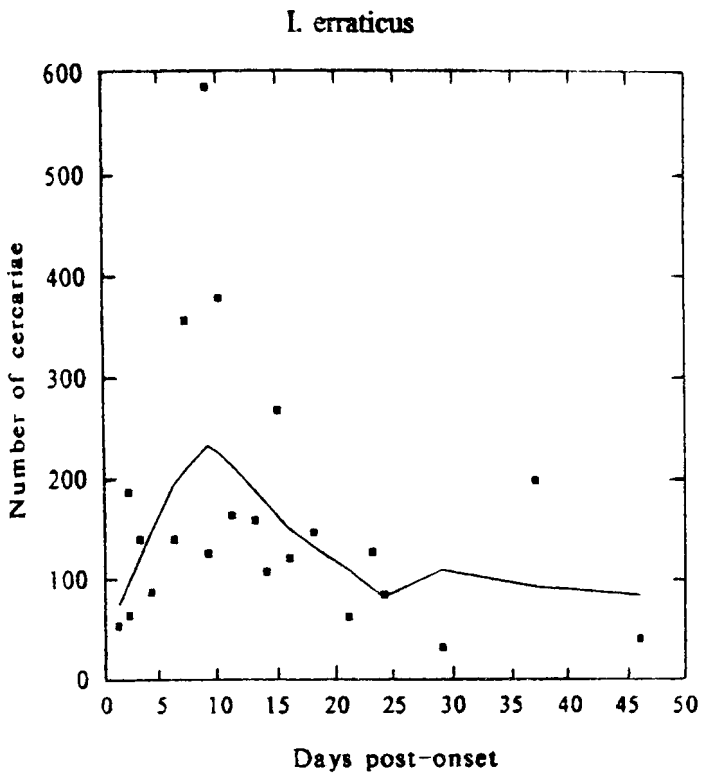
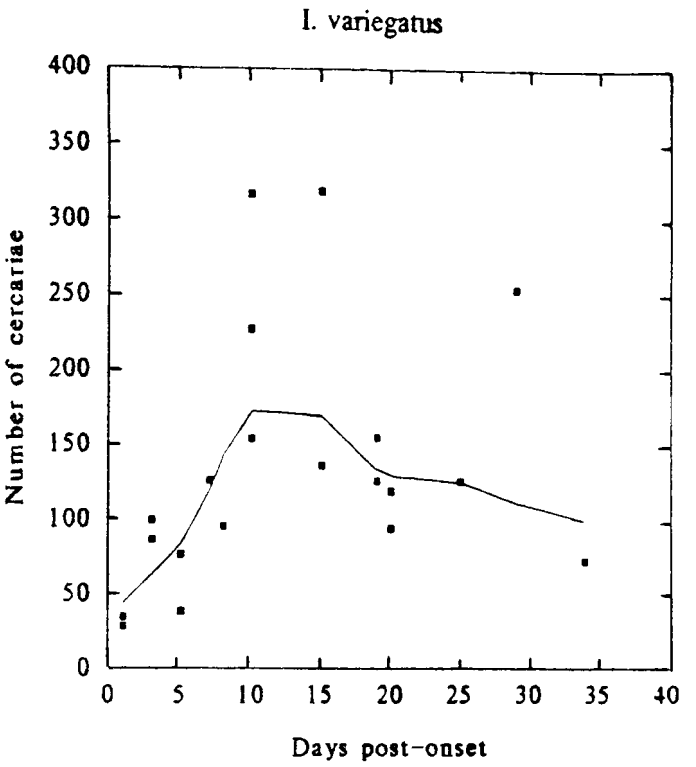
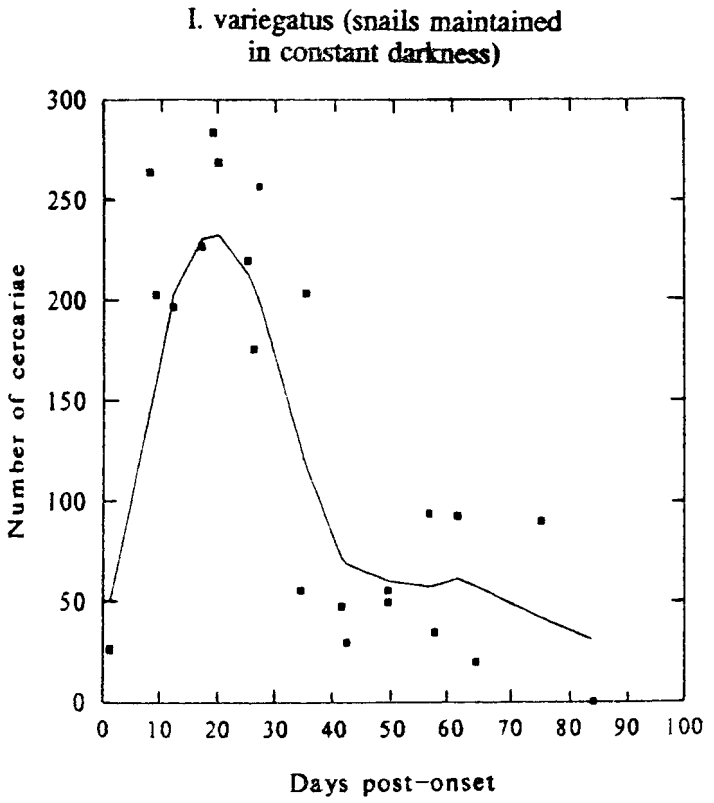
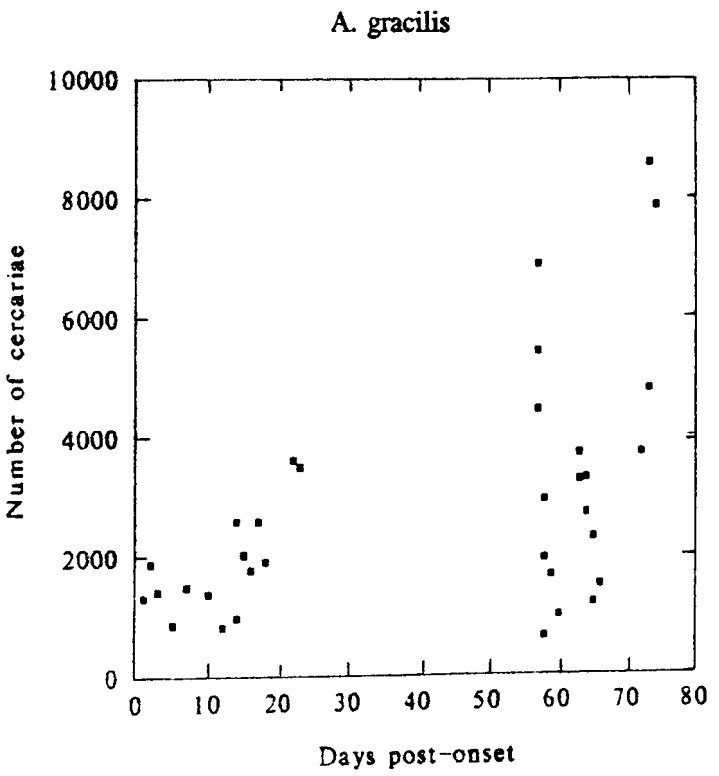


Fig. 105. The relationship between cercarial emergence during a 24 hour period and time elapsed after onset of release. Unless otherwise stated snails were maintained in light:dark cycling conditions.



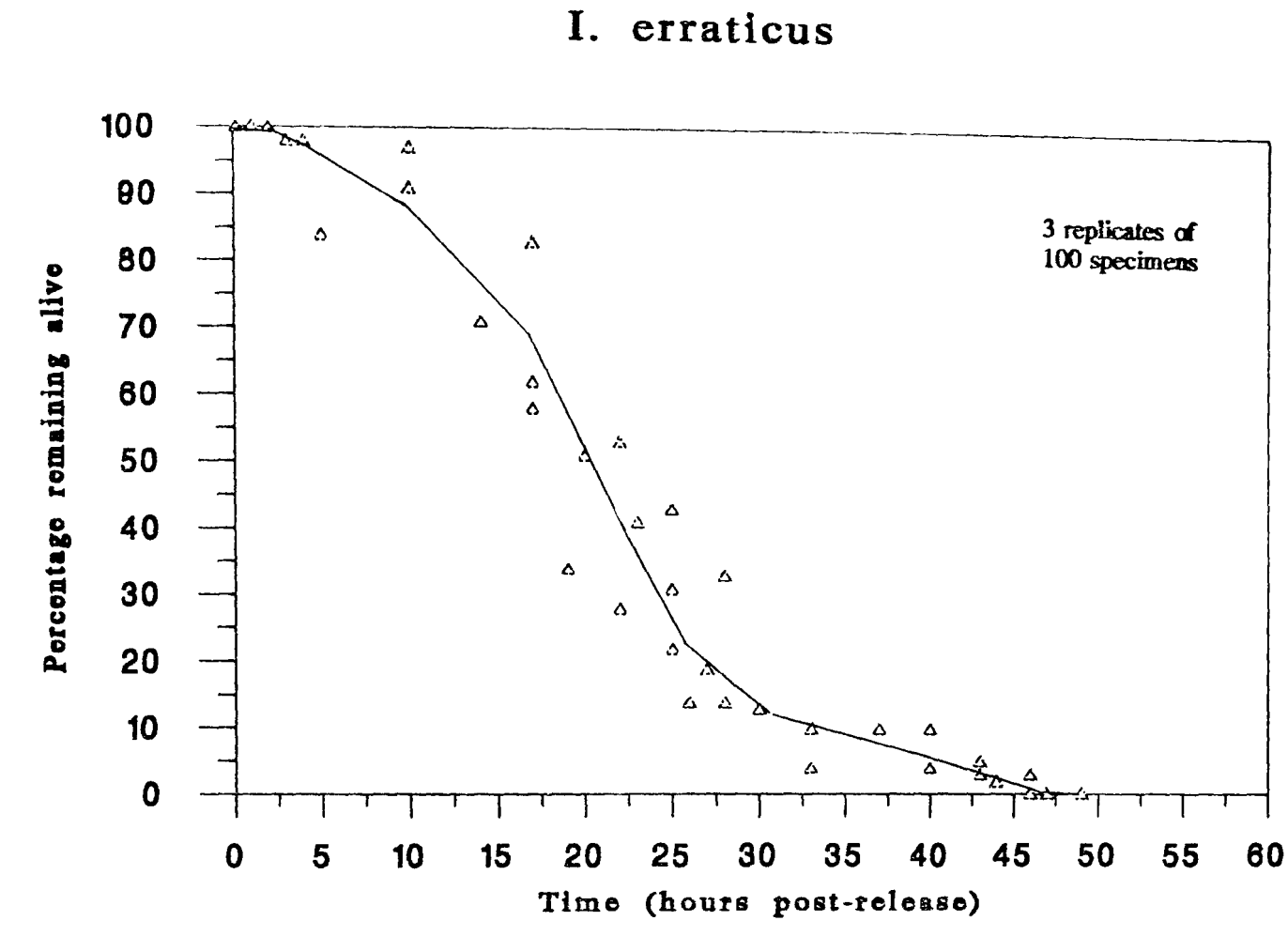






Best-fit lines through points were generated by "Systat" graphics.

Fig. 106. Longevities of strigeid cercariae at 15°C.



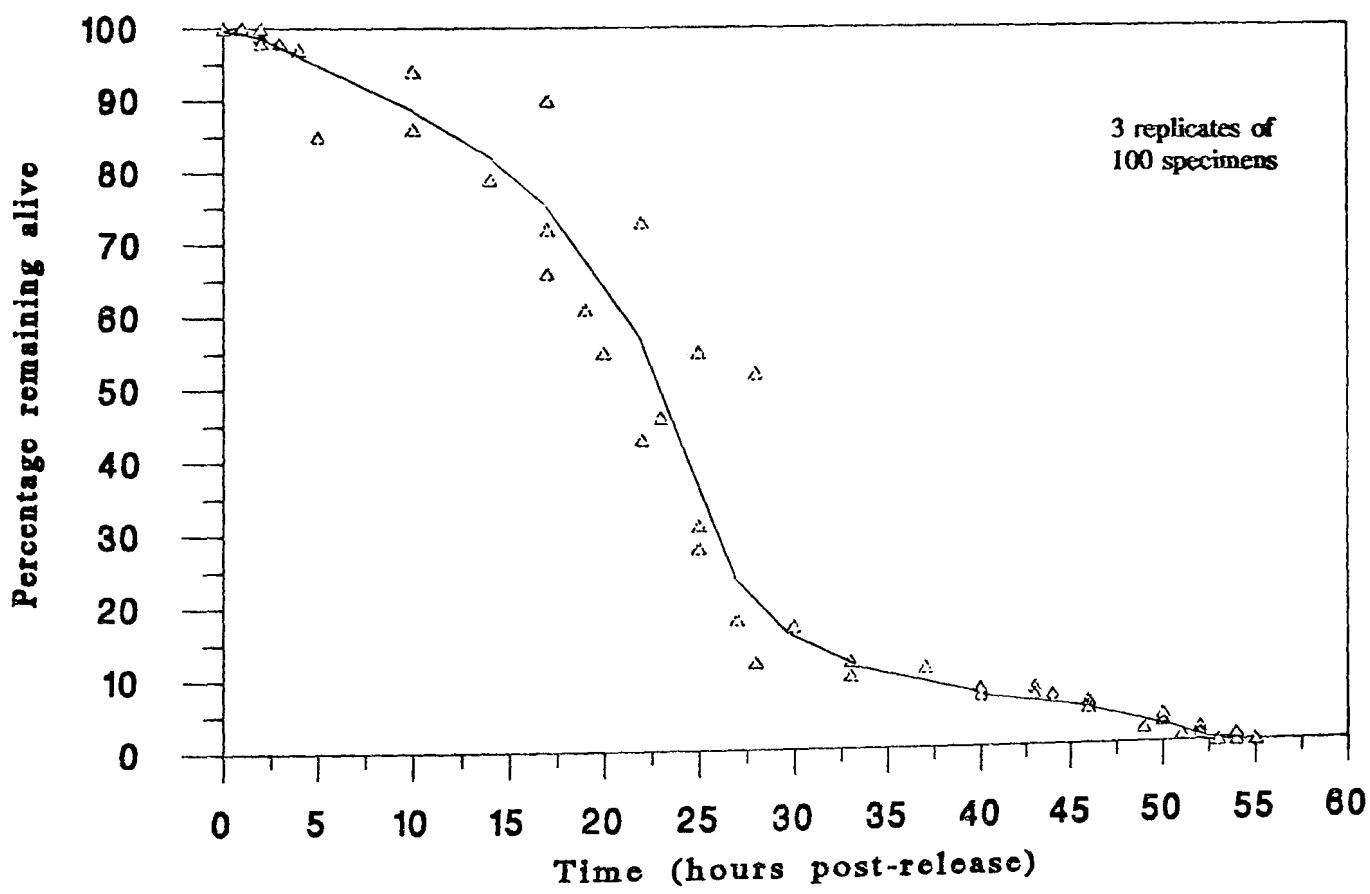
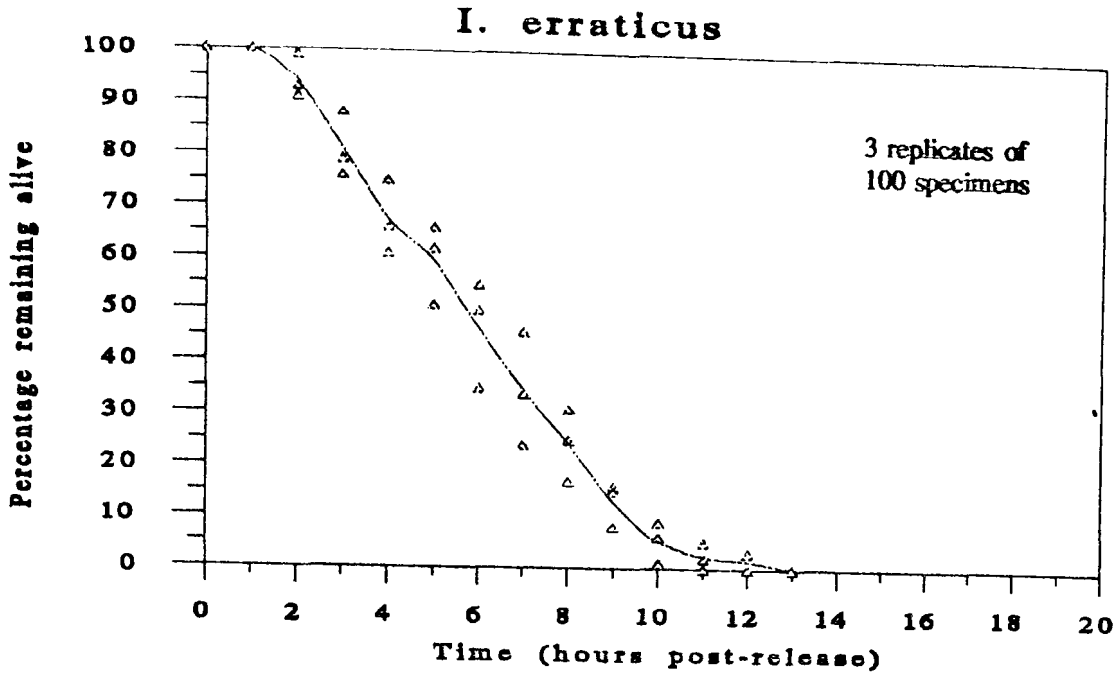
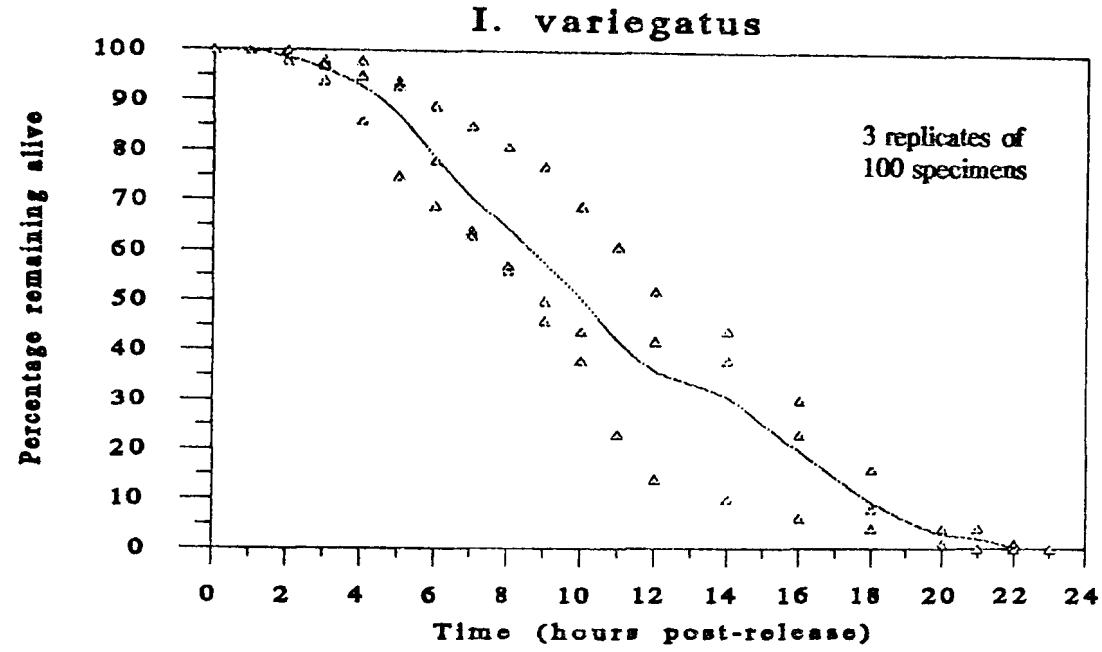


Fig. 107. Longevities of strigeid cercariae at 20°C.





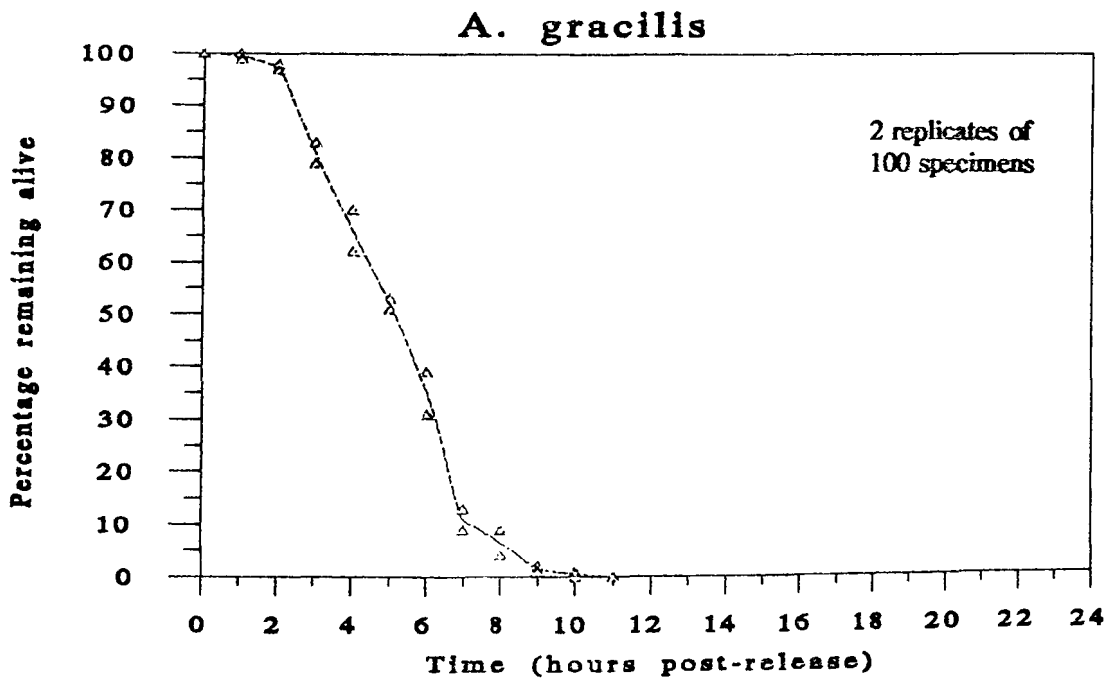
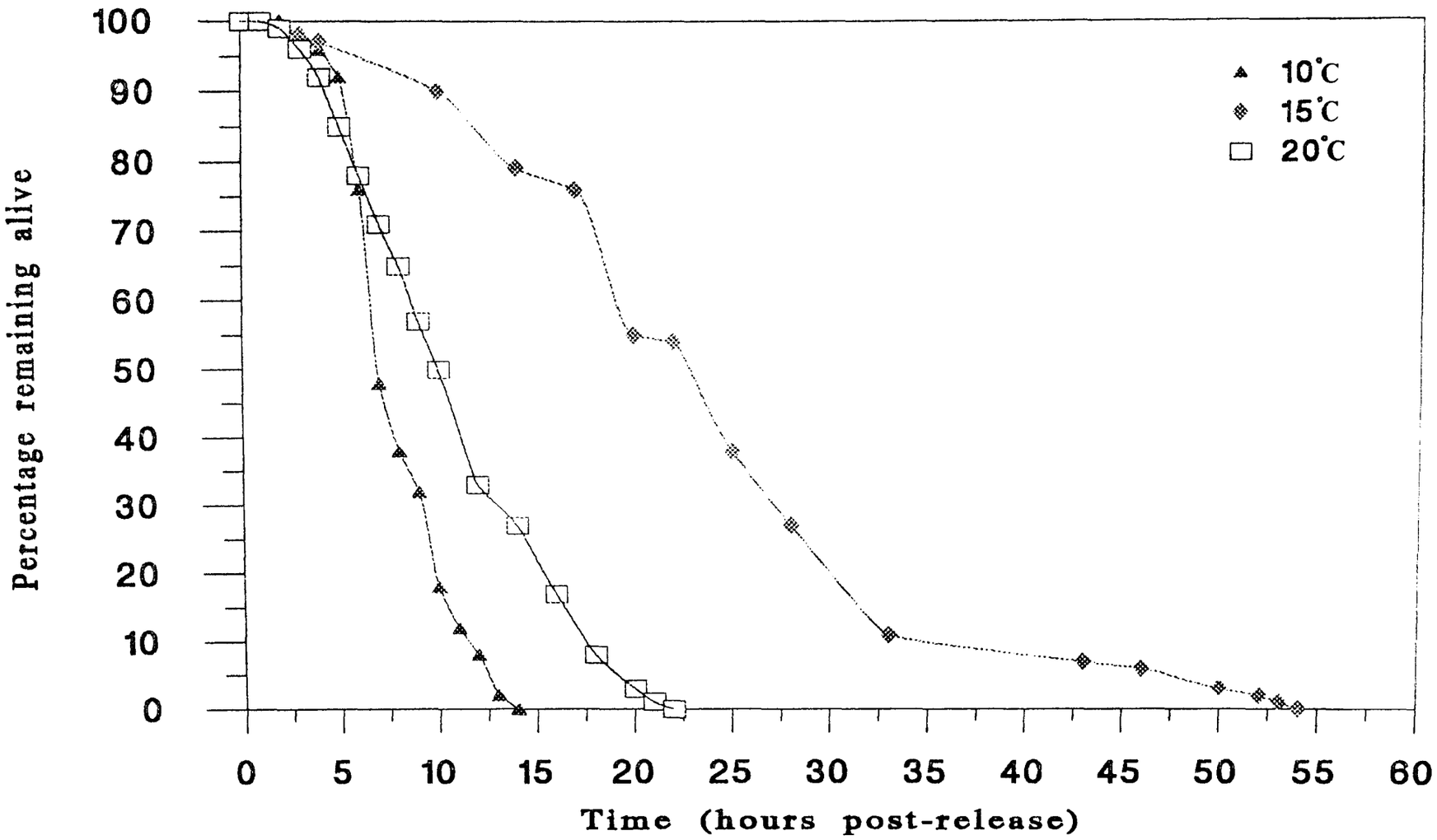


Fig. 108. Longevity of *I. variegatus* cercariae at different temperature regimes. Each point represents the mean of 3 replicates (100 specimens per replicate).



DISCUSSION

5.1. MOLLUSCAN STAGES.

5.1.1. Natural infections.

From 1,409 monitored snails only 19 (1.3%) released strigeid cercariae. Of these infections, 0.9% of *L. peregra* bore what were thought to be *A. gracilis* and 0.1% of *V. piscinalis* shed *I. variegatus* cercariae. No natural infections of *I. erraticus* or *A. annuligerum* were observed. However, this information must be considered in relation to the fact that the collections were not performed as a structured survey and consequently sample dates and sizes were erratic. Abundant *V. piscinalis* samples, in particular, were only obtained early in the year (April and June) which may have preceded infections.

Swennen *et al.* (1979) observed *Ichthyocotylurus* cercariae emerging from *V. piscinalis* specimens in the Ijsselmeer, Netherlands, during the months of July and August. The prevalence of infection was found to be low (2.4%), with *I. erraticus* accounting for 1.2% and both *I. platycephalus* and *I. variegatus* 0.6% of these infections. Similarly, Odening *et al.* (1970) and Odening & Bockhardt (1971) recorded natural infections of *V. piscinalis* with *Ichthyocotylurus* spp. to be low, with prevalences of less than 1% recorded for the Mugglesee, Germany. Odening and his co-workers did not, however, indicate any seasonality in their findings.

The prevalence of *A. gracilis* infections in *L. peregra* was found by Blair (1974) to be low, 0.5%. He observed cercariae releasing snails during the months of May (9), June (4) and September (1). In this study four of the five infections were similarly recorded in the early Summer months. The molluscan host of *A. annuligerum* remains unknown and a comprehensive survey of a water source known to harbour infected fish needs to be performed.

The *Australapatemon* sp. released from *V. piscinalis* specimens is worthy of note, as only one other record exists for a similar cercaria emerging from this genus of snail

(Odening, 1969). The cercaria observed in this study possessed 14 flame-cells, 2x4 post-acetabular penetration glands, long digestive caeca, 6 pairs of caudal bodies, 1 pair of unpigmented eye-spots, a post-acetabular transverse excretory commissure, plus typical strigeid resting posture and body spination. The unidentified *Australapatemon* sp. recorded by Odening (1969) was also released from *V. piscinalis*. His description differed from the present material in that the cercaria possessed 8 caudal bodies, no unpigmented eye-spots and both a pre- and post-acetabular commissure.

5.1.2. Experimental infections: development from miracidia to cercaria.

Strigeid cercariae were successfully obtained from experimentally infected naïve snails and developmental periods were found to differ markedly at 20°C for the two genera studied, with *A. gracilis* cercariae (mean of 32±3.5 days) typically emerging several weeks before either *I. erraticus* (mean of 42±6.8 days) or *I. variegatus* (mean of 53±7.0 days). A comparison of the results provided in Table 54 also suggests that cercarial development and release is more rapid for *I. erraticus* than the other two *Ichthyocotylurus* spp. for which data exists. The developmental durations recorded by Olson (1970) and Swennen *et al.* (1979) for *I. erraticus* did not indicate snail maintenance temperatures, rises in which can markedly accelerate developmental rates, as seen by Mattheis & Odening (1980) for *I. platycephalus* (from 28 days at 28°C to 81 days at 20°C). Elevated temperatures were also observed by Brady (1989) to decrease the developmental period of *Diplostomum* spp. within experimentally infected *L. peregra* hosts. However, Waadu & Chappell (1991) noted a longer developmental period for *D. spathaceum* in *L. peregra* at 25°C compared to 20°C. Although, they did record accelerated rates when the temperature was raised from 14°C to 20°C.

Olson (1970) noted that 24 days were required for the development of *I. erraticus* mother-sporocysts (temperature not provided), while Odening & Bockhardt (1971) and Mattheis & Odening (1980) described developmental durations of 4 and 6 weeks, respectively, for *I. variegatus* and *I. platycephalus* at 20°C. These authors did

not indicate the level of development attained at these times, and in this study mother-sporocysts bearing germ-balls were observed as early as 13 days p.i for *I. erraticus* and after 18 days for *I. variegatus*. The young mother-sporocysts were recovered from the region between the head and the foot of infected snails, the same site as recorded by Olson (1970) and Swennen *et al.* (1979) for this life-stage of *Ichthyocotylurus* spp. Daughter-sporocysts that contained cercariae, recovered from recent host mortalities or from healthy cercariae-releasing snails (dissected for karyological studies, see Chapter 7), were always located within the host's digestive gland.

Experimental infections of *A. gracilis* in *L. peregra* were particularly successful with 63% of challenged snails releasing cercariae. Indeed, this return was greater than any obtained by Brady (1989) for the miracidia of four *Diplostomum* spp. (13-40%) introduced to this snail species; although Staples (1984) was able to obtain a 100% infection of *L. peregra* with *D. spathaceum*. The developmental duration for *A. gracilis* from miracidia to cercarial release which ranged from 28 to 38 days at 20°, was recorded here for the first time. Several authors have briefly described the sporocyst stages of this species (Crocombe, 1959; Vojtek, 1964a) but further details could not be ascertained in this study due to the paucity of material available and its requirement for other investigations.

5.2. TAXONOMIC STUDIES OF CERCARIAE.

5.2.1. Light microscopic observations of cercaria.

It was found that hot 5% formalin resulted in a more satisfactory fixation of strigeid cercariae than hot 10%, with specimens maintaining a more natural posture. However, as can be seen from Tables 56, 57 and 58, different authors have applied a range of fixation techniques. In the case of *A. gracilis* cercariae, measurements taken from specimens fixed in hot 10% formalin were also included, as Blair (1974) had used this medium, enabling a more accurate comparison of results.

Morphological measurements recorded for *Ichthyocotylurus* spp. agreed with

those of previous authors, even though different fixation methods were employed (see Tables 56 and 57). Although, the total cercarial length (sum of body, tail-stem and furcal lengths) of *I. erraticus* exhibited a larger size range in the present study, with smaller individuals also present and the body breadth of this cercarial species was found to be narrower compared to previous authors. This latter result may be due to the point at which this feature was measured by the various authors. In this case it was taken at the anterior margin of the ventral sucker, which was not necessarily the widest point. A comparison of the dimensions of *I. erraticus* and *I. variegatus* cercariae did not provide any obvious measurement which could be useful in discrimination of these species.

There is some discrepancy between authors regarding the position of penetration glands for *I. erraticus* and *I. variegatus* cercariae, which, according to Blair's (1977) key, should be, and were found to be, post-acetabular. Swennen *et al.* (1979) reported the anterior pair in *I. erraticus* to be level with the ventral sucker, while Odening & Bockhardt (1971) observed this pair to be pre- or post-acetabular in *I. variegatus*. Such variation may have resulted from observations of live specimens where the glands are highly mobile due to body contractions. The only author to record more than two pairs of penetration glands was Olson (1970) for *I. erraticus*. This may represent intra-specific variation of the type noted by Niewiadomska (1970b), or possibly result from confusion, given the tortuous and often distended nature of the collecting ducts. Consequently, the work of Swennen *et al.* (1979) and the present study suggest that the specific criteria pertaining to *I. erraticus* in 5b of Blair's (1977) key ("possession of an additional pre-acetabular pair of penetration glands" which was based on Olson's (1970) observations) should be deleted. The internal morphological characteristics, which are at present used to discriminate *Ichthyocotylurus* cercariae are the number of caecal septa and the presence or absence of unpigmented eye-spots. Of these two features, only the latter was found to be reliable and even then the distinguishing presence of eye-spots in *I.*

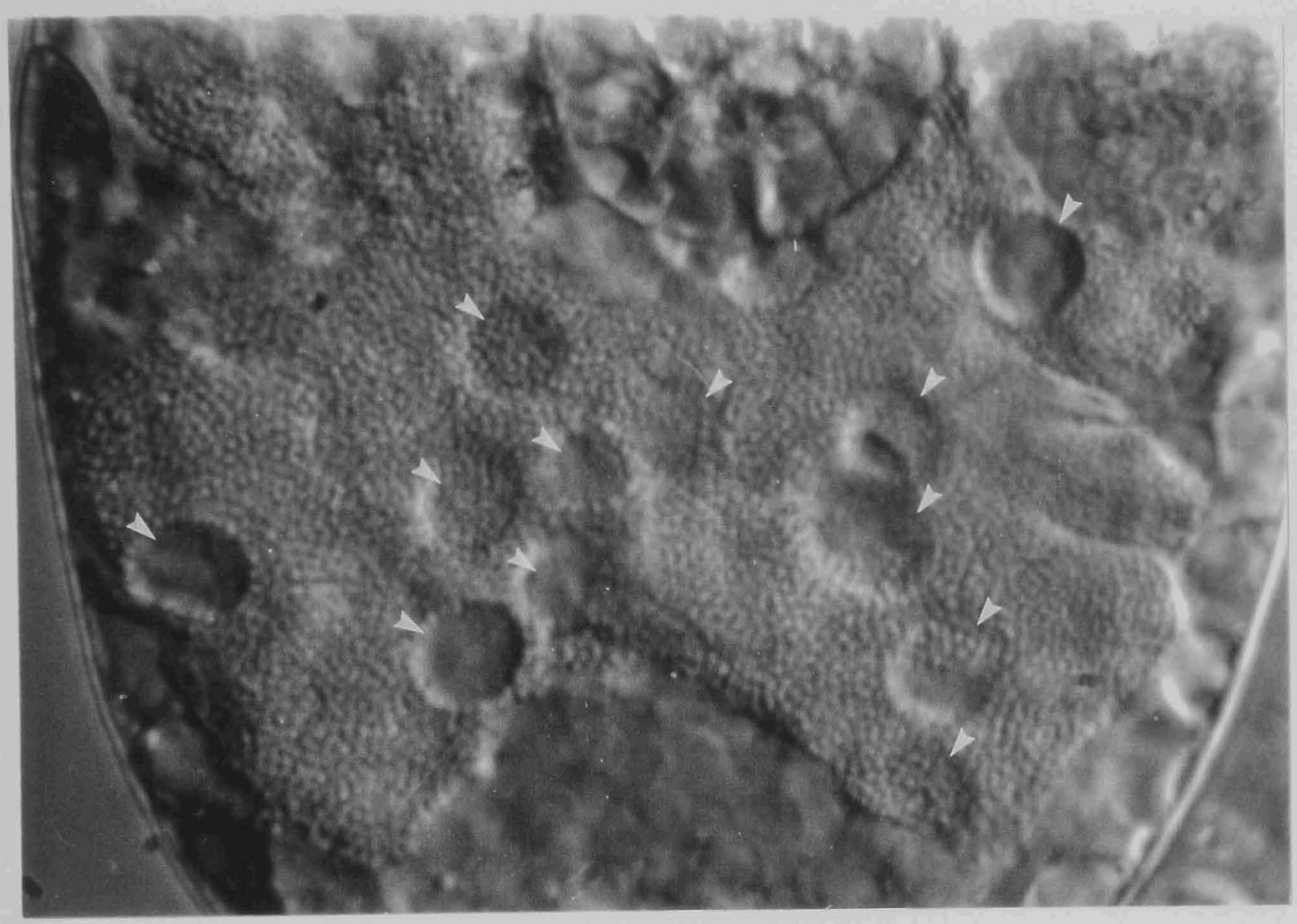
variegatus was often difficult to discern. The number of septa dividing the caeca was consistently recorded in the present study and by Swennen *et al.* (1979) as six in *I. erraticus* cercariae. This number was observed to be 6-10 (typically 6 or 7) in the present study, 10-12 by Odening & Bockhardt (1971) and 7-8 by Swennen *et al.* (1979) for *I. variegatus*; and 8-11 for *I. platycephalus* by Odening *et al.* (1970). The variation noted in *I. variegatus* from the present Scottish population makes this feature of questionable diagnostic value.

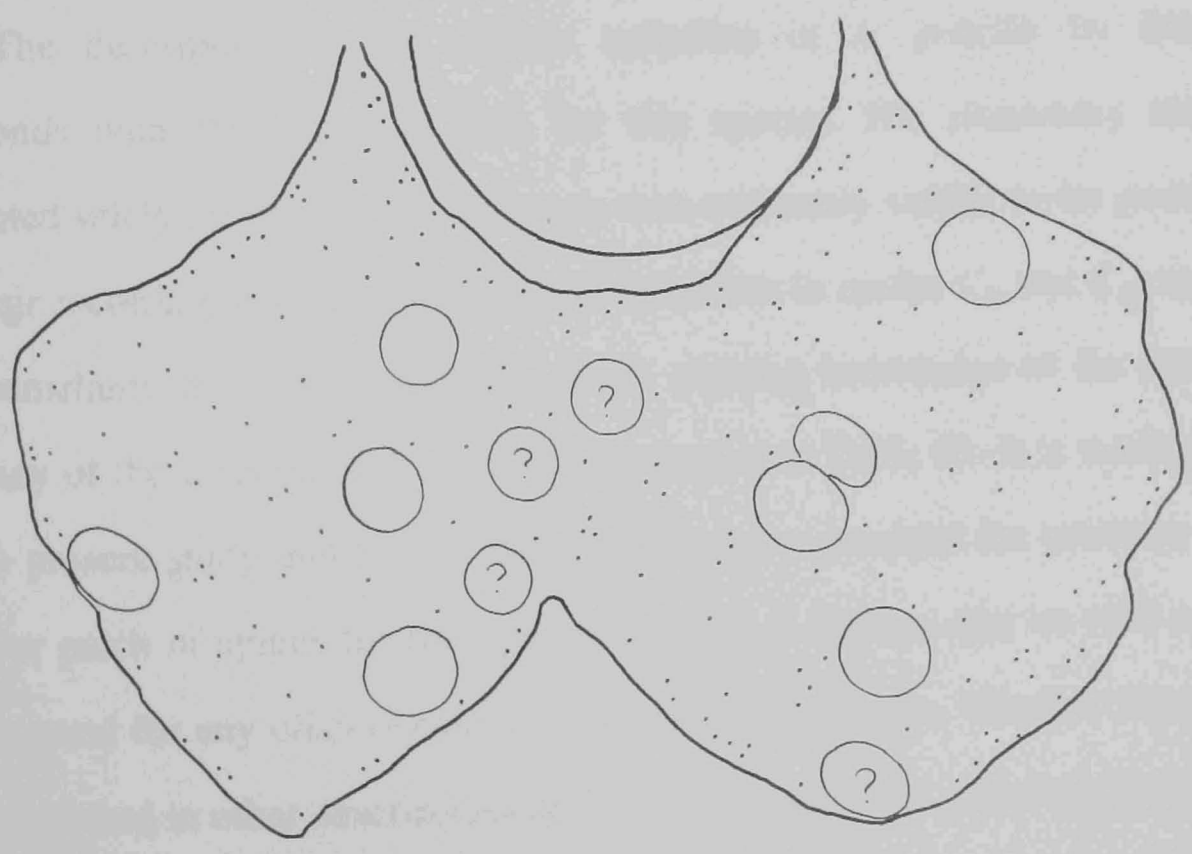
For *A. gracilis*, with the exception of Sten'ko (1977) who reported rather small dimensions, the morphological measurements recorded by the authors in Table 58 corresponded closely. Particular similarities were seen between Vojtek (1964a) and the results of the present study for material fixed in 5% formalin (A in Table 58), and between Blair (1974) and the material of the present study fixed in 10% formalin (B in Table 58). The only major difference observed in cercarial characteristics for *A. gracilis* cercariae was in the number of post-acetabular penetration glands recorded by Blair (1974, 1976, 1977). He described six rather than four pairs (all other authors) but acknowledged that "distinct boundaries between the cells are not apparent" and that no more than four collecting ducts could be discerned on each side. This total he based upon six pairs of nuclei which he stated were visible within the glandular mass. In this study, initial observation (new wet mounts) of live specimens suggested the presence of four pairs of glands, the boundaries of which became less clear with time. As water continued to evaporate, causing compression of the live specimens, the nuclei became more visible and these often appeared to total 12 (Fig. 109). Whether, boundaries separating glands are consistently not visible and six glands do drain to four ducts (six pairs of glands) or, the extra apparent nuclei represent the impression of other structures (four pairs of glands) is unclear and requires more specific study.

Collectively, light microscopical observations of the morphology of *I. erraticus* and *I. variegatus* cercariae were not found to provide adequate information for the

confident separation of the two species. Consequently, behavioural aspects were studied and other taxonomic techniques applied in an attempt to isolate diagnostic criteria.

Fig. 109. Photomicrograph of the penetration glands of an *A. gracilis* cercaria. Arrowheads indicate what are thought to represent nuclei.





5.2.2. Discrimination of *Ichthyocotylurus erraticus* and *I. variegatus* cercariae by Principal Components Analysis of metrical features.

Multivariate analysis has not previously been applied to the dimensions and positions of morphological characters in an attempt to separate similar cercariae of different species, although it has, as discussed in 5.2.4, been performed on the number and distribution of sensilla. Unfortunately, this technique failed to discriminate the two cercarial species, emphasising their morphological similarity, and no features of taxonomic use were isolated. The two methods of data standardisation employed were not found to affect the analyses.

5.2.3. Chaetotaxy and Scanning Electron Microscopy of cercariae.

Silver staining and SEM observation of specimens has enabled the chaetotaxy and armature of two *Ichthyocotylurus* spp. to be studied in detail for the first time. Differences were recorded in the armature of these species, with *I. erraticus* generally possessing more spines in the pre-oral tuft and a greater number of post-oral rows; but, although the mean numbers of these features differed, there was a large overlap in their ranges and they were not found to be significant ($P \geq 0.05$).

The description of the surface spination of *A. gracilis* by Blair (1974) corresponds with that recorded here for this species. His chaetotaxy results were represented solely by figures, but these too were extremely similar to the present results, with Blair recording a single extra ventral sensillum in cycles C_{II} and C_{III} and one less dorsal sensillum in C_I. A comparison of the existing knowledge of the armature and chaetotaxy of the cercaria of *A. gracilis* is provided in Table 63. It is worth noting that only the present study and that of Blair (1974) have recorded the presence of a post-acetabular patch of spines for this species. Indeed, it appears that no such spines have been observed for any other cercariae of the genus *Apatemon*. Whether this feature has been overlooked in other descriptions or is absent is unknown, although the latter option seems more likely as most authors do indicate ventral sucker spination (see review of Combes, 1980).

Figures in Combes' *World Atlas of Cercariae* (1980) indicate that the presence of a post-oral collar of spines and sparse body spination is ubiquitous among the studied strigeid cercariae. A pre-oral tuft of spines, however, was either absent or not observed in certain genera (e.g. *Strigea* spp.).

All descriptions of *Ichthyocotylurus* cercariae (see Table 62) record five pairs of sensilla postero-laterally on the tail-stem. In each case antero-lateral sensilla were also present: *I. erraticus* - two pairs, Swennen *et al.* (1979) and present study; *I. variegatus* - three pairs, Odening & Bockhardt (1971) and two pairs, present study; *I. platycephalus* - three pairs, Odening *et al.* (1970). Odening & Bockhardt (1971) also observed a mid-lateral pair of sensilla on each side of the tail-stem of *I. variegatus*. The discrepancies for *I. variegatus* cercariae between Odening & Bockhardt (1971) and the present study (Odening & Bockhardt recorded an additional pair of antero-lateral sensilla and the presence of a mid-lateral pair) may be due to the limitations of light microscopical observations on unstained specimens. It is possible that dorsal/ventral sensilla (not observed by Odening & Bockhardt, 1971) might have been confused with lateral sensilla because of the long cilia involved. Bayssade-Dufour (1979) suggested that members of the Strigeidae possess only postero-lateral sensilla, while diplostomid species have two groups of lateral sensilla, anterior and posterior. The presence of antero-lateral sensilla in *Ichthyocotylurus* spp. weakens her proposal that the main difference in cercarial chaetotaxy between the Diplostomidae and Strigeidae is the position of the lateral sensilla on the tail-stem. Kostadinova (1993) noted the lack of complete chaetotaxy descriptions of strigeid cercariae and commented on the variability of lateral tail-stem sensilla within this family, resulting in its unsuitability as a character for differentiation at this level. There does, however, appear to be some taxonomic value to this feature at the subgeneric/generic level, since *Apatemon* (*Apatemon*) spp. possess no antero-lateral sensilla (Blair, 1974; Bayssade-Dufour, 1979; present study), those of *A.* (*Australapatemon*) spp. (Cort & Brook, 1928; Iles, 1959) and *Cotylurus* spp. (Basch, 1969; Zavicek & Valenta, 1964; Shigin, 1974; Richard, 1982; Zazornova, 1987) tend

to be evenly spread along the tail-stem and *Ichthyocotylurus* cercariae bear anterior and posterior groups (Odening & Bockhardt, 1971; Odening *et al.*, 1970; Swennen *et al.*, 1979; and present study).

The number and arrangement of sensilla on the ventral sucker exhibit a high degree of constancy in the families Schistosomatidae: $3S_1 + 1S_2 = 4$ (see Richard, 1971; Bayssade-Dufours, 1979) and the Diplostomidae: $3S_1 + 6S_2 = 9$ (see Richard, 1971; Blair, 1974; Niewiadomska, 1987; Brady, 1989; Niewiadomska & Kiseliene, 1990). However, descriptions of these families are taken primarily from single genera, *Schistosoma* and *Diplostomum*, respectively, and may be of generic rather than familial significance. There appears to be far less uniformity in the number of ventral sucker sensilla amongst members of the Strigeidae with known chaetotaxy patterns: *Apatemon (Apatemon) gracilis* - three (Blair, 1974; present study); *A. (Australapatemon) minor* Yamaguti, 1933 - six (Blair, 1974; Shigin, 1974); *Apharyngostrigea* sp. - eight (Shigin, 1974); *Cotylurus hebraicus* Dubois, 1938 - ten (figure) or 14 (text) (Zazornova, 1987); *C. brevis* Dubois & Rausch, 1950 - ten (Richard, 1982); *C. cornutus* (Rudolphi, 1808) - 12 (Shigin, 1974); and *Ichthyocotylurus erraticus* and *I. variegatus* - ten (present study). It seems that cercariae of the Strigeidae exhibit variation between some subgenera as well as similarities between different genera in relation to this feature. The distribution of ventral sucker sensilla ($4S_1 + 6S_2 = 10$) recorded for most *Cotylurus* and all *Ichthyocotylurus* spp. is not even unique to these closely related genera, also being reported by Kostadinova (1993) for the diplostomid *Codonocephalus urniger* (Rudolphi, 1819).

The results obtained in the present study have indicated that chaetotaxy and SEM do not provide a method for the confident discrimination of *I. erraticus* and *I. variegatus* cercariae. Other detailed chaetotaxy studies are required before it is possible to distinguish groups of sensilla common to genera or which might discriminate species within the family Strigeidae.

Mohandas (1971) was able to distinguish three types of papillae (sensilla) on

silver-stained cercariae. These appeared as disc-like rings, with or without "setae" (cilia). Even earlier, Wagner (1961) observed raised papillae bearing "setae" on the body surface of the cercaria of *Schistosoma mansoni* and "stalked papillae" on the anterior margin of the oral sucker. These findings were later described in greater detail by Robson & Erasmus (1970) using scanning and transmission electron microscopy. They discovered that the "stalked papillae" consisted of a proud conical bulb, terminating apically in a sheath bearing a cilium. SEM images enabled Køie (1973) to find cilium-like processes on the surface of *Neophasis lageniformis* (Lebour, 1910) cercariae and similar simple sensilla were described by Rees (1971) on the cercaria of *Parorchis acanthus* (Nicoll, 1906). Pariselle & Matricon-Gondran (1985) identified some six types of surface receptors on *Nicolla gallica* (Dollfus, 1941) cercariae, several with complex concentric sets of "villi" originating from a single collar. Multiciliate sensilla were also recorded by Fried & Fujino (1987) on *Echinostoma revolutum* (Frölich, 1802) cercariae.

Scanning electron microscopy revealed four types of sensilla present on *I. erraticus*, *I. variegatus* and *A. gracilis* cercariae, differing in external structure. All of these types were visible in silver stained preparations but could only be differentiated with the SEM. This technique also enabled Niewiadomska & Nasincova (1990) to identify four forms of sensilla in the cercaria of *Diplostomum paracaudum* (Iles, 1959). Three of their forms closely resemble those described here: sensilla with a long cilium and loose, short collar; sensilla with a cilium of intermediate length; and sensilla with a short cilium and tight collar. They did not observe any aciliate sensilla but recorded a type with a short cilium and no collar. The latter form was located around the margins of the oral orifice and bears some similarity to the five sensilla of the C_I cycle with poorly defined collars recorded for *Ichthyocotylurus* spp. It appears that no author has so far identified multiciliate sensory receptors for cercariae of the Superfamily Strigeoidea. Niewiadomska & Nasincova (1990) have suggested that variation in the outer sensilla structure corresponds to differences in internal structure, as was found in TEM investigations of sensory endings of *D. pseudospathaceum* Niewiadomska, 1984

cercariae by Czubaj & Niewiadomska (1992). Niewiadomska & Nasincova (1990) found that the distribution of different sensilla types was stable in *D. paracaudum*; this was also true of the species examined here. Similarities in sensilla types between *D. paracaudum* and cercariae in the present study are not surprising if they reflect function, as in each case the next host is a freshwater fish.

5.2.4. Discrimination of *I. erraticus* and *I. variegatus* cercariae by Principal Components Analysis of sensilla number and distribution.

Although the chaetotaxy pattern recorded for *I. erraticus* and *I. variegatus* cercariae was identical, multivariate analysis on the number and distribution of sensilla did enable the successful discrimination of the two species. Separation was achieved according to 95% confidence limits on the centroid. While this indicated distinct differences between the two populations, it would not necessarily provide the correct identification of a specimen newly introduced to the analysis. *I. erraticus* cercariae were found to have ventral surface sensilla which were less spread along the body and fewer tail-stem sensilla than *I. variegatus* cercariae, but larger distances between dorsal surface sensilla across the body (see Table 64). Although present, the differences in these measurements showed a wide overlap in their ranges and did not, unfortunately, provide individual features which could be used for the discrimination of the two species. The multivariate techniques applied to the sensillary distribution on the surface of *Schistosoma* cercariae by Bayssade-Dufour *et al.* (1989), Cabaret *et al.* (1990) and Albaret *et al.* (1993) were found to be of greater diagnostic utility. Their analyses using chaetotaxic indices provided discrete groupings, enabling the confident identification of the vast majority of specimens. The differences in utility recorded between the present study and the authors above are more likely to result from the cercariae investigated, i.e. less variation in sensillary position of the *Ichthyocotylurus* spp. than the *Schistosoma* spp. investigated, than the technique employed, as PCA has proved highly sensitive in earlier chapters of this study (see 2.2.4 and 3.2.2; Results) and by previous workers (see Chapter 2.2; Introduction).

5.2.5. Transmission electron microscope observations of *I. variegatus* cercarial sensilla

On the basis of internal structure, three distinct forms of uniciliate sensilla were identified for the cercaria of *I. variegatus*. These different types corresponded in position to the forms distinguished by SEM and comprised: SEM types a and b; SEM type c; and SEM type d. Although, types a and b were seen to share a common internal arrangement they are still considered to differ due to the characteristic length of their cilium.

Sections obtained of types a and b sensilla were rather thick and further preparations of an improved quality are required for a detailed description of these sensory receptors. A similar sensillum to types a and b was shown in a figure by Morris (1971, fig. 10) for the cercaria of *Schistosoma mansoni.* Sensilla described as type d shared several common features with types a and b; the presence of ciliary rootlets and an electron-dense plate. However, type d sensilla lacked a tegumentary collar, while possessing an electron-dense lining which was bound to the tegument by a cap-like desmosome. There does not appear to be any similar cercarial sensory receptor so far described in the literature. Type c sensilla bear some similarities to uniciliate receptors described by other authors, but differ from most in having three electron-dense rings (rather than one or two) within the nerve bulb and the presence of the sheath-like collar.

Neither aciliate or multiciliate receptors were observed for *I. variegatus* cercariae, although confirmation of their absence would require the examination of a greater number of specimens. From the descriptions available in the literature, it appears that ciliated types of sensory receptors dominate in cercariae, while in metacercariae and adults nonciliated forms are equally common. If sensillary structure reflects function, then these subtegumental non-ciliate forms, which are thought to be receptive to pressure or stretching (Bennett, 1975; Hoole & Mitchell, 1981; Ip & Desser, 1984; Torii *et al.*, 1989), would be of great value during penetration and migration within the second intermediate host, and perhaps they are rapidly acquired along with the other changes in tegumental structure which occur at this time (McLaren, 1980). The function

most commonly attributed to uniciliate sensilla is that of 'tangoreceptors' which are sensitive to water borne vibrations (Hockley, 1973; Nuttman, 1971; Gibson, 1974; Pariselle & Matricon-Gondran, 1985), although rheo- and chemosensory roles have also been postulated for the various forms, particularly when the cilium is modified (Edwards, Nollen & Nadakavukaren 1977; Hoole & Mitchell, 1981). Monogenean multiciliate sensilla were considered by Lyons (1972) to be chemosensory and this is generally thought to be true of similar structures in cercariae (Nuttman, 1971; Fujino, Ishii & Choi, 1979; McLaren, 1980; Zdárská, Našincová, Štěrba & Valkounová, 1987). No multiciliate receptors have as yet been demonstrated for adult digeneans.

5.3. BEHAVIOURAL STUDIES.

5.3.1. Cercarial emergence strategies and swimming behaviour

The swimming behaviour of *A. gracilis* noted here corresponds with that described by Blair (1974) in so far as the cercaria was very active, swimming tail first, pausing rarely and then for less than a second. However, the mechanism observed for this swimming action appeared rather more complex than that proposed by Blair (1974). He stated that "It swims tail first by strong lateral flexing of the tail-stem, the furcae being used as oars, and the body held extended". This description omits that, whilst following a straight (not helical) path the cercaria rotates about its long axis with the furcae acting like a propeller and performing a spinning 'frontcrawl-like' stroke. Whether such a motion could be created by purely lateral tail-stem flexions would depend upon the angle of the flattened furcae as they cut through the water and would require further study to elucidate.

The resting posture of this cercarial species, shown in Table 67, differs from that indicated by Crocombe (1959) for *Cercaria duodecaglandis* (which he considered to represent *A. gracilis*) in that he recorded no ventral bend to the body. Crocombe (1959) noted that *C. duodecaglandis* exhibited no response to light, in contrast to the negative phototaxis observed here for *A. gracilis*. These behavioural differences of *C.*

duodecaglandis raise further doubt as to the identity of this cercaria (see 5.1; Introduction). The negative phototaxis demonstrated by *A. gracilis* cercariae may play some role in their ability to locate a suitable second intermediate host, several of which are bottom dwelling fish species (see Chapter 2.1; Results).

Both *Ichthyocotylurus* spp. studied were found to emerge from the terminal ctenidial lamellae of infected *V. piscinalis*. The only previous description of cercariae emerging from *V. piscinalis* was made by Wesenberg-Lund (1934). This author described the furcocercaria as *Cercaria longiremis* and gave details of its emergence from the terminal lamellae. It would be interesting to monitor the release of other cercarial species (e.g. the Xiphidocercaria sp. and unidentified *Australapatemon* sp., see 5.1.1; Results) from infected *Valvata piscinalis* to see if the gill is the sole emergence route from this snail.

Migration of *Ichthyocotylurus* cercariae, from the snail's digestive gland to the site of emergence (ctenidium), was found to occur via the vascular system. Such a utilisation of the host's blood vessels represents the most commonly observed emergence route for cercarial species (see 5.3; Introduction).

The swimming behaviour observed for *Ichthyocotylurus* cercariae, consisting of a resting phase where the cercaria slowly sinks through the water column and a short active phase where the cercaria rises diagonally to a higher level, would enable the cercaria to patrol a large proportion of the water column with the minimum expenditure of energy. The presence of a shadow response may be important, particularly during the resting phase, in the location of the fish second intermediate hosts (see 5.3.2; Discussion).

The cercarial resting postures indicated by Olson (1970) and Swennen *et al.* (1979) for *I. erraticus* show a lateral flexure of the body, not ventral as recorded here (Table 67). These observations may be equivalent and just show different representations of such a posture. A similar discrepancy was present between the figures of Odening & Bockhardt (1971) and the present study for *I. variegatus* cercariae. The

posture displayed by *I. platycephalus* cercariae appears to differ from the other *Ichthyocotylurus* spp. in not showing any flexure of the body (Odening *et al.*, 1970).

5.3.2. Cercarial release patterns

All three strigeid species were found to exhibit cercarial emergence patterns which correlated with the imposed light:dark cycle. Both *Ichthyocotylurus* spp. demonstrated a latent period before the daily onset of emergence and similar delays have been observed by other authors. The amount of stimulation (or removal of inhibition) required may be minimal, emergence beginning almost simultaneously with the onset of light/darkness, as occurs in *S. curasoni* and *S. bovis* (see Mouchet *et al.*, 1990). Alternatively, a lengthy period of stimulation may be required, with emergence not occurring until the middle or latter half of the productive conditions: *S. intercalatum* from Zaïre and *S. intercalatum* from Cameroon, respectively (Pagès & Théron, 1990).

Swennen *et al.* (1979) recorded that naturally infected *V. piscinalis* regularly produced 100 *Ichthyocotylurus* cercariae a day (species not specified) and that the maximum number observed was 190. Their experimental infections were less productive with only 30-40 cercariae released daily. Here, experimental infections were found to liberate daily, an average of 154 *I. erraticus* and 127 *I. variegatus* cercariae.

Unfortunately, the data obtained on the shedding patterns of these two species provided no basis for their discrimination. The common rhythm recorded suggests that light acts as a trigger for the release of mature cercariae. After several productive hours this supply of cercariae declines with a corresponding decrease in emission. During the hours of darkness maturation proceeds and a new batch is available for release with the renewal of the stimulus.

As stated in the results, there was no significant emergence pattern recorded from *I. variegatus* infected snails that were maintained in constant darkness. However, it is interesting to note, that maximum and minimum numbers of these cercariae were released at a particular time, 3-5pm and 7-9am, respectively (Fig. 104), and that both

of these periods were 2 hours later than the equivalent peak and trough recorded for *I. variegatus* under L:D cycling conditions. Although the circadian rhythm was not as marked, these results initially suggested, before the application of statistical tests, that the cycle had been displaced. Nevertheless, the statistical tests indicated, that for *I. variegatus*, in the absence of a release stimulus, the dichotomy of maturation and release phases is lost and cercarial emergence becomes random. Ginetsinskaya (1988) stated that certain cercariae totally stop emerging into the water after infected molluscs are placed in darkness. Cercariae of other species may gradually become adapted to darkness, but the number of larvae emerging from the mollusc never reaches the same level observed in conditions of light (Rees, 1947). In laboratory experiments, Lewis, Welsford & Uglem (1989) found that exposure of *Proterometra macrostoma* and *P. edneyi* infected snails to constant light or constant dark resulted in a disruption of their emergence cycles, with equal numbers released during the first and second 12 hour periods corresponding to light and dark in controls. The daily shedding rate of *P. edneyi* was not affected by either L:L or D:D, while numbers were decreased by 42% for *P. macrostoma* under conditions of constant dark. In the present experiment, the number of *I. variegatus* cercariae emerging was unaffected by the absence of light.

A. gracilis cercariae were also found to exhibit a circadian release rhythm, but with the majority emerging nocturnally and without a latent period before onset. This pattern of release suggests that darkness acts as the stimulus for release of *A. gracilis* cercariae. Light was not seen to inhibit emission with sizable numbers still being produced during the first few hours of this period; rather, numbers decreased as the mature cercarial population was depleted. The total number of *A. gracilis* cercariae liberated during a 24 hour period was far higher than recorded for the *Ichthyocotylurus* spp., with an average of 2,913 released.

Unfortunately, due to the low subsample sizes (resulting from the deaths of

monitored snails) and the variation in numbers released from individual snails, definite trends in cercarial production throughout the duration of the infection could not be identified. Fig. 105 suggests that *I. erraticus* cercarial emission was greatest around 10 days after the onset of release, while the highest daily output of *I. variegatus* cercariae occurred between days 10 and 20 post-onset. With all three species, cercarial release was generally terminated by the death of the snail host. Cercarial emergence limited by snail mortality was also reported by Fryer & Probert (1988) for Nigerian bulinids infected with two strains of *S. haematobium* and by De Bont, Vercruysse, Van Aken, Southgate & Rollinson (1991) for *Indoplanorbis exustus* infected with *Schistosoma* spp. However, Webbe & James (1972) and Pflüger, Roushdy & El-Emam (1984) both recorded molluscan "self-cure" from *S. haematobium* infections. The cercarial output of *S. haematobium* through the course of infections in *Bulinus truncatus*, *B. senegalensis* and *B. globosus* was found to peak within a few weeks of the onset of shedding, before declining until the hosts' death (Fryer & Probert, 1988). These authors found that the durations of infection varied according to the strain of parasite and host species utilised. In studying the dynamics of *S. rodhaini* cercarial emergence from *Biomphalaria glabrata*, Touassem & Théron (1989) observed alternating periods of high and low productivity throughout the duration of infection. This they believed to be due to the staggered recruitment of cercariae-producing daughter sporocysts. Such waxing and waning in cercarial production has also been described for *S. mansoni* (see Théron & Jourdane, 1979) and *S. haematobium* (see Kechemir & Théron, 1989). However, in both these cases, temporary decreases in cercarial emission was thought to result from periods of sporocyst production when the daughter sporocyst population is replenished.

During the monitoring of cercarial release an incubator fault resulted in a rise in the maintenance temperature to 25°C for a 2 hour period. This event affected 7 *V. piscinalis* releasing *Ichthyocotylurus* spp. The subsequent counts showed an immediate increase in cercarial emergence, with a peak some 8 hours later at between 1-3am (dark

period). The numbers recovered at this peak were of an even greater magnitude than the normal light maximum. This accidental result indicated the importance of temperature, as well as light on the emergence of these cercariae. These counts were not included in the pooled data and the specimens were not re-monitored for 10 days. Ginetsinskaya (1988) suggested that a marked increase in temperature breaks the daily rhythm and an almost uniform number of cercariae emerge during the day and the night. Temperature changes will not only affect fully-developed cercariae and the development of larvae within parthenitae, but also the physiological and behavioural responses of the host, which in turn might affect shedding patterns.

The distinct patterns of release exhibited by the strigeid species studied are likely to be related to the ways these cercariae are transmitted to the next host in the life-cycle. *A. gracilis* cercariae utilise a range of fish as secondary intermediate hosts (see Chapter 2.1; Introduction and Results). Of these, stone loach were the only source of metacercariae which established in the experimental avian hosts employed and subsequently provided the necessary quantities of eggs required for further life-cycle studies (see Chapter 3). Consequently, all of the experimentally raised cercariae originated from metacercariae excised from stone loach. These cercariae later appeared to be specific for the same fish host (see Chapter 6). Given the origin of these cercariae and their apparent host specificity, the nocturnal emission, observed negative phototaxis and constant swimming behaviour (see 5.3.; Results) may be important in their initial location of these bottom dwelling fish hosts. By contrast, *I. erraticus* and *I. variegatus* cercariae penetrate and develop within fish which are commonly active during day-light hours, i.e. salmonids and percids, respectively. The diurnal emergence patterns of these cercariae, their apparent lack of a phototactic response and their swimming behaviour (see 5.3.1; Results) would all tend to increase their chance of exposure to suitable piscine hosts.

5.3.3. Cercarial longevity.

Maximum longevity of *I. variegatus* cercariae was recorded at 15°C, with survival being compromised at temperatures of 5°C, 10°C and 20° (Fig. 107). This suggests that 15°C is close to the optimal temperature for the survival of this species. Although the longevity observed was greater at 5°C than 10°C, this did not reflect infectivity, as the majority of cercariae were inactive at the lower temperature, with swimming only occurring when the water temperature was raised. *I. variegatus* cercariae were found to survive longer at 20°C than *I. erraticus*, which in turn exhibited a greater longevity than *A. gracilis*. The reason for the shorter longevity of *I. erraticus* at this temperature is unclear, as both species exhibit similar swimming behaviour (see 5.3.1; Results) and appear to possess the same glycogen reserves in their six pairs of caudal bodies. However, the brief longevity of *A. gracilis* cercariae reflects their extremely active swimming behaviour. Interestingly, the longevities of both *Ichthyocotylurus* cercariae were similar at 15°C (Fig. 106).

Taskinen, Valtonen & Gibson (1991) studied the longevities of two species of bucephalid cercariae emerging from *Anadonta* specimens in Finland. Their findings showed life-spans at 20°C of up to 29 and 40 hours with 50% surviving 17 and 28 hours, respectively. However, they commented that cercariae were only active and infective for the initial few hours. At this temperature the strigeid species investigated here showed shorter longevities but remained active for the majority of this period. Whether this continued activity reflected infectivity would require experimental investigations to ascertain.

In summary; cercariae of *I. erraticus*, *I. variegatus* were successfully raised experimentally from miracidia of known identity and origin within naïve (experimentally raised) *Valvata piscinalis* hosts. *A. gracilis* cercariae were similarly obtained from laboratory reared *Lymnaea peregra*. Cercarial developmental periods within the molluscan host were found to be temperature dependent and markedly different for the

strigeid genera investigated, with both *Ichthyocotylurus* spp. requiring several weeks longer than *A. gracilis* at 20°C. Indeed, these members of the two genera were found to differ greatly, both morphologically and behaviourally. The *Ichthyocotylurus* spp. exhibit a distinct diurnal emergence rhythm from their molluscan host, being shed during the hours of daylight, while *A. gracilis* cercariae demonstrate a reciprocol pattern emerging during the hours of dark. Behavioural contrasts between the two genera were also observed in longevities, emergence strategies (route of exit) and swimming behaviour (recorded on Kontron image analysis equipment).

Cercariae of the two *Ichthyocotylurus* spp. were extremely similar, the only cercarial features found to be of diagnostic use were: the presence or absence of eye-spots; their differing developmental periods from miracidium to cercaria; the number and distribution of sensilla when compared by PCA; and their differing longevities at 20°C. Characters thought to be of value in differentiating between strigeid cercariae at the species level, including the armature, chaetotaxy pattern and resting posture, did not differ between these two species.

Ichthyocotylurus cercariae were found in naturally infected *V. piscinalis* which constitutes the first record in Britain of cercariae of this genus.

CHAPTER 6: EXPERIMENTAL INFECTION OF FISH

INTRODUCTION

The migration of *Diplostomum* cercariae (often referred to as diplostomules in their tail-less migratory state, see *inter alia* Whyte, Allan, Secombes & Chappell, 1987) through the fish host to their site of localisation, the eye, is well documented (for a review see Brady, 1989 and the later work of Höglund, 1991). Such studies on strigeid species appear to be limited to that of Johnson (1971) for *I. erraticus*. He exposed young rainbow trout (average length of 4cm) to known suspensions of *I. erraticus* cercariae, killed the fish at intervals post-infection and examined transverse serial sections for the presence of migrating forms. The results obtained indicated that penetration typically occurred in the pectoral area, particularly on the gills, at the base of operculum and fins. Migration to the pericardial cavity was nearly complete eight hours after exposure; a similar period to that observed for *Diplostomum* spp., and the migratory route was predominantly via the circulatory system, although the loose connective tissues were also utilised.

Once the cercariae have reached their 'preferred site' development to the infective metacercarial stage begins. The developmental period is considered to be primarily determined by the host species and water temperature (see review of Chubb, 1976), although intensity of infection (competition) may also be influential (Nasir, 1960). Detailed accounts of this process exist for *I. variegatus* (see Odening & Bockhardt, 1971) and *I. platycephalus* (see Odening *et al.*, 1970) metacercariae, while that of Olson (1970) for *I. erraticus* described only the time required for encystment, and there have been no such observations made for *I. pileatus*. Descriptions of the development of *Apatemon* spp. in fish are limited to those for *A. gracilis*. Crocombe (1959) recorded the development of pre-cystic metacercariae, believed to represent *A. gracilis*, in some depth, as did Vojtek (1972), while Blair (1974) also observed pre-cystic metacercariae of this species.

Specific studies on the physical/physiological barriers pertaining to strigeid

specificity for their fish hosts have not been performed, and whether cercariae fail to penetrate an unsuitable host or fail to migrate/develop after penetration and subsequently die, remain largely unanswered. Information on this subject is confined to several incidental observations following attempted experimental infections. For example, Crocombe (1959) observed cercariae, that were believed to be *A. gracilis*, to penetrate guppies *Lebistes reticulatus* and bullheads, but to only develop within the latter. Similarly, Blair (1974) noted *A. gracilis* cercariae to penetrate but develop no further within experimentally infected 3-spined-sticklebacks, while successful establishment of metacercariae occurred with the same cercariae in rainbow and brown trout. This case is particularly interesting as 3-spined-sticklebacks are recorded natural hosts for the species (Blair, 1974, 1976). Another case of successful penetration without establishment was reported by Combes & Nassi (1977) for *A. graciliformis* cercariae in male guppies; the guppy being a natural host for this strigeid species in Guadeloupe, French West Indies. In studying the life-cycle of *A. graciliformis* they found that the cercariae preferentially infect female fish, but could be made to penetrate males in experimental challenges, within which they soon died. These examples demonstrate that there is, at least in some cases, a physiological barrier to establishment; although, the additional presence of physical barriers may have been "masked" by the extreme conditions of experimental infections, with small water volumes and high cercarial challenges.

Experimental infections in fish were performed for the completion of the laboratory maintained life-cycles of *I. erraticus*, *I. variegatus* and *A. gracilis*. It was hoped that the infections would confirm the viability of experimentally raised cercariae, while demonstrating the metacercarial host and site specificities of these species. Successful infections would yield metacercariae of a known life-history, and consequently experimentally reared material would have been obtained for all life-stages.

MATERIALS AND METHODS

Source of experimental fish.

Fish to be used for experimental infections were, whenever possible, naïve, i.e. they had not encountered previous cercarial challenges. Such a guarantee was only possible for specimens raised in a hatchery with a domestic water supply, treated and filtered in a system known to be completely mollusc free. Typically, however, experimental fish were obtained from hatcheries fed by river water (which might contain cercariae) or from their natural habitat. These latter sources were those considered to be free from metacercarial infections (as indicated by previous negative records for samples taken during the course of this study). Nevertheless, fish were reinvestigated prior to infection by examining subsamples of at least 20 specimens. Occasionally, these criteria could not be met and specimens least likely to be infected, e.g. below a particular age/size (stone loach), or harbouring very low intensities of infection (salmon parr) had to be employed. In fish populations known to harbour metacercariae subsamples of at least 20 fish were taken to indicate levels of prevalence and intensity of infection. The results of experimental infections performed with fish bearing natural infections were subsequently compared statistically to levels recorded for the naturally challenged subsample. Details of the sources of experimental fish are provided in Table 66.

Infection and maintenance of fish.

The fish were maintained in covered plastic tanks (length 70cm, width 50cm, depth 40cm) in a flow-through system at 14-18°C with continuous aeration. An appropriate grade of commercial fish food was fed daily *ad libitum*.

Fish were infected in a smaller tank (length 35cm, width 15cm, depth 15cm) with minimal aeration. A known quantity of cercariae (estimated using 3 x 1ml subsample aliquots) were added to the tank. Cercariae were from experimentally

infected *Valvata piscinalis* and *Lymnaea peregra* hosts and of a known identity (see Chapter 5.1; Materials and Methods). Infections were carried out at about 15°C, when both the tank water and the cercarial suspension had equilibrated to this temperature. The duration of the challenge was for 1 hour, after which the fish were returned to clean tanks maintained at 14-18°C. Details of the infections performed are given in Table 67, including any deviations from the protocol stated above.

Examination of fish.

Fish were examined for the presence of metacercariae several weeks post-infection (p.i.) when encystment was expected to be completed, indicating successful metacercarial development. Early examination p.i. of fish, as an indicator of experimentally acquired metacercariae in non-naïve hosts, was not considered to be of particular value because pre-cystic specimens were also recorded from subsamples. Consequently, evidence of successful experimental infections in salmon parr and stone loach relied on the statistical comparison of burdens from the "control" and experimentally infected fish.

Treatment of metacercariae.

Time constraints precluded detailed morphological examination of the metacercariae obtained, but body dimensions were recorded for 10 specimens of each species reared. Excystment of metacercariae was performed using artificial digests as described in Chapter 2.2.1, the specimens being fixed in Berland's fluid and mounted as stated in Chapter 2.2.2.

Table 66. Details of experimental fish hosts.

Host species	Source	Source status	Fish size	Subsample examined prior to challenge	Prevalence (%), range and intensity of infection (mean)
Brown trout	Hatchery at the Institute of Aquaculture, University of Stirling	Naïve	9.5-11.6	20	0
Rainbow trout	Loch Awe (cage fish farm), Strathclyde	Previously -ve	12.5-16.0	25	0
Salmon parr	SOAFD hatchery, River Almond, Perthshire	Low intensities of infection previously present	8.3-10.2	34	32%, 1-3 (1.3)
Perch	Due Loch, Stirlingshire	Previously -ve	8.1-10.8	22	0
Stone loach	Fish farm lade, River Almond, Perthshire	Low prevalence and intensity of infection previously recorded from this size class	<4.0	22	14%, (1.0)

Table 67. Details of experimental infections performed.

Fish host	No of fish	Cercarial species	No of cercariae
Brown trout	14	*A. gracilis* [a]	c. 300
	14	*I. erraticus* [b]	c. 300
Rainbow trout	5	*A. gracilis* [a]	c. 500
	5	*I. erraticus* [b]	c. 500
Salmon parr	20	*A. gracilis* [a]	c. 500
	8	*I. erraticus* [b]	c. 450
Perch	4*	*I. variegatus* [c]	c. 350
	4	*I. variegatus* [c]	c. 250
Stone loach	7	*A. gracilis* [a]	c. 150

Superscript letters: cercariae derived from stone loach metacercariae (a); cercariae derived from powan metacercariae (b); cercarial origin unknown - derived from either Scottish ruffe or Scottish perch metacercariae (c).

* Fish were maintained in an outside tank at ambient temperatures (late August '93).

RESULTS

I. erraticus

Experimental infections using *I. erraticus* cercariae, derived from natural metacercarial infections in powan, were attempted with 3 species of fish host: rainbow trout, brown trout and salmon parr. No metacercariae were recovered from the cercarial challenge to 14 naïve brown trout; similarly, there was no evidence of successful experimental infections in salmon parr. Of 8 salmon parr exposed to 450 cercariae 4 fish were found to contain metacercarial cysts. However, the 7 cysts recovered were all readily identifiable as *A. gracilis,* having originated from natural infections.

Far greater success was achieved with experimental infections in rainbow trout. A fish dying on day 18 p.i. harboured 95 metacercariae: 86 pre-encysted and free within the pericardial cavity; 6 encysted, of which 4 were attached to the ventricle and bulbus arteriosus and 2 to the inside of the pericardium; and 3 pre-encysted specimens which were associated with the pyloric caeca. The examination of a second mortality the following day revealed 71 metacercariae, the majority (42) encysted and 36 of these attached to the inner surface of the pericardium. One rainbow trout was later found to have jumped from the tank and was unsuitable for examination. The 2 remaining fish examined on days 24 and 27 p.i. contained 78 and 58 metacercariae, respectively; all encysted, with the exception of a single specimen located among the pyloric caeca of the first fish. Even without the data of the fifth rainbow trout specimen, some 302 metacercariae were recovered from the approximately 500 cercariae to which the fish were exposed, representing a recovery of 60%.

I. variegatus

Two sets of infections using experimentally raised *I. variegatus* cercariae were attempted with perch. In the first experiment 4 fish were exposed to 350 cercariae. These perch were subsequently maintained at ambient Autumn temperatures and all fish

died within 5 weeks p.i. Three of the 4 fish harboured metacercariae, but intensities of infection were low (9, 5 and 6) considering the size of the cercarial challenge. All metacercariae recovered were located on the inner surface of the swimbladder or in the body cavity close to this organ. No encysted specimens were observed, although metacercariae excised from the fish examined at day 34 p.i. were smaller and more developed than the others.

In the second experimental infection 4 perch were exposed to 250 cercariae and then maintained at 14-18°C. The first fish was examined at 17 days p.i. and found to contain 18 metacercariae, of which 14 were encysted, all specimens being attached to the inner surface of the swim bladder. Upon dissection at 8 weeks p.i., the remaining 3 perch harboured 19, 32 and 7 encysted metacercariae, respectively. All specimens recovered were adhered to the inner surface of the host's swimbladder. In total 76 metacercariae were obtained from the experimentally infected perch; 30% of the cercariae introduced to the infection tank.

A. gracilis

Experimental infections using *A. gracilis* cercariae, derived from metacercariae in naturally infected stone loach, were attempted with 4 species of fish host: brown trout, rainbow trout, salmon parr and stone loach. Of these, only the challenges to stone loach experimental hosts appeared to establish.

A. gracilis metacercariae were recovered from experimentally infected salmon parr, but the numbers recovered (9 from 20 fish) were actually slightly lower than recorded from the non-challenged subsample (mean of 1.3), providing no evidence for new acquisitions. No metacercariae were obtained from 5 rainbow trout or 14 brown trout experimentally infected with approximately 500 and 300 cercariae, respectively.

Seven stone loach were exposed to 150 *A. gracilis* cercariae; 1 fish was lost to the flow-through system, but the other 6 all harboured significantly ($P<0.001$) larger intensities of metacercariae than the 'control' subsample. Two stone loach examined on

day 18 p.i. contained 10 and 7 metacercariae; most of these were recovered from the body cavity and all were pre-encysted. The 4 remaining fish, dissected at 5 weeks p.i., yielded 37 metacercaria (36 encysted), with burdens of between 3 and 16 specimens. The distribution of these metacercariae from the later infections was: cranial cavity (10), 1 pre-encysted; pericardial cavity (3); humour of the eye (1); and body cavity (23). Collectively, 36% of introduced cercariae were recovered as metacercariae.

Table 68 provides body dimensions recorded for 10 specimens of experimentally raised: *I. erraticus* metacercariae, from a rainbow trout at 27 days p.i.; *I. variegatus*, from perch at 8 weeks p.i.; and *A. gracilis*, from stone loach at 5 weeks p.i.

Table 68. Range and mean (in parentheses) of body dimensions recorded for *I. erraticus*, *I. variegatus* and *A. gracilis* metacercariae raised in experimental fish hosts. Measurements were taken from 10 specimens of each species and are in micrometres.

Metacercarial species	Body length	Forebody length	Body breadth
I. erraticus	301-404 (363)	-	250-321 (284)
I. variegatus	399-534 (472)	-	334-379 (352)
A. gracilis	668-809 (749)	507-642 (586)	250-334 (286)

DISCUSSION

Encysted metacercariae were recovered from experimental infections performed with all three species of experimentally raised cercariae, demonstrating their viability. Measurements of the metacercariae obtained revealed body dimensions that corresponded to those recorded for specimens recovered from naturally infected hosts (see Chapter 2.2.2; Results). However, the range of body dimensions recorded for the experimentally raised metacercariae were narrower than those from natural infections. This is likely to result from the experimental metacercariae being of a more similar age.

I. erraticus cercariae used for experimental infections were derived from metacercariae recovered from naturally infected powan. No physiological barrier was found to exist in rainbow trout to these cercariae, with infections establishing in all fish examined. However, attempted infections in brown trout and salmon parr all failed. There are no records in the literature of natural *I. erraticus* metacercarial infections in British salmon parr and this fish species may represent an unsuitable host. Natural host records do exist for *I. erraticus* in brown trout (Wootten, 1973a,b; Campbell, 1973), though not confirmed by life-cycle data, and the failure of experimental infections in this host are more puzzling.

I. erraticus metacercariae excised from an experimentally infected rainbow trout (maintained at 14-18°C) at 18 days p.i. were predominantly (94%) pre-encysted. At 19 days p.i. 59% of metacercariae from another experimentally infected rainbow trout were encysted, while at 27 days p.i. all specimens were found to be encysted. These results compare well to those of Olson (1970) who observed *I. erraticus* encystment to occur between 14 and 21 days p.i. at the higher maintenance temperature of 21°C; also in experimentally infected rainbow trout. These respective developmental times indicate the accelerated maturation resulting from elevated temperatures. The *I. erraticus* metacercariae obtained in the present study were predominantly located within the

pericardial cavities of experimentally infected rainbow trout. The site specificity to the pericardial cavity corresponds with that recorded here and by other authors for natural infections in all fish hosts (see Chapter 2.1). However, in two of the four experimental infections, low numbers of metacercariae were also recovered from the body cavity, associated with the pyloric caeca. This additional site of cysts was occasionally observed for natural infections of *I. erraticus* in powan (present study) and in experimentally infected rainbow trout (Olson, 1970). All such cases were recorded from heavily infected fish and the phenomenon appears to be a result of burden size. However, in contrast, Wootten (1973a) noted *I. erraticus* cysts in "unusual sites" within naturally infected rainbow trout from Hanningfield reservoir, Essex, but he recorded these cysts from "light" infections.

In initial experimental infections with *I. variegatus* cercariae the perch were maintained at ambient temperatures and metacercariae failed to encyst by day 34 p.i. This slow maturation rate was believed to be due to the low water temperature in the small static tank, which would have dropped significantly at night from its warm (c. 18°C) daytime levels. An experimentally infected perch maintained at 14-18°C was found to harbour mainly encysted *I. variegatus* metacercariae by 17 days p.i., demonstrating a slightly more rapid development than seen for *I. erraticus*.

All attempted *I. variegatus* cercarial infections established in perch (remaining specimens examined at 8 weeks p.i.), with encysted metacercariae adhering solely to the inner surface of the swimbladder, the site typically recorded for naturally infected fish of this species. Encysted *I. variegatus* metacercariae were recovered by Odening & Bockhardt (1971) from the swimbladder and body cavity walls of experimentally infected *Mesogonistius chaetodon* after two weeks p.i. at 20°. However, they stated that development to infective metacercariae could take up to 29 days in this host. It must be remembered that the infectivity of the metacercariae attained in the present study was not investigated and encystment does not represent evidence of this. Consequently, the

newly encysted *I. variegatus* metacercariae obtained at 17 days p.i. may not have been infective.

The intensities of *I. variegatus* infection attained in the present study were not high (7-32 metacercariae) and the percentage recovery from cercariae challenged (76 from 250; 30%) were lower than obtained for *I. erraticus* in rainbow trout (302 from 500; 60%). Experimental infections of ruffe with *I. variegatus* cercariae would have made an interesting comparison to the results gained for perch infections, but a source of uninfected/lightly infected ruffe was unavailable. Would the ruffe have exhibited larger burdens from challenges of the same magnitude (naturally infected ruffe were always found to be heavily infected, see Chapter 2.1; Results), i.e. are they more susceptible to infection? Or, if recoveries were of a similar size, would the low burdens have indicated a preferential infection site in ruffe; one that was masked by the high intensities in naturally infected fish?

Developmental periods, from cercarial invasion to infective metacercariae, recorded by Odening *et al.* (1970) for *I. platycephalus* were similar (maximum of 4 weeks) to those observed by Odening & Bockhardt (1971) for *I. variegatus*. However, fish experimentally infected with *I. platycephalus* were maintained at the elevated temperature of 20-28°C, indicating that maturation of this species is somewhat slower, possibly explained the greater size attained by this metacercaria (see Chapter 2.2.2).

Experimental infections with *A. gracilis* cercariae, derived from metacercariae excised from naturally infected stone loach, showed an unexpected host specificity. Attempted infections in stone loach were all successful, while challenges to other known hosts (see Chapter 2.1), salmon parr, rainbow trout and brown trout, all failed. Inverse results were obtained by Blair (1974, 1976). He experimentally infected a range of fish species with *A. gracilis* cercariae emerging from a naturally infected snail. These infections successfully established in rainbow and brown trout, but no metacercariae were recovered from stone loach. Such host specificity results raise doubts as to the homogeneity

of this species, a possibility that is supported by previous observations in the present study; the collective evidence for which will be considered later in Chapter 8.

A. gracilis metacercariae from two experimentally infected stone loach were still found to be pre-encysted at 18 days p.i. A proportion of the experimentally raised metacercariae of both *Ichthyocotylurus* spp. were found to be encysted by this time, suggesting that a longer maturation period is required for *A. gracilis* metacercariae.

Detailed monitoring of the maturation of *A. gracilis* metacercariae was undertaken by Crocombe (1959) in experimentally infected bullheads and Vojtek (1972, as *A. cobitidis*) in experimentally challenged *Proterorhinus marmoratus*. Crocombe's fish were kept in outdoor aquaria at ambient temperatures and encystment was not noted prior to day 57 p.i. Maintenance temperatures were not indicated by Vojtek (1972), but he observed that encystment began at about 30 days p.i. and was complete by 38 days. Later, Blair (1974, 1976) noted 68 pre-encysted and 39 encysted *A. gracilis* metacercariae in experimentally infected rainbow trout, which were maintained at 15°C, at four weeks p.i. Examination of infected stone loach in the present study at five weeks p.i. revealed 36 of 37 metacercariae to be encysted. These metacercariae were predominantly located in the cranial and body cavities of their hosts, although smaller numbers were also recovered from less typical sites, such as the humour of the eye and pericardial cavity. This broader distribution of cysts was also seen in some heavy natural infections of stone loach. Percentage recovery of metacercariae (36%) was similar to that obtained for *I. variegatus* experimental infections.

In summary: experimentally raised cercariae were found to be infective; host specificities were observed for *I. erraticus* and *A. gracilis*, being particularly stringent for the latter species; site specificities recorded were as observed in natural infections; metacercarial maturation periods (for encystment) were highly temperature dependent, being comparable for the two *Ichthyocotylurus* spp. and rather longer for *A. gracilis* specimens.

CHAPTER 7: KARYOLOGICAL STUDY OF STRIGEIDS

INTRODUCTION

According to Blaxhall (1983), "Cytogenetics relies on the preparation of a karyotype where individual chromosomes can be accurately identified. A karyotype is the characterisation and analysis of a chromosome complement at metaphase within the nucleus of a given species. This defines the chromosomal numbers, size, type and morphological characteristics". Cytogenetic methods of investigation have contributed significantly to the elucidation of controversial problems in taxonomy, as karyotype parameters remain constant throughout ontogenesis and exhibit discrete species-specific characteristics.

According to White (1973), the first studies on helminth karyotypes began near the end of the 19th century. Nevertheless, cytological analysis of cestodes and trematodes is still in its infancy. Of over 40,000 trematode species noted by Cheng (1973), cytological information is available on just several hundred, and for many of these, data are limited solely to chromosome numbers. White (1977) stated that the haploid chromosome number of most animal species lies between 6 and 20. In trematodes the range is rather narrower, with 93% (246 of 264) of those studied possessing a haploid complement of between 6 and 11, and for over 56% of these, 10 or 11 chromosomes (Baršiené, 1993).

Although no digenean families have been extensively studied, among the most widely and thoroughly investigated are: the Plagiorchiidae (see Britt, 1977; Baršiené & Grabda-Kazubska, 1988a, 1988b, 1991; Baršiené & Orlovskaya, 1990, 1991; Baršiené, 1993); the Schistosomatidae, unique for their heterosexuality and heterogeneity of chromosome numbers (14, 16, 18, 19, 20) (see Short & Menzel, 1960; Raghunathan & Bruckner, 1975; Short, Menzel & Pathak, 1979; Grossman, Mckenzie & Cain, 1980; Baršiené, 1993); and the Echinostomatidae (see Mutafova & Kanev, 1986; Mutafova, Kanev & Angelova, 1986; Mutafova, Kanev & Vassilev, 1987; Richard & Voltz, 1987; Richard, Voltz & Vassilev, 1986; Baršiené & Kiseliene, 1990; Baršiené, Kiseliene &

Grabda-Kazubska, 1990; Mutafova, 1993; Baršiené, 1993). In each of these studies the authors elucidated both the number and morphology of the parasite's chromosomes, while several workers discussed karyotypic features pertaining to particular taxa (e.g. Baršiené & Kiseliene, 1991), or proposed possible mechanisms involved in the evolution of the chromosomes described (e.g. Mutafova, 1994).

Such investigations on named strigeid species appear to be limited to that of Baršiené, Petkevichute, Stanevichute & Orlovskaya (1990). They used the parthenitae from naturally infected snails in North-West Chukotka to describe the karyology of four strigeid species belonging to three genera; *Cotylurus cornutus*, *Apatemon* (*Australapatemon*) *fuligulae*, *Ichthyocotylurus pileatus* and *I. erraticus*. The later text by Baršiené (1993) also included the chromosome morphology of three further *Cotylurus* spp. and three *Apatemon* spp. (subgenus not indicated).

It was hoped that the application of this technique to the experimentally reared parthenitae in the present study would enable the first karyological descriptions of *I. variegatus* and *A. gracilis*, while providing an accurate means of discriminating between the molluscan stage of *I. erraticus* and *I. variegatus*, the cercariae of which are morphologically very similar and occupy the same molluscan host species.

MATERIALS AND METHODS

7.1. Source of sporocysts.

Cytological analysis of digeneans is performed either upon cells extracted from the testes of adults or, more typically, from parthenitae (sporocysts or rediae). In this investigation sporocysts of *I. erraticus* and *I. variegatus* were obtained from experimentally infected *Valvata piscinalis* and *A. gracilis* from *Lymnaea peregra*. The snails were infected and maintained as described in Chapter 5.1; Materials and Methods.

7.2. Preparation of mitotic chromosomes.

Experimental infections in snails were known to have been successful with the onset of cercarial release. At this point germ balls within the daughter sporocysts are rapidly undergoing mitosis. These snails were carefully removed from their (operculate, *V. piscinalis*) shells (to maximise exposure and uptake of the alkaloid) and placed into a 0.1% solution of colchicine made up in artificial spring water (A.S.W) at room temperature. Periods of incubation in this medium were either 3-4 hours or 16 hours. The colchicine blocks cell division at metaphase by inhibiting microtubule polymerisation and hence spindle formation, resulting in the accumulation of cells bearing condensed diploid chromosomes. Following treatment, sporocysts were dissected out of the snail's digestive gland in a solution of isotonic KCL, placed in distilled water for 5-10 minutes and then fixed in Carnoy's fluid (1 part acetic acid to 3 parts ethanol), which was changed after 30 minutes, 1 hour and 24 hours. The parthenitae were teased apart and then ground with a glass rod in 45% acetic acid to yield an homogenous cell suspension. Using a drawn-out pipette, the suspension was dropped from a height of approximately 30cm onto a slide heated to 40°C . Each drop was allowed to evaporate for 5-10 seconds before being withdrawn. This technique resulted in a monolayer of cells in concentric rings upon the slide. The slides were allowed to dry overnight and then stained in 10% Geimsa.

The same procedure was followed for uninfected snails as a "control"; cell suspensions being created from the digestive gland, the site of sporocyst localisation.

7.3. Determination of chromosome number.

Chromosomes of more than 50 metaphase spreads, prepared from sporocysts originating from at least three infected snails, were counted for each species. The chromosome number whose frequency occurrence was highest was considered to be the diploid number for that species. Variations in chromosome number (both hypo- and hyper-diploid) were considered to be the result of errors inherent in the preparation technique.

7.4. Morphometric measurements of the chromosome.

Karyotypes were made from the photographs of individual metaphase plates. Individual chromosomes of each karyotype (from at least 5 karyotypes) were measured with a slide calliper. The relative length (L^R) of each chromosome was calculated as the percentage of the haploid complement length: L^R= 100CL/HCL; where CL is the individual chromosome length and HCL is the combined length of all chromosomes in the haploid complement. The centromeric position of each chromosome, expressed as the centromeric index (I^C), was calculated on the basis of the percentage of the small arm in relation to the total length of the chromosome.

The level of interspecific differences between chromosomes were determined by performing Student's t-tests on their relative lengths and centromeric indices. Idiograms were constructed which show diagrammatically the absolute length or relative length of a chromosome in relation to the total haploid genome.

Classification of chromosomes

The karyotypes of different species have been described according to the classification proposed by Levan, Fredga & Sandberg (1964) on the basis of centromeric

index (I^C). The karyotypes were presented according to the length of the chromosomes in descending order and increasing number. So, the 1st chromosome is the longest and the 10th the shortest in any karyotype. Although this classification of chromosomes is generally accepted, very few authors have adopted the nomenclature suggested by Levan *et al.* (1964): median, submedian, subterminal and terminal chromosomes. Most workers have preferred to apply the more established metacentric-acrocentric names to this classification system. In this study the nomenclature of Levan *et al.* (1964) was not applied, as it is too confusing for the reader to follow comparisons with other work when different nomenclature is used.

The number of chromosome arms in the karyotype, the "nombre fondamental" (N-F) of Matthey (1945), was also included for each species description.

Nomenclature used in present study

Centromeric index: (short arm/total length) x 100	Chromosome designation	Symbol	Description
50	Metacentric	M	Bi-armed
37.5-50.0	Metacentric	m	
22.5-37.5	Submetacentric	sm	
12.5-22.5	Subtelocentric	st	Single-armed
0.00-12.5	Acrocentric	a	
0	Telocentric	T	

RESULTS

Both snail incubation periods in colchicine provided preparations with cells in metaphase. However, the shorter period (3-4 hours) produced low numbers of particularly well-defined chromosome spreads, while the longer exposure (16 hours) gave a larger number of less well-defined spreads. This applied equally to both molluscan hosts.

7.1. Chromosome number.

Diploid chromosome number data obtained from strigeid parthenitae cells and molluscan host digestive gland cells are given in Tables 69 and 70, respectively. These include the number of cells with different chromosome numbers and their frequency in percentages. These data are also represented graphically in Fig. 110.

Table 69 indicates, that for all three strigeid species studied, most of the parthenitae cells (over 61%) possess 20 chromosomes, suggesting that this is the diploid chromosome number for each of these parasites. The variation in chromosome number was similar in each case, i.e. *I. erraticus* (16-21), and *I. variegatus* and *A. gracilis* both (16-22). The distribution of the chromosome frequency is quite skewed, particularly for *I. erraticus* parthenitae cells. Collectively, 27.3% of the cells observed exhibit hypodiploidy compared to 6% of the cells which have a hyperdiploid chromosome number (Fig. 110). This suggests that the technique for chromosome preparation is more likely to cause the loss of a chromosome rather than fragment them.

Table 70 shows the distribution of chromosome numbers recorded from molluscan host cells. Digestive gland cells of *V. piscinalis*, the molluscan host of both *Ichthyocotylurus* spp., have a diploid chromosome number of 20 (70% of cells). Metaphase cells of *L. peregra*, the snail host of *A. gracilis* parthenitae, were found to typically contain 34 elements (60.4%). The variation in chromosome number about the mode was normally distributed for *V. piscinalis* cells (17-22 chromosomes) but skewed

for *L. peregra* (29-35 chromosomes) with a hypodiploid number accounting for 18 of the remaining 19 cells (Fig. 110).

7.2. Chromosome morphology.

Metrical data and the classification of the chromosomes are presented in Tables 71-73. The classification of many of the chromosomes is borderline due to an overlapping of their 95% confidence limits. When chromosomes fall into the submetacentric-subtelocentric (sm-st) classification class they are considered to be single-armed.

I. erraticus (Table 71, Figs 111, 112)

The karyotype consists of 4 single (Nos 3, 4, 5, and 6) and 6 bi-armed (Nos 1, 2, 7, 8, 9 and 10) pairs of chromosomes. There are 4 pairs of metacentric (Nos 1, 2, 9 and 10), 2 pairs of submetacentric (Nos 7 and 8), 3 pairs of subtelocentric-acrocentric (Nos. 3, 4 and 5) and a single pair of submetacentric-subtelocentric chromosomes (No. 6). The chromosome length varies between 2 and 7.5μm with the first 5 pairs accounting for 70.3% of the total haploid genome length. The diploid arm number (N-F of Matthey, 1945) in this species is 32.

I. variegatus (Table 72, Figs 113, 114)

The karyotype consists of 4 single (Nos 2, 3, 4 and 5) and 6 bi-armed (Nos 1, 6, 7, 8, 9 and 10) pairs of chromosomes. There are 4 pairs of metacentric (Nos 1, 8, 9 and 10), 1 pair of submetacentric (No 6), 4 pairs of subtelocentric (Nos 2, 3, 4 and 5) and 1 borderline metacentric-submetacentric pair (No 7) of chromosomes. The chromosome length varies between 1.7 and 5.5μm with the first 5 pairs accounting for 67.3% of the total haploid genome length. The N-F is 32.

A. gracilis (Table 73, Figs 115, 116)

The karyotype consists of 5 single (Nos 2, 3, 5, 6 and 9) and 5 bi-armed (Nos 1, 4, 7, 8 and 10) pairs of chromosomes. There are 3 pairs of metacentric (Nos 4, 8 and 10), a single pair each of submetacentric (No 7) and acrocentric (No 3), a pair of borderline metacentric-submetacentric (No 1), 3 pairs of borderline submetacentric-subtelocentric (Nos 2, 6 and 9) and a single pair of borderline subtelocentric-acrocentric (No 5) chromosomes. The chromosome length varies between 3.0 and 11.0μm with the first 5 pairs accounting for 71.4% of the total haploid genome length. The N-F is 30.

V. piscinalis (Figs 117, 118)

The karyotype consists of 10 bi-armed pairs of chromosomes. There are 8 pairs of metacentric (Nos 1 to 4, 6, 7, 9 and 10) and 2 pairs of submetacentric (Nos 5 and 8) chromosomes. The chromosome length varies between 2.5 and 6.0μm with the first 5 pairs accounting for 59.2% of the haploid genome length. The N-F is 40.

The karyotype differences between *Ichthyocotylurus* species and *Valvata piscinalis* (the snail host possessing only bi-armed chromosomes) enabled any contamination by host tissue to be readily identified.

L. peregra (Fig. 119)

The karyotype was not studied in detail because *L. peregra* cells could be readily distinguished by their large chromosome number; but it was found to consist mainly of metacentric and submetacentric chromosomes.

Although possessing the same number of chromosomes in diploid sets, the species studied here exhibited distinct differences in their karyotypes (Tables 71-73 and Figs 112, 114 and 116). Idiograms, based on the relative length of chromosomes of the three species examined, demonstrate this visually (Fig. 121). The level of these

interspecific differences was determined by performing Student's t-tests on the relative lengths and centromeric indices of their chromosomes and are demonstrated in Table 74. These results showed that the differences were species-specific. Other idiograms, based on the absolute length of the parasite chromosomes indicated the differences in their size between the 3 species (Fig. 120).

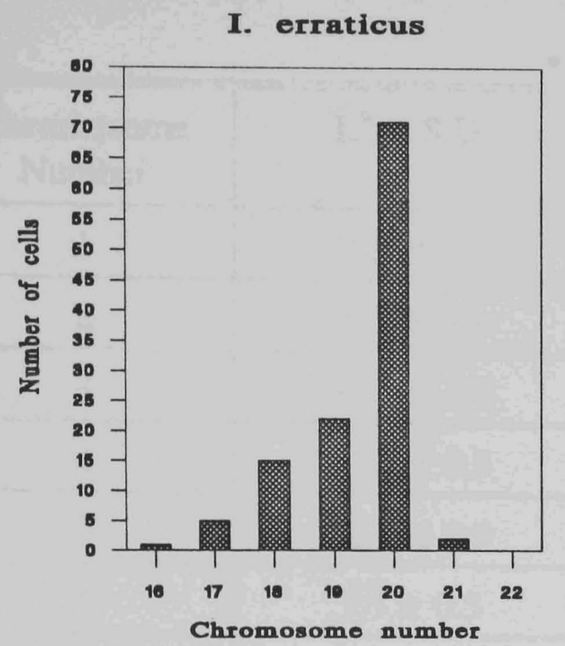
Table 69. Frequency distribution of the number of diploid chromosomes in the strigeids studied.

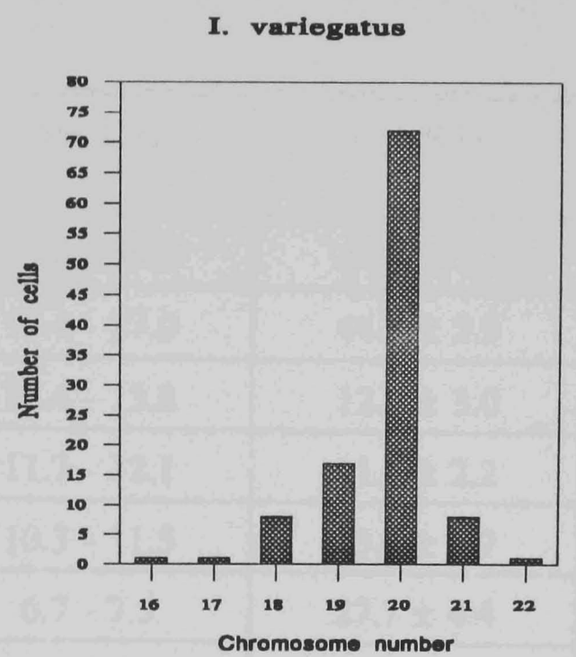
Species		Number of chromosomes							
		16	17	18	19	20	21	22	Total
I. erraticus	No. of cells	1	5	15	22	71	2	0	116
	%	0.86	4.31	12.93	18.97	61.21	1.72	0	100
I. variegatus	No. of cells	1	1	8	17	72	8	1	108
	%	0.93	0.93	7.41	15.74	66.67	7.41	0.93	100
A. gracilis	No. of cells	1	1	6	7	55	4	2	76
	%	1.32	1.32	7.9	9.21	72.37	5.26	2.63	100
TOTAL	No. of cells	3	7	29	46	198	14	3	300
	%	1.00	2.33	9.67	15.33	66.00	4.67	1.00	100

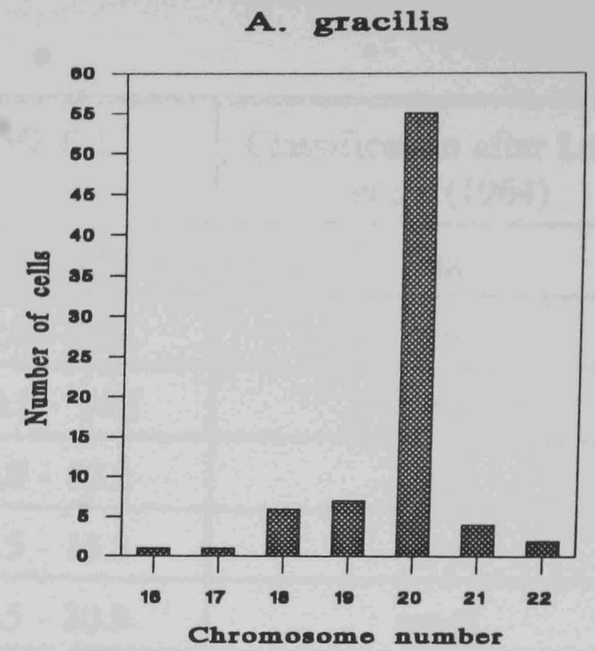
Table 70. Frequency distribution of the number of diploid chromosomes in the molluscan hosts studied.

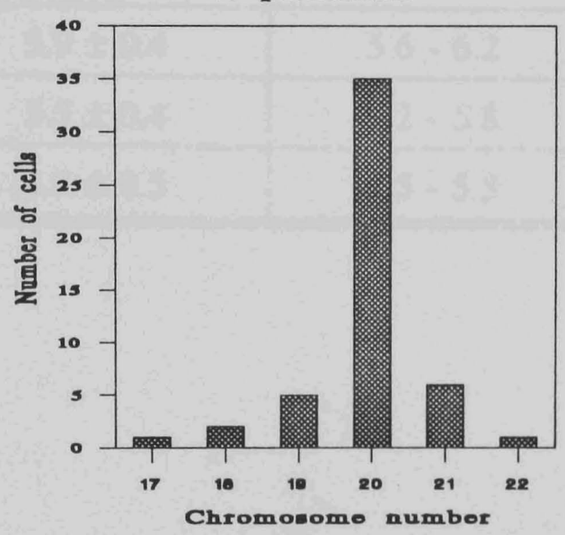
Species									
V. piscinalis	Number of chromosomes	17	18	19	20	21	22	23	Total
	No. of cells	1	2	5	35	6	1	0	50
	%	2.00	4.00	10.00	70.00	12.00	2.00	0	100
L. peregra	Number of chromosomes	29	30	31	32	33	34	35	Total
	No. of cells	1	1	2	6	8	29	1	48
	%	2.08	2.08	4.17	12.5	16.67	60.42	2.08	100

Fig. 110. Frequency distribution of diploid chromosome number in the strigeids and molluscs studied.









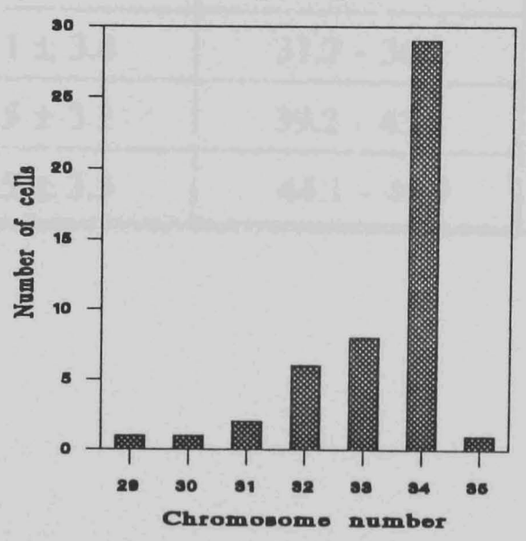


Table 71. Metrical data on the chromosomes of *Ichthyocotylurus erraticus* and their classification.

Chromosome Number	$L^R \pm$ S.D.	95% C.L.	$I^C \pm$ S.D.	95% C.L.	Classification after Levan *et al.* (1964)
1	18.1 ± 1.5	17.0 - 19.2	44.5 ± 1.5	43.4 - 45.6	m
2	16.2 ± 1.1	15.4 - 17.0	44.1 ± 2.8	42.1 - 46.1	m
3	13.1 ± 1.0	12.4 - 13.8	12.3 ± 3.0	10.1 - 14.5	st-a
4	11.9 ± 0.3	11.7 - 12.1	11.6 ± 2.2	10.0 - 13.2	st-a
5	10.9 ± 0.9	10.3 - 11.5	13.4 ± 2.7	11.5 - 15.3	st-a
6	7.1 ± 0.5	6.7 - 7.5	27.7 ± 4.4	24.5 - 30.9	sm-st
7	6.5 ± 0.6	6.1 - 6.9	28.2 ± 2.8	26.2 - 30.2	sm
8	5.9 ± 0.4	5.6 - 6.2	34.1 ± 3.4	31.7 - 36.5	sm
9	5.5 ± 0.4	5.2 - 5.8	41.5 ± 3.2	39.2 - 43.8	m
10	4.9 ± 0.5	4.5 - 5.3	44.5 ± 3.3	44.1 - 46.9	m

Fig. 111. Metaphase spread of *I. erraticus* chromosomes.

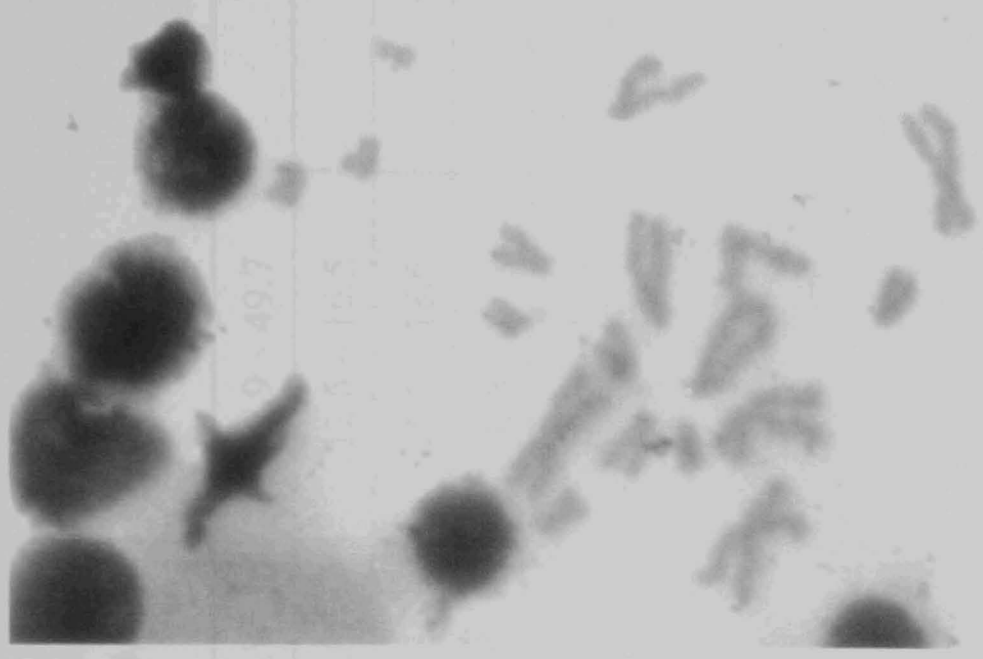


Fig. 112. Representative karyotype of *I. erraticus* chromosomes.

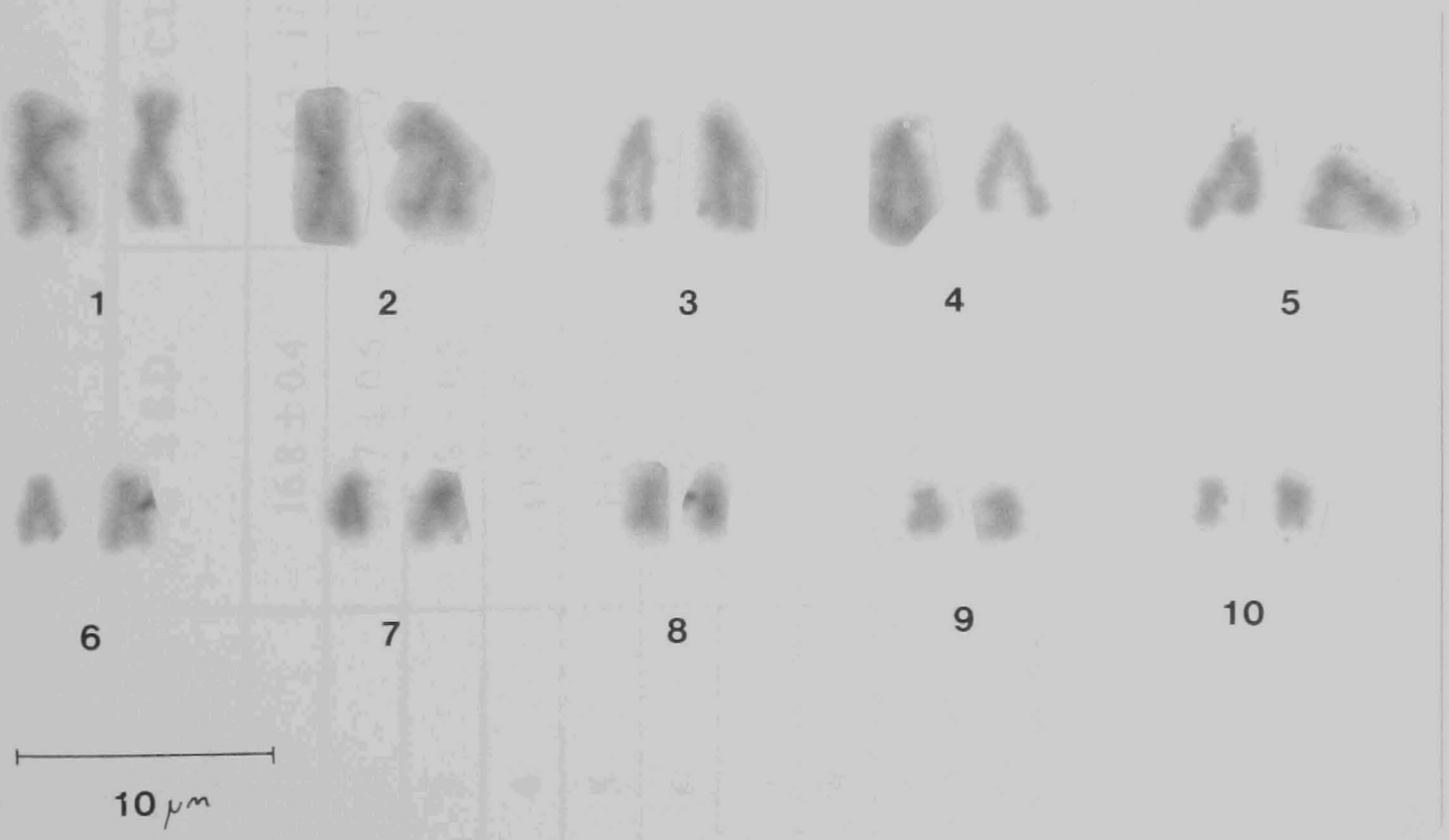


Table 72. Metrical data on the chromosomes of *Ichthyocotylurus variegatus* and their classification.

Chromosome Number	$L^R \pm$ S.D.	95% C.L.	$I^C \pm$ S.D.	95% C.L.	Classification after Levan *et al.* (1964)
1	16.8 ± 0.4	16.3 - 17.3	48.8 ± 0.7	47.9 - 49.7	m
2	14.7 ± 0.6	13.9 - 15.5	14.3 ± 1.8	12.5 - 16.5	st
3	12.8 ± 0.5	12.2 - 13.4	17.1 ± 1.2	15.6 - 18.6	st
4	11.9 ± 0.3	11.5 - 12.3	19.0 ± 1.9	16.6 - 21.4	st
5	11.0 ± 0.6	10.2 - 11.8	17.8 ± 1.6	15.8 - 19.8	st
6	8.0 ± 0.5	7.4 - 8.6	30.8 ± 2.0	28.3 - 33.3	sm
7	7.2 ± 0.4	6.7 - 7.7	39.4 ± 4.2	34.2 - 44.6	m-sm
8	6.5 ± 0.4	6.0 - 7.0	38.8 ± 1.8	36.6 - 41.0	m
9	6.0 ± 0.2	5.7 - 6.3	46.5 ± 2.0	44.0 - 49.0	m
10	5.1 ± 0.4	4.6 - 5.6	46.5 ± 1.3	44.9 - 48.1	m

Fig. 113. Metaphase spread of *I. variegatus* chromosomes.

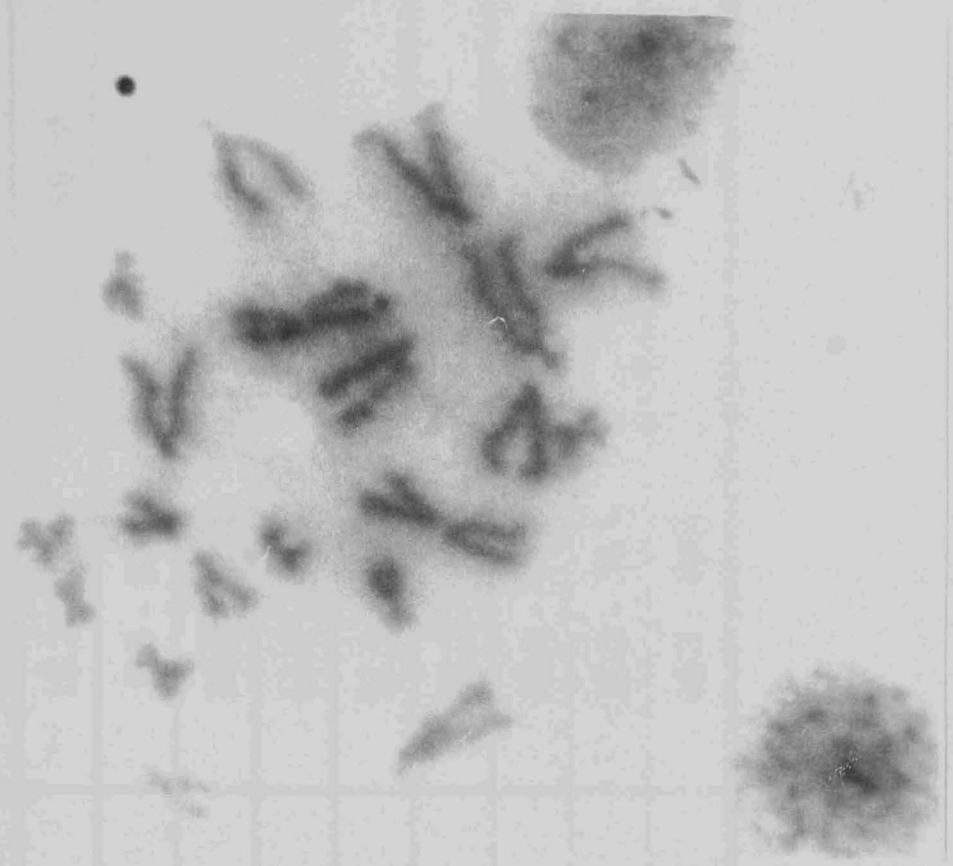


Fig. 114. Representative karyotype of *I. variegatus* chromosomes.

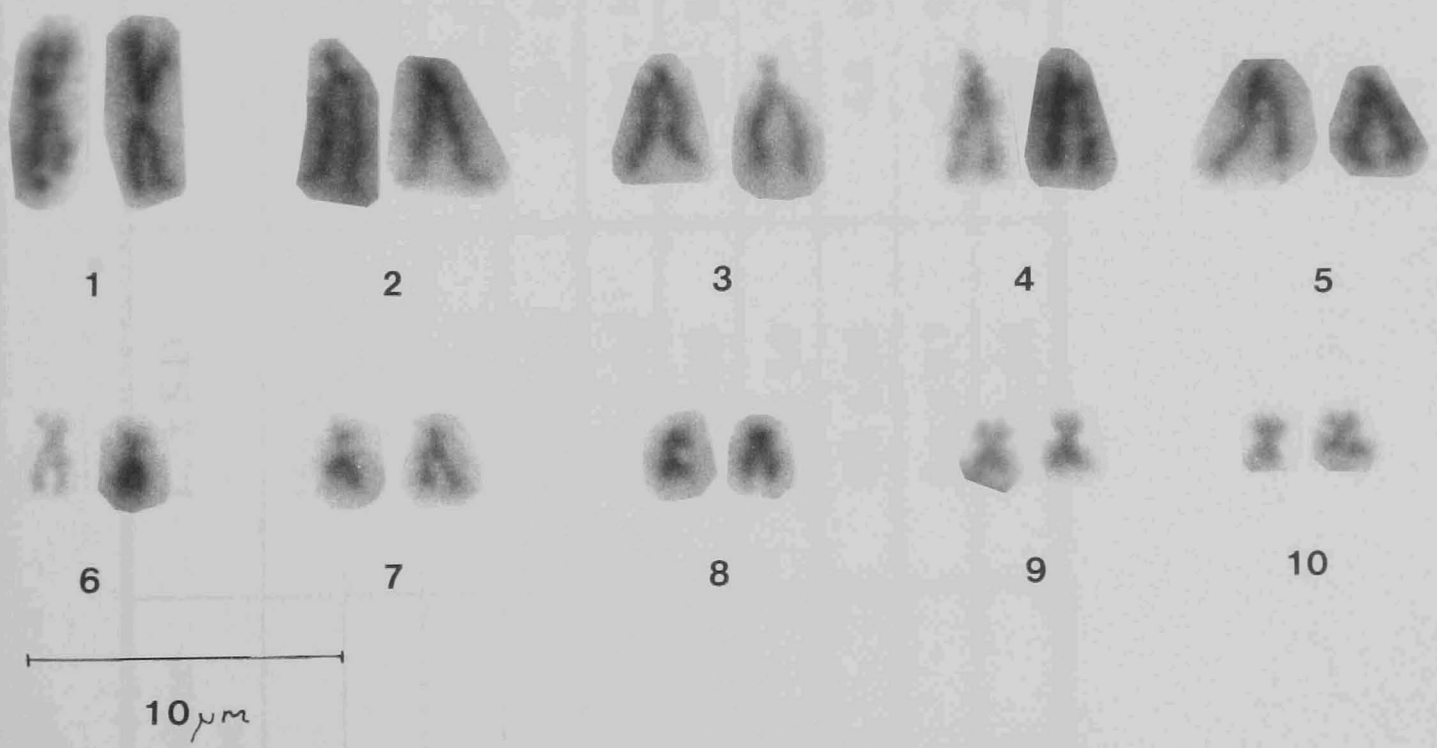


Table 73. Metrical data on the chromosomes of *Apatemon gracilis* and their classification.

Chromosome Number	$L^R \pm$ S.D.	95% C.L.	$I^C \pm$ S.D.	95% C.L.	Classification after Levan *et al.* (1964)
1	16.8 ± 1.4	15.0 - 18.6	37.1 ± 0.9	36.0 - 38.2	m-sm
2	15.3 ± 1.5	13.4 - 17.2	21.7 ± 2.8	18.2 - 25.2	sm-st
3	13.8 ± 1.1	12.4 - 15.2	10.6 ± 0.9	9.5 - 11.7	a
4	13.0 ± 1.5	11.1 - 14.9	41.3 ± 2.1	38.7 - 43.9	m
5	12.3 ± 1.5	10.4 - 14.2	13.3 ± 1.7	11.1 - 15.4	st-a
6	7.4 ± 0.5	6.8 - 8.0	24.8 ± 2.3	21.9 - 27.7	sm-st
7	6.5 ± 1.0	5.3 - 7.7	30.4 ± 2.6	27.2 - 33.6	sm
8	5.6 ± 0.6	4.8 - 6.4	44.2 ± 2.9	40.6 - 47.8	m
9	5.4 ± 0.7	4.5 - 6.3	26.9 ± 2.7	23.5 - 30.3	sm-st
10	4.0 ± 0.8	3.0 - 5.0	47.9 ± 1.1	46.5 - 49.3	m

Fig. 115. Metaphase spread of *A. gracilis* chromosomes.

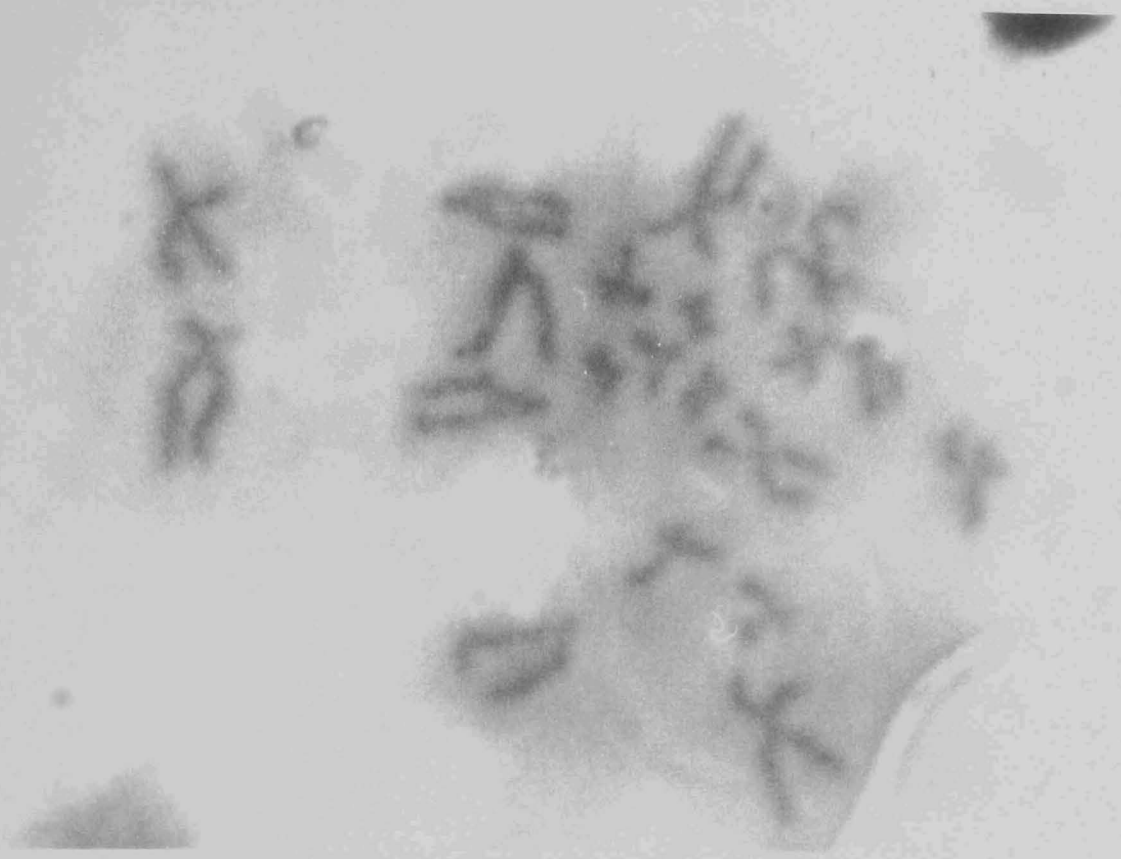


Fig. 116. Representative karyotype of *A. gracilis* chromosomes.

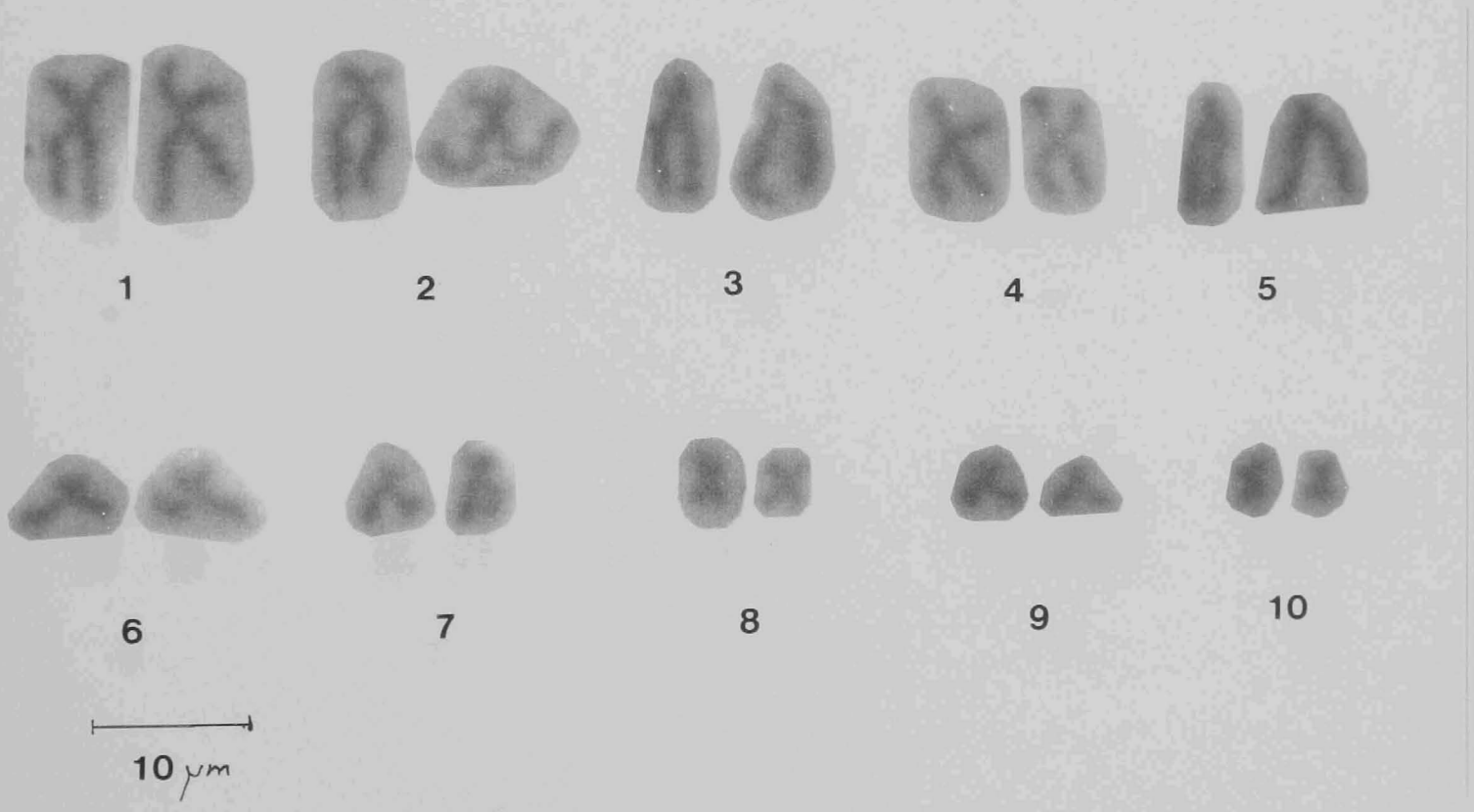


Fig. 117. Metaphase spread of *V. piscinalis* chromosomes.

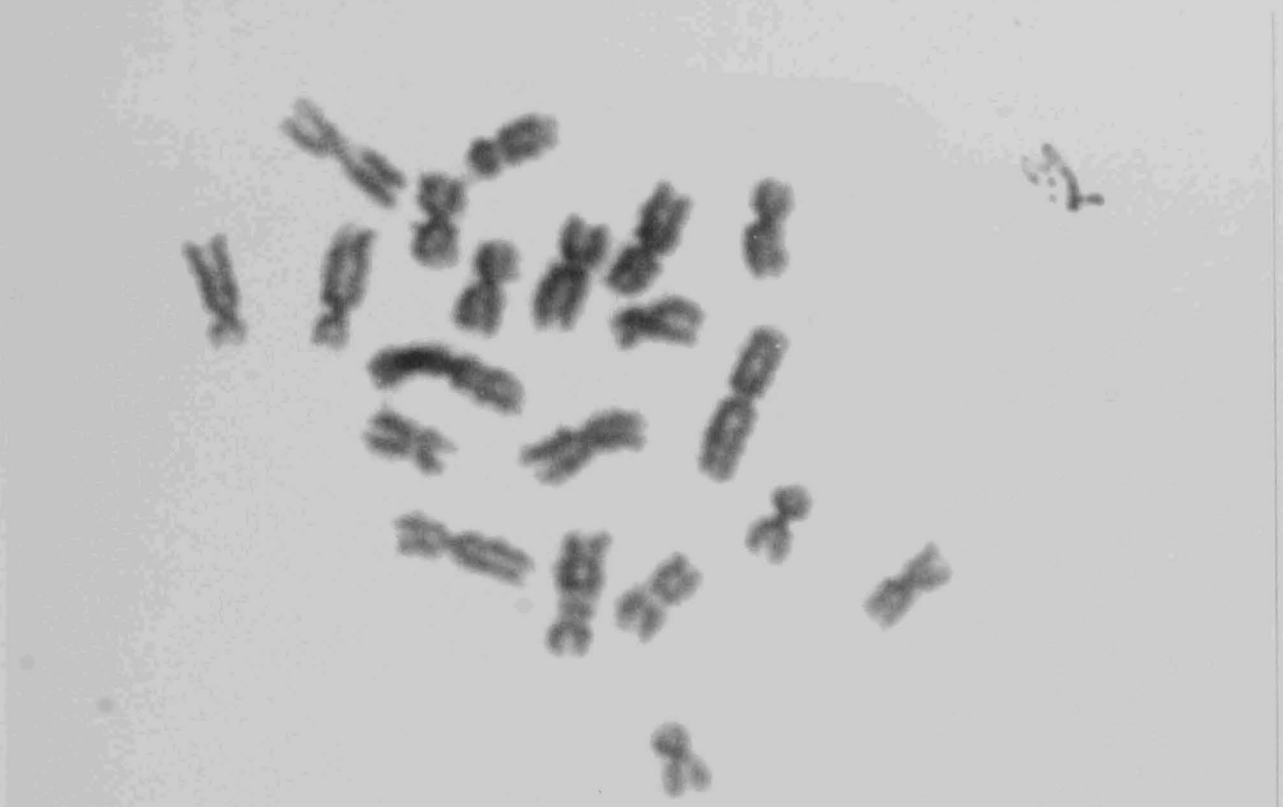


Fig. 118. Representative karyotype of *V. piscinalis* chromosomes.

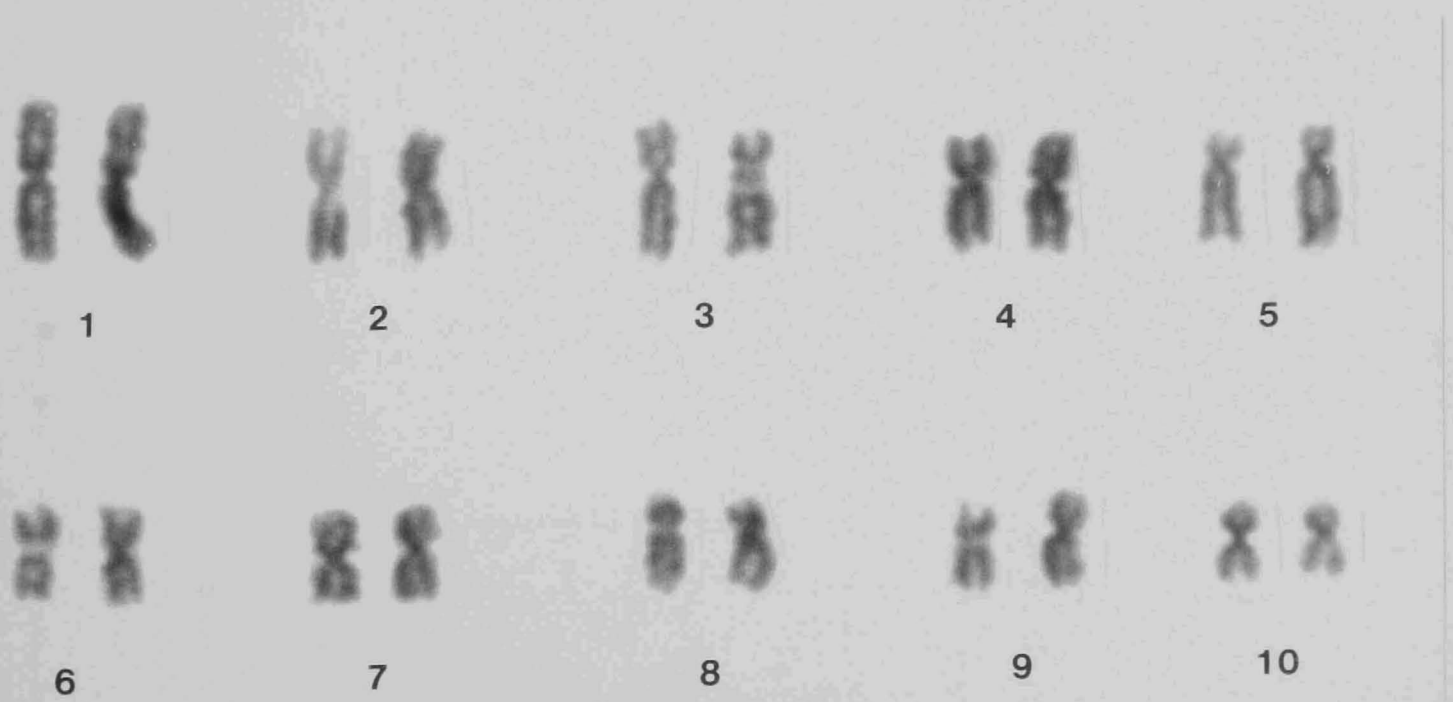


Fig. 119. Metaphase spread of *L. peregra* chromosomes.

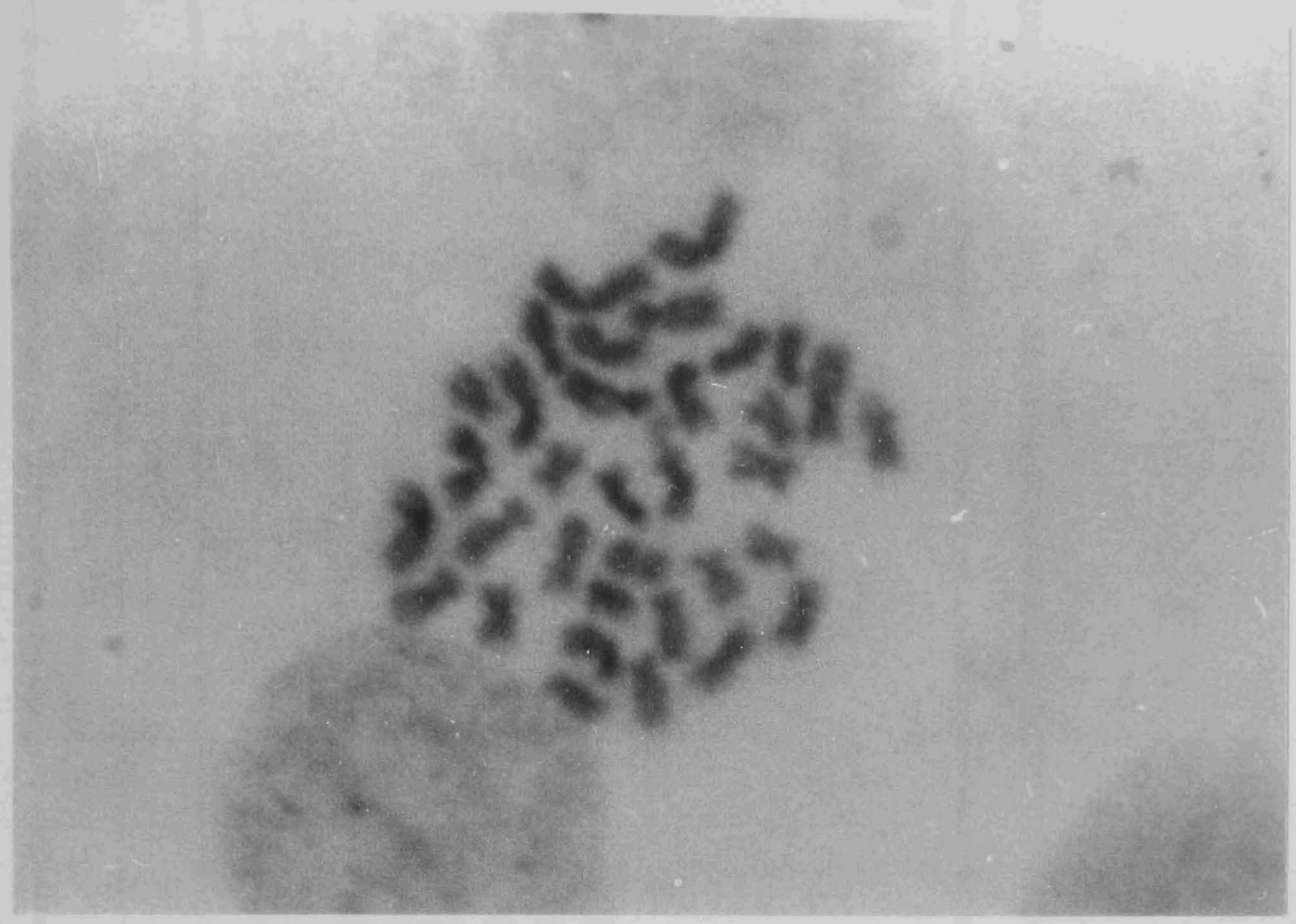


Table 74. Significant differences in relative size and centromeric indices of chromosomes of the strigeid species studied (Students t-test).

Strigeid species	*I. variegatus*		*A. gracilis*	
	L^R	I^C	L^R	I^C
I. erraticus	C1. 2.63c C2. 3.23b C6. 3.25b C7. 2.81c C8. 3.15c C9. 3.40b	C1. 7.61a C2. 24.77a C3. 4.50a C4. 6.88a C5. 3.95b C7. 6.38a C8. 7.23a C9. 3.70b		C1. 11.82a C2. 14.49a C4. 25.43a C8. 5.80a C9. 9.33a
I. variegatus			C8. 3.06c C10. 2.5c	C1. 22.39a C3. 8.05a C4. 9.02a C5. 4.27b C7. 4.71b C9. 13.09a

L^R - relative length of chromosomes; I^C - centromeric index; upper case letters indicate chromosome number; lower case letters indicate significant differences at - a: p,0.001, b: p,0.01, c: p,0.05.

Table 75. Known karyotypes of strigeid species.

Chromosome No.	Baršienė *et al.* (1990)				Present study		
	Cotylurus cornutus	*Apatemon (Australapatemon) fuligulae*	*Ichthyocotylurus erraticus*	*I. pileatus*	*I. erraticus*	*I. variegatus*	*Apatemon (Apatemon) gracilis*
C1	a	st	m	a	m	m	m-sm
C2	a	st	a	a	m	st	sm-st
C3	a	sm	a	a	st-a	st	a
C4	a	a	a	st-a	st-a	st	m
C5	a	a	a	st	st-a	st	st-a
C6	st	sm	sm	sm-st	sm-st	sm	sm-st
C7	st-a	sm	sm	st	sm	m-sm	sm
C8	st	sm	sm	m	sm	m	m
C9	st	m	m	m-sm	m	m	sm-st
C10	m	m	m	m	m	m	m
Number of arms	22	32	32	26	32	32	30

Lowercase letters: Chromosome classification according to Levan *et al.* (1964).

Fig. 120. Absolute chromosome lengths from centromere origin (0) in: a - *I. erraticus*, b - *I. variegatus*, c - *A. gracilis*.

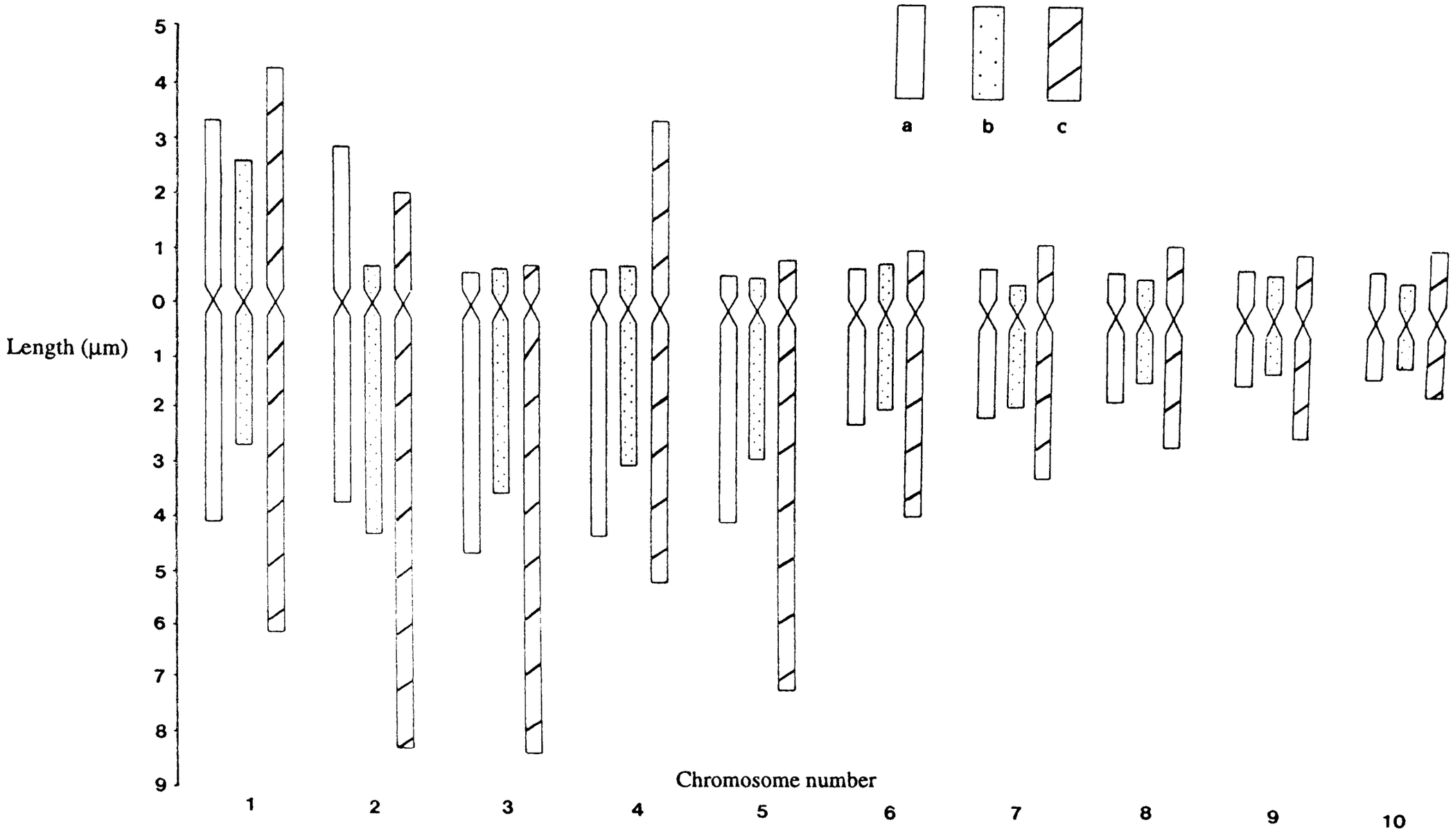
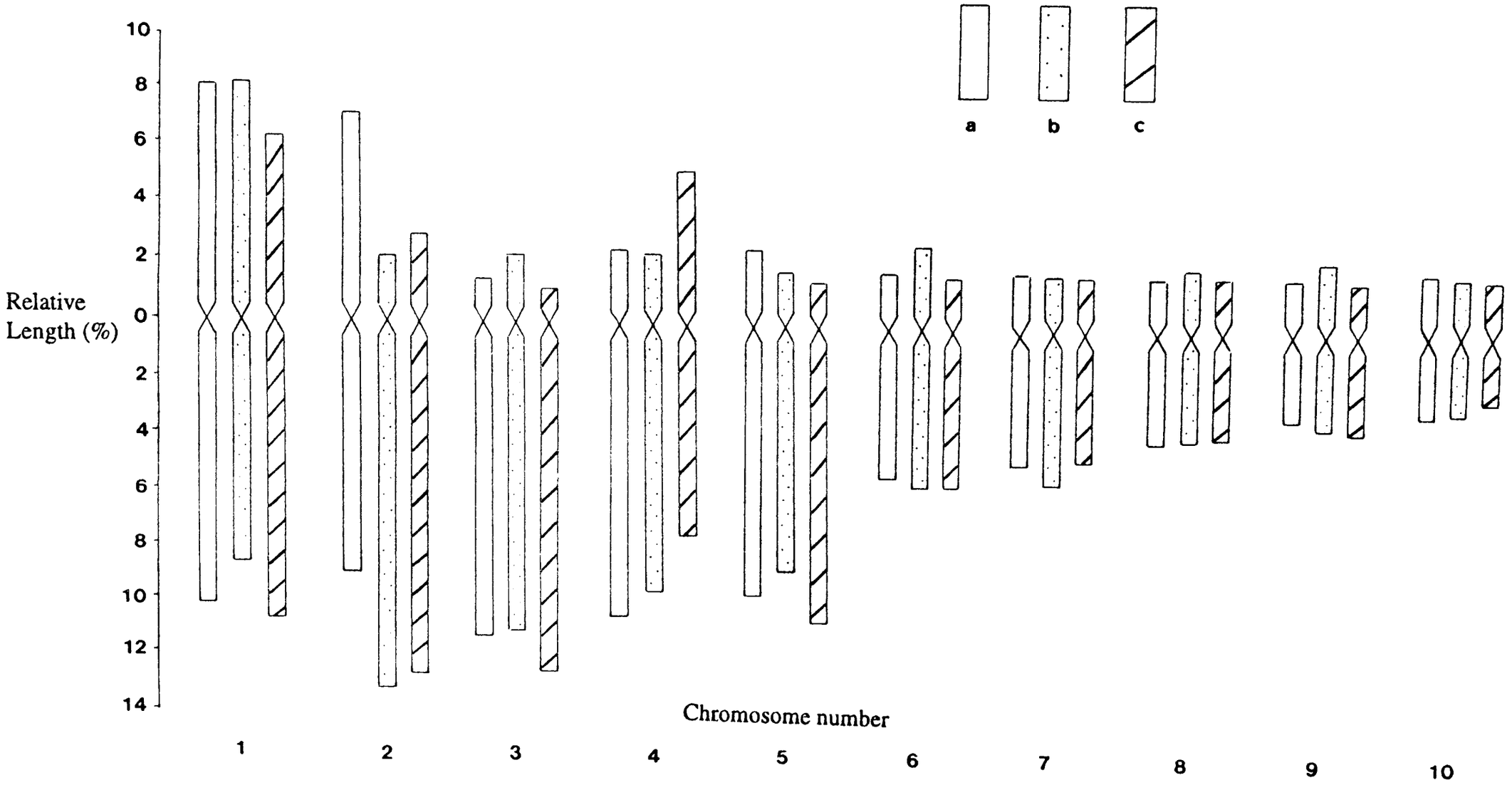


Fig. 121. Relative chromosome lengths from centromere origin (0) in: a - *I. erraticus*, b - *I. variegatus*, c - *A. gracilis*.



DISCUSSION

The snail incubation period in colchicine was found to affect the number and quality of metaphase spreads ultimately recorded, with the shorter duration (3-4 hours) providing more well-defined chromosome sets for the construction of the karyotype.

Karyotypes were obtained for the three strigeid species whose life-cycles were completed experimentally, *Ichthyocotylurus erraticus*, *I. variegatus* and *Apatemon gracilis*.

The number of chromosomes in metaphase spreads was the same for each species (2n = 20), and this number was also recorded for the strigeid species, *A. fuligulae*, *Cotylurus cornutus*, *I. erraticus* and *I. pileatus* examined by Baršiené *et al.* (1990). Twenty chromosomes appears to be the standard for somatic cells of the family Strigeidae. However, this generalisation is based on just six species from three genera and a larger number of karyotypes, including those of species from other genera, are required before such a conclusion can be made.

Other digenean families have demonstrated that the number of chromosomes comprising diploid sets is not strictly conserved, sometimes even at the level of genus. Members of the Paramphistomidae possess 14 [e.g. *Paramphistomum cervi* (Schrank, 1790)], 16 [e.g. *Cotylophoron cotylophoron* (Fischoeder, 1901)] or 18 [e.g. *P. microbothrium* (Fischoeder, 1901)] chromosomes in somatic cells (see Subramanyam & Venkat Reddy, 1977). The Plagiorchiidae may have a diploid complement of 18 [e.g. *Eustomos chelydrae* (MacCallum, 1921) and *Glypthelmins quieta* (Stafford, 1900); see Britt, 1947], 20 [e.g. *Leptophallus nigrovenosus* (Bellingham, 1844); see Baršiené & Grabda-Kazubska, 1988] or 22 [e.g. *Haplometra cylindracea* (Zeder, 1800) and *Opisthioglyphe ranae* (Frölich, 1791); see Baršiené & Grabda-Kazubska, 1988] and the Echinostomatidae typically 20 (*Echinoparyphium* spp.; see Mutafova *et al.*, 1987) or 22 (*Echinostoma* spp.; see Baršiené & Kiseliene, 1991).

While exhibiting the same diploid chromosome number, distinct differences were recorded in the chromosome morphology of the three strigeids investigated and statistical analysis (Table 74) showed these to be species-specific. These differences were most readily seen in the form of an idiogram indicating relative chromosome lengths (Fig. 121). It is interesting to note that differences in the chromosome morphology of *I. erraticus* and *I. variegatus* were as extensive as those between *A. gracilis* and the individual *Ichthyocotylurus* spp. Indeed, it was the two *Ichthyocotylurus* spp. that exhibited the greatest differences between their relative chromosome lengths. These *Ichthyocotylurus* spp. could be readily discriminated without the use of statistics by their second chromosome pair. In *I. erraticus* this pair was bi-armed and *I. variegatus* single-armed (Figs 112 and 114). Similarly, the fourth chromosome pair of *A. gracilis* was bi-armed but single-armed in both *Ichthyocotylurus* spp. (Figs 112, 114 and 116).

The snail host (*V. piscinalis*) of the *Ichthyocotylurus* spp. was, like the parasites, found to possess 20 chromosomes in somatic cells. Fortunately, the karyotypic differences between the *Ichthyocotylurus* spp. and *V. piscinalis* were marked; all chromosomes of the snail being bi-armed, which enabled any contamination by host tissue to be readily identified.

Fig. 120 shows that the absolute lengths of individual chromosomes differ greatly between the species investigated. These results should be treated with caution, as the degree of chromosome spiralization varies depending upon the point in metaphase at which cell division was stopped. It does appear, however, that *A. gracilis* (2.8-10.8μm) possesses longer chromosomes than *I. erraticus* (2.0-7.4μm), which have longer chromosomes than *I. variegatus* (1.7-5.5μm). Baršiené *et al.* (1990) observed a narrower size range for their strigeids (3.8-7.2μm), while Baršiené & Grabda-Kazubska (1988) recorded chromosome lengths of 2.0-14.2μm for plagiorchiids and Baršiené & Kiseliene (1990) 1.8-11.1μm for echinostomatids.

The karyotype of *I. erraticus* was described by Baršiené *et al.* (1990) using

parthenitae from naturally infected *Valvata helicoidea* in north-west Chukotka, Siberia. Their results differed markedly from the karyotype recorded here for *I. erraticus*. It is possible that interpopulational variation exists in the chromosome morphology of *I. erraticus* from geographically distant samples, especially when utilising different molluscan hosts. Such variation was noted by Baršiené & Kiseliene (1991) for *Echinostoma revolutum*, originating from *Lymnaea stagnalis* and *L. auricularia*. Nevertheless, the karyotypic differences recorded for the two *I. erraticus* populations were extreme and, given the difficulties in discriminating *I. erraticus* and *I. variegatus* by external cercarial morphology alone, it is also possible that the material of Baršiené *et al.* (1990) was not conspecific (present study identification confirmed by adult morphology and known life-history). The differences between their *I. erraticus* karyotype and that described here for *I. variegatus* are minimal and could easily be accounted for by measuring techniques (See Table 75).

When comparing the known chromosome morphologies of strigeid species from this study and elsewhere, several similarities were observed. In each diploid set there was a size division between the first and last five pairs. Elements of the large chromosome group (pairs 1-5) were predominantly single-armed and elements of the small chromosome group (pairs 6-10) bi-armed. The exception to this was that of *Cotylurus cornutus*, whose karyotype consisted of nine single-armed pairs; see Table 75.

It was proposed by White (1971), and is now widely assumed in studies on chromosome evolution, that a "primitive karyotype" consists typically of a large number of single-armed (subtelocentric and acrocentric) chromosomes. Thus, Grossman & Cain (1981) considered the karyotypes of *Megalodiscus temperatus* (Stafford, 1905) and *Philophthalmus gralli* Mathis & Leger, 1910, which are composed primarily of acrocentric chromosomes, to show lesser degrees of chromosomal evolution than karyotypes of members of the Schistosomatidae, which possess both metacentric and acrocentric chromosomes (Short & Menzel, 1960; Short & Menzel, 1979; Grossman *et*

al., 1980). Similar conclusions were made by Baršienė & Kiseliene (1990) for the karyotype of *Neoacanthoparyphium echinatoides* (Filippi, 1854), which they suggested was more primitive than other known echinostomatids with 20 chromosomes. Thus, it appears that the chromosomes comprising strigeid karyotypes, with a high proportion of bi-armed (metacentric/submetacentric) elements, have evolved considerably from the primitive form.

Changes in chromosome form were believed by Grossman & Cain (1981) to result most commonly from centric fusion, pericentric inversions, changes in the amount of heterochromatin and euchromatin, and through other chromosomal rearrangements. Often a likely route for the evolution of chromosomes within a family or genus can be visualised; key indicators include: karyotypic variation between related species whose diploid complements differ (e.g. Baršienė & Grabda-Kazubska, 1988); atypically large chromosomes (e.g. Grossman & Cain, 1981); the relative lengths of chromosomes comprising the haploid genome (White, 1973); the chromosome arm number (e.g. Mutafova, 1993); and the presence and distribution of heterochromatin, which can be seen using C-banding techniques (e.g. Baršienė & Kiseliene, 1991). One of the most commonly observed evolutionary pathways occurring in the digenean genome results from a Robertsonian translocation. This change in form involves the fusion of two non-homologous, single-armed chromosomes to form a single bi-armed element, with the concomitant loss of a unit from the genome. Such events were thought to be responsible for interspecific differences in chromosome sets of *Phyllodistomum* spp. (Baršienė & Orlovskaya, 1990), echinostomatids (Mutafova, 1993) and plagiorchiids (Baršienė & Grabda-Kazubska, 1988). It appears unlikely that Robertsonian translocations were involved in karyotypic differences observed for strigeid species for several reasons: all strigeid species described were found to possess the same number of chromosomes (2n = 20), resulting in no evidence of a past reduction in the number of chromosomes comprising the diploid set; several species do not possess a large bi-armed chromosome (e.g. *C. cornutus* and *I. pileatus*; see Baršienė *et al.*, 1990); and, when species do have

large bi-armed chromosomes, none were found to be atypically large.

The idiogram of relative chromosome lengths, shown in Fig. 121, suggests that another common chromosome rearrangement, pericentric inversions, may have been responsible for some of the karyotypic differences between strigeids, and particularly the metacentric elements of *A. gracilis* (C1 and C4), *I. erraticus* (C1 and C2) and *I. variegatus* (C1). These pairs of chromosomes have large increases in the length of their short arms and a reciprocal decrease in the length of their long arms. This type of re-arrangement increases arm numbers but has little effect upon relative chromosome length. Among the strigeid species which have been studied, the fundamental karyotype (N-F) ranges from 22 for *C. cornutus* (see Baršiené *et al.*, 1990) to 32 for *A. fuligulae* (see Baršiené *et al.*, 1990), *I. erraticus* and *I. variegatus* (present study). Mutafova (1993) believed that pericentric inversions were responsible for the development of some chromosome short arms in echinostomatid species.

The small chromosome pairs (C6-C10) of studied strigeid species (Baršiené *et al.*, 1990 and present study) were predominantly bi-armed, which suggests that major reorganisation has occurred in these elements. Given their size, changes in the form of these chromosomes may have resulted from minor duplications, deletions, inversions or translocations. It is suggested above that fusions (Robertsonian translocations) were unlikely to have been involved in the development of strigeid karyotypes; although such an event might have explained the discrepancy in size between chromosomes 5 and 6 (the loss of an analogue of intermediate size). An alternative explanation for this division of the diploid sets into two size groupings could be that chromosome 6 (and possibly 7) was involved in translocations with smaller units, resulting in the uniformity in size of these latter pairs.

Further work on other strigeid species is required before any firm conclusions can be made on the chromosomal evolution of this group. In relation to this, the use of C-banding techniques might be of value in determining the presence and distribution of heterochromatin within individual chromosomes.

In summary, chromosome number and morphology was described for *I. erraticus*, *I. variegatus* and *A. gracilis*, the identity of which was known through life-cycle studies. The strigeids described here and from all other sources have been found to possess 20 chromosomes in diploid sets. Karyotypes obtained from the cercariae-releasing parthenitae enabled the ready discrimination of *I. erraticus* and *I. variegatus* cercariae which had proved problematic by comparisons of gross morphology. This technology may also be useful for discriminating between locally obtained *I. platycephalus* and *I. variegatus* specimens and so confirm the validity of the latter species.

CHAPTER 8: GENERAL DISCUSSION AND CONCLUSIONS

GENERAL DISCUSSION AND CONCLUSIONS

The survey of metacercarial infections in fish included many sites from mainland Britain and though by no means exhaustive, discovered the presence of just four strigeid species, two belonging to the genus *Ichthyocotylurus* and two to the subgenus *Apatemon (Apatemon)*. On the basis of the available keys and the descriptive works listed in Chapter 2; Introduction, these species were tentatively identified as *I. erraticus*, *I. variegatus*, *A. gracilis* and *A. annuligerum*. Material obtained from Finland was identified using the same methods applied to the British metacercariae and appeared to contain all four *Ichthyocotylurus* spp. recognised by Odening (1979), *I. erraticus*, *I. variegatus*, *I. platycephalus* and *I. pileatus*. Records of *I. platycephalus* metacercariae from Europe and North America have been predominantly from the Percidae and Cyprinidae (see Chapter 2.1; Historical perspective). Although cyprinids were only examined from a few sources in the present study, none were found to be infected. Previous tentative reports of this species from Britain conform more to the knowledge gathered currently (see Chapter 2.1 and 2.2.2; Results) and by previous authors (see *inter alia* Odening & Bockhardt, 1971) for *I. variegatus*. No British reports exist of the remaining known member of this genus, the smallest species, *I. pileatus*.

These metacercariae were used to experimentally raise adults. Morphological and site specificity data obtained from these adults confirmed the identities conferred to *I. erraticus* and *A. gracilis* metacercariae, while strongly supporting the validity of *I. variegatus* metacercariae which has been the subject of some dispute. Principal components analysis (PCA) was also successful in separating these species. Furthermore, metacercariae from sources that were not confirmed experimentally clustered with the confirmed species groups. Though the Finnish specimens of *I. pileatus* and *I. platycephalus* metacercariae did not establish as adults in the experimental definitive hosts and therefore there were no adults available for the examination of their morphology, nevertheless, PCA of these metacercariae clearly distinguished them from

the other *Ichthyocotylurus* spp. (Fig. 23). The discrimination of *I. pileatus* from other *Ichthyocotylurus* metacercariae by PCA was achieved even without the suggested addition to Odening's (1979) key for this species, i.e. "length of tribocytic organ comprises at least 1/3 of body length". This ratio, originally proposed as a feature by Bauer (1987), was however, found to be of considerable value in distinguishing *I. pileatus* from *I. erraticus* metacercariae of similar dimensions when using traditional morphological comparisons. Without the refinements suggested as a result of the current work in Chapter 2.2.2; Results, the key of Odening (1979) was unable to provide a confident identification of all the *Ichthyocotylurus* specimens recovered in the present study.

Had adult *I. platycephalus* specimens been obtained it would have been interesting to apply PCA to attempt to discriminate this species from *I. variegatus*. Similarly, the techniques subsequently used on other life-stages (miracidium, parthenita, cercaria) would have been performed on *I. platycephalus* to identify characteristic features. In particular, karyology should be applied to the parthenita of this species in the future to enable a comparison of its chromosome morphology with that now known for *I. variegatus*. However, even without this information, all the data acquired for *I. variegatus* at the metacercarial and adult stages supported the validity of this as a separate species from *I. platycephalus*, as does the work of Odening & Bockhart (1971), Swennen *et al.* (1979) and Odening (1979). Furthermore, the data gathered for *I. variegatus* failed to agree with, or contradicted, the descriptions provided for *I. platycephalus* by Hughes (1928), Dubois (1968), Odening *et al.* (1970) and McDonald, 1981. The salient features are listed below:

1. Metacercariae considered to be *I. variegatus* were typically recovered from the inner surface of the swimbladder of naturally and experimentally infected hosts, not the pericardial cavity which is the most commonly recorded site of *I. platycephalus* cysts.

2. Metacercariae were never found to possess a ventral sucker of comparable length to the tribocytic organ which is a characteristic of *I. platycephalus* metacercariae.

3. The adults were site-specific to the small intestine and rectum of experimental gull hosts and were never located within the cloaca or bursa Fabricius. The latter regions are the predominant location of *I. platycephalus* adults, which, although occasionally recorded from the rectum, are never present in the small intestine.

4. All adults possessed 'closed' forebodies, with superficially placed suckers. Illustrations of *I. platycephalus* in the literature suggest that the suckers are deep set within an 'open' forebody, though no measurements were available for comparison. The differences in forebody form and arrangement of suckers for the two species probably represent adaptations to the topography of their respective specific niches in the digestive tract of definitive hosts.

The existence of *I. platycephalus* in Britain, known to be present in Eire (see Chapter 2.1; Introduction), could be further investigated by performing an extensive sampling programme of potential fish hosts from uninvestigated sources. Apart from percids the most likely British hosts for the metacercaria of this species include bream, silver bream, pike and zander.

Metacercariae of both *I. erraticus* and *I. variegatus* were found to mature rapidly within the definitive host. The developmental rate of *I. variegatus* was particularly remarkable, growing from about 0.4mm to over 6mm in approximately a week. Egg production was difficult to quantify accurately because the proportion of introduced metacercariae which did not reach maturity was unknown, but was very high for both species, particularly for the larger *I. variegatus*. A single millilitre of faeces from a gull infected with 200 *I. variegatus* metacercariae was found to contain approximately

48,000 eggs. However, this massive output of eggs was balanced by the short life of these species, which was recorded as a maximum of 45 days for an *I. erraticus* infection and a maximum of 30 days for an *I. variegatus* infection.

Adult worms of *A. gracilis* were successfully raised in Aylesbury and mallard ducklings but not gulls. These experimental hosts did not prove, however, to be particularly suitable definitive hosts because adults in most infections exhibited a reduced hindbody size and eggs produced were never released. Only a single source (from stone loach) resulted in egg releasing adults. All infections with metacercariae believed to be *A. annuligerum* failed to establish in these hosts which was not unexpected, as similar attempts to raise the adult in hosts other than buzzards have also failed (Odening, 1970; Blair, 1974) and this species may prove to be specific for birds of prey. Without adults and subsequent life-stages the identification of *A. annuligerum* specimens relied on its specificity to perch eyes, a host from which no other *Apatemon* metacercariae have been recorded, and the morphological differences between these specimens and *A. gracilis* demonstrated by PCA.

With the recent advances in *in vitro* culture techniques (see Strain & Irwin, 1995) attempts could be made to raise *A. annuligerum* adults in this manner, hopefully providing viable eggs and consequently miracidia with which to challenge a range of potential molluscan hosts. Alternatively, an extensive survey of molluscs is required to isolate the first intermediate host of *A. annuligerum* which remains unknown; identification of the cercariae being via experimental infections in perch and ideally the subsequent culture of adults.

Several interesting irregularities in the morphology and host specificities of *A. gracilis* were recorded in the present study and by other authors, which, when considered collectively, raise questions as to the homogeneity of the species as currently accepted. These are listed below:

1. Light microscopical examination indicated that *A. gracilis* metacercariae were morphologically similar, regardless of host origin. However, such similarities are also observed with the light microscope for *A. gracilis* and *A. annuligerum* metacercariae. PCA demonstrated that *A. gracilis* specimens excised from non-salmonids exhibited morphometrics intermediate between salmonid metacercariae and those considered to represent *A. annuligerum.*

2. Experimental infections using metacercariae from stone loach were markedly more successful in ducklings than rainbow trout and salmon parr material, attaining a greater size, showing an increased longevity and releasing eggs in the host's faeces. Even though the experimental birds employed were not recognised natural hosts, it is peculiar that the development of the non-salmonid metacercariae should consistently surpass that of the salmonid material. Comparable results were obtained by Crocombe (1959) and Watson & Pike (1993) for *A. gracilis* infections in ducklings; Crocombe recorded the successful development of bullhead metacercariae to adults, while Watson and Pike were unable to raise adults from rainbow trout material. In contrast to the latter authors and the present study, Blair (1974, 1976) obtained adults of a uniform development, all of which produced viable eggs, regardless of metacercarial source (salmonid and non-salmonid).

The developmental differences observed here and by previous authors may simply result from degrees of metacercarial 'sensitivity' to an unsuitable host, factors which might have been influenced by the host's diet or the age of metacercariae introduced. Nevertheless, it would be interesting to observe if metacercariae from all sources develop equally well within the recognised definitive host, *Mergus* spp.

3. Particularly interesting results were obtained in the present study for *A. gracilis* cercarial challenges to a range of known natural fish hosts. Cercariae derived from stone loach material penetrated and developed into encysted metacercariae in stone loach, but

not in rainbow trout, brown trout or salmon parr. Previous experimental infections performed by Blair (1974, 1976) used *A. gracilis* cercariae emerging from naturally infected snails. These infections also showed a distinct, but reciprocal, host specificity, being successful in rainbow and brown trout, but not in stone loach.

Together, the PCA results, developmental differences in the same experimental host and piscine host specificities all suggest the presence of two (or more) species which are at present all considered to be *A. gracilis*. In hindsight it would have proved informative to have examined chromosome morphology, using the testes of the variously derived *A. gracilis* adults, to ascertain if genetic differences were present. The possibility of an *A. gracilis* species complex should certainly be explored in further work on this group. Comparisons of the morphology and behaviour (release patterns) of known origin cercariae, karyology of known origin parthenitae, comprehensive experimental infection trials and genetic techniques, might all be employed to this end.

This confusion for the most studied member of the subgenus *Apatemon* (*Apatemon*) emphasises the limited knowledge presently available for the taxa. Indeed, some recent authors still maintain that *A. gracilis* may also utilise leeches as the second intermediate host (Spelling & Young, 1986), thus indicating the morphological similarities between metacercariae across the whole genus which gives rise to this disorder. Experimental completion of the life-cycles of *Apatemon* spp., such as *A. fuligulae* (metacercaria and adult described), are needed to identify ontogenic characters of systematic importance at the species level. While life-cycle studies must be performed for species like *A. somateriae*, whose identity is conferred solely on the basis of adult morphology and which is recorded from the non-piscivorous eider (Dubois, 1968). Consequently, with future life-cycle information the latter species may actually prove to belong to the subgenus *Australapatemon*.

Increased knowledge on all ontogeny of *Apatemon* spp. may provide new evidence for the raising of the subgenus to the full generic level, as originally proposed

by Sudarikov (1959) and clarify the significance of cercarial host specificity at this taxonomic level. Hopefully, the new knowledge acquired in the present study for the life-stages of *A. gracilis* (derived from stone loach material) will enable discrimination at the species level, while aiding the evaluation of criteria for higher taxa when equivalent information is obtained for other species.

The miracidial morphology was found to be identical in all three strigeid species examined, with no differences observed in the arrangement of internal organs, epidermal plates or sensilla. Indeed, the miracidium is the only life-stage at which *A. gracilis* could not be readily discriminated by its morphology from the *Ichthyocotylurus* spp. Nevertheless, miracidial development within the egg was more rapid for *A. gracilis* than for either of the *Ichthyocotylurus* spp. Elucidation of the taxonomic value of the absence of cephalic glands and presence of a gland of unknown function that is situated and opens posteriorly, in strigeid miracidia (latter feature also described for other strigeoids) will require further studies on strigeid and related miracidia.

The mounting of miracidia for chaetotaxy examination in glycerine was found to be extremely beneficial to their observation, allowing the specimens to be "rolled" within the medium and the epidermal plates counted in relation to a landmark. Such a method is far more reliable than descriptions made from observations at different planes of focus, and enabled the description of an unpaired mid-ventral plate in the second tier of the *A. gracilis* miracidium which was not recorded by Vojtek (1964a) for this species. The sensilla pattern was fully revealed by this technique and when compared to works on other strigeoids was seen to be highly conserved across the superfamily. It was found that the available nomenclature for the distribution of miracidial sensilla derived by Dimitrov *et al.* (1989) was not adequate to fully describe the pattern recorded for these strigeid species and amendments to their system are proposed. Deciliation of miracidia was achieved by osmotic shock, a technique which had not previously been applied to this life-stage and which proved to be more successful than the commonly used method

of sonication. This removal of the locomotory cilia enabled SEM observation of the surface structures which confirmed the arrangement of sensilla and revealed sensillum form.

The longevity of *I. variegatus* miracidia was short (<11 hours under experimental conditions), necessitating the rapid location of its *Valvata* spp. host. Experimental observations suggested that host location by this species may be preceded by an initial dispersal phase, after which the miracidia responded to the presence of a substance(s) emitted from susceptible snails. This water borne "miraxone" caused an increase in the turning response of miracidia, resulting in their accumulation in the vicinity of the snail and thus an enhanced possibility of contact.

Ichthyocotylurus cercariae were found to be released from a naturally infected *V. piscinalis* specimen which constitutes the first record in Britain of cercariae of this genus (see 5.1.1; Results). All previous confident host records for cercariae of this parasite genus have been made from *Valvata* spp. Naïve *V. piscinalis* were also successfully used for experimental infections (see 5.1; Results). *A. gracilis* is predominantly recorded from lymnaeid snails, and naïve *Lymnaea peregra* proved, in the present study, to be efficient experimental hosts.

Cercarial developmental periods within the molluscan host were found to be temperature dependent and markedly different for the strigeid genera investigated, with both *Ichthyocotylurus* spp. typically requiring several weeks longer than *A. gracilis* at 20°C.

Cercariae being specially adapted to a free-living existence are traditionally considered to possess many useful features for the discrimination of taxa; these include morphological structures, many of which are subsequently modified or lost, physiological specificities and behavioural traits. Differences in *I. erraticus* and *I. variegatus* cercarial morphology were previously thought to include the presence (*I. variegatus*) or absence (*I. erraticus*) of unpigmented eye-spots, the number of septa

dividing the digestive caeca, arrangement of sensilla and pattern of armature (see Olson, 1970; Odening & Bockhardt, 1971; Swennen *et al.*, 1979). Of these, only the first criterion was found to be reliable, but even this feature, the presence of eye-spots in *I. variegatus*, was often difficult to discern. Fine details of the surface of these cercarial species had previously only been investigated with the light microscope; the "sensory hairs" being recorded from unstained preparations. Silver-staining and SEM observation revealed that the chaetotaxy maps of these species were identical, contrary to previous descriptions, and that variations seen in the armature were not characteristic. PCA was applied to the sensilla distribution in an effort to find discrete differences. This examination demonstrated species differences with separation provided by 95% confidence limits on centroids, but discrimination of individual specimens was not conclusive and the technique is considered to be of limited practical application. Many more sensilla were recorded on the tail-stem and furcae of *I. erraticus* and *I. variegatus* cercariae than observed by previous authors, who were only able to visualise those bearing a long cilium. At present morphological discrimination of *I. variegatus* and *I. platycephalus* cercariae is based solely on the distribution of tail-stem sensilla and the pattern of the armature (see Odening *et al.*, 1970; Odening & Bockhardt, 1971; Swennen *et al.*, 1979). Given the current findings it is likely that descriptions in the literature of the distribution of sensilla and armature (light microscopical) of *I. platycephalus* will prove incomplete and may actually correspond closely to those redescribed here for *I. erraticus* and *I. variegatus*. The cercaria of *I. pileatus* has yet to be described and any characteristic morphological features are unknown. Thus, it appears that, morphologically there are few good diagnostic criteria available for distinguishing *Ichthyocotylurus* cercariae. Consequently, members of this genus are in the unusual position of being more readily identified by metacercarial than cercarial morphology.

Within the Strigeidae the features of cercarial armature and chaetotaxy appear to have more significance at the subgeneric/generic level, e.g. *Ichthyocotylurus* spp.

possess both antero-lateral and postero-lateral tail-stem sensilla, while they are evenly spread along the tail-stem of *Cotylurus* and *Apatemon* (*Australapatemon*) spp., and occur only distally in *A. gracilis*, the sole member of the subgenus *Apatemon* (*Apatemon*) to have its sensillary pattern demonstrated (for references see Chapter 5.2.3; Discussion). The post-acetabular patch of spines described in the present study and by Blair (1974) for *A. gracilis* cercariae does not appear to have been recorded for any other *Apatemon* species. This feature was not noted by other authors for *A. gracilis* (see Table 56) and a similar oversight may have been made for the remaining cercariae described for the subgenera *Apatemon* (only *A. graciliformis*) and *Australapatemon*. Consequently, the systematic significance of this character is at present unknown, but warrants future study.

Karyological examination of experimentally raised parthenitae known to be releasing *I. erraticus*, *I. variegatus* and *A. gracilis* cercariae demonstrated convincingly distinct differences in their chromosome morphology and readily enabled their discrimination. The most visible difference between the two *Ichthyocotylurus* spp. occurs in the second largest chromosome pair, which are bi-armed in *I. erraticus* and single-armed in *I. variegatus*. This characteristic was found to be obvious from microscopical examination of the chromosome 'spreads' and did not require statistical analysis. The karyology study emphasised the importance of performing karyotypic "controls", i.e. ascertaining the karyotype of the snail host, by the finding that *V. piscinalis* and the *Ichthyocotylurus* spp. investigated both exhibit 20 chromosomes in diploid sets. Although the technique is quick, inexpensive and reliable once the karyotype is known, the initial description requires material of known identity and the examination of many 'spreads' to ensure the correct complement of chromosomes is considered. A limitation, which remains untested, is the possibility of variation in a species karyotype resulting from geographical isolation. Nevertheless, as the technique can also be applied to the testes of adults (Baršiené, 1993), karyotyping of *I. variegatus* and *I. platycephalus* adults, raised from metacercariae excised from locally infected fish, would enable the

confirmation of species identities and satisfy the controversy surrounding the status of these two species.

The specificity of cercarial species to their next intermediate host, which was proposed by Blair (1977) as a diagnostic criterion, was strongly supported by the results obtained in this study for *I. erraticus* and *I. variegatus*, indicated in Chapter 2.1; Natural infections and in Chapter 6; Experimental infection of fish. In the present study *I. erraticus* metacercariae were recorded from the pericardial cavities of naturally infected British powan, gwyniad, rainbow trout and grayling, with additional specimens recovered from Finnish whitefish and vendace; all members of the Salmonidae. Specimens recovered from gwyniad and grayling represent new British host records. The list of British hosts should also include brown trout (few of which were examined here) as reported by Wootten (1973a,b) and Campbell (1973). Both of these authors noted clustering of cysts on the ventricle of the heart in heavier infections, often with cysts piled on top of each other. This arrangement of cysts is far more typical of *Ichthyocotylurus* infections within the pericardial cavity than *A. gracilis*, where heavy infections tended to form a monolayered "blanket" adhering to the pericardium and bound together by connective tissue as a result of the host response (see Tables 2, 4).

I. variegatus metacercariae were found to be restricted to the Percidae and located predominantly on the inner surface of the swimbladder. Unfortunately, from a discriminatory perspective, *I. platycephalus* also utilises percids (and cyprinids), while *I. pileatus* has been recorded from salmonids and percids (see 2.1; Historical perspective). Site-specificities within the fish host may be typical for an *Ichthyocotylurus* species, but this feature alone was not found to provide a reliable means of identification. There was also some evidence gathered here to suggest that the site-specificity of these metacercarial species broadens with increasing intensities of infection (see Chapter 2.1; Results).

A far more extensive host range was observed for *A. gracilis* metacercariae than

the two *Ichthyocotylurus* spp., with specimens obtained from three families of fish; the Cobitidae (stone loach), Cottidae (bullhead) and Salmonidae (rainbow trout, salmon parr and arctic charr). The infection in arctic charr represents a new British host record. Additional hosts were also identified by Blair (1974, 1976) as the three-spined-stickleback and brown trout (experimental host). Site specificities of *A. gracilis* were found in the present study to vary according to host species (see also Crocombe, 1959; Blair, 1974, 1976; Wootten & Smith, 1980). This broad range of hosts and site specificities for *A. gracilis* metacercariae provides a sharp contrast to the apparently oioxenic *A. annuligerum* and highly specialised life-cycle demonstrated experimentally by Combes & Nassi (1977) for *A. graciliformis* (see Chapter 2.1; Historical perspective). Particularly puzzling regarding *A. annuligerum* is the lack of a clear link between its known hosts; perch and buzzards. What advantage this pairing imparts to the parasite is unclear. Equally mystifying is why no cysts of this species were ever recovered from ruffe; a species which is closely related to perch, occupies the same habitat, exhibits similar behaviour and is presumably equally liable to consumption by the definitive host.

Another mechanism for identification of *Ichthyocotylurus* spp. at the cercarial stage relies on behavioural phenomena. This study recorded a common resting posture for all three species of strigeid cercariae, the characteristic feature being the ventral curvature of the body. The postures of these species have all been described before (in illustrations) and differ in each case from those recorded here (Table 75). All previous figures of *I. erraticus* (see Olson, 1970; Swennen *et al.*, 1979) and *I. variegatus* (see Odening & Bockhardt, 1971) show a lateral flexion of the body, though this may be due to representational differences in the drawings, while Crocombe (1959) observed no body curvature for the cercariae he considered to represent *A. gracilis.* The resting posture for *I. platycephalus* recorded by Odening *et al.* (1970) showed a straight body. Without a more detailed examination (chaetotaxy/SEM) of the surface structures of *I. platycephalus,* which may or may not prove characteristic, this feature represents the

only distinction between *I. platycephalus* and *I. variegatus* cercariae.

The experimentally raised cercariae were seen to exhibit strict circadian release patterns from their molluscan hosts, which almost certainly enhance the possibility of infecting their particular fish hosts. A marked difference in strategy was recorded for the two genera, with both *Ichthyocotylurus* spp. emerging during daylight hours and *A. gracilis* with the onset of darkness. These differences are likely to be linked to their respective swimming behaviour, which was also seen to differ. *Ichthyocotylurus* spp. swim periodically in upward bursts before pausing, spreading the furcae and drifting downwards; while *A. gracilis* swims almost continually and away from the light. The daylight emergence and zig-zagging across the water column would enhance the possibility of the *Ichthyocotylurus* spp. encountering their active predatory hosts. In contrast, the behavioural traits of *A. gracilis* would increase the chance of contact with their hosts while the fish are immobile on the substratum during the hours of darkness. Alternatively, this may represent an adaptation for maximising contact with predominantly bottom dwelling hosts, e.g. stone loach and bullheads. A consequence of their respective swimming behaviour was the reduced longevity of *A. gracilis* cercariae, particularly compared to that of *I. variegatus* cercariae. Further differences between the two genera existed in the daily production of cercariae and duration of cercarial release. Experimentally infected *L. peregra* specimens emitted an average of 2778 *A. gracilis* cercariae daily over the course of the infection, which was seen to last for up to 80 days. A far lower average number of *I. erraticus* (154) and *I. variegatus* (127) cercariae were released each day and the duration of release from experimentally infected *V. piscinalis* typically lasted no longer than 30 days. It seems likely, given these differences in cercarial output, that the mode of transmission of the two *Ichthyocotylurus* spp. is more efficient than that of *A. gracilis*.

SEM examination of life-stages enabled a comparison of the number and types of sensilla particular to ontogeny. All sensilla observed were uniciliate, although

variation was noted in the structure displayed at the different life-stages. *Ichthyocotylurus* miracidia were found to possess at least three forms of sensilla; the first terminating in a crescent-shaped structure; the second, the "lateral papilla", comprising a large dome with highly folded surface; and the third, ending in a short cilium surrounded by a low collar. The retraction of the terebratorium prevented visualisation of these apical sensory structures which could be investigated in the future using TEM. Three forms of sensillum were common to the cercariae of both strigeid genera, the types differing in cilium length and the nature of the collar. Both *A. gracilis* and the *Ichthyocotylurus* spp. exhibited a fourth sensillum type with a short cilium, however, the presence of a tall tightly investing collar around the cilium was only observed for the *Ichthyocoylurus* cercariae. The four types of sensilla recorded for *Ichthyocotylurus* cercariae were also visualised using TEM and differences in external appearance reflected variation in internal structure. One particular form (type d, see Fig. 95) was atypical of any previously described for cercariae. Only two sensilla types were recorded for the metacercariae of both genera, with a short cilium emerging either from the tegument or from a raised dome. The numbers of sensory structures present were seen to increase from miracidia to metacercaria, but very few were observed on the surface of adults and these were restricted to a single form. However, due to the 'cupped' nature of the forebody, the sensilla positioned on the inner (ventral) surface could not be viewed. This could be rectified by preparing adults for histological section (in wax), cutting through to expose the inner surface of the cup, removing the wax and then processing for SEM. A complete examination of the sensory structures present in these strigeid life-stages would require extensive use of TEM to investigate the presence of subtegumental non-ciliate receptors which have been recorded for metacercariae in other families (see Bennett, 1975; Hoole & Mitchell, 1981). The greater variety of surface sensillum types exhibited by the free-living stages reflects the complex behaviour involved in their location, recognition, penetration and ultimate migration through the intermediate hosts.

This study applied a range of both traditional and little used modern techniques to all available life-stages of strigeids found infecting fish in the United Kingdom. Morphological, behavioural and karyological investigations were carried out and all shown to contribute to discrimination at the species and generic levels. The description of complex patterns of sensilla using chaetotaxy and of fine surface structures under SEM revealed many similarities at the species level, but provided evidence for their use in the separation of higher taxa. All comparisons of morphology were enhanced by the ability of principal components analysis to discern subtle variation, while karyological investigations provided precise species determination. There is much scope for the use of the latter two techniques for other systematic problems within the digenea.

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APPENDIX

APPENDIX

Definition of the family Strigeidae Railliet, 1919 according to Shoop (1986).

Diagnosis: Body bisegmented; forebody cup-shaped with a bilobed tribocytic organ; oral sucker, pharynx, and bifurcate caeca to the posterior end of the body usually present; pseudosuckers present, vestigial or secondarily absent; acetabulum present; vitelline distribution whole body or only in the fore- or hindbody; reproductive organs in hindbody; testes tandem and lobed; cirrus sac absent; paraprostate present; hermaphroditic duct present; genital pore dorso-subterminal; excretory pore terminal; eggs large and operculate; mesocercarial stage sometimes present; tetracotyle-type metacercaria; paranephridial plexus with 3 major longitudinal vessels, transverse commissures 3 or less; adult parasitic in intestinal tract of birds, more rarely mammals.

Definitions of the genera *Apatemon*, *Ichthyocotylurus* and *Cotylurus*, reproduced with slight amendments* from Blair (1974) and based on the work of Dubois (1968, 1970a), Niewiadomska (1971a), Odening (1969) and Dubois & Nassi (1977).

Genus *Apatemon* Szidat, 1928

Strigeid with bisegmented body; pharynx present; forebody variously cup-shaped, lacking lateral extensions, small multilobed proteolytic gland, (typically*) lacking vitellaria. Hindbody sac-shaped, subreniform or subcylindrical; generally curved or lacking a collar, separated from the forebody by a constriction. Ellipsoidal or reniform ovary in the anterior 2/5ths of the hindbody; testes of various shapes and generally orientated obliquely, may be bi- or trilobed (with the lobes directed anteriorly) or multilobed. Copulatory bursa of medium size, with a terminal opening containing a genital cone at the base of which the uterus and ejaculatory duct unite to form the hermaphroditic duct.

Subgenus *Apatemon* Szidat, 1928

Apatemon with poorly developed genital cone traversed by a narrow, straight, hermaphroditic duct with little associated musculature. Cercariae with rudimentary gut caeca; six, eight, or more postacetabular penetration gland cells. Excretory formula 2[(2)+((2)+(1))] = 10; postacetabular excretory commissure; metacercariae in fishes.

Subgenus *Australapatemon* Sudarikov, 1959

Apatemon with well developed and muscular genital cone, traversed by a muscular, and often convoluted, hermaphroditic duct. Cercaria with gut terminating in two long caeca. Eight (rarely six) postacetabular penetration gland cells. Excretory formula 2[(2)+((4)+(1))] = 14; with one or two excretory commissures. Metacercariae in leeches.

Diagnosis common to both *Ichthyocotylurus* Odening, 1969 and *Cotylurus* Szidat, 1928 genera.

Strigeids with bisegmented body; pharynx present. Forebody variously cup-shaped or hemispherical, lacking lateral extensions; proteolytic gland poorly developed and rather diffuse; forebody may contain a few vitellaria. Hindbody variously cylindrical, lacking a collar, and separated from the forebody, to which it is often attached eccentrically, by a distinct constriction. Ellipsoidal or reniform ovary situated in the anterior half of the hindbody. Testes may be trilobed with the lobes directed posteriorly (one dorsal and two lateral), or multilobed. Copulatory bursa of moderate size with a subterminal genital pore; genital cone absent, but a genital bulb present, at the base of which the genital ducts emerge dorsally into the copulatory bursa. Cercariae with excretory formula 2[(2+2)+((2+2)+(2))] = 20, preacetabular excretory commissure.

Genus *Ichthyocotylurus* Odening, 1969

Testes trilobed or multilobed. Cercariae with two pairs of postacetabular

penetration gland cells. Metacercariae in fishes, *enveloped in unperforated cyst of various thicknesses.

Genus *Cotylurus* Szidat, 1928

Testes with three lobes. Cercariae with two pairs of preacetabular penetration gland cells. Metacercariae in snails and leeches, *enveloped in tightly investing thick walled cyst which is typically pear-shaped and perforated.