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The immune response of the grey mullet, Chelon labrosus (Risso, 1826), to Cryptocotyle lingua (Creplin, 1825), (Digenea)

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The immune response of the grey mullet, *Chelon labrosus* (Risso, 1826), to

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partial fulfilment of the requirements for the Award of Doctor of Philosophy

Department of Biological Sciences, Polytechnic South West

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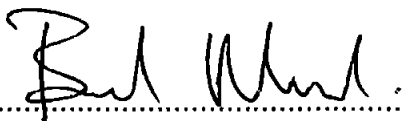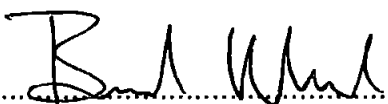
Declaration

This work has not been accepted and is not concurrently being submitted for any other degree and is a record of work carried out by the candidate himself.

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All experimental procedures applied to live animals during the course of this study were performed under Home Office Licence ELA 24/6876.

Abstract

The immune response of the grey mullet, *Chelon labrosus* (Risso, 1826), to *Cryptocotyle lingua* (Creplin, 1825), (Digenea). By Bradbury Patrick Wood.

The ability of thick-lipped grey mullet to mount both humoral and cellular immune responses to *Cryptocotyle lingua* has been demonstrated here for the first time. Of the 3 procedures for immunisation investigated, namely exposure to live cercariae, intraperitoneal (ip) injection of whole killed cercariae and ip injection of a cercarial sonicate, the former gave the highest primary antibody titres with a peak response at 4 weeks post infection. Peak titres occurred at 5 and 7 weeks after immunisation by ip injection of sonicated cercariae and whole killed cercariae respectively. An enhanced secondary antibody response was observed following challenge by ip injection of sonicated cercariae. Cercariacidal activity, apparently involving complement, was detected in normal serum and enhanced in immune serum. However, the mechanism for complement activation remains uncertain. Parasite antigens were identified following separation by SDS-PAGE and immunoblotting using mullet and rabbit antisera. Mullet failed to respond to certain parasite proteins recognised by rabbit antiserum and *in situ* immunogold-silver staining demonstrated that mullet, immunised by exposure to live cercariae, were unable to recognise the metacercarial cyst wall. Migration and polarisation of pronephric leucocytes *in vitro* was enhanced following immunisation but cellular adherence to encysted and unencysted parasites was not observed. *In vitro* studies further demonstrated a possible role for fish serum in stimulating metacercarial encystment. The intensity of melanisation of the host capsule in metacercarial infections was increased in fish held in total darkness and with increased temperature. The rate of development of the metacercarial tegument and cyst wall and the associated host response was similarly affected by temperature and initial development was inhibited by prior immunisation via ip injection of sonicated cercariae. There was however, no evidence of protection against cercarial infection following such immunisation. These results are discussed in relation to mechanisms of immunity, metacercarial survival strategies and control of metazoan infections in fish.

Publications

Parts of this thesis have been published in the following papers:

Wood, B P and Matthews, R A (1987). *In vivo* study of the effect of light and temperature on the melanisation of cysts of *Cryptocotyle lingua* (Creplin, 1825) in the mullet, *Chelon labrosus* (Risso, 1826). *Aquaculture* 67 (1/2), 121.

Wood, B P and Matthews, R A (1987). The immune response of thick-lipped grey mullet, *Chelon labrosus* (Risso, 1826) to metacercariae of *Cryptocotyle lingua* (Creplin, 1825). *Journal of Fish Biology* 31 (Supplement A), 175-183.

Acknowledgements

To Dad,
Forever,
Butch.

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Introduction

Representatives of Heterophyidae including *Cryptocotyle lingua* have been recorded as pathogens of public health importance (Babbot *et al*, 1961), particularly species of the genus *Heterophyes* in mullet cultured in tropical and sub-tropical areas (Paperna and Overstreet, 1981). *C. lingua* shows a low degree of specificity for the fish host and consequently metacercariae are recorded from many marine species, particularly in coastal waters. Infections are particularly pathogenic to immature fish (Sindermann and Rosenfield, 1954a) and can reduce the value of food fish by spoilage (Sindermann, 1970). They are therefore viewed as a potential threat to mariculture in cage systems. Indeed, increasing problems from parasitic infections in aquaculture systems have recently been highlighted by Chappell (1990). However, relatively little information is available concerning immune responses of fish to metacercarial infections, most work being concerned with the detection of humoral antibody and localised tissue reactions. Furthermore, Chappell (1990) has stated that considerably more information relating to immune responses of fish to metacercarial infections must be obtained before the feasibility of the application of vaccines can be assessed.

Because of the potential threat of *C. lingua* and the relative paucity of investigations of host-parasite relationships in fish, especially concerning immune mechanisms, this parasite was selected for the present study. Furthermore, *C. lingua* is widespread in the Northern Hemisphere, is easily maintained in the laboratory and many aspects of it's general biology previously been investigated. The grey mullet, *Chelon labrosus* (Fig 1), was employed here as host for three reasons; mullet are commercially important species notably in Mediterranean regions, they are freely available and some

Figure 1 Thick-lipped grey mullet, *Chelon labrosus*

fundamental aspects of their immune responses have been studied within this Department by Mughal (1984) and Mughal and Manning (1986).

Transmission (Fig 2; Stunkard, 1930) associated with the marine environment, includes sea birds, notably the herring gull, as definitive hosts, *Littorina littorea* as first intermediate host and fish, including the grey mullet as second intermediate hosts. Rediae develop in the snail host and give rise to free-swimming cercariae (Fig 3a). Cercariae penetrate the fish host and the developing metacercariae encyst, usually in the dermis of the skin, causing the familiar black spots (Fig 3b).

The aims of the present study were to further our understanding of the interactions of helminth parasites and their marine hosts with particular emphasis on immune responses and with a view to more effective control of these diseases.

Figure 2 Life cycle of *Cryptocotyle lingua* (after Stunkard, 1930)

GREY MULLET

CERCARIA

EATEN

METACERCARIA

LITTORINA LITTOREA

HERRING GULL

EGGS IN FAECES
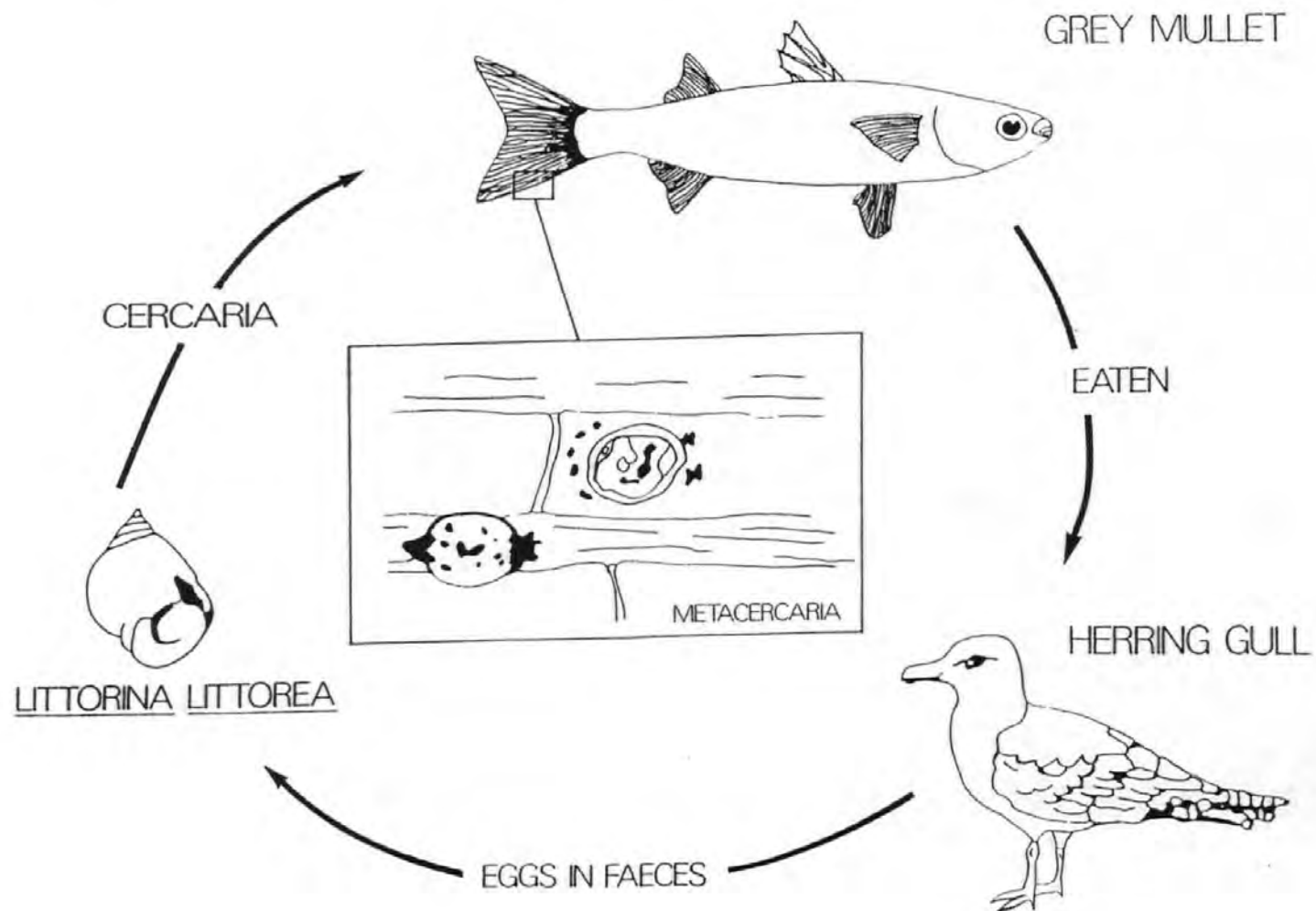
Figure 3 Life cycle stages of *C. lingua*

(a) Cercaria. (Bar= 150μm).

(b) Metacercaria. (Bar= 200μm).

a

b
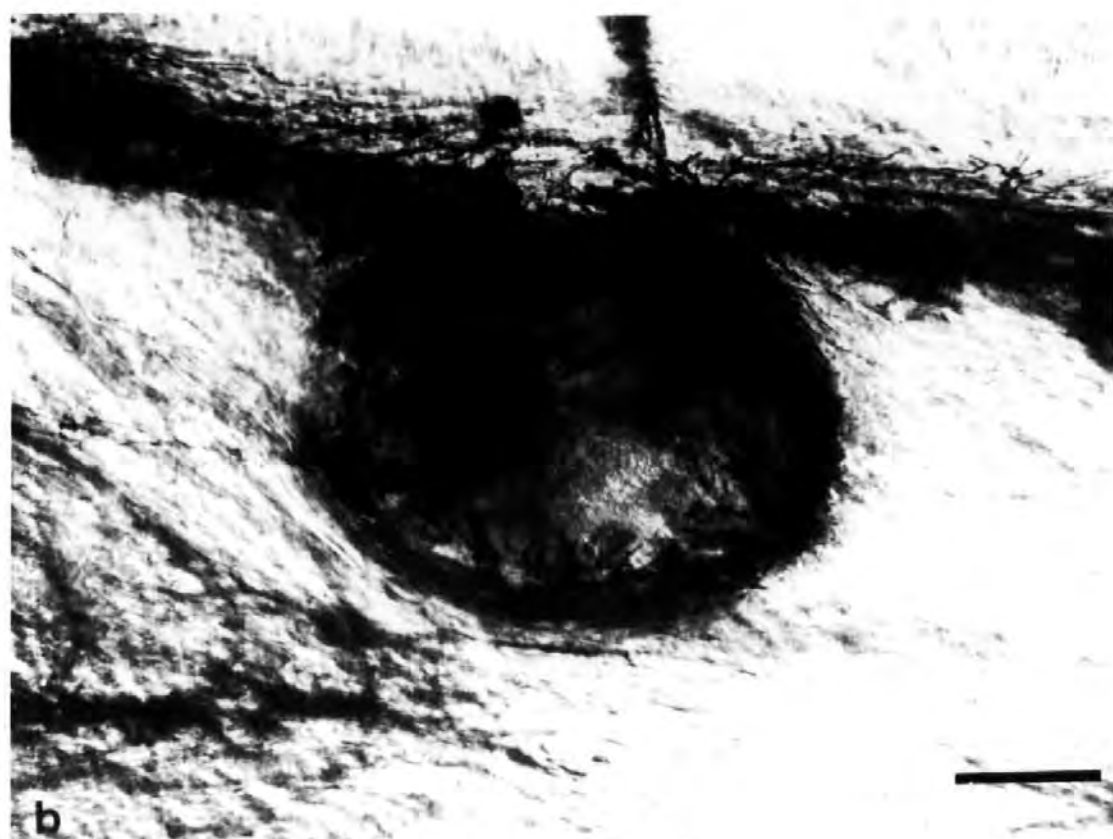
Review

In reviewing the literature it was found convenient to organise the information under the following headings: Mullet, Macroparasitic Diseases of Cultured Mullet, Biology of *C. lingua*, Immune Response to *C. lingua*, Immune Responses of Fish to Metazoan Parasites and the Immune System of Fishes.

Mullet

Grey mullets belong to the Mugilidae, a chiefly tropical and sub-tropical group with the British Isles being close to it's northern most limits. The biology of this group has been reviewed by Thompson (1968) and more recently by de Silva (1980) and Oren (1981). Aspects of the biology of *C.labrosus* and *L.ramada* have been studied by Erman (1961) and also Lasserre and Gallis (1975). Three species of grey mullet have been recorded in the South West of England, namely the thick-lipped grey mullet, *Chelon labrosus* (Risso, 1826) (syn. *Crenimugil labrosus*), the thin-lipped grey mullet, *Liza ramada* (Risso, 1826) and the golden grey mullet, *Liza auratus* (Risso, 1810). Mullet represented almost a third of all fishes sampled by Kelly (1986) around the west coast of England. The thick-lipped grey mullet constitutes almost 93% of the mullet species present in South Devon (Hickling, 1970a). Additionally, this species is found elsewhere in the Southern regions of Great Britain and Northern Europe, and is closely related to the striped grey mullet, *Mugil cephalus*, which has a worldwide distribution including North and South America, Asia, the Mediterranean and Black seas (Wheeler, 1978).

The recent increased scientific interest in the Mugilidae is due to the potential cultivation of many species especially in the tropics and sub-tropics but also in more temperate regions of Europe. A recent report by the Aquaculture

Development and Co-ordination Programme of the United Nations (1987) announced that the production of mullet in the Mediterranean region was expected to rise by 85% from 11,000 tonnes in 1985 to 20,400 tonnes in 1992. This rise is due mainly to the expected expansion of aquaculture of the species in Italy, Tunisia and Egypt during the next 5 years.

Graham (1981) studied the feasibility of farming grey mullet in Great Britain and the culture of these fish has been reviewed by many including Korringa (1976), Chen (1976), Pruginin *et al* (1975), Hickling (1970b, 1971) and Iversen (1968). Although in many areas of the world, for example, Italy, Israel and Egypt, grey mullet is considered a delicacy (Ling, 1970), it is not generally eaten in Britain or North America. Farming of this species is not usually intensive, although it constitutes an important component of polyculture systems (Pruginin *et al*, 1975). The high quality flesh, hardiness and low position of mullet in the food chain make them an ideal species for culture. However, many difficulties have been experienced in their artificial propogation and availability of sufficiently large numbers of fry is still the major limiting factor in Taiwan.

Macroparasitic diseases of cultured mullet

Outbreaks of disease during the culture of these fish is a major barrier to the expansion of this industry (Paperna, 1975). Whilst parasitic diseases pose one of the greatest problems, studies of the parasites of mullets have only been made in a few regions of the Eastern world (Paperna and Overstreet, 1981; Overstreet, 1975; Paperna, 1964; Reshetnikova, 1955) and Western Europe and Great Britain (Anderson, 1981a, 1981b, Lee and Cheng, 1970). Digenea are the most common helminths infecting this species and Paperna and Overstreet (1981) cited over two hundred references concerning these parasites. Several representatives of the family Heterophyidae are known to cause severe problems in cultured fish (Korringa, 1976; Paperna, 1975). Surveys of commercial size mullets sold in Israel, Egypt and Northern Sinai (Paperna, 1975, 1964a) and the Nile Delta (Khalil, 1937) indicate 100% infections with metacercariae of heterophyiids. Extensive studies on the epidemiology of these parasitic infections have been made in Eygpt (Kuntz, 1957; Kuntz and Chandler, 1956; Khalil, 1937, 1933), Israel and Northern Sinai

(Paperna and Lahev, 1971; Paperna, 1964a; Witenberg, 1924), the Phillippines (Vazques-Colet and Africa, 1938) and Japan (Asada, 1928).

Infections with digenean metacercariae can affect all stages of fish cultured in enclosed ponds (Paperna and Overstreet, 1981) but juvenile fish are more readily debilitated than are the adults. Studies of the pathological changes in mullet caused by such larval trematodes are very limited. Lee and Cheng (1970) described necrotic degradation of myofibres caused by metacercariae of *Stellantchasmus falcatus* in *Mugil cephalus*. The definitive hosts of these parasites are piscivorous birds and mammals such as cats and dogs, although zoonosis is associated with the adult stage (Healy, 1970). In areas where raw or improperly prepared fish is eaten, heterophyiids constitute a considerable public health problem (Taraschewski, 1984; Paperna and Overstreet, 1981; Paperna, 1975). *Heterophyes heterophyes* is the predominant species which infects mullet in the Mediterranean (Paperna and Overstreet, 1981) and infection rates of this species in humans of up to ninety per cent have been recorded (Khalil, 1937, 1933). Similar statistics have been given by many authors including Kuntz and Chandler (1956) and Witenberg (1924). Normal symptoms of such infections in humans are vomiting, diarrhoea and in more severe cases, dysentry (Kahlil, 1937). Nonetheless, complications can result in lesions of internal organs and the central nervous system (Africa, *et al*, 1937,1935) and Deschiens *et al* (1958) reported adult *H. heterophyes* in the human brain.

Biology of *Cryptocotyle lingua*

The life cycle of *Cryptocotyle lingua* (Creplin, 1825), the heterophyid investigated in the present study, was described by Stunkard (1930) although earlier studies had been made by Linton (1915) and Jagerskiold (1898). Eggs are produced within 3 days in the definitive host (Rees, 1978). The definitive hosts are usually gulls and terns, notably the herring gull, *Larus argentatus* Pontopp, with adults having been reported in the alimentary canal (Threlfall, 1981; Irvin and Prentice, 1976; Harris, 1964). However any fish-eating mammal may become infected. Adult stages of *C. lingua* have also been reported in humans, cats and dogs (Ching, 1978; Miyamoto and Kitsumi, 1978; Babbot *et al* 1961; Christensen and Roth, 1949; Christensen *et al*, 1946; Willey and

Stunkard, 1942; Cameron *et al*, 1940; Price, 1932). This parasite has also been recorded from the brown bear, *Ursus arctos* L. in Russia (Rausch *et al*, 1979) and in the bald eagle, *Haliaeetus leucopephalus* L. (Smith, 1978). Stunkard (1930) successfully experimentally infected white rats, guinea pigs and kittens, attaining patent infections in these hosts. Rothschild (1939) however, was unable to infect albino rats despite successfully infecting gulls. The degree of pathogenicity varies between different hosts and although little effect is seen in gulls (Willey and Stunkard, 1942) extensive damage to the intestinal mucosa of dogs was evident (Christensen and Roth, 1949; Cameron, 1945; Willey and Stunkard, 1942).

The most common first intermediate host for *C. lingua* is the edible winkle, *Littorina littorea* but it has also been recorded in *Littorina saxatalis* (James, 1968), *Littorina obtusa* (Iddon, 1973) and *Hydrobia ulvae* and *H. ventrosa* (Ginetsinskaya, 1961). In contrast to most other digenea the eggs which are transmitted in the faeces of the definitive host, must first be ingested by the snail host before hatching to release the infective miracidia (Christensen and Roth, 1949).

A wide range of marine teleosts act as second intermediate hosts for *C. lingua*, whose metacercariae show a very low host specificity. Linton (1940) lists seventeen species of fish infected with this parasite in North America and the following have been recorded as hosts in European waters: cod, *Gadus callanias* (Chapman and Hunter, 1954; Christensen and Roth, 1949; Hsio, 1941), plaice, *Pleuronectes platessa* (van den Broek, 1979; Cottrell, 1975; McQueen *et al*, 1975; Iddon, 1973; MacKenzie, 1971, 1968; Christensen and Roth, 1949), flounder, *Platichthys flesus* (van den Broek, 1979; Mawdesley-Thomas and Young, 1967; Christensen and Roth, 1949), cunner, *Tautogolabrus adspersus* (Stunkard, 1930), herring, *Clupea harengus* (Sindermann and Rosenfield, 1954b), rock gunnell, *Pholis gunnellus* (Gorman and Moring, 1982), whiting, *Merlangius merlangus* and pouting, *Trisopterus luscus* (Evans *et al*, 1983; van den Broek, 1979), dab, *Limanda limanda* (Koie, 1983).

Grainger (1977) recorded the metacercariae of *C. lingua* in several species of gadoid fish and Rothschild (1939) showed that the goby, *Gobius ruthensparri*

and the ballan wrasse, *Labrus bergylta*, amongst several species served as hosts. This parasite is known also to infect the sand goby, *Pomatoschistus minutus* and the mullet, *Chelon labrosus* (Pulsford, 1980 and the present study). The cercariae will penetrate and encyst in most fish with which they make contact although some species are more susceptible than others (Rothschild, 1939). That they can infect freshwater fish such as the common carp, *Cyprinus carpio*, has been demonstrated by R A Matthews (pers. comm.) following immersion of the caudal fin in a suspension of cercariae.

A number of studies have been made of the larval stages of *C. lingua* although these have largely been confined to the rediae. Studies on the ecology of winkles infected with *C. lingua* have been carried out by Lauckner (1984), Hughes and Answer (1982), McDaniel (1969), Lambert and Farley (1968), Sindermann (1966), Sindermann and Farrin (1962), Rothschild (1939) and Skunkard (1930). The longevity of such infections was reported by Rothschild (1942) and Meyerof and Rothschild (1940) whereby an infected winkle released approximately 1,600 cercariae daily for seven years. Physiological studies of *C. lingua* infections in winkles have been made by Jones and Kamel (1984), Zavras and James (1979), Irwin et al (1978), Moore and Halton (1977), Davies and Farley (1973), Watts (1970), McDaniel (1969), McDaniel and Dixon (1967) and Kruppa *et al* (1967). Willey and Gross (1957) discussed the pigmentation of the foot of *L. littorea* as a means of recognition of infection with *C. lingua*.

Sites close to fishing harbours have shown high levels of incidence of *C. lingua* of up to 46.53% due to the presence of many definitive hosts (Matthews *et al*, 1985). A low incidence of infection of 0 - 10% is however more common (Hughes and Answer, 1982; Robson and Williams, 1970). Sindermann and Rosenfield (1954b) discovered that emergence of cercariae ceased when sea temperatures fell below 10°C but was usually in the region of 3,000 cercariae per snail per day at 15°C. Cable (1934, 1931) made a study of gametogenesis in the adult and the germinal development of the larval stages of *C. lingua* but they have been shown to tolerate low salinity conditions (Stunkard and Shaw, 1931) enabling them to survive the habitats of the first intermediate host. Robson and Williams (1970) demonstrated a positive correlation between the

presence of sea birds and incidence of infection. They also stated that incidence of infection was highest in autumn and lowest in summer and that larger snails produced greater numbers of cercariae.

Histochemical studies of the cercariae have been made by Bhutta (1974) and Janoff and Ford (1965), who found that the cystogenous glands contained acid-glycerophosphatase which they proposed caused lysis of the glands and thus enabled the release of secretory material during encystment. Hunter and Hunter (1937) discovered an histolytic ferment released by *C. lingua* cercariae which may be used to facilitate penetration of the fish host. These results supported the findings of Stunkard (1930) that cercarial secretions caused disintegration of fish skin. Chapman (1974) observed the behaviour of the cercariae and found them to be positively phototropic. The same author described the fine detail of the tail of the cercariae (Chapman, 1973) and Chapman and Wilson (1973) demonstrated, by the use of cinematography, the method of propulsion of the cercaria. McDaniel *et al* (1976) examined the effect of carbon dioxide upon the adult *C. lingua*.

Several studies of the ultrastructure of both larval and adult stages of *C. lingua* have been made. Krupa *et al* (1969, 1968, 1967) examined the ultrastructure and histochemistry of the rediae and cercariae. Aspects of cercariae and adult ultrastructure were studies by Rees (1979a, 1979b, 1978, 1975a, 1975b, 1974) and Rees and Day (1976). Koie (1977) utilised scanning electron microscopy to examine the topography of cercariae, metacercariae and adults.

Much less attention has been given to the metacercarial stage of C. lingua. Day (1976) described changes in the tegument during metamorphosis of cercaria to metacercaria. The ultrastructure of the genital rudiments of the metacercaria of *C. lingua* was examined by Rees (1979a) and Pulsford (1980) made an ultrastructural study of the development of the metacercaria of the same species in the mullet, *Chelon labrosus.* Early work on the development of the metacercaria of *C. lingua* was carried out by Stunkard (1930). Iddon (1973) and McQueen *et al* (1973) both undertook histochemical studies on different developmental stages of the metacercariae of *C. lingua* in the plaice. They found the chemical composition of the cyst differed to that of another

heterophyid, *Stallantchasmus falcatus* (Lee and Cheng, 1970), although their physical structures were similar. The metacercaria have been successfully excysted using a pepsin/trypsin digest (McDaniel, 1966) but no success has been achieved at inducing cercariae to encyst *in vitro* (Laurie, 1974).

A common site of infection of many metacercaria including *Diplostomum spathaceum* (Haas, 1969), *D. flexicardum* (Smyth, 1966), *Cercaria X Baylis* (Erasmus, 1959) and *Cercaria bessiae* (Krull, 1934) is the eye. A condition known as "pop-eye" occurs resulting in blindness and usually leading to death. Such a condition was reported to have been caused by *C. lingua* metacercariae infecting the herring, *Clupea harengus* (Sindermann and Rosenfield, 1954b). The metacercariae of *C. lingua* more commonly infect superficial layers of the skin or musculature. A melanin bearing cell component is usually associated with the cysts in such infections. This condition is often described as "black-spot" and has been reported from a large number of fish/parasite systems. Examples include cysts of *V. aubloplitis* (Hoffman and Putz, 1965; Hunter and Hunter, 1941) *Neascus pyriformis* and *N. lorgicellis* (Chandler, 1951). Many authors have described such pigmentation in fish infected with *C. lingua* metacercariae including van den Broek (1979), Roberts, (1975a, 1975b), McQueen *et al* (1973), Iddon (1973), MacKenzie (1968), Mawdesley-Thomas and Young (1967), Sindermann and Farrin (1962), Chapman and Hunter (1954) and Hsio (1941). Furthermore, Thulin (1985) noted that heavily infested cod, *Gadus morhua*, displayed a green colouration, which he suggested was due to a combined reaction of different chromatophores. An early report of abnormal pigmentation in whiting due to trematode larvae (Gamble and Drew, 1911) is also probably caused by *C. lingua*. Rothschild (1939) noted that the cysts of *C. lingua* metacercariae in the spotted goby, *Gobius ruthensparri* showed no signs of pigmentation whereas those of *C. jejuna*, a closely related species, were heavily melanised. DeVeen (1969) suggested that this feature might be used to study different populations of the plaice.

Metacercariae of *C. lingua*, are potentially serious pathogens of the fish host (Sindermann, 1970, 1966) to the extent that massive metacercarial infections will blind and kill immature herring, *C. harengus* (Sindermann and Rosenfield,

1954a,b). The results of MacKenzie (1971, 1968) who studied the ecological aspects of metacercarial infections in the plaice in field experiments, support these findings. The expansion of mariculture using cage systems has seen an increased incidence of *C. lingua* infections in farmed species. This parasite has been reported to be a problem in salmon farms sited close to rocky shores (Needham, 1980) and to the culture of salmonid and gadoid species in northern Europe. Apart from mortalities of young fish, the parasite also causes reduced growth and spoilage of marketable stocks by disfigurement.

Immune response to *C. lingua*

There have been very few studies of fish host responses to *C. lingua*, these being Iddon (1973), McQueen *et al* (1973) and Cottrell (1977, 1976, 1975, 1974), all of which used the plaice as host.

Iddon (1973) showed that repeated exposure of plaice to cercariae of *C. lingua* lead to a reduced infection rate upon challenge, suggesting that protection could be induced. However, she did not investigate whether this was due to innate factors or acquired immunity. Furthermore she detected cercariacidal activity in the mucus which was absent in the serum.

Cottrell (1977, 1976, 1975, 1974) further investigated the immune response of plaice to *C. lingua* and demonstrated that this host produces specific humoral antibody to *C. lingua* when exposed to cercariae or immunised by intraperitoneal injection with a soluble cercarial preparation. However, no antibody was detected in the cutaneous mucus of these fish. Immune serum was highly toxic for *C. lingua* cercariae and was directed against the tegument of the parasite. The same author also showed that temperature had an effect on this immune response. Plaice held at 5°C were unable to produce specific antibody against the parasite whilst a positive response was recorded after 40 days at 15°C and 20 days at 25°C.

The effect of temperature on the inflammatory response of plaice to metacercariae of *C. lingua* has also been investigated (McQueen *et al*, 1973). They reported that the development of the parasite and of the host response

was slight at 15°C and inhibited further at 5°C. This they suggested may be due to a highly evolved state of parasite adaptation and host tolerence. This is in contrast to infections of mullet, M. cephalus, with metacercariae of another heterophyid, S. falcatus which resulted in muscle necrosis and focal haemorrhage (Lee and Cheng, 1970). McQueen et al (1973) also observed myofibrillar necrosis which they suggested was associated with a bacterium which was possibly introduced with the parasite.

Immune responses of fish to metazoan parasites

Most studies of immune reactions in fish have been concentrated upon those induced by bacteria and viruses or other experimental antigens. Very little work has been carried out on the immune responses of fish to eukaryotic pathogens, least of all metazoan parasites. A brief review of such responses was made by van Muiswinkel and Jagt (1984) and Woo (1987) has reviewed fish immune responses to protozoan parasites. Early work by Reuling (1919) gave rise to the first evidence for the development of immunity of a fish to a parasite. This author found that the short and long-nosed gar, Lepisosteus osseus and L. platostomus, developed immunity to glochidia of mussels when repeatedly exposed to the larvae. This immunity was suggested to have been due to the production of antibodies. Nigrelli (1935b), Nigrelli and Breder (1934) and Jahn and Kuhn (1932) demonstrated that marine fish can acquire immunity to infections with Benedia (Epibdella) melleni, an ectoparasitic monogenea and that some species of fish developed a greater degree of protection than others. Jahn and Kuhn (1932) suggested that humoral immunity may be involved. Further work by Nigrelli (1935b) attributed this protection to a property of the mucus but the same author was unable to immunise fish by injections of either sera from immune fish or fresh or dried parasite material (Nigrelli, 1937). This suggested that it might be the attachment of the parasite to the skin of the fish which was important in inducing immunity.

More recently, Paperna (1964b, 1963) suggested that reduced reinfection rates of Dactylogyrus vastator and D. extensus in carp, Cyprinus carpio may be due to the development of an acquired immunity. He , together with Wunder (1929) and Putz and Hoffman (1964) described a proliferation of gill epidermis,

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which resulted in the loss of parasites. The host response of the stickleback, *Gasterosteus aculeatus leiurus* to infection with with *Gyrodactylus alexanderi* was studied by Lester (1972). He observed a reduction in levels of infestation with time and attributed these not to a humoral response but to a cellular component. Protection was shown to be short lived, lasting not more than 4 weeks. Lester and Adams (1974) using the same host parasite system, suggested that it was the host response which prevented both host and parasite death by controlling parasite infection levels. Scott (1985), Scott and Robinson (1984) and Scott and Anderson (1984) demonstrated acquired immunity in guppies, *Poecilia reticulata*, to *Gyrodactylus bullatarudis*. These workers showed that protection lasted for up to 6 weeks although no immunological investigations were undertaken and they offered no suggestion of a possible mechanism for this protection.

Experimental infections of *Eupomotis gibbosus* with *Cercaria bessiae* (Krull, 1934) showed that this parasite was pathogenic to young fish whereas older fish were refractory to further infection after repeated exposures. Hoffman (1956) detected no signs of immunity to *Crassiphiala buboglossa* after repeated exposure of fathead minnows, *Pimephales primelas primelas* to this parasite. Furthermore, Spall and Summerfelt (1970) and Ferguson (1943) were unable to confer immunity in cyprinid fish to *Posthodiplostomum minimum*. Meade and Harvey (1969) however, investigated the effect of parasitism of *Lepomus machrochirus* by this species upon the relative concentrations of serum globulins. Such serum had lethal effects on both cercariae and excysted metacercariae but encysted metacercariae were unaffected (Harvey and Meade, 1969) and heating the serum to 56°C for 30 minutes inhibited it's lethal effect, suggesting complement was involved.

O'Rourke (1961) demonstrated the presence of plasma proteins in fish mucus which he postulated might be the basis of a protective mechanism against parasitic invasion. Subsequent work by many authors supports this finding and has shown that specific antibodies are found in fish mucus in addition to other protective agents including lysosyme, C-reactive protein (CRP) and complement (Harris, 1972; Bradshaw *et al*, 1971; Fletcher and Grant, 1969).

Furthermore, Goven *et al* (1980) detected specific antibodies in the mucus of catfish, *Ictalurus punctatus* following intraperitoneal injection with *Ichthyophthirius multifiliis*. These results were supported by St. Louis-Cormier *et al* (1984) who reported that rainbow trout, *Oncorhynchus mykiss* (syn. *Salmo gairdneri*), injected with sheep erythrocytes, produced specific antibody in cutaneous mucus. Infected plaice were shown by Cottrell (1977, 1976, 1975, 1974) to produce specific humoral antibody to *Rhipidocotyle johnstonei* but no activity was detected in the mucus of these fish.

Recent studies on salmonids infected with the eye fluke *Diplostomum spathaceum* have included attempts at short term immunisation against such infections (Speed, 1976) and testing the susceptibility of different salmonid species to the parasite (Speed and Pauley, 1984). Evidence for the development of acquired immunity in carp fry to *D. spathaceum* and *D. paracaudum* was presented by Razmashkin (1985). Speed and Pauley (1985) could not detect serum antibody in rainbow trout injected intraperitoneally with whole or sonicated *D. spathaceum* metacercariae. However, these fish did show an increased survival time compared with controls, when challenged by natural exposure. Speed and Pauley (1984) obtained similar results when fish were immunised orally with live cercariae. Stables and Chappell (1986) were also unable to detect specific antibody following immunisation of rainbow trout with live infections or intraperitoneal injection of whole killed cercariae of *D. spathaceum*. In contrast to these results, Bortz *et al* (1984) detected specific antibody to *D. spathaceum* metacercarial antigens in fish immunised by either natural infection or intraperitoneal injection of soublised metacercariae. They also described an enhanced secondary antibody response following a booster immunisation. They used the enzyme linked immunosorbent assay (ELISA) to detect antibody to the parasite antigens. Whyte *et al* (1987) also using the ELISA technique, detected antibody production in rainbow trout which had been injected intraperitoneally with either sonicated cercariae or diplostomules. Furthermore, they reported cross reactivity between sera raised to each of these stages, suggesting the presence of common antigens.

McArthur (1978) using passive haemagglutination and McArthur and Sengupta

(1982) using the ELISA technique were able to detect specific humoral antibody in New Zealand short-finned eels, *Anguilla australis schmidtii* to the intestinal digenean, *Telogaster opisthorchis*.

Molnár and Berczi (1965) produced evidence to suggest that specific antibodies against *Ligula intestinalis* were produced in infected *Abramis brama* but whether this afforded protection was not stated. Sweeting (1977) however, could not detect precipitating antibody in roach, *Rutilus rutilus* infected with the same parasite although he did find an increase in relative levels of gamma globulin.

Kennedy (1969, 1968) suggested that acquired immunity could account for the seasonal incidence in infection of dace, *Leuciscus leuciscus* by the intestinal cestode *Caryophyllaeus laticeps*. Rejection of this parasite by the same host was seen in experimental infections (Kennedy and Walker, 1969) but Harris (1973a) was unable to detect circulating or skin sensitising antibodies.

Conversely, Harris (1972, 1970) found that the same fish, *L. leuciscus* did not reject the acanthocephalan, *Pomphorhychus leavis* but did produce a precipitation reaction in both experimental and natural infections. Separation of these precipitins suggested that they were IgM. Again, Harris (1973a) was unable to detect skin sensitising antibody to this parasite. Precipitating activity in plaice serum to an antigen extract of *Proleptus obtusus* was detected using immunoelectrophoresis (Harris and Cottrell, 1976) and shown to be due to specific immunoglobulin production. This parasite was specific to elasmobranchs, notably the dogfish, *Scyliorhinus canicula* and the detection of precipitins in the plaice was attributed to seriological cross reaction between helminth antigens. McVicar and Fletcher (1970) also demonstrated a specific serum reaction of the ray, *Raja radiata* which was toxic to the cestode, *Acanthobothrium quadiparatitum*. CRP, toxic to *Bothriocephalus scorpii* was detected in infected turbot, *Scophthalmus maximus* (Fletcher *et al*, 1980). The host specificity of *Schistocephalus solidus* was suggested to be due to the production of cytotoxic antibodies, causing death of plerocercoids (Orr *et al*, 1969), whereas Bråten (1966) proposed that some species posessed an innate immunity to this parasite. Renaud *et al* (1984) isolated protein components of

B. scorpii and identified antigenic proteins by immuno-electrophoresis using sera from infected brill, *Scophthalmus rhombus* and turbot, *S. maximus.*

Hoffman *et al* (1986) noted that, unlike in minnows, *Phoxinus phoxinus* and char, *Salvelinus alpinus* where infections remain, burbot, *Lota lota* were able to induce the rejection of plerocercoids of *Trianophorus nodulosus* by immunological mechanisms. Taniguchi (1970) immunised rabbits with extracts of *Anisakis* and found antigenic cross-reactivity between proteins from adult and larval forms of the parasite. A similar study was undertaken by Tsuji (1975) using serum from infected mackrel and dolphins.

Shields and Goode (1978) suggested that rejection of the copepod, *Laernaea cyprinacea* by the goldfish, *Carassius auratus*, may be due to an immune response. Similarly, Shariff (1981) proposed that the gradual disappearence of *L. piscinae* from infected bighead carp, *Aristichthys noblis* may have been caused by the development of an immune response. Shariff and Roberts (1989) further demonstrated that the localised response to *L. polymorpha* contained greater numbers of lymphocytes and eosinophillic granular cells.

There have been several studies on the localised tissue and cellular responses of fish to metazoan parasites. An early study by Arey (1932) described the cellular response of both normal and immune fish to invasion of the gills by glochidia of *Lampsilis anodontoides*. An increased cellular infiltration was observed in immune hosts and eosinophillic cells were identified in the capsule surrounding the glochidia. Fustich and Millemann (1978) found that glochidia of *Margaritifera margaritifera* were sloughed from the gills by the development of an extensive hyperplasia in coho salmon, *Oncorhynchus kisutch* but no reaction was seen in infected chinook salmon, *Oncorhynchus tshawytscha.*

Inflammatory responses of fish to metacercarial infections vary greatly in intensity. Lee and Cheng (1970) described muscle necrosis and focal haemorrhage together with a large inflammatory response in mullet, *M. cephalus* infected with the heterophyid *S. falcatus.* The digenean trematode *Nanophyetus salmincola* is also pathogenic to salmonid fish (Millemann and

Knapp, 1970), haemorrhage and an infiltration of pigment bearing cells being associated with such infections. Pike and Burt (1983) described an intense host response in the yellow perch, *Perca flavescens* to metacercariae of *Apophallus brevis*. This reaction comprised a boney capsule surrounded by a thick layer of melanocytes and connective tissue.

An ultrastructural study of the cellular response of plaice to *R. johnstonei* (Pulsford and Matthews, 1984) revealed a chronic inflammatory lesion around the metacercariae. This granulomatous response included monocytes, macrophages, epithelial cells, multinucleated cells and fibrocytes. These authors suggest that the presence of lymphocytes including plasma cells may be indicative of a cell-mediated response. Chronic inflammation also occurs in response to miracidia and eggs of *Sanguinicola kiamathensis* in the gills of cutthroat trout, *Salmo clarkii* (Evans, 1974). Erasmus (1959) observed phagocytosis of post penetration larvae (diplostomules) of *Cercaria X baylis* (*D. spathaceum*) and when damage occured to the lens of rainbow trout infected with *Diplostomum* spp, an inflammatory reaction resulted (Shariff et al, 1980), whereas, if the lens remained intact, there was no such response. Furthermore, Ratanarat-Brockelman (1974) reported that inflammation and phagocytosis of the same parasite occured only if they did not complete migration to the lens.

Several other workers have reported minimal inflammation to metacercariae. Higgins *et al* (1977) observed small amounts of collagen together with a few leucocytes surrounding metacercarial cysts of *Bucephalus haimeanus* in the goby, *P. microps*. Leong and Howell (1971) reported a minimal response of *Gambasia affinis* to infection with *Stictodora lari*. No significant host inflammatory or immunological response could be seen in *Cyprinoden variegatus* infected with either *Ascoctyle chandleri* (Lumsden, 1968) or *A. pachycytis* (Stein and Lumsden, 1971). In both cases there was a small infiltration of fibroblasts and associated collagen fibres and Stein and Lumsden (1971) suggested that the cyst wall may be sequestering metacercarial antigens. Encapsulation by connective tissue is a characteristic response to metacercariae of *Clinostomum marginatum* (Hunter and Dalton, 1939) and *Uvulifur ambloplitus* (Hunter and Hamilton, 1941) although melanisation is only evident in the latter.

Hunter and Hunter (1940) observed that cysts of _P_. *minimum* within the liver were surrounded by flattened hepatocytes although Hoffman (1958) disputed this, stating that they were in fact surrounded by connective tissue. Mitchell (1974) and Mitchell *et al* (1983) studied the inflammatory response of *Lepomis* spp. to the same parasite and observed a fibrous capsule surrounding the metacercariae, together with some necrosis. An inflammatory response to *P. cuticola* was also recorded by Lucky (1970).

Sommerville (1981) studied the tissue response and encystment of *Stephanochasmus baccatus* in four species of flatfish. She found the nature of the response was similar in all species but that the rate and intensity differed. Capsules consisted of an inner layer of leucocytes and an outer fibrous layer. Leong and Howell (1971) showed that fish, *Gambusis affinis* experimentally infected with the heterophyid *Stichodora lari* mounted no response until 35 days post infection when a minimal host cell encapsulation was observed. Howell (1973) implanted metacercariae of this parasite, which had encysted *in vitro*, into the abdominal cavity of the same fish host and noted that there was no host response until 21 - 23 days after infection when cysts became encapsulated. Fish innoculated in the same way with glass beads, encapsulated them within 3 days and this lead Howell to propose that either the cyst wall incorporates fish host material thus disguising itself from the host response or that the surface of the cyst is unsuitable for the attachment of host cells.

Berezantsev (1975) investigated the inflammatory reponse of fish to *P. cuticola* and *Diphyllobothrium latum* and found that the response in natural hosts was slight, comprising fibrous connective tissue and pigmented cells. An enhanced response was only seen upon death of the larvae when leucocytes, macrophages and giant cells were identified. This author concluded that the development of the capsule seemed to provide an equilibrium between host and parasite to enable extended parasite survival. When plerocercoids were implanted in goldfish, *C. auratus*, an unnatural host, a violent cell reaction occured followed by death of the host.

Infections of the monogenea *Lamellodiscus major* and *Haliotrema spariensis* in the sparid *Acanthopargus australis* were reported to induce and increase neutrophil numbers in the gills (Roubal, 1986).

The host response of several fish species to plerocercoids of the tapeworm, *Ligula intestinalis* has been studied by several workers including Sweeting (1977) and Arme and Owen (1970, 1968). However, no response could be detected in the gudgeon, *Gobio gobio*, infected with this parasite (Arme, 1975). The ultrastructure of cells involved in the response to *L. intestinalis* has been investigated in adult roach, *R. rutilus* (Hoole and Arme, 1983a, 1982) and in roach fry (1983b) and cell types identified as macrophages and neutrophils. Serum facilitated the adherence of roach leucocytes to plerocercoids of this parasite *in vitro* (Hoole and Arme, 1986). Adherence was increased in the presence of immune serum and the authors suggested that complement was the mediator of this adherence. Furthermore, adhering macrophages and neutrophils were shown to cause considerable damage to the tegument of the parasite. More recently, Hoole and Arme (1988) suggested a possible role for C-reactive protein in the adherence of roach leucocytes to plerocercoids of *L. intestinalis*. Studies by Sadkovskaya (1953) and Shpolyanskaya (1953) have shown there to be an increase in the number of blood monocytes and polymorphonuclear leucocytes in fish infected with *L. intestinalis*.

Inflammatory responses caused by other cestodes in their fish hosts have also been reported (Sharp *et al*, 1989; O'Neill *et al*, 1987; Sakanami and Moser, 1986; Hoffmann *et al*, 1986; Eiras *et al*, 1986; Moser *et al*, 1984; Otto and Heckmann, 1984; Hayunga, 1979; Scott and Grizzle, 1979; Stromberg and Crites, 1974; Esch and Huffines, 1973). These responses consisted mainly of connective tissue hyperplasia together with the infiltration of leucocytes. Eiras *et al* (1986) observed lymphocyte infiltration to the plerocercoids of *Megathylacus brooksi* and *Jauela glandicephalus* in *Paulicea lutkeni*. The Antartic silverfish, *Pleuragramma antarcticum*, exhibited an inflammatory response to an unidentified plerocercoid which consisted of connective tissue and phagocytic cells but no damage to the parasite was observed (O'Neill *et al*, 1987).

The inflammatory response of fish to acanthocephalan infections occur in varying degrees (Taraschewski, 1989a,b,c, 1988; Hine and Kennedy, 1974; Esch and Huffines, 1973; Chaicharn and Bullock, 1967; Bullock, 1963; Prakash

and Evans, 1960; Venard and Warfel, 1953 and Wurmbach, 1937). Chronic inflammation occured in trout infected with *Acanthocephala jacksoni* but other species of fish infected with different species of acanthocephalans showed only very slight host responses.

The cellular response of whiting, *Merlangius merlangus*, to species of *Contracaecum* and *Anisakis* was characterised by the presence of neutrophils, macrophages, fibroblasts and melanin granules in the capsules surrounding parasites (Elarifi, 1982). An inflammatory reaction to *Spinitectuscarolini* has also been seen (Jilek and Crites, 1982). Hauk and May (1977) noted macrophages and lymphocytes incorporated in the granulomatous lesions surrounding *Anisakis* sp in the herring *Clupea harengus (pallasi)*.

There have been several reports of inflammation in response to copepod infections in fish (Roubal, 1986; Boxshall, 1977 and Joy and Jones, 1973), consisting of the characteristic proliferation of fibrous connective tissue. Interestingly, infections of the cornea of the eye, an avascular site, in bighead carp, *A. noblis* by *Lernaea piscinae*, caused a severe inflammatory response (Shariff, 1981). He proposed that such a reaction may be due to the development of an immune response to the parasite within the body of the host.

There have been very few studies on the effect of parasitic crustacea on their fish hosts although Nair and Nair (1983) demonstrated that infestations of *Alitrophus typhus* on *Channa striatus* caused a decrease in the number of blood leucocytes in the host.

Apart from studies of localised tissue reactions, there has been very little work on cell-mediated responses of fish to parasites. The work of Hoole and Arme (1988, 1986) on the adherence of leucocytes to plerocercoids *in vitro* has already been mentioned above. Berezantsev and Opnin (1976) studied the chemotaxis of fish host leucocytes to plerocercoids of *D. latum* and *S. solidus*. Using the *in vitro* technique of Boyden (1962), they discovered that these parasites inhibited chemotaxis of host cells. Most recently, Whyte *et al* (1989) investigated the effect of *D. spathaceum* on macrophage respiratory burst activity in rainbow trout.

The immune system of fishes

The immune responses of fish have been extensively reviewed by Ellis (1989, 1988, 1978), Lamers (1985), Dorson (1984), van Muiswinkel *et al* (1978), Corbel (1975) and Cushing (1970). Fish have been shown to possess an extensive defense system which can be divided into two broad categories, namely non-specific and specific mechanisms. The former can be elicited by a range of different factors including infectious agents and non-antigenic materials. Specific mechanisms involve the stimulation of the lymphoid system, resulting in the production of a response directed against a particular molecular structure. These two categories are considered separately below.

Non-specific defense mechanisms

Non specific defense mechanisms of fish have been reviewed by Ellis (1981) and Ingram (1980). The first line of defense of the fish are the epithelial surfaces of the skin, gills and gut and the mucus layer covering these. The skin mucus of channel catfish, *Ichtalurus punctatus* (Ourth, 1980) and plaice (Fletcher and White, 1973a) have been shown to contain lysozyme. Furthermore Harrell *et al* (1976) detected complement in mucus of rainbow trout and Ramos and Smith (1978) reported low levels of C-reactive protein (CPR) in skin mucus.

A range of non-specific humoral factors have been reported in the body fluids of fish and these include transferrin, lectins, enzyme inhibitors, lytic enzymes, interferon, "natural antibodies", CRP and complement. CRP is a serum component of many teleosts and in the presence of Ca^{++} ions reacts with phosphorylcholine molecules, which are common components of surface structures of many pathogens. This reactivity makes CRP an important protective substance (Baldo and Fletcher, 1973). Fish CRP can cause agglutination of pathogens and activation of complement with subsequent opsonisation of phagocytosis and is therefore a most important component prior to the activation of a specific response.

Although complement is involved in antigen specific as well as non-specific mechanisms, it will be considered here for convenience. Complement is

probably the most important of the factors mentioned above because of its multiple role. Fish complement is an enzyme cascade system, comprising approximately 12 proteins, present in the serum and tissue fluids and is biochemically similar to its mammalian counterparts (Nonaka *et al*, 1981a). Its activity has been shown to be thermolabile, becoming inactivated at about 45°C (Rijkers, 1982; Sakai, 1981). Furthermore, fish complement requires Ca^{++} and Mg^{++} ions and is not reactive with immunoglobulin from other species (Rijkers, 1982; Fletcher, 1982). Nonaka *et al* (1981b) isolated and characterised 2 complement components of serum from rainbow trout and found them to be analogous to mammalian C3 and C5. The role of complement in chemotactic attraction of fish leucocytes has also been demonstrated to be important in inflammatory reactions (Suzuki, 1986; Obenauf and Hyder Smith, 1985; Griffin, 1984).

Nonaka *et al* (1981a) demonstrated that teleost complement could be activated by both the classical and alternative pathways. The classical pathway, requiring the presence of Ca^{++} ions was shown to cause lysis of sheep erythrocytes (Nonaka *et al*, 1981a; Giclas *et al*, 1981). Complement from non-immune fish serum can be activated by the alternative pathway by bacteria (Ourth and Wilson, 1982a,b), insulin and zymosan (Kaastrup and Kock, 1983; Nonaka *et al*, 1981b).

Non-specific cellular mechanisms of fish involve a variety of cells including macrophages, neutrophils, basophils, mast cells, natural cytotoxic cells and fibrocytes. Phagocytic cells comprise the reticular-endothelial cells of the kidney and spleen and may be found in the gills and heart (McKinney, *et al*, 1977; Ellis *et al*, 1976).

Inflammatory responses are common in fish (van Muiswinkel and Jagt, 1984; Roberts, 1978; Finn, 1970) especially in response to parasitic infections as mentioned earlier. Both acute inflammation (Finn and Nielsen, 1971a,b) and chronic inflammation responses (Timur and Roberts, 1977; Timur *et al*, 1977) can occur.

Activation of fish leucocytes by lymphokines plays an important role in

inflammatory responses. Evidence for the activation of such cells by lymphokines has been put forward by Howell (1987), Hunt and Rowley (1986a,b) and Smith and Braun-Nesje (1982).

Specific defense mechanisms

This line of defense is characterised by its specificity and memory formation. The cells involved have been extensively reviewed by Ellis (1981) and include lymphocytes, plasma cells, mononuclear phagocytes and granulocytes (Boomker, 1981; Davina et al, 1980 and Ellis, 1976). Melanomacrophages may be found at sites of extensive phagocytosis as well as in the lymphoid organs of most teleosts. They are thought to be analogous to mammalian germinal centers and their phylogeny, ontogeny and functional significance has been extensively investigated (Agius and Agbede, 1984; Agius, 1983, 1981a,b, 1980, 1979; Agius and Roberts, 1981 and Roberts, 1975).

The lymphoid organs of teleost fish include the thymus, spleen, head kidney (pronephros) and trunk kidney (opisthonephros) (Fänge, 1982; Corbel, 1975). In addition, large numbers of lymphoid cells are also present in the intestinal mucosa (Davina et al, 1980; Zapata, 1979a). Wardle (1971) described the presence of lymph vessels in the plaice, but they appear to be absent in the rainbow trout (Vogel and Claviez, 1981).

The thymus is the first lymphoid organ to appear during ontogeny (van Loon et al, 1982; Grace and Manning, 1980; Ellis, 1977b) and appears to be a primary lymphoid organ with no antigen processing or phagocytosis occuring there (Ellis, 1980; Ellis et al, 1976).

The spleen becomes lymphoid late in ontogeny (Grace and Manning, 1980; Ellis, 1977b) and there are considerable differences in the morphology between species (Haider, 1966). Antigen binding and antibody producing cells have been detected in the spleen (Rijkers et al, 1980c) and Secombes et al (1982a) described the appearance of pyroninophillic cells after antigenic stimulaton. Lymphoid cell accumulations are often seen around ellipsoids and melanomacrophage centres (Zapata, 1982; Ferguson, 1976a and Sailendri and Muthukkaruppan, 1975a).

The kidney is a very important lymphoid tissue in teleosts and appears to be both a primary and secondary lymphoid organ. It is divided into pronephros and mesonephros, the former being totally lymphoid and having lost its excretory function. In the mesonephros, the lymphoid tissue is situated in the inter-tubular spaces (Zapata, 1981b, 1979b; Grace and Manning, 1980; Ellis *et al*, 1976). The kidney is thought to be analogous to the mammalian bone marrow (Rijkers, 1980c; Zapata, 1979b) with a high number of antigen-processing cells being present (Rijkers *et al*, 1980c).

The structure of fish immunoglobulin has been reviewed by several authors including Dorson (1981), Marchalonis (1977), Corbel (1975) and Carton (1973). Generally speaking, fish immunoglobulin is a tetrameric form similar to mammalian IgM, with a molecular weight in the range 608-720Kd. In addition to the serum, fish immunoglobulin has been demonstrated in the mucus of the skin and intestine and also in bile.

Antibody resposes of fish have been shown to be similar to those seen in mammals. Macrophages are required at the onset of the response (Smith and Braun-Nesje, 1982) and this is followed by exposure of the antigen (Secombes *et al*, 1982b; Ellis, 1980), production of interleukins (Caspi and Avtalion, 1984 and Grondel and Harmsen, 1984) and lymphocyte proliferation in the lymphoid organs to produce specific antibody (Manning *et al*, 1982).

Serum antibody titres generally increase exponentially, reach a plateau and then decay (Rijkers *et al*, 1980c). Enhanced secondary (anamnestic) cellular and humoral responses have been observed in fish (Rijkers, 1982; Rijkers *et al*, 1980b; Botham *et al*, 1980).

Evidence suggests that fish possess cell mediated immunity but there have, to date been very few studies in this area. Graft rejection has been reviewed by Rijkers (1982) and Botham *et al* (1980) and delayed-type hypersensitivity reactions have been reputed in response to bacteria (Bartos and Sommer, 1981) and parasites (Good and Papermaster, 1964). Macrophage migration inhibition in response to *A. salmonicida in vitro* was demonstrated by Smith *et al* (1980) and Chilmonzcyk (1978) showed that peripheral blood leucocytes were

stimulated by viral antigens *in vitro*. Most recently, studies on macrophage activation mechanisms have been made by Graham and Secombes (1988) and Chung and Secombes (1988, 1987), including the involvement of fish lymphokine (Graham ans Secombes, 1990). The few studies made clearly suggest that cell mediated immunity can exist in fish but the mechnisms and significance of these responses has yet to be determined.

Finally, the local or mucosal immune system which involves the surface structures of the fish including the skin, gills and intestine plays an important role in the immune responses of fish. Only skin associated immunity will be discussed here.

Both non-specific and specific defense factors have been reported in the skin mucus, including; immunoglobulin, lysozyme, CRP and complement (Harris, 1972; Bradshaw *et al*, 1971 and Fletcher and Grant, 1969). Lymphoid cells have been observed in the skin (DiConza and Halliday, 1971) including granulocytes and macrophages (Ferri and Macha, 1982; Phromsuthirak, 1977; Mittal and Munshi, 1971) and lymphocytes and plasma cells (Peleteiro and Richards, 1988, 1985; St-Louis Cormier and Anderson, 1984). Antigen uptake in the skin is poor (Hockney, 1985), although local processing of antigen may be possible due to the presence of macrophages and Langerhans-like cells (Mittal *et al*, 1980).

Materials and Methods

Thick-lipped grey mullet, *Chelon labrosus* (Risso, 1826)

Collection and maintenance

0-group mullet weighing 1 - 3 g were collected from the River Tamar at St. John's Lake, Cornwall. Older fish weighing 500 - 1000 g and aged 5 - 8 years were caught from the Looe estuary, Cornwall during August, 1985. All fish were caught with the aid of a seine net, 0.5 cm and 3 cm mesh nets being used for 0-group and older fish respectively. 0-group fish were transported to the aquarium in a 100 l polythene tank containing estuarine water, aeration being maintained by means of a portable air pump. Larger fish were transported in sealed plastic bags, each half filled with oxygenated river water and kept cool on ice. Fish were allowed to acclimate gradually to sea water in isolation tanks for 2 - 6 weeks, this period serving also for quarantine when bacterial and fungal infections were treated. The quality of sea water was maintained by biological filtration units (Eheim Ltd, West Germany). After quarantine mullet were transferred to 500 l tanks containing recirculating, well aerated sea water maintained at pH 6.5 and 12°C (± 2°C). Salinity was maintained at 35 parts per thousand, nitrites at < 0.05 mg l^{-1}, nitrates at < 10.0 mg l^{-1} and ammonia at 0.01 - 0.05 mg l^{-1}. For experimental work, 0-group fish were transferred to small aquaria with under-gravel filtration and containing well aerated sea water. Temperature controlled water baths at 10°C, 20°C and 25°C were used to hold aquaria containing 0-group fish. Salinity and pH were monitored and maintained at optimum levels described above. Fish were allowed a further acclimation period of 2 - 3 weeks prior to commencement of experimental work.

Fish were fed daily on trout pellets (Ewos Baker Ltd., West Lothian, Scotland)

using No. 2 for 0-group and No. 5 for older fish, the diet being supplemented with fresh fish at weekly intervals and in addition goldfish flake (Aquarian Fish Foods Ltd, UK) for 0-group fish.

Aquaria were monitored daily and care was taken to ensure the maintenance of good hygiene with regular removal of uneaten food from aquaria and weekly monitoring of water quality. Fish were handled with soft mesh nets to avoid damage to the body surface. Mullet occasionally developed bacterial infections especially immediately after collection and transportation. These were usually treated with Myxazin (Waterlife Research Ltd, Heathrow, UK) or latterly Broad Spectrum Bactericide (Technical Aquatic Products, Bristol, UK). More severe infections, due mainly to *Vibrio sp*, were treated with Oxalinic acid added to the feed at a concentration of 10 mg g^{-1} fish and added to the water directly at a concentration of 10 mg l^{-1}.

Handling Procedures

Anaesthesia. Large mullet were generally anaesthetised prior to handling to minimise stress and to reduce scale damage. Of the two anaesthetics used, namely Ethyl-m-aminobenzoate (MS222; Sigma Ltd, Poole, UK) and Ethyl-p-aminobenzoate (Benzocaine; Sigma Ltd), the latter was preferred as it is considered to cause lower levels of stress to fish especially during the initial stages of anaesthesia. Both anaesthetics were used at a concentration of 0.05 g l^{-1} sea water, however, due to the insolubility of Benzocaine an initial step involving its dissolution in a small volume of absolute alcohol was required. Fish were killed by overdose in anaesthetic.

Screening. Metacercarial stages of *C. lingua* encyst superficially beneath the skin of the fish host and are visible on micrscopic examination of the body surface. A simple method for screening mullet for natural infections of *C. lingua* was therefore undertaken by direct observation with the aid of a stereo microscope. Uninfected fish were isolated and kept free from exposure to *C. lingua* until required for experiments when they were re-examined to detect any young infections which might not easily have been identified at the time of collection.

Blood collection. Mullet weighing less than 5 g were killed and the tail region between the caudal and ventral fin severed using a sterile scapel. Blood was collected from the caudal blood vessels directly into a microhaematocrit tube. Larger fish were bled aseptically through the dorsal blood sinus situated beneath the vertebral column in the caudal region. A needle was inserted in this region, the sinus located and blood drawn into a sterile syringe of 1, 2 or 5 cm^3 capacity fitted with a 23 or 27 gauge needle depending on the size of the fish. Blood required for cells was collected in anticoagulant. Both Heparin (Sigma Ltd, Poole, UK) and Alsevers solution (Gibco, Paisley, Scotland) were used routinely, however the latter was preferred as cells were less damaged and could be stored for longer periods in this solution. Cells for microscopic examination were collected in 3.5% tri-sodium citrate as cell morphology was found to be better preserved in this anticoagulant.

Aging. The age of 0 and 1-group mullet was estimated by measuring the mean length of 20 fish and relating this to the age on the basis of modal length as described by Graham (1981). In the case of older fish, a scale was removed, placed on a microscope slide and mounted in tap water with a coverslip. This was then viewed using a microfische projector, with a magnification of x43 enabling the yearly rings to be counted from the nucleus of the scale to the periphery.

Cryptocotyle lingua

Collection of cercariae from Littorina littorea

Cercariae of *C. lingua* were obtained from the naturally infected first intermediate hosts, *Littorina littorea*, collected from the foreshore. Collections were made between May and October 1984 - 1987 from coastal sites at Cawsand Bay, Cornwall and Newton Ferrers, Devon. No collections were made during the winter months when the parasite is known to regress and no cercariae are shed. Prior to screening for infected specimens, winkles were placed in a dry, covered container overnight at 15°C as this increased the probability of cercarial shedding the following day. They were then washed in tap water and placed in glass covered 250 cm^3 beakers containing fresh sea

water, 4 winkles per beaker. The winkles were incubated at 15°C under cold temperature white fluorescent lighting to stimulate cercarial emergence. Beakers were checked frequently for the presence of released parasites. After 8 hours the sea water in each beaker was replaced and if no cercariae were observed after 24 hours the winkles were returned to the sea. Winkles from beakers containing cercariae were sub-screened by placing them individually in 50 cm^3 beakers and incubating them under the same conditions for a further 12 hours. Infected winkles were transferred to a perspex aquaria containing sea water to a depth of approximately 15 cm, water quality being maintained by means of under-gravel filtration and airstones. The temperature was maintained at 15°C ± 2°C and day length controlled using fluorescent lighting which was automatically timed to provide light for 14 hours each day thus simulating summer day length. Large stones were placed in aquaria such that they break the water surface to simulate the rockpool environment and enable winkles to climb out above water level. Winkles were fed with fresh seaweed, usually *Ulva sp* which survived well under laboratory conditions.

Infected winkles maintained in the laboratory were induced to shed cercariae throughout the winter months. However, when regular collections were made from individual winkles, shedding generally halted after a period of 3 - 4 months and a resting period was required. Examination by dissection indicated that infections were still present although rediae contained only immature cercariae. Nevertheless, these were usually discarded in preference for fresh stock. Within the period of active shedding it was possible to harvest significant numbers of cercariae at daily intervals using the following procedure.

Infected winkles were removed from their holding tank and placed overnight in a dry, covered container at 4°C. They were then transferred to a 2 l glass covered tank containing fresh sea water and placed under a cold temperature white fluorescent light at room temperature. Although sea water was constantly aerated, it was replaced at frequent intervals until the emergence of cercariae to maintain the temperature at approximately 15°C and to remove solid waste products. The collection and concentration of cercariae was undertaken by one of two methods, using either a light source or a series of sieves. The

photopositive response of cercariae enabled their concentration to a point light source. This simple method was particularly valuable when low numbers of cercariae were required as it was less likely to cause mechanical damage to cercariae. When large numbers were required however, water containing released cercariae was collected and passed through a series of sieves (Endecotts Ltd., London, UK) of aperature ranging from 200 μm to 32 μm. This served to remove debris present and concentrate the cercariae which were retained by the 32 μm seive. Cercariae were then washed with fresh sea water and resuspended to the desired concentration. Cercariae collected for infection experiments were used within 6 hours of their release, previous studies confirming their viability within this period. Those not required for infection of fish were stored at -70°C for subsequent use as parasite antigens. Cercariae collected for antigen preparation were stored at -70°C enabling stockpiling until required quantities were obtained.

Cercarial numbers were estimated with the aid of a stereo microscope and using a "Sedgewick Rafter" counting cell (Graticules Ltd., Tonbridge, UK)

Recovery of rediae from winkles

Rediae of *C. lingua* were required for the investigation of parasite antigens as described later. The shell of infected winkles was first carefully removed with the aid of a vice to expose the digestive gland. They were then placed in sterile filtered sea water in a petri dish and the digestive gland gently teased apart to release the rediae. Rediae were transferred, using a pasteur pipette, with the aid of a stereo microscope to fresh sea water in a centrifuge tube. They were then washed three times (100 *g*, 5 min) and prepared for SDS-PAGE analysis.

Experimental infection of mullet

Mullet were experimentally infected with *C. lingua* using one of three methods involving exposure of the whole body or selected areas to a suspension of cercariae.

For routine infections the total body immersion method was preferred. The following protocol was strictly adhered to. Fish were placed in tanks of 2 l

capacity containing 1.5 l well aerated sea water and a known number of cercariae. The fish were then exposed to infection for 6 h in the dark at 20°C ± 2°C. Dark conditions were selected in view of the photopositive response of cercariae and concentration towards a light source. Following the infection period fish were transferred to clean sea water. In a preliminary study, 4 fish were infected in this way and after the infection period, the remaining water was filtered. The remaining cercariae were counted and the proportion of cercariae actually penetrating the fish estimated at 98 - 99%.

It was often convenient to expose only specific sites of the fish surface to infection, in particular when preparing material for microscopy and also when testing for viability. One of two methods was used, namely caudal fin immersion or selective body site exposure.

A convenient method of restricting cercariae to a particular region of the fish body surface was the adhesion of a small piece of polyethylene tubing with super glue. The cercarial suspension was then added to the tube and sufficient time allowed for cercariae to penetrate before fish were returned to holding tanks to recover. Fish were anaesthetised throughout this procedure and kept on moist tissue paper. The adhered tubes were left in place and usually fell off after a few days, nevertheless, the site of infection could still be identified due to scarring left from the adhesive.

An alternative method was that of caudal fin immersion. Anaesthetised fish were placed on a layer of moist tissue paper with the caudal fin immersed in a small volume of sea water in a solid water glass. A known number of cercariae suspended in sea water was then added to the watch glass. Penetration of fish tissue by cercariae was monitored with the aid of a stereo microscope. When penetration was complete fish were returned to fresh, well aerated sea water for recovery.

Methods for transformation of cercariae to metacercariae

In addition to experimental infection of mullet, other methods were investigated for the transformation of cercariae to metacercariae using *in vitro* and *in ovo* methods. These are described below.

Artificial membrane methods

The following two methods employed coagulated mullet serum and supplemented agar respectively as artificial membranes to induce cercariae to encyst. In both cases, cercariae were first washed in sterile filtered sea water (100 *g*, 5 min) and added to media using a sterile pasteur pipette. Cercarial activity was monitored using a stereo microscope for up to 24 h.

Coagulated mullet serum (after Smyth and Smyth, 1980). 1.0 cm^3 volumes of normal mullet whole serum were heated at 80°C for 30 - 60 min in a sterile square watch glass to coagulate the serum.

Supplemented agar (after MacInnis and Voge, 1970). 1.75 g of Oxoid ionagar, No. 2 (Consolidated Laboratories, Chicago, USA) was dissolved in 100 cm^3 distilled water in a steam bath. The agar was allowed to cool to 40°C and then mixed thoroughly with the supplement in 25 cm^3 distilled water. This mixture was poured into sterile plastic petri dishes (Sterilin, Teddington, UK) and allowed to solidify. Supplements used were as follows: The amino acids; aspartic acid, glutamic acid, valeric acid, butyric acid, lactic acid, arginine and lysine, which were added in 5 mM concentrations; Glucose, at the same concentration; Lyophilised eel mucus added at a concentration of 0.4 g l^{-1}; and extract from whole mullet. To prepare the whole mullet extract, a fish (wet weight 1.4 g) was sacrificed and minced in Young's Teleost Saline (YTS; Appendix A) using a hand homogeniser. The extract was thoroughly filtered and added to the agar as described above.

Skin membrane method

Metacercariae were collected after cercarial penetration of isolated pieces of fish skin using a modification of the method described by Clegg and Smithers (1972) for schistosomula. An area of skin approximately 1 cm^2 was excised from freshly killed fish and the attached muscle tissue and scales removed by careful rubbing with a scalpel blade. The thinned piece of skin was then placed in the plastic penetration apparatus (Fig 4) made from a microcapped centrifuge tube. The lower container (B) was carefully filled with supplemented Leibovitz L15 minimal culture medium (MCM; Appendix A) or YTS supplemented with 1% mullet whole serum. The prepared skin was placed dermal side downwards over the culture medium (liquid) and held in place by chamber A which could be clipped securely onto tube B. The surface of the skin was rinsed with sterile filtered sea water to remove any culture medium and the top chamber filled with a cercarial suspension in sea water. A light was positioned beneath chamber B and the apparatus left at room temperature for periods of up to 6 hours to allow ceracriae to penetrate the skin. Only very low numbers (Approximately 1%) of cercariae were able to migrate through the skin into the culture medium. Most of those penetrating the skin encysted within the tissue and were removed by artificial digestion.

In vitro method

A variety of other media were used to induce the *in vitro* encystment of metacercariae in liquid media. The following media were tested; glucose in YTS, glucose in sea water (SW), sucrose in YTS, sucrose in SW, mullet serum in YTS, mullet serum and glucose in YTS, glucose in supplemented culture medium (SCM; Appendix A), glucose in MCM, mullet serum in SCM, mullet serum in MCM, mullet serum and glucose in SCM and mullet serum and glucose in SCM.

Cercariae were collected and washed three times by centrifugation (100 *g*, 5 min), initially in sterile filtered sea water followed by two changes of the medium under test and the concentration adjusted to 500 cercariae cm^{-3}. 50 µl of the appropriate diluent (SW, YTS, MCM or SCM) was added to each well along one row of a sterile flat bottomed microtitre plate (Sterilin Ltd, Teddington, UK), the
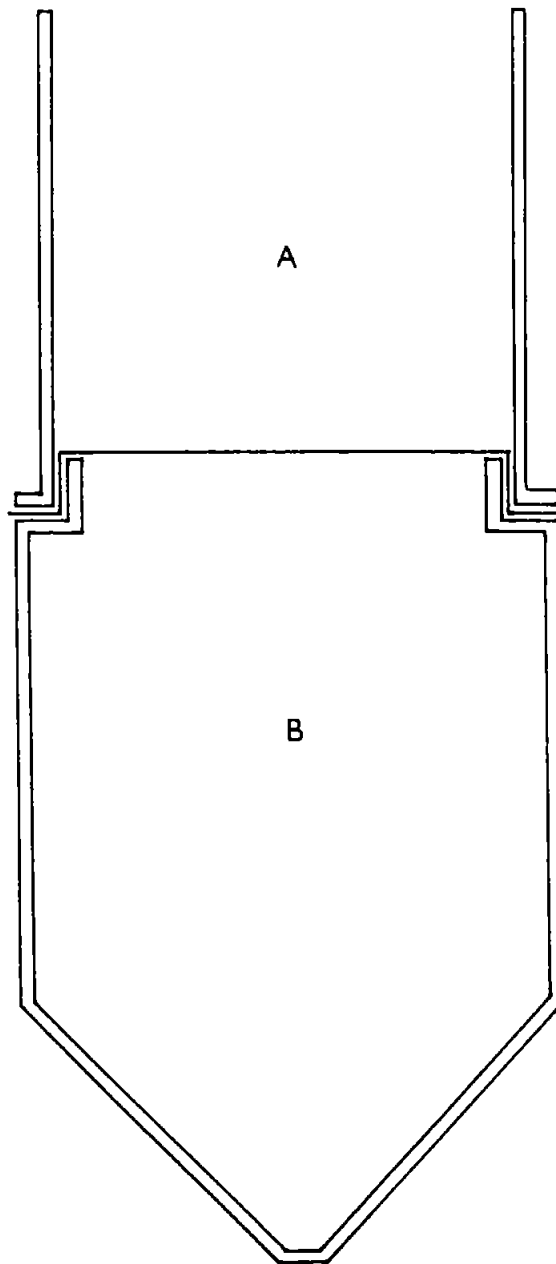
Figure 4 Skin penetration apparatus

A- upper chamber containing suspension of cercariae in sea water.

B - lower chamber containing supplemented culture medium.

Two chambers separated by a thin layer of mullet skin.

same volume of the factor under test added to the first well of the row and serially diluted along the row. Factors tested were glucose (20 mM), sucrose (20mM) and normal pooled mullet serum. 50 µl of the cercarial suspension was pipetted into each well, the plate covered to prevent contamination and evaporation, and incubated at room temperature for up to 24 h. Plates were examined during this period every hour up to 6 h and then at 12 h, 18 h and 24 h using an inverted microscope and % encystment calculated.

In ovo method

Fertilised chicken eggs were obtained 5 days post-fertilisation and incubated at 38.5°C (±1°C) and 70-75% humidity. Cercariae were implanted on the chorioallantoic membrane by one of the following 2 methods.

The first method involved exposing the chorioallantoic membrane by creating a false air space on the side of the egg. Each 8 day old egg was candled to ensure fertility and to mark the position of the embryo, air space and area of the membrane, at a point furthest from the embryo, to be dropped. Holes were drilled over the air space and the area of the chorioallantoic membrane to be dropped. The holes were swabbed with 70% alcohol and the shell membrane over the air-space punctured. A drop of 0.85% saline was added to the hole over the chorioallantoic membrane where the membrane was still intact. A small flat blade (a fountain pen nib was found to be ideal) was inserted at an acute angle and gently lifted to approximately 45°. Suction was applied over the air space and the holes sealed with sellotape. The eggs were then recandled to ensure the correct realignment of the chorioallantoic membrane.

The second method was a modification of the technique used by Zwilling (1959) designed to make a window in the egg before the chorioallantoic membrane had formed, thus reducing trauma. Each 5 day old egg was candled and a small hole drilled on the upper region close to the pointed end of the egg, leaving the shell membrane intact. The hole was swabbed with 70% alcohol to remove dust and a 5 cm^3 syringe fitted with a sawn off 18 gauge needle was used to draw off 1.5 - 2.0 cm^3 albumen. The needle was inserted so that it penetrated towards the pointed end of the egg to avoid damage to the embryo. Keeping this hole uppermost, a rectangle (approximately 1 cm^3) was cut at the

site previously marked, swabbed with 70% alcohol and the piece of shell removed. The shell membrane was torn away and the resulting window sealed with sellotape. The egg was then returned to the incubator to allow the chorioallontoic membrane to form under the window.

Cercariae were washed once in sterile filtered sea water (100 g, 5 min) and twice in sterile Locke's antibiotic solution (Appendix A) before being implanted on the chorioallantoic membrane on the day after the membrane had been exposed. Between 10 and 1000 cercariae per egg were implanted and development monitored with the aid of a stereo microscope for up to 3 days post inoculation. After this time all cercariae had failed to develop and the technique was developed no further.

Recovery of metacercariae from fish tissues

Metacercariae were recovered from fish tissues by artificial digestion. A convenient method involved cutting the body musculature and skin into small pieces and incubating these in trypsin (0.25%) in Rinaldi's buffer (Appendix A) at 37°C. Tissue digests were maintained in 50 cm^3 conical flasks placed in an orbital shaker for up to 24 h. Metacercariae released on breakdown of the tissues and were removed from the digestion mixture with a pasteur pipette and resuspended in YTS.

Measurement of growth of metacercariae *in situ*

Metacercariae in the caudal fin were measured by placing anaesthetised fish on a microscope slide and covering the fin with a coverslip mounted in sea water. Cysts in the fin tissue were then examined using the x10 objective of a light microscope and size measured with the aid of an eyepiece graticule. Maximum length and width were measured and from these the circumference estimated using the following parabolic equation, assuming the circumference of each cyst to be composed of two parabolas,

$$c = \sqrt{4w^2+l^2} + \frac{l^2}{2w} \times \ln \frac{2w+\sqrt{2w^2+l^2}}{l}$$

where c is circumferential length, l is maximum cyst length and w is maximum cyst width. Calculations were made on a BBC microcomputer using the BASIC program described in Appendix C.

Serum preparation and storage

Antiserum was prepared in fish, rabbits and rats. The collection of blood from fish has already been described. Rabbits were test bled from the ear and rats from the carotid artery after open chest surgery under anaesthesia, terminal bleeding of both rabbits and rats being carried out by exsanguination. Blood collected from animals was placed in sterile glass bottles and was allowed to clot at room temperature for 30 - 60 min. The clot was allowed to retract by leaving the blood at 4°C overnight. Serum was removed using a sterile pasteur pipette, transferred to 10 cm³ centrifuge tube (Sterilin Ltd, UK) and centrifuged at 600 g for 5 min to sediment any blood cells. Smaller volumes of serum were transferred to microcapped centrifuge tubes and centrifuged in a single speed microcentrifuge (James A Jobling, Stafford, UK) for 2 min.

Serum for testing was used fresh as freezing is thought to reduce antibody levels (Rio and Reco, 1971). Sera was stored at -20°C or -70°C in 50 μl or 250 μl aliquots. Once thawed, serum was used immediately and never refrozen.

Preparation of antigens

A variety of antigens were used in the investigation including human gamma globulin, sheep and rabbit erythrocytes and parasite antigens including whole cercariae and solublised cercarial fractions. The preparation of these is described below.

Human gamma globulin (HGG).

HGG, Cohn fraction II (Koch Light Laboratories, Colnbrook, UK) was prepared by dissolving 50 mg cm⁻³ antigen in 0.85% saline (Appendix A) and emulsifying with an equal volume of Freund's complete adjuvant (FCA) or Freund's incomplete adjuvant (FIA; Gibco, Paisley, Scotland).

Red blood cells (RBC).

Both sheep and rabbit RBC were used. Suspensions of sheep RBC in Alsevers solution were obtained from Tissue Culture Services, Slough, UK. Rabbit RBC were collected in either Alsevers solution (Gibco, Paisley, Scotland) or heparin (Sigma Ltd., Poole, UK) solution to prevent clotting, the former being used in preference as it was more effective in preventing lysis. Cells could be stored for longer periods in Alsevers than in heparin solution. Preparation of sheep and rabbit RBC antigens was the same. Erythrocyte suspensions were washed thrice (650 g, 5 min) in phosphate buffered saline (PBS, pH 7.2) (Appendix A) to remove anticoaggulant and to allow concentrations of the suspensions to be adjusted as necessary (20% v/v for fish; 50% v/v for rabbits and rats).

Whole cercariae.

Cercariae killed by freezing in liquid nitrogen, were washed (300 g, 5 min) thrice in sterile YTS, once in Locke's antibiotic solution and twice more in YTS. Suspension concentrations were then adjusted as necessary in YTS.

Solubilized cercariae.

A cercarial suspension of known number was washed thrice in either sterile YTS or culture medium (300 g, 5 min) and resuspended in 1 - 2 cm^3. The material was kept chilled on ice and homogenised in an ultrasonic disintegrator (MSE Soniprep 150; Crawley, UK) in 30 second bursts at an amplitude of 14 microns. The resulting homogenate was centrifuged (400 g, 30 min), the supernatant decanted and assayed for protein and the concentration adjusted as required. The soluble antigen was used freshly prepared or stored at -20°C until required.

Determination of protein concentration

Three methods for assaying protein were used; Ultraviolet absorption at 280 nm, Coomassie Brilliant Blue G-250 (Bradford, 1976) and a modification of the Lowry proceedure using a total protein diagnostic kit (Sigma Ltd., Poole, UK).

Administration of antigens by injection

0-group mullet were injected using an Agla micrometer glass syringe (Wellcome, Beckenham, Kent) fitted with a 30 gauge needle. Rabbits, rats and large mullet were injected with a 1 cm^3 or 2 cm^3 sterile plastic syringe fitted with a 23 or 25 gauge needle. Antigen was administered by intraperitoneal injection for fish and by the intraperitoneal or subcutaneous route for rabbits and rats.

Raising antisera

Antisera were raised against HGG, Sheep RBC and Rabbit RBC in fish. In rabbits and rats, antisera were raised against fish immunoglobulin M and whole serum and to solublised cercariae of *C. lingua*. The methods employed in raising antisera in fish, rabbits and rats are described below.

Fish

Mullet anti-HGG

Mullet weighing 500 - 1000 g were anaesthetised and injected intraperitoneally with HGG at the rate of 0.6 cm^3 g^{-1} body weight (25 μg g^{-1} body weight). HGG was administered in FCA for the initial injection and in FIA subsequently. A second injection was made 3 weeks after the initial one and fish were test bled 3 weeks after the second injection. The serum titre to HGG was measured using passive haemagglutination. Fish were given a further injection of HGG in FIA if the titre was not sufficiently high (1:1000 or greater) and tested again after 6 weeks. When the titre was sufficiently high fish were bled out and serum collected and stored at -20°C in 250 μl aliquots.

Mullet anti-RBC

Antisera was raised in mullet to both sheep RBC and rabbit RBC using a similar method. Mullet weighing 500 - 1000 g were injected intraperitoneally once every two weeks with 0.5 cm^3 of a 20% suspension of washed RBC in PBS pH 7.2. When haemagglutination titres of fish serum were 1:1000 or greater, fish were bled, serum collected and stored at -20°C in 250 µl aliquots until required.

Rabbits and rats

Eleven male Dutch rabbits (OLAC Ltd, Bicester, UK), aged 6 months and weighing 2 - 3 kg, together with 17 male Wistar rats born at Plymouth Polytechnic, aged 3 months and weighing 250 - 350 g were used to raise antisera as follows.

Anti-mullet immunolglobulin M (IgM)

Anti-mullet IgM antiserum was raised in rabbits and rats by the following methods:

HGG/mullet anti-HGG immune complexes. Complexes of HGG with mullet anti-HGG antibodies were prepared *in vitro* following the method of White *et al* (1975). The optimal concentration of mullet anti-HGG antisera was determined using the interfacial ring test (Campbell *et al*, 1963). 50 µl of immune serum was pipetted into a small Durham tube and 50 µl of HGG solution carefully layered over it. A visible precipitin ring forms within a few minutes after which time the tubes were left overnight and centrifuged (1000 *g*, 5 min). A range of HGG concentrations were used, the lowest concentration giving the heaviest precipitate being taken as being optimal. Remaining antiserum was then incubated in a 10 cm^3 plastic centrifuge tube (Sterilin, Teddington, UK) with an equal volume of HGG at optimal concentration for 2 h at room temperature (RT) and overnight at 4°C. Precipitated immune complexes were recovered by centrifugation (250 *g*, 5 min), the supernatant being discarded, weighed and washed thrice (250 *g*, 5 min) with 0.85% saline. The complexes were then dissolved in antigen excess (HGG at 10 times the optimal concentration) and emulsified with an equal volume of adjuvant for injection. Initial injections were

made in FCA and subsequent injections in FIA. Rabbits were injected subcutaneously with 1 cm^3 of antigen preparation, 0.5 cm^3 in each flank. Rats were injected intraperitoneally or subcutaneously with 0.5 cm^3 antigen preparation.

Injections were made once every two weeks and animals test bled for serum after 6 weeks. Serum specificity was tested by immunoelectrophoresis against mullet whole serum. A single precipitin arc extending through the gamma and slow beta region of the immunoelectrophoretogram was taken to indicate specificity. Animals were then terminally bled, serum collected, aliquoted into small volumes (50 - 250 μl) and stored at -70°C or in liquid nitrogen until required.

RBC coated with mullet anti-RBC antibody. A modification of the method described by Ellis (1980) for raising rabbit anti-plaice IgM was undertaken using either sheep RBC coated with mullet anti-sheep RBC antibody or rabbit RBC coated with mullet anti-rabbit RBC antibody. Heat inactivated (45°C, 30 min) mullet anti RBC serum was incubated with a 20% suspension of washed RBC in PBS pH 7.2 (0.5 cm^3 serum with 10 cm^3 RBC) for 1 hour at room temperature. Agglutinated cells were thrice washed (650 *g*, 5 min) in PBS pH 7.2 to remove lysed cell debris and excess serum. Coated rabbit RBC were introduced into animals by one of two procedures depending on the presence or absence of adjuvant and route of administration as follows. Rabbits were injected subcutaneously with 1.0 cm^3 of the suspension, 0.5 cm^3 in each flank. Rats were injected with 0.5 cm^3 either subcutaneously or intraperitoneally. Injections were given every 14 days, animals test bled after 12 and 14 weeks and serum specificity tested as described above. Alternatively, coated rabbit RBC were administered in an equal volume of Freund's adjuvant, initial injections being made in FCA and subsequent injections in FIA. Rabbits were injected subcutaneously in multiple sites along the back with 2.0 cm^3 of this suspension and rats received 0.5 cm^3 either subcutaneously or intraperitoneally. Administrations were made every 2 - 4 weeks and animals test bled 2 weeks after each booster injection. Sera was tested for specificity as described above. Coated sheep RBC were similarly administered in adjuvant.

When positive, animals were terminally bled, serum collected and stored at -70°C or in liquid nitrogen until required.

Mullet bile / rat anti-mullet whole serum immune complexes. Rats were immunised with immune complexes formed between mullet bile and rat antiserum raised against mullet whole serum as follows.

Mullet bile was electrophoresed against rat anti-mullet whole serum and the resulting precipitin lines were cut out of the gels of 8 immunoelectrophoresis plates. These were emulsified in 1 cm^3 sterile PBS using a 1 cm^3 Potter homogeniser and mixed with an equal volume of adjuvant. Each rabbit was immunised with 2 cm^3 of this mixture by sub-cutaneous injection in multiple sites along the back. Initial administration was made in FCA and a booster injection given in FIA 14 days later. Rabbits were test bled after a further 14 days and serum tested by electrophoresis against mullet whole serum. A single precipitation arc in the beta and slow gamma region indicated specificity for mullet IgM. When positve, rabbits were terminally bled, serum collected and stored at -70°C or in liquid nitrogen until required.

Rat anti-mullet whole serum

Mullet whole serum was emulsified with an equal volume of adjuvant and each rat injected subcutaneously with 0.5 cm^3 of this emulsion. Initial injections were made in FCA and subsequent injections in FIA, 4 - 6 weeks after the first injection. Rats were test bled 4 - 6 weeks after the second injection and serum tested for specificity against mullet whole serum by immunoelectrophoresis (Fig 5). Positive serum was collected after terminal bleeding and stored in 50 µl aliquots at -70°C.

Rabbit anti-*C. lingua* solubilised cercariae

A solubilised cercarial suspension containing the equivalent of 10^5 cercariae cm^{-3} was emulsified with an equal volume of adjuvant. Each rabbit was injected sub cutaneously in 4 sites with 1 cm^3 of this emulsion. Initial injections were made in FCA, subsequent injections being made in FIA at 4 week intervals with 4 injections administered in total. Rabbits were test bled after the final injection and serum tested for specificity using Ouchterlony double diffusion. Precipitin

Figure 5 Immunoelectrophoretogram of mullet whole serum against rat anti-mullet whole serum
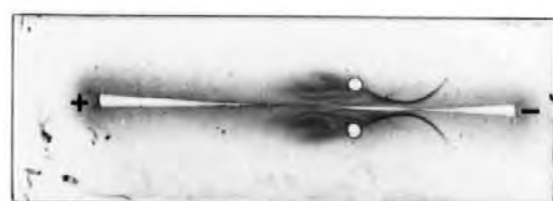
lines were observed down to a 1 in 4 serum dilution (Fig 6). When positive, rabbits were terminally bled, serum collected and stored in 250 µl aliquots at -70°C or in liquid nitrogen until required.

Detection of antigen/antibody reaction

Immuno-electrophoresis

Between 2 and 3 µl of mullet whole serum was applied to the wells of agarose plates (1% w/v agarose in barbitone buffer pH 8.2, Appendix A) and electrophoresis was conducted in barbitone buffer, pH 8.2, for 2 hours at 100 volts using a Shandon Southern 600X 100 electrophoresis unit. Troughs were filled with antiserum under test and allowed to diffuse against the electrophoresed serum overnight. Gels were pressed beneath one wet and five dry filter papers with a 1 kg weight placed on top of a glass plate. The pressing was repeated twice at 5 min intervals, followed by 2 x 10 min washes in 0.1 M sodium chloride and pressing repeated twice more. Gels were dried in a cool air stream, stained in Coomassie Brilliant Blue staining solution (Appendix A) for 5 min and destained as necessary.

Passive haemagglutination

Serum antibody levels to soluble protein antigens were titrated using the technique of passive haemagglutination described by Stavitsky (1954) using tanned sheep RBC. Serum complement and natural haemolytic activity was removed by heat inactivation (45°C, 30 min) and naturally occuring agglutinins were removed by incubation with an equal volume of sheep RBC (Gibco, Paisley, Scotland) for 1 h at room temperature. Sheep RBC were thrice washed (650 *g*, 5 min) in PBS pH 7.2 before use. Erythrocytes were tanned by mixing a 2.5% (v/v) suspension in PBS pH 7.2 with an equal volume of 0.005% (w/v) tannic acid in saline (Appendix A) for 15 min at 37°C. Cells were then washed (650 *g*, 5 min) in PBS pH 7.2 and resuspended to 2.5% in the same buffer. These tanned cells were then coated with antigen. Uncoated cells were used as controls.

To coat cells with antigen one volume of antigen at a protein concentration of

Figure 6 Precipitation of solublised cercarial antigens with rabbit anti-*C. lingua* serum using the Ouchterlony technique

Central well (A) contains solublised cercariae.

Outer wells 2, 4, 8, 16, 32, 64 contain serial two-fold dilutions of rabbit anti-*C. lingua* serum.
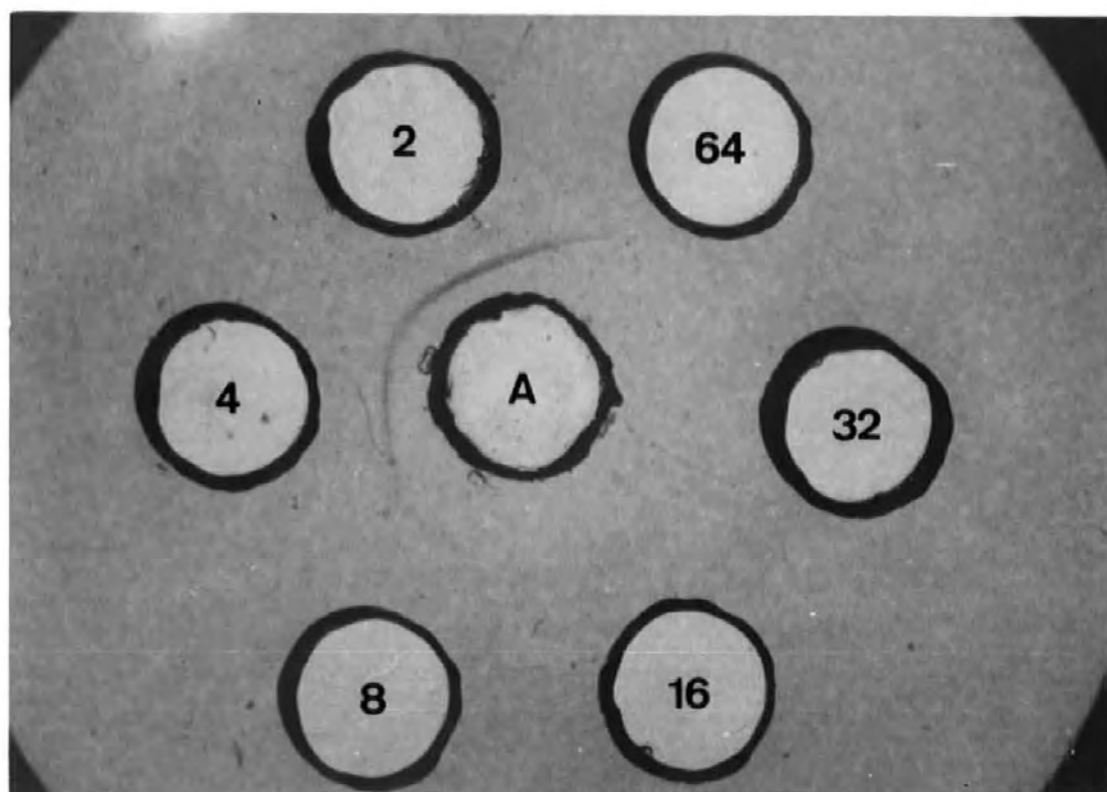
1.0 mg cm^{-3} for HGG or 6.0 mg cm^{-3} for solublised cercariae was added to one volume of 2.5% tanned sheep RBC and 4 volumes PBS pH 6.4 (Appendix A) and incubated at room temperature for 15 min. Controls in which antigen was omitted and replaced with 1 volume PBS pH 6.4 were employed.

The mixture was washed (650 g, 5 min) with a 1% solution of heat inactivated and absorbed normal mullet serum in PBS pH 7.2 (serum diluent) to stabilize and prevent spontaneous agglutination of tanned cells during titration. Cells were resuspended in serum diluent to 0.25%.

Titration was carried out in Cooke microtitre plates with 'U' shaped wells (Sterilin, Teddington, UK). 50 μl of serum diluent was added to each well and then test serum samples were serially diluted, twofold along either 2 or 3 rows (24 or 36 wells). 50 μl of coated sheep RBC were then added to each well.

Controls set up similarly included; uncoated tanned sheep RBC, normal mullet serum, serum diluent alone (no serum) and PBS pH 7.2 alone (neither serum nor serum diluent present). Plates were gently agitated, covered, incubated for 3 hours at room temperature and left overnight at 4°C before being read. The last well showing complete agglutination was taken to be the end-point. Representative serum samples from each experiment were simultaneously tested to ensure comparability of results using this assay.

Direct haemagglutination

Direct haemagglutination was carried out to measure serum antibody levels against either sheep red blood cells or rabbit red blood cells. The method used was the same as that for passive haemagglutination with the exception that the red blood cells were not tanned. Plates were read in the same way and the end-point taken to be the last well showing complete agglutination.

Cercarial agglutination

Into each well of a microtitre plate (Sterilin Ltd) was placed 50 µl of sterile YTS. An equal amount of heat inactivated (47°C, 30 min) serum under test was serially diluted along 2 rows of the plate and 50 µl of antigen (500 cercariae cm^{-3}) was added to each well. Plates were left at room temperature for 3 h and then examined for agglutinating activity using a stereo microscope. The final well showing agglutination of cercariae was taken as the end point. Mean -log^2 antibody titres were compared using a student t-test (P<0.05).

Double immunodiffusion

The method of Ouchterlony (1948) was used to detect antibody reaction to parasite antigens. A 1% agar solution in PBS was prepared and 15 cm^3 added to each petri dish and allowed to solidify. Wells, 6 mm in diameter, were cut and 20 µl antigen added to the central well. Antiserum was serially diluted two-fold in the surrounding wells, each well receiving a total of 20 µl. Controls using non-immune serum were emlpoyed. Plates were incubated at room temperature and then examined for immunoprecipitation.

Complement fixation test

This was undertaken to test the ability of normal (non-immune) and immune mullet serum to fix complement and the effect of this on live cercariae. Serum under test (50 µl) was heat inactivated (47°C, 30 min), diluted by serial two-fold dilutions in sterile YTS along 2 rows of a microtitre plate (Sterilin Ltd, Teddington, UK) and 50 µl of live cercariae (500 cercariae cm^{-3}) added to each well. Plates were incubated at room temperature for 30 min after which time 50 µl complement was added to each well. Sera taken from uninfected mullet and diluted to 50% in YTS was used as a source of complement. Serum from infected mullet however was occasionally used to investigate the anomaly of decreased response with increased titre. Plates were incubated for a further 3 h when 50 µl 0.5% trypan blue in YTS was added to each well and cytotoxic activity estimated on the basis of the percentage of cercariae taking up stain.

Chelation of fish sera

In order to determine the role of complement in lysis of cercariae, fish sera was treated with the following chelating agents; 40 mM Ethylenediaminetetra-acetic acid (EDTA, disodium salt; Sigma, Poole, UK), 40 mM Ethylene glycol-bis (β-Amino-Ethyl ether) N,N,N',N'-tetra-acetic acid (EGTA; Sigma, Poole, UK) with 0.5 M $MgCl_2$ added, 40 mM EDTA with 0.5 M $MgCl_2$ and 40 mM EDTA with 0.5 M $CaCl_2$.

Equal volumes of serum and chelating agent were mixed in a microtitre plate and incubated at room temperature for 30 min. The same volume of cercariae, suspended in 1.1% saline (Appendix A) was then added and plates incubated for a further 3 hours after which time cercariae were stained by adding 0.5% trypan blue in 1.1% saline. Cercarial survival was estimated on the basis of the percentage of cercariae taking up stain.

Immunoblotting

Proteins separated on 12% SDS-polyacrylamide gels were transferred onto nitrocellulose membranes using a Western electroblotting technique. Gels and membranes were always handled with gloves to prevent contamination. When gel electrophoresis was complete, 3 pieces of 3 mm chromatography paper (Whatmann Ltd, Maidstone, UK) and one piece of nitrocellulose membrane (Hybond-C extra; Amersham International, Little Chalfont, UK) were cut to a size slightly larger than the gel and then soaked in Tris-glycine buffer (Appendix A). The 2 sponges from the electroblotting apparatus (Trans-Blot Cell; Bio-Rad Laboratories Ltd, Watford, UK) were similarly soaked and the Trans-Blot Cell filled with buffer. One piece of soaked chromatography paper was layed on a clean surface and the gel placed on top of this, followed by the nitrocellulose membrane and then the other 2 pieces of chromatography paper. Any air bubbles were carefully removed and this assembly placed between the 2 sponges and loaded into the Trans-Blot Cell with the nitrocellulose membrane nearest to the positive electrode. The apparatus was run at 30 v for 24 h and the nitrocellulose membrane immunostained as described below. The gel was stained with coomassie blue to ensure that all proteins had been transferred to the nitrocellulose membrane.

Immunostaining of transferred proteins was performed using an immunogold-silver system. All incubations were made at room temperature in a small covered perspex vessel containing 20 - 50 cm^3 of the appropriate solution under constant agitation. The nitrocellulose membrane was first incubated in 5% BSA for 20 min to block any non-specific binding sites. This was then replaced with the antisera under test at a dilution of 1:100 and incubated overnight. The membrane was then washed 3 times (10 min each) with 0.1 M Tris-HCl buffer pH 7.2 containing 0.1% BSA and incubated overnight in rabbit anti-mullet IgM antiserum at 1:200 dilution. This step was omitted when the primary antibody under test was not of mullet origin. The membrane was again washed and then incubated overnight in a 1:200 dilution of goat anti-rabbit antiserum to which had been conjugated with 10 nm gold particles (Janssen Pharmaceuticals, Wantage, UK). Following this, the membrane was washed 3 times in 0.1 M Tris-HCl (pH 7.2) containing 0.1% BSA (10 min each), followed by 3 washes in 0.1 M Tris-HCl (pH 7.2) and then 3 washes in distilled water. The gold signal was then enhanced using a silver staining process (IntenSE; Janssen Pharmaceuticals, Wantage, UK) following the manufacturers instructions. A permanent photographic record of the stained membrane was then made and a diagrammatic representation of stained protein bands prepared from this.

In order to react the same nitrocellulose membrane with further antisera, the first was removed by incubating in 0.1 M glycine-HCl (pH 2.2) containing 50 mM KCl using a modification of the method described by Legocki and Verma (1981). The membrane was incubated in this buffer for 48 h, after which time all the stain had dissapeared. It was then neutralised by washing 3 times (15 min each) in 0.1 M Tris-HCl (pH 7.2) and reacted with the next antiserum as described above. This process was repeated up to 4 times, enabling 5 (3 experimental and 2 control) antisera to be tested using only one membrane. The method is particularly applicable for investigations where only minimal amounts of antigen are available, as in the present study.

Detection of cellular responses

In order to study the cellular responses of fish to *C. lingua* the assays described below were performed using pronephric leucocytes.

Collection of pronephric leucocytes

The pronephros was used for cellular based assays as it contains relatively high levels of leucocytes. The pronephros was removed from exsanguinated fish and placed into a solid watch glass containing ice-cold Leibovitz L15 culture medium with L-glutamine (Gibco, Paisley, UK) and supplemented as described in Appendix A. The osmolality of mullet serum was measured using a Wescor 5100C vapour pressure osmometer (Chemlab Instruments Ltd, Hornchurch, UK) and found to be 355 mmolkg^{-1}. The osmolarity of all cell culture media used was adjusted accordingly using NaCl.

The tissue was disrupted by gently forcing it through a sterile stainless steel gauze (140 μm mesh) using a scalpel blade. The resulting cell suspension was washed 3 times with culture medium (200 *g*, 5 min) in a refrigerated centrifuge at 4°C, numbers estimated using a haemocytometer and the concentration of viable cells adjusted as required after trypan blue exclusion.

Assays of cellular response

Under-agarose migration

The migration of pronephric leucocytes towards solubilised cercariae was assessed using a modification of the under-agarose migration assay (Nelson *et al*, 1981). Type V agarose (Sigma, Poole, UK) was dissolved in double distilled water by boiling in a water bath, cooled to 50°C and added to an equal volume of HEPES (Sigma, Poole, UK) buffered 2x Leibovitz L15 culture medium (Flow Laboratories, Rickmansworth, UK) pH 7.5 containing 0.5% gelatin at the same temperature. Preliminary studies using a range of agarose concentrations showed the optimum to be 1% agarose, therefore all subsequent assays were performed using this concentration.

Agarose medium (5 cm^3) was transferred onto acid cleaned gelatin coated

microscope slides and allowed to solidify at 4°C. Wells, 3 mm in diameter and 3 mm apart were cut in the agarose in groups of 3 (Fig 7). Each well was filled with 10 µl of fluid, one outside well receiving the solubilised preparation of cercariae in culture medium (125 µg cm^{-3} protein), the other outer well received culture medium alone and the cells under test were placed in the central well at a concentration of 5 x 10^7 cells cm^{-3}. Slides were incubated in a humidification chamber at room temperature for 3 h after which time they were fixed by flooding each slide with absolute methanol. After 30 min this was repeated and slides left at 4°C overnight to complete fixation. The agarose was allowed to dry, then carefully removed and slides air dried and stained with 10% Giemsa for 15 min. Chemotaxis was quantified (Fig 7) by measuring the distance cells migrated from the margin of the central wells towards the well containing antigen (Distance A) and subtracting from this the random migration represented by the corresponding distance towards the control well (Distance B) to give the Chemotactic Differential (A-B). Mean values were compared using a student t-test (P<0.001).

Cell polarisation

A modification of the method described by Haston and Shields (1985) was used to assess the potential chemotactic effect of cercarial proteins on fish leucocytes. Pronephric cells (10^7 cells cm^{-3}; 0.9 cm^3) were mixed with 0.1 cm^3 of either solubilised cercariae (125 µg cm^{-3} protein) or culture medium (negative control) and incubated at room temperature for 1 h. Cells were then fixed for 30 min with 1 cm^3 2.5% gluteraldehyde in culture medium, washed and a proportion of polarised cells counted using a haemocytometer. Leucocytes which normally possess a spherical morphology, adopt an elongated form in response to chemotactic substances (Fig 8). Results were expressed as the ratio of polarised cells in a suspension incubated with antigen to those incubated with culture medium alone (Polarisation Index). Mean results were compared using a student t-test (P<0.05).
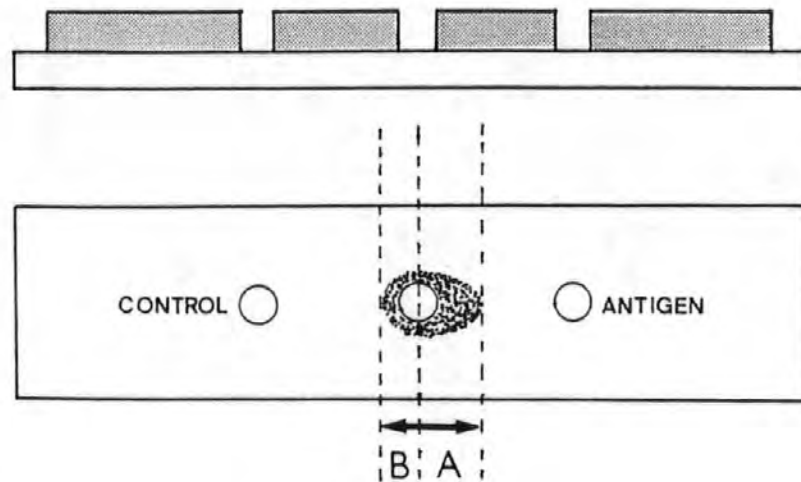
Figure 7 Apparatus for the under agarose migration assay

Distance **A**: migration towards antigen source.

Distance **B**: migration towards control.

Chemotactic Differential = **A-B**.

Figure 8 Polarisation of mullet leucocytes

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(a) Normal (unpolarised) leucocytes. (Bar= 40μm)

(b) Polarised leucocyte from suspension in solublised cercariae extract.
(Bar= 40μm).

a

b
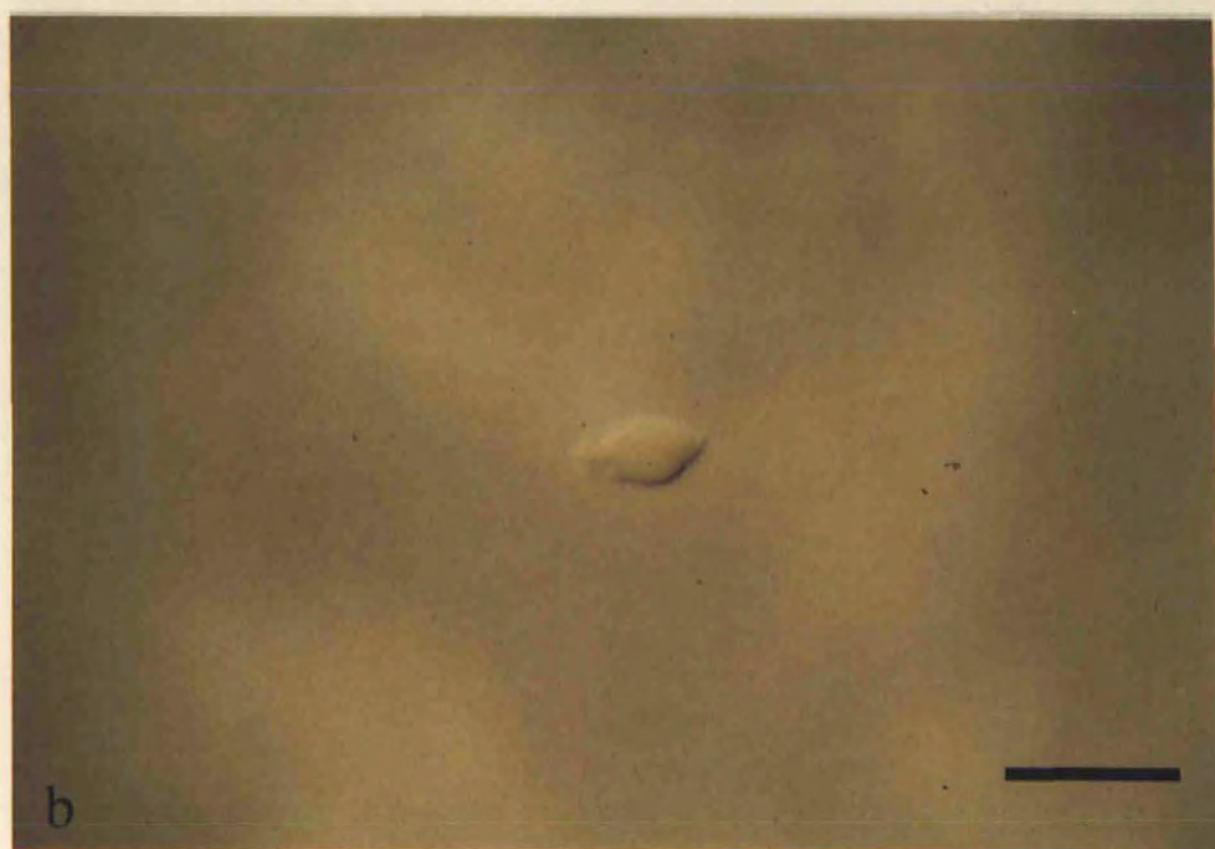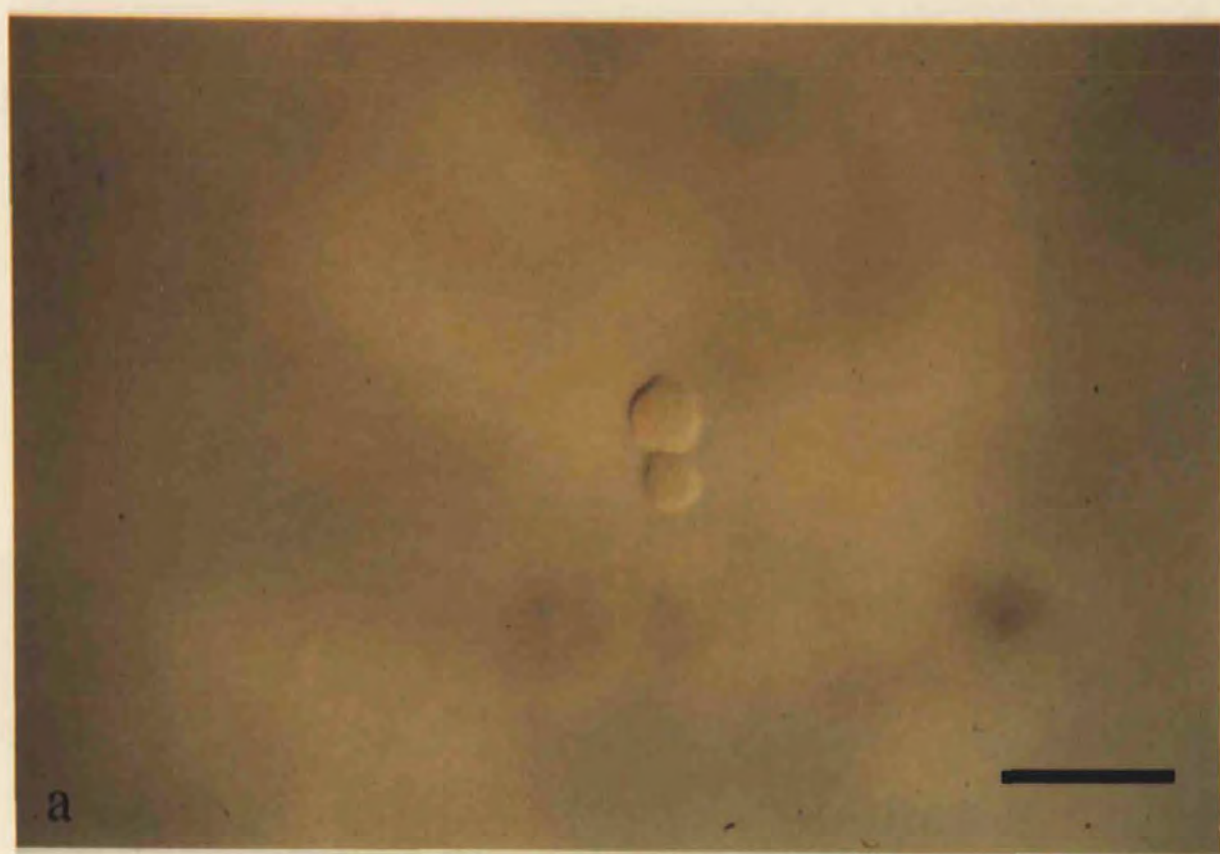
Cellular adherence

Live cercariae or cercariae killed by freezing at 20°C were washed 3 times in sterile filtered sea water and then 3 times in culture medium and suspended to 500 cercariae cm^{-3}. To each well of a flat bottomed microtitre plate was added 50 µl leucocyte suspension (10^6 cells cm^{-3}) and serum (1:200 dilution) both from either infected or control fish. Controls lacking serum and/or cells were employed. Cercariae (50 µl) were then added to each well, the plate incubated at room temperature and examined for adherence of cells to parasites and parasite damage between 2 h and 24 h with the aid of an inverted micrscope.

Migration inhibition assay

A modification of the capillary tube assay described by George and Vaughan (1962) was employed. Capillary tubes (Bilbate, Daventry, UK) were filled with cell suspensions (10^7 cells cm^{-3}), sealed at one end with critoseal (Sherwood, St Louis, USA) and centrifuged at 200 g, 5 min to pack cells at the plug surface. Each tube was then cut at the buffy coat interface and the section containing the cells placed in a well of a leucocyte migration tray (Sterilin, Teddington, UK) and secured with a spot of sterile petroleum jelly. Wells were immediately filled with 400 µl of either culture medium containing solubilised cercarial antigens (125 µg cm^{-3} protein) or culture medium alone (controls), sealed with a coverslip and incubated at room temperature for 18 h. The extent of migration was then assessed. Migration fans were either viewed directly using a binocular microscope or coverslips were first removed and fans fixed in gluteraldehyde vapour for 4 h after which time the medium was carefully removed and cells stained using 10% Giemsa. The diameters of migration fans were then measured using an eyepiece graticule.

This technique was not employed routinely in experimental work due to the lack of consistant cell migration observed even under identical conditions. However, with further development it is felt that this assay may offer a useful method of quantifying early phase cell mediated responses to parasitic infections of fish.

Analysis of protein fractions

Gel filtration

In order to determine the IgM fraction, mullet sera from both control (unimmunised) fish and from fish immunised with rabbit red blood cells, were fractionated by gel filtration through Sephadex G-200 or Sepharose 6B gels. Degassed gels were packed into a Wright gel filtration column (Wright, Stonehouse, UK) using 1.0 M NaCl and 0.1 M Tris-HCl buffer, pH 8.0 containing 0.02% sodium azide at a flow rate of 2 - 3 l h^{-1} maintained by a peristaltic pump (Pharmacia Fine Chemicals AB, Uppsala, Sweden). The serum samples in 10% surcrose were applied to the column via a Pharmacia SA-5 sample applicator (Pharmacia) and eluted fractions collected using a Pharmacia Frac-100 fraction collector.

Absorbance of the eluant at 280 nm was automatically monitored using an LKB Unicord II ultraviolet absorbitometer and recorded using a Pharmacia REC-481 single channel recorder. Blue dextran was added to the samples as a marker.

Eluant fractions of mullet anti-rabbit RBC antiserum showing absorbance peaks were tested for anti-rabbit RBC activity using direct haemagglutination. Those fractions showing positive results were precipitated by the addition of ammonium sulphate.

Polyacrylamide-gel electrophoresis (PAGE)

Sodium dodecyl sulphate (SDS) slab PAGE was carried out using the procedure of Laemmli (1970) to determine the protein profiles and molecular weights of extracted parasite components. A 12% separating gel and a 4.5% stacking gel were used. The composition of stock solutions and volumes used are described in Appendix B.

The separating gel was degassed, poured into the gel mould, overlayed with water-saturated n-butanol (BDH, Bristol, UK) and allowed to polymerise at room temperature for 1 h. The n-butanol was then washed off using distilled water

and the degassed stacking gel poured onto the separating gel. A well former was inserted and the gel allowed to polymerise as above, after which time the apparatus was wrapped in cling film to prevent desication and stored overnight at 4°C.

Parasite samples were prepared by concentrating whole parasites by centrifugation at 200 *g*, 5 min and by adding the pellet to an equal volume of loading buffer (Appendix B) containing 2-mercaptoethanol (2ME) and SDS. This mixtue was homogenised using an ultrasonic disintegrator (MSE Soniprep 150) in 30 second bursts of an amplitude of 14 microns and then boiled for 10 - 15 min. Samples were used fresh or stored at -20°C until required. Wells were loaded with 10 - 40 µl samples or 10 µl of a protein molecular weight standard (Sigma, Poole, UK) using a 100 µl Hamilton syringe (Hamiltom, Nevada, USA).

Electrophoresis was carried out at a constant current of 20 mA for the first 30 min followed by 30 mA until the leading front was approximately 1 cm from the bottom of the gel. Gels were then carefully removed and stained using 0.2% Coomassie brilliant blue (R-250) overnight, destained and photographed. (Staining and destaining solutions are given in Appendix B).

Histology

Light microscopy

Tissue sections

Material for light microscopy was fixed for 3 days in 10% neutral buffered formalin (Appendix A) and dehydrated via an ascending alcohol series. For wax embedding, material was then cleared in xylene and embedded in parafin wax. Sections were cut at 5 - 7 µm and floated onto albumin coated microscope slides using a water bath at 50°C and heat dried at 37°C overnight before being brought to water for staining. Resin embedding of material was undertaken using JB4 (International Enzymes Ltd., Windsor, UK), HEMA (TAAB, Reading, UK) and Lowicryl (Chemische Werke Lowi GmbM, Waldkraiburg, FRG), a low temperature methacrylate resin, polymerised by ultra-violet light following manufacturers instructions. Resin sections were cut at 1 - 2 µm using either a

Reichert-Jung 1140 Autocut glass knife microtome or a Reichert-Jung Ultracut ultramicrotome with glass knives. Glass knives were made using a Reichert-Jung or LKB 7801 glass knife maker. Each semi-thin section was transferred to a drop of water on a gelatin coated microscope slide and heat dried at 37°C for 30 min before being brought to water for staining.

For routine examination sections were stained by one of the following methods, details of which are given by Carlton (1967), Pearse (1972) or Gray (1957): Ehrlich's Haematoxylin and Eosin; Mallory's triple stain; Calcium was detected by staining with Alizarin Red S. Tyrosinase was detected using the 3, 4, dihydroxyphenylalanine (DOPA) methods of Becker *et al* (Pearse, 1972) and latterly a modification of the technique described by Fitzpatrick *et al* (1950) using DOPA in place of l-tyrosine and a temperature of 20°C instead of 37°C. Staining times often had to be increased for resin sections in order to obtain satisfactory results. After staining all sections were dehydrated, cleared and mounted using DPX mountant (Raymond A Lamb, London, UK).

Blood smears

In view of the rapidity of clotting in mullet blood, it was necessary to use an anticoagulant for the collection of blood for smears. Tri-sodium citrate (3.2%) was chosen as cells were found to be damaged least in this anti-coagulant. Thin films were made on acid cleaned microscope slides, air dried as quickly as possible and fixed in absolute methanol for 5 min. Smears were stained with May Gründwald and Giemsa (MGG) for routine observations and the following histochemical stains used as described by Hayhoe and Flemans (1982); Periodic Acid Schiff (PAS), Sudan black B. After staining, slides were air dried and mounted using DPX (Raymond A Lamb, London, UK). Human blood cells were used in parallel as positive controls. Differential leucocyte counts were carried out on smears by counting a minimum of 100 cells from each of 5 fish.

Electron microscopy

Transmission electron microscopy

Material for transmission electron microscopy was cut into small pieces (approximately 1 mm^2) and fixed in 3% gluteraldehyde (EM grade; Agar Aids, Essex, UK) in 0.2 M sodium cacodylate buffer pH 7.2 for routine examination and 4% paraformaldehyde (Agar Aids, Essex, UK) + 1% gluteraldehyde in the same buffer for immunochemistry. Material for routine examination was post-fixed using 1% osmium tetroxide (Agar Aids, Essex, UK) in 0.1 M sodium cacodylate buffer pH 7.2, dehydrated and embedded in one of the following resins under manufacturers instructions: Spurr resin (TAAB, Reading, UK); Araldite resin (Polaron Equipment Ltd., Watford, UK) or Epon resin (Polaron Equipment Ltd.).

Material for immunocytochemistry was embedded routinely without post fixation in either Lowicryl resin (Chimische Werke Lowi GmbM, Wablkraiburg, FGR) or LR Gold (London Resin Co., Oxford, UK) using the progressive lowering of temperature (PLT) method described in the manufacturers instructions. Both of these resins polymerise at -25°C to -35°C using ultraviolet light (for Lowicryl) or blue light (for LR Gold). Resin embedded material was sectioned on either a Reichert-Jung Ultracut ultramicrotome or a Sorvall Porter-Blum MT2-B ultramicrotome using glass knives made with an LKB 7800N glass knife maker. To locate the area of interest, semi-thin sections (1 - 2 μm) were taken and stained with 1% methylene blue/1% borax and viewed under a light microscope. Ultra thin sections were then cut and collected in a water boat. Sections showing gold to silver interface patterns (60-100 nm thickness) were floated onto copper or nickle 200 mesh grids (Polaron Ltd., London, UK) and allowed to dry.

Sections were stained with lead citrate (Reynolds, 1963) and saturated aqueous or alcoholic uranyl acetate and viewed on either a Philips 300 or a Jeol JEM 100CX transmission electron microscope.

Scanning electron microscopy

For routine scanning electron microscopy material was fixed, post-osmicated and dehydrated to absolute alcohol as for transmission electron microscopy and then critically point dried in liquid carbon dioxide using a Samdri pvt-3 critcal point dryer (Tousimis Research Co, Maryland, USA). Tissue was mounted on metal stubs with double sided tape, coated with gold (13 nm) using a Polaron SEM coating unit (Model ES100, Polaron Equipment Ltd, Watford, UK) and viewed with a Jeol JSM T20 scanning electron microscope.

X-ray micro-analysis

Tissue processed for the detection of calcium using x-ray micro-analysis was left unfixed, dehydrated, freeze-dried and viewed using a Jeol JSM 35-C scanning electron microscope with facilities for x-ray micro-analysis. In addition, material which had already been processed for routine transmission electron microscopy was used for x-ray micro-analysis. Grids were first coated with a thin layer of carbon in an Edmunds sputter coating unit and sections analysed using a Jeol 200CX transmission electron microscope.

Immunocytochemistry

Immunocytochemical staining was carried out using a colloidal gold immunolabelling system at both light and electron microscopical levels. For light microscopy, lowicryl resin sections were incubated in a 1:10 dilution of normal goat serum (Dako Ltd, High Wycombe, UK) for 20 min to block any non-specific binding sites. These were then drained without washing and incubated with the antiserum under test (1° antibody) for 2 - 3 h. The dilution of the 1° antibody varied according to it's source; antisera derived from mullet was used at 1:10 and those from rabbits at 1:20. Sections were then washed 3 times (5 min each) in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.1% BSA and 0.5% Tween 20 and incubated in rabbit anti-mullet IgM antiserum (link antibody) at 1:10 dilution for 2 - 3 h. This step was omitted when the 1° antibody was not of mullet origin. Sections were again washed in buffer and then incubated for a further 2 h in a 1:20 dilution of goat anti-rabbit antiserum which had been conjugated with 10 nm gold particles (Janssen Pharmaceuticals, Wantage, UK).

Following this, sections were washed 3 times in buffer and then 3 times in distilled water. The gold signal was then enhanced using a silver staining process (IntenSE, Janssen Pharmaceuticals, Wantage, UK) following the manufacturers instructions. Sections were counterstained using 0.01% toluidine blue, mounted in DPX and viewed by routine and epipolarised light microscopy. All incubations were performed at room temperature in a humidified chamber to prevent evaporation. Controls employed included:

(i) Omitting 1:10 normal goat serum

(ii) Omitting 1° antibody

(iii) Replacing 1° antibody with normal antiserum

(iv) Omitting rabbit anti-mullet IgM link antibody (where applicable)

For electron microscopy, lowicryl resin sections mounted on nickle grids were immunostained as for light microscopy with the following exceptions. Primary antisera were used at dilutions of 1:20 (mullet) and 1:50 (rabbits). The rabbit anti-mullet IgM link antibody (where applicable) was used at a dilution of 1:20 and the gold conjugated goat anti-rabbit antiserum used at 1:20. These incubations were all made for 2 h at room temperature by floating grids, section side down, on droplets of antisera on sheets of either parafilm or dental wax. An extra washing step in 0.1 M Tris-HCl buffer pH 8.4 containing 1% BSA and 0.5% Tween 20 (3 x 5 min each) was also included immediately before and after incubating with the gold conjugated antiserum. Gold labelled sections for electron microscopy were not enhanced with silver staining but were counterstained with uranyl acetate and lead citrate as for routine microscopy.

Photomicrography

For light photomicrography, sections and live material were photographed on either a Zeiss Photomic II photomicroscope, an Olympus Vanox-T model AHBT photomicroscope, an Olympus BH-2 fitted with an epipolarising unit and an Olympus C-35AD camera attachment (for epipolarised microscopy) or a Zeiss standard microscope fitted with a Zeiss MC63 automatic camera attachment using Pan F (50 ASA) or FP 4 (100 ASA) monochromatic film (Ilford Ltd., London , UK) with a green filter or Ektachrome (100 ASA) colour transparency

film (Kodak Ltd., Hemel Hempstead, UK) with a blue filter. For photography of live metacercariae developing in the caudal fin of mullet, anaesthetised fish were placed on a microscope slide and a coverslip mounted over the fin using sea water.

Electron micrographs were taken using Kodak Plate film for electron microscopy (Eastman-Kodak Co., New York, USA). Plates were developed using Kodak D19 developer (4 min, 20°C), fixed (15 min, 20°C), washed for 30 min in running tap water and allowed to dry. Photomicrographs were printed on Rapidoprint paper (Agfa-Gevaert, West Germany), processed using a Rapidoprint DD37 automatic processor (Agfa-Gevaert, West Germany), fixed, washed thoroughly in running tap water and glazed.

Results

Section 1: Aspects of the immune system of the grey mullet

Although some fundamental studies of aspects of the immune system of mullet have been undertaken by Mughal and Manning (1986, 1985) and Mughal (1984), further investigations were also necessary here as a basis for interpreting host responses to *C. lingua*. These studies included the partial characterisation of serum, description of the lymphoid system and the effects of antigen presentation on the immunogenicity of mullet immunoglobulin.

Partial characterisation of mullet serum

Mullet antiserum raised against rabbit erythrocytes was fractionated by gel electrophoresis and the antibody titre of each fraction to rabbit erythrocytes was measured using the direct haemagglutination assay.

From the results (Fig 9) it can be seen that agglutination was only associated with the first of 4 protein peaks of the serum profile and agglutination titres obtained are given in Table 1. That Peak 1 represents the immunoglobulin component of the serum was further confirmed on treatment with ammonium sulphate which produced a precipitate only with eluant samples from this peak.

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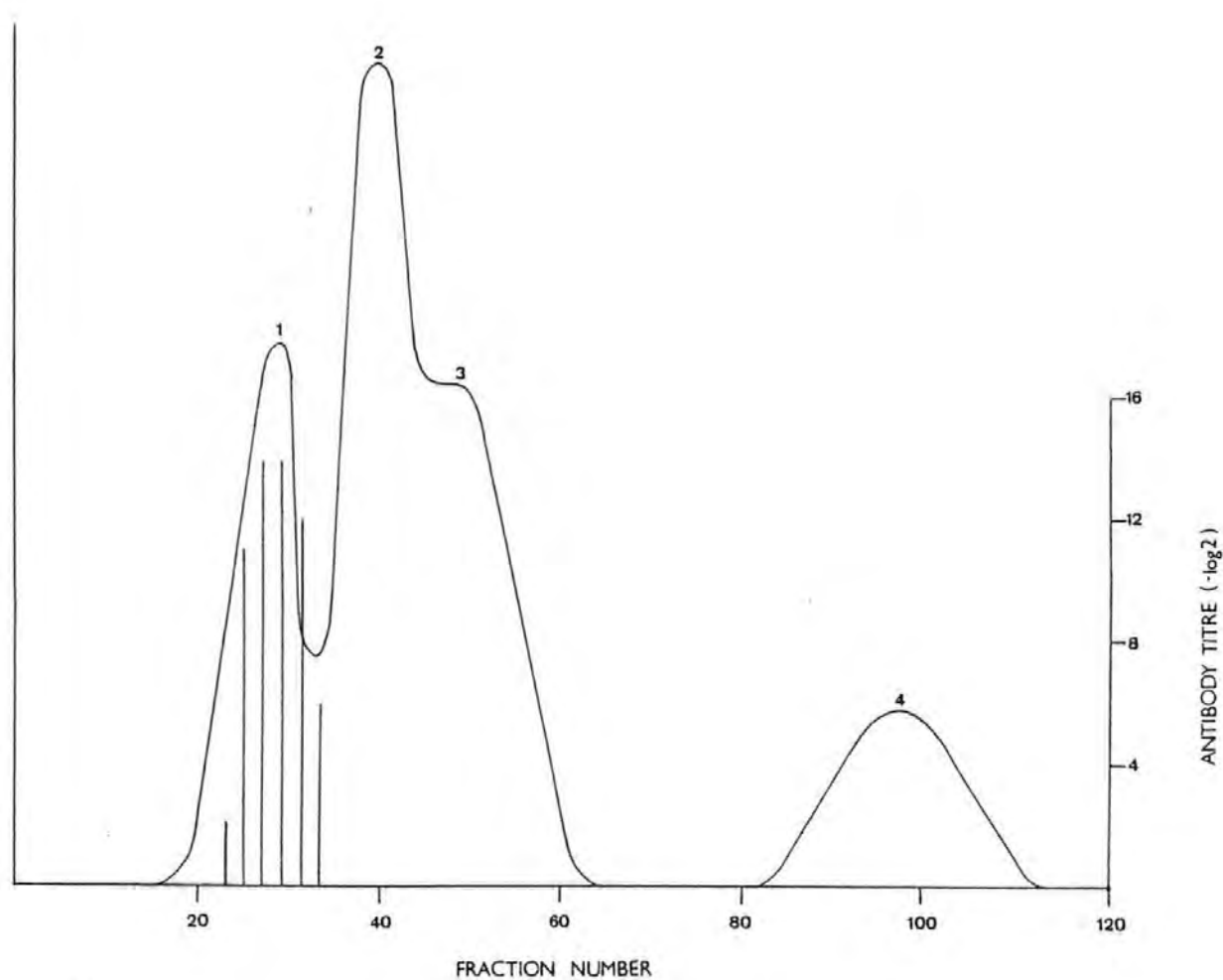
Figure 9 Fractionation of mullet serum on Sephadex G200

Vertical bars represent antibody titres of fractions associated with Peak 1.

Table 1 Agglutination titres associated with Peak 1 after gel filtration

Fraction Number	Antibody Titre
22	0
23	2
24	7
25	11
26	11
27	14
28	14
29	14
30	14
31	12
32	7
33	6
34	4
35	0

Cells and tissues of the lymphoid system

Peripheral blood leucocytes

Thrombocytes, lymphocytes, monocytes and granulocytes identified within the peripheral blood of normal 1-group grey mullet are described below and these findings summarised in Table 2.

Thrombocytes. Thrombocytes (Fig 10a, 11a) represented 40% of the leucocyte population. These ovoid cells were frequently vacuolated and exhibited the presence of PAS positive cytoplasmic granules distributed either uniformly or with a degree of aggregation at the cell poles (Fig 10b, c). In the spiky form of this cell (Fig 10a) the cytoplasm was drawn out at one or both poles into tapering filaments up to 14.5μm in length.

Lymphocytes. Lymphocytes were the most abundant cell type representing 53% of the leucocyte population (Fig 10d, 11b). The majority of these had diameters of not more than 8.0μm and were morphologically similar to those described for other fish species, being characterised by large nuclei with a relatively thin layer of cytoplasm. These cells were often marginally indented. Larger lymphocytes 10.9μm in diameter with a greater ratio of cytoplasm to nucleus and plasma cells were also identified (Fig 11c).

Monocytes. These showed features typical of vertebrate monocytes (Fig 10e, 12a) with frequently vacuolated cytoplasm which exhibited weak PAS activity (Fig 10f). These cells constituted only 5% of the total leucocyte count.

Granulocytes. Only one type of granulocyte was observed at the light microscopical level and only occasionally, representing 2% of leucocyte types present. These cells, tentatively characterised as neutrophils, had an eccentric nucleus and large pale staining cytoplasm (Fig 10g) and did not display PAS or sudan black B activity although human granulocyte controls were positive. At the electron microscopical level this cell was shown to contain round or oval electron leucent granules (Fig 12b) and was designated a Type 1 granulocyte. Two further granulocyte types, observed in association with the localised tissue response to metacercarial infections, are described later.

Table 2 Leucocytes of *Chelon labrosus*

Leucocyte Type	Relative Count (%)	Size (μm)	PAS	Sudan Black B
Lymphocyte	53	8.0	−	−
Thrombocyte	40	11.0 x 8.7	+	−
Monocyte	5	11.3	+	−
Granulocyte	2	12.9	−	−

Figure 10 Peripheral blood leucocytes of *Chelon labrosus* I

(a) Thrombocytes in the ovoid (O) and spikey (S) forms. MGG (Mag= 1000).

(b) Thrombocyte exhibiting uniform distribution of PAS-positive cytoplasmic granules. PAS (Mag= 1000).

(c) Thrombocyte exhibiting PAS-positive granules aggregated at the cell poles. PAS (Mag= 1000).

(d) Lymphocyte (L). MGG (Mag= 1000).

(e) Monocyte (M). MGG (Mag= 1000).

(f) Monocyte (M). PAS (Mag= 1000).

(g) Type 1 granulocyte. MGG (Mag= 1000).
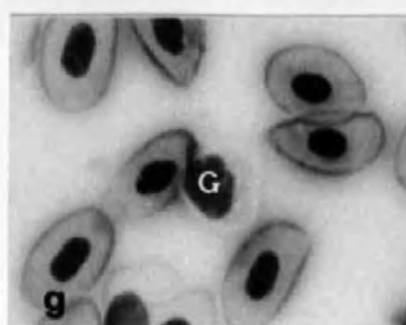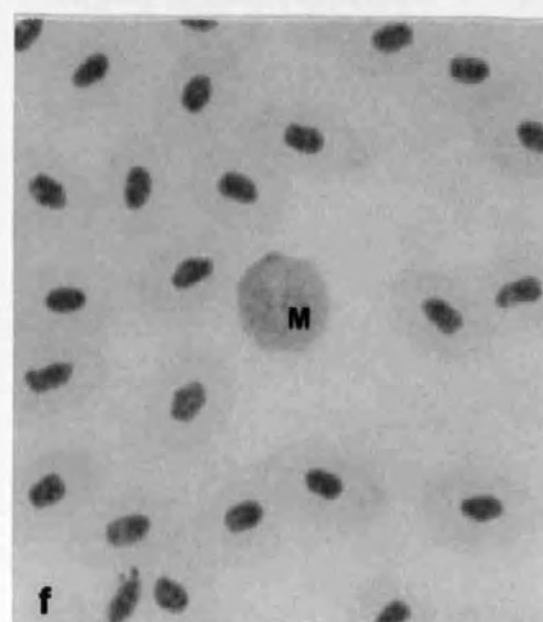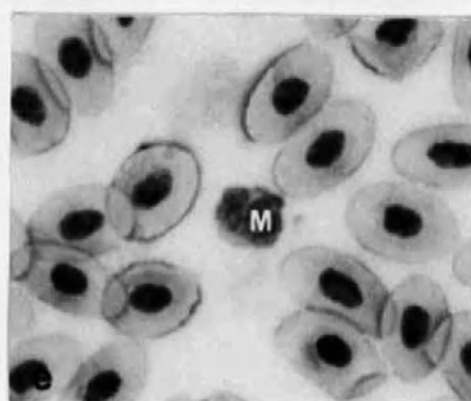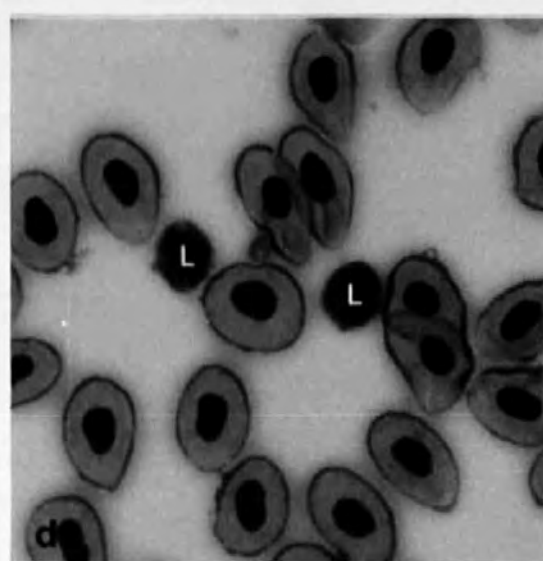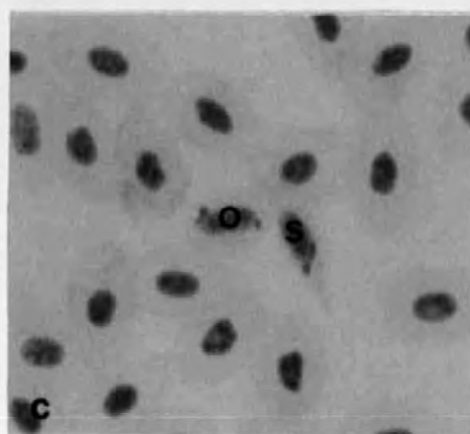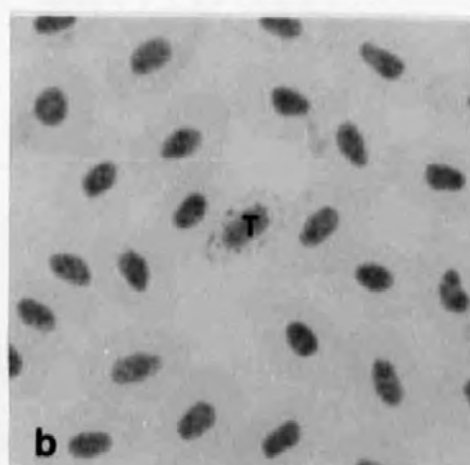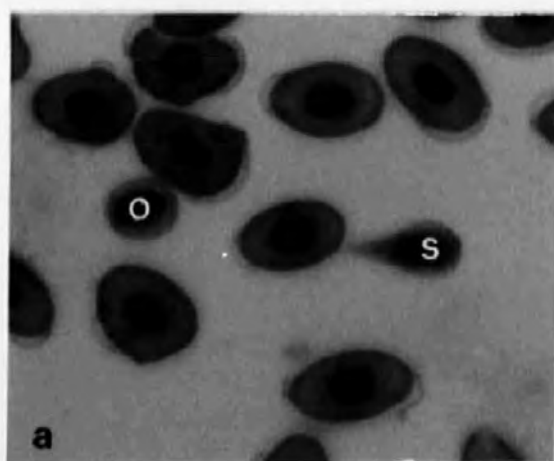
Figure 11 Peripheral blood leucocytes of *Chelon labrosus* II

(a) Thrombocyte (T) adjacent to an erythrocyte (E). (Mag= 12000).

(b) Lymphocyte (L) adjacent to an erythrocyte (E). (Mag= 20000).

(c) Plasma cell (P) surrounded by lymphocytes (L) and an erythrocyte (E).
     (Mag= 16000).
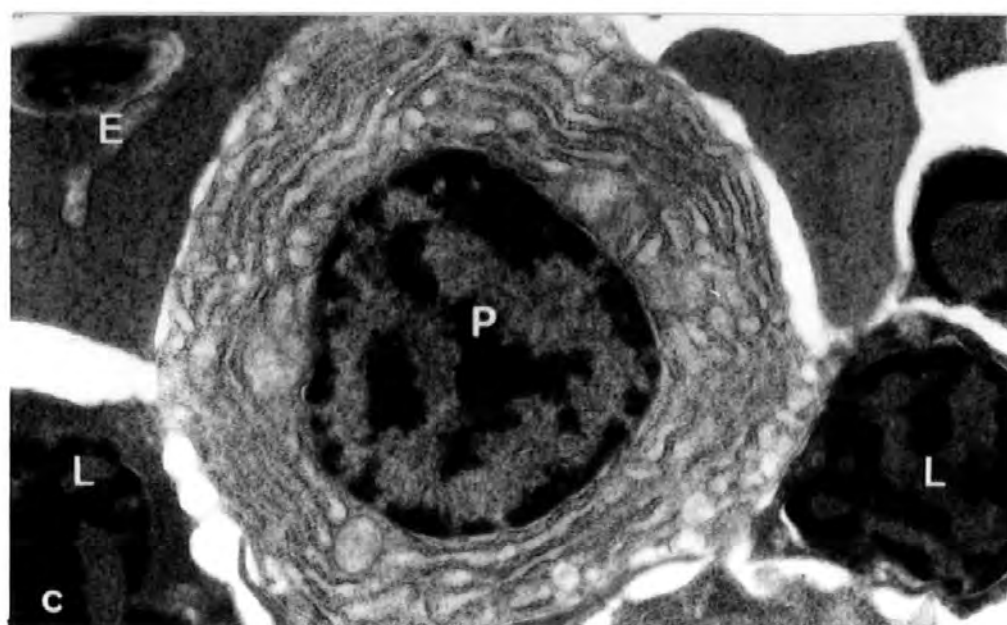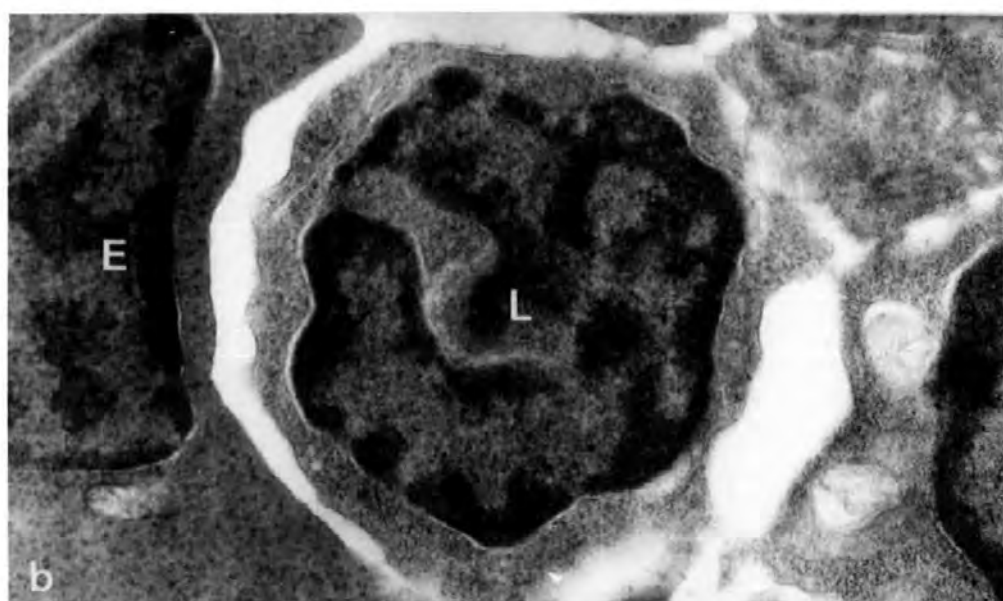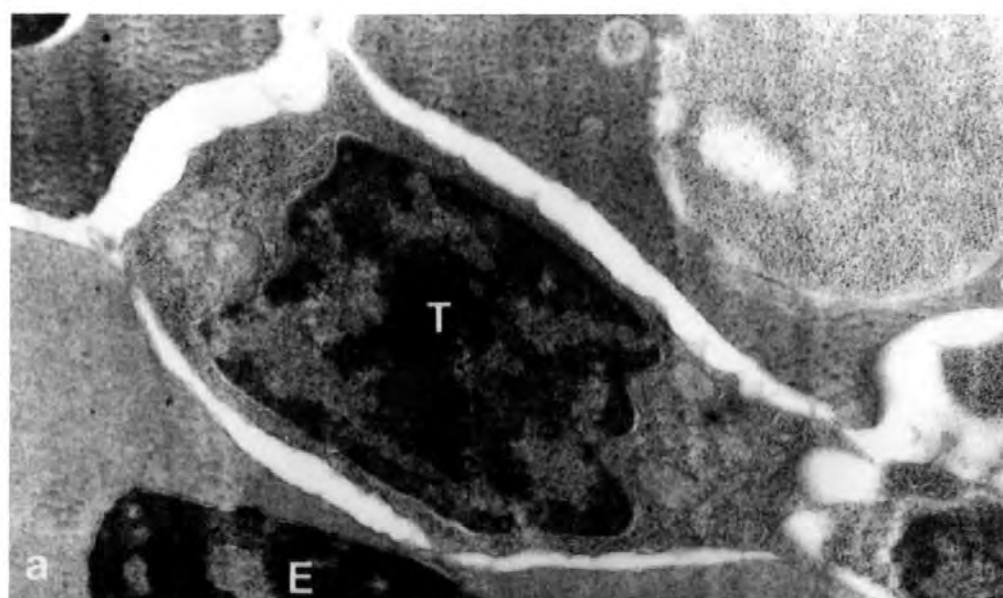
Figure 12 Peripheral blood leucocytes of *Chelon labrosus* III

(a) Monocyte (M). (Mag= 19000).

(b) Type 1 granulocyte (G). (Mag= 12500). Insert shows granule detail.
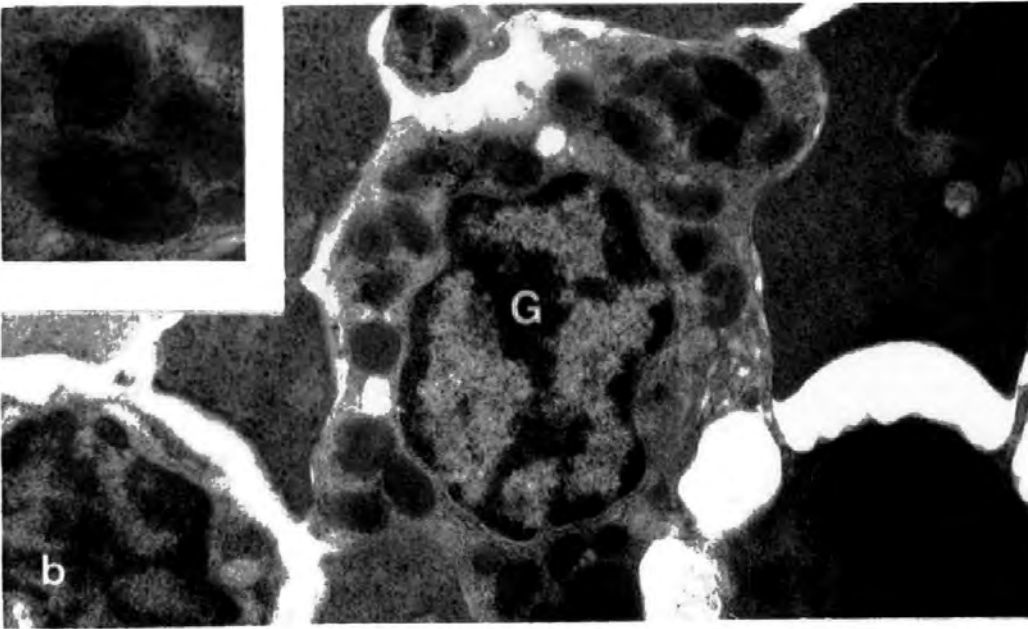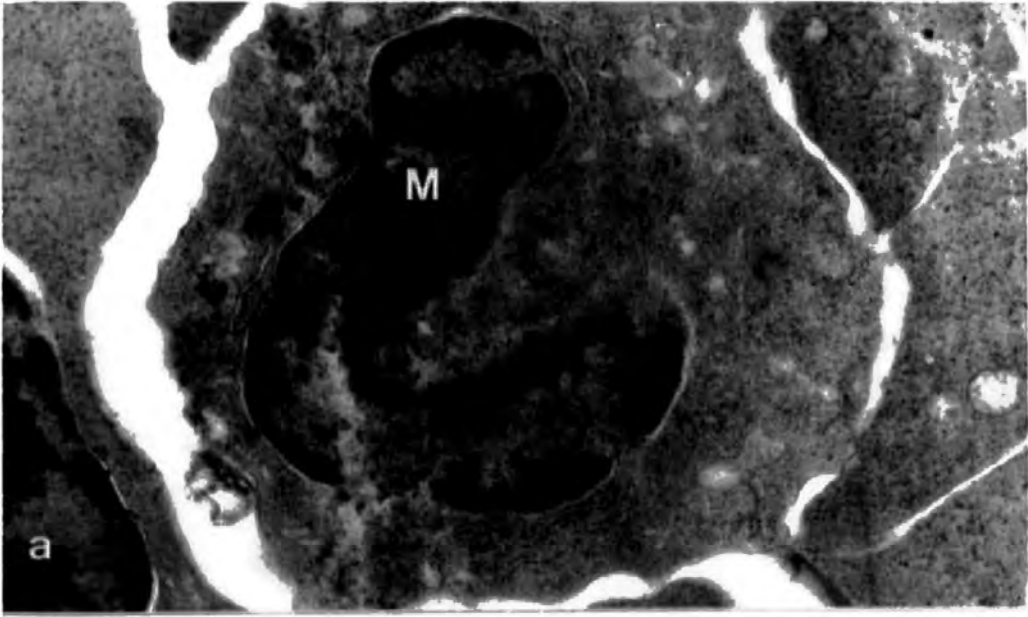
Lymphoid tissue

Lymphoid tissues of 1-group grey mullet were found to be similar to those described for other fish species and their essential features are described below.

Thymus. The bilobed thymus is located dorsal to the gill arches under the operculum (Fig 13a). It is permiated by a network of fibrous traberculae and encapsulated in a fibrous tissue sheath. As described in other fish, immature thymocytes were densely packed in the cortex with more mature cells in the medulla. Structures similar to the Hassall's corpuscles of mammals were evident in medullary tissue and cells containing eosinophillic inclusions were seen most frequently in the cortex (Fig 13b). Melanomacrophages and lymphocytes were also identified.

Spleen. Areas of lymphoid tissue (white pulp) were clearly differentiated from red pulp (mainly erythrocytic) the former occurring in nodules surrounding melanomacrophage centres and ellipsoid sheaths both of which were dispersed throughout the tissue (Fig 14a). Melanomacrophage centres were encapsulated in a thin epithelial layer in the spleen with a network of reticulin fibres providing support to the red pulp and allowing the passage of blood through the organ.

Kidney. The kidney was located ventral to the vertebral column being separated from the body cavity by the peritoneum. Haemopoietic and lymphoid components of the kidney were chiefly confined to the pronephros (Fig 14b) which extended approximately one third of the organ and contained each leucocyte type. The proportion of lymphoid tissue decreased in the mesonephros, with the increase in excretory tissue (Fig 15a) and the opisthonephros consisted largely of kidney tubules interdispersed with small accumulations of lymphoid cells in the intertubule spaces (Fig 15b).

Single melanomacrophages and melanomacrophage centres were present throughout the pronephros (Fig 14b) with smaller centres present in the intertubule spaces of the mesonephros (Fig 15a) but no such accumulations were evident in the opisthonephros. As in the spleen, lymphoid cells of the

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Figure 13 Thymus of *Chelon labrosus*

(a) Location of thymus adjacent to the gill cavity (GC). Cortex (C) and medulla (M) are clearly differentiated. G = gills. H&E. (Bar= 800μm).

(b) Thymus cortex (C) and medulla (M). E = eosinophillis cells. H&E. (Bar= 200μm).
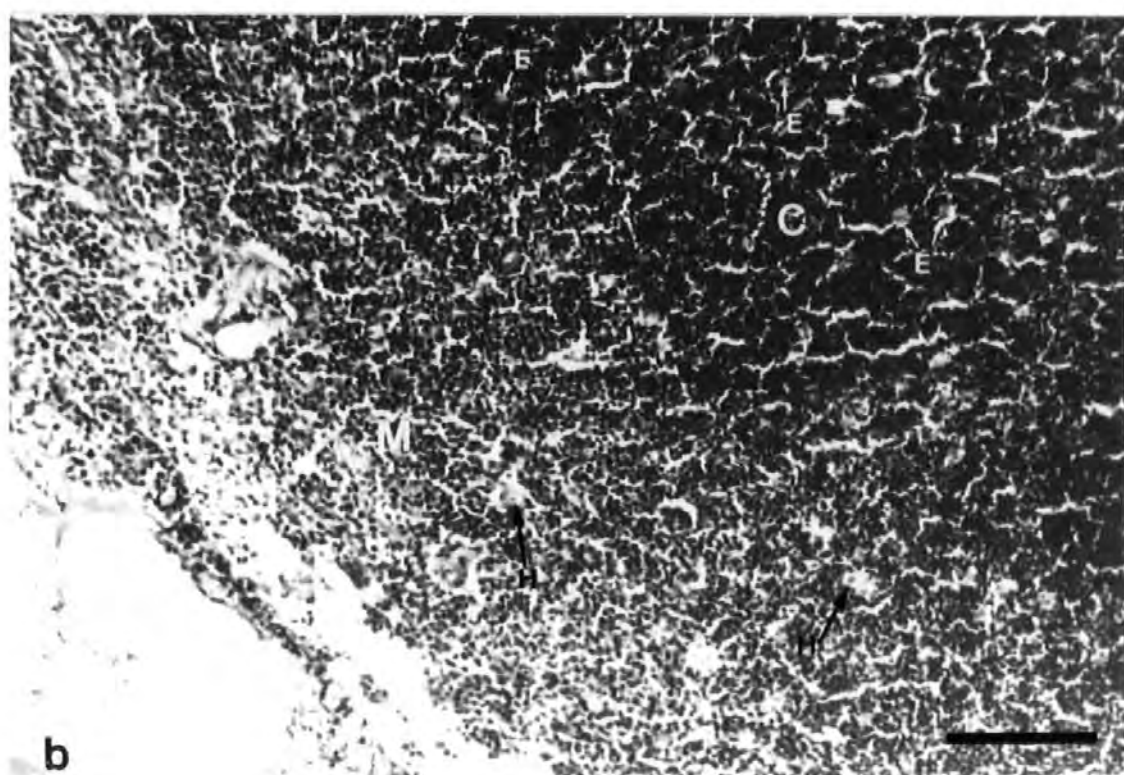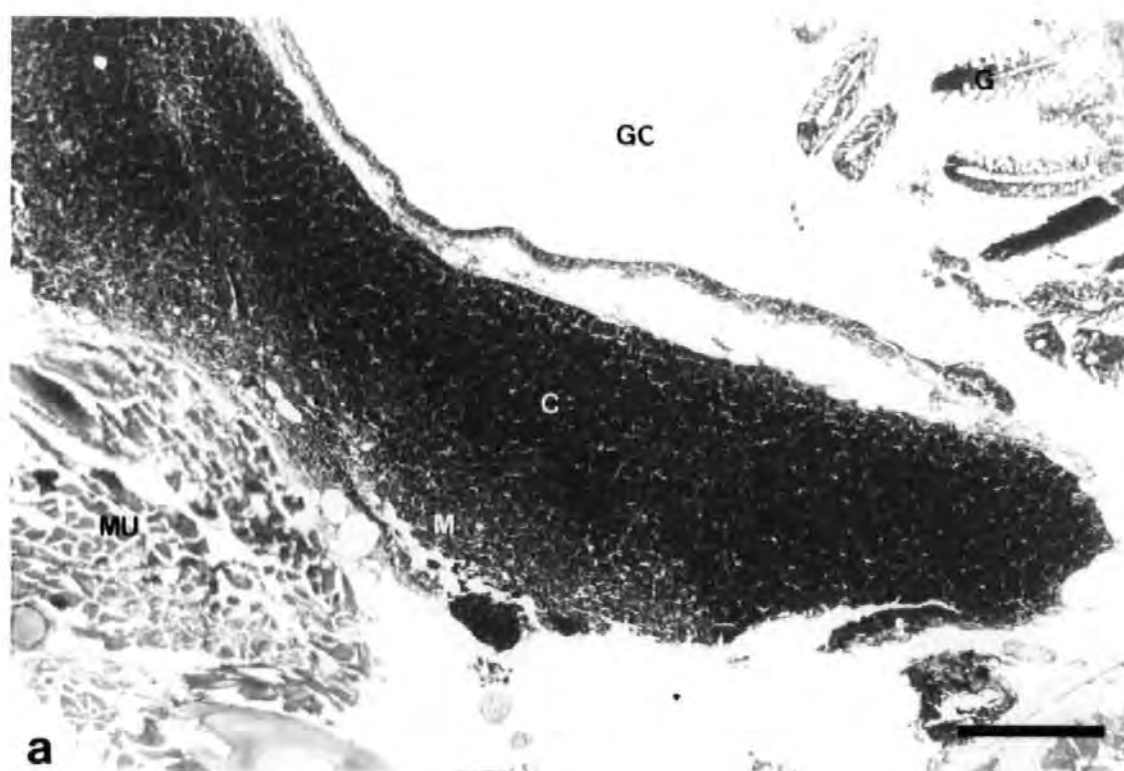
Figure 14 Spleen and pronephros of *Chelon labrosus*

(a) Spleen showing lymphoid tissue sheaths (L) surrounding blood sinuses (S) and melanomacrophage centres (M). E = ellipsoids. H&E. (Bar= 200μm).

(b) Pronephros showing dense accumulations of lymphoid tissue. S = blood sinus. H&E. (Bar= 200μm).
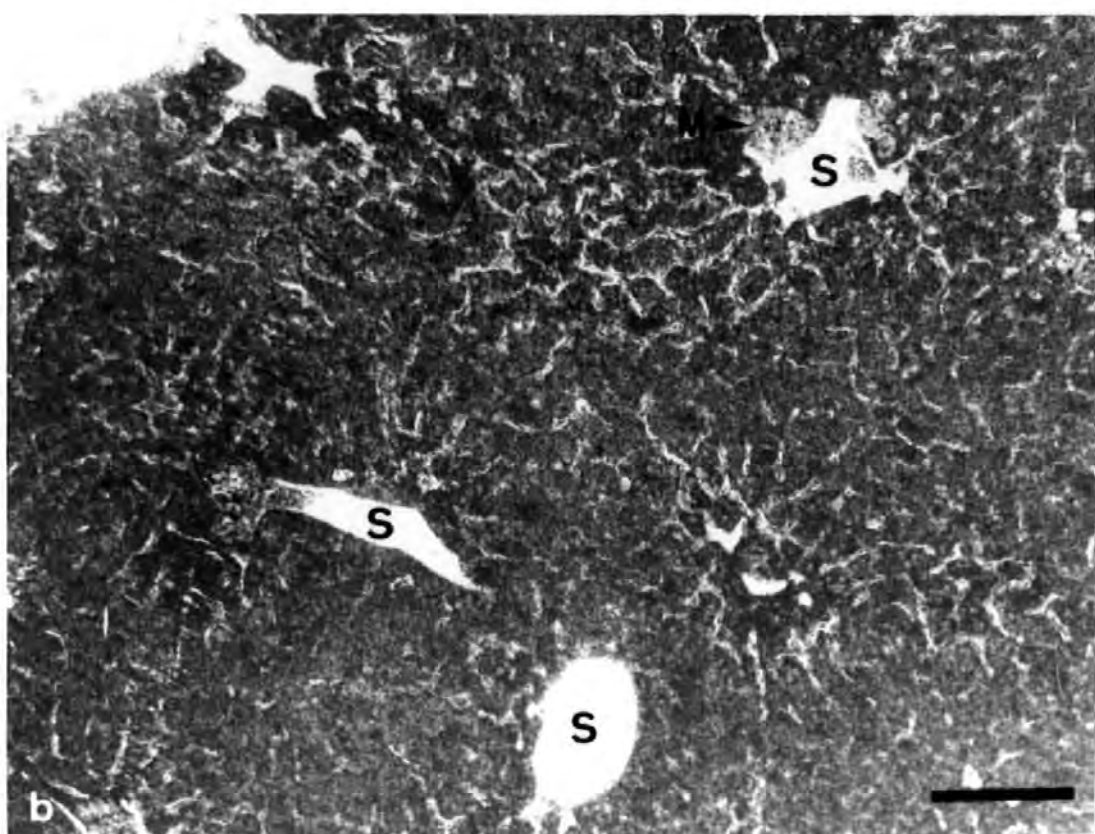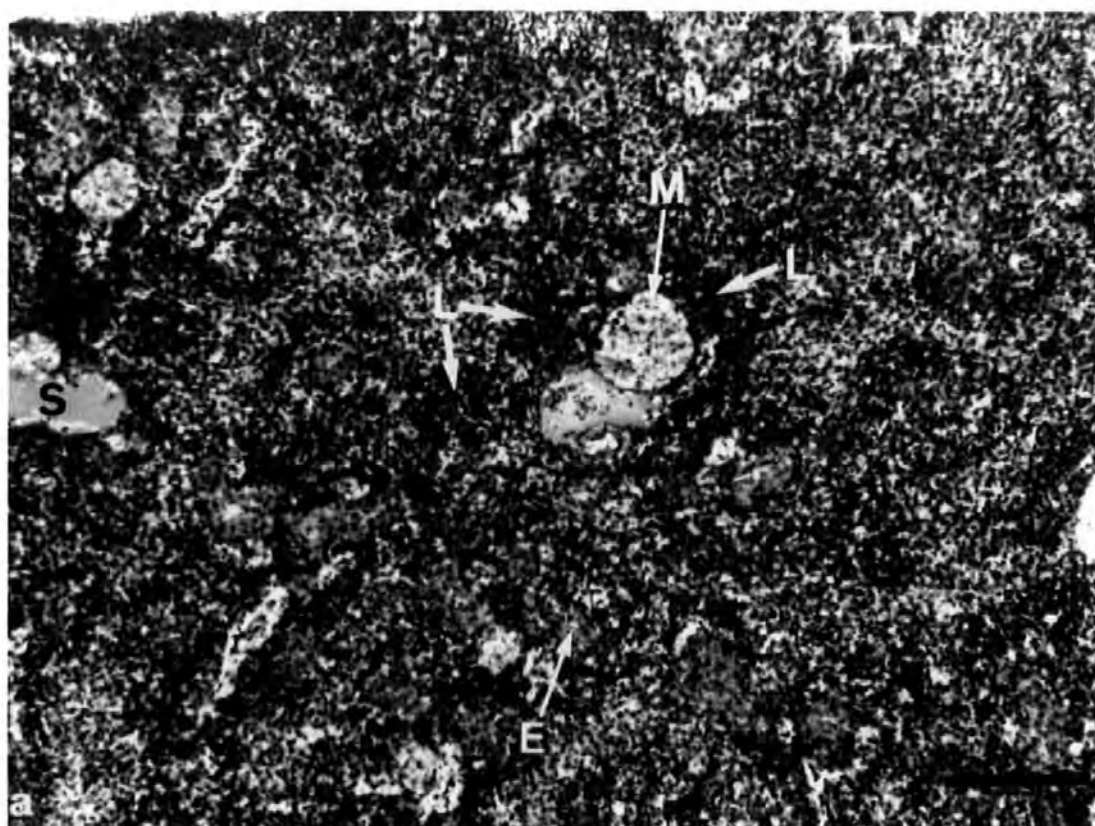
Figure 15 Meso- and opistho-nephros of *Chelon labrosus*

(a) Mesonephros showing lymphoid tissue accumulations (L) in inter-tubule spaces. H&E. (Bar= 100μm).

(b) Opisthonephros consisting almost entirely of kidney tubules. S = blood sinuses. H&E. (Bar= 200μm).
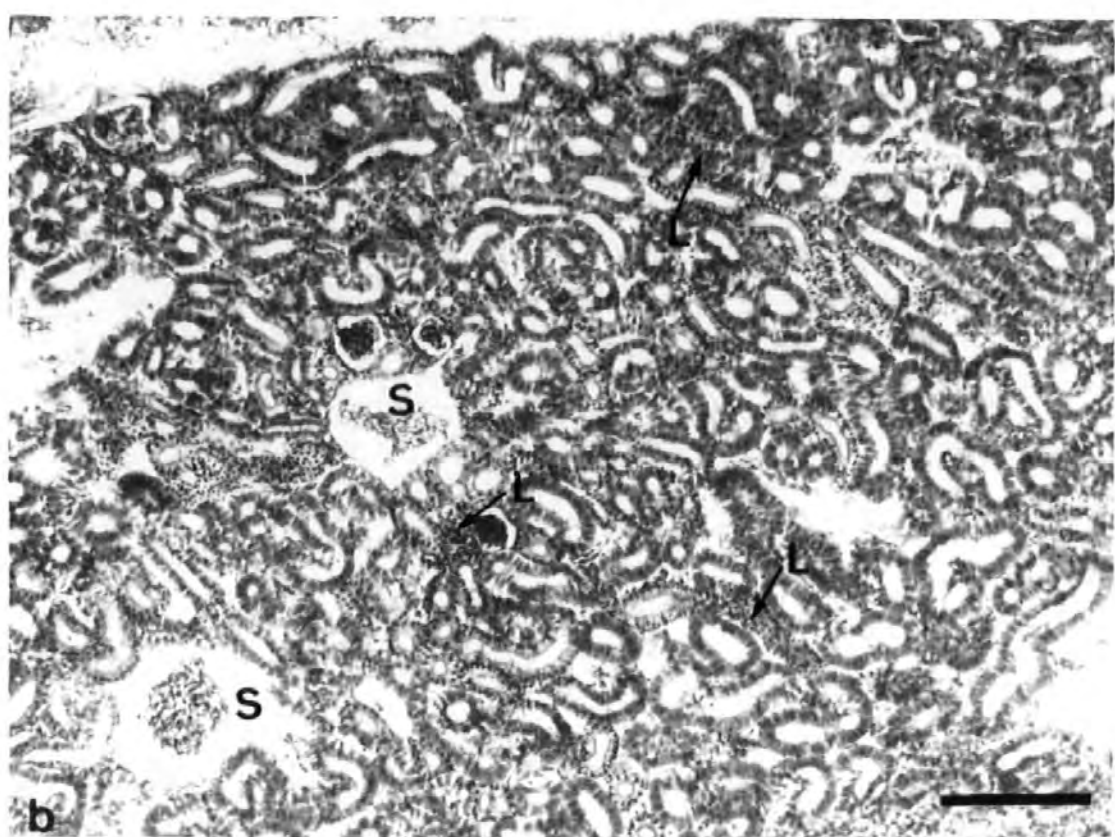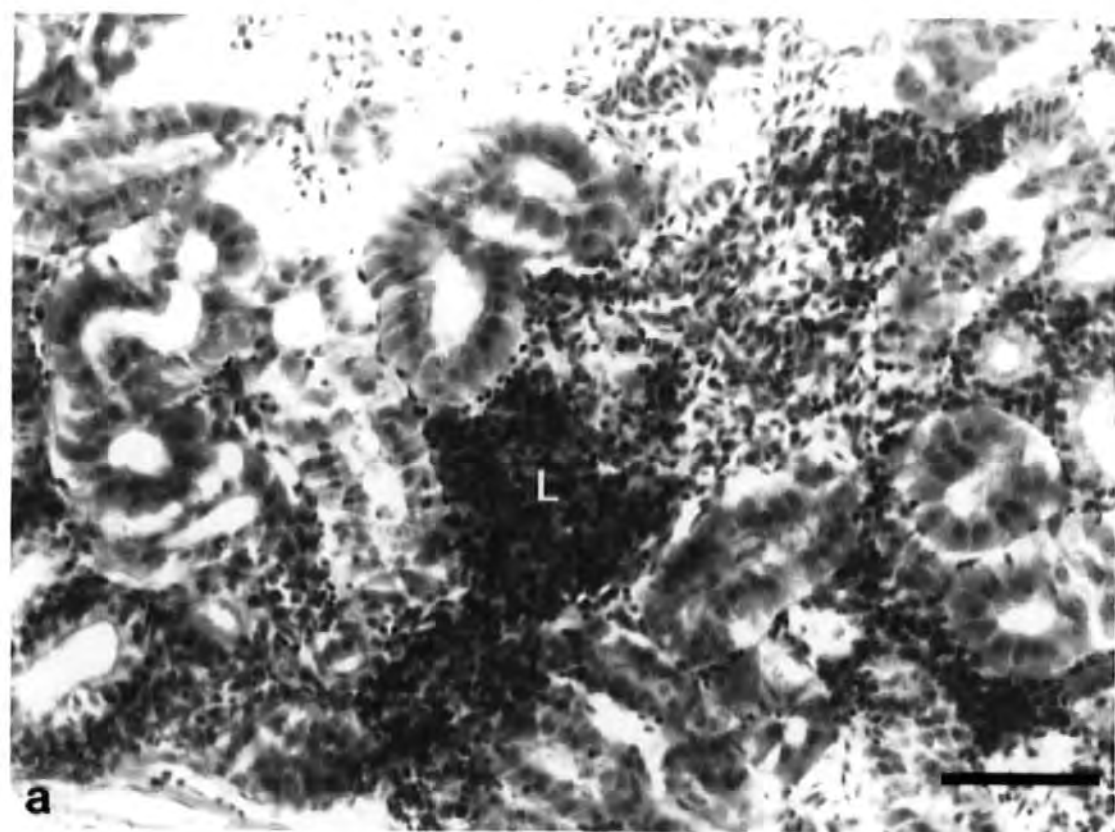
white pulp were associated with the melanomacrophage centres and with blood sinuses, forming a sheath around each (Fig 14b).

Liver. A few leucocytes, associated mainly with the sheaths of melanomacrophage centres were the only lymphoid cells detected in the liver.

Skin. Cells containing IgM were recorded in the epidermis of the skin with the aid of immunogold-silver staining (Fig 16a, b), although their identity remains uncertain. IgM was also detected in the mucus (Fig 16b).

Effect of antigen presentation on the immunogenicity of mullet immunoglobulin to rabbits and rats

Nine male dutch rabbits aged 6 months each weighing 2 - 3 kg and 13 male Wistar rats, aged 3 months each weighing 250 - 350 g were immunised with mullet IgM complexed using one of the following procedures:

(i) Rabbit RBC coated with mullet anti-rabbit RBC antibody

(ii) Sheep RBC coated with mullet anti-sheep RBC antibody

(iii) Mullet bile-rat anti-mullet whole serum immune complexes

(iv) HGG-mullet anti-HGG immune complexes

2 - 3 rabbits and 2 - 4 rats were used for each procedure with the exception that rabbits only were used in (iv). The effect of route of presentation was also investigated, introduction of antigen to rabbits being by subcutaneous injection either in each flank of the hind limbs or in multiple sites along the back; and to rats either by subcutaneous or intraperitoneal injection. Furthermore, the effect of Freund's adjuvant was assessed. The combinations of procedures used together with results are shown in Table 3.

Only the immunisation procedures including coated sheep RBC and HGG immune complexes were successful in raising antibody to mullet immunoglobulin and these only in the presence of Freund's adjuvant (Fig 17a). However, antibody raised using HGG immune complexes was not specific solely for mullet immunoglobulin with several unidentified precipitin lines being detected in immuno-electrophoresis (Fig 17b). None of the other antigens used

Figure 16 IgM-containing cells in the skin of *Chelon labrosus*

(a) IgM-containing cells (I) in the skin. Immunogold-silver/methylene blue. (Bar= 20μm).

(b) Small arrows indicate detection of IgM in skin mucous layer. I = IgM-containing cell. Immunogold silver/methylene blue. (Bar= 20μm).

a
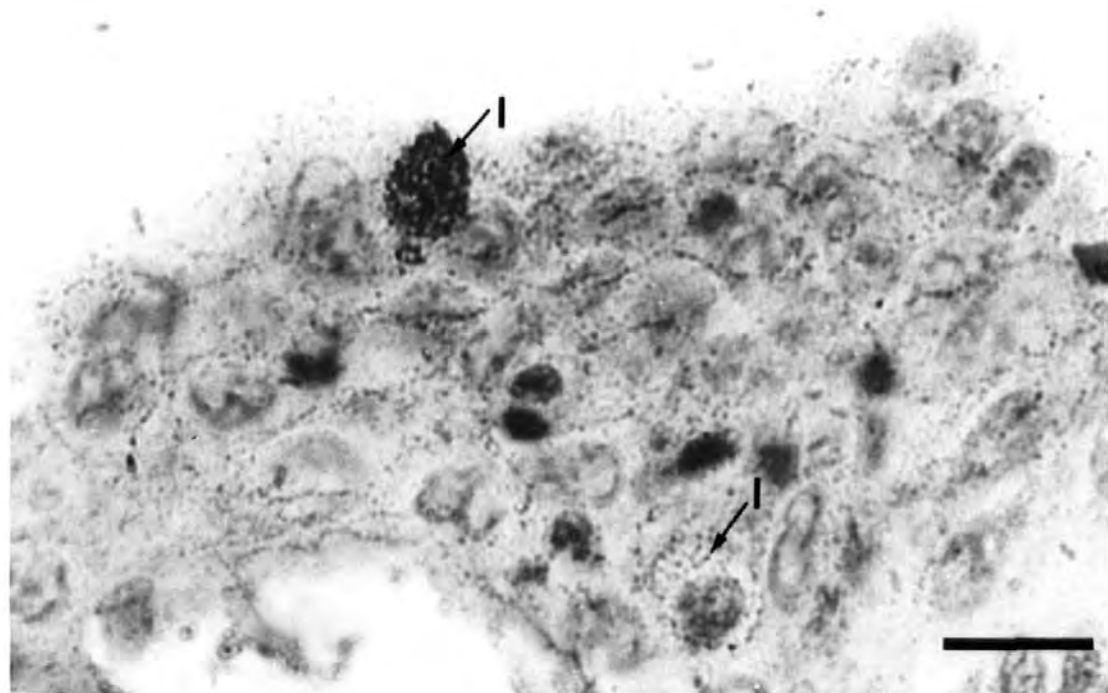
b
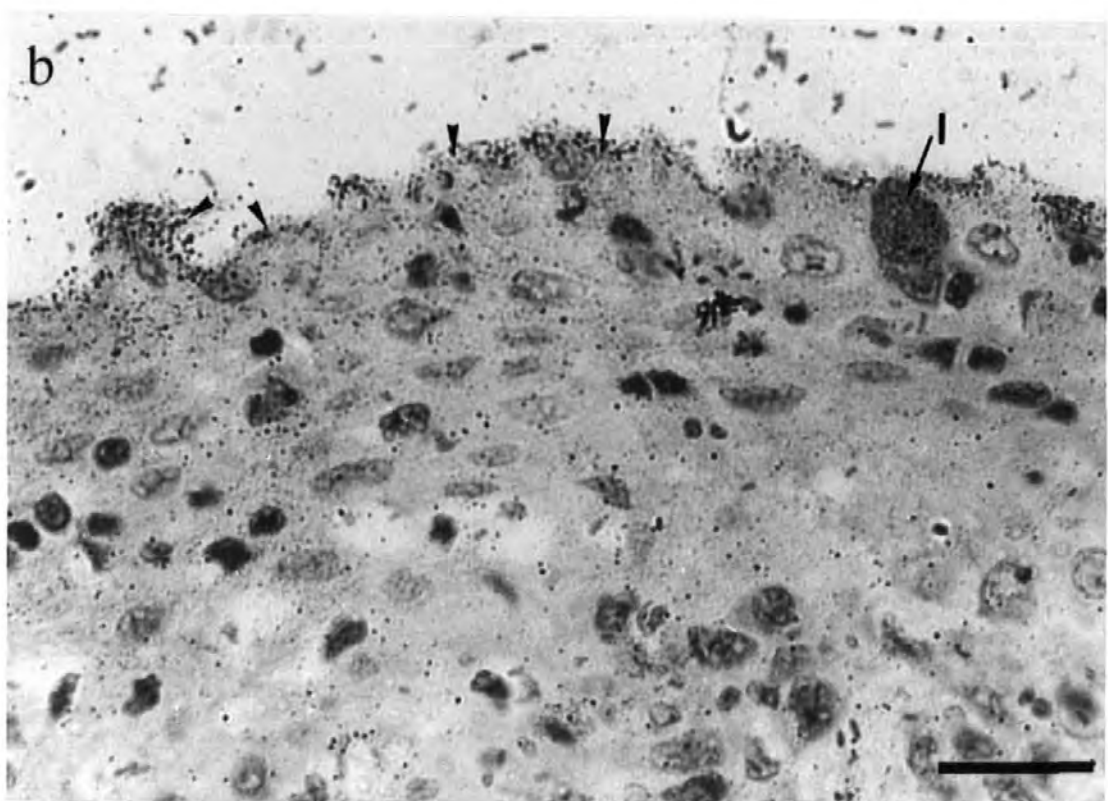
Table 3 Effect of antigen presentation on the immunogenicity of mullet
IgM

Immunogen	Animals	Route	Freund's Adjuvant	Administrations	Positive Response After (Weeks)	Specificity of Serum
Coated Rabbit RBC	Rabbit 1	SCF	−	5		
	2	SCF	−	5		
	3	SCMS	+	4		
	Rat 1	SCF	+	4		
	2	SCF	−	5	−	Negative
	3	IP	−	5		
	4	IP	−	5		
	5	IP	+	4		
	6	IP	+	4		
Coated Sheep RBC	Rabbit 1	SCMS	+	2	6	Single Precipitin Line
	2	SCMS	+	4	−	Negative
	Rat 1	SCF	+	4	−	
	2	SCF	+	3	9	Single Precipitin Line
	3	IP	+	2	6	
	4	IP	+	2	6	
HGG Immune Complexes	Rabbit 1	SCMS	+	2	4	Non−Specific Lines Also Present
	2	SCMS	+	2	4	Negative
Bile Immune Complexes	Rabbit 1	SCMS	+	2	−	
	2	SCMS	+	2	4	Single Precipitin Line (Not IgM)
	Rat 1	SCF	+	2	−	
	2	SCF	+	2	−	Negative
	3	SCF	+	2	−	

Figure 17 Rabbit anti-mullet IgM immuno-electrophoretograms

(a) Antisera raised using coated sheep erythrocytes. Note single precipitin line.

(b) Antisera raised using HGG immune complexes, displaying several unidentified precipitin lines.

(c) Antisera raised using mullet bile with a single precipitin line not corresponding to IgM.
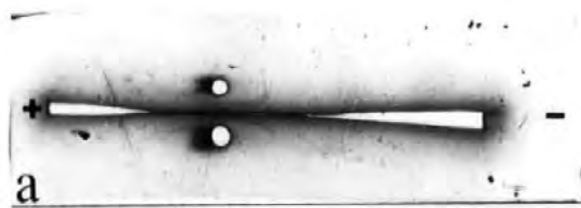
were successful in producing a humoral response within the 14 weeks of the experiment. Precipitin lines detected using immuno-electrophoresis with serum from rabbits injected with mullet bile & rat anti-mullet whole serum immune complexes did not appear in the beta/slow gamma region (Fig 17c) and therefore did not correspond to immunoglobulin M.

Section 2: Immune responses of grey mullet to *C. lingua*

Three investigations described below were undertaken to assess immunological responses of grey mullet to *C. lingua*. Parasite antigen was presented via one of 2 routes and in one of 3 different forms, namely, exposure to live cercariae or intraperitoneal injection with either whole killed or solublised cercariae.

Primary immune response to intraperitoneally injected whole killed cercariae

Seventy 0-group mullet, each weighing approximately 2 g, were divided into 2 equal groups and maintained at 20°C ± 2°C. Group A received 100 whole killed cercariae suspended in 40 μl YTS administered by intraperitoneal injection. The remaining fish (Group B) were injected with the same volume of YTS and served as controls. Samples of 5 fish from each group were killed and bled at weekly intervals between 3 and 9 weeks post injection. Serum from each sample was pooled and antibody titres against *C. lingua* assessed using passive haemagglutination.

Specific humoral antibody was detected against cercarial proteins in the immunised fish (Fig 18). This response increased steadily from day 1 and peaked at 7 weeks post injection with a $-\log_2$ titre of 10. The response then decreased slightly to give a $-\log_2$ titre of 8 at 8 weeks post injection but had increased again to $-\log_2$ 10 at the conclusion of the experiment at week 9. Fish from the control Group B, injected with YTS alone, gave $-\log_2$ titres of zero except at weeks 6, 7 and 8 when titres were 2, 2 and 1 respectively.

Figure 18 Primary antibody response following intraperitoneal injection
of whole killed cercariae

(●) Immunised fish.

(■) Control (unimmunised) fish.

Each point represents pooled antisera from 5 fish.
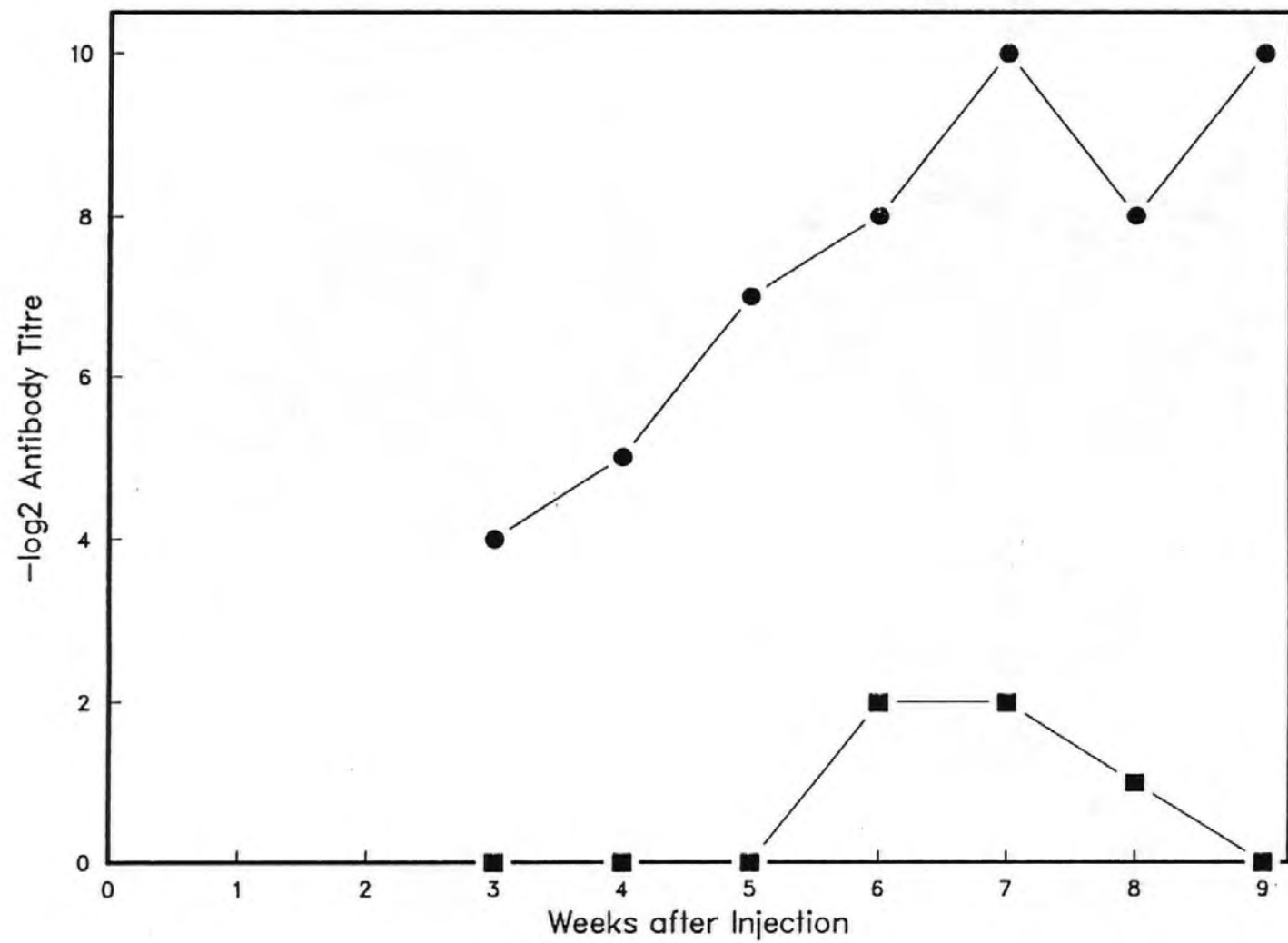
Primary immune response to metacercarial infection

Fifty 2-group mullet, each weighing 60 - 100 g, were maintained at 20°C ± 2°C. Of these, 40 were each exposed to a single infection of 20,000 cercariae of *C. lingua*. The remaining 10 fish were left untreated and served as controls.

4 infected fish together with a single control were killed at weekly intervals up to the conclusion of the experiment at week 10. Serum was prepared from individual fish and the pronephros removed for cellular assays. Spleen and mesonephros were also removed for histological and histochemical investigations including the application of immunogold techniques for detection of parasite antigens.

Humoral antibody levels were assessed by passive haemagglutination and direct cercarial agglutination using both live and killed cercariae. Complement activity was also assessed together with examination of cellular polarisation, migration and adherence in response to parasite antigen *in vitro*.

Mullet exposed to infection levels of 20,000 cercariae were shown to produce humoral antibody against cercarial proteins when tested by passive haemagglutination (Fig 19), this response peaking 4 weeks post infection with a mean -$\log_2$ titre of 16.0 ± SE 1.0. Uninfected controls gave a negative response. Direct agglutination tests using live cercariae (Table 4) suggest a significant difference in antibody titres between uninfected controls (-$\log_2$ 17.0 ± SE 1.3) and experimental fish (maximum titre -$\log_2$ 21.5 ± SE 0.7) up until week 4 when titres of the latter were no longer significantly higher than controls. When direct agglutination tests were performed using cercariae killed by freezing at -20°C (Table 5) no significant differences occurred between titres of experimental fish and uninfected controls ($p <= 0.01$).

Cercariacidal activity was detected by *in vitro* tests using antibody at a 1:200 dilution collected at week 4. Tegumental damage was recorded with disruption of the outer surface layers within 6 - 12 hours of exposure to immune serum (Fig 20a). Encysted metacercariae were unaffected (Fig 20b). Serum from uninfected controls was also found to be cercariacidal but only at dilutions down to 1:4. The number of cercariae successfully encysting *in vitro* was reduced in the presence of immune serum, falling from 25% to 13%.

Figure 19 Primary antibody response detected using passive haemagglutination following exposure to live cercarial infection

Vertical bars represent standard error of the mean (n=4).
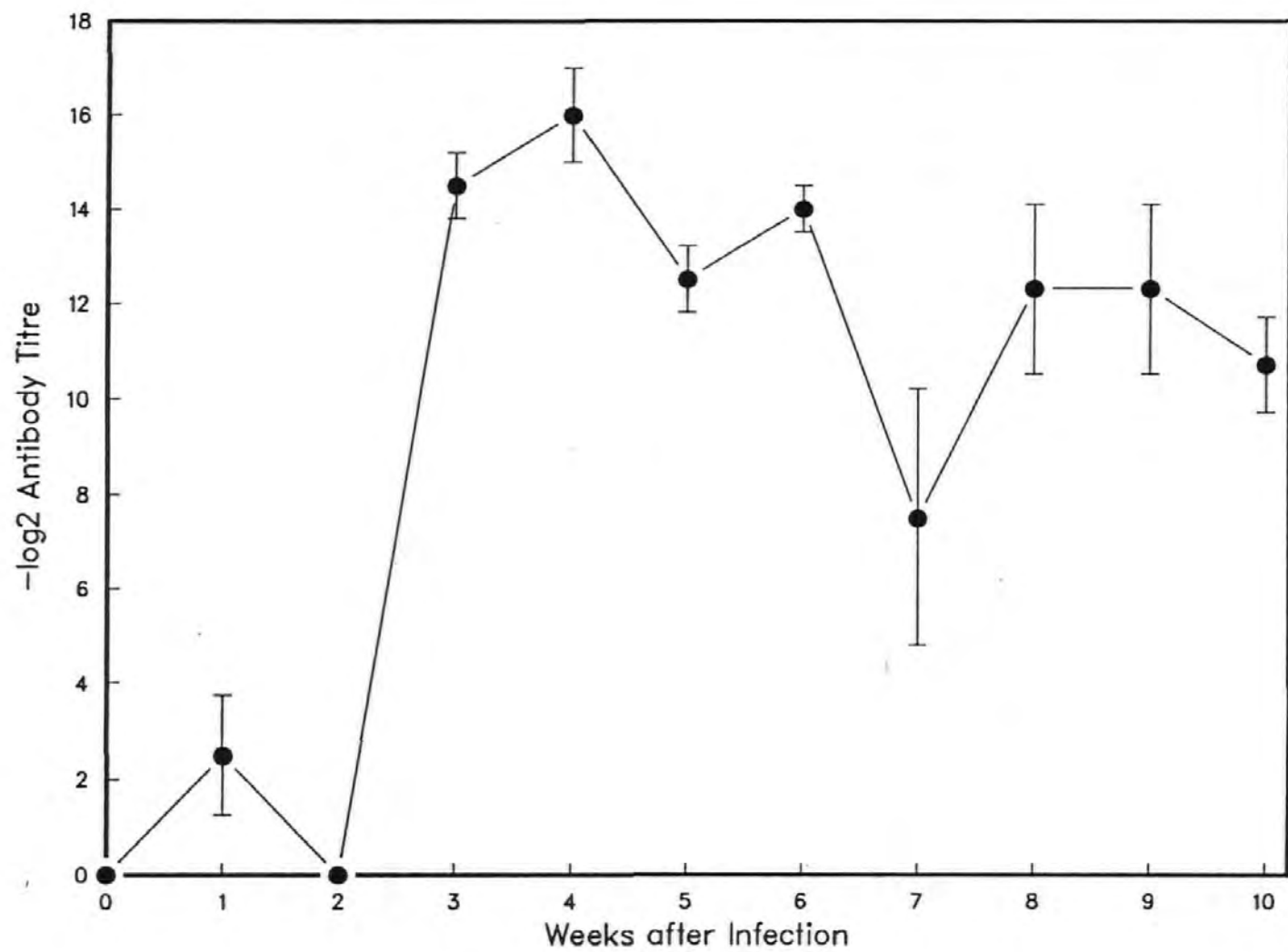
Table 4 Antibody levels detected by agglutination of live cercariae following exposure to live cercarial infection

Weeks after Infection	Sample Size	Antibody Titre
CONTROL	10	17.0 ± 1.3
1	4	21.0 ± 1.7
2	4	21.5 ± 0.7
3	4	21.0 ± 1.7
4	4	20.7 ± 1.6
5	4	15.7 ± 1.6
6	4	19.0 ± 0.0
7	4	18.0 ± 0.0
8	4	17.3 ± 1.6
9	4	16.5 ± 1.2
10	4	11.0 ± 1.5

Figures represent mean -$\log_2$ titres ± standard error.

Table 5 Antibody levels detected by agglutination of dead cercariae
following exposure to live cercarial infection

Weeks after Infection	Sample Size	Antibody Titre
CONTROL	10	19.4 $\pm$ 1.2
1	4	22.0 $\pm$ 1.4
2	4	21.5 $\pm$ 0.7
3	4	19.0 $\pm$ 1.0
4	4	19.7 $\pm$ 1.1
5	4	19.3 $\pm$ 1.0
6	4	17.5 $\pm$ 0.7
7	4	19.0 $\pm$ 0.0
8	4	20.0 $\pm$ 1.8
9	4	20.3 $\pm$ 1.6
10	4	18.3 $\pm$ 1.3

Figures represent mean -$\log_2$ titres $\pm$ standard error.

Figure 20 Tegumental damage sustained in immune serum *in vitro*

(a) Unencysted metacercaria incubated in immune serum in culture medium, showing extensive tegumental damage. (Bar= 20μm).

(b) Encysted metacercaria incubated in immune serum in culture medium, apparently undamaged. (Bar= 20μm).
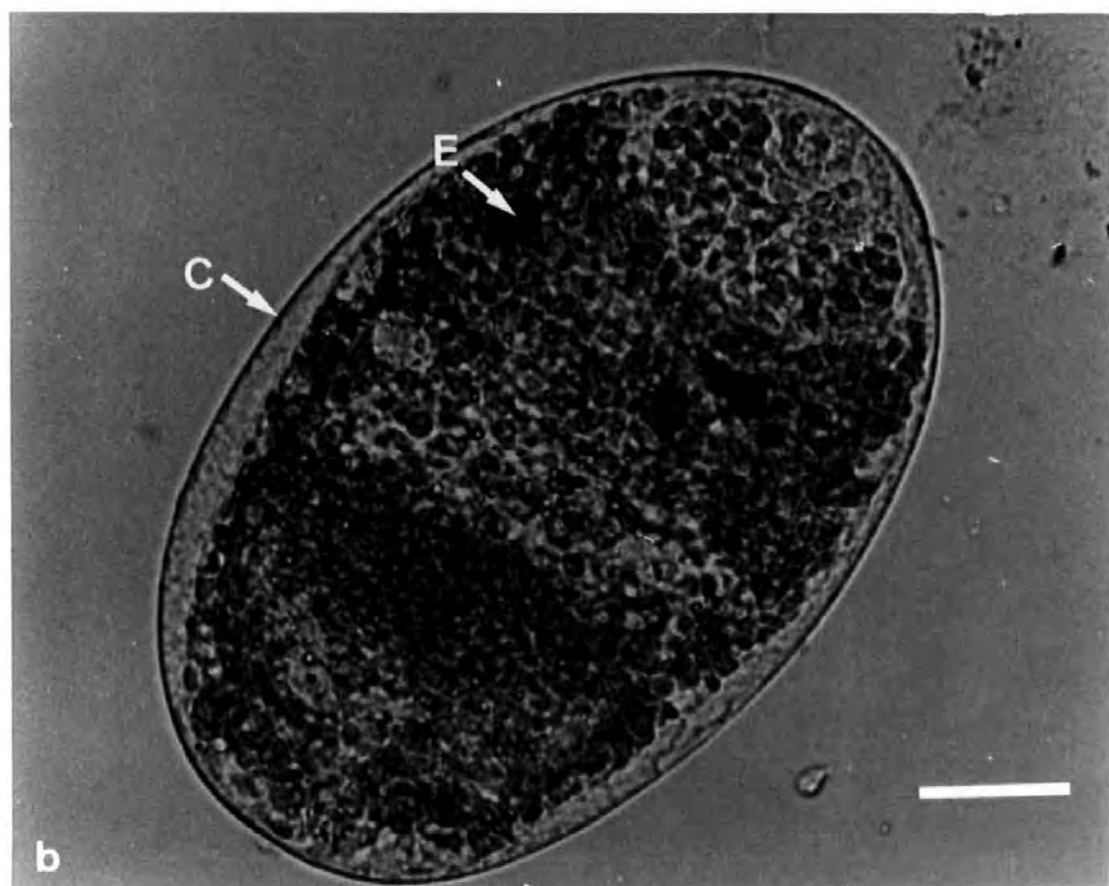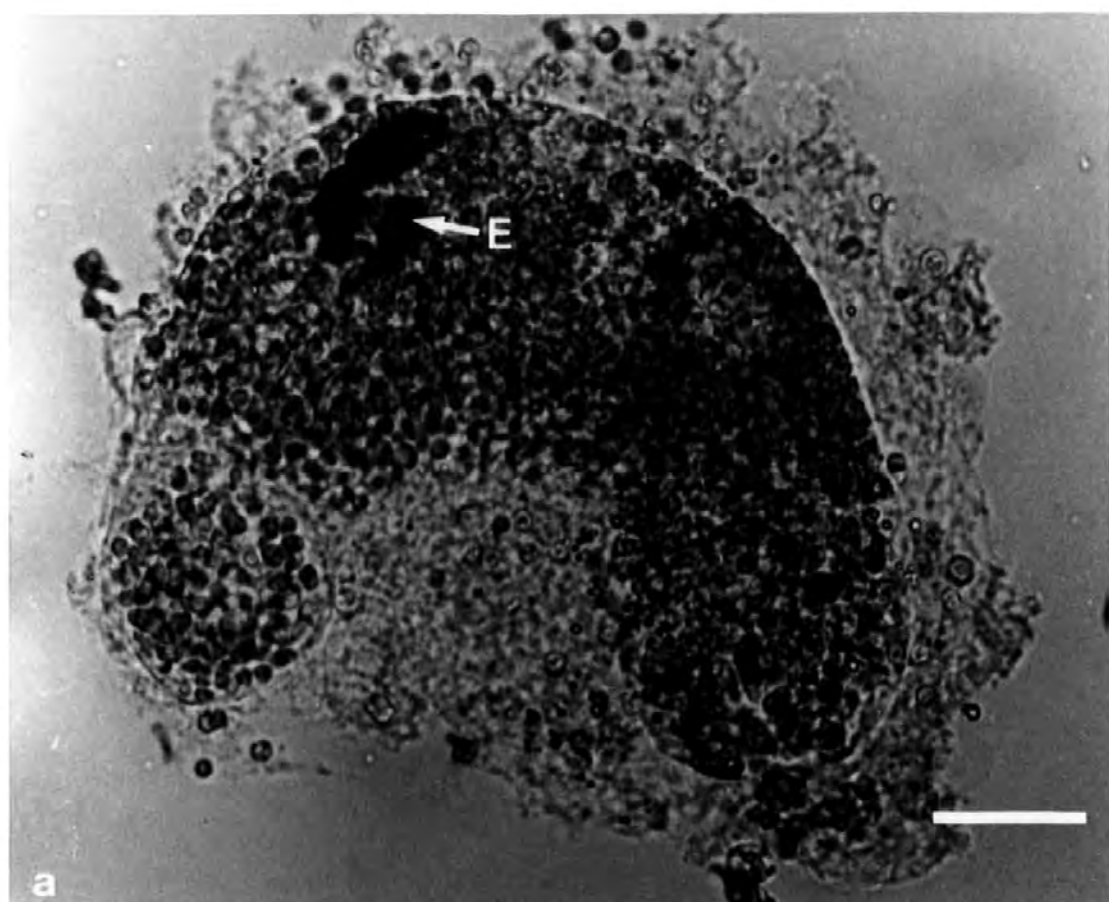
Results of the complement fixation test using immune and control sera are shown in Figure 21. The percentage survival of cercariae in all inactivated test sera, where complement was subsequently added, decreased with a decrease in concentration of the initial (heat inactivated) serum, no difference being recorded between the three regimes investigated. When incubated in inactivated serum alone (without the addition of complement) percentage survival increased to 100% in $-\log_2$ dilutions higher than 4.

The ability of pronephric leucocytes to migrate towards a cercarial antigen source, expressed as chemotactic differential (Fig 22) was significantly higher ($p<=0.001$) in immunised fish throughout the experiment and maximal at 2 weeks. The response gradually decreased to zero by week 7. Leucocyte suspensions from both experimental and control fish, incubated with cercarial antigen, consistantly showed an increased proportion of polarised cells when compared with those incubated in culture medium alone (polarisation index) as shown in Table 6. The polarisation index was not however, significantly higher ($p<=0.01$) in suspensions from experimental fish and showed no obvious trend during the course of the experiment. Adherence of cells to cercariae or metacercariae *in vitro* was not observed at any time under the conditions investigated here.

No histological changes in lymphoid tissues from infected fish were observed during the course of the experiment. However, immunocytochemical staining of kidney and spleen using rabbit anti-*C. lingua* antiserum demonstrated the presence of *C. lingua* antigens in infected fish. In the case of spleen, antigen was dispersed in the tissue throughout the 10 week period of the experiment and the staining intensity was relatively weak suggesting low levels of antigen. In the pronephros however, parasitic material was detected in reticulo-endothelial cells lining blood vessels at 2 and 5 weeks post infection and at 10 weeks was also present in melanomacrophage centres.

(o) Inactivated control serum only.

(●) Inactivated control serum followed by complement from control serum.

(■) Inactivated control serum followed by complement from immune serum.

(▲) Inactivated immune serum followed by complement from immune serum.

Each point represents % cercarial survival from 100 observations.
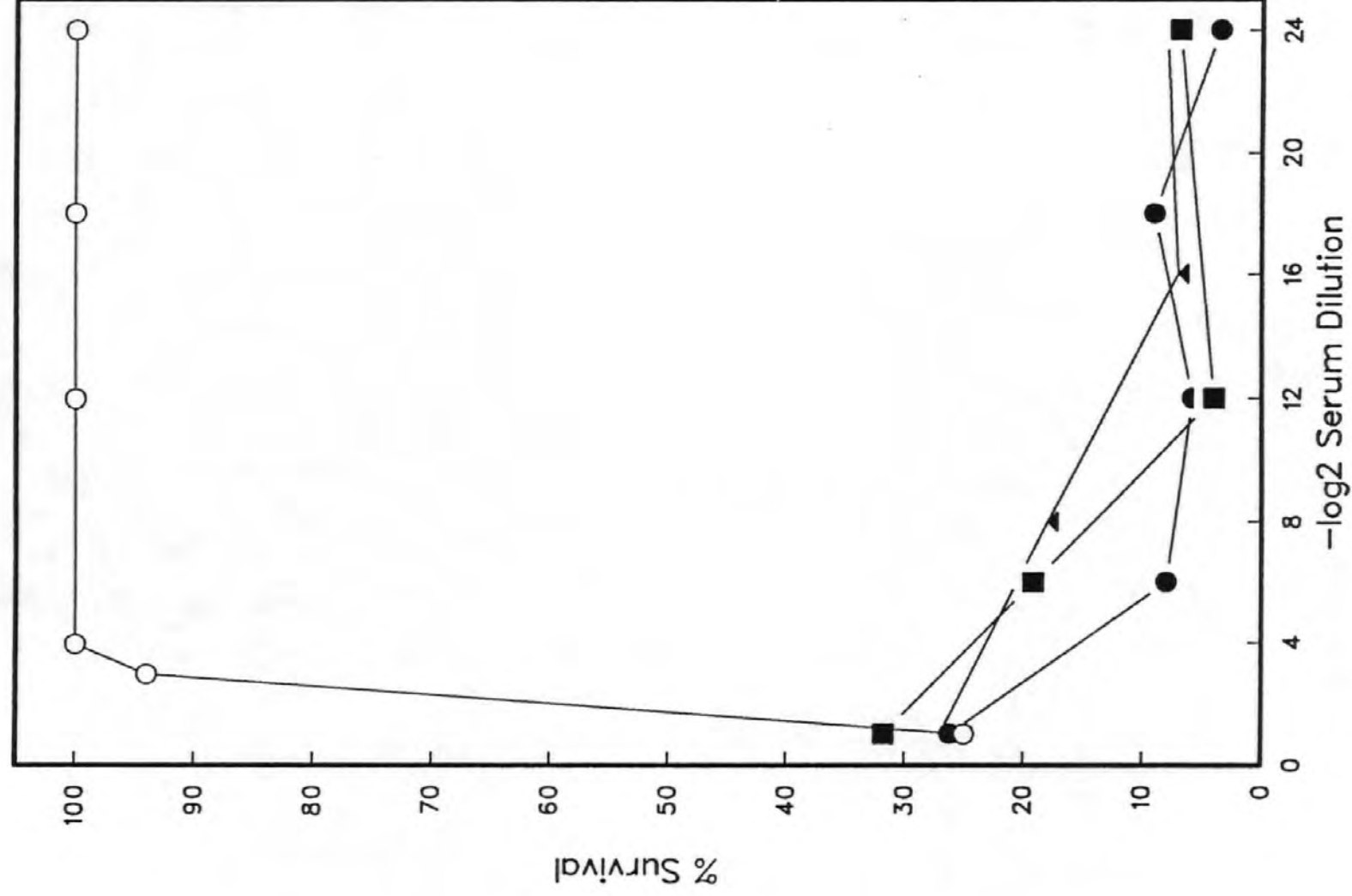
Figure 22 Chemotaxis of pronephric leucocytes following immunisation by
live cercarial infection

Vertical bars represent standard error of the mean (n=4).
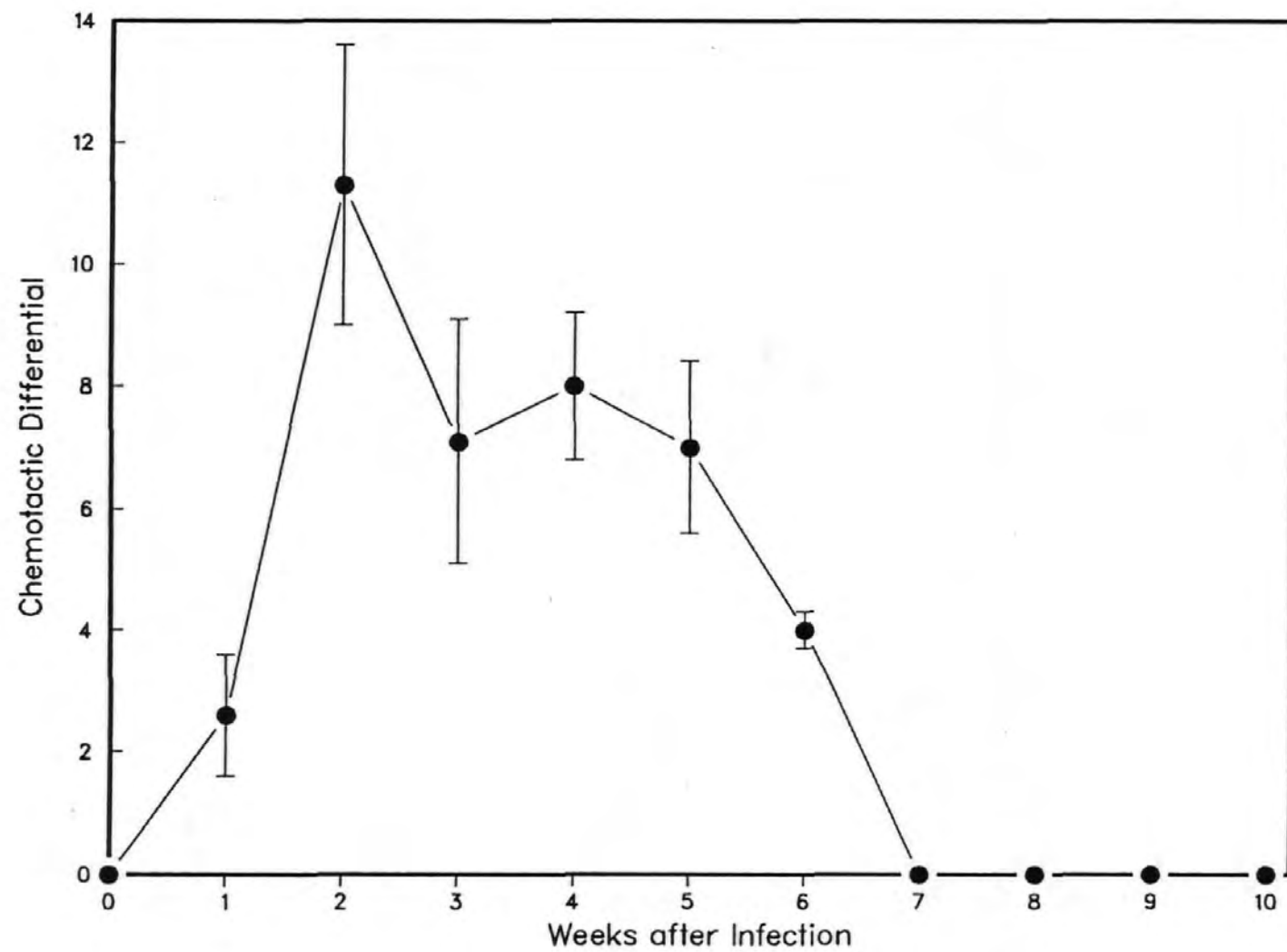
Table 6 *In vitro* polarisation of pronephric leucocytes from fish
immunised by live cercarial infection

Weeks after Infection	Sample Size	Polarisation Index
CONTROL	10	1.30 ± 0.40
1	4	1.06 ± 0.22
2	4	1.43 ± 0.85
3	4	1.09 ± 0.07
4	4	1.23 ± 0.12
5	4	1.42 ± 0.27
6	4	1.11 ± 0.09
7	4	1.45 ± 0.56
8	–	NOT DONE
9	4	1.61 ± 0.14
10	4	1.36 ± 0.08

Figures represent the mean polarisation index ± standard error.

Primary and secondary immune response to intraperitoneally injected sonicated cercariae

235 2-group fish, each weighing 60 - 100 g, were maintained at $20^{\circ}C \pm 2^{\circ}C$ and divided into three Groups A, B and C consisting of 115, 70 and 50 fish respectively. Fish in Groups A and B were injected intraperitoneally with 0.2 cm^3 sonicated cercariae in YTS containing the equivalent of 5×10^4 cercariae cm^{-3} (10^4 cercariae fish^{-1}) at the commencement of the experiment (week 0). At week 8, 70 fish from Group A were given a second injection of 0.2 cm^3 YTS alone. At the same time Group B received a second injection of antigen at the same dose as the first. Control Group C each received a single injection of 0.2 cm^3 YTS alone at week 0 of the experiment.

Six fish from each of Groups A and B and 2 fish from Group C were killed at weekly intervals up to the conclusion of the experiment at week 18. Fish from Groups A and C were first sampled at week 1 and those from Group B at week 9. Serum was collected from individual fish and tested for antibody against cercarial proteins using passive haemagglutination and for cercariacidal activity using the complement fixation test. Leucocyte suspensions were made from the pronephros and used in cellular assays including *in vitro* adherence to parasites, under-agarose migration and *in vitro* polarisation. Spleen, pronephros and mesonephros were also taken for histological and immunocytochemical investigation.

Results showed that mullet injected with sonicated cercariae responded by producing humoral antibody against cercarial proteins (Fig 23). Furthermore, an enhanced response occurred when fish were given a challenge injection at week 8. The response after the initial injection (primary response) increased steadily to a peak mean -$\log_2$ titre of 12.5 $\pm$ SE 1.6 at week 5 and then decreased to give a mean -$\log_2$ titre of 5.0 $\pm$ SE 2.2 at the conclusion of the experiment at week 18. Elevated antibody titres were obtained from fish which had received a challenge injection at week 8, these titres increasing to 14.5 $\pm$ SE 0.7 at week 10 and then further to 18.5 $\pm$ SE 0.7 at week 15 before declining to 7.5 $\pm$ SE 0.7 at week 18. Controls injected with YTS alone gave a mean -$\log_2$ antibody titre of 3.0 $\pm$ SE 1.7, however, titres of zero were recorded for most individuals.

Figure 23 Primary and secondary antibody response following intraperitoneal injection of sonicated cercariae

(■) Response following one administration of antigen at Week 0.

(●) Response following an additional antigen administration at Week 8.

Vertical bars represent standard error of the mean (n=6).
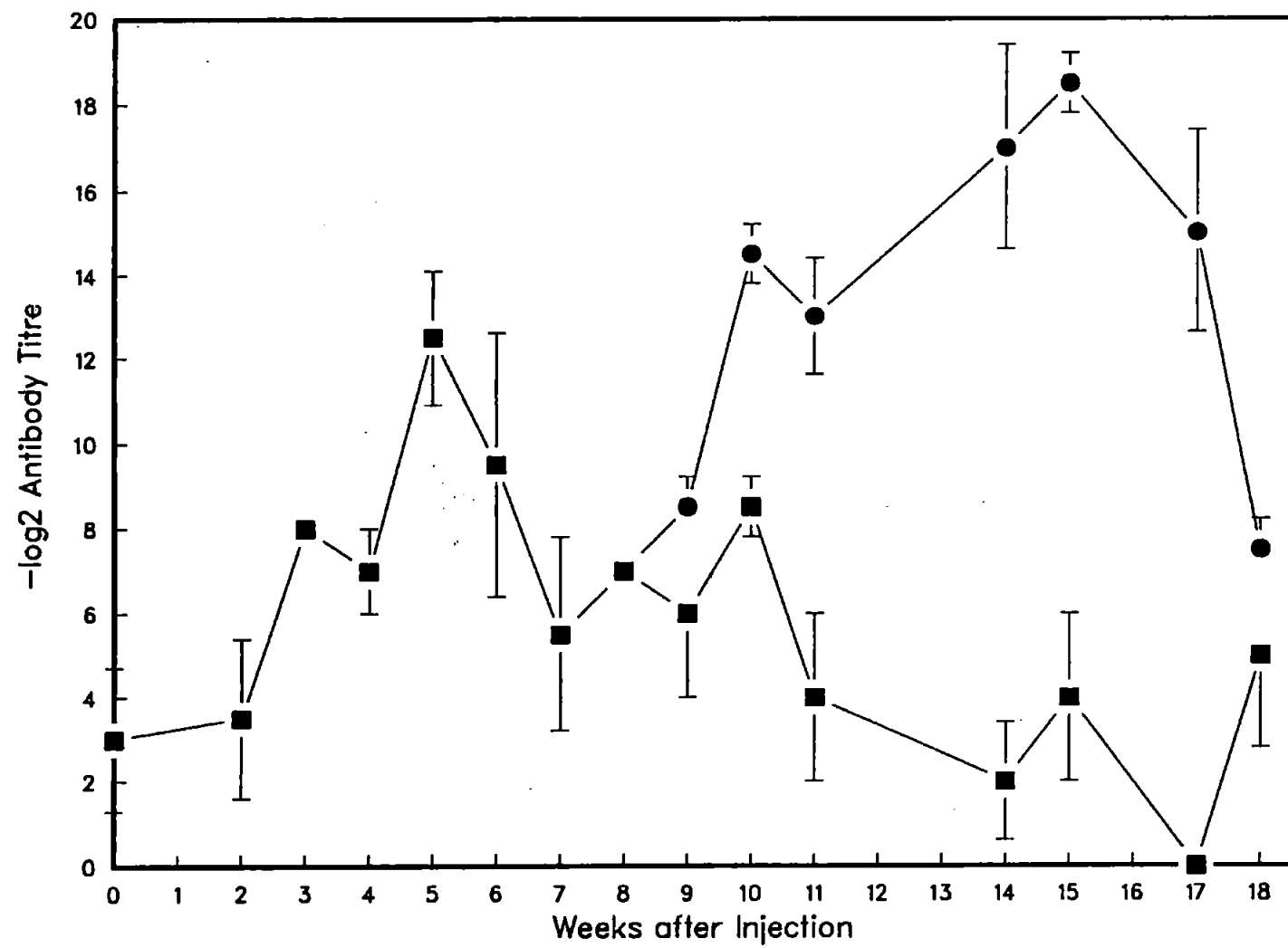
The complement fixation test using immune and control sera showed that the percentage survival of cercariae in inactivated test sera, where complement was subsequently added, decreased with a decrease in concentration of the initial inactivated serum (Fig 24). When incubated in heat-inactivated control serum alone, the percentage survival increased to 100% in $-log_2$ dilutions higher than 6.

Results of the under-agarose migration assay (Table 7) indicate that the chemotactic differential ranged between zero and 1.05 in experimental fish but was zero in all control fish. There was however, no apparent trend in this response in experimental fish during the course of the investigation.

The polarisation index of pronephric leucocytes from experimental and control fish are shown in Table 8. In all cases except week 5, indicies greater than or equal to 1.00 (ie. a higher proportion of cells were polarised in the presence of cercarial antigen than in culture medium alone) were obtained with cells from experimental fish. However, these indicies were never greater than the range of indicies obtained from control fish (range = 0.58 - 2.12).

Adherence of leucocytes taken from experimental or control fish to cercariae or metacercariae *in vitro* was not observed at any time during the investigation.

Histological examinations during the course of the experiment revealed no changes in lymphoid tissues taken from experimental animals (Groups A and B), when compared with controls. Immunocytochemical staining using rabbit anti-*C. lingua* antiserum detected *C. lingua* antigen in the pronephros at 2, 6, 8 and 10 weeks post injection (Groups A and B). Antigen appeared to be dispersed throughout the tissue. Labelling was most intense in fish sampled at week 10, having received a second injection of parasite material at week 8 (Group B). No parasite antigen could be detected in spleen or mesonephros until 10 weeks post injection when a weak response was seen in both tissue types taken from fish from Group B and in only the mesonephros from fish in Group A.

Figure 24 Cercarial survival *in vitro* in serum taken following immunisation by intraperitoneal injection of sonicated cercariae

(●) Inactivated control serum only.

(▲) Inactivated control serum followed by complement from control serum.

(◻) Inactivated primary immune serum followed by complement from primary immune serum.

(○) Inactivated primary immune serum followed by complement from secondary immune serum.

(◻) Inactivated secondary immune serum followed by complement from secondary immune serum.

Each point represents 5 cercarial survival from 100 observations.
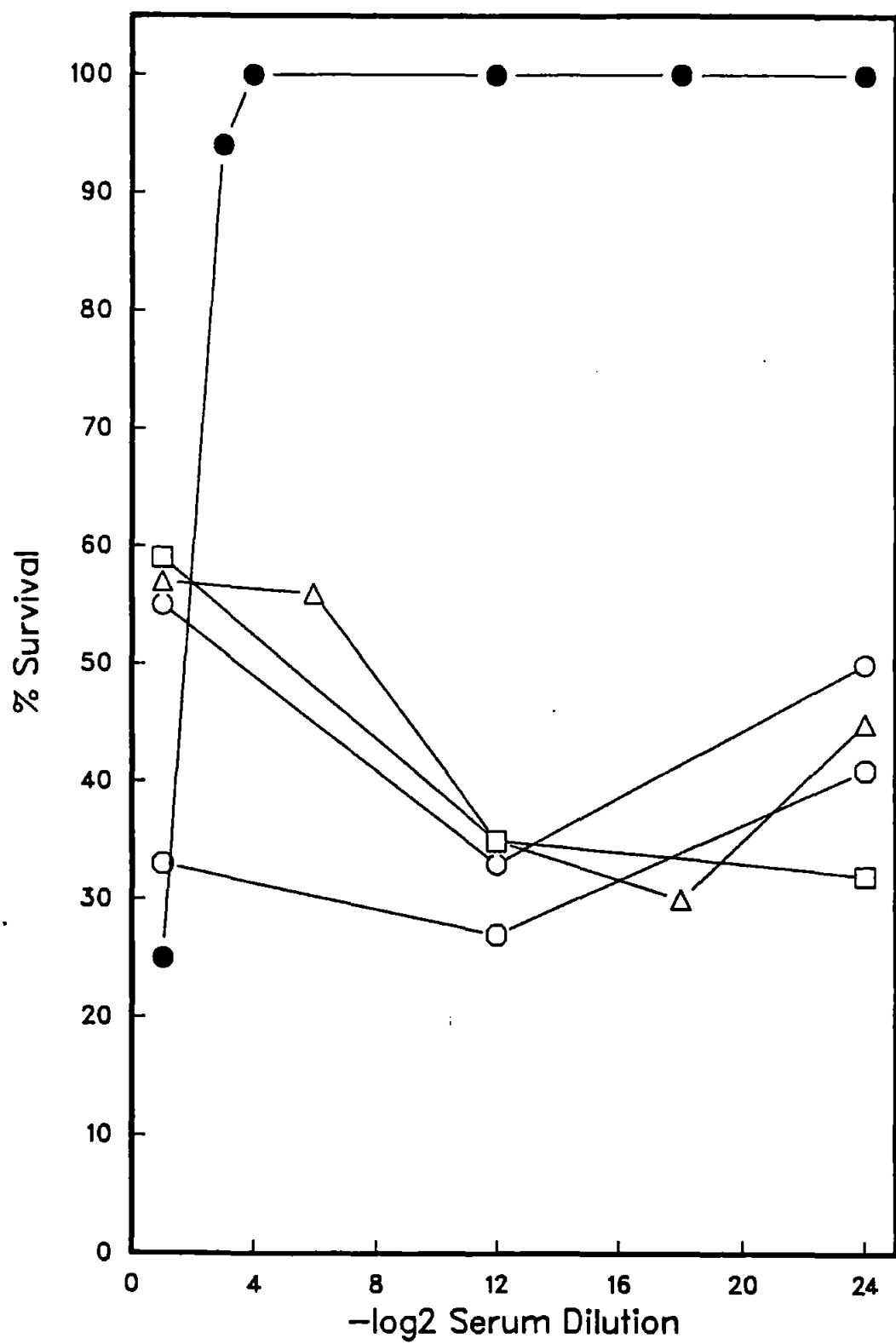
Table 7 Chemotaxis of pronephric leucocytes following immunisation by intraperitoneal injection of sonicated cercariae

Weeks after 1st Injection	2nd Injection of Antigen	Sample Size	Chemotactic Differential
CONTROL		5	0
1		4	0.55 ± 1.10
2		4	1.05 ± 1.30
3		4	0.90 ± 1.40
4		4	0
5		4	1.00 ± 1.20
6		4	0
7		4	0
8		4	0.10 ± 0.50
9		4	0
9	✔	4	0.69 ± 1.20
10		4	0
10	✔	4	0
11		4	0
11	✔	4	0.70 ± 1.30
18		4	0
18	✔	4	0

Figures represent mean chemotactic differential ± standard error.

Table 8 *In vitro* polarisation of pronephric leucocytes following immunisation by intraperitoneal injection of sonicated cercariae

Weeks after 1st Injection	2nd Injection of Antigen	Sample Size	Polarisation Index
CONTROL		5	1.24 ± 0.57
3		1	1.93
4		1	1.03
5		1	0.96
6		1	1.21
7		1	1.19
8		1	1.10
9		1	1.44
9	✔	1	1.16
10		1	1.44
10	✔	1	1.07
11		1	1.69
11	✔	1	1.25
18		1	1.00
18	✔	1	1.00

Figures represent the mean polarisation index ± standard error.

Lytic activity of sera from immunised and non-immunised mullet against cercariae

Serum samples were taken from fish previously immunised above by intraperitoneal injection of sonicated cercariae and exposure to infection levels of 20,000 cercariae respectively.

Cercariae were incubated in the appropriate serum treated with either EDTA or EGTA & Mg^{++}, in heat treated (45^oC, 30 min) serum or in untreated serum for 3 hours at room temperature ($20^oC \pm 2^oC$). Cercarial survival was assessed by staining with trypan blue.

From results shown in Table 9 it can be seen that cercariacidal activity was detected in both immune and non-immune sera with killing ranging from 62% - 69%. This activity was, however, lost when sera was first incubated at 45^oC for 30 min or treated with EGTA & Mg^{++}, levels of survival being similar to those in saline controls. Furthermore, serum killing was reduced in the presence of EDTA, although this salt itself appeared to have a cercariacidal effect in the concentration used here, killing 5 - 19 % of cercariae when incubated together with saline controls.

Non-specific lytic activity of serum from various teleost species against cercariae

Tests for non-specific lytic activity against cercariae of *C. lingua* were carried out on serum samples from the following species of fish; turbot (*Scophthalmus maximus*), dab (*Limanda limanda*), ballan wrasse (*Labrus bergylta*), bass (*Dicentrarchus labrax*), carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). In each case cercariae were incubated for 3 hours at room temperature ($20^oC \pm 2^oC$) in either saline controls or in serum previously treated by the addition of saline, EDTA or EGTA & Mg^{++} or by heating at 45^oC for 30min. Cercarial survival was assessed using trypan blue staining at the end of the 3 hour incubation period. From the results shown in Table 10, it can be seen that cercariacidal activity was detected only in sera from bass and rainbow trout, with killing of 75% and 70% respectively in untreated serum. This activity was greatly reduced in sera from these fish treated with EDTA, with only

Table 9 Killing (%) of cercariae in sera from immunised and unimmunised
mullet

Treatment of Sera	Control	Serum Source			
	Saline	Non−immune	Sonicated Cercariae Primary Response	Sonicated Cercariae Secondary Response	Live Challenge
None (Saline)	0−10	62	65	69	66
Heated 45°C, 30 min	−	0	0	0	0
EDTA	5−19	19	39	37	22
EGTA + Mg^{++}	0−11	11	7	5	11

Table 10 Killing (%) of cercariae in various teleost sera

Treatment of Sera	Control	Marine Species				FW Species	
	Saline	Turbot	Dab	Ballan Wrasse	Bass	Carp	Rainbow Trout
None (Saline)	0	0	0	0	75	0	70
Heated 45°C, 30 min	–	–	–	–	0	–	0
EDTA	5	–	–	–	5	–	5
EGTA + Mg^{++}	0	–	–	–	0	–	0

5% killing in each case and no activity was detected in the presence of sera treated with EGTA & Mg^{++}. Untreated sera were, however, completely inactivated on heating to 45°C for 30min.

Section 3: The host-parasite interface

In attempting to interpret events at the host-parasite interface, studies were undertaken on metacercarial development, notably the tegument and cyst wall, the localised host response and the effect of environmental factors on this response. These aspects are described below. The term cyst is used to refer to material of parasite origin and capsule to that originating from the host in response to the parasite.

Localised tissue response in relation to growth and development of metacercariae

125 0-group mullet, each weighing 1 - 3 g, were divided into 4 groups, three of these A, B and C each containing 25 fish and the remaining Group D 50 fish. Temperatures were maintained at 15, 20 and 25°C for Groups A, B and C respectively and 20°C for D. Following acclimation for 2 weeks, the first 3 Groups were infected with C. lingua by exposure of the caudal fin only to 10 - 20 cercariae. Penetration and encystment were monitored and the exact location of individual metacercariae mapped for each fish. Group D were immunised with 10 µl of a solublised parasite suspension containing the equivalent of 10^5 cercariae cm^{-3} administered as a single intraperitoneal injection. 5 weeks post immunisation, when the humoral antibody response was at its highest level with mean -log$_2$ titres of 12.5 ± SE 1.6, Group D were exposed to cercariae as above.

Growth of individual metacercariae, expressed as cyst size, was followed over a period of 30 days in vivo for each infection regime, measurements of individual parasites being made in situ in the living fish. Some fish were also killed at significant stages in parasite development and material taken for routine histological studies. In addition, material was prepared at this stage for later investigations of the cellular immune response to metacercariae in situ.

The rate of growth of metacercariae in non-immunised fish varied with temperature (Fig 25) although in all cases growth curves followed a similar pattern with an initial rapid growth phase being followed by a plateau.

Initial growth rates of metacercariae were similar in fish maintained at 20°C and at 25°C, both having a mean circumferential length of 0.54 mm after 6 days. Further growth at 25°C was faster until 16 days post infection, after which time the growth rates were similar. In contrast, parasite growth was slower at 15°C, there being a significant difference ($p <= 0.05$) in mean circumferential length after 8 days although eventually, after a further 14 days, they reached a similar size to cysts in fish held at the higher temperatures.

Metacercariae grew more slowly for the first 3 days in immune fish held at 20°C than those in un-immunised fish at the same temperature, there being a significant difference ($p <= 0.05$) in mean circumferential length. Nevertheless, cysts from fish in each Group attained the same size by the end of the study at day 30 when the mean circumferential length of the metacercaria was 0.61 mm. Mean cyst measurements and their standard deviations are given in the Appendix D.

The ultrastructure of the body wall and epidermis of the cercaria have been described by Rees and Day (1976) and Rees (1974). Of significance in the present study are the membrane bound secretion bodies which have been conveniently categorised into 10 types by these authors, 5 from the syncytial layer of the tegument and 5 from the cystogenous glands. In the present study, these have been denoted E1-5 and C1-5 (Table 11).

On contacting the fish host, cercaria attach by their oral sucker and immediately shed the tail. Penetration of the skin is accomplished within minutes, followed by a relatively short migration through the tissues. Five subsequent stages of development of the metacercaria were identified (Table 12), namely encystment, which usually occurs in the dermis; secretion; tegumental reorganisation; absorption and the pre-adult form. The effects of temperature and immunisation on the rate of metacercarial development are outlined in Table 12. The presence of secretion bodies during the stages of metacercarial development is summarised in Table 13.

Figure 25 Effect of temperature and immunisation on metacercarial growth
rate

(▵) 15°C.

(o) 20°C, unimmunised.

(●) 20°C, immunised.

(▫) 25°C.

Each point represents mean circumferential length (n=20). Standard deviations
of these values are given in Appendix D.
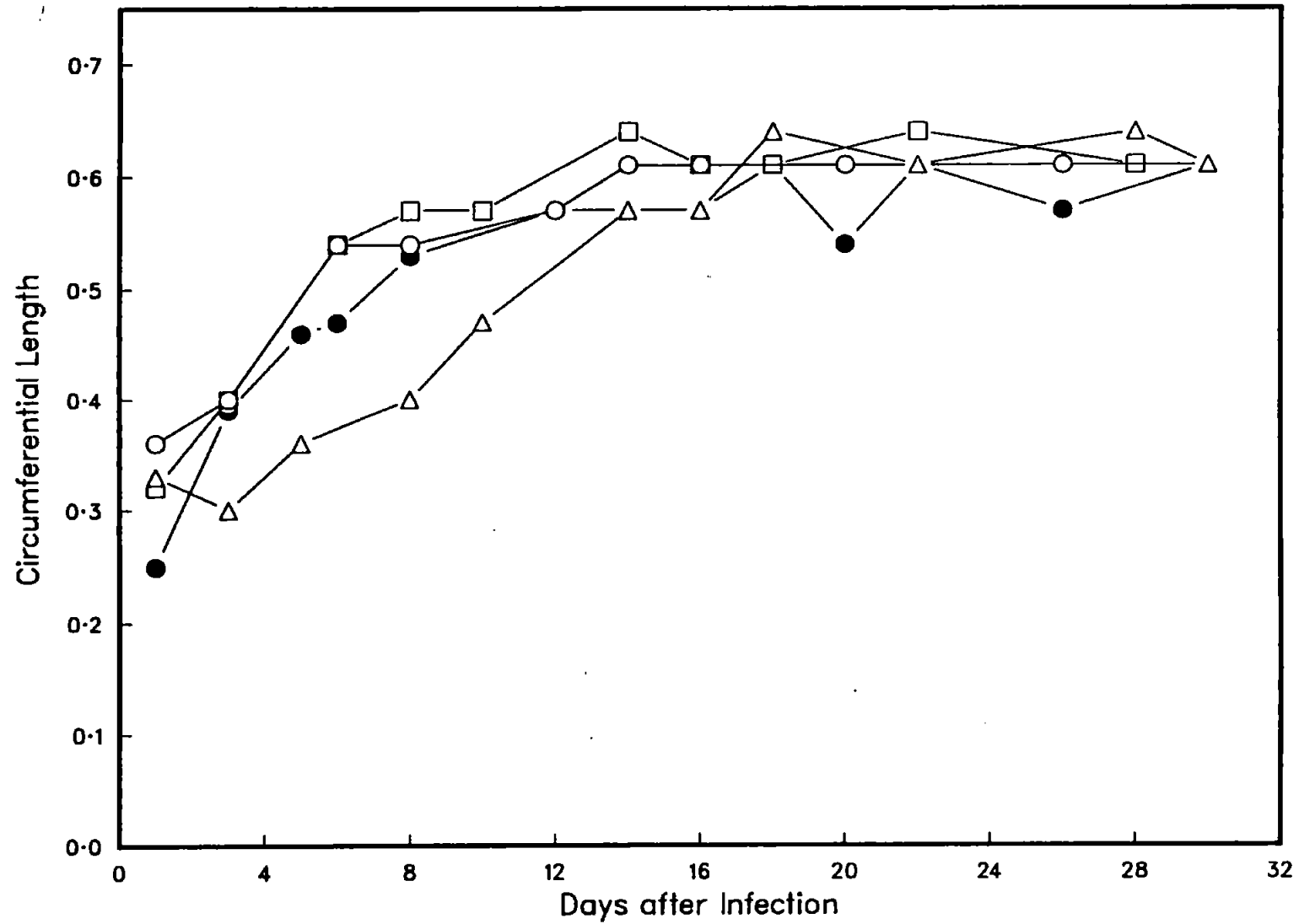
Table 11 Secretion bodies of *C. lingua* (after Rees and Day, 1976)

	Type	Size	Shape	Contents
Epidermal Secretion Bodies	E1	Large	Elongate	Granular
	E2	Small		Fibrillar
	E3	—	Round or Oval	
	E4	Large		Electron Dense
	E5			Electron Lucent
Cystogenous Secretion Bodies	C1		Oval	Electron Dense (larger than Esb4)
	C2	Small	—	Electron Lucent
	C3	Large	Round or Oval	
	C4	Small	—	Granular
	C5		Spherical	

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Table 12 Effect of temperature and immunisation on the rate of metacercarial development

Group	Time Post Infection (Days)						
	4	8	12	16	20	24	28
A	SE		RE	AB	PA		
B	SE	RE	AB	PA			
C	SE	RE	AB	PA			
D	SE	RE	AB	PA			

SE - Secretion (including penetration, migration and initial encystment).

RE - Reorganisation of the tegument.

AB - Absorption.

PA - Pre-adult.

Table 13 Location of secretion bodies in metacercaria at different stages of development

	Type	Metacercarial Development Stage				
		Encystment	Secretion	Reorganisation	Absorption	Pre—Adult
Epidermal Secretion Bodies	E1	Tegument	Tegument	—	—	—
	E2	"	Tegument/ Lumen	Tegument	Tegument	—
	E3	Tegument/ Lumen	Tegument/ Lumen	—	—	—
	E4	Tegument/ Sub—tegument	Tegument/ Lumen	—	—	—
	E5	Sub—tegument	—	—	—	—
Cystogenous Secretion Bodies	C1	"	Tegument	—	—	—
	C2	"	Tegument/ Lumen	Tegument	—	—
	C3	"	Tegument/ Sub—tegument	Tegument/ Lumen	—	—
	C4	"	Tegument/ Lumen	Tegument	—	—
	C5	"	Tegument/ Lumen	"	—	—
	C6	—	—	—	—	Tegument
	C7	—	—	—	—	"
Membranous Bodies		—	Lumen	—	—	—

Cyst wall formation was observed on histological examination of infected fish from Groups A, C and D 1 h post infection. It comprised 2 acellular layers; an outer, relatively thin electron dense layer and a thicker, granular layer (Fig 26a). The outer layer was noticeably more compact and electron dense in fish from Groups C and D (Fig 26b) and in some cases was markedly folded (Fig 26c). In contrast, metacercariae examined 1 h post infection from Group B had not yet become encysted (Fig 26d).

During the period of encystment, membrane bound secretion bodies were widely dispersed, occurring in the cyst wall, cyst lumen and in surrounding host tissue in fish from all Groups. Epidermal secretion bodies E1, 2 and 3 were still present at this time in the tegumental syncytium of metacercariae from all 4 Groups of fish (Fig 27a,c), with large numbers of E3 appearing in the cyst lumen. The sub-tegumental layer contained type E4 in individuals from Groups B and D (Fig 27b) and type E5 in all 4 Groups (Fig 27a,c). Disruption of the tegumental surface was evident, resulting from the release of secretion bodies (Fig 28a). Cercarial spines were visible in all individuals examined at 1 h post infection (Fig 26d, 27a,c). Throughout this period of development, metacercariae were observed to be very motile within the cyst.

Histopathological investigation indicated evidence of host tissue necrosis associated with the parasite migratory path and site of encystment. This necrosis was particularly noticeable in fish from Group B where cyst wall formation had not yet occurred (Fig 28b). Capillaries were frequently damaged causing haemorrhage (Fig 28c). Some material of parasitic origin, including intact E1 secretion bodies was present outside the cyst wall in the surrounding necrosed host tissue (Fig 27a, 28b). Cellular debris was abundant and a few host cells thought to be macrophages were seen to contain phagocytosed host and parasite material (Fig 28d,e). Up to 1 h post infection, there was no further evidence of a leucocytic host response even in fish immunised prior to infection (Group D). The first association of fibrotic tissue with the parasite cyst wall was evident within 1 h of infection. At this stage there appeared to be some degree of intermixing of parasite and host material at the cyst-capsule interface (Fig 27a).

Figure 26 Cyst wall formation at 1 h post infection

(a) Cyst wall (OL-outer layer; IL-inner layer) and tegument from Group A. (Mag= 28000).

(b) Cyst wall (OL-outer layer; IL-inner layer) from Group C. (Mag= 40000).

(c) Marked folding of the cyst wall in Group C metacercaria. T - tegument, L - lumen. (Mag= 13000).

(d) Metacercaria from Group B prior to encystment. HT - host tissue, S - cercarial spine. (Mag= 19000).
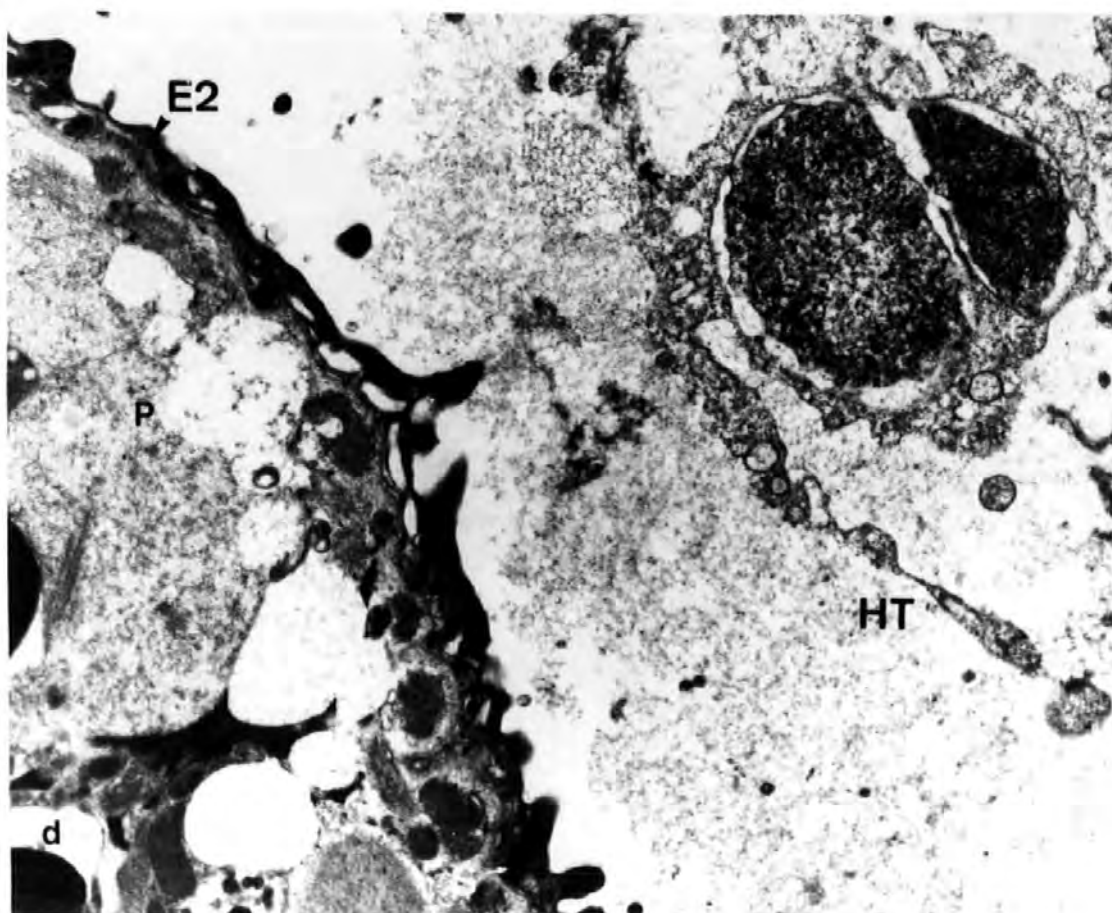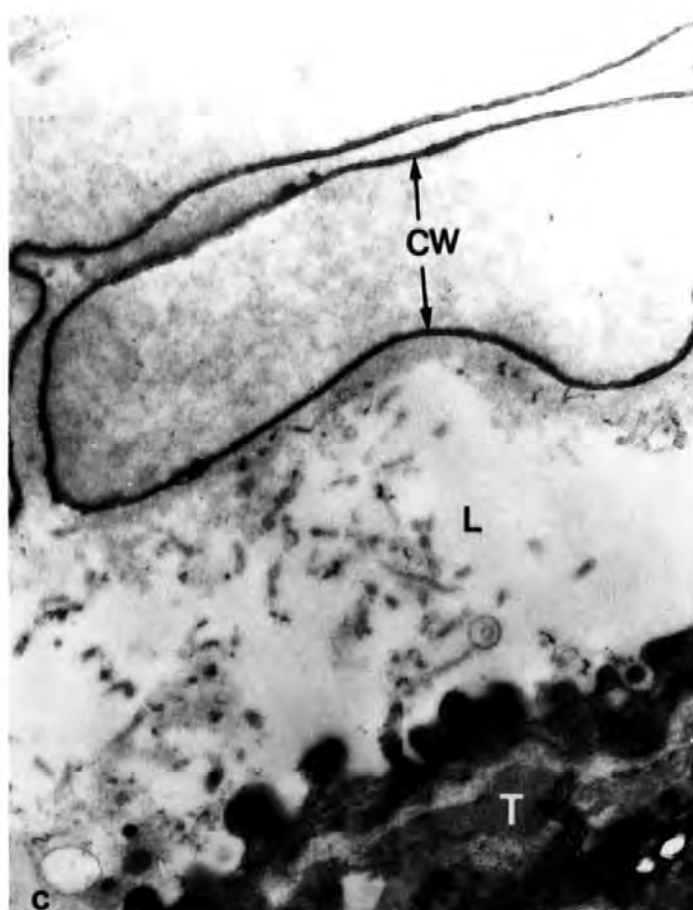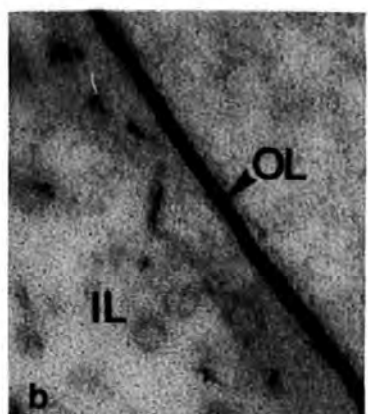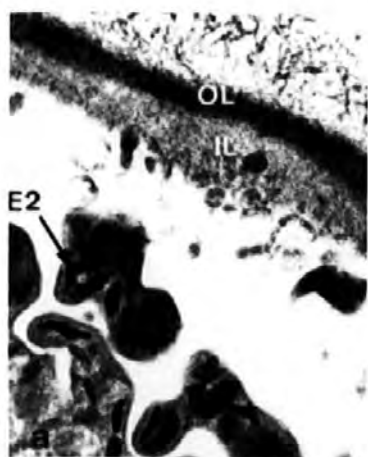
Figure 27 Secretion from the tegument at 1 h post infection

(a) Release of secretion bodies from the tegument in Group A. Parasite material
      is present outside the cyst wall (CW) in the surrounding host tissue (HT). S -
      cercarial spine. (Mag= 18000).

(b) Secretion bodies in the tegument in Group D. (Mag= 16000).

(c) Secretion bodies within the tegument in Group B. S - cercarial spines.
      (Mag= 16000).
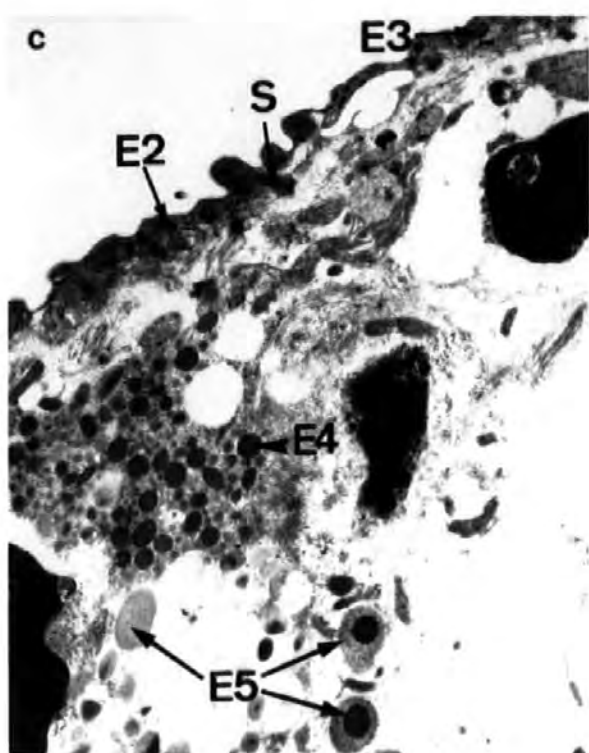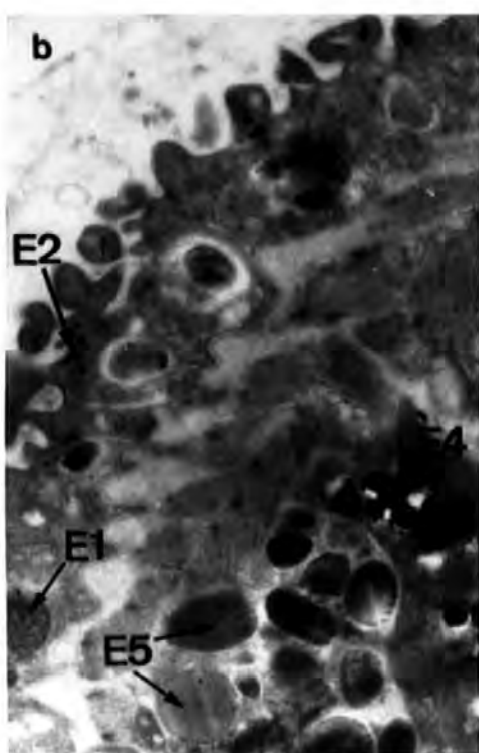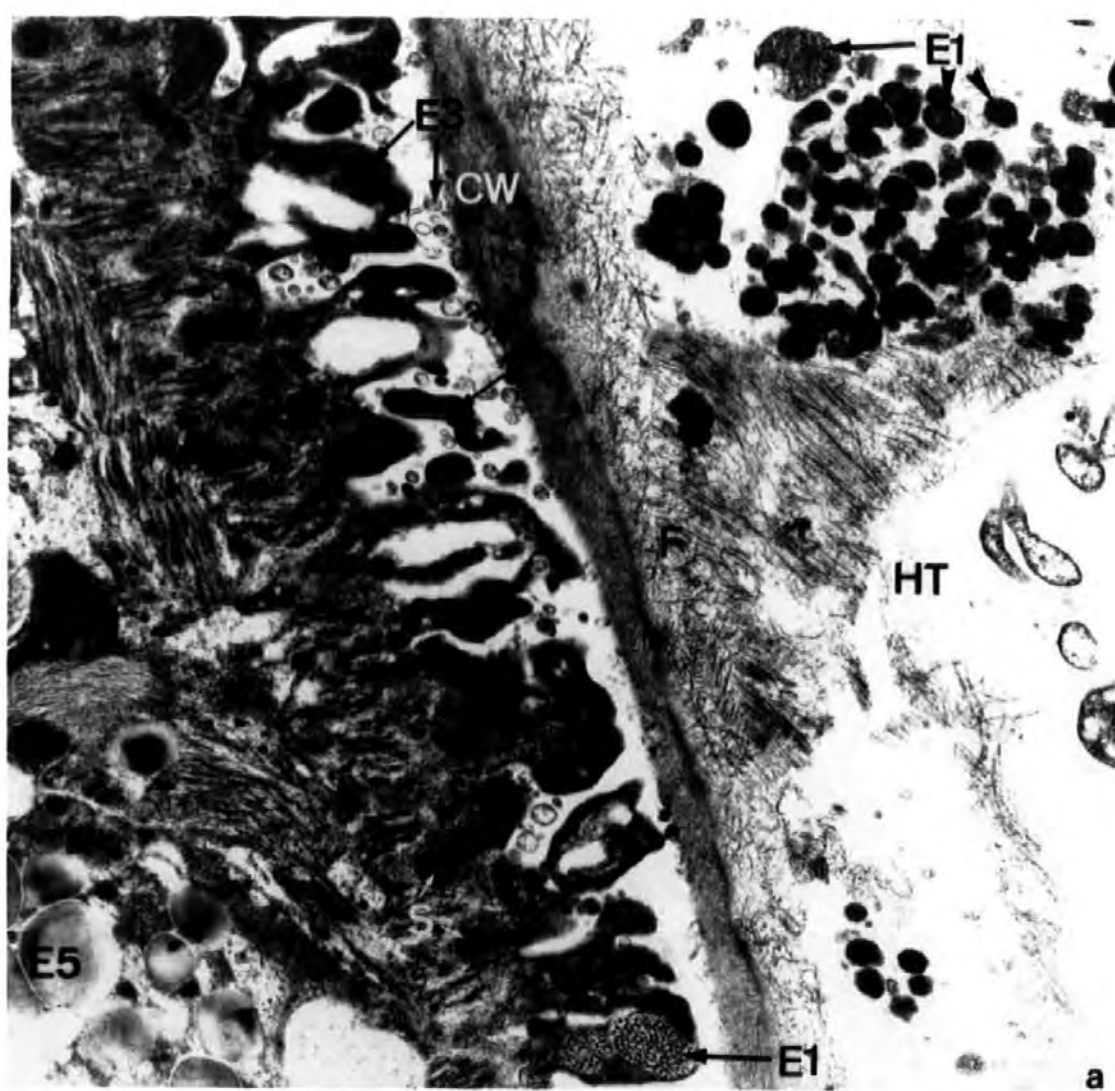
Figure 28 Host-parasite interface at 1 h post infection

(a) Depression in the tegument resulting from the release of a secretion body. Group B. (Mag= 19000).

(b) Metacercaria from Group B prior to encystment. Surrounding host tissue (HT) including a rodlet cell (RC) is heavily necrosed. P - parasite tegument. (Mag= 19000).

(c) Haemorrhaging in host tissue immediately adjacent to a metacercarial cyst in Group C. (Mag= 4000).

(d) & (e) Phagocytic host cells, presumed to be macrophages, in the host capsule in Group A. (Mag= 40000 - d; 20000 - e).

a

b

c

E

E2
E3

P

E4

R

HT

d

e
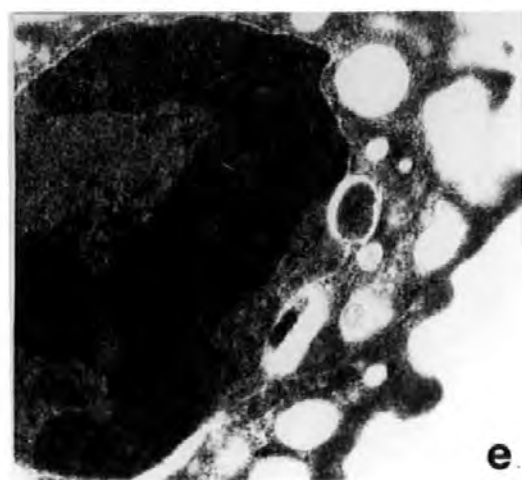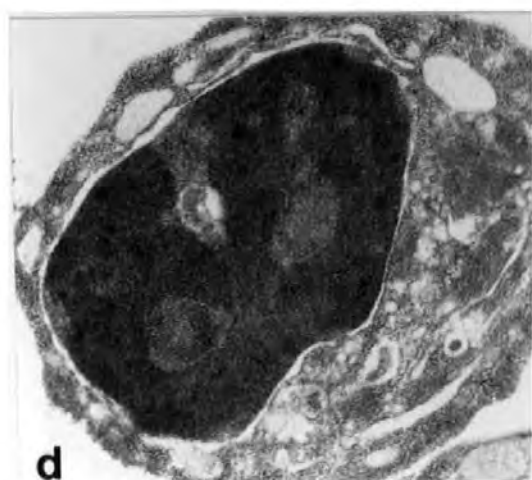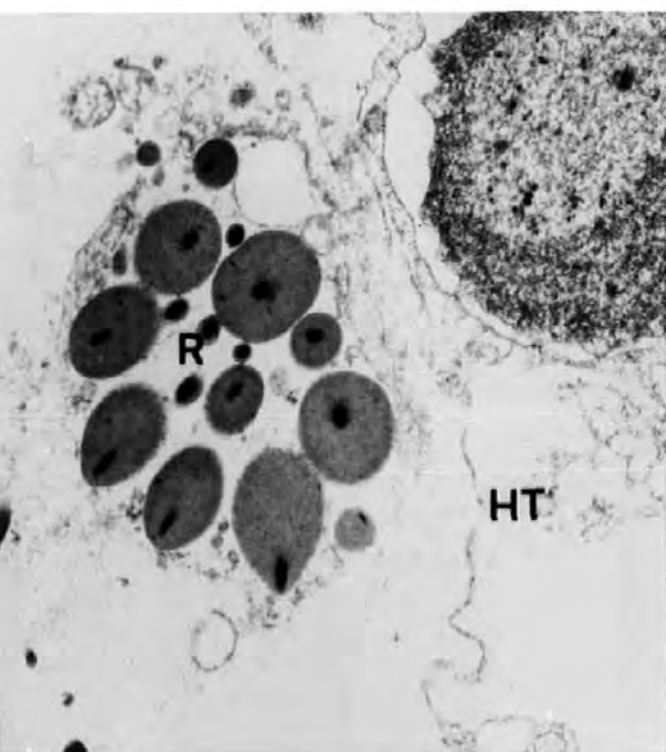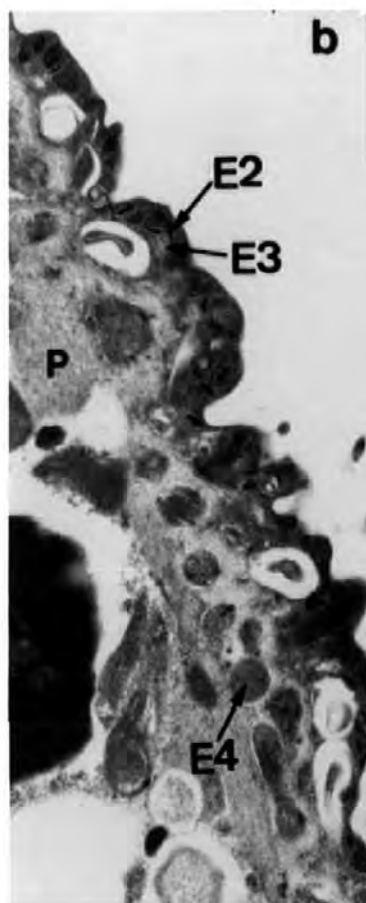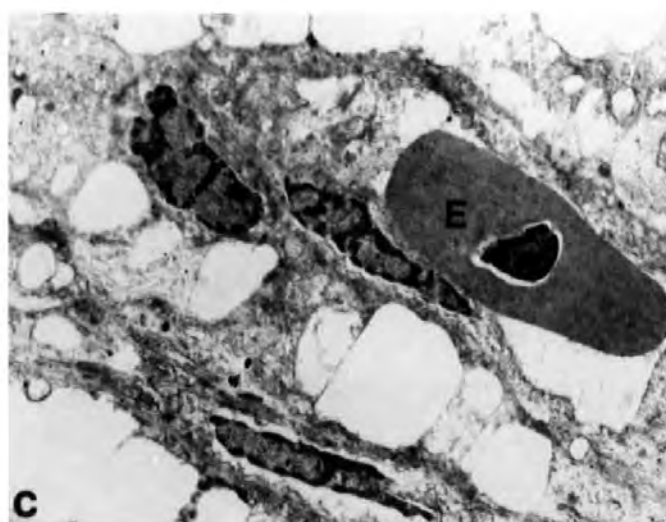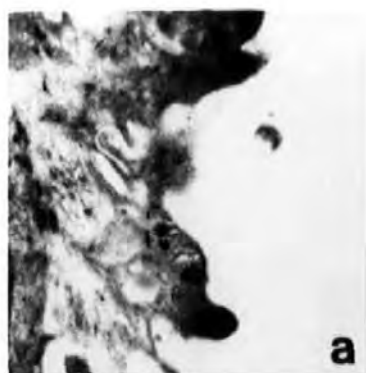
At 6 h post infection, intact secretion bodies E1 and 3 were still present in the cyst lumen of parasites with types E1-4 being situated in the tegument from all 4 Groups (Fig 29a,b,c). Evidence of continued active secretion was apparent with bodies being released into the cyst lumen including E2, 3 and 4 and large membranous bodies in parasites from all 4 Groups (Fig 29a,b, 30a,b). Furthermore, C1 type bodies were seen in the tegument from Group A (Fig 29a) and types C1 and C3 from Group B (Fig 30b). Some of the secretory components of metacercariae appeared to be incorporated into the inner cyst wall (Fig 29a, 30c) and pass through this layer to be incorporated at the host-parasite interface (Fig 30a). By this time the cyst wall itself had thickened, with the outer layer beginning to take on a bilayered appearance in parasites from Groups B, C and D (Fig 30a,d) but not in those from Group A (Fig 29a). At this stage there was evidence of a localised host response with the first observation of fibroblasts in the capsular region. Some leucocytes were also present within the capsule including Type 2 granulocytes within the developing fibrous network (Fig 31a) and macrophages (Fig 31b). This granulocyte type contained 2 granule types, which were either fibrillar or elctron dense with an electron lucent core. Necrosis of surrounding host tissue was still evident.

There was little change after 12 h. Active secretion was still observed (Fig 32a,b) with cystogenous secretion bodies C2 and 4 detected in the tegument syncytium for the first time in individuals from Group C. The cyst wall was more developed, especially at the higher temperatures (Fig 32c, 33), with the inner cyst wall being thickest in Group C and thinnest in Group A. Host responses at this time were characterised by fibrosis (Fig 33) and leucocyte infiltration in fish from all Groups, predominant cell types being lymphocytes and macrophages.

After 24 h, the outer layer of the cyst wall had developed a more electron dense inner face in all Groups (Fig 34a,b,c,d). Further material secreted by the parasite appeared to be incorporated into the cyst wall in individuals from Groups A and B (Fig 34a,b). Increasing numbers of host cells were found surrounding cysts including macrophages, some of which appeared to be ingesting cyst material, and fibroblasts.

The host response had greatly increased after 48 h in immunised fish (Group

Figure 29 Secretion from the tegument at 6 h post infection.

(a) Secretion bodies present in the tegument and cyst lumen in Group A. F - fibrous layer of host capsule, CW - cyst wall. (Mag= 12000).

(b) Secretion bodies in the tegument of metacercaria from Group B. S - cercarial spine. (Mag= 33000).

(c) Secretion bodies in the tegument and cyst lumen in Group D. HT - host tissue, CW - cyst wall. (Mag= 13000).
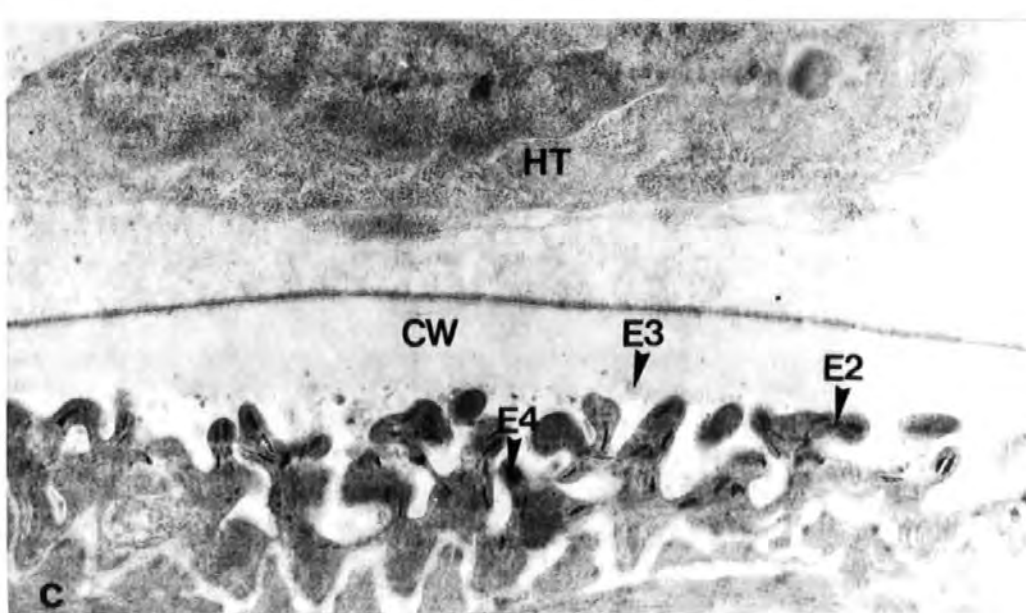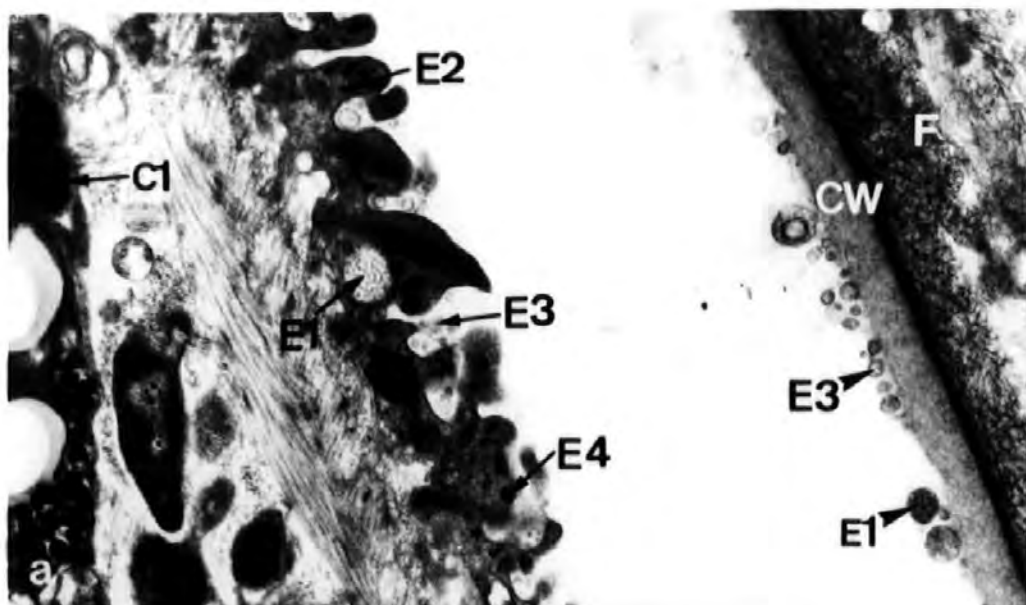
Figure 30 Cyst wall structure and secretion at 6 h post infection

(a) Membranous body (M) in the lumen adjacent to the cyst wall (CW) in Group B. The bilayered structure of the outer laer (OL) of the cyst wall can be seen. F - fibrous host tissue. (Mag= 40000).

(b) Secretion bodies in the tegument of a metacercaria from Group B. (Mag= 26000).

(c) Release of secretion bodies into the cyst lumen and their incorporation into the cyst wall in Group D. (Mag= 14000).

(d) Incorporation of parasite secretory material into the inner layer (IL) of the cyst wall in Group D. The outer layer (OL) of this wall shows a bilayered structure. (Mag= 26000).
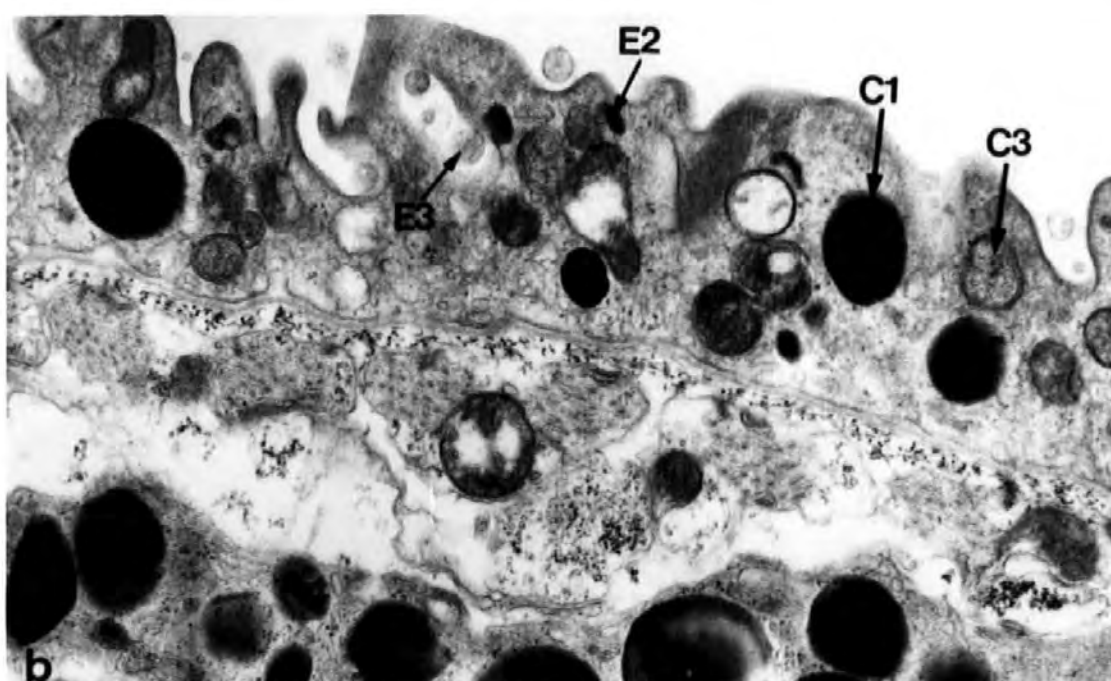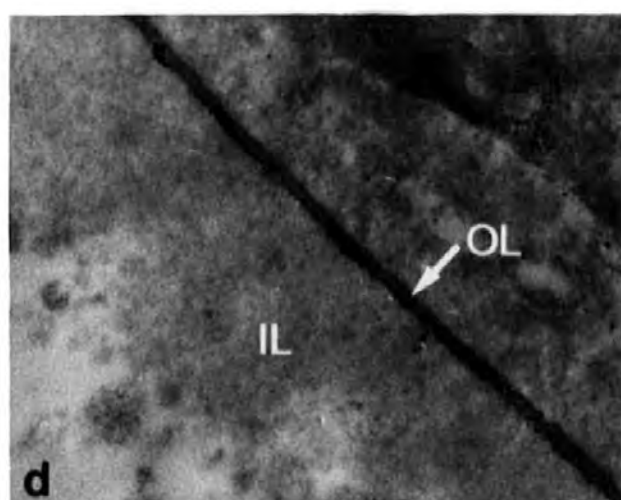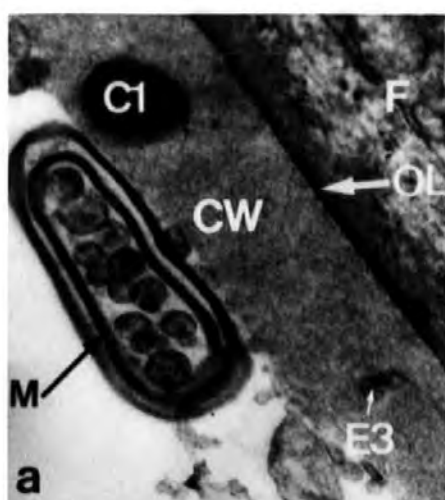
Figure 31 Leucocytes in the host capsule at 6 h post infection

(a) Type 2 granulocytes in the fibrous network of the host capsule in Group A. (Mag= 12000).

(b) Macrophage in the host capsule immediately adjacent to a metacercarial cyst in Group D. (Mag= 20000).
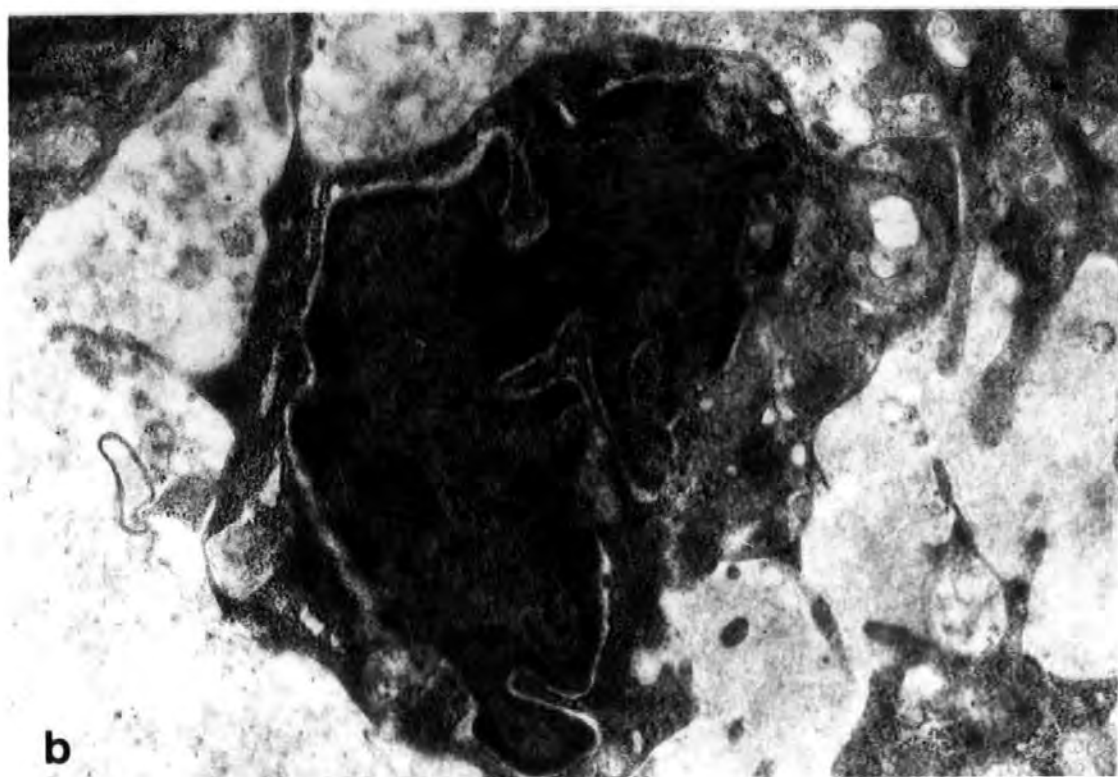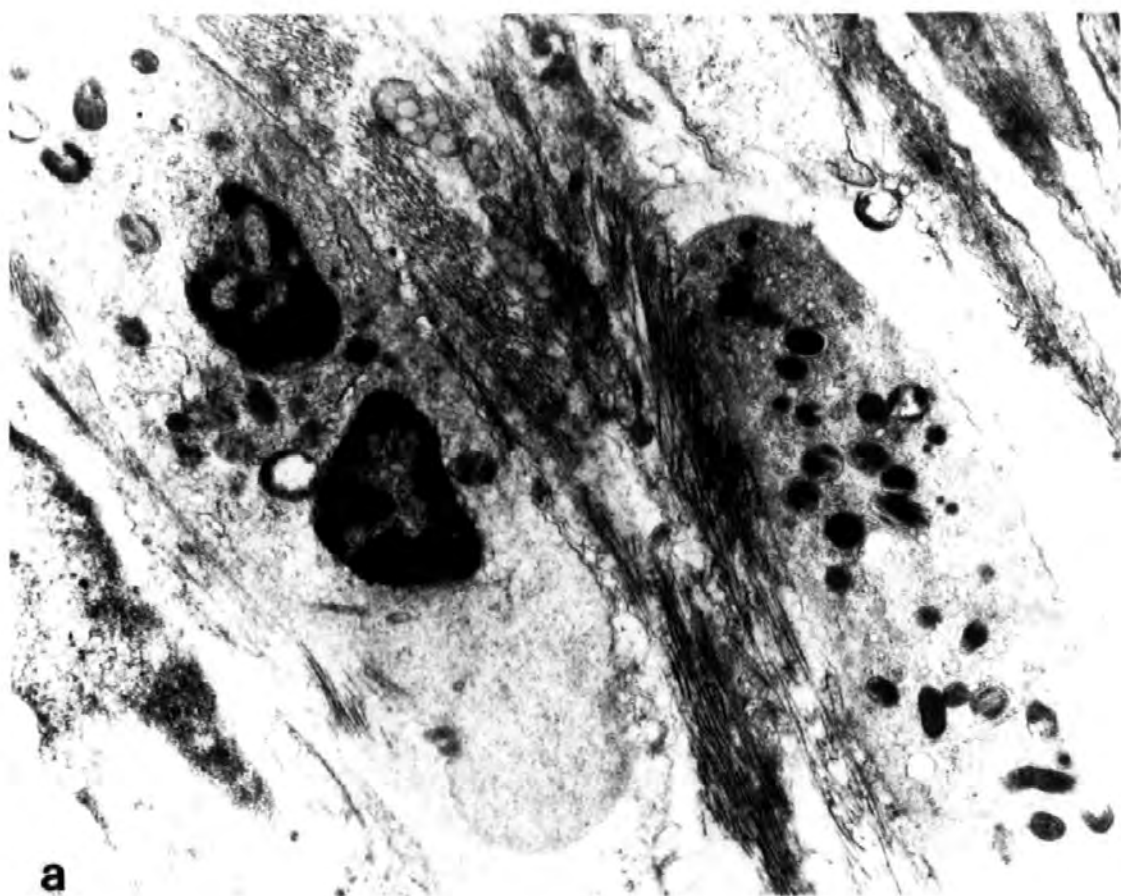
Figure 32 Tegument and cyst wall at 12 h post infection

(a) Active secretion through the tegument of a metacercaria in Group C. (Mag= 22000).

(b) Secretory bodies in the parasite tegument (P) and lumen (L) of the metacercaria in Group A. CW - cyst wall, F - fibrous layer of host capsule. (Mag= 22000).

(c) Tegument (T), cyst wall (CW) and host capsule (H) in Group D showing more developed cyst wall structure. (Mag= 16000).
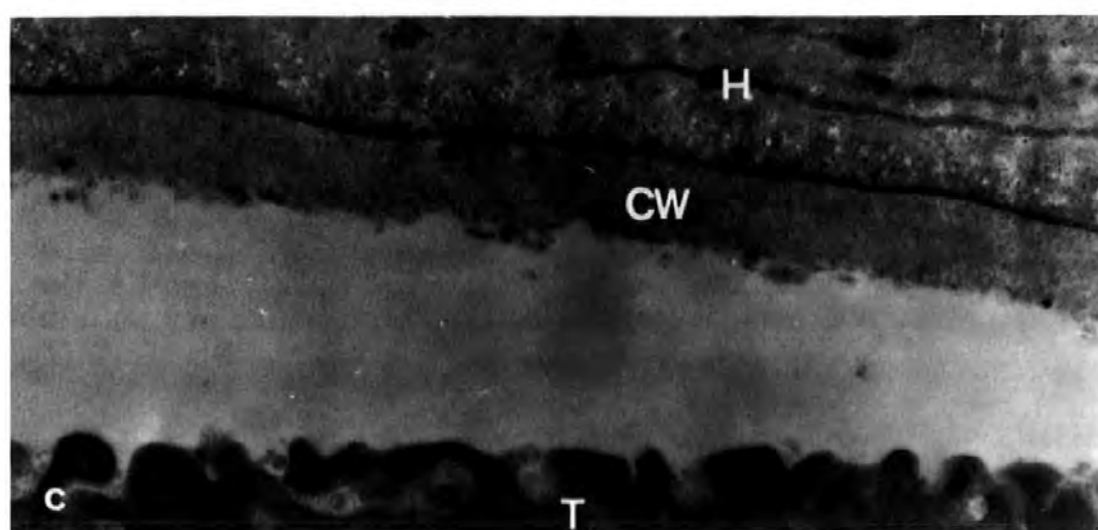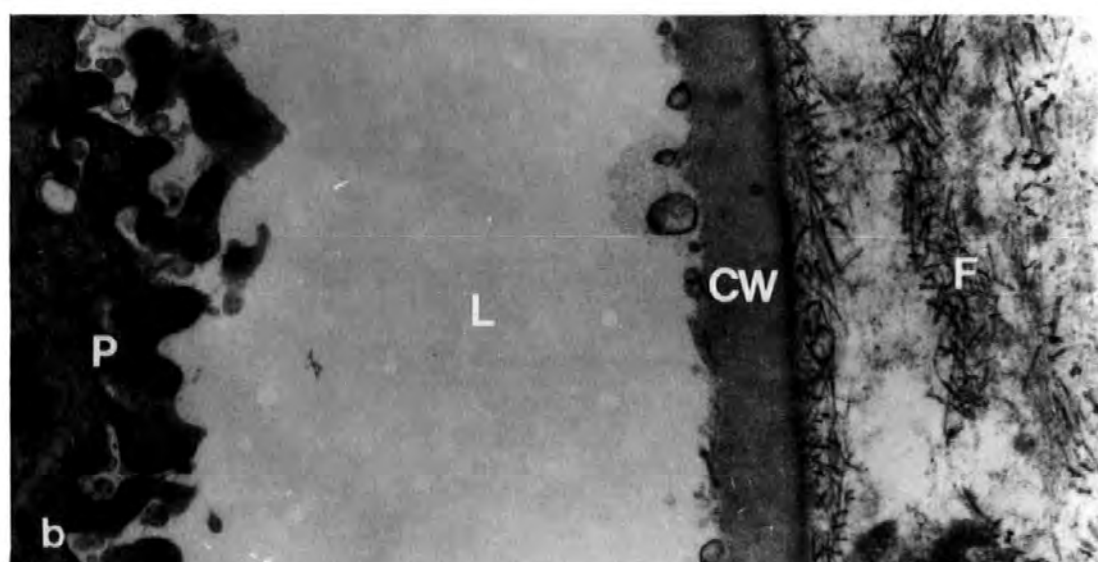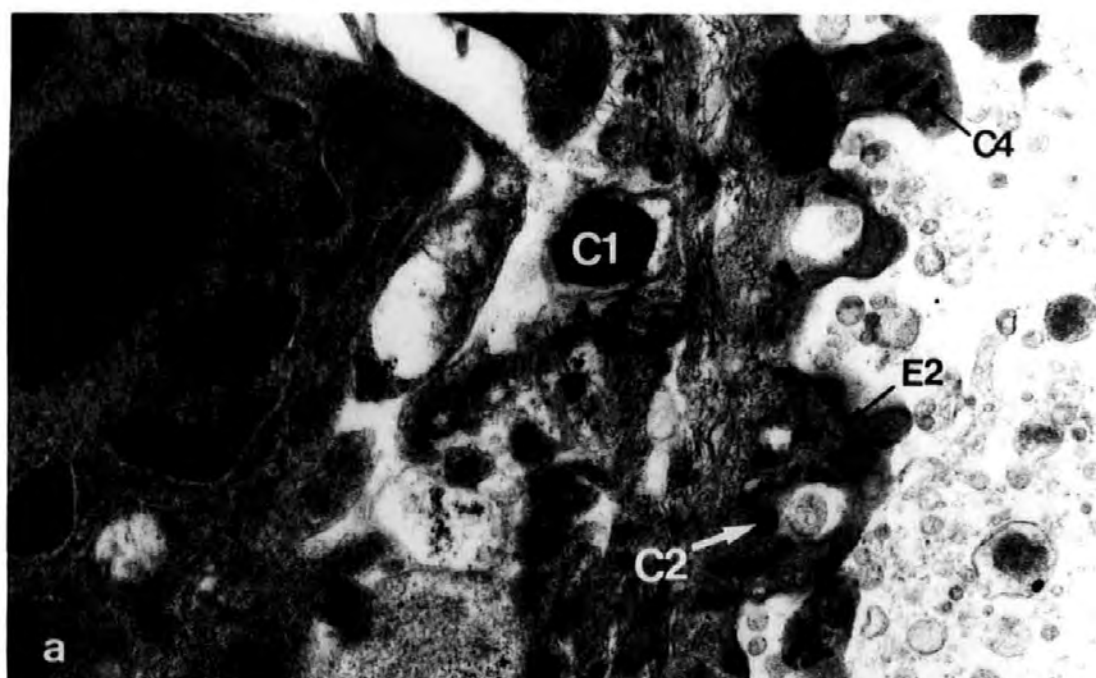
Figure 33 Host-parasite interface at 12 h post infection

Tegument (T), cyst wall (CW) and fibrous layer (F) of the host capsule in Group C, showing further development in the cyst wall structure. (Mag= 16000).

Figure 34 Host-parasite interface at 24 h post infection

(a) Continued secretion into the lumen (L) in a metacercaria from Group A. (Mag= 24000).

(b) Macrophage adjacent to the cyst wall (CW) in Group B. (Mag= 14000).

(c) Cyst wall (CW) and host tissue (HT) in Group C. (Mag= 12000).

(d) Cyst wall (CW) and host tissue (HT) in Group D. (Mag= 24000).
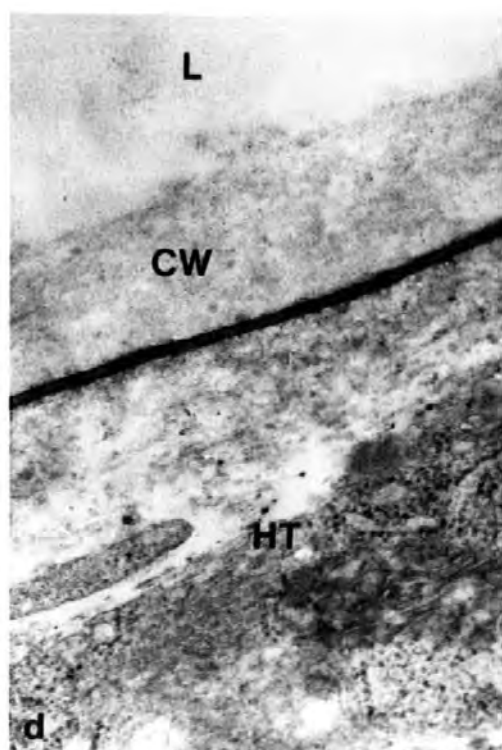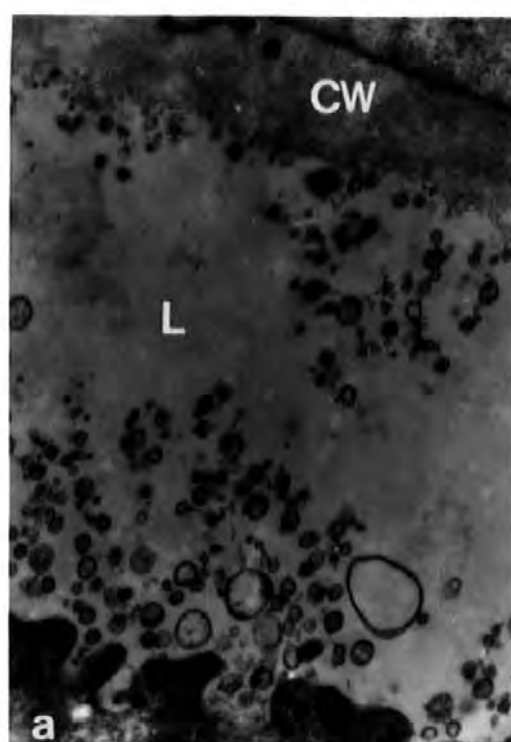
Figure 35 Leucocytes in the host capsule at 24 h post infection

(a) Macrophage (M) and lymphocyte (L) in the host capsule in Group D. (Mag= 9500).

(b) Macrophage in the host capsule in Group D. (Mag= 13000).

(c) Lymphocyte in host capsule in Group D. (Mag= 12000).

(d) Macrophage in host capsule in Group D. (Mag= 8000).
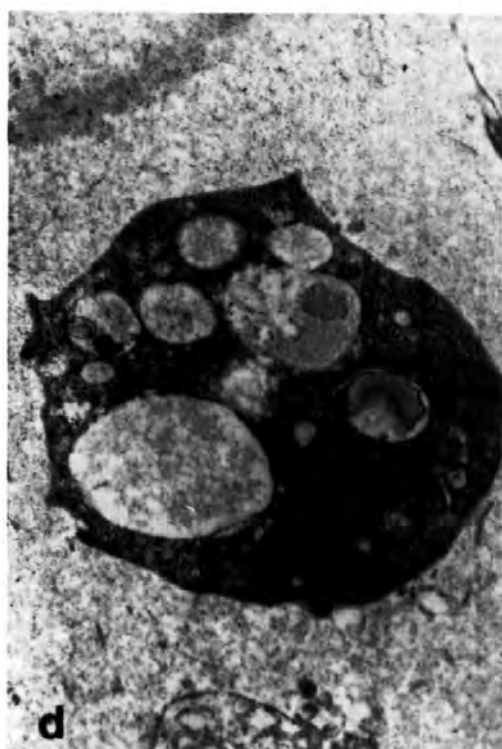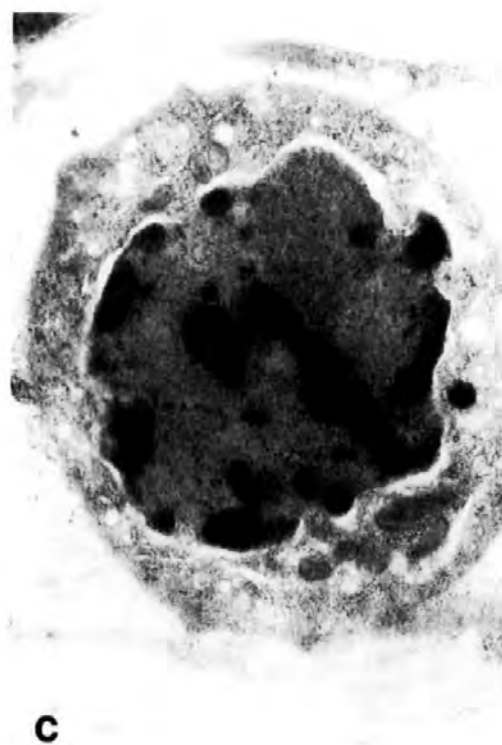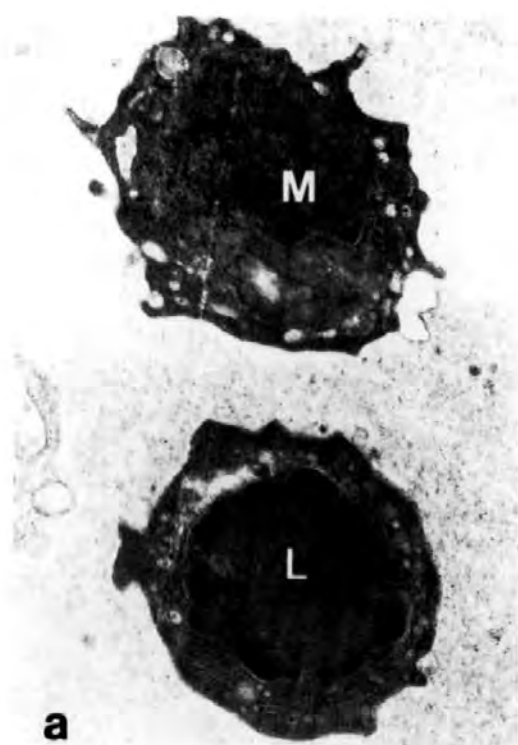
D) with increased cellular infiltration (Fig 35a,b,c,d). Macrophages and lymphocytes predominated, the surfaces of the latter being highly microvillous. At this stage the parasite tegument was characterised by a loss of cercarial spines (Fig 36a,b) and the presence of secretory bodies including types C2, 4 and 5 and E1 and 2. The outer layer of the cyst wall clearly demonstrated a bilayered form with a new outermost thin electron dense layer evident in Group D samples (Fig 36c).

After 4 days, differences in the rate of development of the host-parasite interface between the 4 groups of fish were more marked. Parasites from those in Group A still appeared to be discharging secretion bodies of the cystogenous types C1, 3 and 4 (Fig 37a). At higher temperatures, the tegument had undergone structural modification, becoming pre-adapted to adult life in the definitive host. The extent of reorganisation was more pronounced in individuals from Group C (Fig 37b) compared to Group D, in which secretory activity was still apparent (Fig 37c, 38a). In Group B, C and D the outer layer of the cyst wall clearly displayed a bilamellar structure (Fig 38a,b) and the cyst wall inner layer had increased in thickness. A noticeable feature first observed in Group C infections at this time was the deposition of an electron dense layer at the exterior surface of the cyst (Fig 38b). A minimal host response was recorded in fish from Group A which were maintained at the lower temperature of 15°C. In contrast, an intense response was apparent in Groups C and D representing fish kept at higher temperatures and immunised fish (Fig 38c). Cell types included lymphocytes (Fig 38b,c, 39a,b,c) and phagocytic cells presumed to be macrophages (Fig 39d, 40a,b,c,d).

At 6 days post infection, the tegument showed fewer signs of disruption with a well developed microvillous form in parasites from Groups C and D (Fig 41a), whereas those in Group B had not developed beyond the stage recorded at 4 days in these groups (Fig 41b). Metacercariae from Group A (at the lowest temperature) showed little advancement in development, material still being incorporated into the cyst wall (Fig 41c). The host response in all groups at 6 days was not dissimilar to that described at 4 days although it appeared more intense in those fish held at the highest temperature (Group C) (Fig 42a,b,c)

Figure 36 Cyst wall structure and secretion from the tegument at 24 h
post infection

(a) Release of secretion bodies from the tegument which is devoid of cercarial
spines in Group B. CW - cyst wall, F - fibrous layer of the host capsule. (Mag=
22000).

(b) Secretion bodies in the tegument which has lost the cercarial spines in
Group C. CW - cyst wall, HT - host tissue. (Mag= 9000).

(c) Cyst wall in Group D, clearly demonstrating the bilayered nature of the outer
layer (OL). IL - inner cyst wall layer, HT - host tissue. (Mag= 45000).
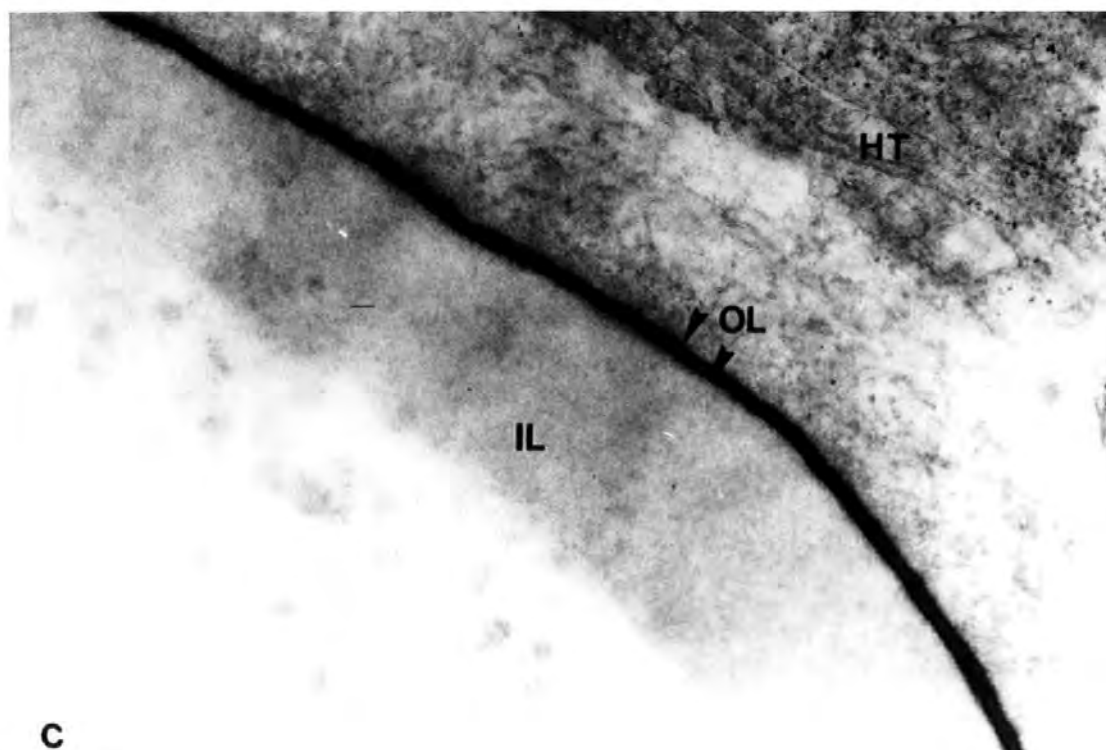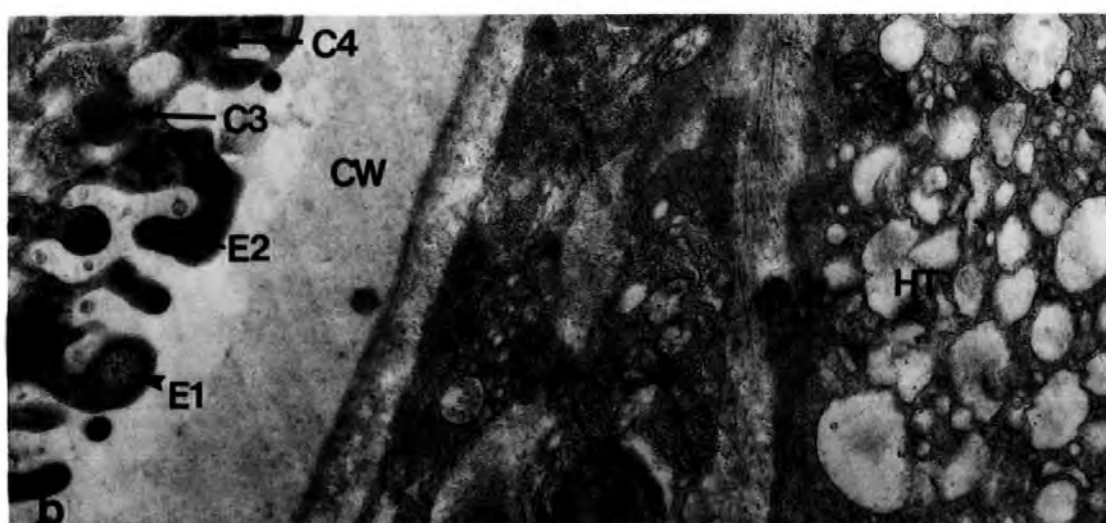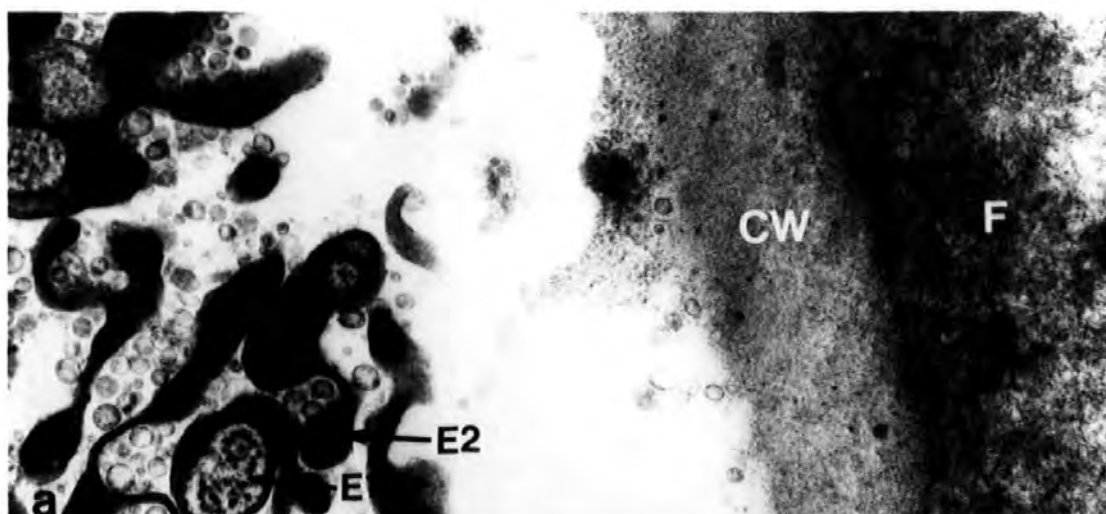
Figure 37 Tegument and cyst wall at 4 days post infection

(a) Continued discharge of secretion bodies in metacercaria from Group A. CW - cyst wall, HT - host tissue. (Mag= 16000).

(b) Structural modification to the tegument (T) during it's reorganisation in Group C. CW - cyst wall, HT - host tissue. (Mag= 13000).

(c) Secretory activity in the tegument (T) of metacercaria from Group D. CW - cyst wall, F- fibrous layer of host capsule. (Mag= 15000).
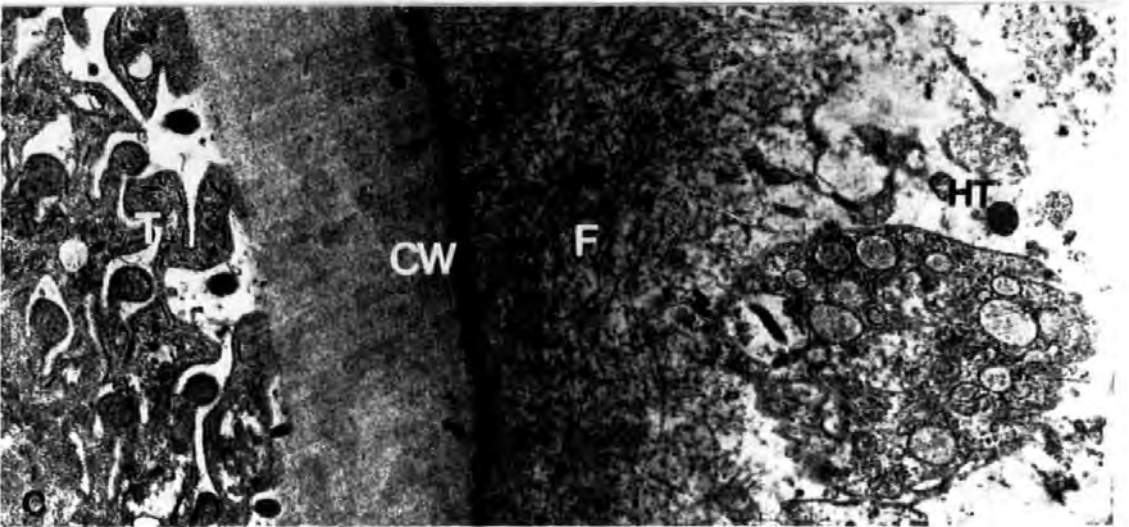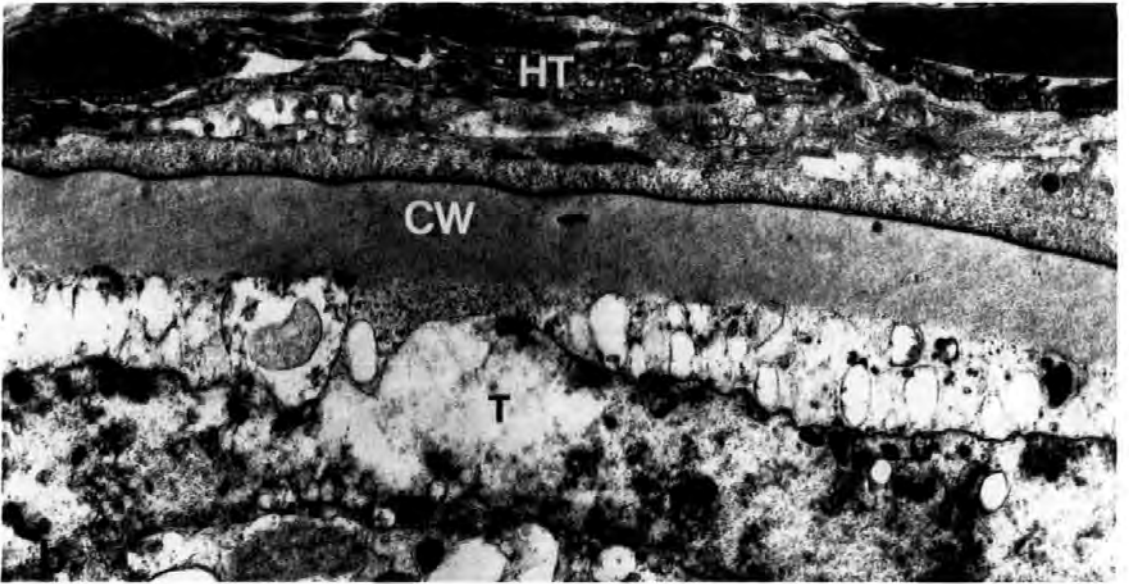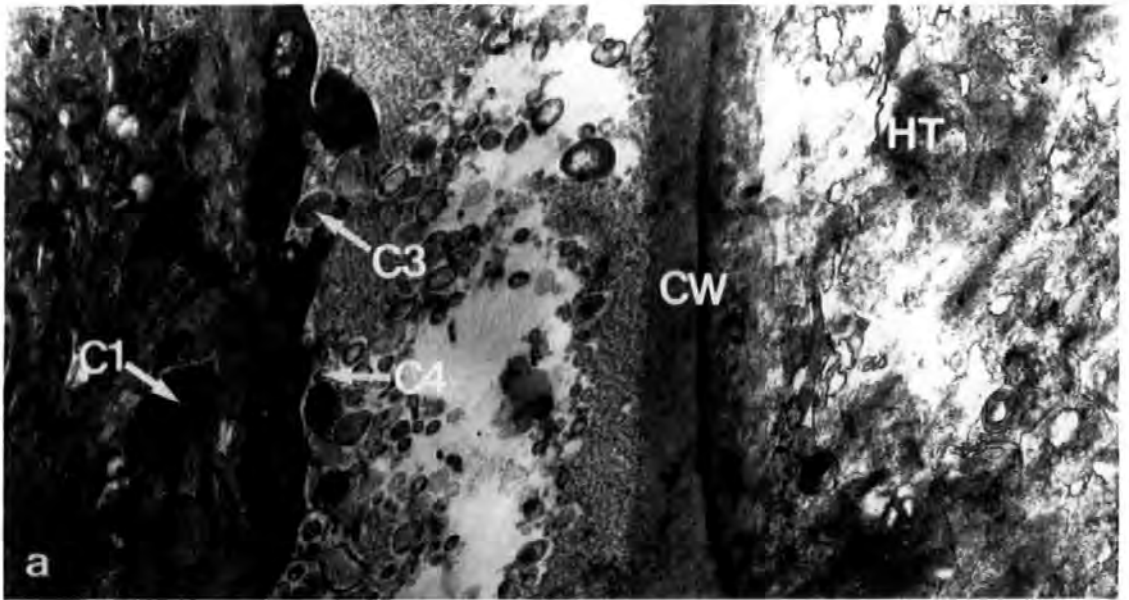
Figure 38 Host-parasite interface at 4 days post infection

(a) Secretion bodies released from the tegument in Group D. F - fibrous layer of host capsule. (Mag= 26000).

(b) Lymphocytes (L) immediately adjacent to a metacercarial cyst in Group C. Note the deposition of an electron dense layer (G) at the exterior surface of the cyst wall (CW). (Mag= 18000).

(c) Intense host cellular response to metacercaria in Group C. (Mag= 4000).
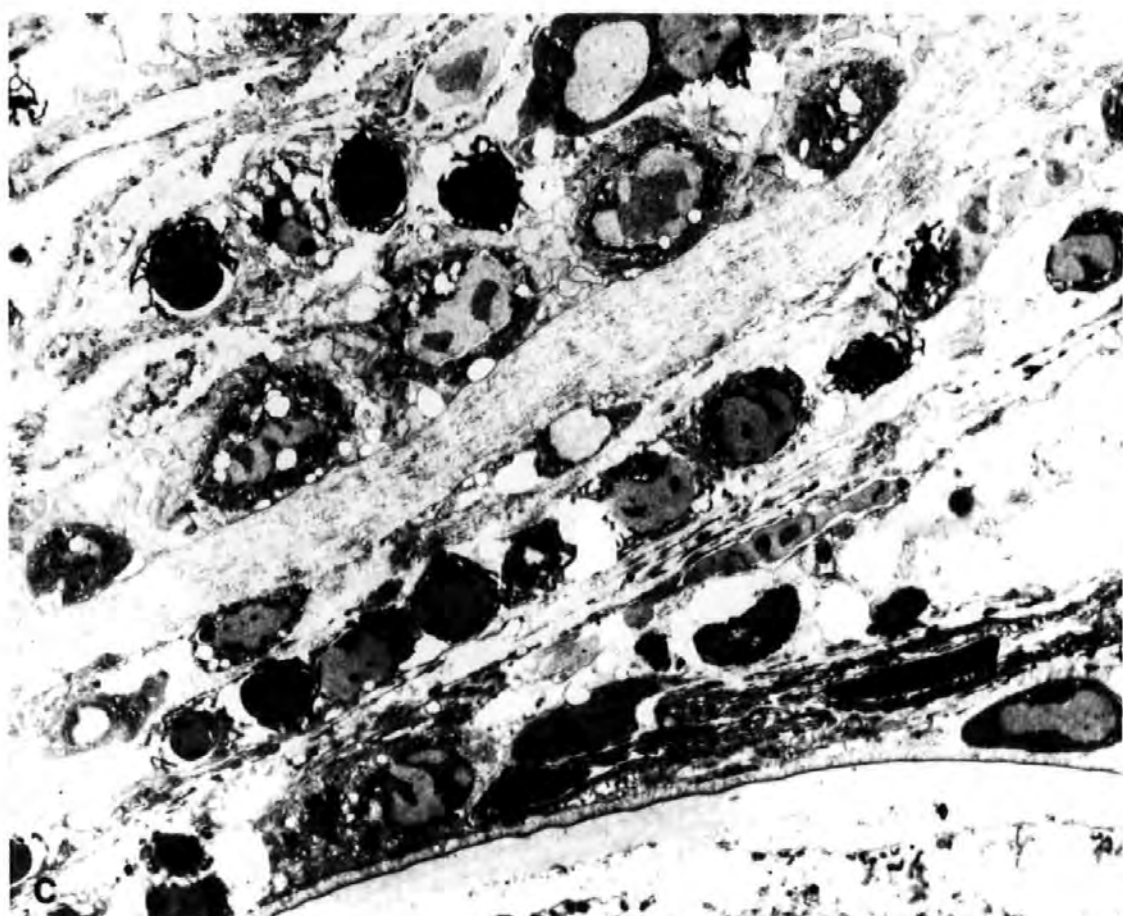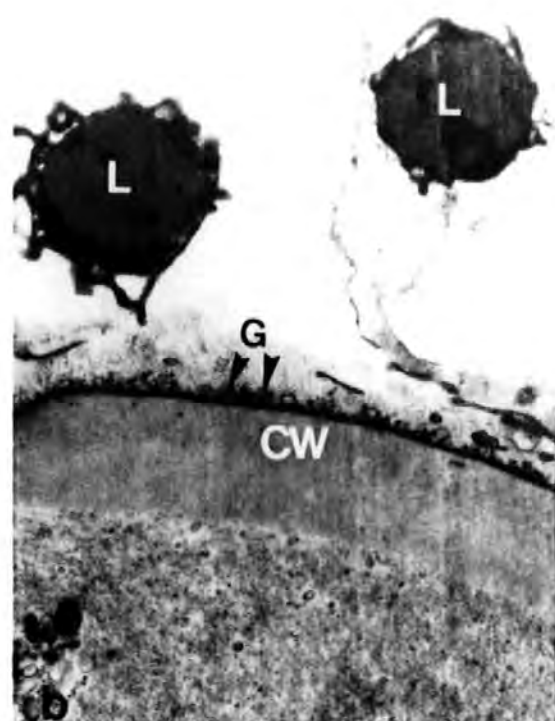
Figure 39 Leucocytes in the host capsule at 4 days post infection

(a) Macrophages (M) and lymphocytes (L) in the host capsule of metacercaria in Group C. (Mag= 15000).

(b) Lymphocytes (L) in the host capsule from Group C. (Mag= 16000).

(c) Lymphocyte in the host capsule from Group D. (Mag= 18000).

(d) Phagocytic cell (P) presumed to be a macrophage and a lymphocyte (L) in the host capsule from Group D. (Mag= 20000).
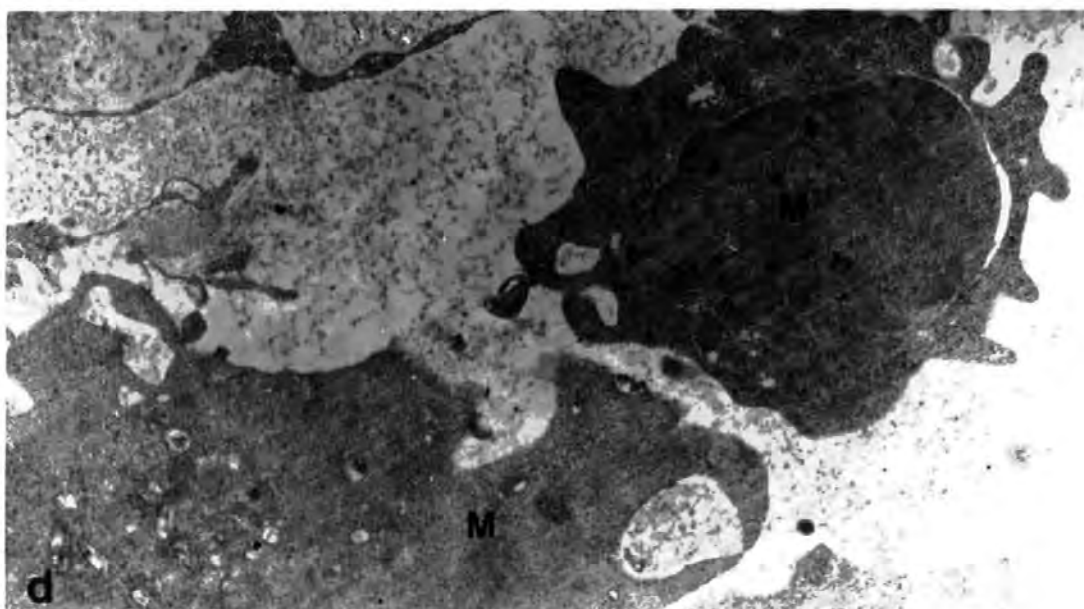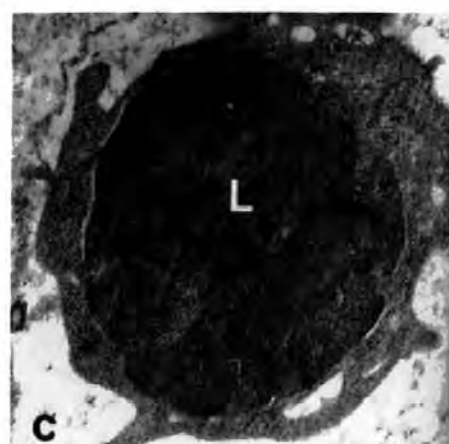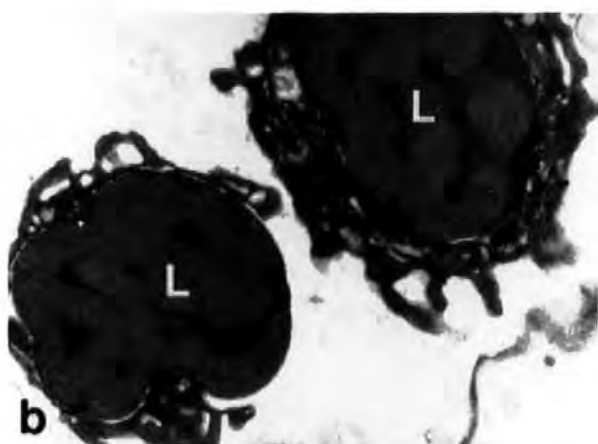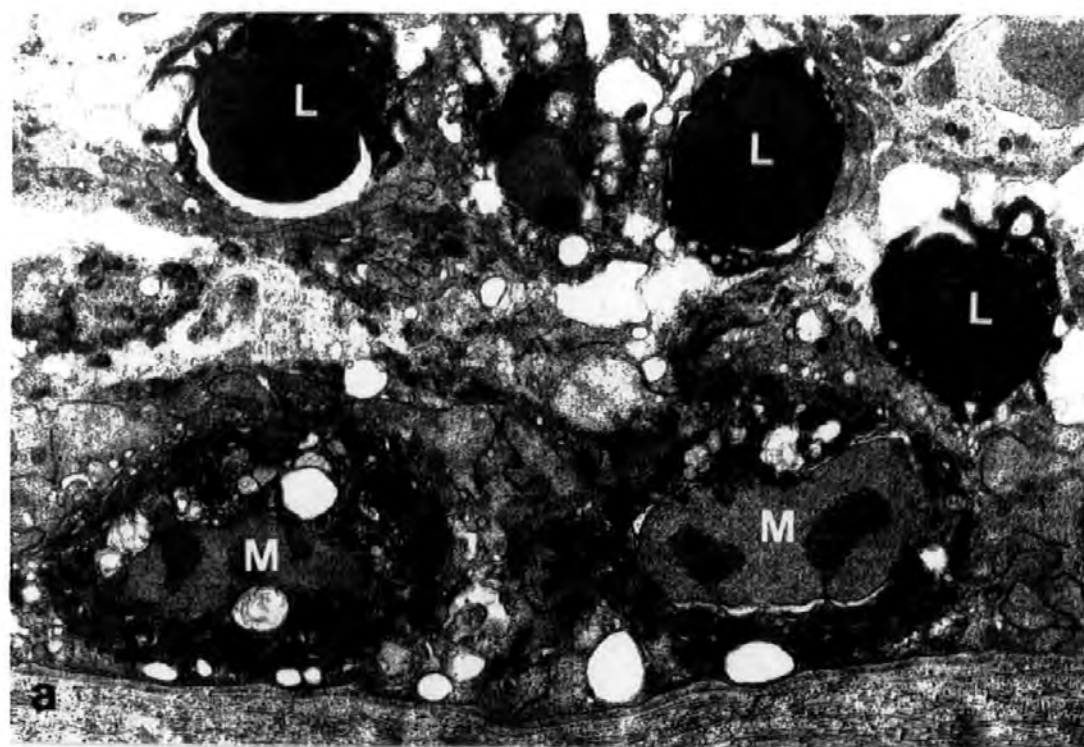
Figure 40 Phagocytic cells in the host capsule at 4 days post infection

(a) Group C. (Mag= 13000).

(b) Group C. (Mag= 15000).

(c) Group D. (Mag= 16000).

(d) Group C. P - phagocytosed material. (Mag= 20000).
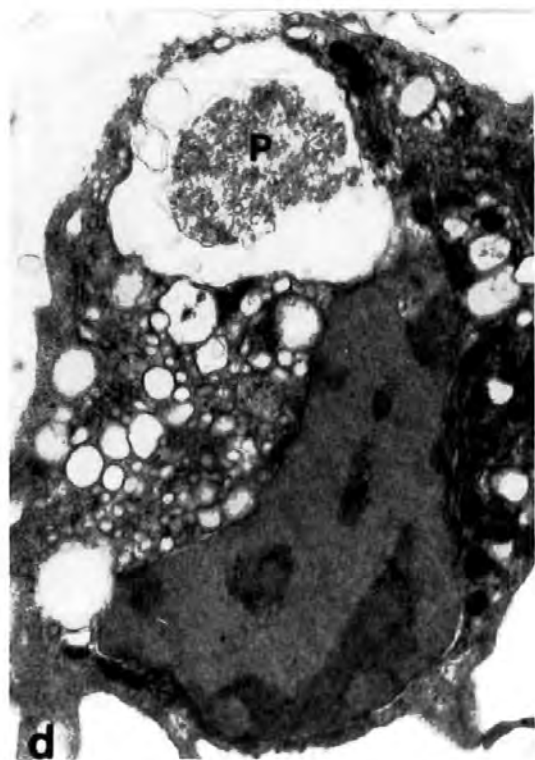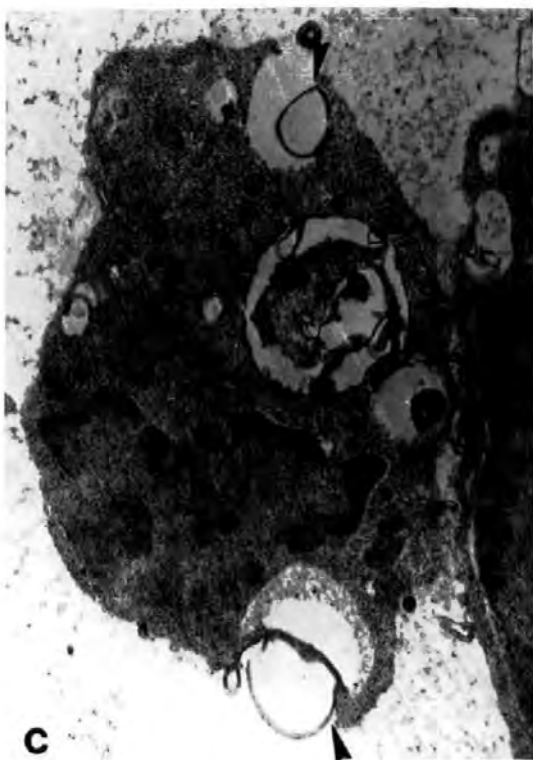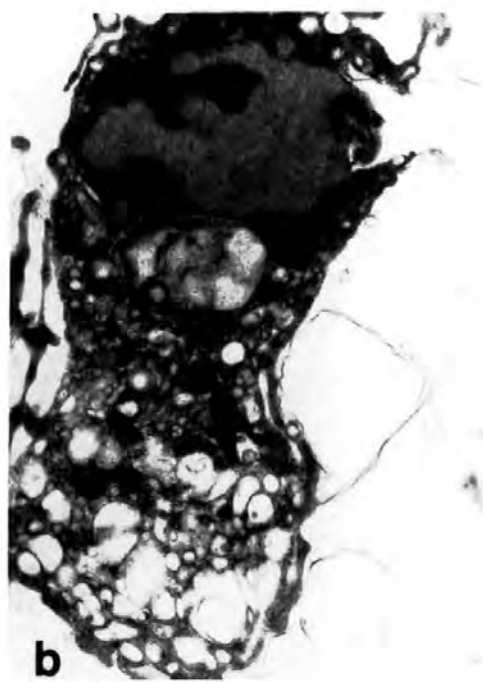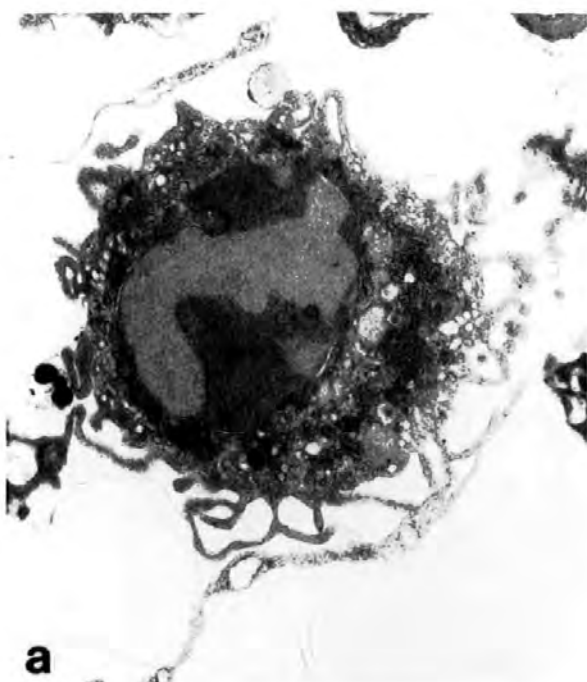
Figure 41 Host-parasite interface at 6 days post infection

(a) Tegument of metacercaria from Group C showing microvillous form. (Mag= 12000).

(b) Structural reorganisation of the tegument from Group B. Melanin bearing (ME) cells associated with the cyst wall (CW) are present in the surrounding host tissue (HT). (Mag= 11000).

(c) Tegument of metacercaria from Group A still in the secretory phase of development. CW - cyst wall, HT - host tissue. (Mag= 15000).

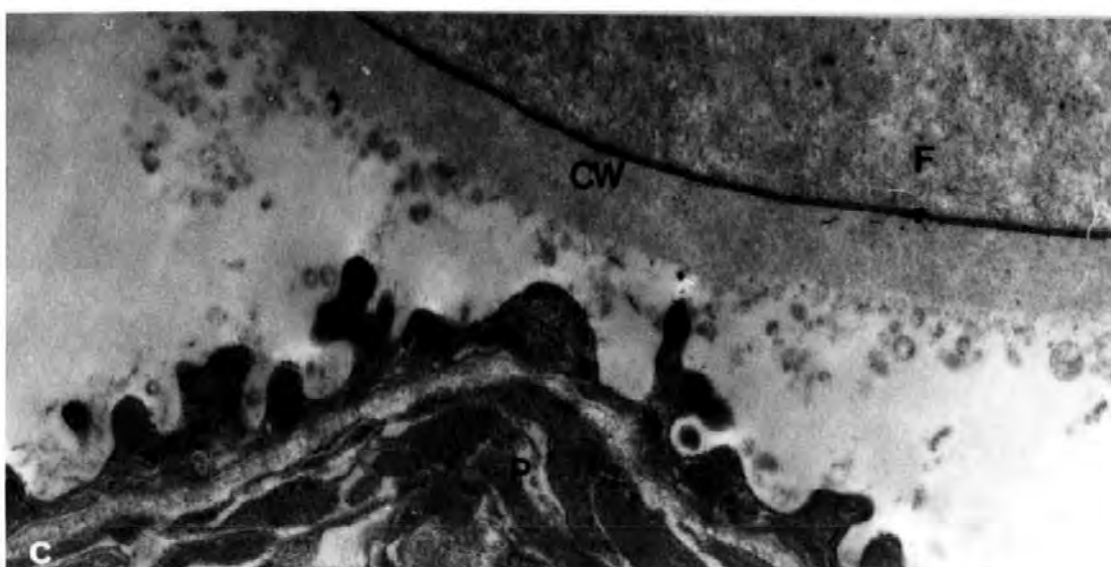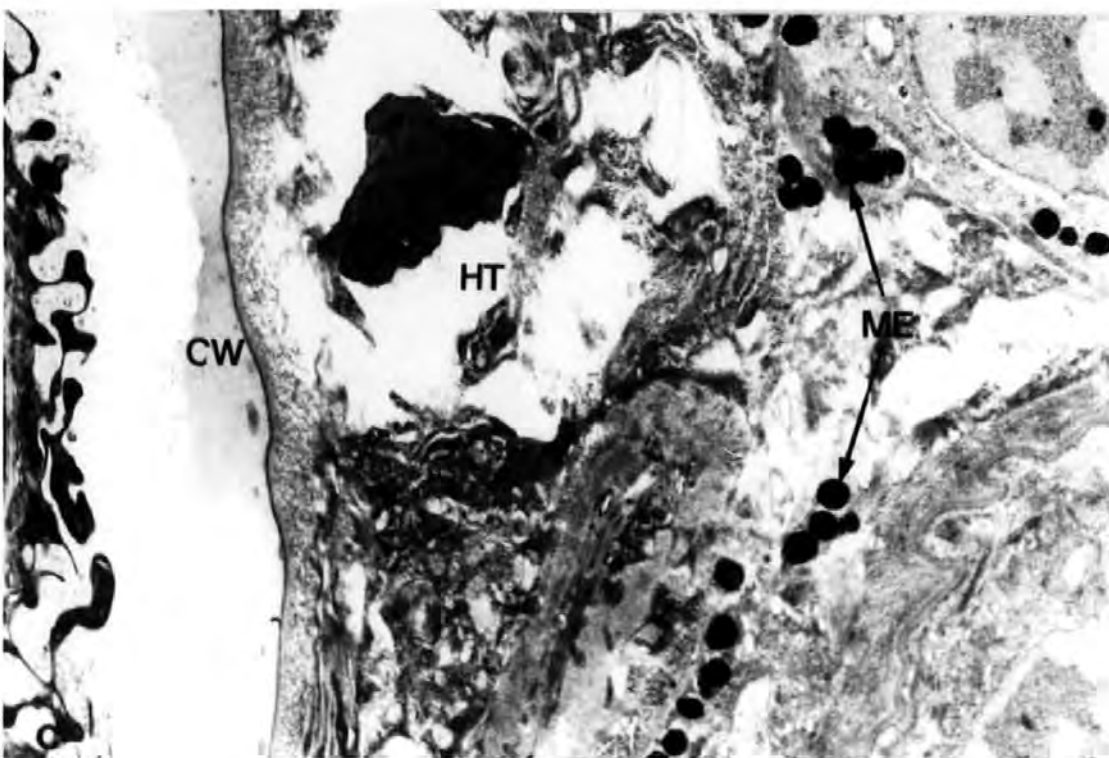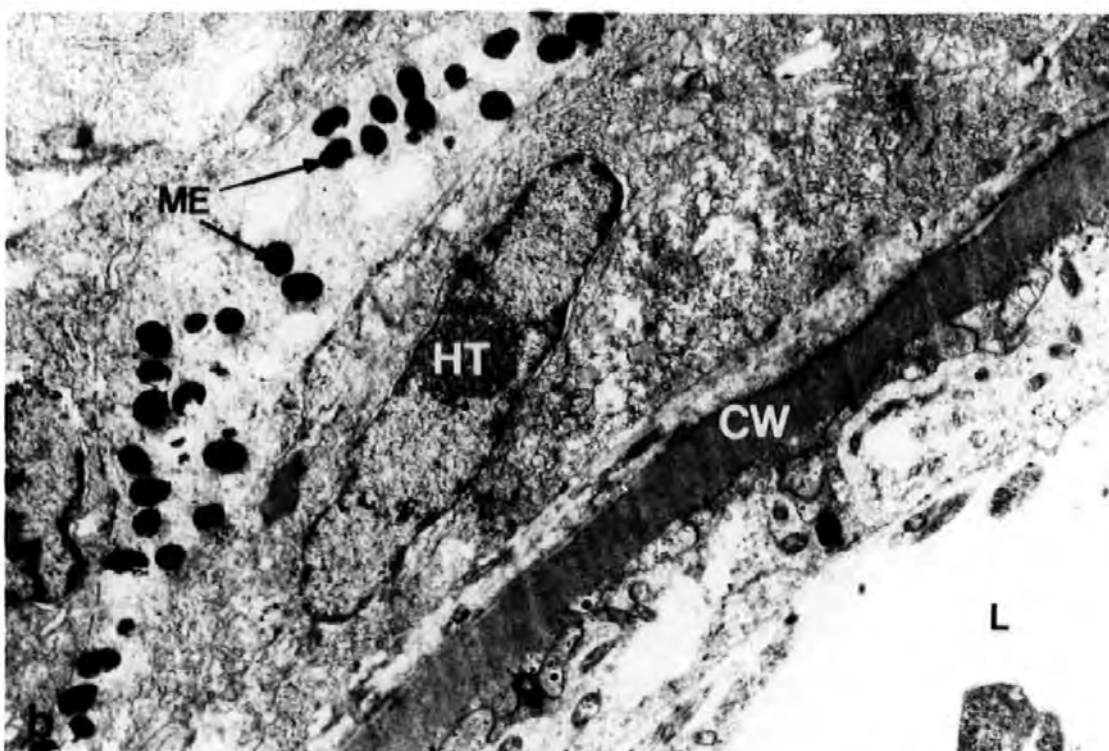**Figure 42** Leucocytes in the host capsule at 6 days post infection

(a) Type 3 granulocyte from Group C. (Mag= 11000).

(b) Type 2 granulocyte from Group C. (Mag= 13000).

(c) Type 3 granulocyte from Group C. (Mag= 16000).

(d) Macrophage from Group D. (Mag= 16000).
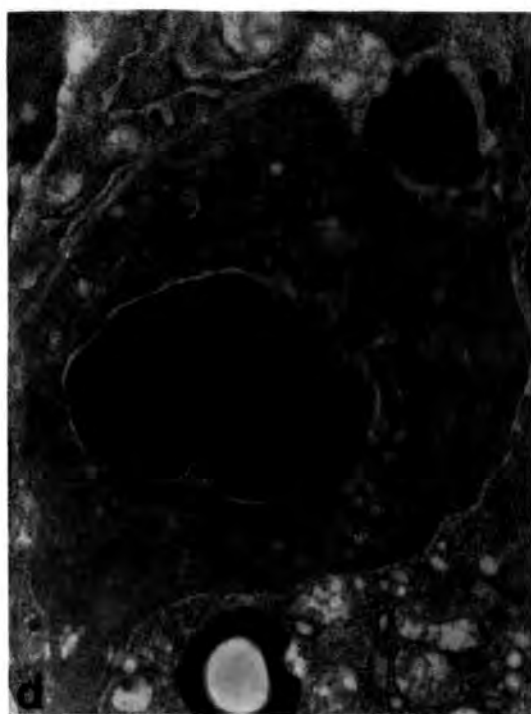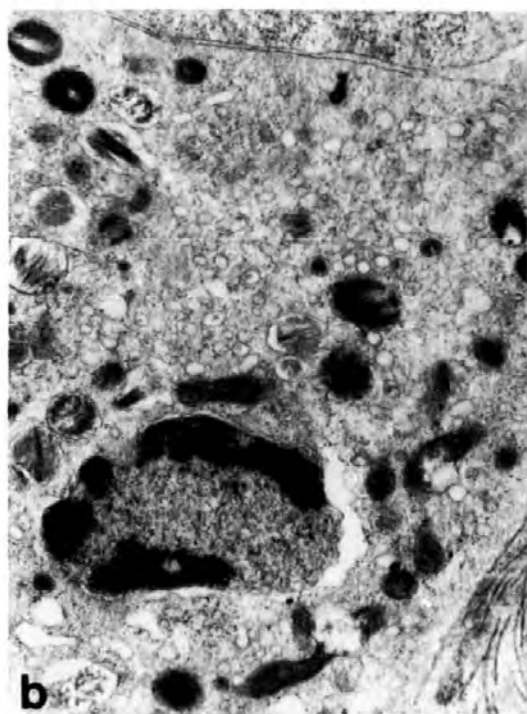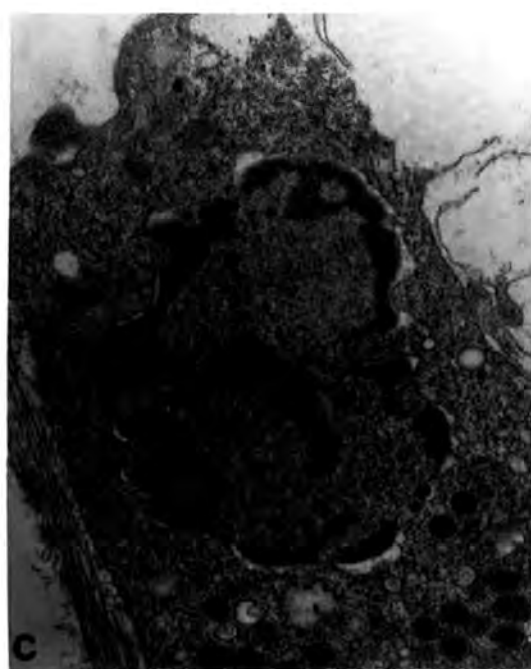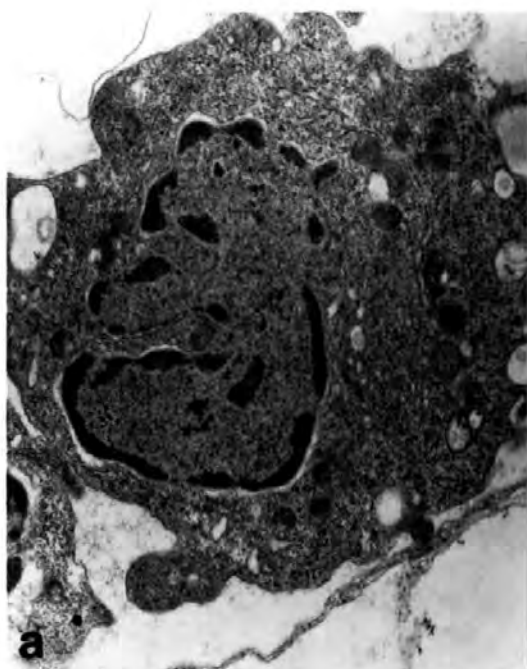
and those immunised prior to infection (Group D) (Fig 42d). One distinctive feature was the appearance of melanin bearing cells in all groups, irrespective of temperature (Fig 41b,c) and the appearence of Type 3 granulocytes (Fig 42c) for the first time. These cells were characterised by the presence of granules within the cytoplasm with an electron dense core and electron lucent outer region.

After 8 days, fish in Groups A and B showed similar host cell responses to those in Groups C and D at day 6 including the presence of Type 2 granulocytes (Fig 43a,b,c,d). Associated with the increased diameter of the cyst, was the compactation of surrounding host cells (Fig 43e).

The tegument of parasites from Group A was still in it's microvillous form at 15 days post infection (44a), however, these had attained their full stage of development at 20 days. The same stage of development was reached earlier in metacercariae from the other groups, ranging from 8 days in Group C to 10 days in Groups B and D. This stage was recognised by the formation of the pre-adult tegument with the loss of microvilli and the formation of new spines (Fig 44b,c). Two new secretion body types were recognised at this time (Fig 44c,d); Type C6, small and granular bodies and C7 with an electron dense core and radiating spokes of electron dense material. Within this period of time, an additional deposition of granular material was observed forming a third inner layer of the cyst wall (Fig 44e) and the amount of granular material deposited at the outer layer of the cyst wall had increased (Fig 44b,e). A fourth granulocyte type (Type 4) was seen for the first time at this stage in Group C fish (Fig 45). The granules of these cells were elongate with a characteristic electron dense spindle.

By 30 days post infection the parasite tegument appeared unchanged (Fig 46a,b) and there was little further change in the host response (Fig 10 47a,b), although plasma cells were recorded peripheral to the capsule (Fig 47c). At this stage there was still evidence of host cell damage resulting from initial migration and encystment. A particular feature of note was the presence of rodlet cells, often in large numbers, in some of the material examined (Fig 48).

Figure 43 Host response to metacercariae at 8 days post infection

(a) Type 2 granulocyte in the host capsule from Group A. (Mag= 11000).

(b) Detail of granules in (a).

(c) Type 2 granulocyte in host capsule from Group B. (Mag= 13000).

(d) Detail of granules in (c).

(e) Compactation of host tissues (HT) surrounding metacercarial cyst. CW - cyst wall, L - lumen. (Mag= 10000).
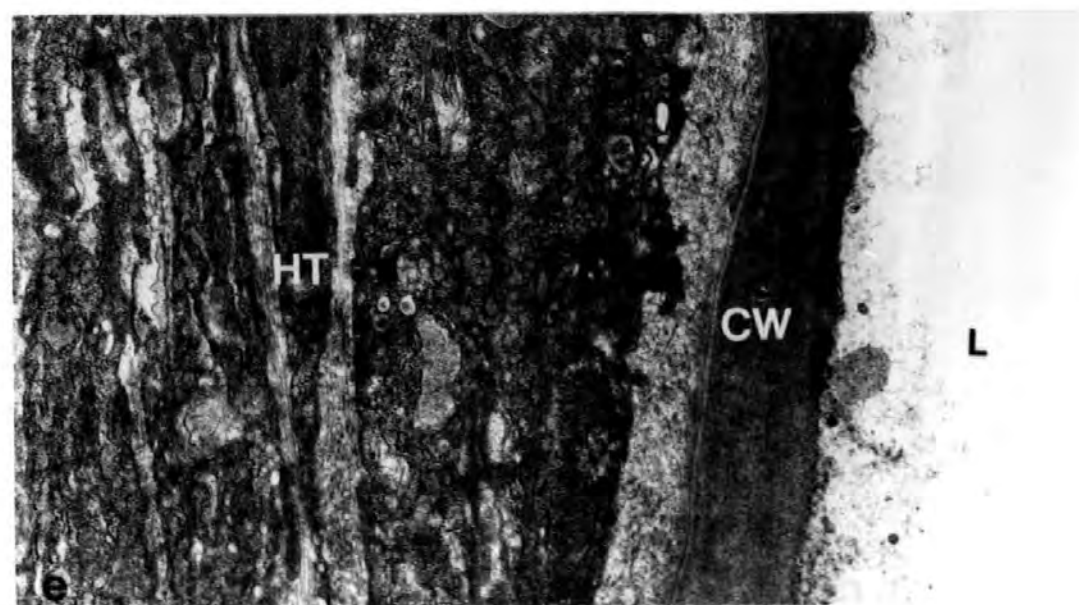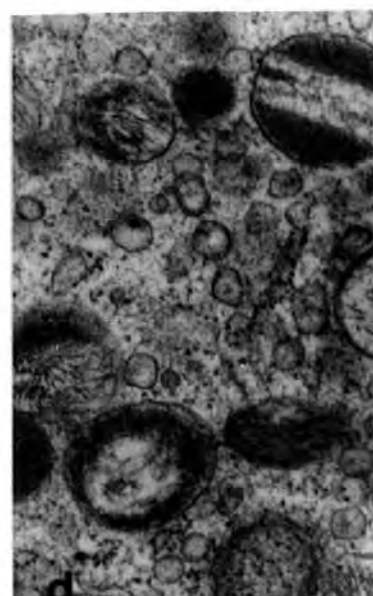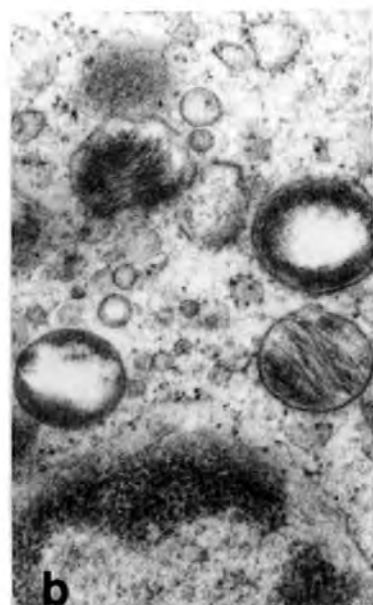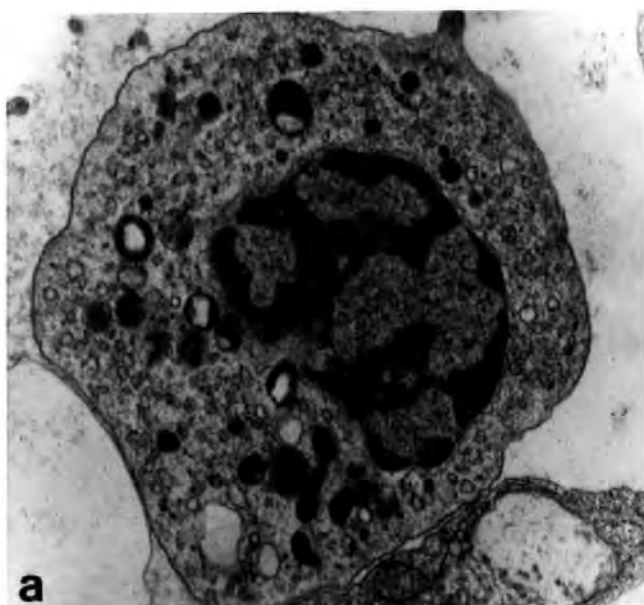
Figure 44 Host-parasite interface at 15-20 days post infection

(a) Tegument from Group A still at the microvillous stage of development at day 15. F- fibrous layer of host capsule, M - microvilli, CW - cyst wall. (Mag= 10000).

(b) Tegument from Group D at the pre adult stage of development after 15 days, showing spines of the future adult (S). CW - cyst wall. (Mag= 13000).

(c) Tegument from Group B at the pre adult developmental stage after 20 days, with adult spines (S) and cystogenous secretion bodies C6 and C7 seen in the tegument. CW - cyst wall, HT - host tissue. (Mag= 11000).

(d) Cystogenous secretion bodies C6 and C7 and adult spines (S) in the tegument from Group B at 20 days post infection. CW - cyst wall. (Mag= 15000).

(e) Four layers of the cyst wall from Group D after 20 days. 1 - innermost diffuse layer; 2 - median granular layer; 3 - outer bilayer; 4 - granular deposits on the exterior surface. (Mag= 32000).
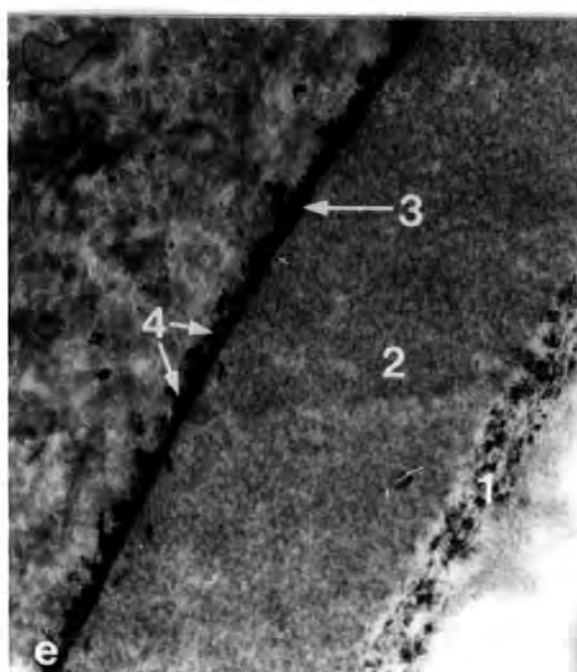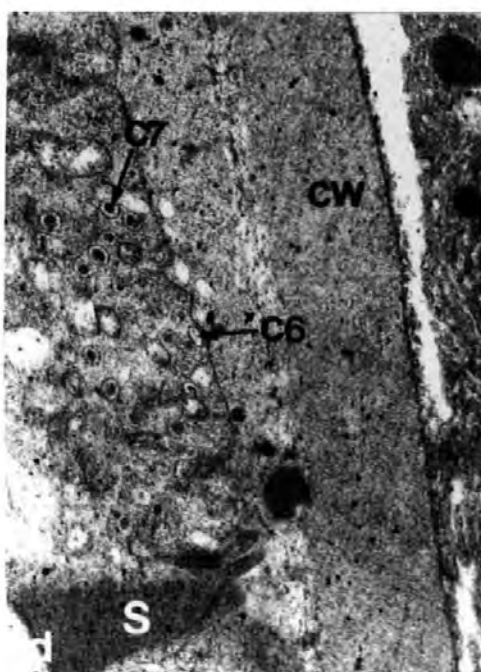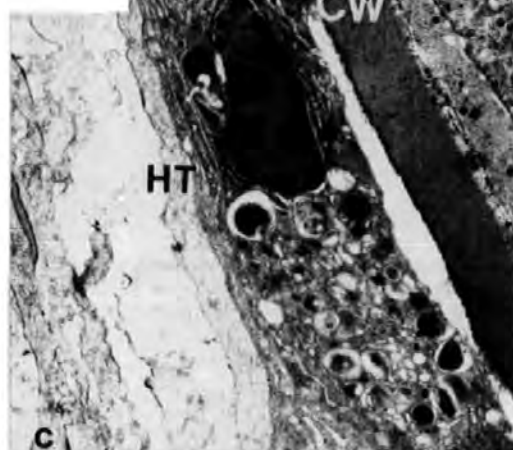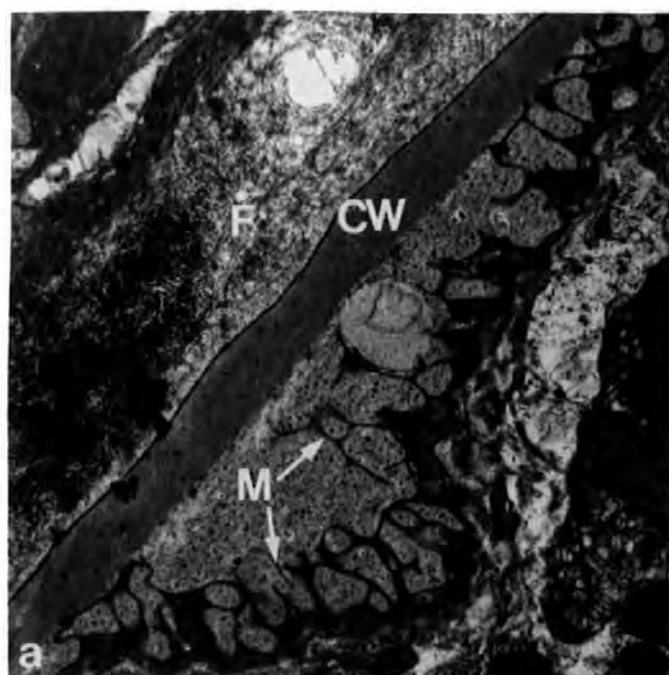
Figure 45 Host capsule at 15 days post infection

Type 4 granulocytes (G) in the host capsule from Group C. (Mag= 12000).
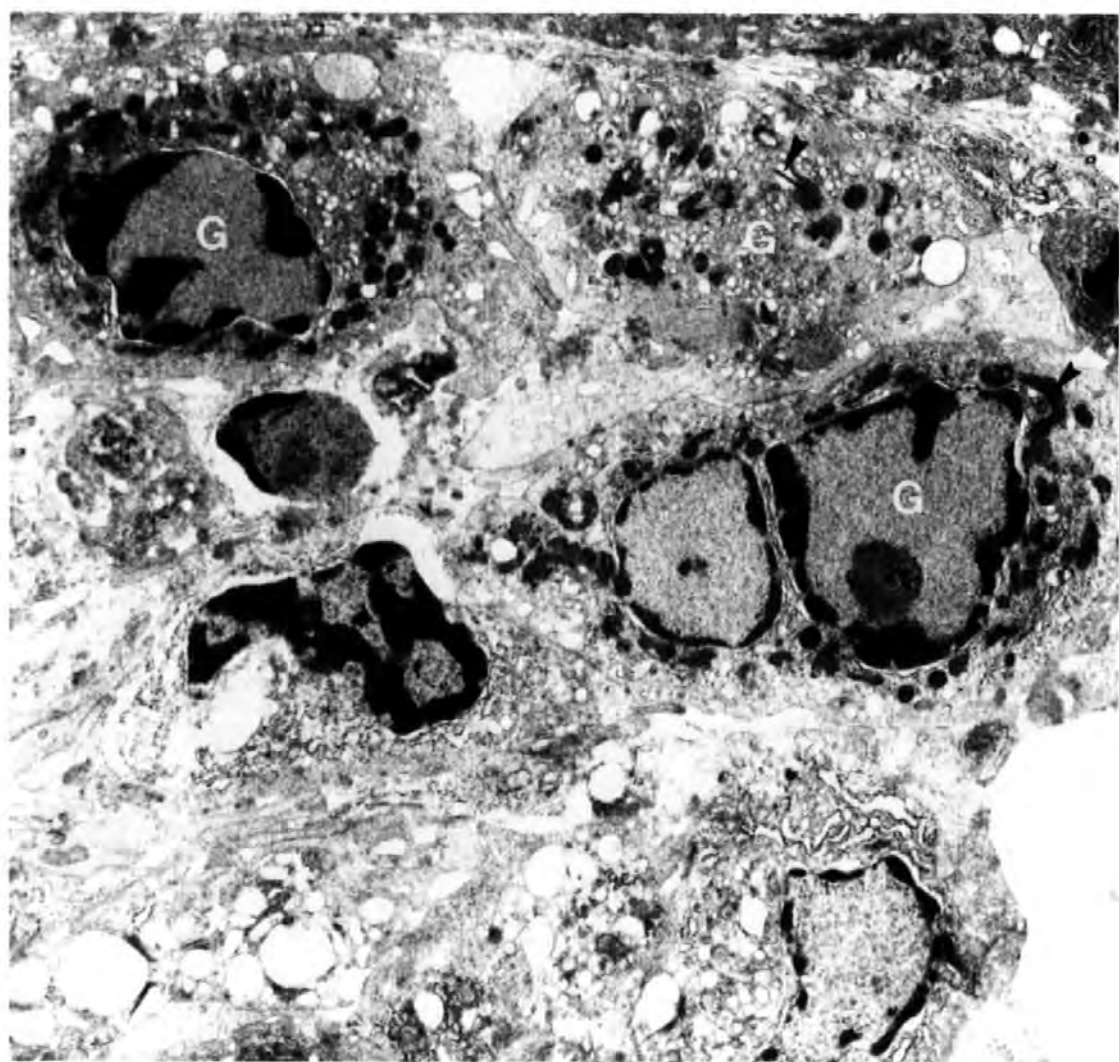
Figure 46 Host-parasite interface at 30 days post infection

(a) Host tissue (HT), cyst wall (CW) and tegument showing presence of secretion bodies and adult spines (S) from Group D. (Mag= 10000).

(b) Fibrous layer of host capsule (F), cyst wall diffuse inner layer (IL), outer bilayer (OL) and granular deposits (G) in the exterior surface and tegument containing secretion bodies and spines of the future adult (S). (Mag= 20000).
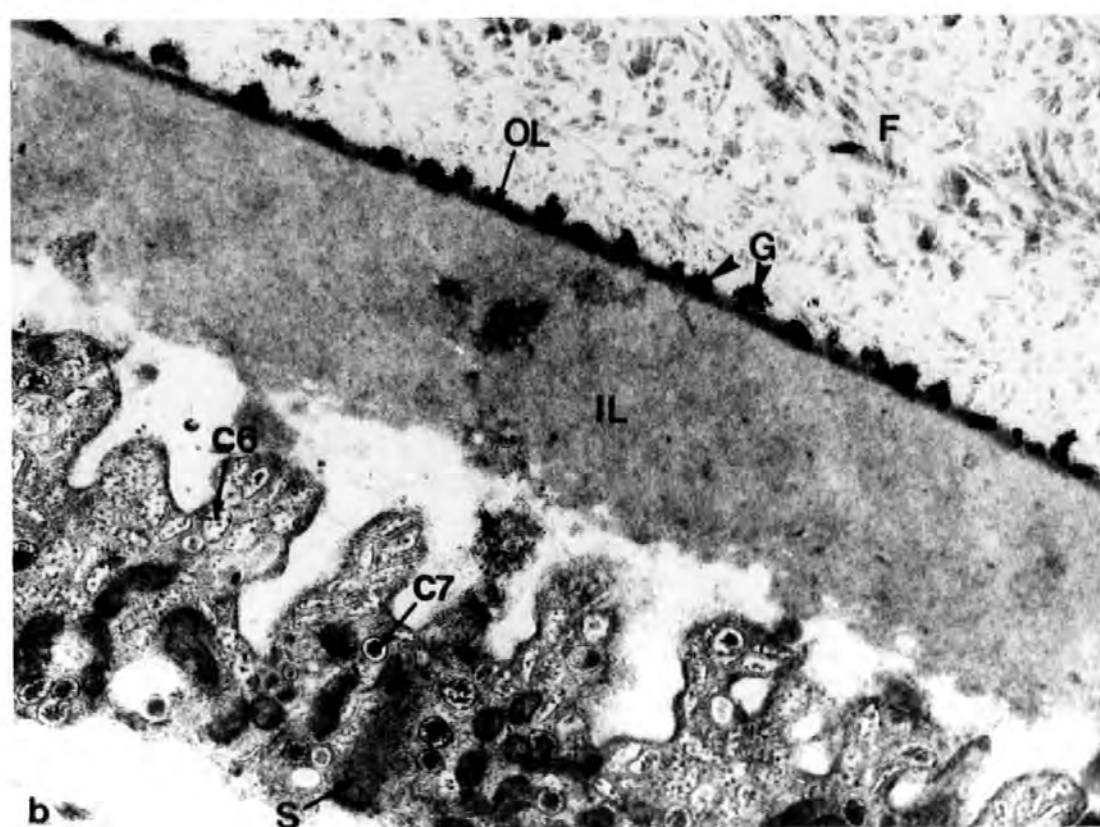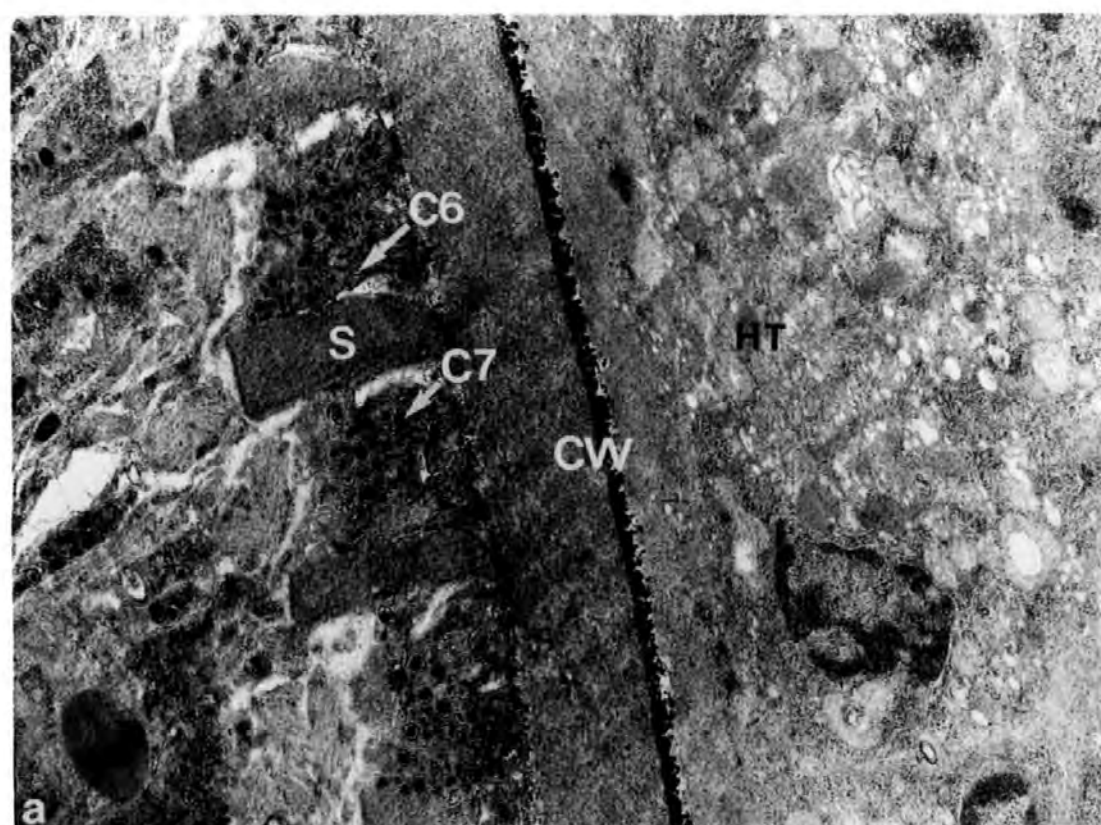
Figure 47 Leucocytes in the host capsule at 30 days post infection

(a) Macrophage in the host capsule of Group D seen actively phagocytosing cell debris (arrow). (Mag= 20000).

(b) A lymphocyte (L) and a polarised lymphocyte (P) from Group D. (Mag= 13500).

(c) Plasma from Group D. (Mag= 14000).
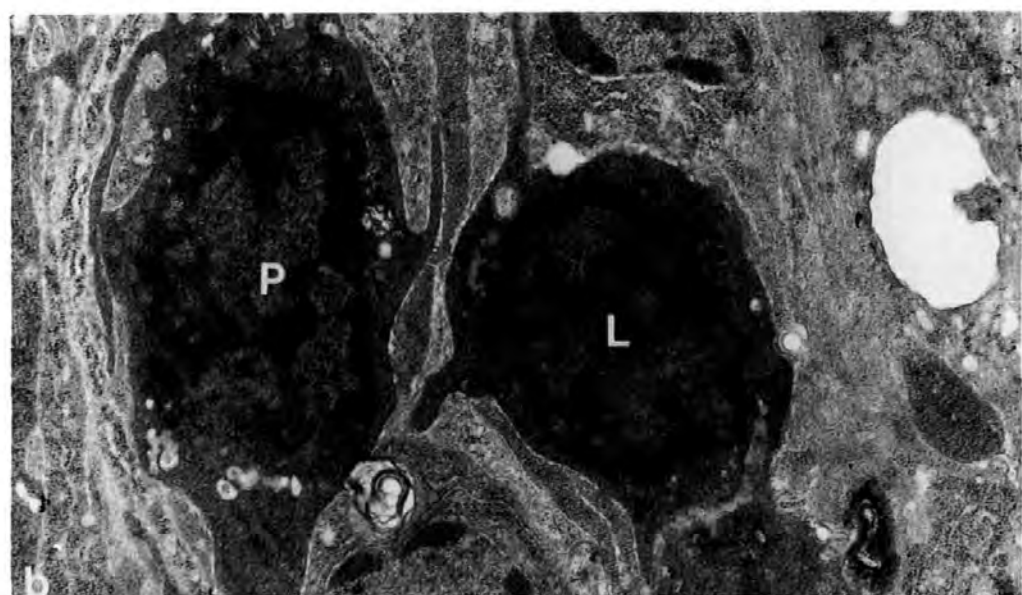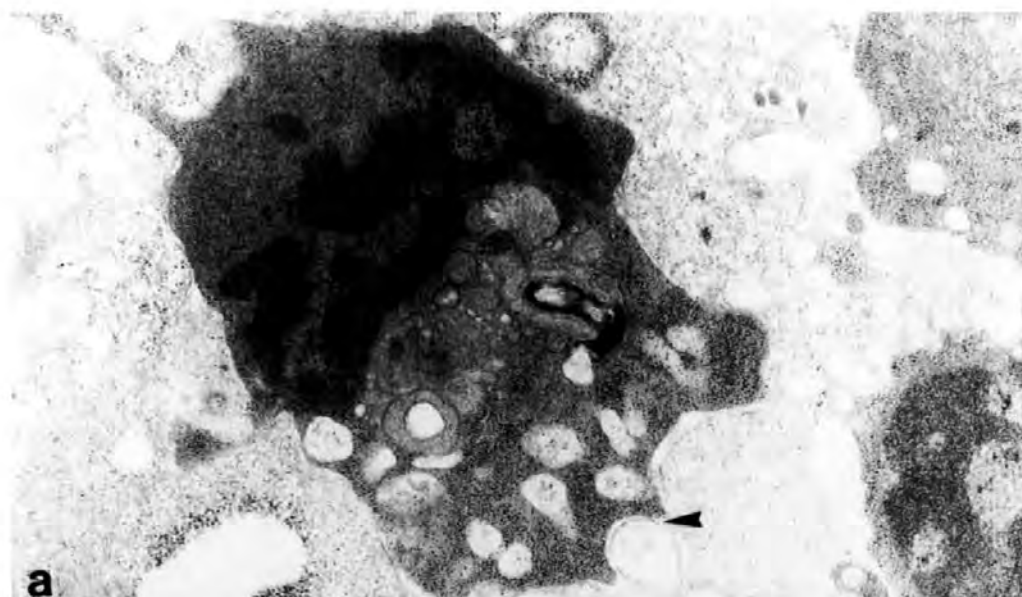
Figure 48 Rodlet cells associated with the host capsule of metacercarial infections

(a) Light micrograph showing numerous rodlet cells (R) associated with the host capsule (HC) in Group B at 15 days post infection. CW - cyst wall, L - cyst lumen. H&E. (Bar= 20μm).

(b) Electron micrograph of a rodlet cell in the host capsule of Group C fish at 10 days post infection. (Mag= 19000).

(c) Electron micrograph of a rodlet cell in the host capsule of Group C fish at 10 days post infection. (Mag= 19000).
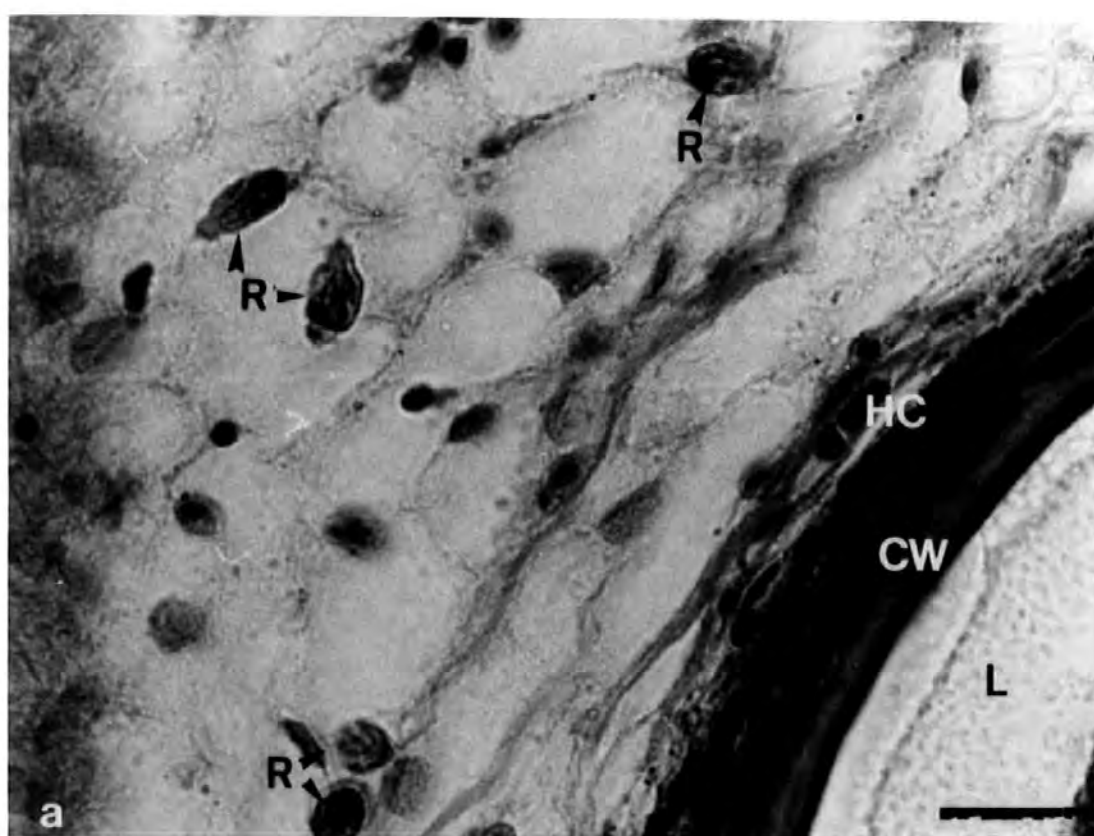
Investigations at 45 days post infection were directed at detection of calcium within the capsule. Calcium detected in the metacercaria was associated with the calcareous corpuscles of the excretory system (Fig 49). Within the host capsule, calcium was associated with melanosomes of melanin bearing cells when measured by x-ray micro-analysis (Fig 50).

Death of metacercariae *in situ* in experimental infections resulted in rapid breakdown of the cyst wall with leucocytic infiltration and removal of metacercarial debris by phagocytic cells (Fig 51).

Effect of light and temperature on the melanin-bearing cell component of the host response

60 0-group mullet were divided into 6 equal groups; A, B and C were maintained under normal conditions of daylight (January-March), approximately 10 h per day, the remainder D, E and F being kept in constant darkness. Controlled temperatures were established for groups as follows; 10°C, A and D; 15°C, B and E; 20°C, C and F. All fish were infected by caudal fin immersion in a suspension of cercariae. Penetration and encystment were monitored and the exact location of individual metacercariae mapped for each fish. A photographic record of *in vivo* development was made over a period of 75 days post infection. Light and electron microscopical studies were made of significant stages and histochemical tests to detect tyrosinase were also undertaken using the DOPA (3,4, dihydroxyphenylalanine) methods of Becker *et al* (Pearse, 1972) and Fitzpatrick *et al* (1950).

Results indicate that melanisation of metacercarial cysts was most intense in fish maintained in total darkness at 20°C after 45 days (Fig 52a). Under darkened conditions the rate of pigmentation was shown to be influenced by temperature, melanin containing cells being associated with cysts within 6 days at 20°C, 10 days at 15°C (Fig 52b) and 20 days at 10°C (Fig 52c). The degree of melanisation increased at each temperature (Fig 53a,b,c) with the appearance of pigment bearing cells around the parasite cyst. In contrast, melanisation was minimal in fish maintained under conditions of normal daylight, pigment bearing cells rarely being observed in association with the parasite cyst at any of the 3 temperatures investigated (Fig 54a,b,c).

Figure 49 Calcification of the host capsule in metacercarial infections

Calcium deposits (C) are present in host tissues and parasite (P). CW - cyst wall, M - melanin bearing cells, B - bone. Alizarin red S. (Bar= 150μm).
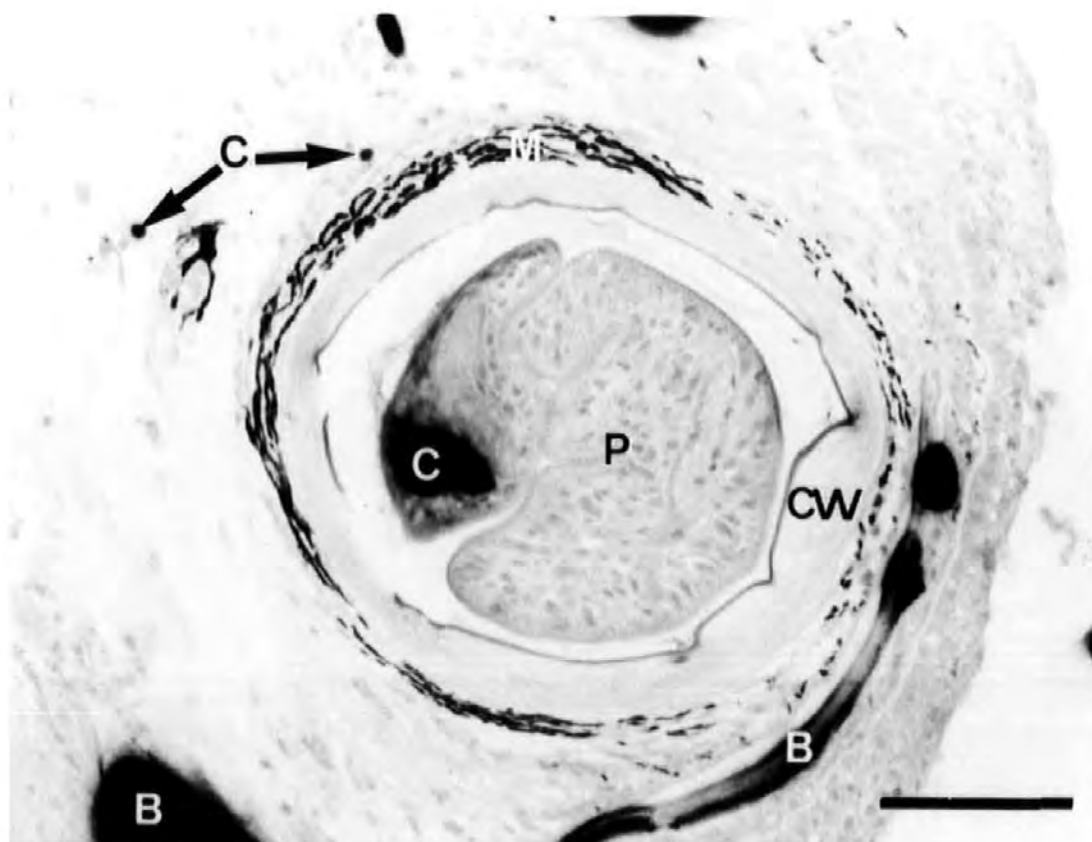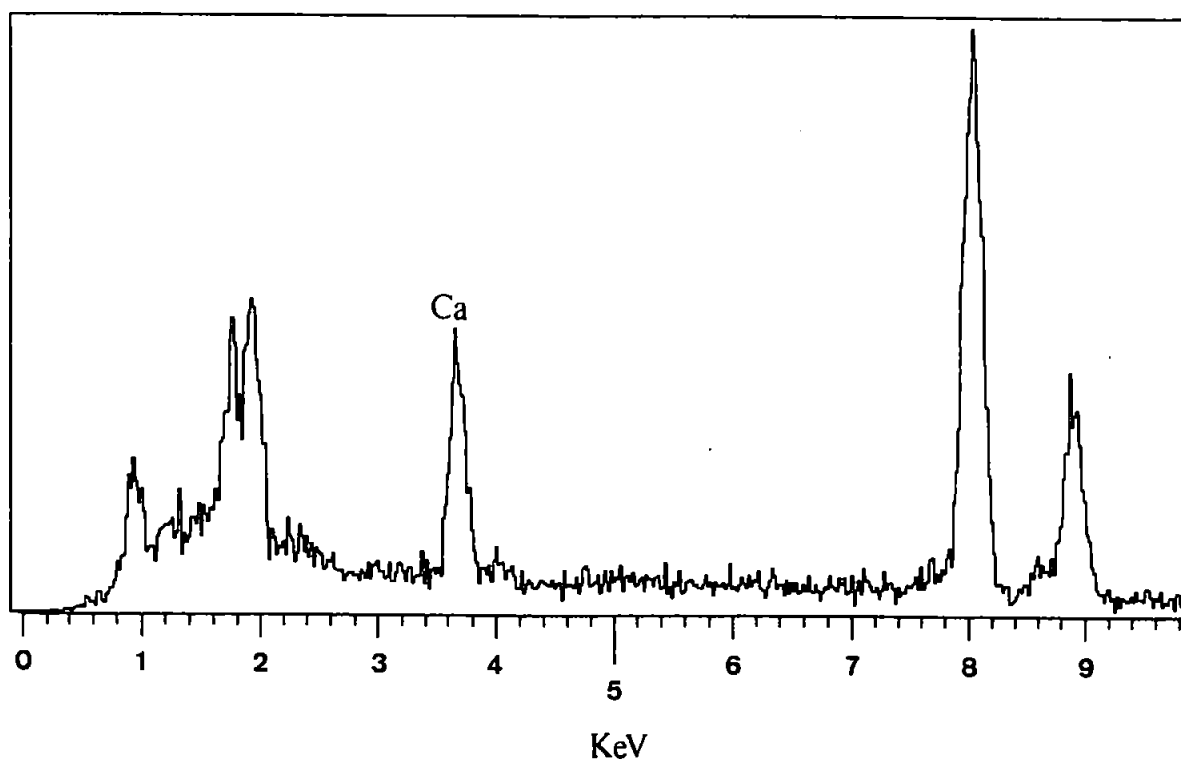
Figure 50 X-ray micro-analysis of the host capsule

Scan of a melanosome of a melanin bearing cell in the host capsule showing a peak (Ca) corresponding with relatively high levels of calcium.

Figure 51 Cellular infiltration after death of the metacercaria

(a) Light micrograph of two adjacent metacercariae showing necrosis of the parasite (P) with breakdown of the cyst wall (C) and leucocytic infiltration. H&E. (Bar= 100μm).

(b) & (c) Electron micrographs showing granulocyte (type 1) degranulation within necrotic metacercarial tissue. (Mag= 20000).
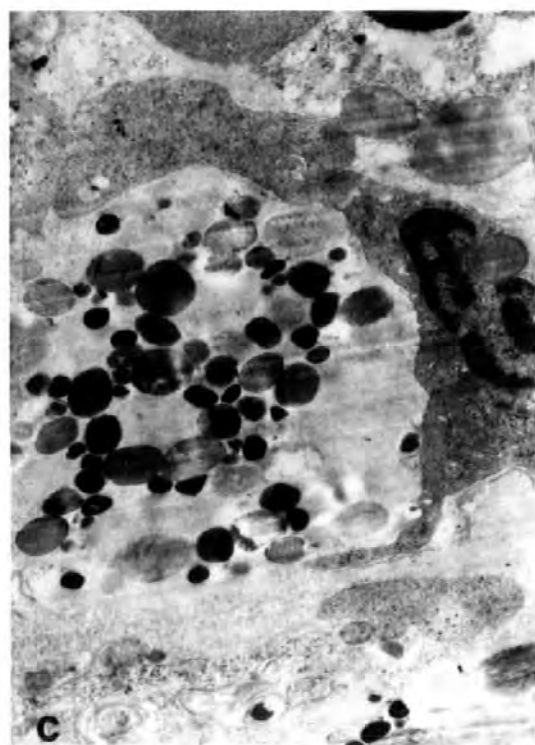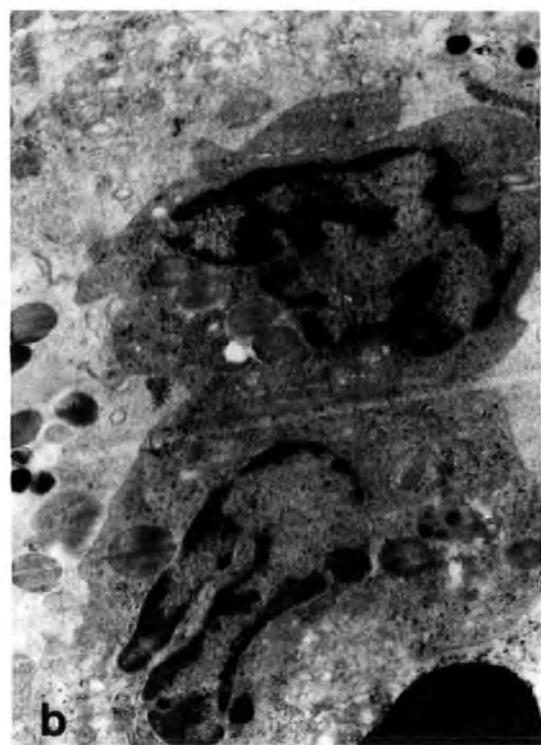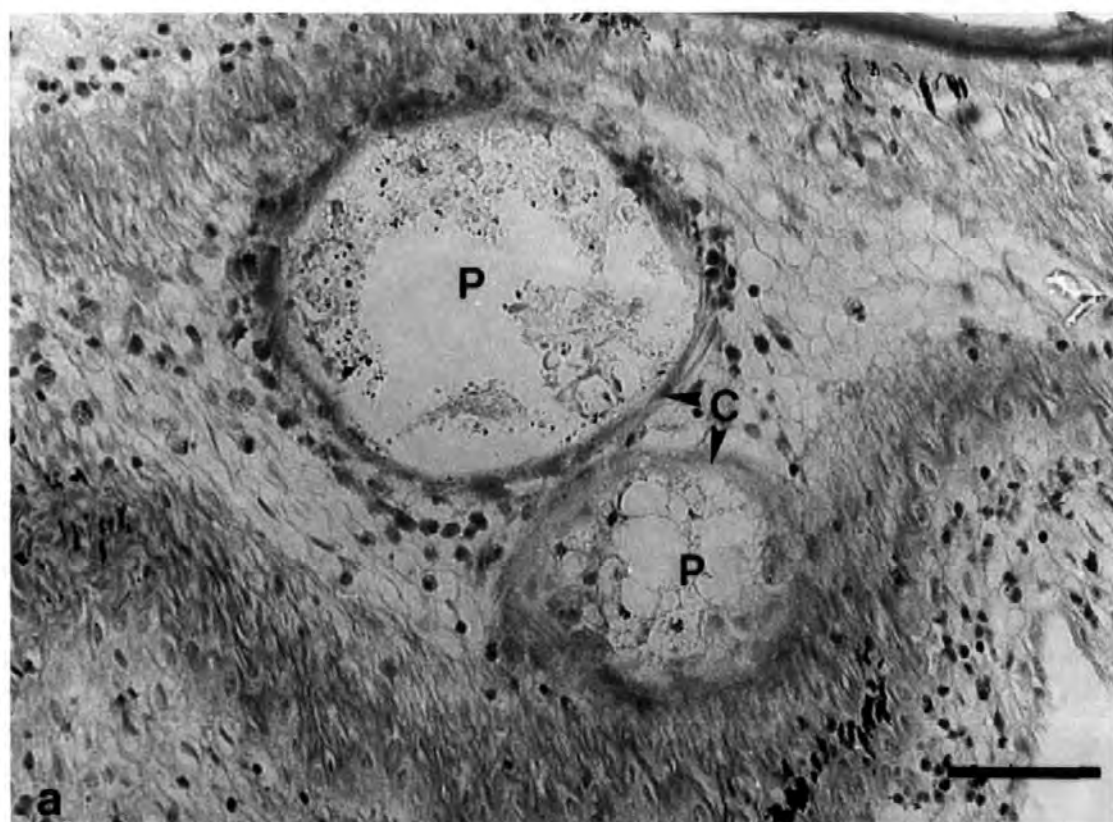
Figure 52 Melanisation of the host capsule in mullet held in total darkness

(a) Melanisation after 45 days at 20°C. (Bar= 100μm).

(b) Melanisation after 10 days at 15°C. (Bar= 100μm).

(c) Mealnisation after 20 days at 10°C. (Bar= 100μm).

M - melanised cyst; E - eye spot; C - cyst wall; FR - fin ray.
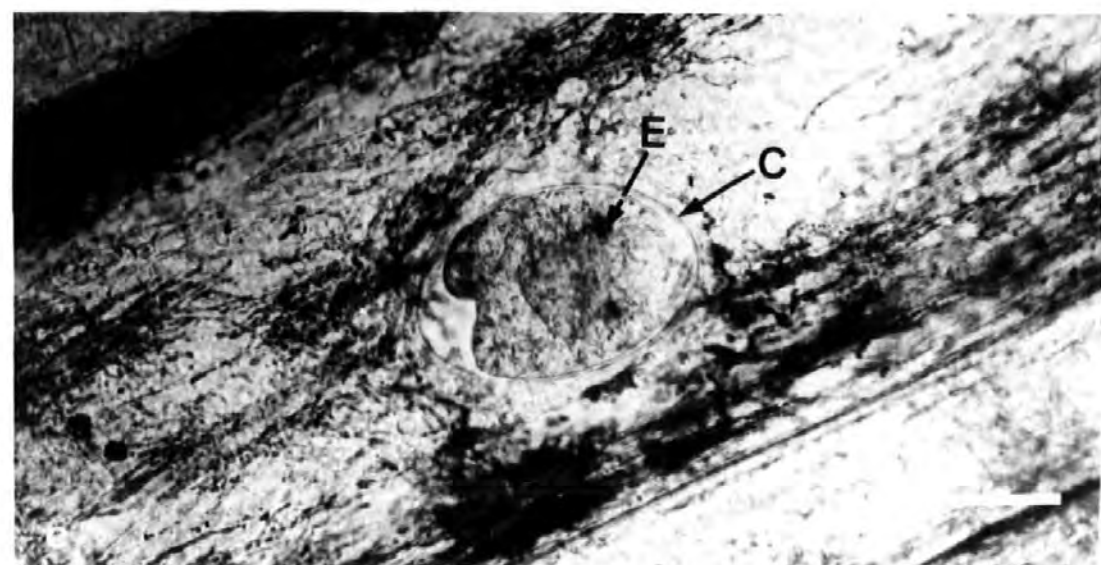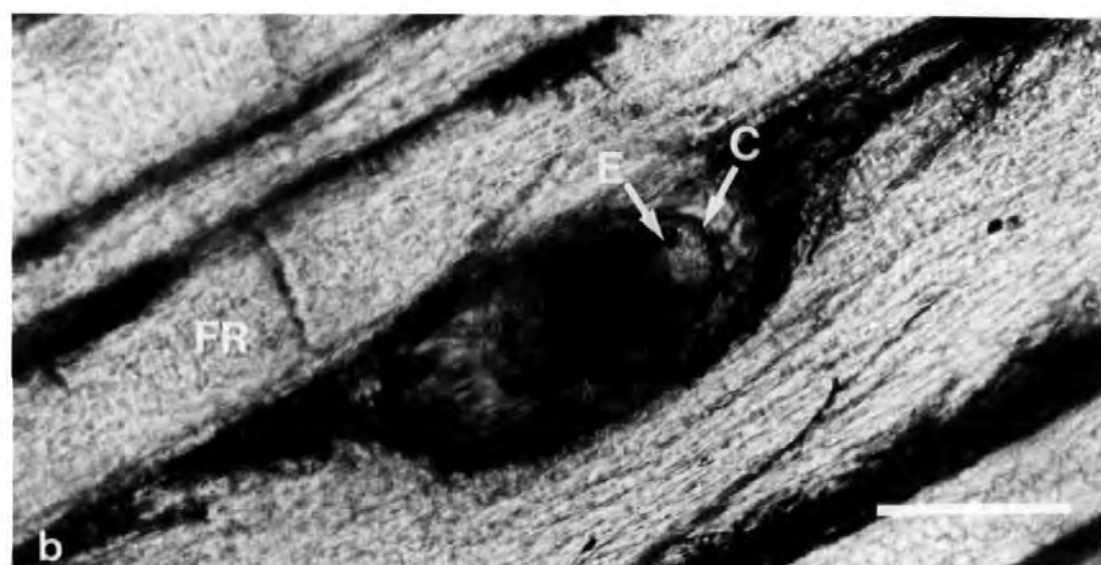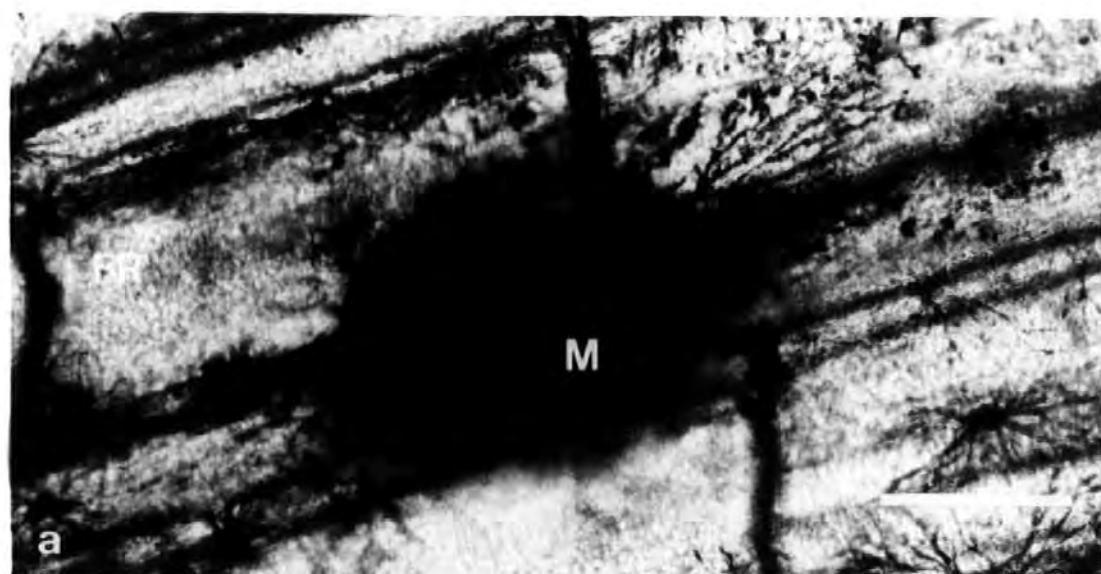
Figure 53 Effect of temperature on the rate of melanisation of the host capsule in mullet held in total darkness

(a) Melanisation after 60 days at 10°C. (Bar= 100μm).

(b) Melanisation after 45 days at 15°C. (Bar= 100μm).

(c) Melanisation after 14 days at 20°C. (Bar= 100μm).

P - parasite; M - melanised cyst; E - eye spot; C - cyst wall; FR - fin ray.
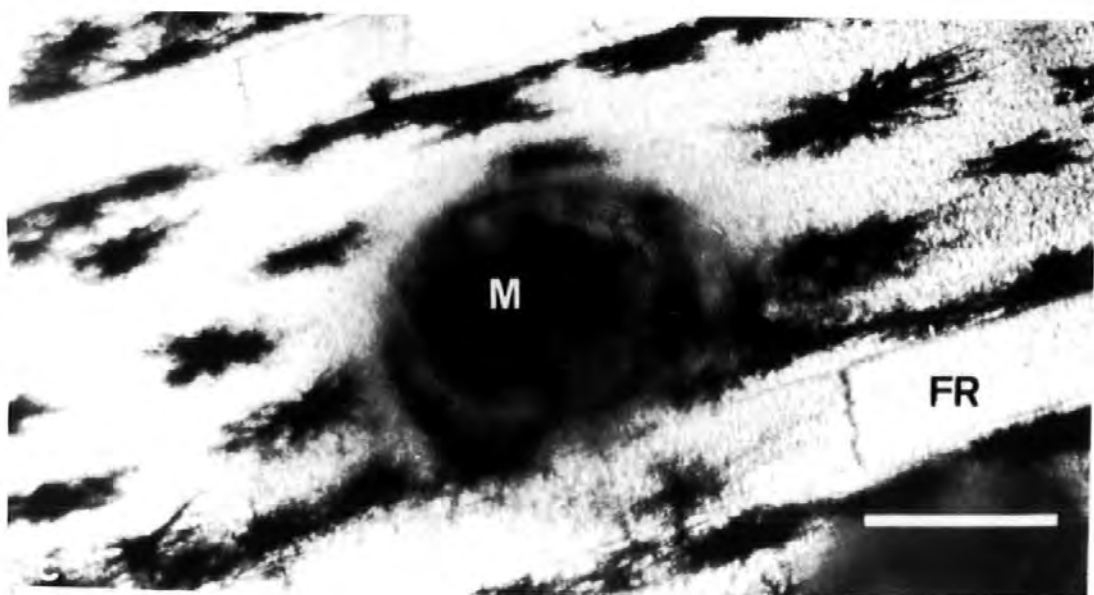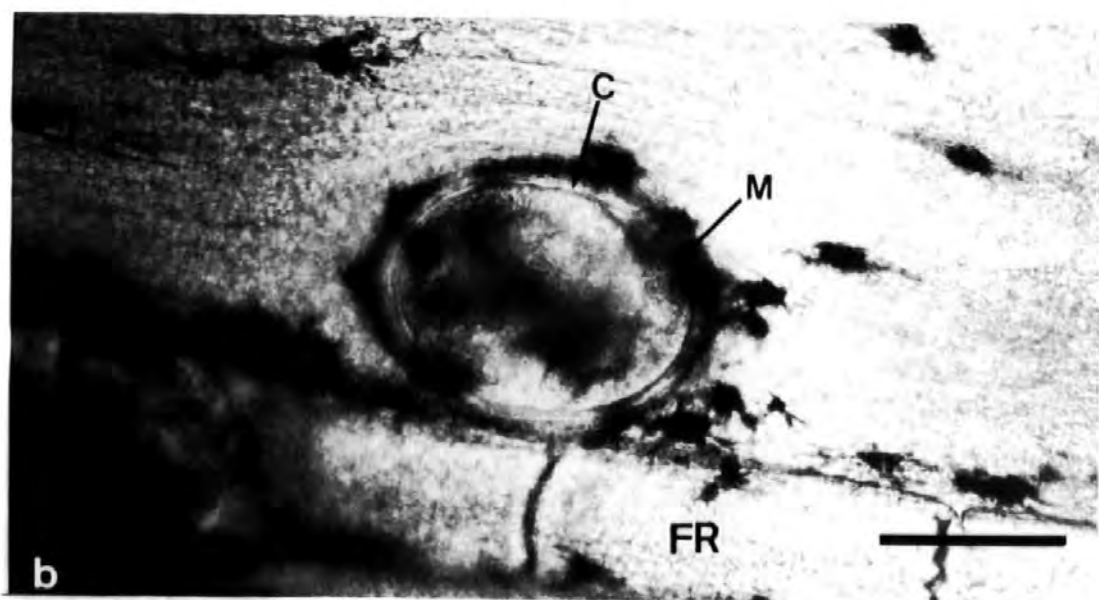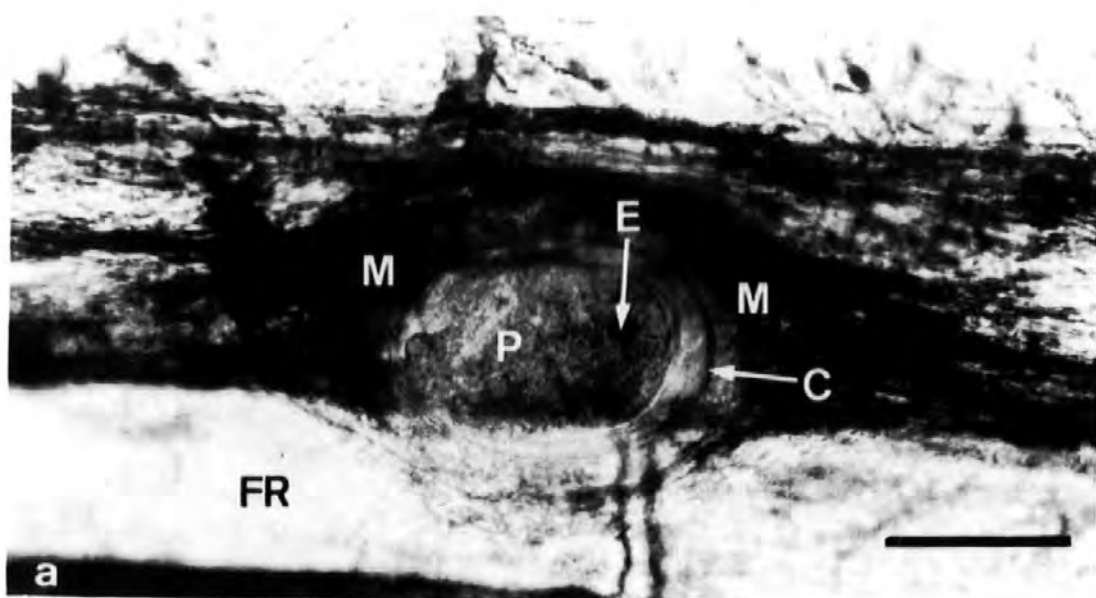
Figure 54 Effect of temperature on the host capsule of mullet held under normal light conditions

(a) After 45 days at 10°C. (Bar= 100μm).

(b) After 45 days at 15°C. (Bar= 100μm).

(c) After 45 days at 20°C. (Bar= 100μm).

P - parasite; E - eye spot; C - cyst wall; FR - fin ray.
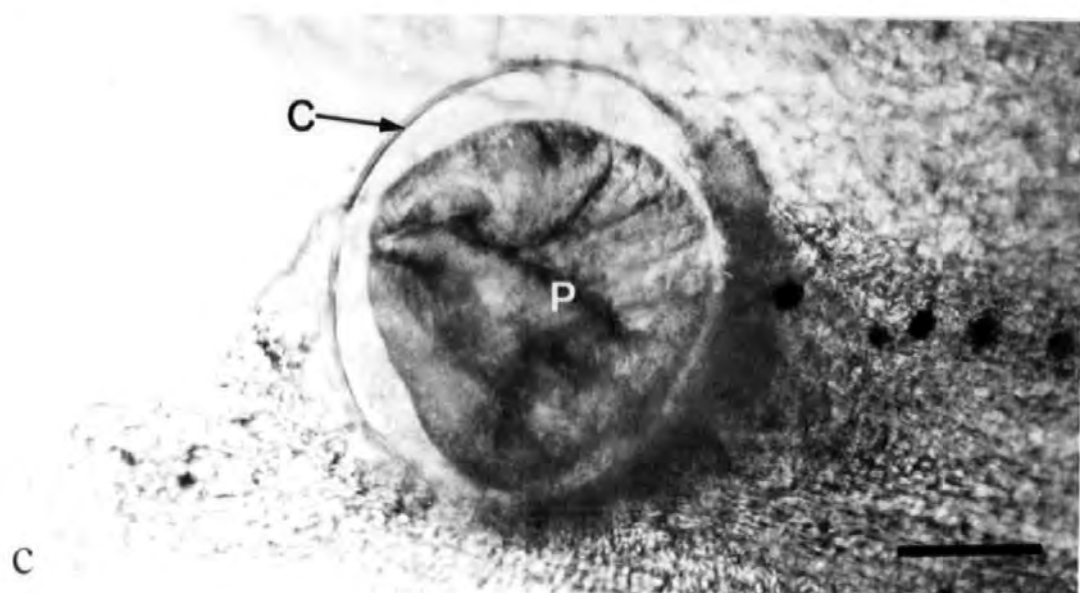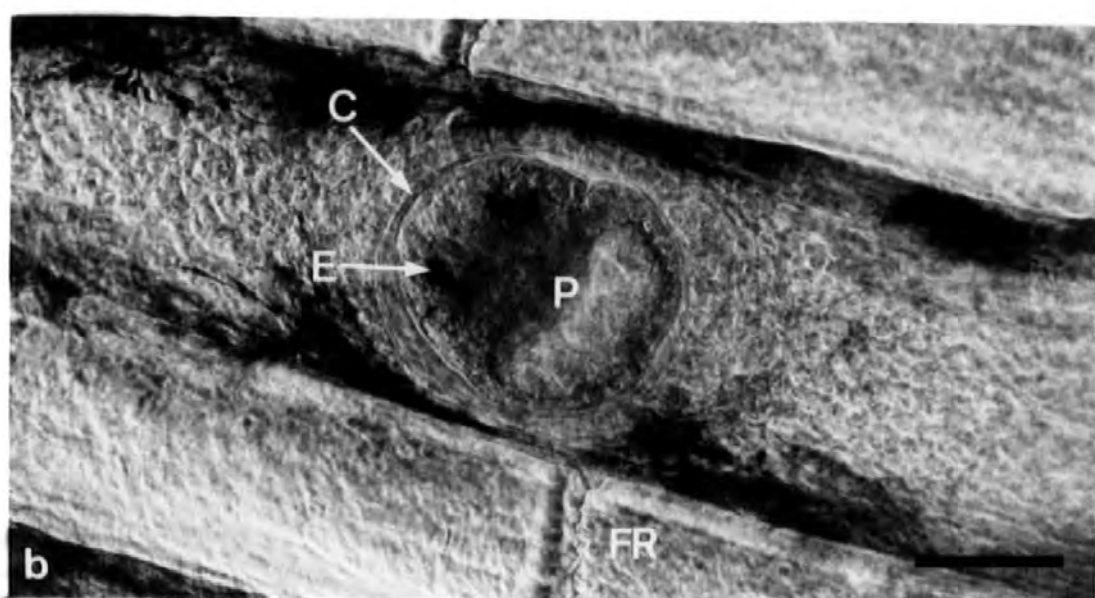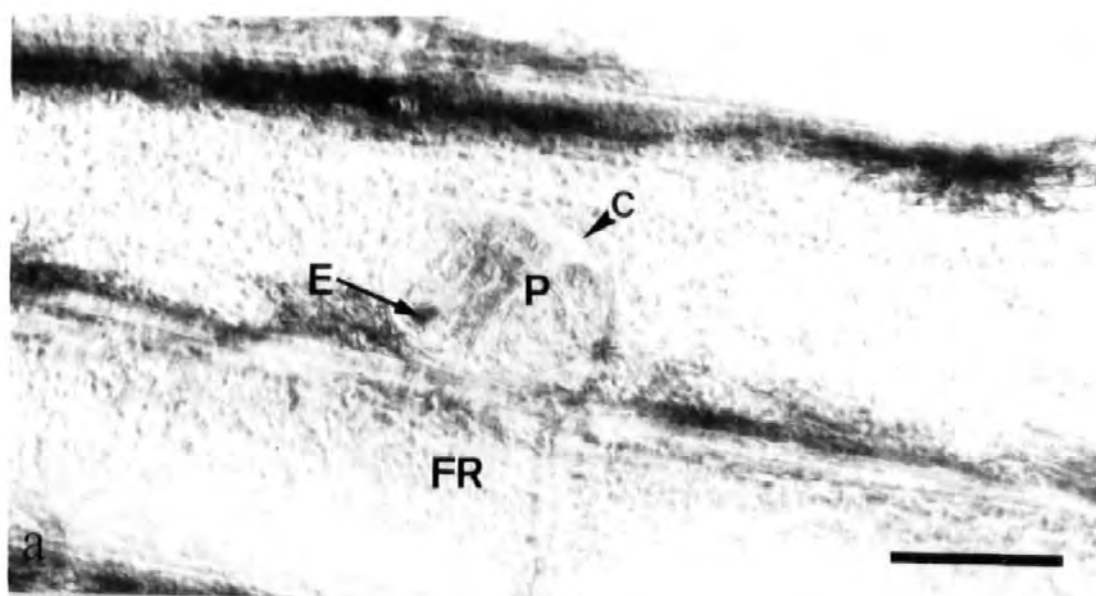
Histological studies at both light and EM levels (Fig 55) confirmed the presence of melanin containing cells within the fibrous host capsule surrounding the cyst wall of the parasite in fish maintained in constant darkness at 20°C after 45 days. These cells were not detected however, in those held in light conditions at the same temperature after 45 days (Fig 56).

The parasite cyst and host capsule from fish maintained in constant darkness gave negative results to the DOPA test of Becker et al (Pearse, 1972). The validity of this test however, is in some doubt here in view of the absence of positive controls. DOPA positive cells (ie those containing tyrosinase) were detected however, using the modification of the method described by Fitzpatrick et al (1950) with samples from fish maintained under normal light conditions and from those held in constant darkness. Positive cells were located in the dermis (Fig 57a) and associated with host capsular material surrounding parasite cysts (Fig 57b) from both groups of fish. Although quantitative studies were not undertaken, there generally appeared to be no significant difference between the number of DOPA staining cells surrounding cysts in fish from either group. Melanin produced by the DOPA reaction and naturally occurring melanin could be differentiated by the relatively pale orange-brown colour of the DOPA pigment compared with the darker brown-black colour of natural melanin.

Section 4: Identification and isolation of parasite antigens

Investigations aimed to identify major antigens in the life cycle of C. lingua, in particular those of cercarial and metacercarial origin relevant to parasite survival within the fish host. Comparative studies of these with the redial stage of the life cycle were made to identify common antigens. As success of the methods involved are dependant upon the quantity and purity of antigen it is necessary first to consider the approaches taken in obtaining the appropriate stages free from host material and in sufficient quantities for the application of electrophoretic techniques. In addition to methods for the isolation of these stages described earlier, namely transformation and encystment of metacercariae in fish skin and in ovo, experiments were undertaken in attempts to artificially induce encystment of metacercariae without exposure to the fish host. These are described first below.

Figure 55 Melanisation of the host capsule in mullet maintained in total darkness

(a) Light micrograph showing hevily melanised host capsule (M) after 45 days at 20°C. Mallory's triple stain. (Bar= 100µm).

(b) Electron micrograph showing a melanin bearing cell (M) in the host capsule at 45 days post infection at 20°C. (Mag= 6000).

P - parasite; CW - cyst wall; FC - fibrous layer of the host capsule.
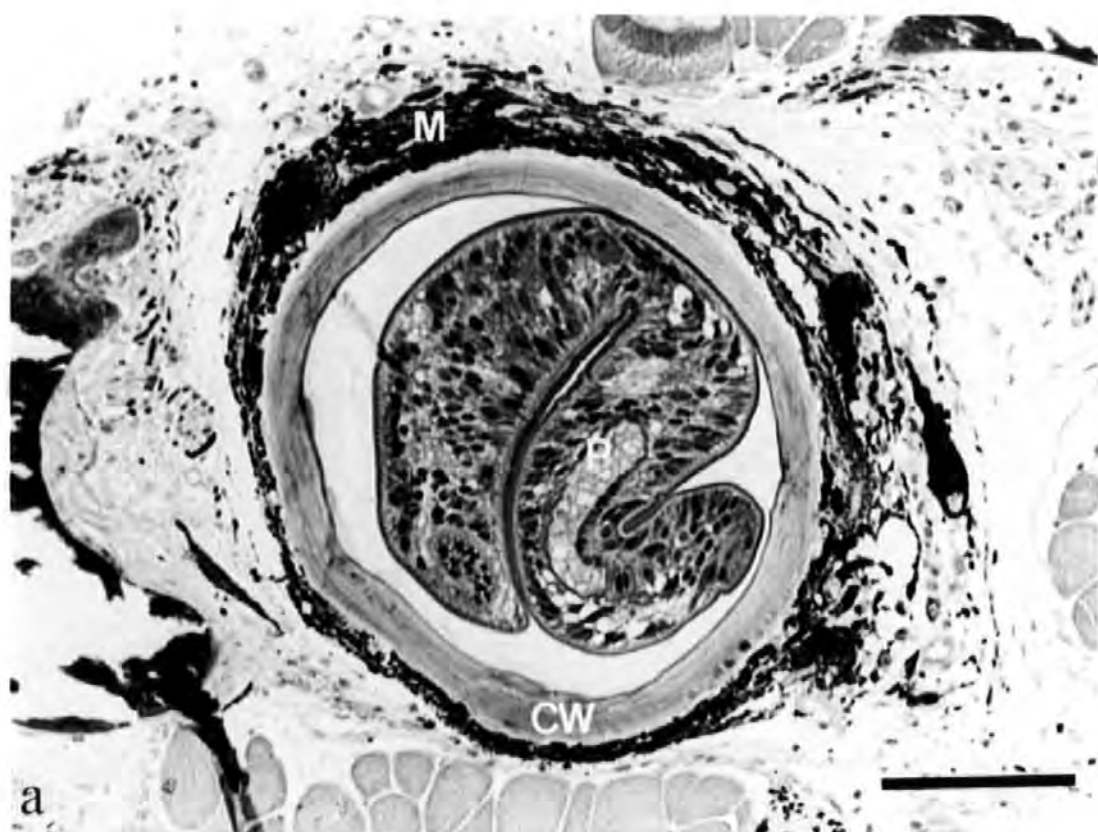
Figure 56 Host capsule of mullet held under normal light conditions

(a) Light micrograph showing the absence of melanin bearing cells within the host capsule after 45 days at 20°C. Mallory's triple stain. (Bar= 100μm).

(b) Electron micrograph of the host capsule after 45 days at 20°C. (Mag= 6000).

P - parasite; T - tegument; CW - cyst wall; FC - fibrous layer of the host capsule.

a

P

CW

b

FC

CW

T

HT
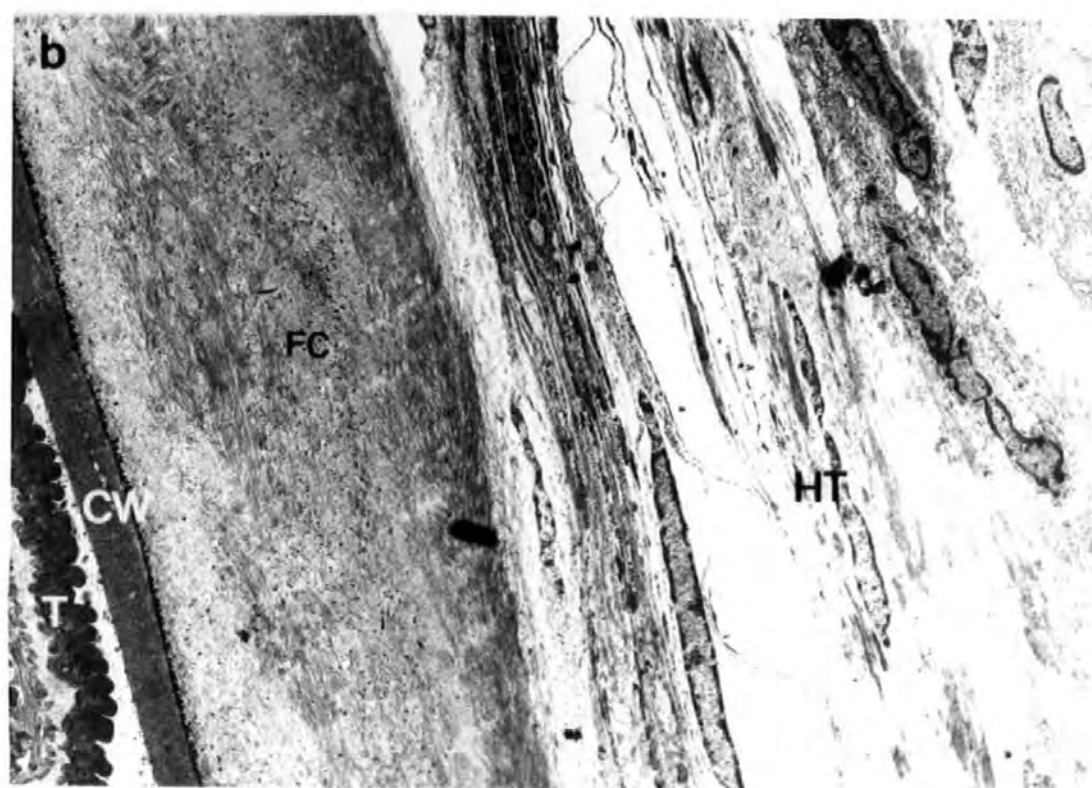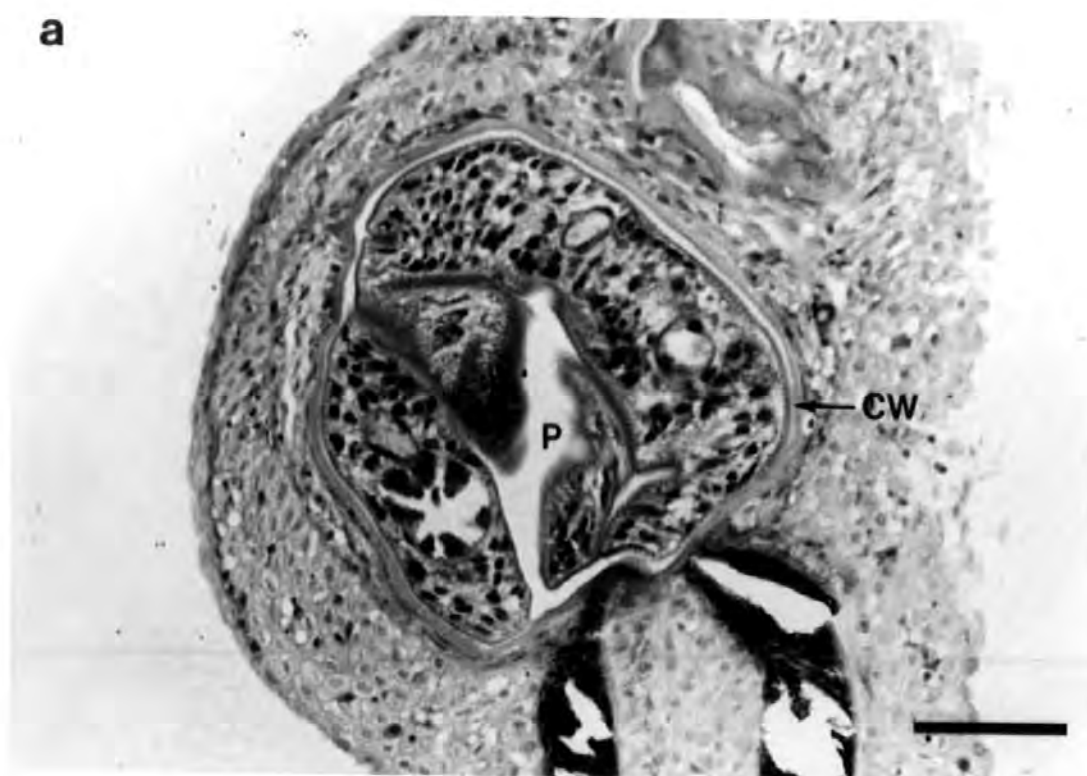
Figure 57 DOPA staining (Fitzpatrick *et al*, 1950) of the host capsule of mullet maintained under total darkness

(a) DOPA positive cells (D) and naturally occuring melanin bearing cells (M) in the dermis after 45 days at 20°C. DOPA/Mallory's triple stain. (Bar= 100μm).

(b) DOPA positive cells (arrowed) associated with the host capsule after 45 days at 20°C. DOPA/Mallory's triple stain. (Bar= 100μm).
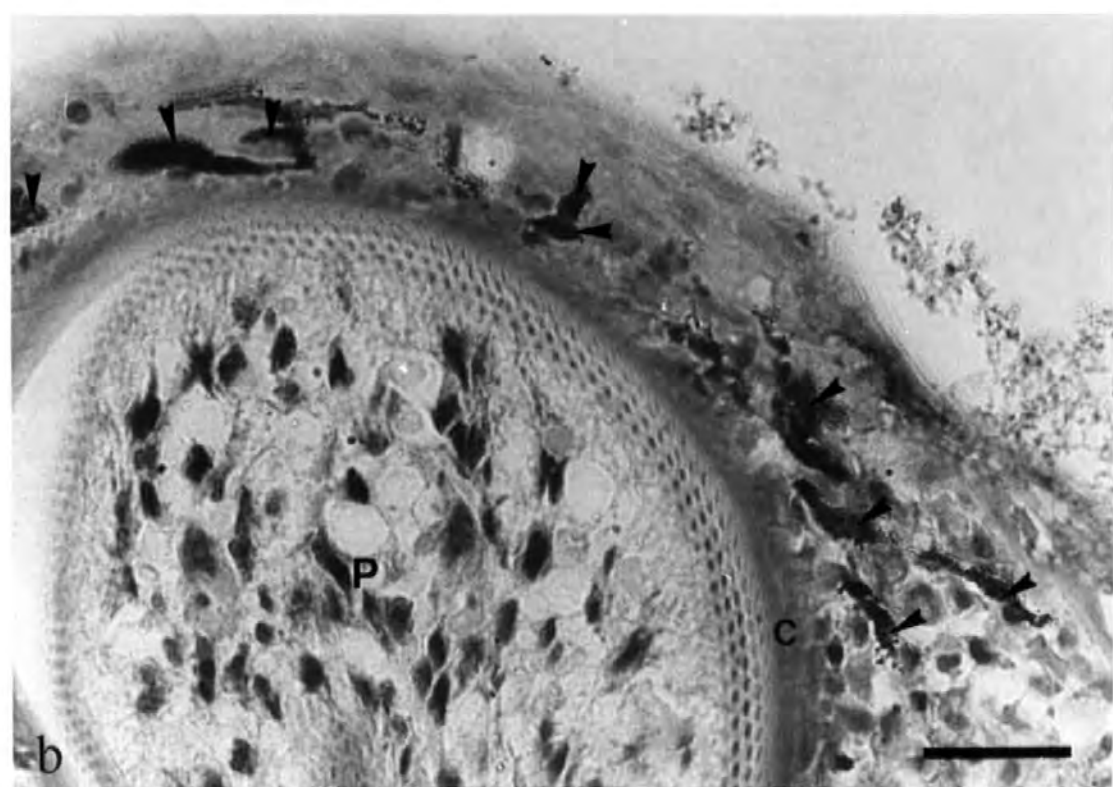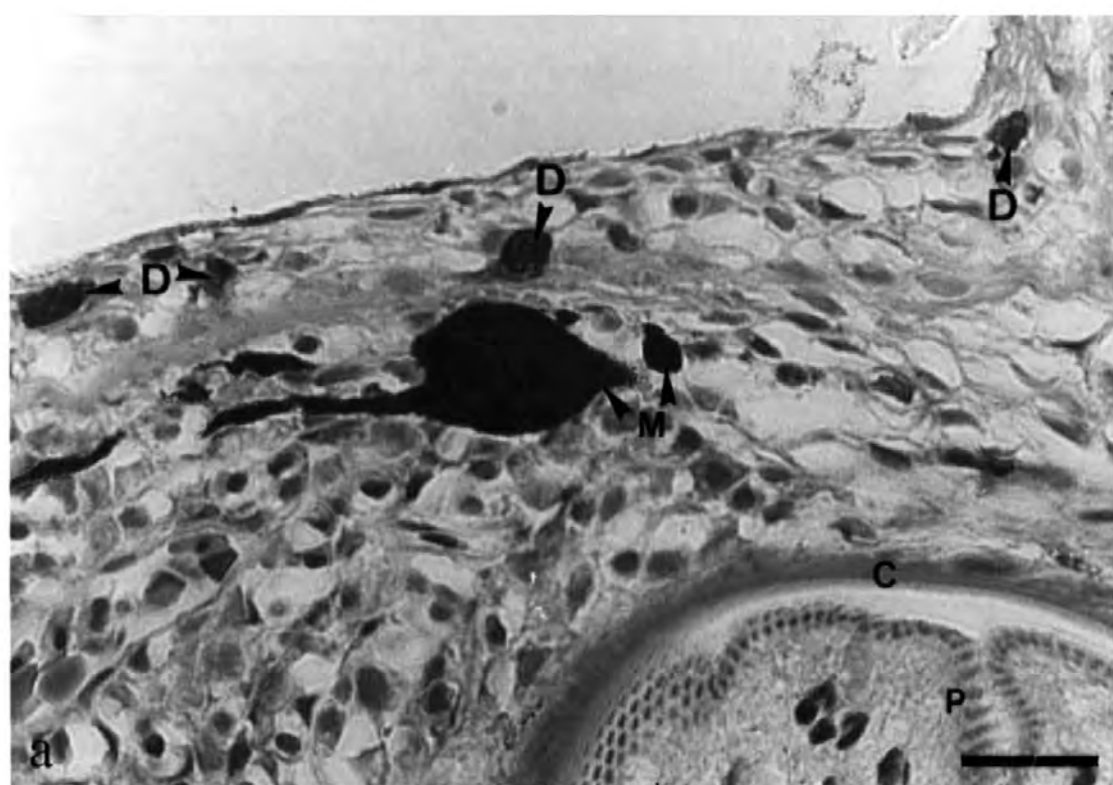
Induction of encystment

Attempts were made to simulate suitable environmental conditions for initiating transformation of cercariae to metacercariae and their encystment. Two general approaches were investigated using either solid or liquid phase media.

Response of cercariae to selected artificial substrates

Suspensions of cercariae in filtered sea water were added to square watch glasses or petri dishes containing either coagulated mullet serum or agar supplemented with one of the 9 substances listed in Table 14. The response of cercariae was monitored over a period of 24 h with the aid of a stereo microscope.

Results (Table 14) indicated that 30% - 40% of cercariae shed their tails but none adhered to or penetrated the coagulated serum. None of the substrates added to agar induced cercariae to encyst. In the presence of whole mullet extract however, 30% - 50% of cercariae shed their tails but none penetrated or encysted in the agar. Cercarial activity ceased within 24 hours of exposure to unsupplemented agar and within 30 min when incubated with agar supplemented with either aspartic, valeric or butyric acid.

Survival and transformation of cercariae in liquid phase media

Two media were investigated, namely minimal Leibovitz L15 culture media and supplemented L15 medium (Appendix A) together with sea water and YTS controls. Experimental media were established by adding serial two-fold dilutions of either glucose (20 mM), sucrose (20 mM) or heat inactivated pooled mullet serum. In addition, further experimental media were investigated, omitting sea water and combining glucose at 2.5 mM and mullet serum for each of the remaining media. Controls were established using sea water, YTS, minimal culture medium and supplemented culture medium, omitting factors under investigation. To each of these experimental media, dispensed in flat bottomed microtitre plates, was added a suspension of 2,000 cercariae cm^{-3} in the appropriate medium. Observations were made with the aid of an inverted microscope at regular intervals over a 12 h period.

Table 14 *In vitro* response of cercariae to solid media

Substrate	Supplement	Survival	Response	% Responding
Coagulated Mullet Serum	None	Active After 24h	Shed Tails	30–40
Agar	None		None	—
	Glucose (5mM)	Motionless		
	Glutamic Acid (5mM)	After		
	Lactic Acid (5mM)	24h		
	Lysine (5mM)			
	Aspartic Acid (5mM)	All Dead		
	Valeric Acid (5mM)	After		
	Butyric Acid (5mM)	30 min		
	Eel Mucus (0.4gl⁻¹)	Motionless		
	Whole Mullet Extract	After 24h	Shed Tails	30–50
Sea Water	None	All Active After 24h	None	—

Table 15 Rate of encystment (%) *in vitro* of metacercariae in liquid media

Media	Supplement	Supplement Dilution					
		1:2	1:4	1:8	1:16	1:32	1:64
Sea Water	Glucose	4	0	0	0	0	0
	Sucrose	0	0	0	0	0	0
Youngs Teleost Saline	Glucose	4	9	15	10	1	0
	Sucrose	2	3	3	4	12	6
	Serum	39	17	12	10	8	11
	Glucose (2.5mM) + Serum	23	15	8	4	5	0
Minimal Culture Medium	Glucose	2	0	3	5	3	2
	Serum	14	15	13	6	19	11
	Glucose (2.5mM) + Serum	38	20	15	7	7	2
Supplemented Culture Medium	Glucose	0	17	6	17	0	2
	Serum	9	11	3	4	8	3
	Glucose (2.5mM) + Serum	36	10	0	0	7	1

Results, summarised in Table 15, show a decrease in encystment with decreasing concentration of fish sera in all media investigated. Furthermore, encystment was highest (36% - 38%) in serum dilutions of 1:2 in YTS, minimal and supplemented culture media containing 2.5 mM glucose. Encystment in sea water only occurred in the presence of a 1:2 dilution of serum or 10 mM glucose. In 5 mM and 10 mM concentrations of glucose and sucrose a high proportion of cercariae appeared distended and ruptured. In lower concentrations (0.625 - 2.5 mM), however, glucose (but not sucrose) induced up to 17% of cercariae to encyst, whereas fish serum alone induced maximum encystment of 39%. Encystment levels of 0 - 8% were observed in all controls.

Antigens of *C. lingua*

Separation and identification of antigens

Protein profiles of extracted components from the redia, cercaria and the metacercaria were determined by SDS-PAGE. In addition, tissues from uninfected snails and fish were investigated as controls and to identify common antigens. Following electrophoresis, proteins were transferred to nitrocellulose paper and antigenic components identified following incubation with the appropriate antisera including 2 prepared from mullet and one from 1 rabbits. These antisera were also used for immunogold staining of sectioned metacercarial material for light and electron microscopical localisation of antigens.

Two mullet antisera were used, the first of which was obtained from fish which had received two intraperitoneal injections of sonicated cercariae. The serum used was taken 15 weeks after the initial injection when the titre was -log_2 18.5. The second mullet antiserum was obtained from fish which had been exposed to infection levels of 20,000 cercariae. Serum was collected 6 weeks after infection when the titre was -log_2 14.0. Normal antisera were used as controls.

SDS-PAGE clearly identified 20 proteins from metacercariae and over 35 proteins from each of the other samples, namely rediae, cercariae, snail and fish (Fig 147). No contamination of rediae and metacercariae with host tissues was apparent from results. Bands A-J represent those proteins which were

Figure 58 Protein profiles of the life cycle stages of *C. lingua* separated by SDS-PAGE

Labelled bands refer to antigens identified by immunoblotting.
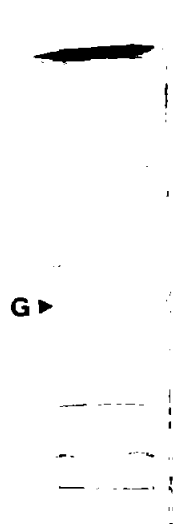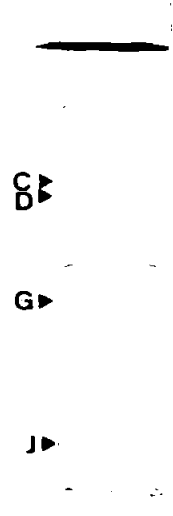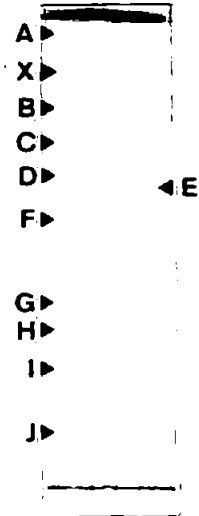
FISH	SNAIL	REDIA	METACERCARIA	MW	CERCARIA	MW
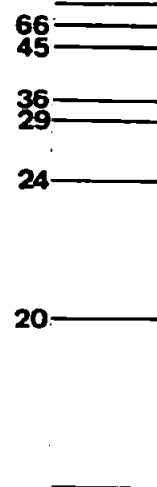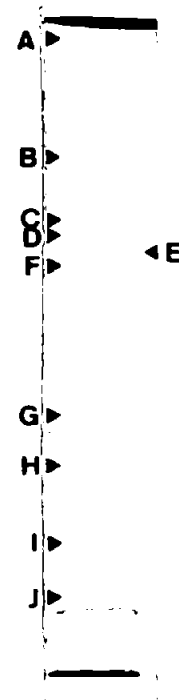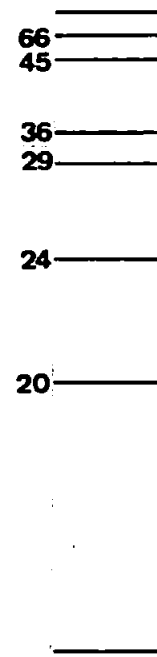
shown to be antigenically similar by immunoblotting with the 3 antisera mentioned above. Their approximate molecular weights, distribution within the life cycle stages investigated and recognition by the antisera tested are given in Table 16.

Immunoblotting demonstrated that mullet antiserum taken from fish exposed to infection levels of 20,000 cercariae responded to 10 metacercarial proteins (Fig 59). 3 of these proteins (Bands A, F and H) were antigenically similar to cercarial proteins and another 2 (Bands D and G) were common to metacercariae, cercariae and rediae, with band G also occuring in snail tissue, although this reaction was relatively weak. Band X stained strongly and was recognised only by this antiserum.

Mullet injected intraperitoneally with sonicated cercariae responded to 8 more cercarial proteins (Fig 60), 4 of which were antigenically similar to metacercarial proteins (Bands B, F, I and J) and 2 (Bands D and G) were common to all three stages of the life cycle investigated.

Rabbit antisera directed against sonicated cercariae recognised 4 more cercarial proteins than mullet antiserum raised by injection, actually responding to 12 proteins (Fig 61). 5 of these were common to metacercarial proteins (Bands B, E, F, H and I) and 4 proteins (Bands C, D, G and J) were also common to rediae and metacercariae.

Overall, proteins D, F and G reacted with all 3 antisera tested and proteins B, I and J were recognised only when the parasite was presented by injection. Protein bands C and E were recognised only by antiserum raised in the rabbit and band X reacted only with antiserum from mullet exposed to a live cercarial challenge. There was no evidence for cross-reactivity between parasite and fish host proteins with any antisera. Controls performed using sera taken from unimmunised animals responded only to those bands (labelled NS) which contained a number of very light and very heavy proteins respectively. This is thought to be due to non-specific binding.

Table 16 Distribution and antiserum recognition of antigens of *C. lingua*

Protein Band	Molecular Weight (kDa)	Presence of Protein Band					Antiserum Recognition		
		Redia	Cercaria	Metacercaria	Snail	Fish	Rabbit anti-cercaria	Mullet anti-metacercaria	Mullet anti-cercaria
X	40	−	−	+	−	−	−	+	−
A	65	−	+	+	−	−	−	+	−
B	36	−	+	+	−	−	+	−	+
C	27	+	+	+	−	−	+	−	−
D	25	+	+	+	−	−	+	+	+
E	24	−	+	+	−	−	+	−	−
F	22	−	+	+	−	−	+	+	+
G	20	+	+	+	+	−	+	+	+
H	<20	−	+	+	−	−	+	+	−
I	<20	−	+	+	−	−	+	−	+
J	<20	+	+	+	−	−	+	−	+

Figure 59 Immunoblot using sera from mullet immunised by live infection

NS - non-specific binding; S - snail; F - fish; R - redia; C - cercaria; M - metacercaria.
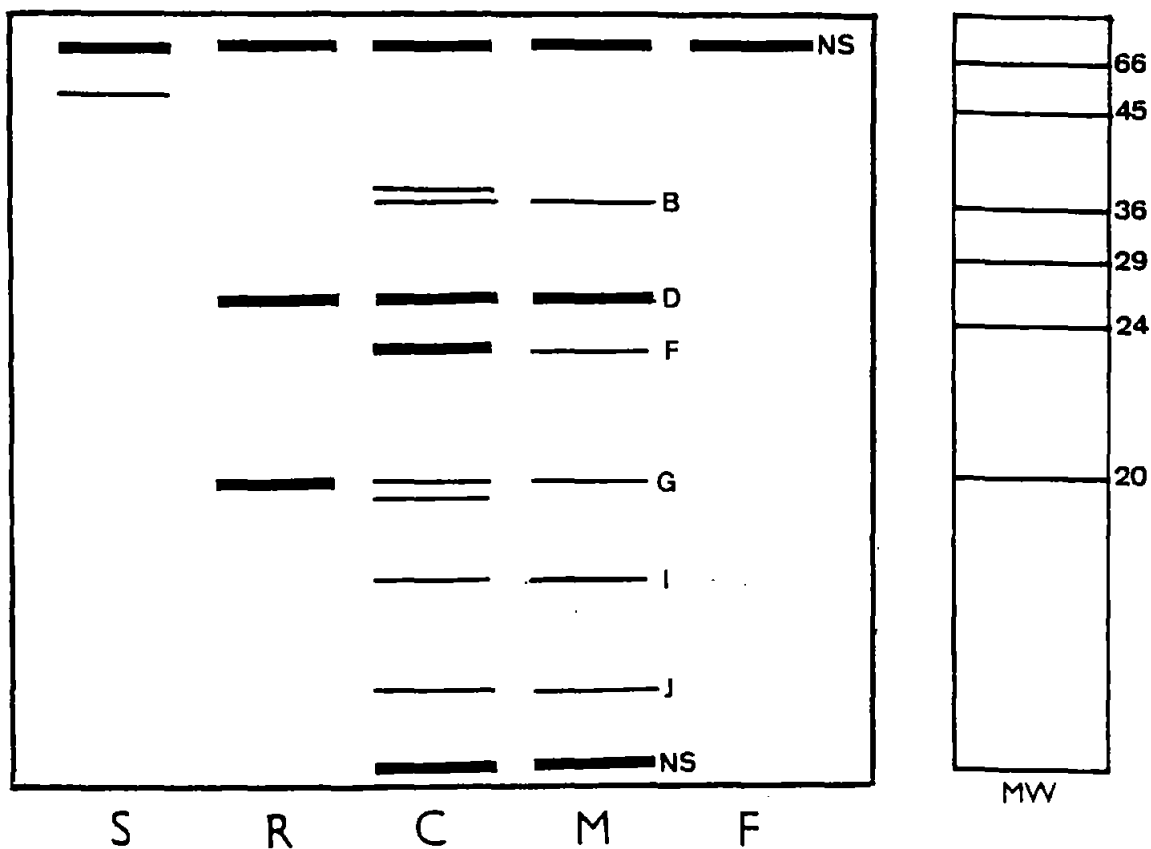
Figure 60 Immunoblot using sera from mullet immunised by intraperitoneal injection of sonicated cercariae

NS - non-specific binding; S - snail; F - fish; R - redia; C - cercaria; M - metacercaria.
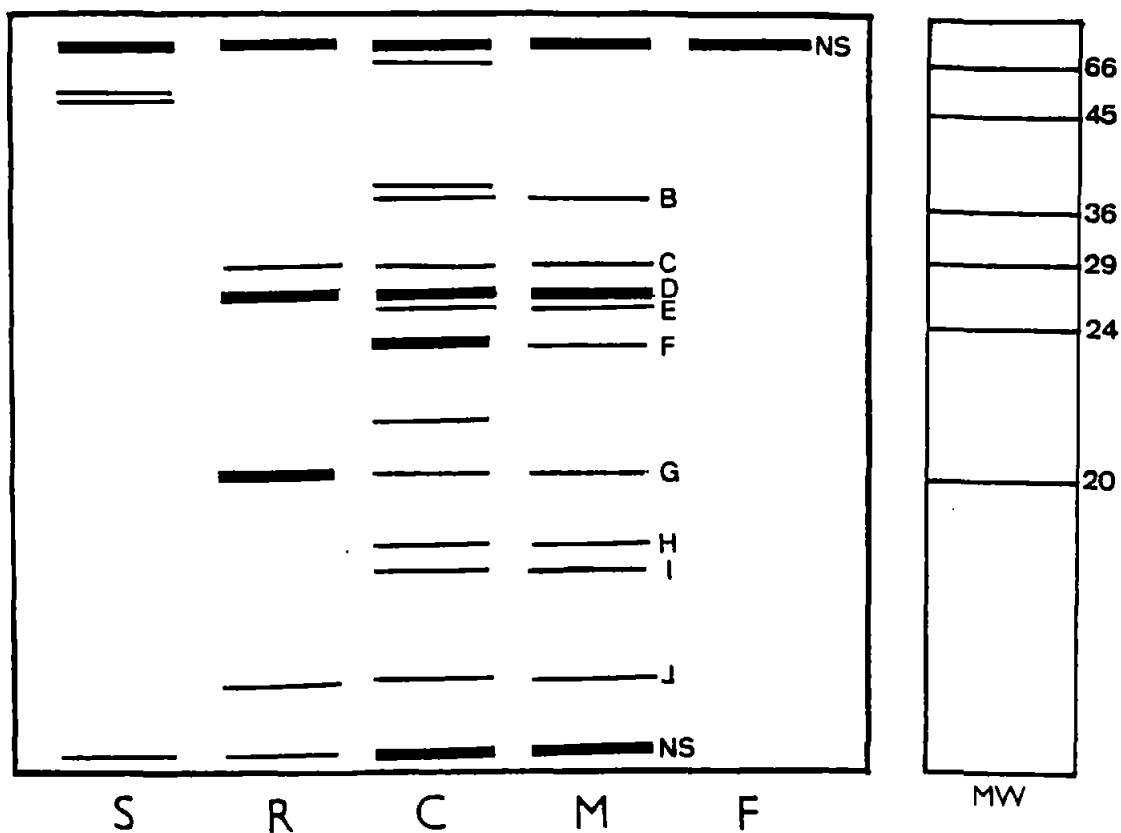
Figure 61 Immunoblot of parasite antigens using rabbit antisera

NS - non-specific binding; S - snail; F - fish; R - redia; C - cercaria; M - metacercaria.

Location of antigens

Antigenic components of metacercariae were located using immunogold labelling techniques on parasite material sectioned for both light and electron microscopy. Antisera used were those described above.

Antiserum from rabbits immunised with sonicated cercariae reacted strongly with metacercarial tissues (Fig 62a). Both tegument and cyst wall gave the most intense reactions and labelling in the region of the suckers was also strong. A reduced response was seen with all other components of the parasite.

Similar reactions were evident using antiserum from fish immunised by exposure to a live cercarial challenge. However, the staining intensity seen at LM level was not as great as that seen with rabbit antiserum (Fig 62b). The tegument again gave the strongest reaction but little or no reaction was evident with the cyst wall. Oral and ventral suckers were also stained but unlike the reaction seen with rabbit antiserum, there was no other generalised staining of the parasite.

Finally, antiserum taken from fish immunised by injection of sonicated cercariae gave the weakest responses of all (Fig 62c). Although non-specific staining was increased, oral and ventral suckers were stained but there appeared to be no reaction with either the tegument or the cyst wall.

Figure 62 Immunogold-silver staining of the metacercaria

(a) Rabbit antisera gives an intense staining reaction with the tegument (T) and, to a lesser extent the cyst wall (C). (Bar= 100μm).

(b) Serum from mullet immunised by live infection reacts with the tegument (T) but no reaction with the cyst wall (C) is evident. (Bar= 100μm).

(c) Serum from mullet immunised by intraperitoneal injection of sonicated cercariae reacts weakly with the metacercarial tegument (T). (Bar= 100 μm).
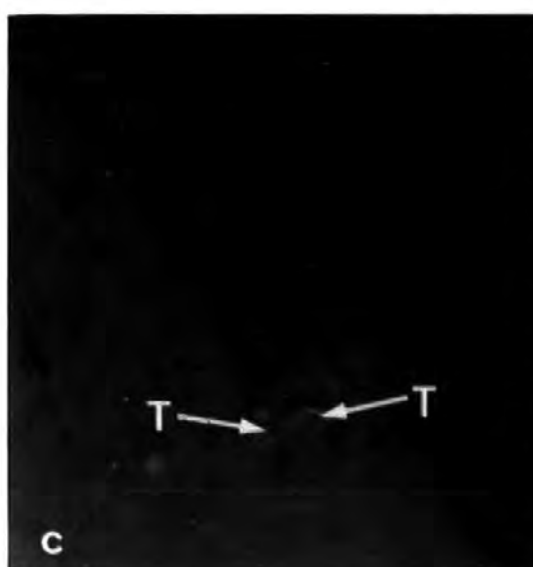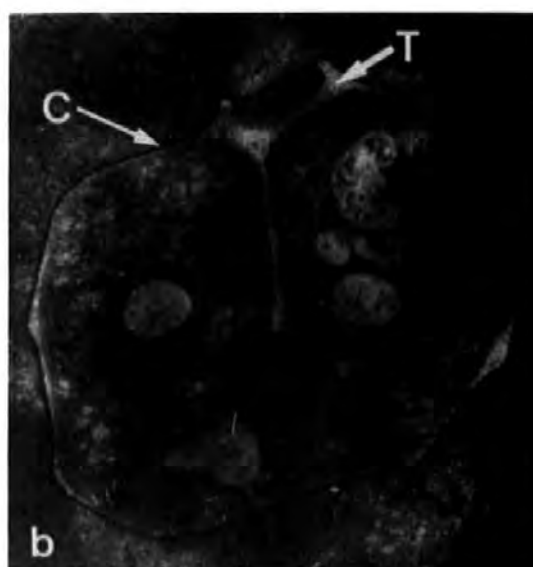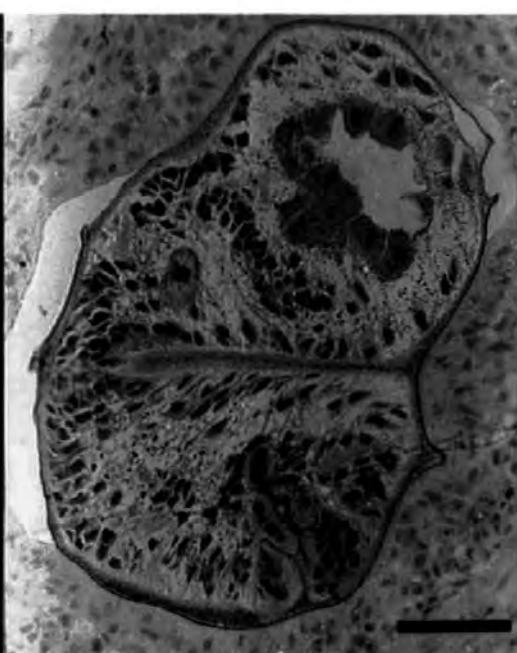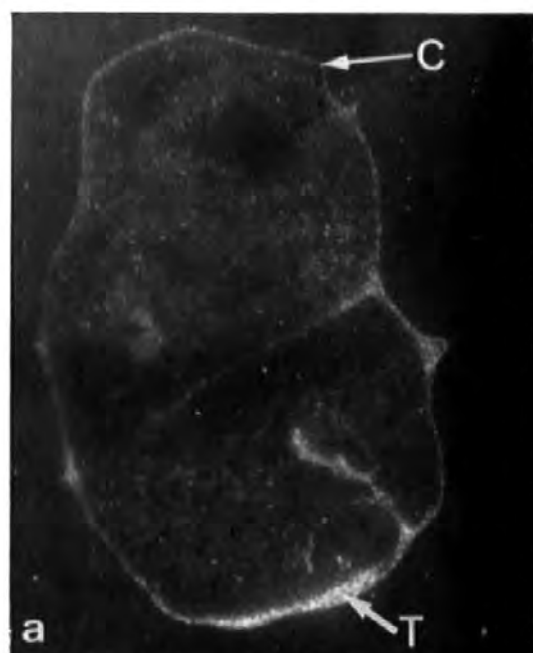
Discussion

Studies on the immune system of the grey mullet revealed that the serum immunoglobulin, leucocytes and lymphoid tissue are similar to those reported in other species of fish. Whole mullet serum, partially characterised by gel filtration, was shown to possess 4 major protein peaks, fractions from the first of these being identified as immunoglobulin on the basis of agglutinating activity and ability to precipitate with ammonium sulphate. Such findings are common with those for most fish species (Hart, 1987; Phillips and Ourth, 1986; Dorson, 1981; Clerx et al, 1980; Sánchez et al, 1980; Hall et al, 1973; Acton et al, 1971; Johnston et al, 1971; Marchalonis, 1971) and confirms that mullet possess immunoglobulin characteristic of fish. Although rabbits responded to a protein component in mullet bile, there was no evidence from electrophoretic analysis to suggest that the protein was IgM. In contrast, Hart (1987), Rombout et al (1986), Lobb and Clem (1981a & b) and Underdown and Socken (1978) did detect immunoglobulin in the bile of a number of fish species.

Specific sera against mullet immunoglobulin (Ig) was successfully raised in rabbits using sheep erythrocytes. In contrast, rabbit erythrocytes failed to induce a response. This was unexpected in view of the work of Ellis (1980) who obtained good results with rabbit erythrocytes coated with plaice antibodies. It can only be concluded that the rabbit erythrocytes were not recognised as foreign thus reducing the antigenicity of the complex and any subsequent response to the antibody coating. Coated sheep erythrocytes however, may induce a stronger response due to their greater foreignness and thereby acting as an adjuvant. Further approaches using immune complexes of mullet sera with HGG were not very satisfactory in producing a specific antisera, rabbits responding to other proteins of fish origin. Other workers have successfully

used immune complexes, namely dinitrophenol-keyhole limpet haemocyanin complexes (Dorson *et al*, 1980; Griffin and Davis, 1978; Cisar and Fryer, 1974) to raise antisera to fish Ig. It seems likely that non-specificity in antisera raised by this method in the present study was the result of other serum proteins becoming trapped within the immune complexes during their formation despite thorough washing and attempts to control the rate of this reaction. Although the method of choice here was that using antibody-coated sheep erythrocytes, future methods may better concentrate on purification of immunoglobulin prior to administration in rabbits (Dorson *et al*, 1980; Ellis, 1977b; Marchalonis, 1971).

The leucocytes in mullet are similar to those in other fish species and are therefore discussed only briefly below as a basis to their possible role within the cellular response to *C. lingua*. Lymphocytes were the most abundant cell type as in most fish species and their ultrastructural features correspond with those of other fish (Roubal, 1986; Blaxhall, 1983; Breazile *et al*, 1982; Morrow and Pulsford, 1980; Ferguson, 1976b). Their response to the PAS reagent was negative, similar to that of turbot (Burrows and Fletcher, 1987) and dogfish (Parish *et al*, 1986). In contrast, PAS positive lymphocytes have been recorded in tilapia, *Oreochromis mossambicus* (Doggett *et al*, 1987), plaice (Ellis, 1976) amongst others. The significance of PAS positive and negative material is unknown. It might be associated with carbohydrate-complexed material related to a phase of lymphocyte activity rather than an evolutionary trait, as turbot and plaice are closely related within the heterosomata. However, more investigations would be required to determine the actual significance of this. Although lymphocytes occured in a wide range of sizes here it is considered inappropriate to make any characterisation on this basis and the author is in agreement with Ellis (1977a) who suggested that such distinction may be arbitrary.

The two forms of thrombocyte recorded here, ovoid and spikey, have been well documented for teleost species by other workers (Burrows and Fletcher, 1987; Dogget *et al*, 1987; Ellis, 1976), the spikey form being related to stress incurred during blood sampling. Their fragility during sampling is likely to be a

reflection of their clotting function in fish (Wardle, 1971). PAS activity observed in mullet thrombocytes in discrete cytoplasmic granules appears to be common to many fish thrombocytes (Burrows and Fletcher, 1987; Dogget et al, 1987; Hine et al, 1986; Ellis, 1976).

Monocytes represented only 5% of circulating leucocytes in mullet and although they have been recorded in many fish species, they are reported to be lacking in brown trout (Blaxhall and Daisley, 1973) and goldfish (Weinreb and Weinreb, 1969). Again, these cells were structurally similar to those in other teleosts (Burrows and Fletcher, 1987; Dogget et al, 1987; Parish et al, 1986; Roubal, 1986; Ferguson, 1976).

Of the four granulocyte types identified in the present study (denoted Types 1-4), only one was observed in the peripheral blood of the mullet, the other three being associated with the inflammatory response to metacercariae of *Cryptocotyle lingua.* The Romanowsky staining characteristics of the Type 1 granulocyte found in the peripheral blood conforms with the general descriptions of a neutrophil. Despite much confusion in the classification of fish granulocytes (Rowley et al, 1988), the neutrophil is well documented for most fish species (Hine and Wain, 1988a & b, 1987a, b & c; Hine et al, 1987; Burrows and Fletcher, 1987; Dogget et al, 1987; Roubal, 1986; Cenini, 1984; Ellis, 1977a; Ferguson, 1976). That no other granulocyte type was observed in peripheral blood supports the findings of Burrows and Fletcher (1987) for the turbot, Ellis (1976) for plaice and Blaxhall and Daisley (1973) for brown trout. However, these findings are in contrast to those of Pulsford (1980) who observed both neutrophils and eosinophils in mullet blood. These differences may be attributed to differences in interpretation of staining reactions and granule morphology. Alternatively, they may reflect genuine variations in the relative populations of these cells due to differences in environmental conditions or immune state of fish examined. Indeed, Suzuki et al (1983) reported that relative numbers of leucocyte types were dependant on both the age of fish and the season when sampling was carried out. The granules from each of the four cell types identified here bear resemblance to those previously described in other teleost species (Rowley et al, 1988). The functional role of granulocytes

within fish in general remains unresolved (Ellis, 1982). Further studies are required to assess the significance of each of these cell types and whether they represent genuinely separate cell populations or different developmental stages.

The present study of the mullet thymus confirms findings briefly described by Mughal and Manning (1986), notably the presence of distinct cortex and medullary zones. These regions have also been described in the antarctic teleost *Harpagifer antarcticus* (O'Neill, 1989), sturgeon *Acipenser transmontanus* (Fänge, 1986), angler fish (Fänge and Pulsford, 1985), viviparous blenny, *Zoarces viviparous* (Bly, 1985) and tilapia, *Sarotherodon mossambicus* (Sailendri and Muthukkaruppan, 1975a). However, this structure is not always well defined in fish (Mughal and Manning, 1986) being absent in rainbow trout (Chilmonczyk, 1985; Grace and Manning, 1980) and salmon (Ellis, 1977b). The occurence of structures resembling Hassal's corpuscles of the mammalian thymus are described here for the first time in the mullet. Although they have been reported in some species of fish (Hibiya, 1982; Sailendri and Muthukkaruppan, 1975a) they may be absent in others (O'Neill, 1989; Chilmonczyk, 1985, 1983; Zapata, 1981a). The significance of the presence of these structures is unclear, however a possible role in mammals for antigen accumulation has been suggested (Kendall, 1981). Another distinctive feature of the thymus was the large number of eosinophillic cells and melanomacrophages distributed throughout the thymus of mullet. These may be indicative of tissue degeneration which is known to occur in older fish of other species (Manning, 1981). The presence of these cells in juvenile fish may be more supportive of the view of Fänge and Pulsford (1985) that they are myoid cells which may play a role in lymphocyte release from the thymus to the circulation. Such cells have also been reported to occur in the thymus of other fish species (Zapata, 1980) as well as the mammalian thymus.

The structural organisation of the mullet spleen appears similar to that of most teleosts (Yasutake and Wales, 1983), consisting predominantly of red pulp with smaller accumulations of white pulp. In the mullet, this white pulp was found in discrete nodes surrounding ellipsoids and melanomacrophage centres similar to

many other fish (Zapata, 1982; Ferguson, 1976; Sailendri and Muthukkaruppan, 1975a). In other fish the white pulp is more diffuse (Lamers, 1985; Ellis *et al*, 1976). The encapsulation of melanomacrophage centres in the mullet spleen within an epithelial layer is similar to that reported in carp (Lamers, 1985).

As in other species of fish, the mullet pronephros is almost entirely haematopoietic in nature, this tissue being replaced by the excretory component in the lower parts of the kidney. Distinct zones of lymphoid tissue surrounding blood sinuses and melanomacrophage centres were apparent in mullet pronephros as in other fish (Lamers, 1985; Zapata, 1979a, Sailendri and Muthukkaruppan, 1975a). Other authors have however described pronephric lymphoid tissue to be diffusely distributed (Zapata, 1981b; Grace and Manning, 1980; Ellis *et al*, 1976). Very small amounts of lymphoid tissue were located in the inter-tubule spaces of the mullet mesonephros, consistant with observations in other teleosts (Zapata, 1979b; Ellis *et al*, 1976).

The presence of antibody-containing cells in the skin of mullet was of particular interest here, supporting the findings of Peleteiro and Richards (1988, 1985) who observed such cells in the epidermis of rainbow trout. These workers did not determine the identity of the cells involved at the ultrastructural level and proposed that they were either lymphocytes or mucous cells. The general appearance of the cells observed here, with high cytoplasm to nucleus ratio, supports the latter. That antibody is secreted at the fish surface was also confirmed in mullet following immunogold-silver staining of skin tissue sections. Previous studies have also claimed to have demonstrated the presence of immunoglobulin in fish mucus (Harris, 1972; Bradshaw *et al*, 1971; Fletcher and Grant, 1969). However, Peleteiro and Richards (1988) have discussed the possibility of cross reaction with non-immunoglobulin components of mucus in rainbow trout, a factor which cannot be ruled out here. Infection of mullet with *C. lingua* was not associated with increased production of antibody-producing cells in the epidermis. Peleteiro and Richards (1988) however, claim that vaccination of rainbow trout with *Vibrio anguillarum* led to a significant increase in immunoglobulin-containing cells, lending some support to the suggestion that they may, in this instance, be of lymphocytic origin. Further studies are required

at the ultrastructural level in order to shed more light on the identity of these cells in fish.

The ability of mullet to mount both humoral and cellular responses to a eukaryotic parasite, namely *Cryptocotyle lingua*, has been demonstrated here for the first time although Mughal and Manning (1986, 1985) and Mughal (1984) have shown that mullet respond immunologically to prokaryotic and single molecule antigens. That many other species of fish have a capability to respond immunologically to a wide range of antigens has been well documented. By comparison, relatively little work has been concerned with responses to eukaryotic antigens, notably macroparasites (van Muiswinkel and Jagt, 1984) with freshwater species attracting most attention. Humoral responses in freshwater fish have been noted to a range of helminth parasites, including Digenea, Cestoda and Acanthocephala but reports of specific reactions in marine teleosts are very few. Previous investigations of cellular responses to metazoan parasites have chiefly been concerned with localised tissue reactions.

That mullet can respond immunologically to *C. lingua*, producing high antibody titres of 16.0 has been shown here for the first time following immunisation with infection levels of 20,000 cercariae. Cottrell (1977) however, recorded relatively low antibody levels to this parasite in plaice. Why there should be a poor response by plaice to this parasite remains unknown as this species has been shown to be capable of mounting immune reactions to other antigens (Ellis, 1980; Fletcher and White, 1973; Fletcher and Grant, 1969). Differences therefore could be attributed to factors other than host species, notably the level of infection and the method of antibody detection. Cottrell (1977) used the Ouchterlony double diffusion method detecting precipitating antibody, the reliability of which in relation to fish antibody has been questioned (Ellis, 1985a). Passive haemagglutination (Stavitsky, 1957) was the primary assay employed here. This assay, modified to detect antibody to cercarial antigens, is a relatively sensitive technique, detecting agglutinating antibody which may predominate in fish. In support of this, it is worth noting that mammalian IgM is known to have good agglutinating properties but poor precipitating ability (Roitt

et al, 1985). In contrast, the Ouchterlony technique is known to be much less sensitive and indeed Cottrell (1977) suggests this may be one explanation for such low titres. Furthermore, it may be that only a low proportion of the antibody produced by the plaice in response to *C. lingua* was capable of precipitating antigen. Although detection of humoral antibody by direct agglutination of live cercariae indicated lower titres, inconsistencies in results were attributed to 3 main features of the parasite. The relatively large size of the parasite together with the high activity of cercariae made interpretation of immobilisation difficult. It is also possible that the release of cystogenous material at the parasite surface, shown here to be induced by serum *in vitro*, may also interfere with agglutination. Immobilisation and direct agglutination assays have been successfully employed with bacterial pathogens by many workers including Lamers (1985), Pyle and Dawe (1985) and Secombes *et al* (1982b), and to protozoan parasites (Clarke *et al*, 1988, 1987; Pyle and Dawe, 1985; Hines and Spira, 1974). In view of the disparity between these organisms and macroparasites such as *C. lingua*, it is not really feasible to directly compare the results of these workers with those obtained here. When killed cercariae were used in this assay, no differences could be detected between infected and control fish. This failure may be due to breakdown of parasite antigens resulting in the inability of serum to agglutinate cercariae.

In view of the low degree of pathogenicity of *C. lingua* and to ensure exposure to the minimum antigen components, a relatively high infection dose of 20,000 cercariae per fish was selected in the present investigation. The dose of *C. lingua* used by Cottrell (1977) was 10, 000 cercariae, half that employed here, which may further explain the lower titres observed in plaice, the temperatures of both studies being the same. It is difficult to assess suitable infection levels as required antigens may be in low concentrations and not all cercariae entering the host develop. Furthermore, in contrast to parasites such as *D. spathaceum* which have a long migratory route within the fish host before reaching their final site of development and may therefore release greater quantities of potentially antigenic enzyme material, *C. lingua* reaches this site very rapidly and is usually encysted within 1h. Thus, infection levels need to be carefully designed to ensure stimulation of the host immune system to specific antigens.

The immune response of mullet was affected by a variety of factors; the route of administration of antigen, the antigen dose and the nature of the antigen itself. This is as expected in immune responses of fish to pathogens (Ellis, 1988). Temperature is also well known to influence the immune response of fish. The choice of 20°C as the experimental temperature maintained continuity with previous studies (Mughal and Manning, 1986, 1985; Cottrell, 1977) and reflected mean water temperatures encountered by mullet species.

Sustained antibody levels with mean titres of 10.7, recorded in mullet up to 10 weeks following immunisation with live cercariae are here associated with the continued survival and development of metacercariae. Such levels are consistent with the continued release of parasite antigens through the cyst wall until the completion of development (pre-adult) within this host at approximately 6 weeks and, to a lesser extent, throughout the remaining period of survival in this host. It is assumed therefore, that the immune system of infected mullet will be constantly primed over a long infection period, possibly in excess of 2 years. However, in primary infections of parasites of short duration such as protozoa including *I. multifiliis* (Houghton, 1987; Hines and Spira, 1974), which leave the host after a relatively short time, antibody titres decline more rapidly.

The response of mullet to freshly killed whole cercariae was significantly weaker than that recorded to live infections, although antibody was detected much earlier at 3 weeks. When injected with sonicated cercariae however, both the time of onset and magnitude of the response were affected with higher titres being achieved at week 3 and peak titres of 12.5 occuring after 5 weeks. Interestingly, the duration of the primary response to sonicated cercariae was not as long lived as that to whole killed cercariae, perhaps reflecting increased time taken in breaking down whole parasites into a manageable size for clearance. These findings support those of Cottrell (1977) who found that plaice, also maintained at 20°C, produced higher antibody levels when injected with sonicated cercariae of *C. lingua* than when infected by skin penetration. The higher titres seen here may be explained by the use of a more sensitive assay or by the fact that Cottrell was detecting precipitating antibody as opposed to agglutinating antibody as discussed above. The results here also

support the findings of Bortz *et al* (1984) who detected antibodies using an ELISA at 3 weeks post immunisation with sonicated metacercariae, after which time titres declined. Furthermore, Whyte *et al* (1987) also using an ELISA system detected antibody responses at 6 weeks post immunisation when sonicated cercariae were used as antigen and 7 weeks in the case of sonicated diplostomules. They found the duration of response to cercariae was extended, with titres remaining at approximately 8.0 until week 11 but in contrast, the response to diplostomules declined rapidly after a peak at week 8. In contrast, rainbow trout maintained at 14°C failed to produce antibody in response to *D. spathaceum* after immunisation by injection of whole killed cercariae or skin penetration of live cercariae (Stables and Chappell, 1986), injection of sonicated metacercariae (Speed and Pauley, 1985) or oral intubation with live cercariae (Speed and Pauley, 1984). The failure of this latter route to induce a response may however, be due to the destruction of parasites in the gut as Matthews (1968) has reported that cercariae of *Bucephalus haimeanus* were killed within the stomach of plaice. The relatively high levels of humoral response recorded here in mullet to *C. lingua* compared to those in rainbow trout to *D. spathaceum* (Whyte *et al*, 1987; Stables and Chappell, 1986) could be attributed to higher temperatures and higher infection levels as investigations of these latter workers were conducted at 14°C, whereas Bortz *et al* (1984) were able to detect a stronger response to *D. spathaceum* in rainbow trout at 20°C. Findings here also demonstrate that the intensity and duration of the antibody response to helminth infection is influenced by antigen presentation, a feature which has long been known to affect the immune response of fish to other antigens (Ellis, 1989; 1988, 1985b, 1978; Lamers, 1985; Dorson, 1984).

An amplified secondary response was detected in mullet which received a second dose of sonicated cercariae 8 weeks after the initial injection. Although higher titres were observed, this response did not possess all the features normally associated with a truely anamnestic one. Such responses are usually of longer duration and much more rapid (Nossal *et al*, 1965). Typical secondary responses in fish have been reported by several workers including Secombes *et al* (1982), Anderson *et al* (1979), O'Neill (1979), Sailenderi and Muthukkaruppan (1975b) and Avtalion (1969). Trump and Hildemann (1970)

however, were only able to detect a change of the kinetics of the secondary response of goldfish, the magnitude of this response remaining at the primary levels. O'Neill (1980) demonstrated that the secondary response of 3 teleost species to MS2 bacteriophage was independant of temperature, although primary reactions were temperature dependant. The effect of antigen dose on the anamnestic response of carp was shown to be similar to that of mammals (Rijkers *et al*, 1980b) in that a low dose led to low primary and high secondary responses whereas a high dose gave rise to high primary but poor secondary antibody responses. These authors also showed that the route of administration of antigen was important in invoking anamnesia. In this respect, Anderson and Dixon (1980) were unable to provide evidence for a secondary response of rainbow trout to *Yersinia ruckeri* antigen when immunised by flush exposure. Immunological memory in carp to HGG could not be induced by direct immersion (Mughal *et al*, 1986), although Lamers and de Haas (1985) were able to demonstrate memory in this species to *Aeromonas hydrophila* when immunised and challenged by the same route. This memory lasted 1 - 8 months but they noticed that the route of challenge was important and had to be the same as the primary route in order to induce anamnesia. These authors proposed that this may be indicative of the possible existence of a local immune system. Mughal and Manning (1986) however, demonstrated that secondary responses mounted by grey mullet to the soluble proteins HGG and keyhole limpet haemocyanin (KLH) were similar whether fish were primed by immersion or injection prior to a challenge by injection. The way in which antigen is presented was also shown to be important by Secombes and Resink (1984).They were able to show that carp immunised with antigen-antibody complexes mounted better immune responses and had improved immunological memory compared to those fish immunised with antigen (HGG) alone.

That the secondary response in mullet to sonicated cercarial antigens was not more rapid than the primary response and that this response was relatively short lived may be a feature of the route of administration of the antigen (intraperitoneally), the antigen dose which was relatively high, or the antigen itself. Further work would be required to ascertain the relative importance of

each of these factors. It is interesting to note that Bortz *et al* (1984) also described increased antibody titres without an effect on the kinetics of the response after a second intraperitoneal injection of sonicated metacercariae of *D. spathaceum* in rainbow trout. In contrast to the present study however, they did report that secondary titres remained elevated for a longer period.

That mullet produce humoral cytotoxins to *C. lingua* has been shown here following incubation of cercariae in immune sera. Severe tegumental damage was recorded in immune sera with negative results being obtained in normal serum at similar dilutions of 1:200. Harvey and Meade (1969) reported cercariacidal activity in both normal and immune fish sera to *Posthodiplostomum minimum*, although no quantitative studies were made and it is assumed undiluted sera were used. In the present study however, at higher concentrations no difference in serum cytotoxicity could be detected between non-immunised mullet and those immunised by either live challenge or injection of sonicated cercariae, with cercarial killing ranging 62-69% irrespective of antibody titre. This supports the findings of Whyte (1989) who found both normal and immune rainbow trout sera was cytotoxic for diplostomules of *D. spathaceum in vitro*. She suggested that this activity was elicited by a cytotoxic antibody acting in conjunction with complement, however, the possibility of activation via the alternative pathway was not dismissed and the involvement of some non-specific factors was also considered. Although the identity of the cytotoxic factor found in mullet remains uncertain, results here do not rule out a possible role for complement, being heat labile and having a lower activity in normal serum (Nonaka *et al*, 1981a). That fish possess complement is well established and the presence of classical and alternative activation pathways have been inferred (Matsuyama *et al*, 1988; Yano *et al*, 1988; Sakai, 1983, 1981; Ourth and Wilson, 1982a; Nonaka *et al*, 1981a,b). The cytotoxicity of mullet sera was found to be almost completely removed in the presence of the chelating agent EDTA and was not restored when Mg^{++} ions were subsequently added, suggesting that if complement was involved it was being activated via the classical and not the alternative pathway (Mayer, 1973). This supports work carried out by Wehnert and Woo (1980) and Bower and Woo (1977) who also detected non-specific cytotoxic factors in serum from several fish species to the

haemoflagellates *Cryptobia salmositica* and *C. catostomi* respectively. Their investigations led them to speculate that lysis was due to alternative complement pathway activation and this was later confirmed by Bower and Evelyn (1988). Furthermore, the digenean *Schistosoma mansoni* is also known to activate mammalian complement via both the classical (Ouaissi *et al*, 1981) and alternative (Santoro *et al*, 1979) pathways in mammalian hosts.

It was of interest that cercariacidal activity could be demonstrated in normal, undiluted sera from a wide range of teleosts including mullet, bass and rainbow trout but not in sera from turbot, dab, ballan wrasse and carp. It would seem unlikely that normal sera contained specific antibody directed against *C. lingua*, especially in the case of a freshwater species such as the rainbow trout but this cannot be totally dismissed in view of the ability of some normal mullet sera to agglutinate *C. lingua* antigen. However, McVicar and Fletcher (1970) suggested that a factor toxic to the tapeworm *Acanthobothrium quadripartitum* present in serum of *Raja radiata* but absent in the natural host *R. naevus* may be complement, activated by the presence of a natural antibody. It may be that non-specific antibody is passively adsorbed to the surface of the cercariae, enabling the resulting complex to activate complement. The existence of such a mechanism for the cytotoxic effect on cestodes of sheep and dog antibody on *Echinococcus granulosus* was however, rejected by Herd (1976) in favour of alternative pathway activation. A possible explanation for the possession of such lytic activity by these fish species could be related to their evolutionary development. Serum from those species considered to be less evolved (Wheeler, 1978), namely rainbow trout, bass and mullet possessed cercariacidal activity which was lacking in the more advanced species; carp, ballan wrasse, turbot and dab. It may be therefore, that a non-specific serum lysin is present in the less-developed species which has been lost during subsequent evolutionary changes.

It is possible that complement activation is being mediated by a non-immunoglobulin serum factor. C-reactive protein (CRP) is known to occur normally in the serum of fish including plaice (Baldo and Fletcher, 1973) and lumpsucker, *Cyclopterus lumpus* (Fletcher *et al*, 1977). This protein has been

characterised in the plaice (Pepys *et al*, 1978) and found to be analogous with human CRP (Pepys *et al*,1980). It reacts in the presence of Ca^{++}, with phosphoryl choline molecules known to be present in many bacteria, fungi and parasitic worms (Fletcher, 1981) and can both agglutinate and precipitate. Most importantly however, is it's ability, once bound, to activate the classical complement pathway (Cooke, 1977). Fletcher *et al* (1980) found that the tapeworm *Bothriocephalus scorpii* contained phosphoryl choline residues which were reactive with turbot, *Scophthalmus maximus,* CRP. Furthermore, Harris and Cottrell (1976) suggested that the precipitating reaction of normal plaice serum with an antigen abstract of *Proleptus obtusus*, an intestinal nematode of the dogfish, may be due to the action of plaice CRP. It is possible that the cercariacidal activity seen in the present study may be due to the binding of an acute phase protein such as CRP to an, as yet, unidentified moity of the cercarial surface, thereby activating the classical complement pathway and causing lysis. Interestingly, Fletcher *et al* (1980) reported that turbot CRP was not toxic for *B. scorpii*. The finding here that turbot serum was not toxic for *C. lingua* cercariae may also reflect the non-toxic nature of turbot CRP.

In contrast to the effect on cercariae, mullet sera, both normal and immune, were found to have no effect on encysted metacercariae *in vitro*, a feature also noted by Harvey and Meade (1969) to *P. minimum*. This feature is attributed to the presence of the cyst wall, which is assumed to provide a protective barrier. Whether these toxicity factors are of significance *in vivo* remains unclear as metacercariae would be encysted within the minimum period in which damage was sustained *in vitro*. It is possible that damage which cannot be detected does occur prior to encystment, leading to death of the encysted worm at a later time. A marked reduction in the number of metacercariae undergoing encystment in the presence of immune serum *in vitro* may however, reflect a reduced survival rate in the fish host on challenge.

A feature of particular interest was the inverse relationship between cercarial survival and serum concentration, contrary to the effect which might be expected in immunological reactions to pathogens. This phenomena was most noticeable in sera taken from fish immunised by exposure to live cercariae but

was also evident in normal sera and sera from fish immunised by injection of sonicated cercariae. A possible explanation, based on an understanding of the parasite infection process is that antibody stimulates the release of cystogenous material which could afford a protective layer at the tegumental surface. In this respect, it might be significant that high concentrations of heat inactivated antibody induced encystment in a small number of cercariae *in vitro*. Alternatively, serum may be binding non-specifically to the cercarial surface, therefore blocking potential binding sites of the non-inactivated serum subsequently added. These findings are supported by those of Cheng *et al* (1966) who similarly found that haemolymph from 7 species of molluscs induced the release of penetration and cystogenous material and encystment of *Himasthla quissetensis*.

The ability of mullet leucocytes to respond to antigens of eukaryotic parasite origin was demonstrated here for the first time using the under-agarose migration technique on pronephric isolates of mullet experimentally infected with live *C. lingua*. This migration was a directional chemotaxis towards the antigen source rather than an enhancement of random migration, known as chemokinesis. Such chemotactic movement has been studied in mammals (Wilkinson, 1982; Snyderman and Goetzl, 1981) but such mechanisms in fish have not been investigated extensively (Ellis, 1977a). Recently, similar responses of fish to bacteria have been described (Weeks *et al*, 1988, 1986; MacArthur *et al*, 1985; Griffin, 1984). Previous studies of cellular migration in fish have been chiefly concerned with responses to known mammalian chemo-attractants (Howell, 1987; Suzuki, 1986; Nash *et al*, 1986; Hunt and Rowley, 1986; Obenauff and Hyder Smith, 1985). An exception to this is a study made by Berezantsev and Opanin (1976) of chemotaxis of fish leucocytes to antigen preparations from *Schistocephalus solidus* and *Diphyllobothrium latum*. In contrast to the present study, they found that *in vitro* leucocyte migration was inhibited by the presence of parasite material and postulated that this inhibition aided survival in their hosts. That migration inhibition was not a feature of cellular responses to *C. lingua* may be attributed to the role of the cyst wall in sequestering the parasite in such infections. In the present study, mullet leucocytes taken from fish immunised by exposure to live parasites showed

enhanced directional migration capabilities when compared with the migration of similar cells from non-immunised fish. This enhanced activity suggests that cells from immunised fish have been 'primed' with the associated production of memory cells. Such memory cells may then be capable of producing lymphokine on challenge with antigen *in vitro*, thereby increasing the non-specific capabilities of macrophages and other migratory cells. It should be noted at this point that the cells involved in the migratory responses in this study were not characterised and their identities are only assumed. Chemotactic cells were identified as neutrophils in the eel, *Anguilla japonica* (Suzuki, 1986), granulocytes in the dogfish, *Scyliorhinus canicula* (Hunt and Rowley, 1986) and neutrophils and macrophages in the plaice (MacArthur *et al*, 1985), all of which are cells which have been associated with the inflammatory responses of fish (Timur and Roberts, 1977; Timur *et al*, 1977; Finn and Neilson, 1971a).

Chemotactic activity in mammals has been associated with the C5a component of the complement cascade (Snyderman *et al*, 1971; Ward and Newman, 1969). In view of the findings of Nonaka *et al* (1981b) that rainbow trout posess a C5 like molecule, it is possible that such a component is involved in teleostean leucocyte migration. Further evidence for the involvement of C5 was given by Obenauff and Hyder Smith (1985) who proposed that the migration of nurse shark, *Ginglymostoma cirratum*, leucocytes to normal rat serum was due to the presence of C5a like receptors on the leucocyte surfaces. That leucocyte migration activity can be affected by prior treatment of fish was also demonstrated by Weeks *et al* (1986) who found that fish previously exposed to toxic pollutants displayed reduced chemotactic activity. Furthermore, MacArthur *et al* (1985) found that migration to chemo-attractants was enhanced in endotoxin-treated rainbow trout.

Interestingly, immunisation of mullet with sonicated cercariae of *C. lingua* did not consistently enhance leucocyte migration when compared with non-immunised controls. It is well established that chemotaxis towards an antigen source by immunocompetent leucocytes is a mechanism for the infiltration of cells to localised sites in mammals. In this respect it is perhaps not suprising that good migration responses were achieved here in mullet immunised with live

parasites and that a weaker response was seen to injected sonicated cercariae. Although the inflammatory response to natural infections of *C. lingua* are relatively slight (McQueen *et al*, 1973), leucocytes were shown during the present study to be associated with metacercarial cysts in mullet. It therefore follows that in cases where metacercarial stages of the parasite persist, cells of the inflammatory response are more likely to be stimulated.

The chemotaxis to *C. lingua* antigens seen here may be indicative of the action of lymphokine-activated cells *in vitro*. In mammals, macrophages activated by lymphokines are the main effector cell in combating macroparasitic infections (Gordon and McLaren, 1988; James, 1986; Kubelka *et al*, 1986; James *et al*, 1983, 1982). The presence of lymphokines in fish has not however been conclusively proven (Ellis, 1989), although there is substantial evidence for their existance. In support of the results presented here are those of Weeks *et al* (1988) who demonstrated that chemotaxis of macrophages of the spot, *Leiostomus xanthurus* was induced by the bacterium *Legionella pneumophila in vitro*.

The peak of the migrative cellular response in mullet infected with *C. lingua* metacercariae by exposure to live cercariae was relatively short-lived and preceded the production of antibody. This supports the findings of Lamers (1985), Rijkers (1982) and Secombes (1981) amongst others, which also indicate that cellular responses precede antibody production in fish.

Two other techniques were employed to measure cellular immune responses of mullet to *C. lingua*, namely cellular polarisation and adherence. The former method revealed that polarisation indices of leucocytes from fish immunised by either exposure to live cercariae or by injection of sonicated antigen were consistently higher than those of leucocytes from non-immune fish, although there was no significant variation in these indices with time post infection. That increased polarisation occured in the presence of parasite antigen demonstrates it's potential to induce cellular responses in the mullet *in vivo*. These findings support those of Hunt and Rowley (1986a,b) who demonstrated that bipolar shape formation occured in dogfish granulocytes in response to leukotriene B4 (LTB4), a known mammalian chemo-attractant. Bipolar shape

formation is known to occur when mammalian leucocytes are exposed to known chemotactic factors (Stickle *et al*, 1985; Jawdwn *et al*, 1981) and is therefore taken as an indicator of the potential of a substance to act as a chemo-attractant. That prior exposure of mullet to parasite antigens enhances the polarisation response of pronephric leucocytes to those antigens provides further evidence for the existence of antigen-specific memory cells in this species. However, an alternative explanation for the increased polarisation in immunised fish may be that an increase in the cell population which exhibits bipolar shape formation occurs in such fish.

In contrast to the findings here, Hoole and Arme (1986) demonstrated that adherence of pronephric leucocytes from uninfected roach, *Rutilus rutilus*, to the surface of plerocercoids of *Ligula intestinalis* occured *in vitro* in normal serum and was increased in immune serum. They concluded that such adherence suggested the presence of complement receptors on the leucocytes of roach. Such receptors are known to occur on mammalian leucocytes and their role in adherence reactions is well established (Butterworth, 1984). However, the presence of Fc and C3 receptors in fish is disputed (Haynes *et al*, 1988; Secombes *et al*, 1985; Johnson and Smith, 1984; Sakai, 1984; Griffin, 1984; Wrathmell and Parrish, 1980). There may be some circumstantial evidence here to suggest the absence of such receptors on mullet leucocytes, however, the absence of adherent cells may be attributable to the protection of the parasite tegument by the cyst wall of the metacercaria or by the premature release of cystogenous material in the case of the cercaria which contrasts with plerocercoid infections (Hoole and Arme, 1986), where direct accessability to the parasite surface membranes might be expected. Corroboration of the inability of mullet leucocytes to adhere to *C. lingua* is provided by the findings of the ultrastructural study of it's development in the immune and non-immune host, which demonstrated that leucocytes are very rarely seen attached or immediately adjacent to the cyst wall of the metacercariae *in vivo*. Furthermore, cellular adherence may be an unlikely defensive strategy in this instance where direct cellular access to the parasite surface is prevented by the cyst wall barrier.

It was suggested earlier that CRP may be involved in the lytic action of mullet serum against *C. lingua*. Hoole and Arme (1988) suggested a possible role for a non-specific serum component in the adherence of roach leucocytes to *L. intestinalis* and that this factor may be CRP due to the inhibitive action of the presence of phosphorylcholine. In light of this, it is perhaps suprising that no adherence of mullet leucocytes to *C. lingua* was seen here if it is accepted that CRP is involved in the activation of mullet complement by this parasite. This non-specific serum component is also thought to be involved in the inflammatory response to *Toxocara canis* within it's mammalian host (Sugane and Oshima, 1983).

That mullet leucocytes appear to be incapable of adhering to the cyst wall of metacercariae may well be a contributory factor in the persistence of *C. lingua* within this host. Such evasion of immune response is essential for *C. lingua* as it's strategy for transmission is dependant upon a long survival period, possibly in excess of 2 years, within the fish host. In this respect it is worth noting that macrophage- and eosinophil-mediated damage to schistosomes occured after adherence to the parasite surface (McLaren *et al*, 1978) and Hoole and Arme (1983a) reported damage to the tegument of *L. intestinalis* after adherence of roach leucocytes. Such damage in mammals is induced by the release of oxidative metabolites produced by a rapid respiratory burst. Similar mechanisms have been recently demonstrated in rainbow trout macrophages in response to *D. spathaceum in vitro* (Whyte *et al*, 1989).

Parasite antigen was detected in the haemopoietic tissue of mullet after immunisation by exposure to live cercariae and by injection of sonicated cercariae. In both cases it was the pronephric kidney which contained most antigen. In fish exposed to live cercariae, antigen appeared initially in the reticulo-endothelial cells surrounding blood vessels and also latterly, at 10 weeks post infection, in the melanomacrophage centres (MMC). These centres, postulated by de Sousa (cited Roberts, 1975a) to be analogues of mammalian germinal centres, have been extensively studied by Agius (1985, 1981a,b, 1980, 1979), Agius and Roberts (1981) and Agius and Agbede (1984). Higher levels of antigen were detected following immunisation by injection with

sonicated cercariae. In these fish, antigen appeared to be distributed throughout the haematopoietic tissue of the pronephros but labelling was most intense after 10 weeks in those fish given a booster injection at 8 weeks post infection. In both cases, only very low levels of antigen were detected in the spleen. These findings suggest that the pronephros is the major organ responsible for trapping parasite antigen in the mullet, irrespective of the route of antigen administration.

It is particularly interesting that antigen was detected in the lymphoid organs of fish with live infections of *C. lingua* metacercariae. Such findings suggest that antigens are released by the parasite via the cyst wall and transported to these organs. Alternatively, it may be that these antigens were released on the death of individual parasites in these fish. No other studies have been undertaken on antigen trapping in the mullet, the work of Mughal and Manning (1986, 1985) concentrating on humoral antibody responses. However, the results here do support those of Lamers (1985) who investigated antigen trapping in the carp using *Aeromonas hydrophila*. This author also found that antigen was initially located in the ellipsoids of the spleen and diffused throughout the kidney, latterly being bound to cell membranes within the MMC of both organs. Trapping of BSA antigen in the plaice occured in the spleen and kidney but was found only intracellularly in the kidney and extracellularly in the spleen (Ellis, 1980). The spleen appeared to be the major site for trapping BSA, contrary to the results presented here for the trapping of *C. lingua* antigens. More recently, Herraez and Zapata (1987) have investigated antigen trapping of *Yersinia ruckeri* in the lymphoid organs of the goldfish, *Carassius auratus* and their results support those presented here, initial antigen accumulation occuring in the peri-ellipsoid macrophages of the spleen and the reticular cells of the kidney, with subsequent localisation in the MMC of both organs. They too reported an increase in antigen intensity after secondary injection of bacteria. Furthermore, Roberts (1975a) found that spores of *Myxobolus pseudodispor* localised in the MMC of the spleen and kidney of roach. Spores of species of *Myxobolus* and coelozoic myxosporeans *Sphaerospora renicola* and *Mitraspora cyprini* have also been reported to accumulate in MMC of the kidney and spleen fish (Dykova, 1984).

Present studies revealed no change in the histological appearance of any lymphoid tissues following administration of *C. lingua* antigen, whether by exposure to live cercariae or injection of sonicated cercariae. In contrast, Harraez and Zapata (1986) noted an increase in the number and size of MMC in the spleen and kidney of goldfish after injection of sheep erythrocytes or phenylhydrazine. Furthermore, Taylor and Hoole (1989) described an increase in the number of melanomacrophages and MMC in the spleen of roach and gudgeon, *Gobio gobio* infected with plerocercoids of *L. intestinalis*. Dykova (1984) also described similar increases in MMC in myxosporean infected fish.

Melanisation of the host capsule is a characteristic feature of natural infections of *C. lingua* in the fish host. Such pigmentation is characteristic of parasites, including *C. lingua* and *Uvulifer ambloptis* (Hunter and Hunter, 1942), whose definitive host is a piscivorous bird. Disruption of the fish's camouflage in this way would be of obvious advantage to the transmission of the parasite by increasing the likelihood of predation. There have been several reports of capsular pigmentation in *C. lingua* infections (Thulin, 1985; Mawdesley-Thomas and Young, 1967; Hunter and Wasserman, 1941; Hsio, 1941; Rothschild, 1939; Stunkard, 1930), indeed it is considered a characteristic feature of such infections. In heavy infections, melanisation may become more general, involving the entire body of the fish (Thulin, 1985; Mawdesley-Thomas and Young, 1967; Hsio, 1941). In contrast however, Rothschild (1939) reported that pigmentation was absent in infections of this parasite in the spotted goby. In the present study, pigmentation was not a consistant feature of experimental infections of *C. lingua* in the mullet. Both ambient temperature and the presence or absence of light were shown to have an effect on pigmentation of laboratory infections, with maintenance in total darkness inducing melanisation, this being further enhanced by elevated temperature. There are three major melanin-bearing cell types in fish, namely melanophores, melanocytes and melanomacrophages. Melanophores are asteroid cells, derived from melanocytes and found in the stratum spongiosum of the dermis. They are under nervous or hormonal control by the fish, are responsible for the colour changes of the skin in reaction to varying light conditions (Fujii, 1969) and have been associated with a variety of pathogenic conditions in fish (Roberts *et al*,

1973; Wasserman, 1965). Melanocytes however, are dendritic cells found in the deeper layers of the stratum spongiosum, in the hypodermis and surrounding blood vessels (Roberts, 1975a). They are responsible for synthesising melanin and are thought by de Sousa (cited Roberts, 1975a) to be primitive analogues of peripheral lymphoid nodules of higher vertebrates. Finally, melanomacrophages are found in haematopoietic tissues of fish and have been extensively studied (Agius, 1985, 1981a, 1980, 1979; Agius and Agbede, 1984; Agius and Roberts, 1981; Ferguson, 1976; Roberts, 1975a) and are thought to play a role in the clearance of foreign material from the circulatory system. However, the relationship between these cells and other melanin bearing cells remains uncertain. The structure of melanin itself has been reviewed by Edelstein (1971) and Mason (1959).

That melanin-bearing cells associated with cysts are influenced by light similarly to melanophores provides some evidence in support of the findings of Chapman and Hunter (1954) who suggested that the pigmented cells associated with *C. lingua* infections in the cunner were indeed melanophores. However, histochemical studies here indicated that these pigmented cells contain the enzyme tyrosinase, responsible for the catalysis of tyrosine to melanin (Fitzpatrick *et al*, 1959), suggesting that they are melanocytes (Roberts, 1975a). The latter author also believes that the pigment-bearing cells associated with *C. lingua* metacercariae are melanocytes despite the previous failure to demonstrate the presence of dihydroxyphenylalanine (DOPA), a melanin precursor, in such cells (McQueen *et al*, 1973; Chapman and Hunter, 1954). If it is assumed that the identity of this cell type is a melanocyte and that these are functioning as part of the fish's immune system, their response to different light conditions is difficult to interpret. It may be that exposure to continued darkened conditions stimulates the production of melanocytes and subsequent production of melanophores, thereby increasing the number of cells potentially available to become associated with the host capsule. However, once associated with the parasite, these cells cease to respond to light, suggesting that they are no longer under the control of the host. A similar loss of control by damaged melanophores has also been reported by Bell (cited in Roberts, 1975a) after exposure to laser beams.

Results presented here support the view of Roberts (1975a) that pigment cells migrate towards the parasite from surrounding host tissues. This author suggested that the parasite may be capable of releasing a specific melanocyte-stimulating factor which exerts an effect on melanocytes, inducing melanisation of the tissue encapsulating the metacercaria. Such factors are known to be involved in the control of colour changes in fish (Fujii and Novales, 1969). In contrast, Hunter (1941) suggested that pigmentation was the result of the release of DOPA by the parasite which is then transformed into melanin by the fish cells. However, the latter account fails to explain the apparent formation of pigmented cysts in areas such as the cornea, where no melanocyte precursors are found. The precise identity of these pigment bearing cells still remains uncertain in the mullet. However, the presence of tyrosinase in these cells and their failure to respond to external stimuli as would be expected with melanophores, suggests that they are most similar to melanocytes.

The inflammatory response of mullet to *C. lingua* is a typical chronic granulomatous inflammatory reaction, containing fibroblasts, macrophages, granulocytes, lymphocytes and plasma cells, although the intensity of the response in mullet is relatively slight. Such reactions have been described in fish in response to foreign particulate matter (Timur *et al*, 1977; Roberts and Bullock, 1976; Finn, 1970) and to other metacercarial infections (Biagianti and Maillard, 1986; Pulsford and Matthews, 1984; Sommerville, 1981; Berezantsev, 1975; Howell, 1973). General tissue responses of fish to parasitic infections, reviewed by van Muiswinkel and Yagt (1984), vary greatly in their intensity and appear to be greatest in those parasites not encysted or sequestered in some way (Sharp *et al*, 1989; Taraschewski, 1989, 1988; Joy and Madan, 1989; Weiland and Meyers, 1989; O'Neill *et al*, 1988, 1987; Rosen and Dick, 1984; Otto and Heckmann, 1984; Hoole and Arme, 1983a & b, 1982; Stromberg and Crites, 1974; Joy and Jones, 1973).

The predominant feature of the localised response here was the development of a fibrous capsule immediately adjacent to the cyst surface, with fibroblasts being prominant throughout infections. Formation of this layer begins almost immediately on infection when the parasite is at the end of it's migratory phase

and is beginning to encyst at 1h post infection. Furthermore, it seems possible that this initial fibrous layer forms a foundation on which the cyst wall is built. Such fibroblastic responses are quite common in metacercarial infections of fish (Pulsford and Matthews, 1984; Sommerville, 1981; Higgins *et al*, 1977; Hoffman, 1956; Hunter and Hunter, 1942) as well as in fungal infections (McVicar and McLay, 1985; Richards *et al*, 1978) and those of mycobacterial origin (Bucke, 1980; Timur and Roberts, 1977). The origin of this cell type in fish remains uncertain, although it has been suggested that macrophages may differentiate into fibroblasts in mammals. Fibrosis is also frequently seen in responses of mammals to parasitic infections such as Schistosomiasis and is the major factor of this disease. Indeed, eggs of *S. mansoni*, known to cause massive granuloma in mammalian hosts, produce chemoattractants and molecules shown to be mitogenic for fibroblasts (Wyler, 1983).

The presence of calcium in the host capsule of *C. lingua* metacercariae was confirmed here for the first time. Calcification, commonly seen in mammalian granuloma and generally associated with fibroblasts, is known to occur in older infections of *C. lingua* in fish (R A Matthews, pers comm) and in *Posthodiplostomum minimum* infections (Mitchell and Croy, 1976). Mineralisation is a pathologic reaction which often occurs in injured tissues and granuloma (Slauson and Cooper, 1982) including reactions to parasitic infections of mammals. These reactions appear to be most extensive when necrosis is minimal (La Via and Hill, 1975), calcium present mainly extracellularly in these pathologic conditions, and the mechanisms involved have been described by Trump *et al* (1980). The detection of calcium in melanosomes is unlikely to be a pathological feature and may reflect the strong cationic binding property of melanin (Edelstein, 1971). These findings further demonstrate that the granulomatous reaction of mullet to metacercariae of *C. lingua* has the components of that recorded in mammals. That only minor deposits of calcium were detected here is probably related to the early stage of infection investigated.

That both antibody-mediated (Type III) and cell-mediated (Type IV) hypersensitivity reactions are involved in the response of mullet to *C. lingua* is

indicated here by observations of the cellular components of the granuloma and from *in vitro* tests. Furthermore, these results support the view of Ellis (1986) that specific immune cells interact with those of the non-specific system during chronic inflammatory responses in fish. That all the cell types observed in the present study were not detected by McQueen *et al* (1973) in association with metacercariae of this parasite in plaice may be due to the limitation of their study to the light level or it may reflect genuine host differences. Macrophages were the predominant leucocyte type present in mullet. These cells are known to be the major antigen presenting cells in mammals (Roitt *et al*, 1985) and Ellis (1986) has suggested that these cells may play a similar role in fish. It may be that these cells are involved here in antigen presentation in the spleen and pronephros, accounting for the detection of parasite antigen in these organs in mullet, therefore serving an important function at the start of infection. Furthermore, their involvement in the migratory response to *C. lingua* antigen seen here *in vitro* must be considered. Granulocytes were associated with metacercariae at all temperatures during the infection. These cells usually appear early at sites of infection and are present for only short periods (Ellis, 1986), a feature not observed here. Granulocytes, notably eosinophils are a common feature of cellular responses to mammalian parasites (Lumsden, 1979). However, their role in fish may differ to that in mammals, although they are known to be an important component of the non-specific defense system of fish (Ellis, 1981; Fletcher, 1981). Their presence in association with cysts of *C. lingua* in mullet may be of importance in mediating the inflammatory response, a role proposed by Ellis (1981) and that they are present in low numbers may explain the relatively weak inflammation observed. This is in contrast to the findings of Sharp *et al* (1989) and Hoole and Arme (1983b, 1982) amongst others, who reported higher numbers of granulocytic cells associated with infections of tissue parasites in fish. However, these latter investigations were carried out on parasites which lack a cyst wall and therefore are in direct contact with host cells. That granulocytes were prevented from coming into contact with the parasite surface by the presence of the cyst wall greatly reduces their effectiveness and may offer a possible explanation for their paucity in the tissue responses seen here.

The occurence of rodlet cells in association with the granulomatous response is reported here for the first time and further questions the significance of these enigmatic cell types. They have been reviewed by Morrison and Odense (1978) and are commonly reported in fish, mainly associated with epithelial tissues including the skin. Whether rodlet cells are of parasitic origin or normal fish cells has been a matter of much debate (Barber *et al*, 1979; Flood *et al*, 1975; Bannister, 1966), although recent studies by Barber and Westermann (1986) provide substantial evidence that the rodlet cell body is of fish origin, the rodlets themselves being derived from an invasive parasite. However, the identity, origin and function of these cells remains uncertain. The findings here may lend some support to the theory that they form part of the leucocyte population (Chaicharn and Bullock, 1967; Catton, 1951; Duthie, 1939) although their significance in *C. lingua* infections in mullet is unknown and it is impossible at present to identify a role for their association with this parasite.

The host inflammatory response was shown here to be temperature dependant, being most rapid at 25°C and slowest at 15°C. This supports the findings of McQueen *et al* (1973) who investigated the effect of temperature on the response of plaice to infections *C. lingua*. Considerable variation in the localised host response was however observed between individual fish at the same temperatures. Similar variations were reported by Iddon (1973) in the reaction of plaice to *C. lingua* and those of McVicar and McLay (1985) on the inflammatory response of rainbow trout to *Ichthyophonus* infections. Such variations were not recorded by McQueen *et al* (1973) also investigating the tissue responses of plaice to *C. lingua*. The inability of some individual mullet to mount an inflammatory response to *C. lingua* may reflect an inefficiency in the immune system. The consequences of such inability to respond are not known, although it would be of interest to monitor the development of parasites within such fish. However, this variation in response must also be considered in relation to other factors of both parasite and host, including the strain of parasite and it's location in the host and the age, sex, weight and history of the host. The immunosuppressive effect of stress may also be relevent and is a possible area for further investigation. The presence of secondary bacterial infections, introduced during parasite penetration may also affect the degree of local

inflammation observed. Although such bacterial infections were reported to be associated with metacercariae of *C. lingua* in the plaice (McQueen *et al*, 1973), there was no evidence for their existence in the present study.

The failure of the local inflammatory response to inflict any damage upon the metacercaria of *C. lingua* and the inability of mullet to eliminate metacercariae suggests that the major purpose of this reaction is to assist in sequestering the parasite from the host. Indeed, Berezantsev (1975) suggested that connective tissue capsules act as biological barriers, being permiable to low molecular weight compounds such as glucose and amino acids but preventing the passage of larger molecules including immunoglobulin. Furthermore, the presence of melanin bearing cells in the host capsule in *C. lingua* infections may also serve to absorb potentially harmful material released by the parasite, in view of the ability of melanin to bind aromatic and cyclic compounds and cations (Edelstein, 1971). Any such absorption would clearly serve to protect the host and further sequester the parasite. Despite such sequestration, antigen release by the encysted metacercaria does occur as the mullet host produces high specific antibody titres and a significant cellular response.

Results here indicate a capability of the metacercaria to survive within the mullet host for at least 2 years. In addition, the metacercaria appears fully developed within 20 days. This rapid development together with longevity of infection serves to maximise the probability of transmission through predation by the definitive host. Continued survival requires an ability to evade the protective mechanisms of the fish host and results here provide evidence that the cyst plays an essential role in this. That the initial cellular response to *C. lingua* in mullet was seen as early as 1 h post infection highlights the need for rapid encystment. The prolific release of secretory material by the metacercaria during encystment provides evidence that the rapid construction of this barrier has the highest priority. That the initial rate of metacercarial development was reduced in immunised fish further demonstrates that rapid encystment is essential for protection and that the parasite is most susceptible during this early stage. It may be that antibody can pass through the cyst wall during a limited period before the cyst wall is fully developed or that the invading parasite

is exposed to antibody during it's migratory phase before encystment, thereby inhibiting initial development prior to recovery by the parasite after encystment is complete.

The cyst wall retains it's integrity throughout development and increases in thickness and complexity, consisting of 4 distinct layers after 30 days. The 3 innermost layers appear to be of parasitic origin but it remains uncertain whether the outermost layer of granular deposits are derived from host or parasite. Previous studies have described the cyst wall of this parasite as bilayered (McQueen et al, 1973; Iddon, 1973). The cyst wall of other species of metazoans show different degrees of complexity. That of Ascocotyle pachycytis was also found to be a four layered structure (Stein and Lumsden, 1971b), whereas that of Ascocotyle chandleri consisted of only three layers (Lumsden, 1968). Lee and Cheng (1970) made a detailed study of the metacercarial cyst of Stellantchasmus falcatus in another grey mullet species, Mugil cephalus, finding the cyst wall to consist of two acellular layers of parasitic origin. The cyst wall of Stictodera lani, another heteropyid, comprises a single thin layer until 35 days post infection when a further inner layer is laid down (Howell, 1973). In contrast, Hunter and Hamilton (1941) considered the outer layer of the cyst wall of the strigeid U. ambloptis to be of host origin and Hunter and Hunter (1942) further postulated that the material of this layer was derived from debris of host cells destroyed by the pressure exerted by the cyst. In contrast, there was no evidence in the present investigation of cell destruction within the compacted host cell layer surrounding metacercarial cysts.

From the results gained here, it is proposed that the cyst wall may act both as a molecular sieve and as a barrier to the host cellular response. Failure to detect IgM, using immunogold-silver staining, within the cyst supports the first proposal that the cyst wall may allow the transference of small nutrient molecules but inhibit the passage of larger ones such as IgM. Clearly, a parasite of such long duration such as C. lingua cannot be self sustaining and therefore must take up nutrients from the host. The appearance of a microvillous tegument after the cyst wall is formed, suggests an absorbative function and uptake of nutrients from the fish host, further supporting the theory

that the cyst wall is selectively permiable. This microvillous stage was evident for periods of up to 10 days, suggesting that during this time there is an increase in absorption, reflecting a period of rapid growth in the metacercaria. Similar findings were reported by Pulsford (1980) for metacercariae of this parasite and by Krupa et al (1967) in the redial surface of C. lingua, which McDaniel and Dixon (1967) demonstrated to absorb glucose. Furthermore, Iddon (1973) detected alkaline phosphatase within the metacercarial cyst of C. lingua. This enzyme is thought to be involved in the active transport of glucose (Smyth, 1966). It is therefore assumed that relatively small molecules such as glucose and amino acids can be taken up across the cyst wall. It may be however, that this permiability is variable, being less permiable to larger molecules initially and therefore less susceptible to serum damage but allowing their passage later in order to obtain appropriate larger molecules. Secondly, the layers of the cyst wall may represent component parts of an evasion mechanism. In situ studies on the location of antigens of the metacercaria of C. lingua using immunogold labelling techniques demonstrated that mullet, in which metacercariae developed after a live cercarial challenge, did not appear to respond to the cyst wall of the parasite. Instead it was the tegument of the contained metacercaria which appeared most antigenic. These results somewhat contrast those of Whyte et al (1987) who noted that the bodily tegument of the cercaria of D. spathaceum was relatively weakly labelled by immune rainbow trout antiserum. It is the cyst wall which forms the barrier between the developing parasite and the surrounding host tissue. As has been demonstrated during this study, C. lingua is capable of stimulating cellular and humoral immune responses of the mullet. However, a characteristic feature of the localised response to encysted metacercaria is the predominance of fibroblasts, with a relative lack of leucocytes (McQueen et al, 1973 and the present study). Furthermore, despite the presence of high antibody titres in immunised fish and the presence, albeit in relatively low numbers, of activated lymphocytes (identified by a highly microvillous margin) and plasma cells, mullet antibody could not be detected either within the cyst or in the surrounding host capsule. With the survival of the metacercaria for periods exceeding 2 years, it would appear that the cyst wall must permit the intertransference of materials but seems also to serve as an immunological barrier, effectively isolating the

metacercaria from the host's response. Similarly, metacercariae of *Diplostomum* develop in the eye of the trout host, an immunologically privileged site. Strategies of evasion of host immune responses, well reviewed by Parkhouse (1984), include antigen mimicry, antigen variation and sequestration. As far as *C. lingua* is concerned, it would appear that the evasive mechanism of the metacercaria in the fish host is that of sequestration. The absence here of parasite proteins of similar antigenic composition to mullet proteins suggests that there is no mimicry of host antigens by the metacercaria. Although such mimicry may be an unlikely strategy in view of the low degree of specificity for this host, the possible existence of metacercarial antigens indistinguishable from mullet proteins cannot be dismissed altogether. A possible change in antigen production associated with different stages of development of the metacercaria may be a further strategy in evading the host immune response. Finally, the host itself seems to further sequester the parasite by laying down a fibroblastic layer around the cyst and it is possible that some fibroblastic tissue is incorporated into the cyst wall during it's formation as results indicated that this layer forms a base on which parasite secretions collect and subsequently fuse together to form the cyst. These features are typical strategies of parasite evasion mechanisms by sequestration (MacKenzie, 1984). Such mechanisms contrast those employed by the adult of *S. mansoni* for example, which is thought to disguise itself by incorporation of host antigens as an evasive strategy (McLaren, 1984), a feature perhaps reflecting the location of this parasite within it's host.

Although mullet antisera here did not react with cyst wall material *in situ*, antisera from rabbits injected with sonicated cercariae gave a good reaction with this material. This provides further evidence that parasite proteins exist to which the mullet is incapable of responding. The question remains as to why these fish do not recognise the cyst wall as foreign. It has been shown here that there appear to be no parasite antigens of a similar nature to those of the host, therefore another explanation is required to account for this non-recognition. Clearly, with such complex parasites, considerable work is required to identify target antigens and further studies are required to isolate and purify such antigens.

In contrast to the findings in mullet tissue however, one protein was detected in snail host tissue using antisera from mullet immunised by live cercarial challenge. It is therefore possible that the redia posesses at least one protein antigenically similar to one of it's snail host. In support of this, Yoshino and Cheng (1978) presented evidence for antigenic mimicry of host proteins by miracidia of *S. mansoni* in it's snail host *Biomphalaria glabrata*. Pauley (1984) also reported antigenic similarities between spores of *Myxosoma cerebralis* and the cranial tissue of the rainbow trout host. It should be noted that only redial antigens also present in the cercaria or metacercaria will have been detected here. It is therefore possible that other antigens common only to the redia and snail exist but were not detected. Such mimicry might be indicative of a long standing evolutionary association between host and parasite, with selection for common antigens. Alternatively, the host may in some way induce the parasite to produce host like antigens or it may be that the parasite is capable of incorporating host antigens into it's body surface thus masking it from the immune response of the host. Such a mechanism is thought to account for the mimicry of host antigens by *Schistosoma*, which has been suggested by Capron *et al* (1968, 1966) and Damian (1967) amongst others, although alternative mechanisms have also been proposed.

As might be expected, the rate of development of *C. lingua* was here shown to be temperature dependant. In this respect these results support those of McQueen *et al* (1973) and Iddon (1973) who investigated *C. lingua* in plaice. Hoffman and Putz (1965) also found development of another heterophyid, *Uvulifer ambloptis*, encysted in the bluegill, *Lepomis macrorhinus* to be temperature dependant. However, it should be noted that it was only the development rate which was affected here, all features of normal development occuring irrespective of temperature, within the 15°C-25°C range investigated here. Considerable variation between individuals at the same temperature was also apparent, most noticeably during the initial phases of infection, suggesting that ambient temperature had not yet influenced the rate of parasite development. However, the effects upon both host and parasite must be considered when assessing the influence of temperature as optima may differ for each. That metacercarial development was slowest at 15°C may not be a

direct effect of temperature upon the parasite but a reflection of an optimum temperature for the host. Generally however, it would appear that the optimum for both host and parasite was at the highest temperature of 25°C where host response was most pronounced and parasite development most rapid. Furthermore, increased parasite activity at elevated temperatures may represent an inherent seasonal mechanism, reflecting an increase in transmission during the summer months when greater numbers of cercariae are released from the first intermediate host. That secretion bodies were still being released in large numbers 6 h post infection at all temperatures is in contrast to the findings of Pulsford (1980) who stated that the tegument was almost devoid of secretion bodies after 90 min. The possibility that host species has an influence on parasite development cannot be ruled out as, in the present study, the tegument was devoid of cercarial spines by 6 days at 25°C and by 10-15 days at 15°C, whereas Pulsford (1980) found that they were absent at 14 days in the plaice and Day (1976) reported that these spines broke down after 10 days in metacercariae encysted in the goby, *Gobio minutus.* However, as neither of these authors stated the temperatures of their experiments, this also is a likely factor. The incorporation of material into the cyst wall continued up to 8-10 days post infection in fish maintained at 20°C and 25°C and until 20 days at 15°C. This is in contrast to the findings of Day (1976) who reported that all such secretion bodies had disappeared after 4 days post infection. This discrepancy may be due to differences in interpretation of epidermal and cystogenous secretion bodies, as many of them are differentiated on size alone. After 8-20 days, depending on temperature, spines of the future adult and scretion bodies present in the tegument at this time were assumed to be required for further development within the definitive host as they were not seen being released for incorporation into the cyst wall. These findings of structural reorganisation of the tegument during metacercarial development, support those of Hunter and Hamilton (1941), Hunter and Hunter (1940) and Hunter (1937) in other larval trematodes of fish. Such reorganisation would prepare the parasite for development within the gut of the definitive host where rapid attachment to the gut mucosae after excystation would be essential for successful establishment.

In vitro studies of metacercarial transformation demonstrated here the importance of fish serum in inducing encystment. Several liquid media and supplements were tested but best results (with 39% encystment) were achieved with YTS supplemented with heated mullet serum. The further addition of glucose to culture media but not YTS, appeared to have a synergystic effect when mullet serum was present, raising encystment levels from 14% to 38%. It is possible that the osmolality of the media is optimum when glucose is present at 2.5mM. These results contrast with those of Laurie (1974) who was unsuccessful in inducing *C. lingua* to encyst in liquid media *in vitro*. He was however, able to obtain encysted metacercariae of *Himasthla quissetensis* using the same techniques, gaining optimum encystment rates in an aqueous mixture of lysine and glucose. It is uncertain whether the induction of encystment is a result of a chemoreceptive function or whether it involves the adsorption and metabolism of larger quantities of inducing agents. Several authors using phlorizin, an inhibitor of glucose adsorption by some helminths, were unable to reduce encystment in glucose solutions (Fisher and Read, 1971; Laurie, 1971, 1961; Phifer, 1960). The results presented here shed no further light on this subject, except to indicate that both glucose and mullet serum in suitable isotonic media are capable of inducing encystment in *C. lingua*.

Encystment of *C. lingua* in solid phase media was not observed here, in contrast to the findings of Smyth and Smyth (1980) who demonstrated encystment of several trematodes of amphibians on penetration of coagulated serum. It would therefore appear that a solid substrate is not required for induction of transformation and encystment of *C. lingua*. Indeed, only liquid media were successful in such induction. This conflicts with the work of Howells *et al* (1974) whose studies of the transformation of *Schistosoma mansoni* indicated that cercarial tail loss was due to the mechanical action of the tail thrashing against the cercarial body attached to the host skin. That tail loss is seen in cercariae of *C. lingua* in liquid media suggests that another biochemical stimulus is responsible for such induction in this parasite. Furthermore, *in situ* observations revealed that the cercarial tail of this parasite is shed immediately on contact with the mullet host, with no mechanical action being involved. It has also been suggested that the separation of body from tail

led to changes in the permiability of the parasite surface and subsequent transformation (Ramalho-Pinto *et al*, 1975; Colley and Wikel, 1974; Howells *et al* 1974). However, the findings here that cercarial tails may be shed without subsequent transformation and encystment suggests that other factors, including a suitable substrate are also required.

A modification of the skin-transformation technique described by Clegg and Smithers (1972) for *S. mansoni* using isolated pieces of mullet skin met with limited success here in that cercariae penetrated the fish skin, encysted within it and could then be extracted by digestion. However, only 1% of cercariae actually passed through the epidermis into the collecting vessel beneath and none of these parasites had subsequently encysted in the medium. These findings are in contrast to those of Clegg and Smithers (1972) who recovered 20-30% transformed cercariae of *S. mansoni*. Furthemore, Whyte *et al* (1988) successfully adapted Clegg and Smithers' technique for use with *D. spathaceum* and were able to obtain transformed diplostumules after passing cercariae through pieces of rainbow trout skin using a similar method to that employed here for *C. lingua*, including the removal of scales and sub-cutaneous muscle. That only very few numbers of *C. lingua* cercariae were able to pass through excised pieces of mullet skin may reflect the natural infection process of this parasite. These cercariae migrate only relatively short distances, normally being found in the epidermis of the fish host. In contrast to this, diplostomules of *D. spathaceum* must migrate through the body of rainbow trout, eventually reaching the eye where the metacercariae develop. Similarly, *S. mansoni* is a parasite with a relatively long post-penetration migration. It is perhaps not unexpected then that post penetration cercariae of *C. lingua* were unable to pass through the skin of mature mullet *in vitro*, getting trapped in the dermis and muscle.

The failure of cercariae to develop at the relatively high temperature of 37°C *in ovo* on the chorio-allantoic membrane of chicken embryo's is possibly not suprising in view of the temperature range of 10-20°C normally encountered by this parasite, although growth studies here have shown that cercariae remain viable at 25°C. However, Fried and Groman (1985) also using this technique,

successfully induced cercariae of another marine trematode, *Himasthla quissetensis* to transform and encyst at 38°C. This parasite is also normally found at lower temperatures of 8-10°C and yet despite this, encysted metacercariae were obtained after 9 days incubation *in ovo*. Other studies using the *in ovo* technique for culturing trematodes have concentrated largely on the transformation of metacercarial to adult stages and often including chemical excystment of metacercariae prior to incubation on the chorio-allantoic membrane. These studies have been extensively reviewed by Smyth and Halton (1983) and Fried (1969). Indeed, the majority of the literature on *in vitro* culture of trematodes has concentrated on the culture of adult or metacercarial stages of mammalian parasites. Several trematodes of freshwater fish including metacercariae have also been successfully cultured *in vitro* (Whyte *et al*, 1988; Leno and Holloway, 1986; Mitchell *et al*, 1978; Kannangara and Saytu, 1974; Basch *et al*, 1973; Voge and Jeong, 1971) but there appears to be a dirth of examples from marine fish, although Stunkard (1930) maintained *C. lingua* metacercariae *in vitro* for up to 14 days, although during this period the worms were seen to gradually degenerate. If long term studies of development of the parasite and host-parasite interactions *in vitro* are to be carried out, these methods will need to be developed further. Such techniques would clearly be of great value also to the development of control methods including chemotherapy and vaccination. One avenue which may warrant further investigation would be the incubation of cercariae on existing fish cell lines *in vitro*, whereby the medium may be adjusted to enhance parasite survival.

Protein profiles of the redia, cercaria and metacercaria of *C. lingua* have been produced here for the first time using SDS-PAGE. These results demonstrate that the metacercaria contains at least 20 different proteins and that the redia and cercaria a minimum of 35 proteins each. Some of these proteins, with molecular weights of 20 kDa up to and exceeding 66 kDa, were common to more than one life cycle stage. Similar results have been reported by several authors including recently Speer and Burgess (1988) who analysed the proteins of *Sarcocycytis cruzi*. Antigens of *C. lingua* have also been identified here for the first time by immunoblotting of parasite proteins separated by SDS-PAGE. Each of the 3 antisera tested, namely that from mullet exposed to live cercarial

challenge or injected with sonicated cercariae or from rabbits also injected with sonicated cercariae, were shown to be capable of responding to parasite proteins but it was the rabbit antiserum which gave the strongest reactions and recognised most proteins. This may be as expected due to the more sophisticated immune system that homiotherms are known to possess. Furthermore, the rabbit is unlikely to have experienced previous exposure to these parasite proteins, making them antigenically more "foreign" and therefore potentially more immunogenic. However, the feasibility that some potentially antigenic proteins could be denatured by the presence of SDS and mercaptoethanol during the separation process must be considered. Two proteins were recognised by the rabbit to which mullet did not respond, irrespective of the route of antigen administration. This supports the findings of Hastings and Ellis (1988) which demonstrated that some proteases of the bacterial fish pathogen *Aeromonas salmonicida* were recognised by rabbits but not by fish. It is feasible that the failure of mullet to recognise these parasite proteins may be a contributory factor to the persistence of metacercariae in the fish host for periods in excess of 2 years.

That common antigens exist between all 3 stages of the life cycle investigated are also shown here for the first time. In support of these findings are those of Tsuji (1975) and Taniguchi (1970) both of whom found some cross reaction between larval and adult stages of *Anisakis* sp using immune rabbit antisera. Furthermore, Renaud et al. (1984) demonstrated that two strains of *Bothriocephalus scorpii* from turbot, *Psetta maximus* and brill, *Scophthalmus rhombus* contained similar antigens but that some antigens were strain specific. Tsuji (1975) and Harris and Cottrell (1976) also reported cross reaction between antigens from different helminth species. More recently, Whyte et al (1987) were able to demonstrate that anti-cercarial sera from rainbow trout experimentally infected with *D. spathaceum*, cross reacted with diplostomule antigens and that there was a cross reaction between anti-diplostomule sera and cercarial antigen, suggesting the presence of common antigens. There have been numerous such studies of mammalian helminth parasites (see Kagan, 1967). Of note, Capron et al (1965) demonstrated common antigens in adults, eggs and cercariae of *S. mansoni*. Capron et al (1966) showed also that

different species of *Schistosoma* shared common antigens. These authors further demonstrated (Capron *et al*, 1968) that trematodes of different genus had similar antigenic profiles. It would appear therefore, that antigen conservation throughout the life cycle of many trematodes, including those of fish, is high. Such conservation has obvious implications in the development of vaccines, whereby certain parasite stages, such as cercariae, can be collected in bulk relatively easily for vaccine use.

Three proteins, one with a molecular weight of 36 kDa and the other two less than 20 kDa, were recognised here only when the parasite was presented by injection in both mullet and rabbits. Such findings are not unexpected and suggest that these antigens may be internal proteins and therefore not exposed to the host when infected by the natural route of skin penetration. It also demonstrates the effect of route of administration on the response to antigens, mentioned earlier. Badley *et al* (1987) also using imunoblotting techniques, reported a similar variation in response of rabbits administered with *Toxocara canis* antigens by different routes.

Three further proteins, with molecular weights of 20, 22 and 25 kDa respectively, reacted strongly with all 3 antisera tested and appeared to be highly antigenic. However, Lucius *et al* (1987) found that the response of three rodent hosts to antigens of larvae of the filaria *Dipetalonema viteae* varied with time, reacting initially with high molecular weight proteins and latterly those of lower molecular weight as determined by immunoblotting. It may be therefore, that sera taken at an earlier or later time during the present study would have reacted with different parasite proteins due to the possible sequential release of different antigens by the parasite during development.

The precise identity of any of the antigens detected in this study can only be speculated at present and further studies are being directed at their isolation and purification for use in use with *in situ* immuno-labelling procedures.

Although mullet are capable of mounting both cellular and humoral immune responses to *C. lingua*, there was no evidence here to suggest that these are protective against further infections, although an enhanced localised cellular

response was recorded in immunised fish. The metacercarial cyst wall would appear to permit the intertransference of materials including secretory and excretory products of parasitic origin, nutrients from the host and possibly antibody. Its structure however, excludes the entry of cellular components of the immune system which are normally associated with destruction of macroparasites. Any effective host response would therefore have to be directed against this cyst wall. Alternatively, responses directed at cercarial proteins involved in attachment and penetration may prevent entry of the parasite to the pre-immune host. However, as these parasites normally encyst within one hour of entry, a rapid response would be essential if it is to be effective, suggesting that a humoral mechanism would be required.

Effective vaccines have been produced against helminth diseases (Sharma et al, 1988), however, such success has not been forthcoming with human vaccines. If vaccination as a prophylactic treatment for helminth infections in fish, including *C. lingua*, is to be considered it is essential that a fundamental understanding of the immune responses of fish to eukaryotic parasites is first obtained. Certain problems specific to eukaryotic vaccines (Mitchell, 1989) must also be overcome, including identification of target antigens and epitopes thereof which induce protective responses; production of such antigens in sufficient quantities; and the application of suitable adjuvants to enhance the production of specific responses. Fish vaccination, although in it's infancy, has met with some success, chiefly against bacterial pathogens (Ellis, 1988a & b, 1985b). However, these vaccines are relatively crude preparations and unlikely to be successfull with helminth parasites. Due to the complexity of helminths, many different epitopes are presented to the immune system of the host, each of which may activate different effector mechanisms (Roitt, 1989). It is necessary, therefore, to identify protective epitopes such as those associated with the cyst wall or penetration enzymes in the case of *C. lingua*. However, chronic parasite infections are known to frequently possess multiple evasion strategies (Mitchell, 1989) and a response directed at the cyst wall may therefore still be ineffective due to the possible presence of further evasive mechanisms. In view of the apparently strongly antigenic nature of the metacercaial tegument of *C. lingua*, "concealed' metacercarial antigens may provide an alternative target for a potential vaccine.

Assuming protective antigens of *C. lingua* could be isolated, sufficient quantities would have to be available if vaccine production were to be considered. The presence of antigens common to both cercarial and metacercarial stages may enable accumulation of significant amounts of protective antigens from cercariae which are available in large numbers. Initial attempts at *in vitro* culture of *C. lingua* gave promising results and further work towards the development of such a system would be of obvious advantage. Furthermore, recombinant DNA technology may provide the first oppurtunity to produce specific protective epitopes in large quantities.

No adjuvants were used in the present investigation, although the incorporation of various adjuvants in mammalian anti-parasitic vaccines has enhanced protection (Bomford, 1989). The addition of an adjuvant to antigen preparations of *C. lingua* may induce a protective response in fish, and this is an area warranting further research.

Perhaps a more novel approach to vaccination would be of value, combining chemotherapy with vaccination for successful protection against helminth infections in fish. Such approaches to vaccines of mammalian helminths have been discussed by Gutteridge (1989). However, with an increasing awareness for the protection of the marine environment, the application of chemotheraputants is unlikely to be met with public favour. Finally, any vaccine must be easily administered; the methods of choice being bath immersion and oral vaccination (Ellis, 1988a).

Clearly, considerable work is required to identify, isolate and purify target immunogens, to produce these in sufficient quantities and to administer them in an effective and easy to manage form. Such work may well be prohibitively expensive for the production of a fish anti-helminth vaccine within the near future. Fish farming, notably mariculture is continually expanding and helminth parasites, including *C. lingua*, have been identified as a potential threat to these systems. However, it is likely to be some time before effective anti-helminth vaccines are produced for fish. Nevertheless, metacercarial infections of *C. lingua* provide an excellent model for the study of fish immunoparasitology and future studies would usefully be aimed at further developing immunogold

labelling techniques at the electron microscopical level with the ultimate aim of identifying and isolating the protective antigens of *C. lingua*.

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Appendix A

Buffers, salines, fixatives and stains

Young's teleost saline

Sodium chloride	5.50g
Potassium chloride	0.14g
Calcium chloride (anhydrous)	0.12g
Distilled water	1.0l

Physiological saline

Sodium chloride	8.5g
Distilled water	1.0l

1.1% Saline

Sodium chloride	11.0g
Distilled water	1.0l

Phosphate buffered saline pH 6.4

Sodium chloride	4.24g
Disodium hydrogen phosphate (anhydrous)	3.42g
Sodium dihydrogen phosphate	5.90g
Distilled water	1.00l

Phosphate buffered saline pH 7.2

Sodium chloride	8.00g
Potassium chloride	0.20g
Disodium hydrogen phosphate (anhydrous)	1.15g
Sodium dihydrogen phosphate	0.20g
Distilled water	1.00l

Rinaldi's saline

Sodium chloride	8.00g
Potassium chloride	0.20g
Trisodium citrate	1.00g
D. glucose	1.00g
Sodium dihydrogen phosphate	0.005g
Distilled water	1.00l

Locke's antibiotic solution

Sodium chloride	9.00g
Potassium chloride	0.42g
Calcium chloride	0.24g
Sodium hydrogen carbonate	0.20g
Glucose	1.00g
Penicillin	20,000IU
Streptomycin	20,000µg
Distilled water	1.00l

Barbitone buffer pH 8.2

Dissolve 4.4g 5'5-dimethylbarbituric acid in 150cm^3 distilled water at 95ºC.

Add 12.0g 5'5-dimethylbarbituric acid (sodium salt).

Adjust pH with sodium hydroxide.

Make up to 1.0l with distilled water.

Tris-glycine buffer for immunoblotting

Tris(hydroxymethyl)methylamine	12.1g
Glycine	56.3g
Methanol (AR quality)	1.0l
Distilled water	4.0l

NB: Do not adjust pH with hydrochloric acid.

10% Neutral buffered formalin

Formalin	100cm^3
Tap water	900cm^3
Sodium dihydrogen phosphate	4.0g
Disodium hydrogen phosphate (anhydrous)	6.5g

Tannic acid solution

Stock solution: 10mgcm^{-3} tannic acid in 0.85% saline.

Diluted 1:200 to 0.005% in 0.85% saline before use.

Immuno-electrophoresis staining solution

Coomassie brilliant blue R-250	1.0g
Ethanol	90cm^3
Acetic acid	20cm^3
Distilled water	90cm^3

Immuno-electrophoresis destaining solution

Same as the staining solution but omitting coomassie brilliant blue.

Minimal culture medium (Liebovitz L15)

Liebovitz L15	1.0l
Sodium hydrogen carbonate	25.0cm^3
Hepes(1.0M, pH 7.3)	8.0cm^3
Penicillin (5000 IUcm^{-3})	3.0cm^3
Streptomycin (5000gcm^{-3})	3.0cm^3

Supplemented culture medium

Minimal culture medium, supplemented as follows:

Foetal calf serum	12cm^3
Whole mullet serum (heat inactivated)	1.2cm^3

Appendix B
SDS-PAGE

Stock solutions

30% acrylamide & 0.8% bis-acrylamide (filter and stored at 4°C)

10% sodium dodecyl sulphate (SDS)

N,N,N',N'-Tetramethylethylenediamine (TEMED)

10% Ammonium persulphate (made up fresh)

Separation gel buffer

3.0M tris-HCl pH 8.9

Spacer gel buffer

0.5M tris-HCl pH 6.7

Electrode buffer

Tris(hydroxymethyl)methylamine	6.0g
Glycine	28.2g
SDS	2.0g
Distilled water	2.0l

Loading buffer

SDS	1.0%
Glycerol	10.0%
2 mercaptoethanol	0.1%

Bromophenol blue   0.002%

Dissolved in 10% spacer gel buffer.

Separation gel (12%)

Acrylamide/bis-acrylamide   16.0cm^3

Separation gel buffer   5.0cm^3

Distilled water   18.4cm^3

SDS   0.4cm^3

TEMED   0.02cm^3

Ammonium persulphate   0.2cm^3

Gel overlayed with water-saturated n-butanol until polymerised.

Spacer gel (4.5%)

Acrylamide/bis-acrylamide   3.0cm^3

Spacer buffer   5.0cm^3

Distilled water   10.4cm^3

SDS   0.2cm^3

TEMED   0.04cm^3

Ammonium persulphate   0.2cm^3

Staining solution

Coomassie brilliant blue R-250   0.2%

Methanol   50%

Acetic acid   10%

Destaining solution

Methanol   10%

Acetic acid   10%

Appendix C

Programme for calculating circumferential length

```
10 REM**CALCULATION OF CIRMCUMFERENCE**
20 REM**FROM LENGTH AND WIDTH**
30 REM**B P WOOD 1987**
40 VDU2
50 PRINT
60 PRINT
70 INPUT "TEMPERATURE: " T$
80 PRINT
90 REPEAT
100   INPUT "DAY: " D$
110   IF D$="Q" THEN GOTO 300
120   PRINT
130   INPUT "LENGTH = " L
140   INPUT "STD DEV = " LS
150   INPUT "WIDTH = " W
160   INPUT "STD DEV = " WS
170   A=SQR(4*(W*W)+(L*L))+(L*L)/(2*W)
180   B=(2*W)/L
190   E=(SQR(2*(W*W)+(L*L)))/L
200   F=LN(B+E)
210   C=A*F
220   AS=SQR(4*(WS*WS)+(LS*LS))+(LS*LS)/(2*WS)
230   BS=(2*WS)/LS
240   ES=(SQR(2*(WS*WS)+(LS*LS)))/LS
250   FS=LN(BS+ES)
260   CS=AS*FS
270   @%=&0002020A
280   PRINT "CIRCUMFERENCE = " C
290   PRINT "STD DEV = " CS
300   PRINT
310   UNTIL D$="Q"
320 PRINT
330 VDU3
340 END
```

Appendix D

Mean cyst measurements

Days after Infection	15°C		20°C (Normal Host)		20°C (Immune Host)		25°C	
	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation	Mean	Standard Deviation
1	0.33	0.04	0.36	0.04	0.25	0.04	0.32	0.04
3	0.30	0.04	0.40	0.04	0.39	0.04	0.40	0.04
6	0.36	0.04	0.54	0.04	0.47	0.07	0.54	0.07
8	0.40	0.04	0.54	0.04	0.53	0.07	0.57	0.04
10	0.47	0.07	0.57	0.04	0.57	0.07	0.57	0.04
14	0.57	0.04	0.61	0.07	0.57	0.07	0.64	0.12
16	0.57	0.07	0.61	0.04	0.57	0.04	0.61	0.04
18	0.64	0.07	0.61	0.07	0.61	0.07	0.61	0.07
22	0.61	0.07	0.61	0.07	0.61	0.04	0.64	0.04
28	0.64	0.07	0.61	0.05	0.57	0.04	0.61	0.09
30	0.61	0.07	0.61	0.05	0.61	0.07	0.61	0.04

The immune response of the thick-lipped grey mullet, *Chelon labrosus* (Risso, 1826), to metacercarial infections of *Cryptocotyle lingua* (Creplin, 1825)

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Grey mullet, *Chelon labrosus*, (60–100 g) have been found to respond immunologically to *Cryptocotyle lingua*, following a single exposure to 20 000 cercariae, by the production of humoral antibody, sensitized pronephric leucocytes and cytotoxic serum factors. Antibody titres measured by passive haemagglutination reached a peak at week 4 with a $-\log_2$ titre of $16 \pm$ s.e. 1·0, and titres of $10·7 \pm$ s.e. 1·0 were still recorded after 10 weeks at the termination of the experiment. Cercarial agglutination was found unreliable as a rapid test. Pronephric leucocytes, sensitive to cercarial antigen when measured by the under-agarose migration method, were detected between weeks 1 and 6, peaking at week 2. *In vitro* polarization was increased when cells were incubated with the antigen, but this increase was not significantly different between control and infected fish. Heat-labile cytotoxic factors of immune sera have been demonstrated to whole cercariae *in vitro*, these factors being associated with structural damage to the tegument. Pronephros cells isolated from immune fish during each week of infection showed no evidence of adherence to cercariae or metacercariae *in vitro*. The results are discussed in relation to both host response and course of infection of the parasite.

I. INTRODUCTION

Cryptocotyle lingua (Trematoda) is widely distributed in the Northern Hemisphere. The life cycle (Stunkard, 1930) associated with the marine environment, includes sea birds, notably the herring gull, as definitive hosts, *Littorina littorea* as first intermediate host, and fish as second intermediate hosts. Representatives of Heterophyidae including *C. lingua* have been recorded as pathogens of public health importance (Babbot *et al.*, 1961), particularly species of the genus *Heterophyes* in cultured mullet in tropical and sub-tropical areas (Paperna & Overstreet, 1981). *Cryptocotyle lingua* shows a low degree of specificity for the fish host and consequently metacercariae are recorded from most marine species, particularly in coastal waters. Infections are particularly pathogenic to immature fish (Sindermann & Rosenfield, 1954) and can reduce the value of food fish by spoilage. They must be viewed, therefore, as a potential threat to mariculture in cage systems.

Relatively little information is available concerning immune responses of fish to metacercarial infections, most work being concerned with the detection of humoral antibody and localized tissue reactions. The ability of fish to produce specific antibody to metacercarial stages, including *C. lingua*, was demonstrated by Cottrell (1977) using the plaice, *Pleuronectes platessa*, as host. More recently Bortz *et al.* (1984) detected antibody production by rainbow trout, *Salmo gairdneri*, against sonicated metacercariae of the eye fluke, *Diplostomum spathaceum*, introduced intraperitoneally. Stables & Chappell (1986), however, were unable to detect similar responses in rainbow trout to cercariae of *D. spathaceum* following natural infection or intraperitoneal injection but did note a reduced re-infection rate

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on challenge. There are many reports of granulomatous reactions to metacercarial infections in fish, the response to *C. lingua* in plaice being described by McQueen *et al.* (1973).

Some fundamental aspects of immune responses of the mullet, *Chelon labrosus*, to injected soluble protein antigens have been studied by Mughal & Manning (1986). The aim of the present study was to investigate dynamics and mechanisms involved in the immune response of *C. labrosus* to *C. lingua*.

II. MATERIALS AND METHODS

FISH

0-group mullet were collected from the estuary of the R. Tamar, Devon in October 1984 and visually screened for metacercariae of *C. lingua* with the aid of a stereo microscope. Those found free of infection were maintained in a closed aquaria system at 20° C isolated from exposure to *C. lingua* for up to 18 months, by which time they were 2-group. All fish were fed daily on trout no. 6 pellets (Ewos-Baker Ltd.) supplemented weekly with fresh fish.

EXPERIMENTAL PROCEDURES

Of 50 2-group mullet weighing 60–100 g, 40 were each exposed to a single infection of 20 000 cercariae of *C. lingua* collected within 6 h of emergence from *L. littorea*. Fish were exposed for 6 h in darkened tanks containing 1·5 l aerated sea water at 20° C. The remaining 10 fish were left untreated and served as controls. One control and 3–4 infected fish were killed by overdose in benzocaine (ethyl-*p*-aminobenzoate; Sigma) at weekly intervals until the conclusion of the experiment at week 10.

For the preparation of serum, individual fish were bled from the caudal vein, using a sterile 1-ml syringe fitted with a 23G needle. Blood was allowed to clot at room temperature ($20 \pm 2°$ C), stored overnight at 4° C to allow the clot to retract, then centrifuged ($1000 \times g$, 5 min). Serum was drawn off and stored at $-20°$ C until required.

The pronephros was removed and placed into ice-cold Leibovitz L15 culture medium with L-glutamine (Gibco Ltd.) containing sodium bicarbonate (0·3%), 1·0 M Hepes buffer (0·8%), 30 iu penicillin, 30 µg streptomycin, foetal calf serum (12%) and normal pooled mullet serum (1%). The tissue was disrupted by gently forcing it through a 200-µm mesh gauze, using a scalpel blade. The resulting cell suspension was washed three times with culture medium ($200 \times g$, 5 min) in a refrigerated centrifuge at 4° C, numbers estimated using a haemocytometer, and the concentration adjusted to 10^7 cells ml^{-1} (unless otherwise stated) after trypan blue exclusion. The following immunological tests were carried out on serum and cell suspensions from individual fish.

PASSIVE HAEMAGGLUTINATION

Sera were tested for agglutinating antibody against solubilized cercarial proteins, using the modified passive haemagglutination method described by Mughal & Manning (1986), tanned sheep red blood cells being coated with solubilized cercariae (6 mg ml^{-1} protein). Mean $-\log_2$ antibody titres were compared, using a *t*-test ($P < 0·05$).

CERCARIAL AGGLUTINATION

Into each well of a microtitre plate (Sterilin Ltd.) was placed 50 µl of sterile Young's teleost saline (YTS). An equal volume of heat-inactivated (47° C, 30 min) serum under test was serially diluted along two rows of the plate and 50 µl of antigen (500 cercariae ml^{-1}) was added to each well. Plates were left at room temperature for 3 h and then examined for agglutinating activity, using a stereo microscope. The final well showing agglutination of cercariae was taken as the end point. Mean $-\log_2$ antibody titres were compared using a *t*-test ($P < 0·05$).

COMPLEMENT FIXATION (CF)

This was undertaken to test the ability of immune and control serum to fix complement and the effect on live cercariae. Serum under test was serially diluted in microtitre plates as described above and 50 µl of live cercariae (500 cercariae ml^{-1}) added to each well. Plates were incubated at room temperature for 30 min and then 50 µl complement was added to each well. Sera taken from uninfected control mullet and diluted to 50% in YTS was used as a source of complement. Sera from infected mullet, however, was used to investigate the anomaly of decreased response with increased titre described below. Plates were incubated for a further 3 h after which 50 µl 0·5% trypan blue in YTS was added to each well and cytotoxic activity estimated on the basis of % cercariae taking up stain.

UNDER-AGAROSE MIGRATION

The migration of pronephric leucocytes towards solubilized cercariae was assessed using a modification of the under-agarose migration assay (Nelson *et al.*, 1981). Assays were performed using 1·0% type V agarose (Sigma) with 0·5% gelatin on acid-cleaned, gelatin-coated microscope slides. Wells, 3 mm in diameter and 3 mm apart, were cut in the agarose in groups of three. Each well was filled with 10 µl fluid, one outside well receiving the solubilized preparation of cercariae in culture medium (125 µg ml^{-1} protein), the other receiving culture medium alone, and the central well the cells under test. Slides were incubated in a humidified chamber at room temperature for 3 h, after which they were fixed in methanol, stained using 10% Giemsa, and chemotactic differentials assessed. Mean values were compared using a t-test ($P < 0.001$).

CELL POLARIZATION

Pronephric leucocytes (0·9 ml) were mixed with 0·1 ml of either solubilized cercariae preparation (125 µg ml^{-1} protein) or culture medium and incubated at room temperature for 1 h. Cells were then fixed for 30 min with 1 ml 2·5% glutaraldehyde in culture medium, washed, and the proportion of polarized cells counted, using a haemocytometer. Results were expressed as the ratio of polarized cells in a suspension incubated with antigen to those incubated with medium alone. Mean results were compared using a t-test ($P < 0.05$).

CELLULAR ADHERENCE

Live cercariae or cercariae killed by freezing at $-20°$ C were washed three times in sterile filtered sea water and then three times in culture medium and suspended to 500 cercariae ml^{-1}. To each well of a microtitre plate was added 50 µl leucocyte suspension (10^6 cells ml^{-1}) and serum (1:200 dilution) both from either infected or control fish. Controls lacking serum and/or cells were employed. Cercariae (50 µl) were then added to each well, the plate incubated at room temperature and examined for adherence of cells to parasites and parasite damage, using an inverted microscope at intervals between 2 h and 24 h.

III. RESULTS

Mullet exposed to infection levels of 20 000 cercariae were shown to produce humoral antibody against cercarial proteins when tested using passive haemagglutination (Fig. 1), this response peaking 4 weeks post-infection with a mean $-\log_2$ titre of 16·0 ± s.e. 1·0. Uninfected controls gave a negative response. Direct agglutination tests using live cercariae (Table I) indicated that there was a significant difference in antibody titres between uninfected controls ($-\log_2$ 17·0 ± s.e. 1·3) and experimental fish (maximum titre $-\log_2$ 21·5 ± s.e. 0·7) until after week 4 when experimental titres were no longer significantly higher than controls.

Cercaricidal activity was detected in *in vitro* tests using antibody at 1:200 dilution collected at week 4. Damage to the tegument was recorded with total
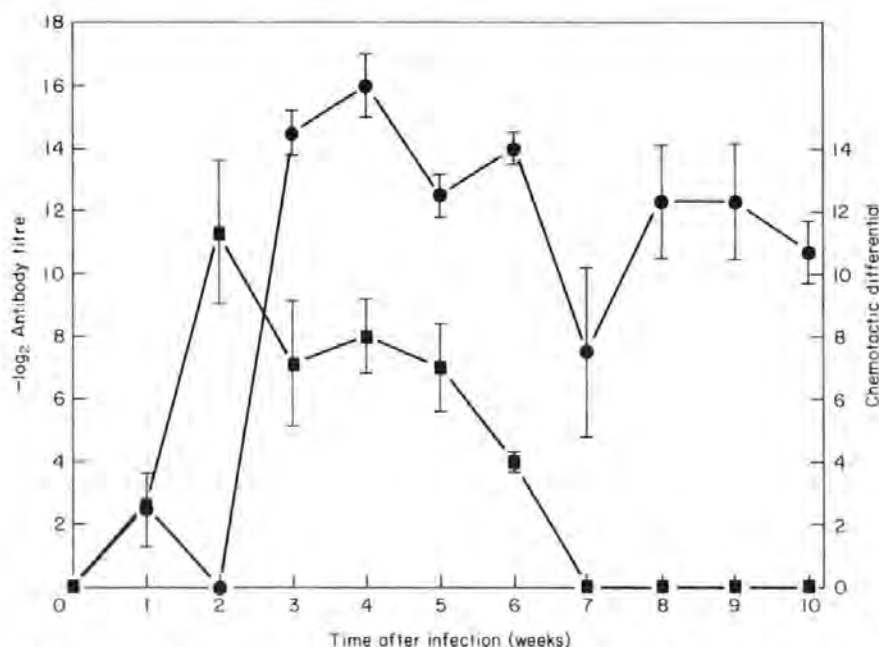
FIG. 1. Primary antibody response detected using passive haemagglutination (●) and chemotactic differential of pronephric leucocytes (■) of mullet after exposure to infection levels of 20 000 *Cryptocotyle lingua* cercariae. Vertical bars represent s.e. of the mean (*n* = 4).

TABLE I. Antibody levels detected using cercarial agglutination after exposure of mullet to infection levels of 20 000 *Cryptocotyle lingua* cercariae

Weeks after infection	Sample size	Antibody titre
Control	10	$17 \cdot 0 \pm 1 \cdot 3$
1	4	$21 \cdot 0 \pm 1 \cdot 7$
2	4	$21 \cdot 5 \pm 0 \cdot 7$
3	4	$21 \cdot 0 \pm 1 \cdot 7$
4	4	$20 \cdot 7 \pm 1 \cdot 6$
5	4	$15 \cdot 7 \pm 1 \cdot 6$
6	4	$19 \cdot 0 \pm 0 \cdot 0$
7	4	$18 \cdot 0 \pm 0 \cdot 0$
8	4	$17 \cdot 3 \pm 1 \cdot 6$
9	4	$16 \cdot 5 \pm 1 \cdot 2$
10	4	$11 \cdot 0 \pm 1 \cdot 5$

Figures represent mean $-\log_2$ titres $\pm$ s.e.

destruction of the surface within 6–12 h in immune serum. Encysted metacercariae were unaffected. The number of cercariae successfully encysting *in vitro* was reduced in the presence of immune serum, falling from 25 to 13%.

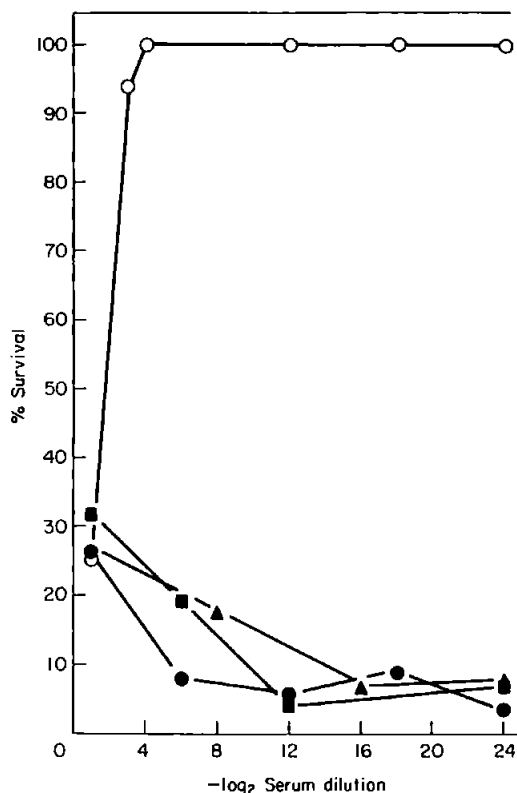FIG. 2. Survival of *Cryptocotyle lingua* cercariae *in vitro* after incubation in inactivated control serum only (O); inactivated control serum followed by complement from control serum (●); inactivated control serum followed by complement from immune serum (■); and inactivated immune serum followed by complement from control serum (▲). Each point represents % cercarial survival from 100 observations.

Results of the CF test carried out using immune and control sera are shown in Fig. 2. In all inactivated test sera where complement was subsequently added, survival of cercariae decreased with a decrease in concentration of the initial serum, no difference being recorded between the three regimes. When incubated in inactivated control serum alone (without the addition of complement) survival increased to 100% in $-\log_2$ dilutions higher than 4.

The ability of pronephric leucocytes to migrate towards a cercarial antigen source, expressed as chemotactic differential (Fig. 1), was significantly higher in experimental fish throughout the experiment and was maximal at 2 weeks, after which it gradually decreased to zero at 7 weeks. Leucocyte suspensions from both experimental and control fish, incubated with cercarial antigen, consistently showed an increased proportion of polarized cells when compared with those incubated in culture medium only (polarization index), as shown in Table II. The polarization index was not, however, significantly higher in suspensions from experimental fish and showed no obvious trend during the course of the experiment. Adherence of cells to cercariae or metacercariae *in vitro* was not observed at any time under the conditions investigated here.

TABLE II. *In vitro* polarization of pronephric leucocytes taken from mullet exposed to infection levels of 20 000 *Cryptocotyle lingua* cercariae

Weeks after infection	Sample size	Polarization index
Control	10	$1\cdot30\pm0\cdot40$
1	4	$1\cdot06\pm0\cdot22$
2	4	$1\cdot43\pm0\cdot85$
3	4	$1\cdot09\pm0\cdot07$
4	4	$1\cdot23\pm0\cdot12$
5	4	$1\cdot42\pm0\cdot27$
6	4	$1\cdot11\pm0\cdot09$
7	4	$1\cdot45\pm0\cdot56$
8	—	Not done
9	4	$1\cdot61\pm0\cdot14$
10	4	$1\cdot36\pm0\cdot08$

Figures represent the mean polarization index $\pm$ s.e.

IV. DISCUSSION

Chelon labrosus has here been shown to produce specific antibody, cytotoxic serum factors and sensitized leucocytes in response to *C. lingua*. As far as we are aware, there are no previous records of immune responses of this host to *C. lingua* or to any other eukaryotic parasite. The highest levels of specific humoral antibody were detected 4 weeks after infection, the timing being in agreement with primary responses recorded in fish by other workers to both parasites (Bortz *et al.*, 1984; Houghton, 1987) and bacterial antigens (Lamers, 1985). Peak titres recorded here $(-\log_2 16\pm$s.e. $1\cdot0)$ were generally higher than those previously reported following single exposure (primary) infections and must be partially attributed to the relatively high antigenic stimulation. In this respect, it might be significant that Cottrell (1977) detected relatively low levels of antibody in plaice to *C. lingua*, using the Ouchterlony technique. Detection of humoral antibody by agglutination of cercariae found some application here in initial screening of serum, but inconsistencies were attributed to parasite size, high activity and possible release of cystogenous material. Sustained antibody levels with mean $-\log_2$ titres of $10\cdot7\pm$s.e. $1\cdot0$ being recorded at the termination of the experiment at week 10 might be as expected with the continued survival and development of metacercariae and the probable release of antigenic material via the cyst wall throughout this period. This appears to be in contrast to primary infections with parasites of short duration, such as *Ichthyophthirius multifiliis* (Houghton, 1987), and following injection of specific antigens (Lamers, 1985).

That mullet produce cytotoxins to *C. lingua* is indicated by severe tegumental damage following incubation of cercariae in immune serum, negative results being recorded in normal serum at similar dilutions of 1:200. Harvey & Meade (1969) reported cercariacidal activity in both normal and immune fish sera to *Posthodiplostomum minimum* although no quantitative studies were made and it is

assumed undiluted sera were used. The identity of the cytotoxic factor remains uncertain; however, the results here do not rule out a possible role for complement, being heat-labile and having lower activity in normal serum (Nonaka *et al.*, 1981). Results from the complement fixation test showed active sera from control fish at a final dilution of 1:3 to be cytotoxic to cercariae, suggesting that, in the absence of specific antibody, the parasite may activate complement via the alternative pathway.

Mullet sera, both normal and immune, were found to have no effect on encysted metacercariae, a feature also noted by Harvey & Meade (1969). As *in vitro* experiments indicated also that a period of at least 6–12 h was required to sustain tegumental damage to cercariae, it seems unlikely that cytotoxic factors play any protective role in primary infections, encystment *in vivo* usually being completed within 1 h at this temperature (B. P. Wood, unpubl. results). A marked reduction in the number of metacercariae undergoing encystment in the presence of immune serum *in vitro*, however, may reflect a reduced survival rate in the fish host on challenge.

A feature of particular interest was the inverse relationship between cercarial survival and serum concentration, contrary to the effect which might be expected in immunological reactions to pathogens. A possible explanation based on an understanding of the parasite infection process is that antibody stimulates the release of cystogenous material which could afford a protective layer at the tegumental surface. In this respect, it might be significant that high concentrations of heat inactivated antibody induced encystment in a small number of cercariae.

The ability of fish leucocytes to respond to antigens of eukaryotic parasite origin has been demonstrated for the first time using the under-agarose migration technique on pronephric isolates of mullet infected with *C. lingua*, although no significant increase in cellular polarization *in vitro* was evident. Weeks *et al.* (1986) and MacArthur *et al.* (1985) have described similar migration responses in fish to bacterial antigens. Previous studies of cellular migration in fish have been chiefly concerned with responses to known mammalian chemo-attractants (Griffin, 1984; Obenauf & Hyder Smith, 1985; Hunt & Rowley, 1986; Nash *et al.*, 1986; Suzuki, 1986; Howell, 1987). The peak of cellular response in mullet infected with *C. lingua* is relatively short-lived and precedes the production of antibody. This supports the findings of Lamers (1985) and Secombes (1981) which also indicate that cellular responses precede antibody production in fish.

Hoole & Arme (1986) demonstrated that adherence of pronephric leucocytes from uninfected roach, *Rutilus rutilus*, to the surface of *Ligula intestinalis* occurred *in vitro* in normal serum but was increased in immune serum. Failure of mullet leucocytes to adhere to *C. lingua* could be attributed to the protection of the tegument by a cyst wall of parasitic origin in metacercariae, and the premature release of cystogenous material in cercariae.

It is generally assumed that the cellular component plays a major role in the immune response to macroparasites (Ellis, 1978), inflammatory reactions and granuloma being a characteristic feature of helminth infections. The response to active infection of *C. lingua* is relatively moderate, including fibroblast and melanocyte infiltration, and is well documented (McQueen *et al.*, 1973). In the present instance, in which mullet were exposed to heavy primary infestations, a large percentage failed to complete development on entry. It might be expected,

therefore, that the presence of any parasite failing to encyst or, on encystment, failing to develop may serve to stimulate the immune system by exposing antigens otherwise sequestered by the cyst wall and increase the capability of the fish to destroy the cysts of healthy parasites.

We thank Miss A. S. Burrows for her assistance with the under-agarose migration assay.

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In vivo study of the effect of temperature and light on the melanisation of cysts of *Cryptocotyle lingua* (Creplin, 1825) in the mullet, *Chelon labrosus* (Risso, 1826)

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Although pigment-bearing cells are a characteristic feature associated with metacercarial cysts of *Cryptocotyle lingua*, little is known of their identity, functions and possible significance. Chapman and Hunter (1954) suggested that these cells were melanophores. Roberts (1975), however, believes them to be melanocytes, cells which De Sousa (cited in Roberts, 1975) suggested may be primitive analogues of peripheral lymphoid nodules of higher vertebrates. The present study was undertaken to clarify the identity of these cells.

Sixty '0' group mullet were divided into six equal groups; A, B and C were maintained under normal conditions of daylight (January–March), approximately 10 h per day, the remainder D, E and F being kept in constant darkness. Controlled temperatures were established for groups as follows; 10°C, A and D; 15°C, B and E and 20°C, C and F. All fish were acclimated for 3 weeks and examined microscopically to confirm the absence of natural infections of metacercariae. They were then exposed to *C. lingua* by caudal fin immersion in a suspension of cercariae. Penetration and encystment were monitored and the exact location of individual metacercariae was mapped for each fish. A photographic record of in vivo development was made over a period of 75 days post-infection. Light and electron microscopical studies were also made of significant stages.

Results indicate that melanisation of metacercarial cysts was most intense in fish maintained in constant darkness at 20°C after 45 days. Under darkened conditions the rate of pigmentation was shown to be influenced by temperature, melanin-containing cells being associated with cysts within 6 days at 20°C, 10 days at 15°C and 20 days at 10°C. In contrast, melanisation was minimal in fish maintained under conditions of normal daylight, pigment-bearing cells rarely being observed in association with the parasite cyst at any of the three temperatures investigated. Histological studies indicate that melanin-containing cells are incorporated into the fibrous host capsule surrounding the parasite cyst wall in fish maintained in constant darkness at 20°C after 45 days. No such cells are present in those held in light conditions at the same temperature after the same length of time.

These results provide some evidence in support of the findings of Chapman and Hunter (1954) in that melanin-bearing cells associated with the cysts are influenced by light similarly to melanophores. If these cells were in fact functioning as part of the fish's immune system, their response to different light levels is difficult to interpret. However, once associated with the parasite, these cells do not respond to light, suggesting they are no longer under the control of the host. Such pigmentation is characteristic of parasites encysting superficially within the skin, whose definitive host is a piscivorous avian. Disruption of the fish's camouflage in this way would be of obvious advantage to the transmission of the parasite.

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