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IMMUNE RESPONSE OF CARP Cyprinus carpio (L.) TO Ichthyophthirius multifiliis (FOQUET), WITH REFERENCE TO EVENTS WITHIN THE EPIDERMIS

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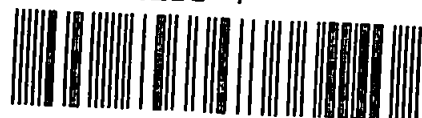
By Martin Leslie Cross B.Sc., M.Sc.,

Thesis submitted to the Council for National
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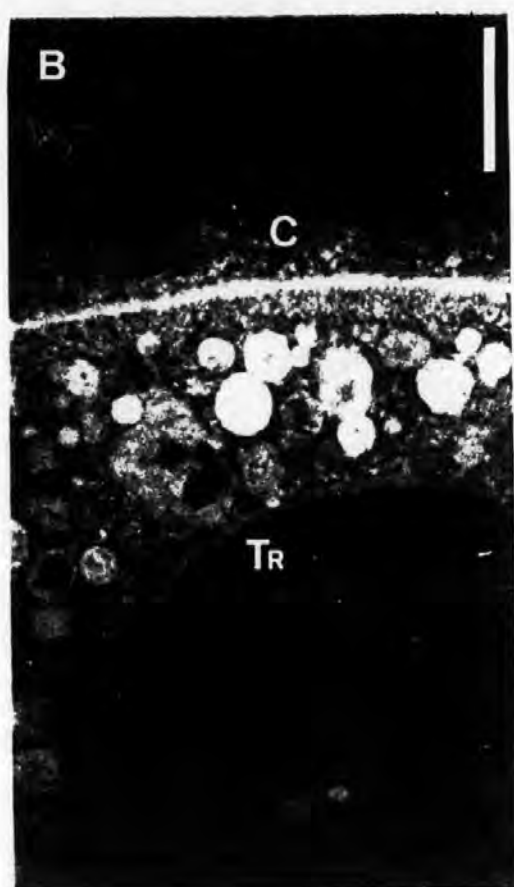


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ABSTRACT

"Immune response of carp Cyprinus carpio (L.) to Ichthyophthirius multifiliis (Foquet), with reference to events within the epidermis."

By Martin Leslie Cross

The in vitro and in situ immune responses of carp Cyprinus carpio to Ichthyophthirius multifiliis were investigated in order to characterise the immune mechanisms involved in protection. 'O' group and adult carp were immunised against I. multifiliis by controlled infection procedures. Sterile immunity was not achieved; theronts were observed to penetrate the skin of immunised fish, although in the majority of cases this did not lead to successful trophozoite establishment. It was concluded that most parasites prematurely exited the epidermis of immunised fish within two hours of penetration as an active survival strategy. Trophozoites remaining in immunised fish beyond two hours post infection were able to complete normal development.

Serum from carp immunised against I. multifiliis displayed specific in vitro theront immobilising activity, and antibody was detected against parasite ciliary membranes and mucocyst organellae; similar activity was not detected in cutaneous mucus. Significant amounts of antibody could not be located at the immediate host/parasite interface of trophozoites in situ in immune skin; prevention of antibody binding may be achieved by means of a mucocyst-derived "sheath" around the parasite and the formation of a layer of necrotic host tissue debris.

Parasite development in immunised fish initiated a localised cellular infiltration, predominated by type III granulocytes ("basophils") and mast cell-like cells, the activity of which may augment further cellular and humoral infiltration. Sites of premature parasite exit from the epidermis of immunised fish were infiltrated by actively phagocytic cells, predominantly macrophages, probably in response to localised antibody/antigen complex deposition. Pronephric leucocytes of immunised fish displayed greater in vitro non-specific phagocytic activity than cells of carp naive to I. multifiliis; the relevance of this to enhanced antigen uptake in immunised fish is discussed.

Based on results of the present study, a model for the mode of protection in fish against I. multifiliis is proposed.

INTRODUCTION

According to Rogers & Gaines (1975), ichthyophthiriasis or "whitespot" causes more damage to fish populations worldwide than any other single parasitic disease. Severe epizootics have been reported in wild fish populations from several countries (Allison & Kelly, 1963; Backer & Crites, 1976; Elser, 1955; Kozel, 1976; Wurtsbaugh et al., 1988). The disease can be highly pathogenic, particularly to young fish, and substantial losses are incurred in the intensive aquaculture and aquarist industries (Bauer, 1962; Nigrelli et al., 1976). Ichthyophthiriasis has a cosmopolitan distribution in freshwater ecosystems, and the disease affects a wide range of teleost fish species, including the economically important cyprinids, ictalurids, salmonids and tilapias (Hoffman & Bauer, 1971).

The causative organism of ichthyophthiriasis is the obligate parasite Ichthyophthirius multifiliis, a holotrich ciliate, belonging to the class Oligohymenophora, subclass Hymenostomata, order Hymenostomatida and suborder Ophryoglenia (Levine, 1980). I. multifiliis has a direct life cycle (figure 1). "Whitespots" visible in the skin of fish (figure 2) are intraepithelial trophozoites. When mature, these rupture the host epithelia and become a temporarily free-living trophont stage, eventually settling on to a substrate

Figure 1. Diagramatic representation of the life cycle of Ichthyophthirius multifiliis. Times shown represent the typical period of each stage of development at 20°C.

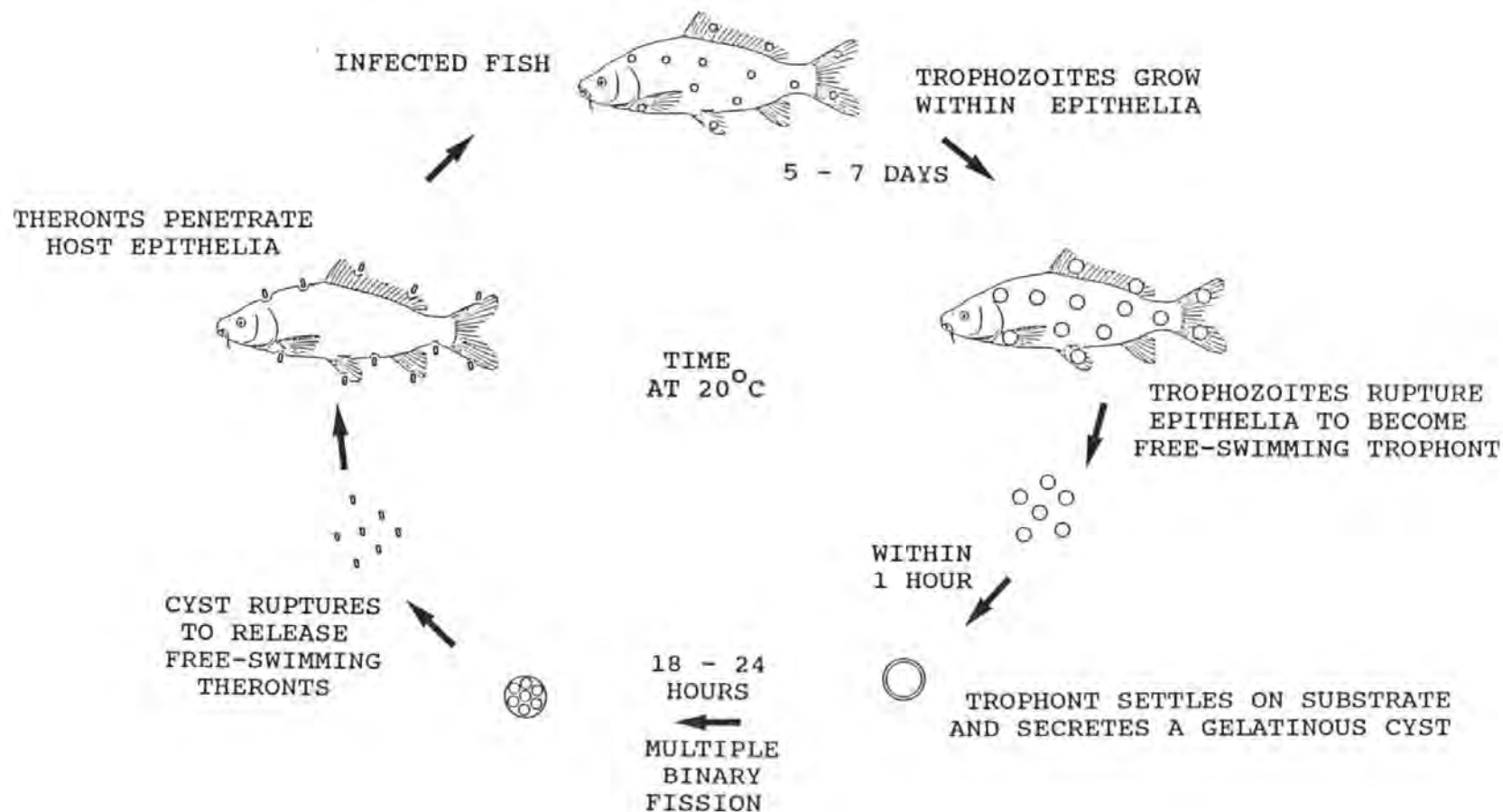
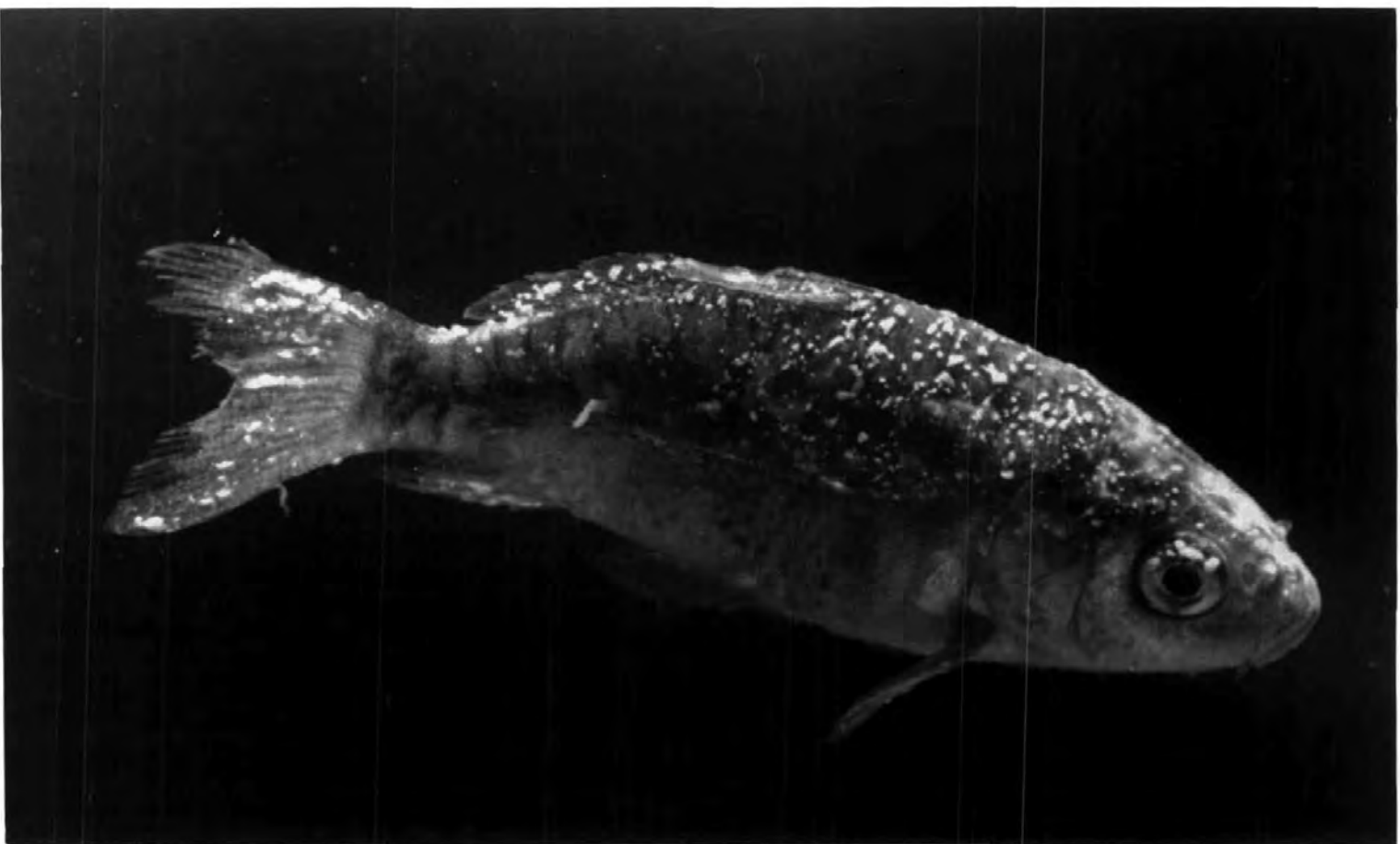
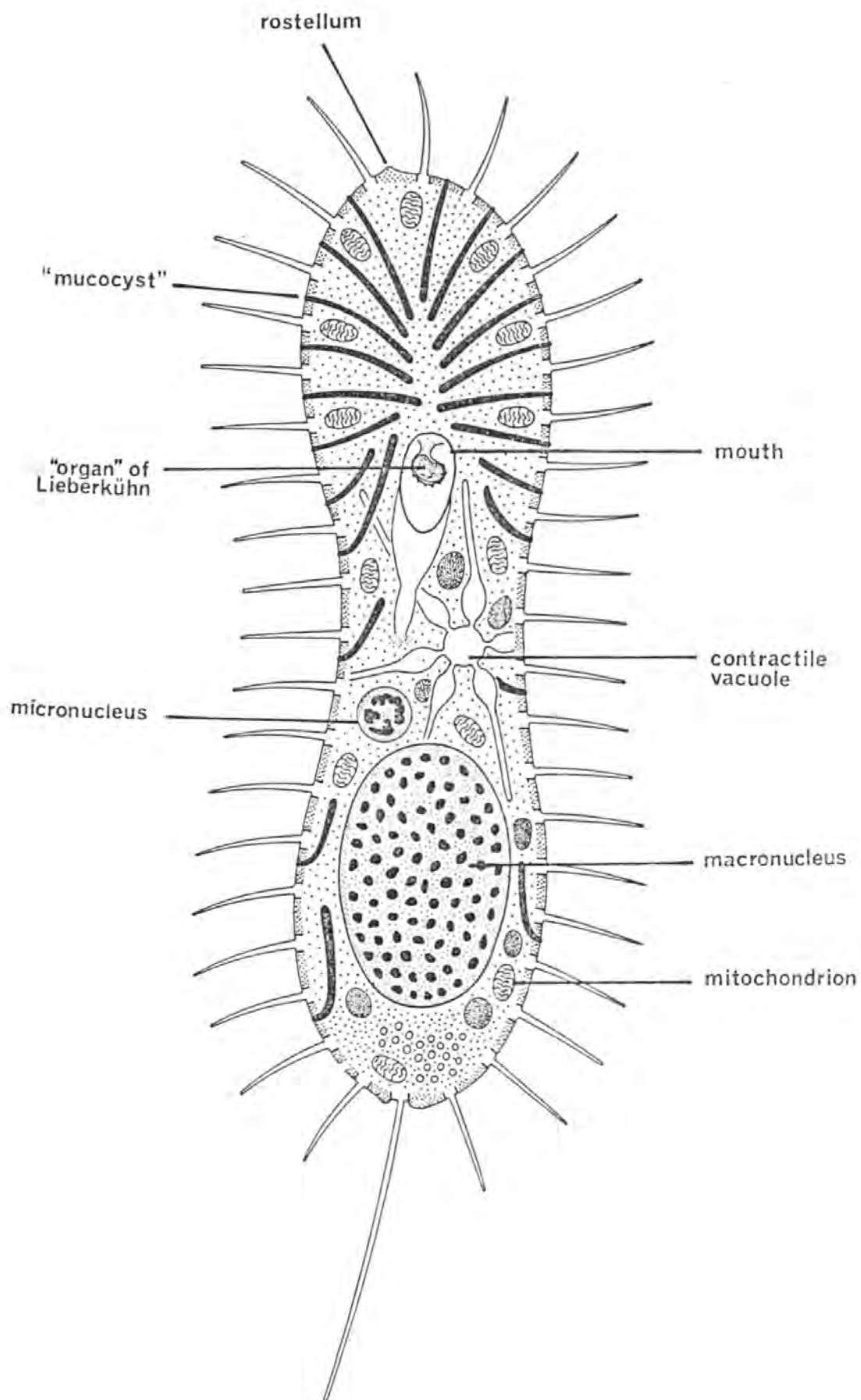


Figure 2. 'O' group carp with ichthyophthiriasis. Each "whitespot" represents a single trophozoite in the epidermis. Note the severe erosion of the fins.



surface. Here the trophont secretes a surrounding gelatinous cyst from mucocyst organelles (Ewing et al. 1983), and undergoes repeated binary fission within the cyst. Following completion of this reproductive stage the cyst ruptures to release free-swimming infective theront stages (figure 3). Up to 1,000 theronts may be released from a single cyst (Beckert, 1975; Leibovitz, 1980; MacLennan, 1935; McLay, 1985). The precise mechanism of host contact is unknown, although it is known that theronts are positively phototactic (Lom & Cerkasova, 1974). Upon contact with the host epithelium, material is extruded from mucocyst organelles and the theront penetrates into the host epithelium by mechanical (and possibly lytic) tissue disruption (Butcher, 1943; MacLennan, 1935; Kozel, 1980). The role of mucocysts in the penetration process is uncertain: Ewing et al. (1985) consider that the extruded mucocyst material adheres the theront to the epithelium to aid penetration, whilst Matthews & Matthews (1984) consider that the extruded material may enzymatically degrade host tissue. The post-penetrated theront (now termed a trophozoite) commences feeding and growth within the epithelium soon after penetration (Ewing et al., 1986). Complete development has been shown to occur at temperatures from 4°C to 25°C (Wagner,

Figure 3. Diagrammatic representation of the infective
theront stage (courtesy of Dr. R.A. Matthews).



1960), and the life cycle proceeds more quickly at higher temperatures. Typically, at 20°C the parasite spends 5 - 7 days in the host, 18 - 24 hours in the reproductive stage, and the theronts have an infective life span of 12 - 24 hours (McCallum, 1982), although infectivity falls over this period.

I.multifiliis inhabits the epithelial layer of the skin (epidermis), gill and buccal cavity tissues. Infections cause severe localised and systemic pathology in the host. In the early stages of infection, surrounding epithelial cells become pyknotic and lose cohesion, forming a layer of necrotic host tissue debris around the developing parasite (McLay, 1985). In the later stages, spongiosis and hyperplasia of the infected epithelia occur, and eventually the epithelial tissue may slough off (Chapman, 1984; Hines & Spira, 1974a; Ventura & Paperna, 1985). In heavy infections, death of the fish usually follows the establishment of secondary bacterial and fungal infections, or as a result of haemodilution and loss of ionic balance due to osmoregulatory failure (Hines & Spira, 1974b). In intensive aquaculture and aquarist shop conditions, the close confinement of large numbers of fish favours transmission of successive generations of the parasite,

and mass mortalities are usually very rapid.

With the rise in intensive aquaculture over the last two decades, increasing attention has been paid to the control of transmissible diseases, which impose a limitation to the further development of the industry (Lamers, 1985). Chemotherapy is currently employed as the major means of controlling ichthyophthiriasis, although the treatments involved are considered to be stressful to the fish, environmentally damaging, expensive and time consuming to administer. Furthermore, these treatments are only effective in killing the parasite in the free aquatic trophont/cyst/theront stages; some mercury-based chemicals are capable of killing trophozoites in situ in the host, although legislation prevents their use in aquaculture (Nousias, 1987). Other methods of control include u.v. sterilisation and electrotherapy (Farley & Heckman, 1980; Gratzek et al., 1983) although these are considered too expensive and unsafe for commercial use. Genetic resistance to I.multifiliis infection has been demonstrated in certain species, and selective breeding has been postulated as a further control means (Bone, 1983; Price, 1985).

Not surprisingly, great attention has been paid to the development of a vaccine against I.multifiliis. A satisfactory in vitro culture system (necessary for the large scale production of antigenic material) has not been established, although attempts have been made to develop a vaccine based on ciliary extracts from the closely related free-living Tetrahymena species (Goven et al., 1980a, 1980b & 1981a; Wolf & Markiw, 1983). To date, however, there is no commercially available whitespot vaccine. As with the development of parasite vaccines in the mammalian field, recombinant DNA technology may offer an alternative approach to vaccine development in the future.

There have been several comprehensive studies on the immune response of fish to I.multifiliis (e.g. Hines & Spira, 1974c; Houghton, 1987; Pyle, 1983). However, many aspects of the immune response have yet to be fully understood. The present study has aimed to investigate aspects of the host/parasite relationship, with a particular view to characterising in vitro and in vivo immunological events in relation to their role in host protection. For this purpose the common or mirror carp Cyprinus carpio was used as a model host, this species being hardy and easy to keep, and of considerable economic importance in western and third world

aquaculture, as well as in the aquarist trade (Michaels, 1986). Furthermore, the basic immunobiology of the carp has been well documented (Lamers, 1985 & 1986; Rombout et al., 1986, 1987 & 1989a-c).

LITERATURE REVIEW

1. IMMUNE RESPONSES TO ICHTHYOPHTHIRIUS MULTIFILIIS

There has probably been more published research concerning the immunology of I.multifiliis than any other eucaryotic parasite of fish. Evidence of a protective immune response to the parasite initially came from observations of parasite population dynamics. Bauer (1953) found that carp (Cyprinus carpio) which had recovered from I.multifiliis infection were, to varying extents, resistant to reinfection, suggesting the establishment of immunity. Similar observations were made under laboratory conditions by Lahav & Sarig (1973), although these authors questioned the role of resistance in modulating infections in aquaculture. Subsequent studies aimed to control the parasite's life cycle more carefully in order to quantitatively characterise the protective response. Beckert & Allison (1964) infected white catfish (Ictalurus catus) by introducing various numbers of trophonts to the aquarium, and reported that the fish were more resistant to a challenge than previously unexposed catfish. Similar observations were recorded by Areerat (1974) after infecting channel catfish (Ictalurus punctatus) by a single exposure to the parasite. Hines & Spira (1973b) exposed adult carp C.carpio to a "high" (400 trophonts per fish) or a "low" (40 trophonts per fish) infection

level, and allowed the infection to proceed in an enclosed environment. They observed that at the low infection level parasites were completely cleared from the the fish by 16 to 18 days and all the fish recovered, whilst at the high infection level large parasitaemias were recorded and all the fish died within 24 days. Similar results were achieved by Subasinghe (1982) using juvenile carp, following repeated exposure of the fish to known numbers of trophonts. These results indicated a threshold level of infection in a given population of fish, above which severe disease outbreaks and mortalities occur, but below which fish recover and develop resistance. This point was further emphasised by McCallum (1986), who mathematically modelled the dynamics of I.multifiliis infections in black mollies, Poecilia latipinna.

The precisely controlled infections used by McCallum (1986) were facilitated by exposing fish to controlled numbers of theronts, rather than adding trophonts of uncertain fecundity to the tank to initiate infections. The development of standardised procedures for the production of theronts (Dickerson et al., 1981; McCallum, 1985) enabled the development of strictly controlled immunisation and challenge regimes for fish, e.g. by Houghton & Matthews (1986), who immunised 'O'

group carp Cyprinus carpio by repeated controlled exposures to known numbers of theronts.

External factors have been implicated in modulating the immune response to I.multifiliis. Wahli et al. (1986) showed that levels of dietary ascorbic acid influenced the level of protection following immunisation in trout, Oncorhynchus mykiss. Houghton & Matthews (1986 & 1990) have demonstrated that the protective immune response of carp Cyprinus carpio may be compromised under conditions of physiological stress. Subasinghe & Sommerville (1989) indicated that immunity in tilapia Oreochromis mossambicus may be transferred maternally to mouthbrooding fry.

The first detailed immunological investigation concerning the mode of protection against I.multifiliis was undertaken by Hines & Spira (1973a & 1974c), who demonstrated specific in vitro immobilising activity against trophonts in serum from previously infected and recovered (re. immune) carp C.carpio. Subsequently, immobilising activity against both theronts and trophonts was shown in serum from several fish species following exposure to the parasite, e.g. carp Cyprinus carpio (Houghton, 1987); channel catfish Ictalurus punctatus (Areerat, 1975; Clark et al., 1987a); tilapia

Oreochromis mossambicus (Subasinghe, 1986); rainbow trout, Oncorhynchus mykiss (Wahli & Meier, 1985; Wahli et al., 1986). At high concentrations immune serum has been shown to be lytic to I.multifiliis in vitro (Clark et al., 1987a). That the in vitro theront immobilising effect is probably due to the production of parasite-specific antibodies by the fish has been demonstrated by Clark et al. (1987b & 1988). Areerat (1974) demonstrated protection against challenge and parasite immobilising activity in serum from channel catfish I.punctatus following intraperitoneal (i.p.) injection of a crude trophont homogenate, indicating a similar immune response to that following infection. Parasite immobilising activity has also been demonstrated in cutaneous mucus from carp, tilapia and trout following infection (Hines & Spira, 1974c; Subasinghe, 1986; Wahli & Meier, 1985).

Extensive studies have been undertaken to characterise the antigens involved in host protection. Goven et al. (1981a) showed that serum from rabbits immunised with either I.multifiliis or the closely related ciliate Tetrahymena pyriformis contained surface-binding antibodies and displayed in vitro immobilising activity against both ciliates. Their results highlighted two

very important principles: First, that the humoral response to I.multifiliis was associated with the cilia and cell surface suggested that theront immobilising activity might be directed against protein components of the surface membrane, analogous to the known "i" antigens from immunological studies on free-living ciliates (Beale & Kascser, 1957; Beale & Mott, 1962; Margolin et al., 1959). Secondly, that antiserum against either ciliate was fully cross-reactive against the surface of the other indicated the presence of shared common antigenic determinants. Cross-reactivity between antigenic determinants of the two ciliates was further demonstrated using polyclonal (Pyle, 1983) and monoclonal (Dickerson, 1986) mammalian antisera. Since no satisfactory in vitro culture technique existed (or now exists) for I.multifiliis, this finding had great implications for the development of a vaccine, based on cross-reactive components of T.pyriformis, which can easily be cultured in vitro.

Further research in this field therefore concentrated on utilising ciliary preparations of Tetrahymena spp. as potentially protective immunogens. Goven et al. (1980a & 1980b; 1981b) immunised channel catfish Ictalurus punctatus parenterally with isolated whole cilia of T.pyriformis and found good levels of protection against

I.multifiliis on challenge. Wolf & Markiw (1983) immunised rainbow trout Oncorhynchus mykiss with cilia of T.thermophila by bath vaccination and demonstrated some degree of protection against challenge with I.multifiliis. However, reservations concerning the use of Tetrahymena cilia as potentially protective antigens were expressed by Dickerson et al. (1984) who showed that the level of protection varied with the strain of ciliate used, and by Houghton (1987) who demonstrated that some strains offered no protection at all. Furthermore, Clark et al. (1988) showed that serum from channel catfish Ictalurus punctatus rendered immune to I.multifiliis following infection displayed good in vitro theront immobilising activity, but they could not detect an antibody response against ciliary extracts of Tetrahymena pyriformis. Their results indicated that the antigens involved in in vitro theront immobilisation and the protective immune response in fish were not common to Tetrahymena. To date, there remains no commercially available vaccine against ichthyophthiriasis.

Interest has remained in characterising the antigens of I.multifiliis relevant to in vitro theront immobilisation and host protection. Theront immobilising activity of serum from channel catfish Ictalurus

punctatus immune to I.multifiliis has been correlated to the level of antibody against integral ciliary membrane proteins in these fish (Clark et al., 1987b & 1988). Dickerson et al. (1987 & 1989) demonstrated in vitro theront immobilising activity in serum from rabbits immunised with isolated ciliary membrane fractions of I.multifiliis. They showed that a 43 KD glycoprotein was the predominant antigen and that this constituted 6% of the whole cell or 60% of the ciliary membrane protein respectively, and considered that this antigen was analogous to the "i" antigens of free-living ciliates. It is clear that natural infection of I.multifiliis in fish can initiate an antibody response to ciliary membrane protein(s) which promotes in vitro theront immobilisation. However, the precise role of this response in host protection is uncertain.

Cellular responses to I.multifiliis infection have received less attention in research than the humoral response, although Houghton (1987) considered that cellular immunity may play a role in host protection. Changes in the proportions of leucocytes in the peripheral blood of infected fish are known to occur, with neutrophilia and lymphocytopaenia being recorded (Hines & Spira, 1973c; McLay, 1985; Trombitskii, 1984). Leucocytes (including lymphocytes, macrophages and

various granulocytes) have also been shown to infiltrate the epidermis of several fish species during infection (Ewing, 1981; Hines & Spira, 1974a; McLay, 1985; Ventura & Paperna, 1985), although the immunological significance of their role is unclear. Graves et al. (1985a & 1985b) showed that natural cytotoxic cells (NCC) from the pronephros of channel catfish Ictalurus punctatus killed Tetrahymena pyriformis in vitro, and their affinity for I.multifiliis was demonstrated by the ability of I.multifiliis to deplete pronephric cell suspensions of NCC s. NCC s from the peripheral blood of heavily infected catfish were shown to display enhanced in vitro killing of mammalian tumor cells, compared to NCC s from either immune or uninfected fish. However, the precise role of cellular elements in protection against I.multifiliis is unclear.

2. IMMUNE RESPONSES TO PROTOZOA OTHER THAN I. MULTIFILIIS

Evidence of protective immune responses in fish to several protozoan parasites has been provided by examining parasite population dynamics, particularly in relation to suppressed immune responses of fish at lower temperatures. Robertson (1979) showed that levels of the flagellate Ichthyobodo necator on farmed salmonids peaked at 8 weeks post first-feeding, but then fell dramatically in older fish, indicating the onset of protective immunity. Regression of infection levels of a myxosporean Henneguya spp. with increasing temperatures has been attributed to enhanced immune responses of their hosts at the higher temperatures (Dykova & Lom, 1978a; Lom, 1969). Barrow (1954) observed that various species of freshwater fish harboured larger numbers of trypanosomes in winter months, and this was correlated with the inability of these fish to produce trypanocidal antibodies at the lower temperatures. Similar observations have been recorded in trypanosome infections of other wild fish species, e.g. plaice Pleuronectes platessa (Cotterell, 1977) and summer flounder Platichthys denatus (Burresson & Zwerner, 1982; Sypek & Burresson, 1983). Fluctuating parasitaemias in other haemoflagellate infections have also been attributed to the development of protective immunity

(Woo, 1979, 1981 & 1987a & 1987b).

Resistance to infection has been shown to occur in fish against several protozoan parasites following infection or injection of parasite material. Klontz et al. (1986) showed that rainbow trout Oncorhynchus mykiss which had survived a clinical infection of proliferative kidney disease were completely refractory to re-infection with the causative agent PKX, whilst Awakuru & Kurahashi (1967) found that fish infected with a microsporidian Pleistophora spp. were resistant to further infection for up to one year. Burresson & Frizell (1986) showed that summer flounder Platichthys denatus immunised with killed Trypanoplasma bullocki were resistant to a live challenge for up to one year.

Antibody production has been recorded against several protozoa. Griffen & Davis (1978) demonstrated circulating antibody in the serum of rainbow trout Oncorhynchus mykiss against spores of the myxosporidian Myxobolus cerebralis. Lytic antibody produced in response to haemoflagellate infection has been recorded in several fish species (Barrow, 1954; Jones & Woo, 1987; Sypek & Burresson, 1983; Wehnert & Woo, 1981); lysis has been considered to be due to complement activation (Jones & Woo, 1987), and in vivo phagocytosis by

macrophages has been demonstrated by Sypek & Burresson (1983) and Jones & Woo (1987), the latter authors considering that specific antibodies were involved in parasite opsonisation. However, several sporozoan infections in fish have been characterised by the inability to detect a host antibody response (e.g. Bartholomew et al., 1989; Halliday, 1974; Lauden et al., 1986; Pauley, 1974; Siau, 1980).

Cellular responses, both localised and systemic, have been reported in response to several protozoan infections in fish. However, whether these responses represent non-specific or specific immune-mediated responses is uncertain. Hoffman (1975) recorded variable levels of dermal and muscular inflammation in response to the ciliate Tetrahymena corlissi in guppies, Poecilia reticulata. Bartholomew et al. (1989) and Yasutake & McIntyre (1986) reported inflammatory reactions involving granulocytes, macrophages and lymphocytes in fish infected with the coelozoic myxosporean Ceratomyxa shasta. Dykova & Lom (1978a) considered that myxosporidians Henneguya spp. could initiate a granulomatous type cellular response following sporulation. Localised granulomatous type infiltration characterised by mononuclear leucocytes has been recorded in response to several myxosporean infections

(Amandi & Fryer, 1978; Copland, 1983; Duhamell et al., 1986; Ferguson & Needham, 1978; Halliday, 1976; Kent & Hedrick, 1985; Taylor & Haber, 1974). In microsporidian infections, breakdown of xenoma cells and release of spores is often followed by intense phagocytosis (Dykova & Lom, 1980; Hauk, 1984; Matthews & Matthews, 1980; Ralphs & Matthews, 1986). Hawkins et al. (1981) recorded localised cellular infiltration into the liver of killifish Fundulus spp. infected with the coccidian Eimeria funduli, involving lymphocytes, actively phagocytic monocytes/macrophages, and three types of granulocytes, at least one of which was observed to degranulate at the infection site. Systemic cellular responses are known to occur during protozoan infections. Increased leucocyte counts, primarily due to granulocytopaenia, have been recorded during haemoflagellate infections (Lowe-Jinde, 1986; Steinhagen et al., 1990).

A further immunological phenomenon often associated with protozoan infections in fish is parasite-induced immunosuppression. Cryptobia salmositica infections are known to suppress the humoral response of rainbow trout Oncorhynchus mykiss to sheep erythrocytes (Wehnert & Woo, 1981) and suppress protection against challenge

with the bacterium Yersinia ruckeri (Jones et al., 1986).
Lauden et al. (1986 & 1987) demonstrated that infection
of winter flounder Pseudopleuronectes americanus with
the microsporidian Glugea stephani or intraperitoneal
injection of parasite antigens reduced serum
immunoglobulin levels and suppressed the ability of the
fish to respond to antigens.

3. SKIN IMMUNOLOGY

In view of the tissue distribution of I.multifiliis being limited to the epithelia, and investigations in the present study being confined to the caudal fin epidermis, it was considered pertinent here to review the immunology of fish skin, with particular reference to the epidermis.

Teleost fish skin, unlike that of mammals, is generally non-keratinized (Whitear, 1986; Yasutake & Wales, 1983; Yokote, 1982). The epidermis comprises living cells which are capable of mitotic division at all levels, although tissue regeneration typically proceeds from the basal epidermal layer (Bullock et al., 1978; Whitear, 1986). The general structure and ultrastructure of the skin of carp (Cyprinus carpio) has been recorded in detail by Hendrickson & Matoltsy (1968a - c).

Epidermal hyperplasia has been recorded in response to various pathogens and chemicals, and is considered by Ellis (1981) to represent one of the major non-specific defence functions of the epidermis. The continual shedding of mucus from the surface of fish skin has been implicated as another important non-specific physical defence mechanism (Ellis, 1981b; Fletcher, 1982; Pickering & Richards, 1980). An increase in mucus

production following handling stress or infection is thought to enhance this protective function (Pickering & Macey, 1977; Wechsler, 1984). Cutaneous mucus has been shown to contain soluble components of the non-specific immune system such as complement-like proteins, C reactive protein and lysozyme, as well as exhibiting broad antimicrobial activity (Austin & McIntosh, 1988; Fletcher & White, 1973a; Harrel et al., 1976; Hjelmeland et al., 1983; Ramos & Smith, 1978; Takahanshi et al., 1986; Wood & Willoughby, 1986).

As well as components of the non-specific immune system, humoral antibody has been demonstrated in cutaneous mucus from several fish species (Bradshaw et al., 1981; Diconza & Halliday, 1971; Fletcher & White, 1973b; Lobb, 1987; Lobb & Clem, 1981a & 1981b; Ourth, 1980; Rombout et al., 1989c; St. Louis-Cormier et al., 1984). Teleost fish contain only one class of immunoglobulin, a tetrameric IgM, although Lobb & Clem (1981a) have demonstrated dimeric forms of the molecule in the mucus of sheepshead Archosargus probatocephalus. Rombout et al. (1986) demonstrated that total immunoglobulin content per mg. of protein in cutaneous mucus was approximately 1/50 the amount in serum from carp Cyprinus carpio. The immunoglobulin present in cutaneous mucus is known to be secreted by epidermal mucus cells, although these cells

are not thought to synthesize immunoglobulin themselves (Peleteiro & Richards, 1988). Peleteiro & Richards demonstrated immunoglobulin in the mucopolysaccharide vesicles of mucus cells, which they considered was derived by endocytosis of intra-epidermal immunoglobulin by these cells.

The precise origin and source of synthesis of mucosal immunoglobulin is uncertain. Fletcher & Grant (1969) provided evidence that cutaneous mucus antibodies were derived from the blood by transudation, since parenteral immunisation of plaice Pleuronectes platessa with mammalian erythrocytes or Vibrio anguillarum bacterin lead to detectable levels of specific antibody in the mucus. Similar results were achieved by St. Louis-Cormier et al. (1984) following parenteral immunisation of rainbow trout Oncorhynchus mykiss with sheep erythrocytes. Contrary to these reports, several authors have been unable to detect antibody responses in cutaneous mucus following parenteral immunisation with antigens (Diconza & Halliday, 1971; Pyle & Dawe, 1985; Rombout et al., 1989c). Lobb & Clem (1981c) injected radiolabelled immunoglobulin into sheepshead (Archosargus probatocephalus) and could only detect low levels of radioactivity in the cutaneous mucus,

suggesting that the majority of cutaneous mucus antibody was not derived from the blood. Further evidence that the blood (i.e. the systemic immune system) is not the major source of cutaneous mucus antibody came from Lobb (1987) and Kawai & Kusuda (1982), who detected specific antibody activity in cutaneous mucus of channel catfish Ictalurus punctatus and ayu Plecoglossus altevelis following immersion vaccination in the absence of a systemic response. The possibility of localised immunoglobulin secretion by intra-epidermal lymphocytes has been suggested by several authors who have detected lymphocyte-like immunoglobulin positive cells in the epidermis of rainbow trout Oncorhynchus mykiss and channel catfish Ictalurus punctatus (Lobb, 1987; Ourth, 1980; Peleteiro & Richards, 1988; St. Louis-Cormier et al., 1984). However, whether these cells are homing primed cells derived from the blood or represent a localised secretory immune system independent of the systemic immune system is not known.

Recent studies by Rombout et al. (1989c) have thrown new light on the origin of cutaneous mucus antibody in carp Cyprinus carpio. They detected specific antibody in cutaneous mucus against particulate antigen (Vibrio anguillarum bacterin) following repeated oral or anal

immunisation but not following parenteral immunisation. They used the phrase "common mucosal immune system" to explain the presence of specific antibody activity in cutaneous mucus following stimulation of the gut-associated lymphoid tissue with antigen. Thus, there is evidence to suggest that in carp at least, cutaneous mucus antibody against certain antigens is derived from gut-associated immune responses, and not the systemic immune system.

The possible role of skin in the uptake of external antigens has received much attention due to its importance with respect to immersion vaccination. An early study in hyperosmotic infiltration vaccination implicated the lateral line region of the body skin in antigen uptake (Amend & Fender, 1976). However, a comprehensive study by Hockney (1985) demonstrated that externally applied particulate or soluble antigens were incapable of entering the blood via the skin of rainbow trout Oncorhynchus mykiss if the epidermis was intact. Similar conclusions were reached by Alexander et al., 1981. However, cells similar in appearance to the Langerhan's cells of mammalian skin (which are involved in antigen uptake) have been described in the epidermis of Monopterus albus (Mittal et al., 1980), whilst

Peleteiro & Richards (1990) considered that phagocytic macrophages may perform this role in the epidermis of rainbow trout. Although these cells are thought to represent a means of uptaking intra-epidermal antigen to the fish, it is now generally agreed that the gills and the gut are the major sites of external antigen uptake (Rombout et al., 1986; Smith, 1982).

Fish skin has been shown to be immunocompetent in terms of cellular immunity as well as humoral. Lymphocytes, monocyte/macrophages and various granulocyte types have all been recorded within the normal undamaged epidermis (Archer, 1979; Bullock & Roberts, 1975; Ferri & Macha, 1972; Iger & Abraham, 1990; Mittal et al., 1980; Ourth, 1980; Peleteiro & Richards, 1985; Percy, 1970; Roberts et al., 1971; St. Louis Cormier et al., 1984). Mast cell types have been recorded in the non-vascularised regions of the dermis and basal layers of the epidermis in several fish species (Liewes, 1978; Bullock et al., 1975). The ability of leucocytes to infiltrate the epidermis of fish during disease or non-specific tissue damage has been well documented (Iger & Abraham, 1990; Iger et al., 1988; Phromsuthirak, 1977; Sharraf & Roberts, 1989). However, Roberts & Bullock (1976) stated that the extent of inflammatory reactions per se in the epidermis is limited by the tissue's avascular nature.

MATERIALS AND METHODS

1. FISH

1.1. SOURCES OF FISH

For all experimental procedures the common carp Cyprinus carpio L. was used. 'O' group carp were obtained at ages from ten days to three months post-hatching from a carp hatchery subsidiary of Munton and Fisons plc., Suffolk. This hatchery had no previous record of ichthyophthiriasis on site. The carp obtained were an inbred line from an original "Dinklespuller" scattered scale pattern parentage. Adult carp were grown on to size from 'O' group fish, or captured by angling from Drake's reservoir, Plymouth.

Fish used for routine in vivo maintenance of I.multifiliis included the cyprinids C.carpio, the crucian carp Carassius carassius L. and the goldfish Carassius auratus L.. These fish were purchased from local aquarists or fish farmers, or captured from local lakes thought to be free from ichthyophthiriasis.

1.2. LABORATORY MAINTENANCE

For all maintenance purposes aerated tap water which had been buffered over limestone at room temperature to pH 6.8-7.5 was used. Fish from disease-free sources were maintained in 50 or 100 litre stock aquaria at room temperature (18°C-25°C over the period of study), which

were well aerated and filtered by Eheim filtration systems. Fish from disease-prevalent sources were maintained as above, following two weeks of quarantine, during which time chemotherapy was applied as outlined in appendix 1.

For experimental procedures involving 'O' group carp, fish were maintained in 10 litre perspex aquaria, filtered by sub-gravel filtration systems and kept at a constant temperature of $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Strict husbandry practices were observed to minimise stress and prevent the introduction of any disease to the system. Half water changes were made fortnightly, and all fish were fed at approximately 1% body weight per day with commercial carp (Paul's Aquaculture) or trout (BP Nutrition) pellets.

When required, fish were anaesthetized in a preparation of benzocaine (ethyl-p-aminobenzoate) solution. This comprised 10 ml. of stock benzocaine solution (20 mg./ml. 90% ethanol) in one litre of water.

2. I.MULTIFILIIS

2.1. SOURCE OF ISOLATES

I.multifiliis was isolated from freshwater fish obtained

from local aquarists and fish farmers. Over the three year period of study eighteen isolates were established in the laboratory, these being maintained for periods ranging from 2 - 21 weeks (table 1).

2.2. IN VIVO LABORATORY MAINTENANCE

2.2.1. Routine maintenance

For routine maintenance of I.multifiliis (passage) infected fish were introduced to 10 litre aquaria at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$ containing several fish previously unexposed to I.multifiliis (naive fish), the number of naive fish depending on the parasite burden of the infected fish, more fish being added if the burden was high to facilitate this infection. I.multifiliis was then allowed to transmit naturally in the aquaria, and the developing infection was monitored visually. Fish subsequently developing ichthyophthiriasis were removed and placed in new aquaria with fresh naive fish to continue the life cycle.

2.2.2. Production and enumeration of theronts

To produce known numbers of infective theronts for experimental purposes the parasite's life cycle was carefully controlled. Passage fish showing mature trophozoites within the skin were gently agitated in a minimal volume of buffered tap water (pH 7.0) in a glass

Table 1: Isolates of I.multifiliis established

DESIGNATED STRAIN CODE	SOURCE OF ISOLATE	DATE OF ISOLATION	PERIOD OF MAINTENANCE
I/1/CW	Goldfish (<u>Carassius auratus</u>); 20°C.	21/10/87	2 weeks
I/2/CW	Trout (<u>Salmo gairdneri</u>); 13°C.	23/10/87	2 weeks
I/3/WW	Tilapia (<u>Oreochromis</u> spp.); 24°C.	23/10/87	5 weeks
I/4/CW	Cichlid (<u>Cichalosoma</u> spp.); 24°C.	10/12/87	9 weeks
I/5/CW	Goldfish (<u>Carassius auratus</u>); 18°C.	24/2/88	2 weeks
I/6/CW	Goldfish (<u>Carassius auratus</u>); 20°C.	5/4/88	8 weeks
I/7/CW	Goldfish (<u>Carassius auratus</u>); 20°C.	7/6/88	17 weeks
I/8/WW	Goldfish (<u>Carassius auratus</u>); 24°C.	17/10/88	4 weeks
I/9/CW	Goldfish (<u>Carassius auratus</u>); 24°C.	20/11/88	2 weeks
I/10/WW	Cichlid (<u>Cichalosoma</u> spp.); 23°C.	27/11/88	21 weeks
I/11/WW	Loach (<u>Misgurnus fossilis</u>); 24°C.	2/5/89	5 weeks
I/12/WW	Goldfish (<u>Carassius auratus</u>); 22°C.	13/6/89	3 weeks
I/13/WW	Goldfish (<u>Carassius auratus</u>); 22°C.	3/7/89	2 weeks
I/14/WW	Cichlid (<u>Hemichromis bimaculatum</u>); 25°C.	7/7/89	2 weeks
I/15/WW	Catfish (<u>Pangassius</u> spp.); 24°C.	21/7/89	3 weeks
I/16/WW	Carp (<u>Cyprinus carpio</u>); 22°C.	10/8/89	2 weeks
I/17/CW	Goldfish (<u>Carassius auratus</u>); 20°C.	10/8/89	2 weeks
I/18/CW	Trout (<u>Salmo gairdneri</u>); 13°C.	30/11/89	18 weeks

(I/n = isolate number; CW = cold water, WW = warm water).

beaker. Trophonts emerging from the skin due to this agitation were carefully collected with a Pasteur pipette, transferred to clean plastic petri dishes and allowed to encyst in a small volume of either fresh buffered tap water (pH 7.0) or "Volvic" mineral water. Encysted trophonts were washed copiously with clean water to remove contaminating host tissue debris, and the petri dishes were then incubated for 24 hours at 20°C in a constant humidity chamber. Emerging theronts (visible under a stereo microscope) were collected, and theront concentration calculated by counting 1ml. of a 1:1 sample of theronts:20% formalin solution in a Sedgewick-Rafter chamber under low power with a compound microscope.

2.2.3. Controlled infection procedures

To standardise infection levels for experimental purposes, controlled infection procedures were employed by exposing fish to known numbers of theronts by one of the three methods outlined below.

2.2.3.1. Immersion infection

'O' group carp were exposed to I.multifiliis by immersion in tanks containing known numbers of theronts for a specified period of time, using a method based on that of Houghton (1987). Theronts were added in the

required amounts to 2 litre tanks containing well oxygenated buffered tap water (pH 7.0) and mixed thoroughly. The required number of fish (usually 10 per tank) were then added to a density of one fish per 150 ml. of water (for fish under approximately 10g. in weight), or one fish per 300 ml. (for fish over approximately 10g.). The tanks were maintained in total darkness at 20°C for two hours during the duration of the exposure, after which time the fish were quickly washed in fresh water and returned to their original 10 litre aquaria at 20°C \pm 2°C. Five days after the exposure fish were individually anaesthetized and the total number of trophozoites which had developed to maturity on the body surface and fins of each fish was counted. Unless otherwise stated, fish were subsequently transferred twice daily over the following five day period to clean aquaria to prevent secondary re-infection by trophonts exiting the epithelia. Any mortalities were recorded.

2.2.3.2. Droplet infection

To produce a controlled infection in a specified region of the fish (in this case the caudal fin), 'O' group carp were infected by topical application of a droplet containing a known number of theronts. Fish were

individually anaesthetized and laid on their right sides on a petri dish, with the gills kept moist by wrapping the head region in damp tissue paper. The caudal fin was splayed out and a 50 µl. droplet of a suspension containing 50,000 theronts/ml. was carefully applied to the surface of the tail. The penetration of theronts into the caudal fin epidermis was monitored under a stereo microscope, and the infection process stopped after two minutes by washing the tail with fresh water. The fish were then returned to their original aquaria at $20^{\circ}\text{C} \pm 2^{\circ}\text{C}$.

2.2.3.3. Addition of theronts to aquaria

Adult carp were infected by adding theronts directly to their aquarium. Prior to exposure the Eheim filtration system and aeration were switched off to reduce disturbance to the theronts, and the water level was reduced to approximately 40 litres. Known numbers of theronts were added to the aquarium and mixed thoroughly, and the exposure was allowed to continue for two hours, after which time the aquarium was re-filled and the aeration and filtration resumed. Five days after exposure the fish were observed in the aquarium for the presence of mature trophozoites in the skin. The infection was then allowed to proceed in the aquarium.

In all of the controlled infection procedures outlined above, the viability of each batch of theronts used was confirmed by routine passage.

2.3. Experimental procedures involving I.multifiliis

2.3.1. In situ parasite mapping

The position and numbers of parasites within a specified region of the body following infection (in this case the caudal fin), could be determined by mapping the infected areas. Using a camera lucida on a Kyowa stereo microscope, maps showing the position of individual parasites within the caudal fin skin of 'O' group carp were drawn, and mean number of parasites per mm² of skin surface area calculated using a Calcomp digitising board linked to a Westward keyboard.

2.3.2. Immunisation of carp for use in experimental procedures

Standard procedures for the immunisation of 'O' group and adult carp were employed. 'O' group carp were immunised by repeated immersion exposures to known numbers of theronts (2,000 or 10,000 per fish) using a procedure based on that used by Houghton (1987) as previously outlined above. Adult carp were immunised by repeated additions of theronts to their aquarium.

3. PREPARATION OF IMMUNOLOGICAL REAGENTS

3.1. PREPARATION OF ANTIGENS

Antigen preparations were made of I.multifiliis, mammalian red blood cells (RBC) and carp IgM, for use as reagents in immunological studies and to raise antisera.

3.1.1. I.multifiliis

Theronts were used to produce sonicate and homogenate preparations as a crude source of parasite antigens. Theronts were collected in clean "Volvic" mineral water, and any cystogenous material was allowed to settle out in a measuring cylinder before decanting the theronts. The resulting theront suspension was centrifuged very gently (about 50 G for 10 minutes) and excess water siphoned off. Concentrated theronts were then mixed with 5 ml of 0.85% saline; 1 ml of this was gently homogenised in a Jencoms micro tissue homogeniser; the other 4 ml. was sonicated using an MSE Soniprep 150 sonicator fitted with a microprobe. After high speed centrifugation (10 minutes at 10,000 G), no appreciable particulate material could be detected in the sonicate preparation; soluble protein content of this was then determined using the micro protein assay of Bradford (1976). Homogenate and sonicate preparations were then

mixed in equal quantities or left individually, and frozen in 100 µl. aliquots at -70°C.

3.1.2. RBC

Sheep RBC (SRBC) or Horse (HRBC) in Alsever's solution (Tissue Culture Services) were washed three times in phosphate buffered saline (PBS) pH 7.2 and resuspended to the required concentration in PBS.

3.1.3. Carp immunoglobulin (IgM)

IgM was purified from heat inactivated whole carp anti-SRBC serum by ion exchange and gel filtration chromatography, using a Fast Pressure Liquid Chromatography (FPLC) system (Pharmacia). Separated serum fractions were collected on a Frac-100 fraction collector (Pharmacia) and absorbance at 280 nm. read by a UV-1 optical unit (Pharmacia).

3.1.3.1. Purification by ion exchange

Proteins were separated using a Mono-Q anion exchange column (Pharmacia) utilising a tris HCl sodium bicarbonate/sodium acetate gradient, modified from a procedure suggested by Dr. N. Garcia-Olono of the Universidad de Oviedo, Spain (see appendix 2 for details). 200 µl. of whole serum or 1.0-1.2 ml. of gel filtration fractions was loaded onto the column, and fractions eluted with a flow rate of 1.0 ml./minute.

Fractions were collected every minute.

3.1.3.2. Purification by gel filtration

Proteins were separated using a Superose 12 column (exclusion limit 1,000-300,000; Pharmacia). The loading buffer was 50 mM tris HCl pH 7.3 containing 0.1 M sodium bicarbonate. 200 μ l. of whole serum was loaded onto the column, and fractions were collected every two minutes with a flow rate of 300 μ l./minute.

3.1.4. Immune complexes

Carp IgM with anti-horse or anti-sheep RBC activity (in whole serum or chromatography-purified fractions) was coated onto the surface of the respective RBC to form immune complexes. 500 μ l. of heat inactivated whole carp anti-S/HRBC serum or 1.0 ml. of purified anti-SRBC serum fractions was incubated with 10 ml. of thrice washed 20% respective RBC in PBS pH 7.2 for one hour at room temperature. The resulting immune complexes were washed five times in PBS by centrifugation (600 G for 5 minutes) and resuspended to the required concentration in PBS.

3.2. RAISING ANTISERA

Antisera were raised in adult carp against I.multifiliis or mammalian red blood cells (RBC), and the latter used

to raise an anti-carp immunoglobulin serum in rabbits by immunisation with IgM-coated RBC, using a method based on that of Ellis (1980).

3.2.1. Carp antisera

3.2.1.1. Anti-I.multifiliis serum and mucus

Adult carp were immunised against I.multifiliis by repeated infections (as previously outlined) or intraperitoneal (i.p.) injection of theront sonicate. Serum and/or mucus samples were then removed from these fish as outlined below for use as reagents in immunological studies.

Blood and mucus samples were removed from three 1 Kg.+ adult carp three weeks after the final exposure to theronts, and from two similarly sized fish naive to I.multifiliis as controls. Sera were pooled from these fish, and mucus was pooled and concentrated for use as immunological reagents.

A single 300 g+ adult carp was anaesthetized and immunised by i.p. injection of 1 ml. of a 1:1 mixture of Freund's Complete Adjuvant (FCA; Sigma):theront sonicate (360 µg. protein/ml.), followed three weeks later by a booster injection of 1 ml. of a 1:1 mixture of Freund's Incomplete Adjuvant (FIA):sonicate. The fish was then

bled three weeks later. Sera was then prepared from this fish for use as an immunological reagent.

3.2.1.2. Anti-RBC serum

Four 1 Kg.+ adult carp were each injected i.p. with 1 ml. of 20% sheep or horse RBC in PBS per Kg. of fish, followed by booster injections at two more fortnightly intervals. Fish were then bled three weeks after the last booster, and sera prepared and pooled.

3.2.2. Rabbit anti-carp IgM serum

Two male Dutch rabbits were pre-bled as a source of control serum. Each rabbit was then injected subcutaneously at multiple sites along the back with 2 ml. of a 1:1 mixture of SRBC/whole serum IgM complex:FCA, followed three weeks later by a booster injection of 2 ml. of a 1:1 mixture of complex:FIA. Test bleeds were then made two weeks after this booster.

Subsequent immunisations were of 2 ml. of a 1:1 mixture of SRBC/chromatography purified IgM complex:FIA per rabbit six months following the last immunisation, followed by a booster injection of 2ml. of neat SRBC/purified IgM complex three weeks later. Further test bleeds were then made ten days after this booster.

3.3. COLLECTION AND PREPARATION OF BLOOD AND MUCUS

3.3.1. Preparation of sera from blood

Blood was collected from adult carp and rabbits and sera prepared as outlined below.

Adult carp were anaesthetized and bled by a syringe fitted with a 23 gauge needle from the caudal vein. 2 ml. of blood per Kg. of fish was removed and allowed to clot for 1 hour at room temperature and overnight at 4°C. Sera were then collected following centrifugation at 600 G for 5 minutes, and samples from individual fish pooled or stored individually in small aliquots at -70°C. If required, sera samples were heat inactivated prior to storage by heating to 47°C for 20 minutes, as suggested by Mughal (1984).

Rabbits were bled from a marginal ear vein, and the blood allowed to clot for one hour at room temperature and overnight at 4°C. Serum was collected following centrifugation at 600 G for five minutes, and 500 µl. aliquots stored at -70°C or in liquid nitrogen.

3.3.2. Preparation of mucus

Cutaneous mucus was collected from adult carp and prepared by concentration as outlined below.

Fish were pursued around their aquarium with a net for five minutes, a procedure quoted as causing "an almost explosive discharge of mucus" (Murray & Fletcher, 1976). Fish were then individually netted in a soft wide-meshed net, lightly anaesthetized, briefly washed in fresh buffered tap water and drained of excess water. Each fish was placed into a clean Sterilin polyethene bag and agitated gently for 30 seconds, causing mucus to be deposited on the inside of the bag. Mucus samples were then collected and pooled, centrifuged at 600 G for 5 minutes and any solid material discarded. The samples were then further centrifuged at 10,000 G for 10 minutes to pellet any cellular debris, and the resulting supernatants dialysed against solid polyethylene glycol (m.w. 20,000) for 4 hours at 4°C, using benzoylated dialysis tubing with a m.w. exclusion limit of 10,000 (Sigma). The dialysed samples were then collected, and the soluble protein content determined using the micro protein assay of Bradford (1976). Each mucus sample was then adjusted to 2.0 mg. of protein/ml. by the addition of buffered tap water pH 7.0. Small aliquots of the purified mucus were then stored at -70°C.

4. TECHNIQUES FOR THE DETECTION OF IMMUNE RESPONSES

4.1. HUMORAL RESPONSES

4.1.1. Agglutination of RBC

The anti-RBC activity of immunised carp antisera was detected by a direct haemagglutination assay (DHA).

50 μ l. aliquots of test heat-inactivated whole carp anti-RBC sera or test chromatography-purified IgM fractions were serially diluted by PBS pH 7.2 in 96 well microtitre u-well plates (Gibco). 50 μ l. aliquots of thrice washed respective RBC in PBS were then added to each well and the plate agitated gently, and incubated for 3 hours at room temperature and overnight at 4°C in a constant humidity chamber. Titre end point was taken to be the last well to show complete agglutination of RBC. Controls were heat-inactivated sera samples from non-immunised fish.

4.1.2. In vitro immobilisation of theronts

The anti-I.multifiliis activity of immunised carp antisera and mucus was detected by a theront immobilisation assay, using a technique based on that of Houghton (1987).

50 μ l. aliquots of test sera or mucus were serially diluted by buffered tap water (pH 7.0) in 96 well flat

bottomed microtitre plates (Gibco). 50 μ l. aliquots from a suspension of 4,000 theronts/ml. in buffered tap water were added to each well, and the plate was incubated for 2 hours at 20°C in a constant humidity chamber. Each well was then observed under low power on a compound microscope for the immobilisation of theronts. Titre end point was taken to be the last well to show 75% or greater immobilisation. Controls were sera or mucus samples from fish naive to I.multifiliis.

4.1.3. Immunoelectrophoresis

The anti-carp IgM activity of immunised rabbit antisera was characterised by immunoelectrophoresis against whole carp serum, concentrated mucus or chromatography purified IgM fractions as the antigens. 1% purified agar gels in 80 mM sodium barbitone buffer pH 8.2 were set on multiple clean glass slides. Wells were filled with the appropriate antigen and proteins separated in a 110 Volt field against a bromophenol blue marker. Troughs were filled with test rabbit antisera or pre-immune rabbit serum as a control, and allowed to diffuse against the antigen for 24 hours at room temperature in a constant humidity chamber. Gels were then washed for 24 hours in 0.85% saline containing 0.05% sodium azide, dried and stained with 0.2%

coomassie blue in methanol/acetic acid solution.

4.1.4. Indirect fluorescent antibody test (IFAT)

The presence of anti-theront surface-binding antibodies in the serum and mucus of adult carp immunised by repeated infections was investigated using a three link immunolabelling system.

50 μ l aliquots from a suspension of 4,000 theronts/ml in buffered tap water were dried onto wells of silicon coated Hendley slides at 37°C. Theronts were fixed in 70% ethanol for 10 minutes according to Dickerson (1986). Slides were washed in PBS pH 7.2 and 20 μ l droplets of test anti-I.multifiliis sera/mucus at dilutions of 1/2 - 1/20 in PBS incubated on each well for 30 minutes at 37°C in a constant humidity chamber. Slides were washed, and labelled using 1/100 rabbit anti-carp IgM serum as a secondary antibody link, and 1/40 goat anti-rabbit IgG FITC conjugate (Sigma) as the label, containing 0.1% Evan's blue. This procedure is summarised in table 2. Washed slides were then mounted under non-fluorescing immersion oil and observed under blue light using a fluorescent microscope. Controls comprised serum or mucus from carp naive to I.multifiliis, pre-immune rabbit serum, or the omission of the primary or secondary antibody links.

Table 2. Summary of immunolabelling procedures

METHOD	IFAT	IGSS/Immunogold labelling		ELISA
ANTIGEN	Whole theront	Semi/ultrathin sections of theronts & trophonts		Theront sonicate
PURPOSE	To detect anti-parasite antibody	To identify parasite antigens	To detect <u>in situ</u> carp IgM	To detect anti-parasite antibody
1# Ab. LINK	Serum/mucus from carp immunised by repeated infections	Serum/mucus from carp immunised by repeated infections or i.p.injection of theront sonicate	Rabbit anti-carp IgM serum	Serum/mucus from carp immunised by repeat infection or i.p. injection
2# Ab. LINK	Rabbit anti carp IgM serum	Rabbit anti-carp IgM serum (IGL) OR rabbit anti-carp IgM gold label (IGSS)	Goat anti-rabbit IgG gold label	Rabbit anti-carp IgM serum
3# Ab. LINK	Goat anti-rabbit IgG FITC label	Goat anti-rabbit IgG gold label (IGL)	-	Swine anti-rabbit IgG perox. label

The efficacy of rabbit anti-carp IgM serum was also determined using a three link immunolabelling system. 20 μ l droplets of HRBC resuspended to 0.1% in PBS pH 7.2 were dried onto wells of silicon coated Hendley slides at 37°C and fixed in 10% methanol. Slides were washed, and each well incubated with 20 μ l droplets of antigen (carp anti-HRBC serum) diluted 1/10 in PBS pH 7.2 for 30 minutes at 37°C in a constant humidity chamber. Slides were again washed and each well incubated with 20 μ l droplets of test rabbit antiserum or pre-immune serum at dilutions from 1/2 - 1/200. Slides were washed, and labelled using 1/40 goat anti-rabbit IgG FITC conjugate (Sigma). Washed slides were then observed as above.

4.1.5. Immunogold labelling

4.1.5.1. Immunogold silver staining (IGSS) at light microscope level

Antigenic sites of I.multifiliis recognised by specific antibodies in the serum and mucus of adult carp immunised by repeated infections were investigated using a two link immunolabelling system.

Semithin tissue sections on glass slides were incubated with 20 μ l droplets of heat inactivated pre-immune

rabbit serum diluted 1/20 in PBS pH 7.2 containing 0.1% bovine serum albumin (BSA) globulin-free fraction V (Sigma) for 20 minutes at room temperature in a constant humidity chamber. Slides were then drained and incubated with 20 μ l droplets of test anti-I.multifiliis serum/mucus at dilutions of 1/2 - 1/20 in PBS/BSA containing 0.05% sodium azide for 24 hours. Slides were washed for 30 minutes in PBS and labelled using 1/8 rabbit anti-carp IgM serum, which had been commercially conjugated with 15 nm colloidal gold (Biocell). This procedure is summarised in table 2. Slides were washed for 30 minutes in PBS and 10 minutes in distilled water, and stained using an Intense-M silver enhancing kit (Jansenn), with the silver reaction being monitored under cross-polarized light using an Olympus CHP microscope with incident light.

Controls comprised using serum or mucus from carp naive to I.multifiliis; carp serum with a raised antibody titre against an unrelated antigen (HRBC); immune carp serum pre-absorbed of specific antibodies by serial incubation with theront sonicate-coated ELISA plates (see section 4.1.6. and Mor & Avtalion, 1990); and omitting the primary and secondary antibody links.

4.1.5.2. Immunogold labelling (IGL) at electron microscope level

Antigenic sites of I.multifiliis recognised by specific antibodies in the serum and mucus of adult carp immunised by repeated infections and i.p. injection of theront sonicate were investigated, using a three link immunolabelling system.

Ultrathin tissue sections on nickel grids were incubated in 20 µl droplets of heat inactivated pre-immune rabbit serum diluted 1/20 in PBS pH 7.4 containing 0.1% BSA for 30 minutes at room temperature in a constant humidity chamber. Grids were drained and incubated with 20 µl droplets of test anti-I.multifiliis serum or mucus at dilutions of 1/2 - 1/20 in PBS/BSA for 1 hour. Grids were washed for 5 minutes in PBS and incubated with 1/20 rabbit anti-carp IgM serum as a secondary antibody link. Grids were again washed and incubated in 50 mM tris HCl pH 7.2 for 3 minutes, followed by tris HCl pH 7.6 containing 0.1% BSA for 3 minutes and tris HCl pH 8.3 containing 1% BSA for 10 minutes. Grids were then drained and labelled using 1/30 goat anti-rabbit IgG 20 nm colloidal gold conjugate (Bioclin) in tris HCl pH 8.3/1% BSA for 1 hour. Grids were washed in tris HCl pH 7.6/0.1% BSA, followed by tris HCl pH 7.2 and distilled water.

The presence of in situ carp IgM in tissue sections was also investigated, using a procedure similar to the system described above, but using a two link system by omitting the primary antibody link step.

These immunogold labelling procedures are outlined in table 2. Controls were the same as those described for IGSS.

4.1.6. Enzyme-linked immunosorbant assay (ELISA)

The presence of anti-theront antibodies in the serum and mucus of adult carp immunised against I.multifiliis by repeated infections and i.p. injection of sonicate was investigated by ELISA, using a three link immunolabelling system.

Reagents were prepared as outlined in appendix 2. For the investigation of mucus samples, 50 μ l aliquots of reagents and test samples were used throughout; for serum samples, 100 μ l aliquots were used. Aliquots of theront sonicate (10 μ g protein/ml) were coated onto wells of 96 well ELISA plates (Gibco) overnight at room temperature in a constant humidity chamber. Plates were washed three times in wash buffer and wells incubated for 2 hours with aliquots of test immune or control mucus or serum, individually serially diluted from 1/2 to 1/256 in incubation buffer. Plates were washed and

wells incubated for 2 hours with aliquots of rabbit anti-carp IgM serum diluted 1/200 in incubation buffer. Plates were again washed and wells incubated with aliquots of swine anti-rabbit IgG peroxidase conjugate (DAKO) diluted 1/1000 according to the manufacturer's instructions for 2 hours. This procedure is summarised in table 2. Plates were then washed four times and 100 μ l aliquots of working strength substrate added to each well. Colour development was stopped after 10 minutes by the addition of 25 μ l of 2.5 M H_2SO_4 to each well. Absorbance at 492 nm was read immediately using a computer-linked Titrtrek Multiskan spectrophotometer.

Where required to reduce the non specific background signal, 2% BSA in incubation buffer was included as a blocking agent following each antibody link step, and rabbit anti-carp IgM serum (secondary antibody link) was partially purified by precipitation of the gamma globulin fraction by the addition of 40% saturated ammonium sulphate solution.

4.2. CELLULAR RESPONSES

The in vitro phagocytic activity by pronephric cells from 'O' group carp immunised against I.multifiliis by repeated infections and previously unexposed carp was compared using a chemiluminescence (CL) assay.

4.2.1. Preparative procedures

4.2.1.1. Pronephric cells (single cell suspension)

Fish were killed by over anaesthetization and bled by caudal fin severance. The pronephros was removed, teased free of contaminating connective tissue in Hank's Incomplete Balanced Salts Solution (HIBSS) and homogenised in fresh HIBSS in a 1 ml Jencoms tissue homogeniser. Tissue debris was allowed to settle and the resulting cell suspension was washed twice by centrifugation at 600 G for 5 minutes in fresh HIBSS. Cells were resuspended in a minimal volume of Hank's Complete Balanced Salts Solution (HCBSS), and viable cell counts made (excluding erythrocytes and small lymphocytes) with 0.1% trypan blue in 0.85% saline. Cells were then resuspended to the required concentration in HCBSS.

4.2.1.2. Phagocytic material

Preparations of I.multifiliis or yeast cell zymosan A (Sigma) were made for use as phagocytic material.

I.multifiliis material was prepared by mixing 40 μ l of a 1:1 mixture of theront sonicate (360 μ g protein/ml):theront homogenate with either 40 μ l of serum from adult carp immunised by repeated infections or 40 μ l of serum from carp naive to I.multifiliis.

This mixture was incubated for 1 hour at room temperature before use. Controls comprised 40 μ l of 0.85% saline mixed with 40 μ l of naive carp serum.

Zymosan was prepared and opsonised (if required) according to the method of Scott & Klesius (1981), using a scaled down version of their procedure to produce 80 μ l of opsonised and non-opsonised zymosan.

4.2.2. Assay procedures

80 μ l aliquots of cell suspensions (2×10^7 /ml for I.multifiliis assays; 10^7 /ml. for zymosan assays) were added to 2 ml flat bottomed glass vials. 20 μ l aliquots of working strength luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) solution (see appendix 2) were gently added to each vial. Background light emission for each vial was monitored on an LKB 1250 luminometer over a 10 minute period, or until the readings had fallen below 0.5 mV. 80 μ l aliquots of the appropriate phagocytic material were then gently added to each vial, and further readings taken every 5 minutes over a 1 hour period. The assays were performed in a completely darkened room, with the only light source being a red photography lamp.

5. HISTOLOGY

5.1. PRE-FIXATION PROCEDURES

Small ($<2\text{mm}^2$) skin and lymphoid tissue samples were removed from fish by biopsy in a small volume of PBS pH 7.2 and fixed immediately. Theronts and trophonts were collected in water as previously described, and fixed by adding an equal volume of X2 concentrated fixative to the sample. Where required, trophonts were incubated in carp serum prior to fixation: trophonts were collected and transferred to 0.5 ml perspex vials in a minimum volume of water, 100 μl of carp serum was then added to each vial at a dilution of 1/4 in mineral water and the vials were incubated for 1 hour at room temperature, before washing copiously with clean water prior to fixation procedures.

5.2. PREPARATION OF MATERIAL FOR ROUTINE LIGHT/ELECTRON MICROSCOPY

Samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.2 for 2 hours at 4°C , washed in cacodylate buffer, and post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at 4°C . Washed samples were dehydrated in an ethanol series (30%-100%), embedded in Spurr's medium resin and polymerized at 70°C for 8 hours.

Semi and ultrathin tissue sections were cut on a Porter Blum ultramicrotome. Semithin sections were dried onto glass slides at 70°C, stained with 1% toluidine blue in borax, and observed using orange and green filters on a compound microscope. Ultrathin sections were mounted on 150 µm mesh copper grids, stained with ethanolic uranyl acetate and lead citrate (Reynolds, 1963) and observed at 60 or 80 KV. on a Phillips TEM 300 electron microscope.

5.3. PREPARATION OF MATERIAL FOR IMMUNOLABELLING AT LIGHT/ELECTRON MICROSCOPE LEVEL

Samples were fixed in 0.4 - 0.8% glutaraldehyde in 0.1 M cacodylate buffer containing 0.2% picric acid for 1 hour at 4°C. Samples were washed in cacodylate buffer, dehydrated in an ethanol series (70%-100%) and embedded in L.R. White medium resin. Polymerization was achieved in gelatin capsules cured at 47°C for 32 hours.

Semi and ultrathin tissue sections were cut as for routine microscopy. Semithin sections were dried onto clean glass slides coated with 1% poly-l-lysine at 37°C. Following immunolabelling and silver staining procedures, sections were counterstained with 1% methylene blue without borax, and observed under epi-polarized light using an Olympus Vanox microscope,

or blue light using a fluorescence microscope. Ultrathin sections were mounted on 150 μ m mesh nickel grids, stained as for routine electron microscopy following immunolabelling, and observed at 80 KV only.

RESULTS

1. ESTABLISHMENT OF PROTECTIVE IMMUNITY

'O' group and adult carp were immunised against I.multifiliis by repeated exposure to known numbers of theronts. The degree of protection established was estimated by observing the number of trophozoites which had completed development to reach maturity within the epidermis five days following exposure. A pool of 120 immunised 'O' group carp was established and subsequently used in further investigative experiments concerning in situ parasite development and the host responses; sera and mucus were removed from three immunised adult carp and used as reagents in in vitro immunological studies.

1.1. Immunity in 'O' group carp

Fish were immunised by repeated immersion exposure to 2,000 theronts per fish at each of three fortnightly intervals, followed two weeks later by a single exposure to 10,000 theronts per fish. 120 'O' group carp were immunised by this procedure in 12 groups of 10 fish per group, at intervals of not more than one week apart. Significantly fewer trophozoites were observed to have developed to maturity following the second and third immunising infections (3.64 ± 5.50 and 1.54 ± 1.54 per fish respectively) than following the first immunising infection (22.98 per fish ± 35.19 ; P values

<0.025 and 0.01 respectively, Student T test; table 3). The establishment of protective immunity following completion of the immunisation procedure was confirmed by challenge. The 120 'O' group carp were pooled, and a random sample of 10 immunised fish (average weight 3.54 g. \pm 0.47 g.) and 10 previously unexposed 'O' group carp (average weight 2.88 g. \pm 0.96 g.) were challenged by immersion exposure with a potentially lethal number of theronts (10,000 theronts per fish). The infection was allowed to proceed on returning fish to their original aquaria. Significantly fewer trophozoites (4.25 ± 5.52 per fish) were observed to have developed to maturity in the immunised fish than in the previously unexposed fish (each more than 200 per fish; $P < 0.05$; table 3), and no mortalities were recorded in the immunised fish compared to 100% mortalities in the previously unexposed fish. It was thus concluded that the immunisation procedure had been successful in establishing protective immunity against I.multifiliis.

Three months following completion of the immunisation procedure 40 fish were removed from the pool of remaining immunised fish, these containing the 10 largest fish present. These fish were boosted by exposure to 10,000 theronts per fish, in 4 groups of 10

Table 3. Mean number of trophozoites developing to maturity in 'O' group carp at various stages of the immunisation procedure, and following a potentially lethal challenge to confirm immunity.

STANDARD IMMUNISING PROCEDURE

	#1 EXPOSURE (2,000 Th. PER FISH)	#2 EXPOSURE (2,000 Th. PER FISH)	#3 EXPOSURE (2,000 Th. PER FISH)	#4 EXPOSURE (10,000 Th. PER FISH)
MEAN NO. TROPHOZ. PER FISH	22.9 ± 35.2	3.6 ± 5.5*	1.5 ± 1.5**	38.7 ± 18.5
NUMBER OF FISH USED	120	120	120	120

(* = Numbers significantly lower than #1 exposure: * = P < 0.025; ** = P < 0.01, Student T test).

	BOOSTER IMMUNISATIONS		CHALLENGE INFECTION	
	#1 BOOSTER (10,000 Th. PER FISH)	#2 BOOSTER (10,000 Th. PER FISH)	IMMUNE FISH (10,000 Th. PER FISH)	NAIVE FISH (10,000 Th. PER FISH)
MEAN NO. TROPHOZ. PER FISH	11.6 ± 6.4	9.3 ± 15.8	4.2 ± 5.5*	> 200
NUMBER OF FISH	40	10	10	10

(* = Numbers significantly lower than previously unexposed naive fish; P < 0.001, Student T test).

Th. = Theronts

Trophoz. = Trophozoites

fish per group, the boosters being administered to each group of fish at the same time. A mean of 11.6 ± 6.4 trophozoites per fish were observed to complete development in these fish (table 3), and no mortalities were recorded; these fish were thus considered to still show a significant level of protection. Three months later, the 10 largest fish were again boosted, by immersion exposure to 10,000 theronts per fish. A mean of 9.3 ± 15.8 trophozoites per fish were observed to complete development in these fish (table 3), and no mortalities were recorded; these fish were thus considered to still be protectively immunised.

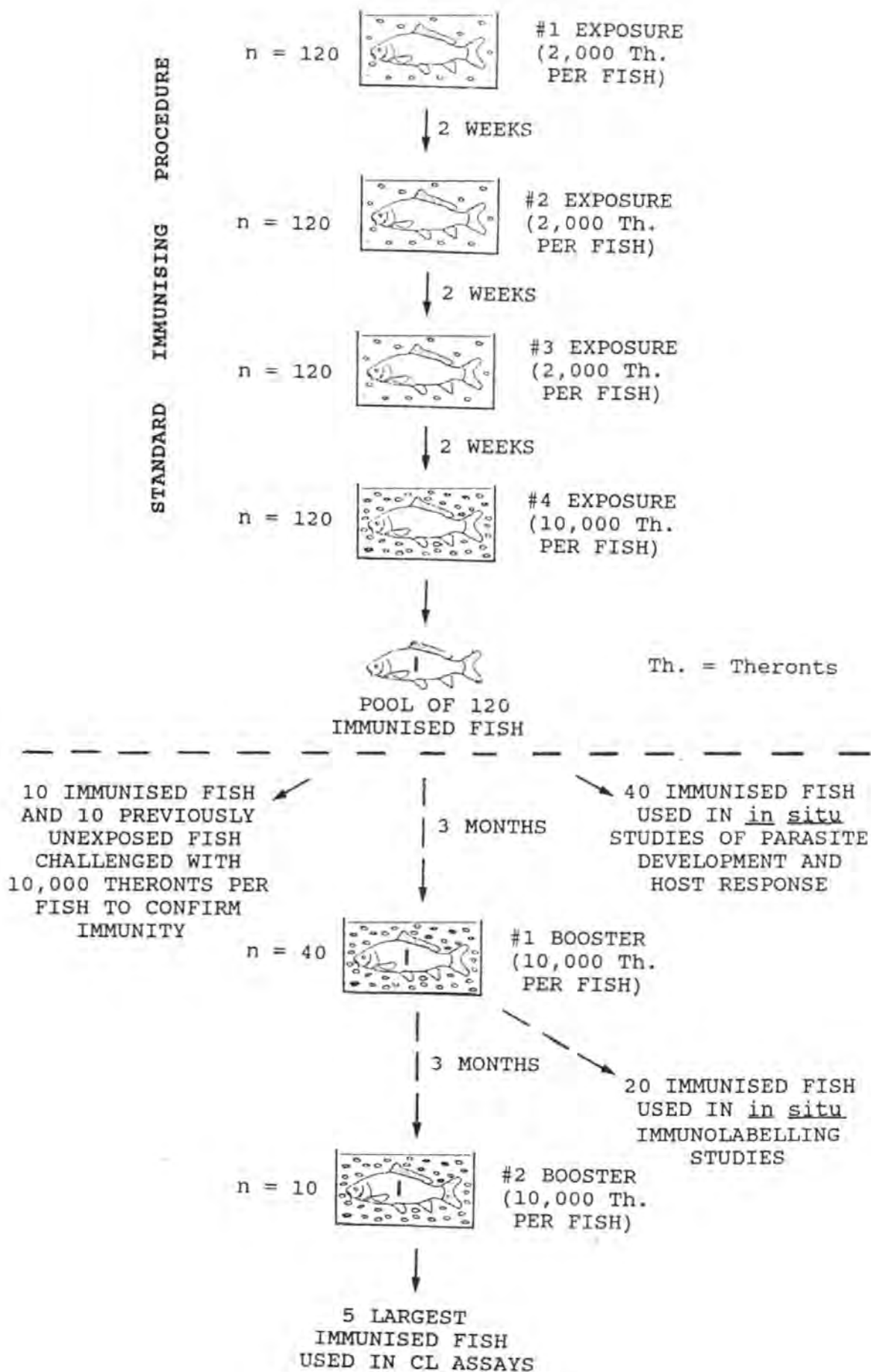
Trophozoites which had developed to maturity were able to complete the life cycle and produce infective theronts as normal at each stage of the immunisation procedure.

The immunisation procedure for 'O' group carp is summarised in figure 4.

1.2. Immunity in adult carp

Adult carp were immunised against I.multifiliis by repeated additions of known numbers of theronts to their aquarium. Three 1 Kg + adult carp were exposed to 100,000 theronts, followed two weeks later by two more fortnightly exposures to 200,000 theronts, and a final

Figure 4. Summary of the immunisation procedure for 'O' group carp.



exposure to 400,000 theronts two weeks after this. Following the final immunising exposure very few trophozoites were observed to develop to maturity in any of the three fish, and it was concluded that the immunisation procedure had been successful in establishing protective immunity in these carp.

The immunisation procedure for adult carp is summarised in figure 5.

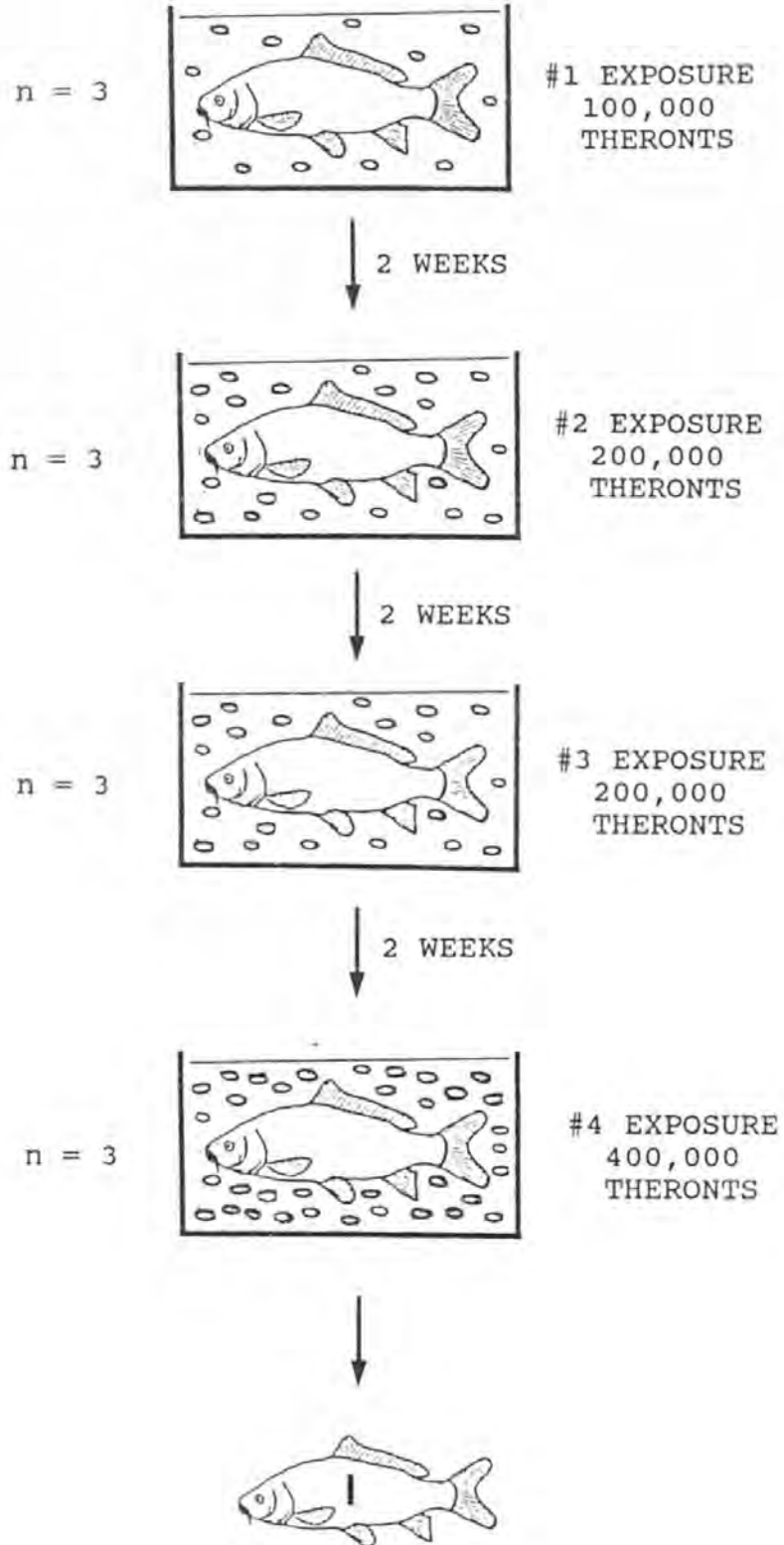
2. PARASITE DEVELOPMENT AND THE HOST RESPONSE

The development of trophozoites and the host response within the caudal fin epidermis of 'O' group carp was investigated, by means of in situ parasite mapping and histological examination at light/electron microscopy level.

2.1. In situ mapping of parasite development

Two groups of 10 immunised 'O' group carp (average weight $3.54 \text{ g} \pm 0.47 \text{ g}$) and 10 previously unexposed fish (average weight $4.14 \text{ g} \pm 0.66 \text{ g}$) were individually infected by droplet infection in two separate experiments. Maps of randomly selected areas (approximately $10 - 20 \text{ mm}^2$) were constructed to show the position and numbers of parasites developing in the epidermis at various time intervals over a 5 day period

Figure 5. Summary of the immunisation procedure for adult carp.



SERA AND MUCUS REMOVED FOR USE AS REAGENTS
IN in vitro IMMUNOLOGICAL STUDIES

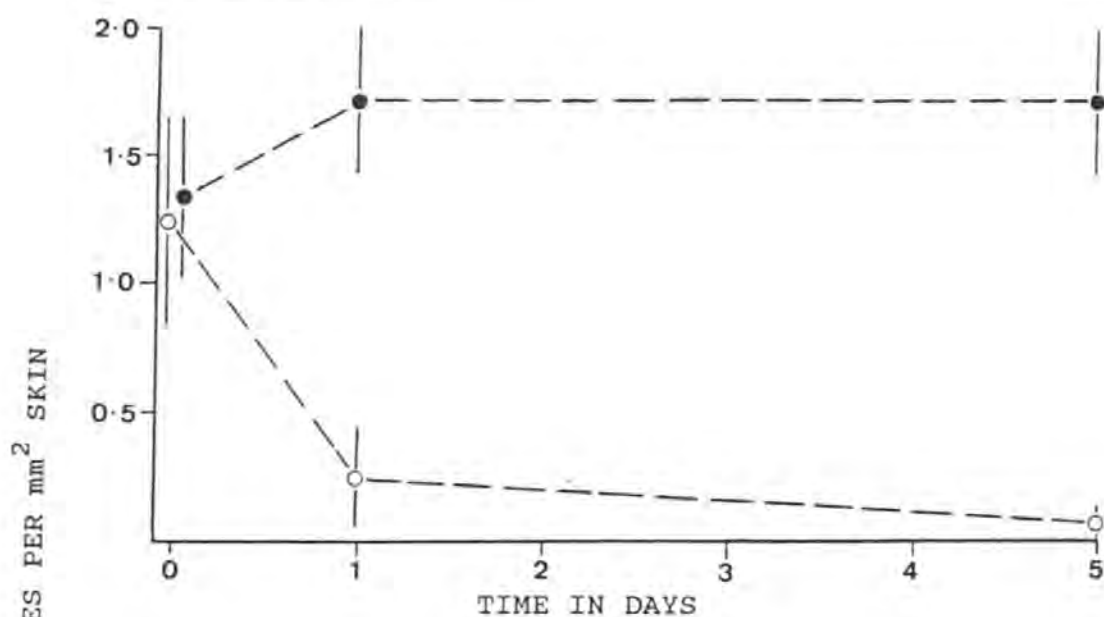
following exposure. In the first experiment mapping was undertaken immediately following exposure (time 0+), and at intervals of 1 day and 5 days following exposure. A significant decrease in the number of trophozoites recorded in the skin of immunised fish was observed 1 day following exposure compared to time 0+, and 84% of parasites could not be re-located (P value <0.025 , Student T test; figure 6 a). In the second experiment mapping was undertaken at time 0+, 2 hours, 8 hours, 1 day and 5 days following exposure. A significant decrease in the number of trophozoites recorded in the skin of immunised fish was observed 2 hours following exposure compared to time 0+, and 79% of parasites could not be re-located (P <0.025 ; figure 6 b), with a further smaller reduction in numbers between 2 hours and 5 days (P <0.025). There were no significant differences between the numbers of parasites recorded in the epidermis of immunised or previously unexposed fish at time 0+.

2.2. Light/electron microscopical study of in situ trophozoite development and the host response

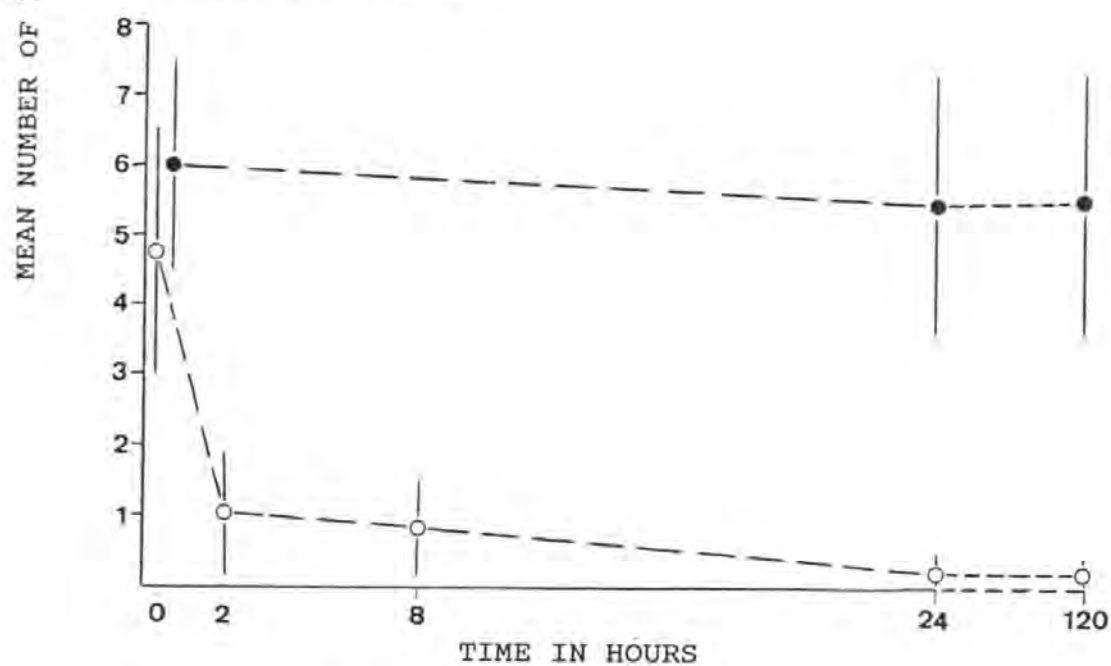
Investigations concerning I.multifiliis were restricted to the caudal fin epidermis of '0' group carp, but it was first considered necessary to compare the structure

Figure 6. Graphs showing the mean number of trophozoites per mm² of tail skin recorded in the caudal fin of immunised and previously unexposed 'O' group carp at various times following droplet infection. The two graphs represent two separate experiments using 10 fish of each group.

a. First experiment



b. Second experiment



● = PREVIOUSLY UNEXPOSED FISH

○ = IMMUNISED FISH

(Figures shown represent means \pm standard error of means [SEM] for 10 fish of each group).

and ultrastructure of the epidermis from different regions of the body from normal non-infected 'O' group carp and from adult carp as well.

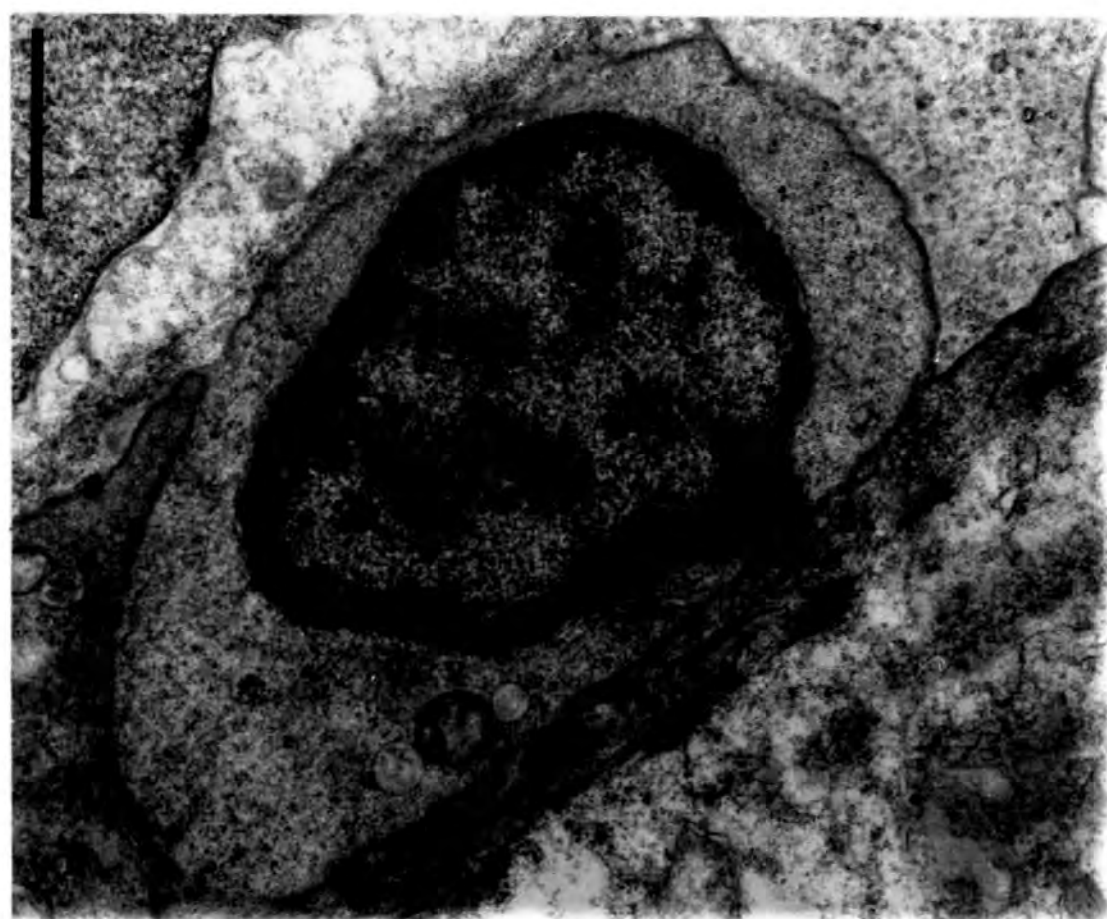
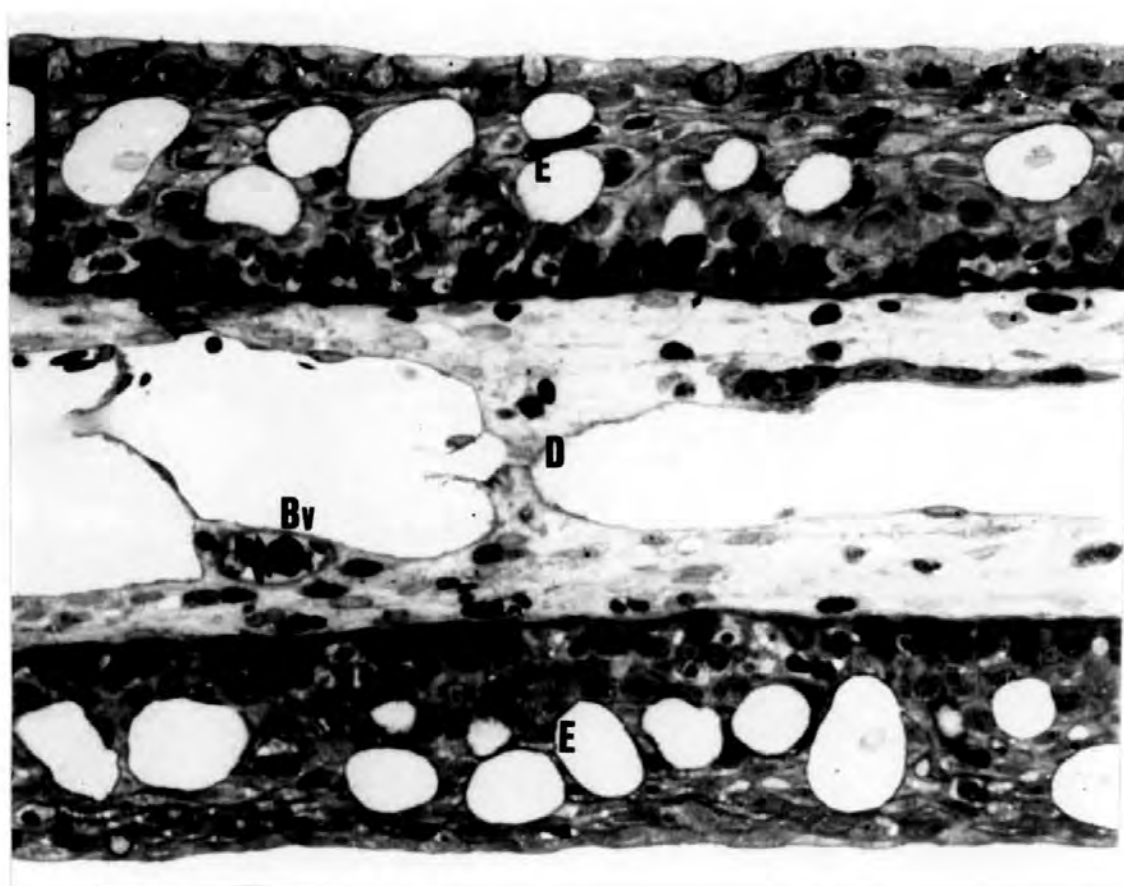
2.2.1. Structure and ultrastructure of carp tissues and cells

Skin from the caudal fin of 'O' group carp comprised two layers, namely the epidermis and dermis, separated by a fibrillar basal lamina (figure 7). There was little difference in caudal fin structure between 'O' group and adult carp, although the epidermis of adult carp was significantly thicker, ranging from 60 μm to 120 μm compared to 30 μm to 70 μm in 'O' group carp. Skin from dorsal and ventral regions of the body was essentially similar in structure to caudal fin skin, but the dermal tissues were underlaid by muscle.

In all cases the epidermis comprised four major resident cell types identified on the basis of ultrastructure (namely filament cells, mucus cells, club cells and chloride cells), with several non-resident leucocyte-like cells also being occasionally recorded. The predominant cell type was the malpighian or **filament cell**, which was observed at all levels of the epidermis. Filament cells were generally oval in shape, and typically measured 6-9 μm by 5-7 μm , with an oval

Figure 7. Light micrograph of 'O' group carp skin from the caudal fin. Bv = blood vessel; D = dermis; E = epidermis. X 500 mag.. Scale bar = 50 μ m.

Figure 8. Electron micrograph of a filament cell from the basal epidermis of an 'O' group carp. BL = basal lamina. X 12,000 mag.. Scale bar = 2 μ m.



centrally positioned nucleus containing randomly dispersed chromatin (figure 8). The surface of filament cells from the peripheral layers of the epidermis were convoluted with microridges, projecting 300 μm - 700 μm beyond the outer face of the cells (figure 9). Filament cells contained occasional mitochondria and lysosomes, but the most distinguishing feature was the abundance of cytoplasmic tonofilaments, often forming extensive desmosomal connections with other adjacent resident cells (figure 10). **Mucus cells** were recorded chiefly in the peripheral layers of the epidermis, where the mucus-containing vesicles were often observed in the process of being discharged (figure 11). Mucus cells were round or slightly ovoid in shape, measuring 8 μm - 14 μm by 6 μm - 12 μm in size, and possessed small eccentrically positioned nuclei, with occasional densely packed rough endoplasmic reticulum (RER). **Club cells** were recorded in the mid epidermal layers. These were large ovoid cells (15 μm - 35 μm by 10 μm - 20 μm) with an amorphous cytoplasm essentially devoid of organelles, but possessing a euchromatic centrally positioned small nucleus (figure 12). **Chloride cells** were infrequently encountered, but always recorded in the peripheral epidermal layer. Chloride cells were round or slightly ovoid in shape, measuring 7 μm - 9

Figure 9. Electron micrograph of a filament cell from the peripheral epidermis of an 'O' group carp. X 10,500 mag.. Scale bar = 2 μ m.

Figure 10. Electron micrograph of tonofilaments and desmosomal junctions between two adjacent filament cells in the epidermis of an 'O' group carp. X 190,000 mag.. Scale bar = 100 nm.

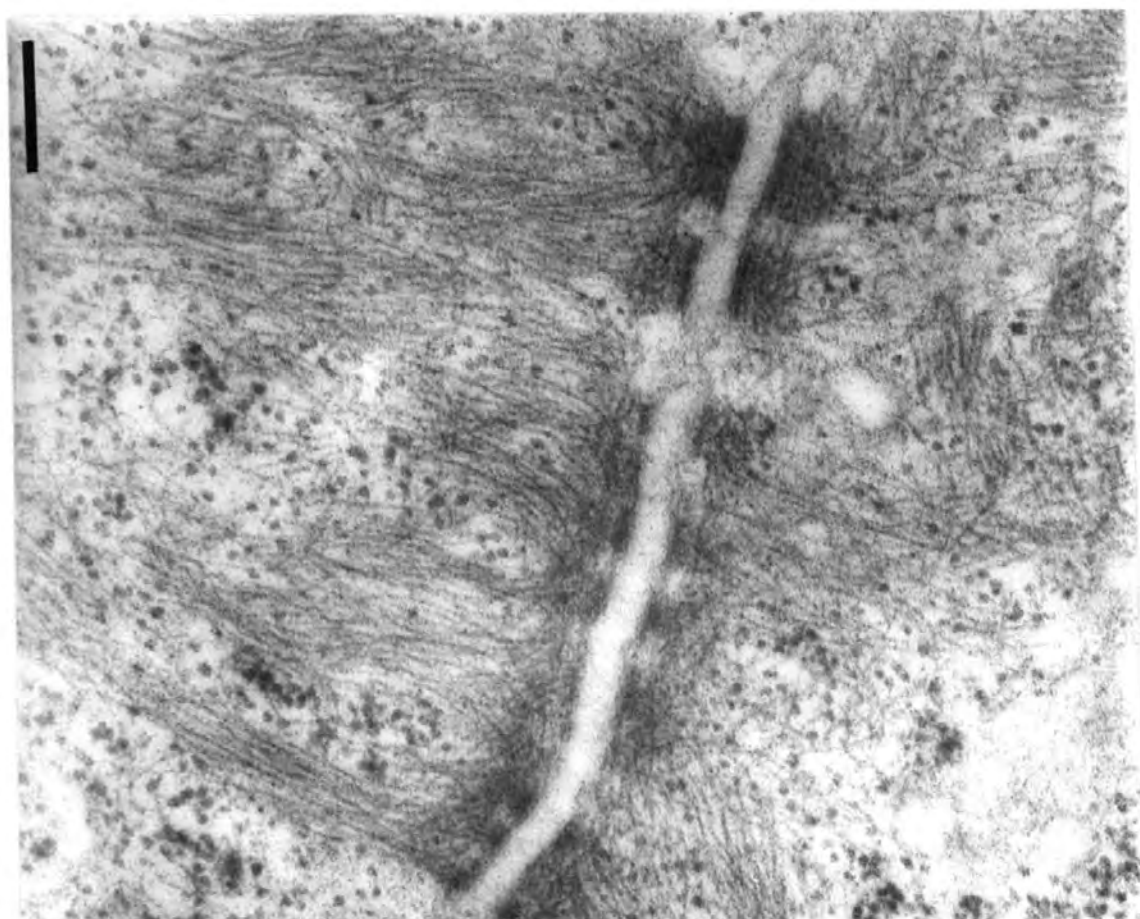
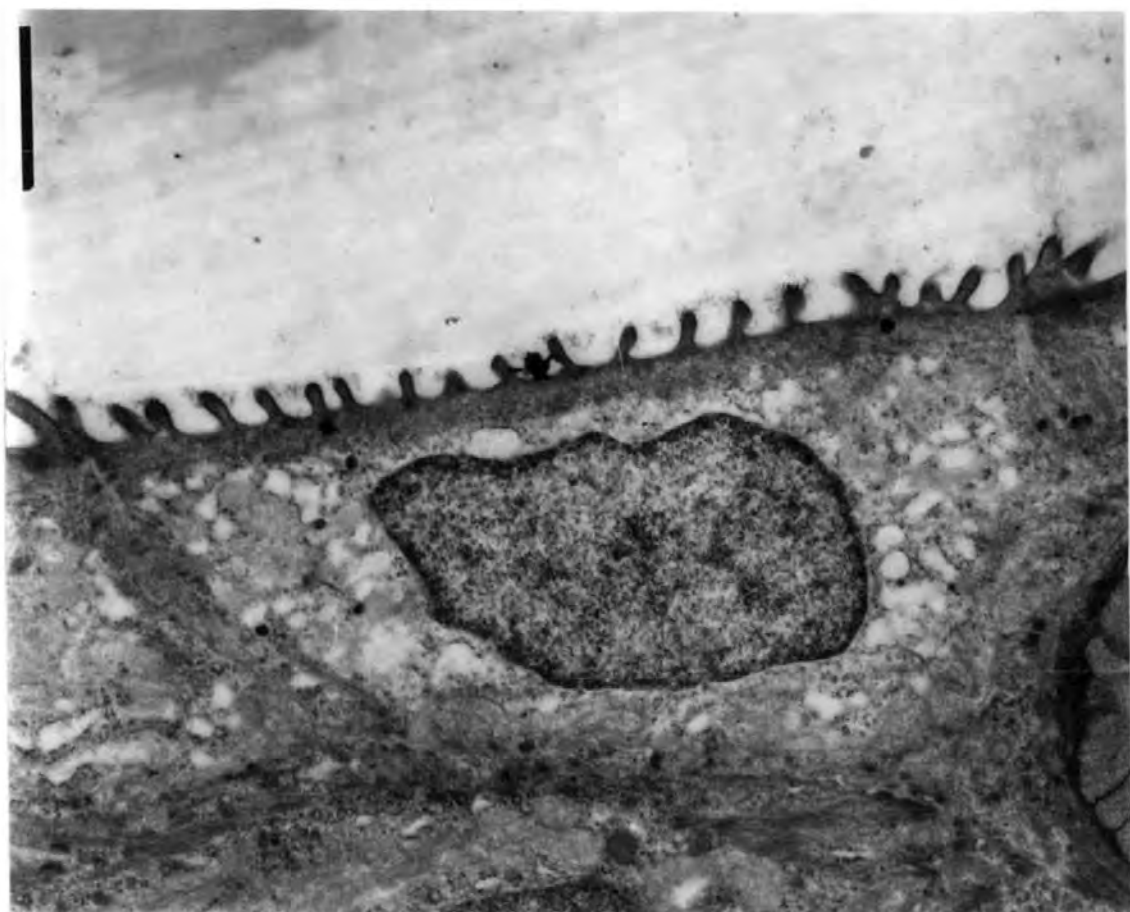
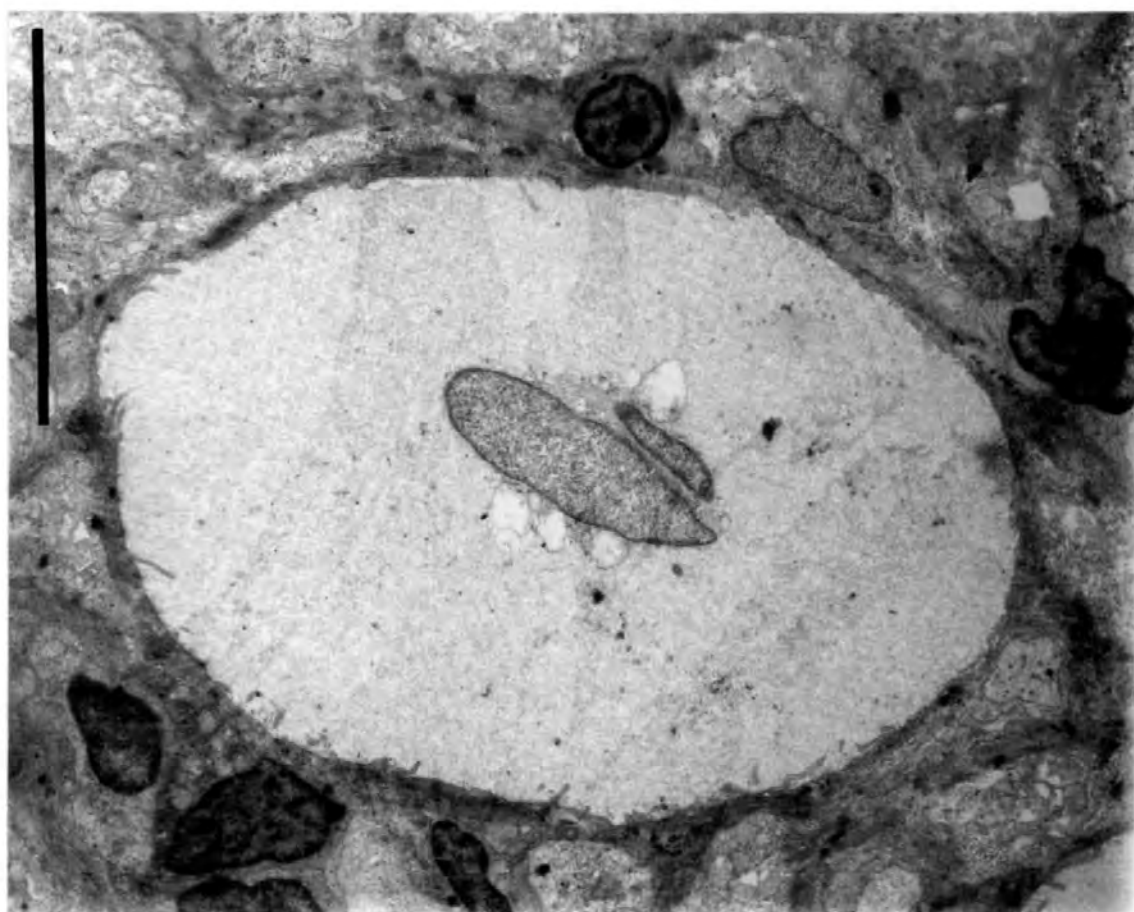
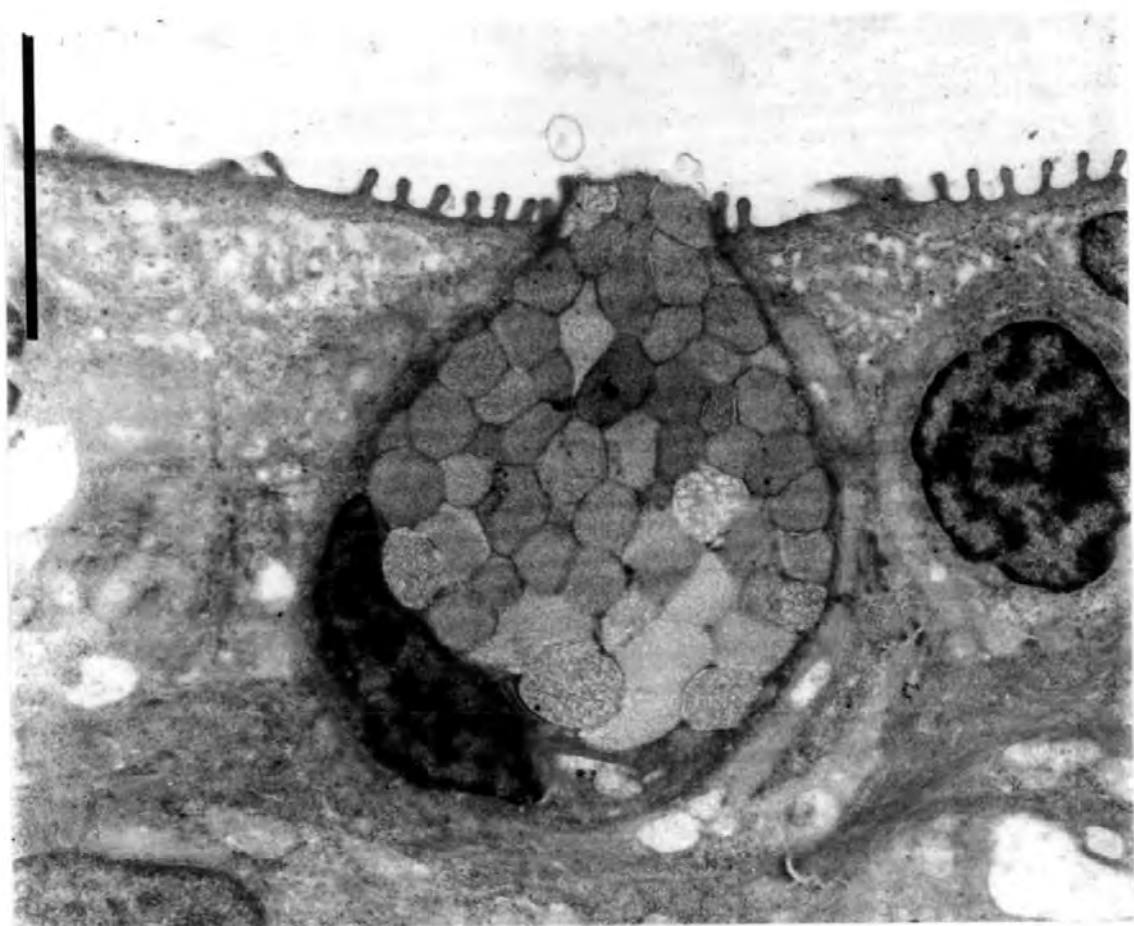


Figure 11. Electron micrograph of a mucus cell from the peripheral epidermis of an 'O' group carp. X 8,200 mag.. Scale bar = 5 μ m.

Figure 12. Electron micrograph of a club cell from the mid epidermis of an 'O' group carp. X 5,000 mag.. Scale bar = 10 μ m.

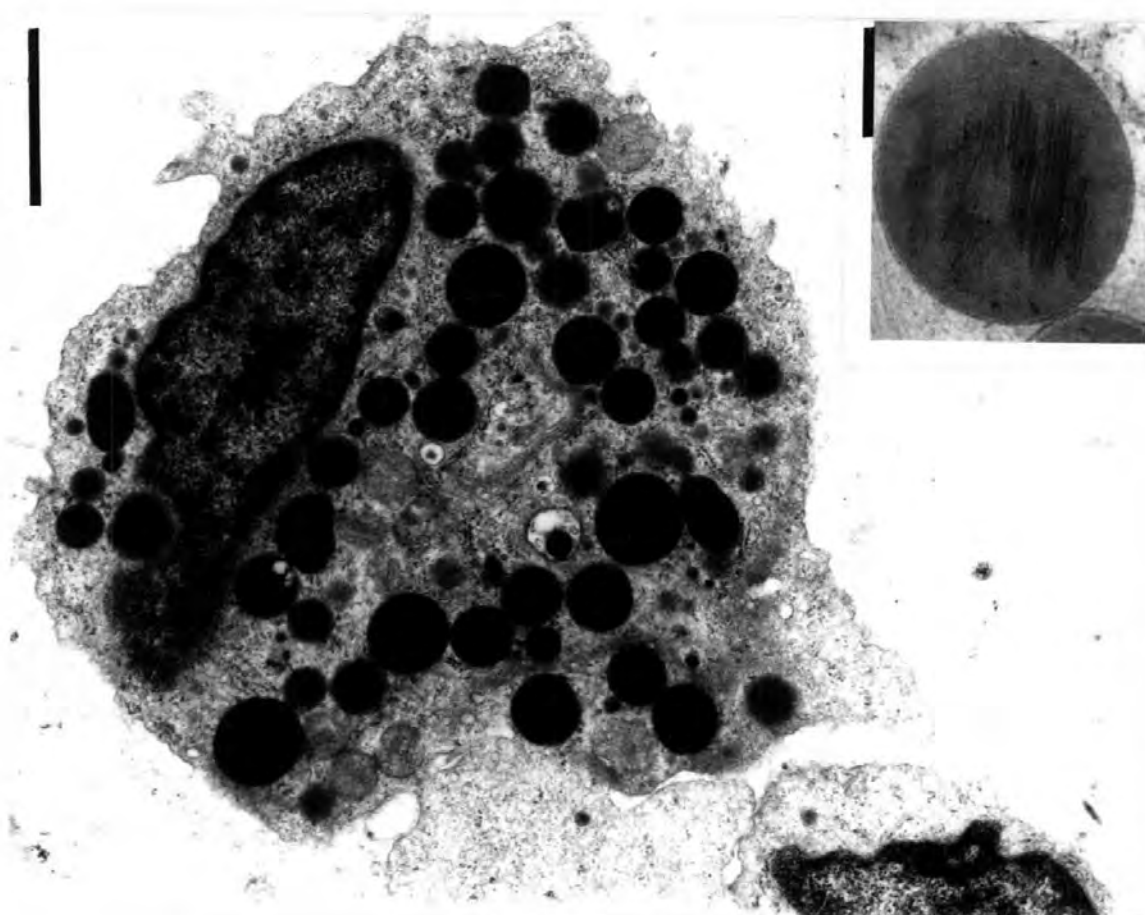
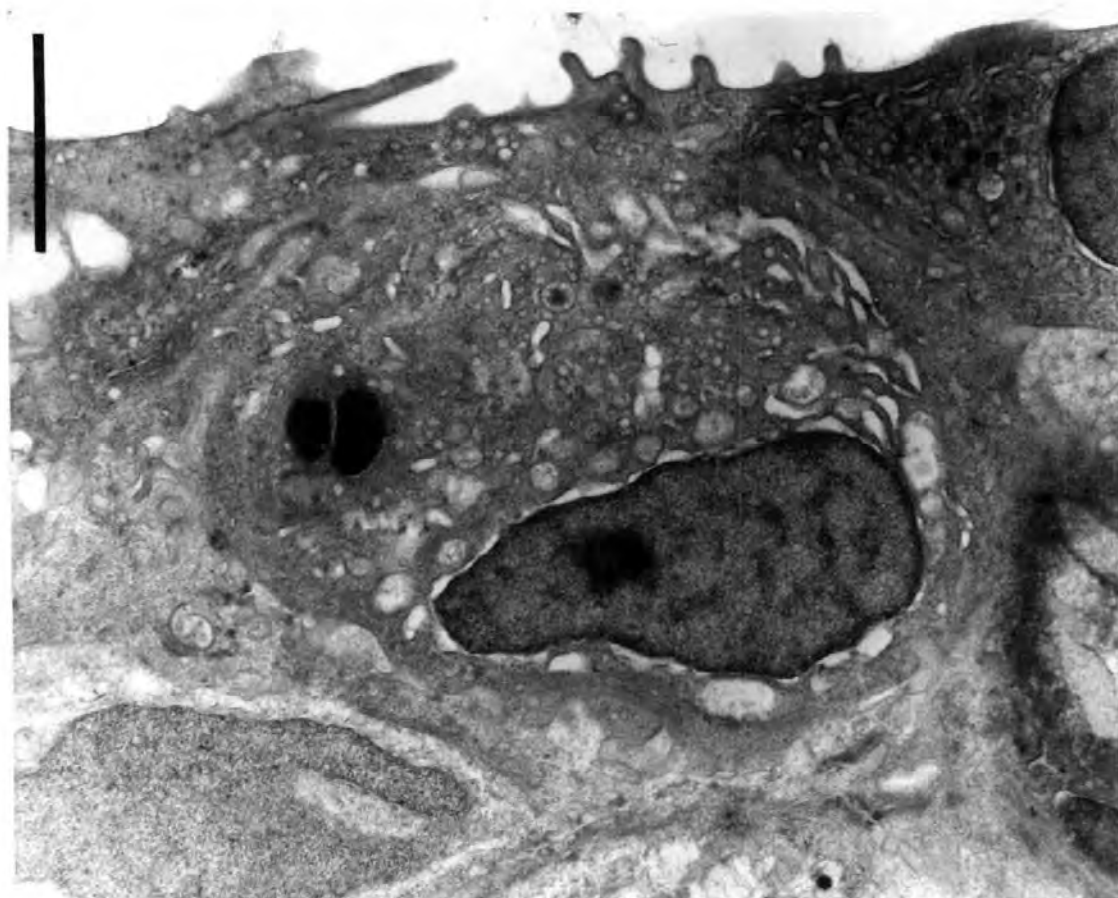


μm by $6 \mu\text{m}$ - $8 \mu\text{m}$ in size, and were extensively vacuolated (figure 13); they possessed an eccentrically positioned euchromatic nucleus and a prominent nucleolus, prominent golgi apparatus and mitochondria, and numerous small cytoplasmic vesicles.

Also recorded present within the epidermis was a leucocyte-like cell which was not recorded from the lymphomyeloid tissues or peripheral blood vessels. This cell type is here termed an **osmiophilic granular cell** (OGC). OG cells did not appear to be resident in the epidermis, and no desmosomal connections with adjacent cells were apparent. These cells were found singly in the mid and basal regions of the epidermis, as well as the non-vascularised regions of the dermis immediately adjacent to the basal lamina. OG cells were round or ovoid in shape, measuring $6 \mu\text{m}$ - $8 \mu\text{m}$ by $5 \mu\text{m}$ - $7 \mu\text{m}$ in size, with an eccentrically positioned often lobate nucleus (figure 14). The cytoplasm contained abundant RER and Golgi apparatus, and several mitochondria. The most distinguishing features of these cells were the electron dense osmiophilic granules. These granules were membrane bound, 500 nm - $1 \mu\text{m}$ in size, with an amorphous matrix containing several crystalline rod-shaped inclusions.

Figure 13. Electron micrograph of a chloride cell from the peripheral epidermis of an 'O' group carp. X 14,500 mag.. Scale bar = 2 μm .

Figure 14. Electron micrograph of an osmiophilic granular cell from a non-vascularised region of the dermis immediately below the basal lamina. X 12,000 mag. Scale bar = 2 μm . Inset: specific granule. X 72,000 mag. Scale bar = 200 nm.



Several leucocytes were identified within the epidermis. These cells were classified on the basis of comparative morphology by examining cells from the lymphomyeloid tissues (spleen and pronephros) and dermal blood vessels. Leucocytes identified within the epidermis included granulocytes here termed type I, II and III cells (respectively representing neutrophils, eosinophils and basophils under the classification of Cenini, 1984, and Temmink & Bayne, 1987), lymphocytes, thrombocytes and monocyte/macrophages (figure 15). All of the cell types mentioned above were also recorded as normal components of the lymphomyeloid tissues and/or peripheral blood vessels.

2.2.2. Localised host response to *I. multifiliis*

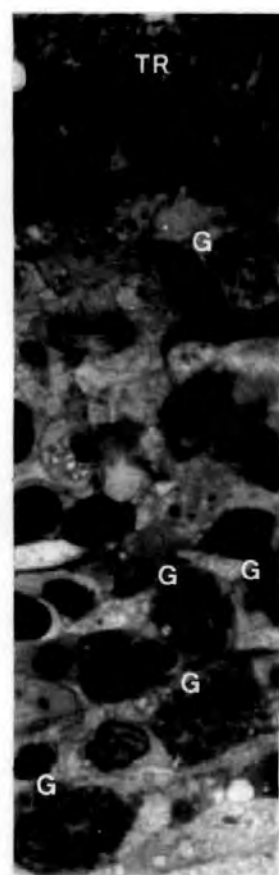
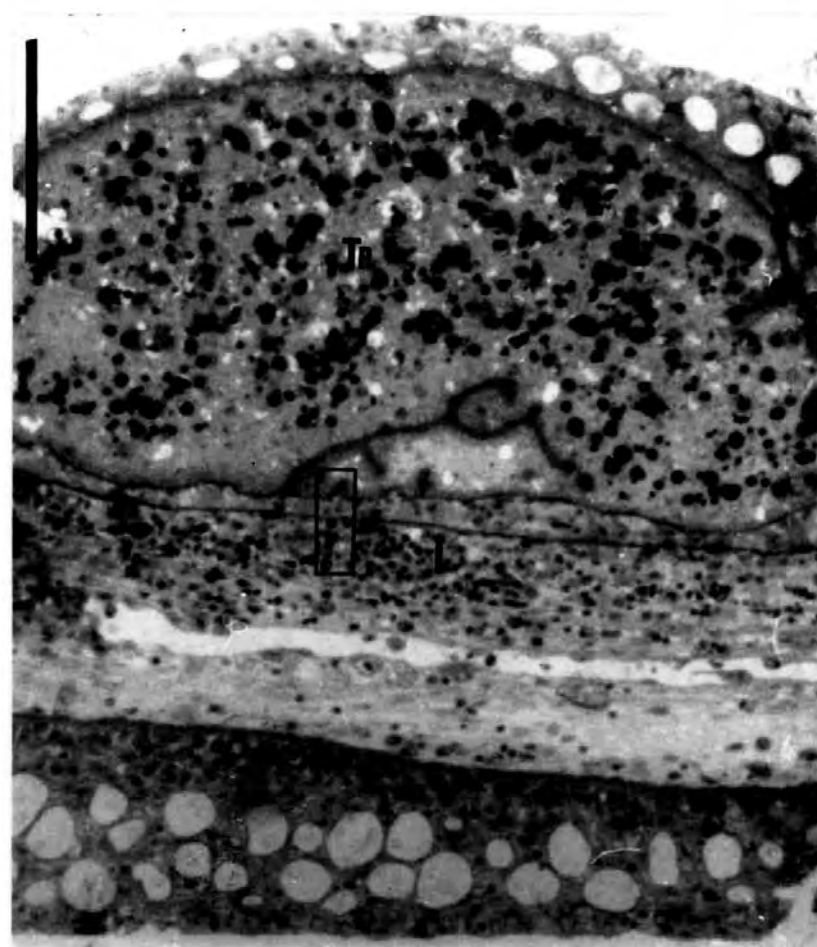
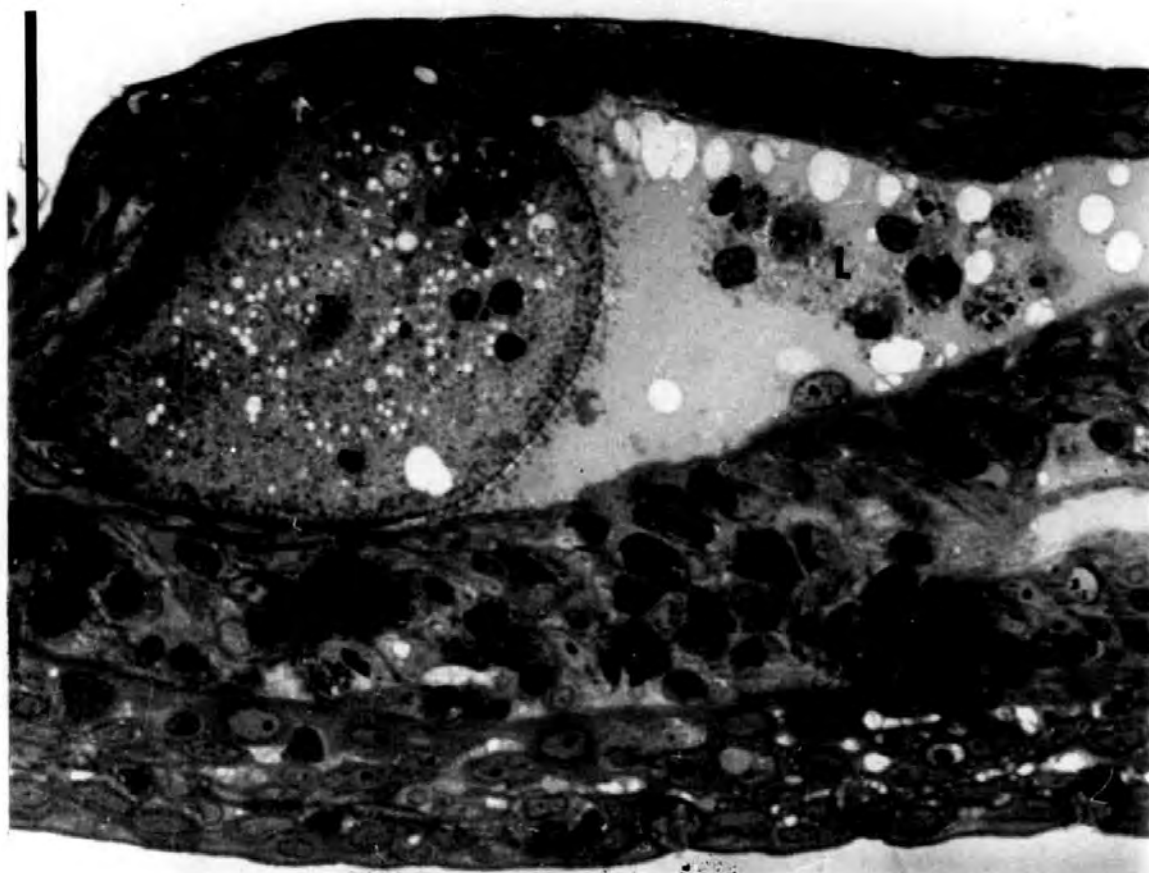
20 immunised 'O' group carp (average weight 3.54 g. \pm 0.47 g.) and 20 previously unexposed fish (average weight 4.14 g. \pm 0.66 g.) were individually infected by droplet infection. The position of approximately 20 parasites in an area of skin chosen at random was recorded by mapping. Biopsies of mapped sites were then taken at various time intervals up to 12 days following exposure, both during the infection and following parasite exit, to be examined histologically.

2.2.2.1. Primary infection

A cellular infiltration to the infection site was recorded. Infiltrating leucocytes (predominantly type I granulocytes) were first recorded in non-vascularised regions of the dermis adjacent to the basal lamina and immediately below an infected site 1 day following infection. By 2 - 3 days leucocytes were recorded in the epidermis in the vicinity of the trophozoite, mostly within layers of tissue debris surrounding the parasite (figure 16). By 5 - 6 days the cellular response was extensive, with large accumulations of leucocytes being recorded below the basal lamina and around the parasite (figure 17). This response was predominated by granulocytes, especially type II cells, with type I and III granulocytes also being recorded in large numbers. Granulocytes were observed in close proximity to the trophozoite surface within the tissue debris layers, although there was no evidence of active cell adherence to the parasite (figures 18 and 19). Lysis of granulocyte membranes and release of granules was observed. There was no evidence of any localised damage to the parasite. All parasites completed development. Five days following mature trophont exit from the epidermis (day 11 - 12), the vacated infection site was diffusely populated by granulocytes (predominantly type

Figure 16. Light micrograph of trophozoite (Tr) in the epidermis of an 'O' group carp 3 days following primary infection. Note leucocytes (L) within a tissue space caused by the parasite. X 660 mag.. Scale bar = 50 μ m.

Figure 17. Light micrograph of a trophozoite (Tr) in the epidermis of an 'O' group carp 5 days following primary infection. Note accumulation of leucocytes (L) in the dermis below the parasite. X 300 mag.. Scale bar = 100 μ m. Inset: Enlargement of boxed area, showing several granulocytes (G). X 1200 mag.. Scale bar = 20 μ m.



I cells), with type II cells, osmiophilic granular cells and a few macrophages also being recorded (figures 20 & 21). Some localised phagocytosis by macrophages, type I granulocytes and resident filament cells was recorded.

2.2.2.2. Infection in immunised fish

In immunised fish, very few of the parasites which had originally been recorded in the epidermis immediately following exposure (at time 0 +) subsequently completed development and reached maturity. A cellular infiltration was recorded in response to the few parasites which did complete development. The time scale of this infiltration largely followed that in the primary infection, with leucocytes first being recorded in the vicinity of the parasite 2 - 3 days post infection. Infiltrating cells were again located primarily within layers of tissue debris surrounding the parasite. The predominant cell types involved were type III granulocytes and osmiophilic granular cells, with other granulocyte types and some macrophages also being recorded (figures 22 and 23). Lysis of infiltrating host cells within the tissue debris layer was again recorded, and granules were released from these cells (figure 24). Granules of type III cells appeared to coalesce before and during cell lysis, forming large aggregations which persisted despite the total degradation of the parent

Figure 20. Light micrograph of a site of mature trophont exit from the epidermis of an 'O' group carp 5 days following initial primary infection. Note diffuse population of granulocytes (enclosed by arrows). X 500 mag.. Scale bar = 50 μ m.

Figure 21. Electron micrograph enlargement of an area from figure 20. I = type I granulocyte, II = type II granulocyte, OGC = osmiophilic granular cell. Note extensive necrosis (arrowed). X 6,200 mag.. Scale bar = 5 μ m.

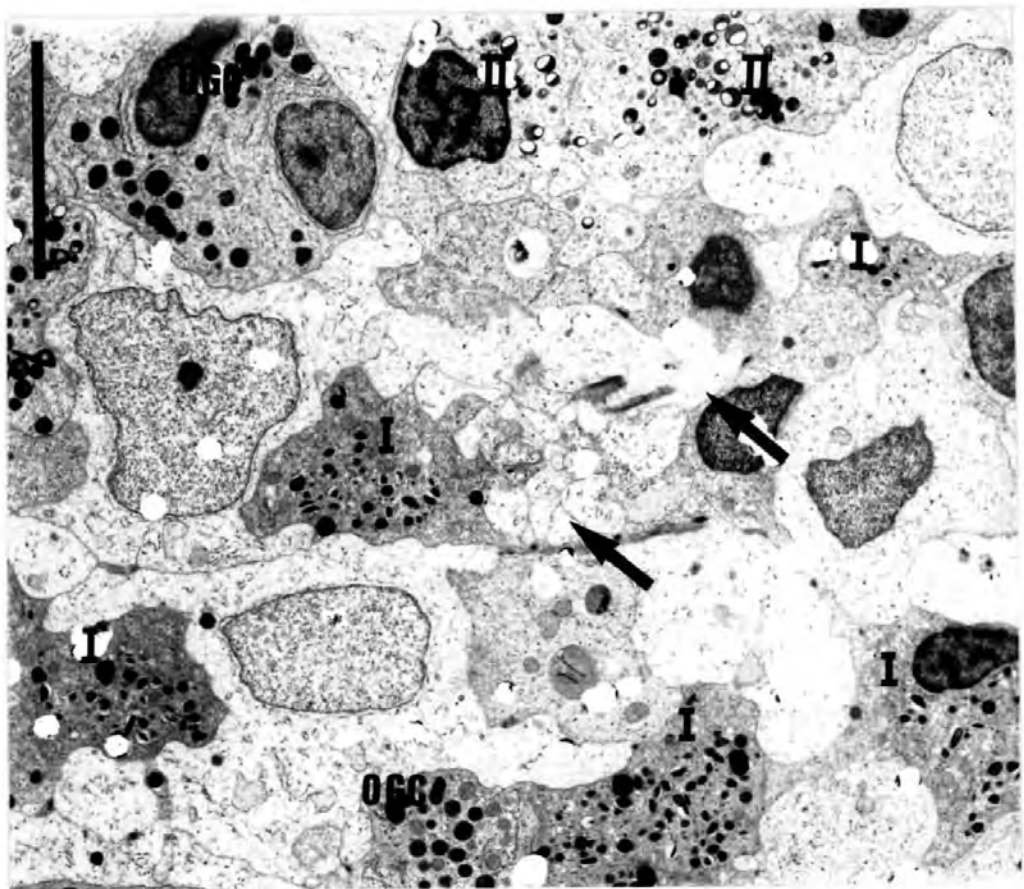
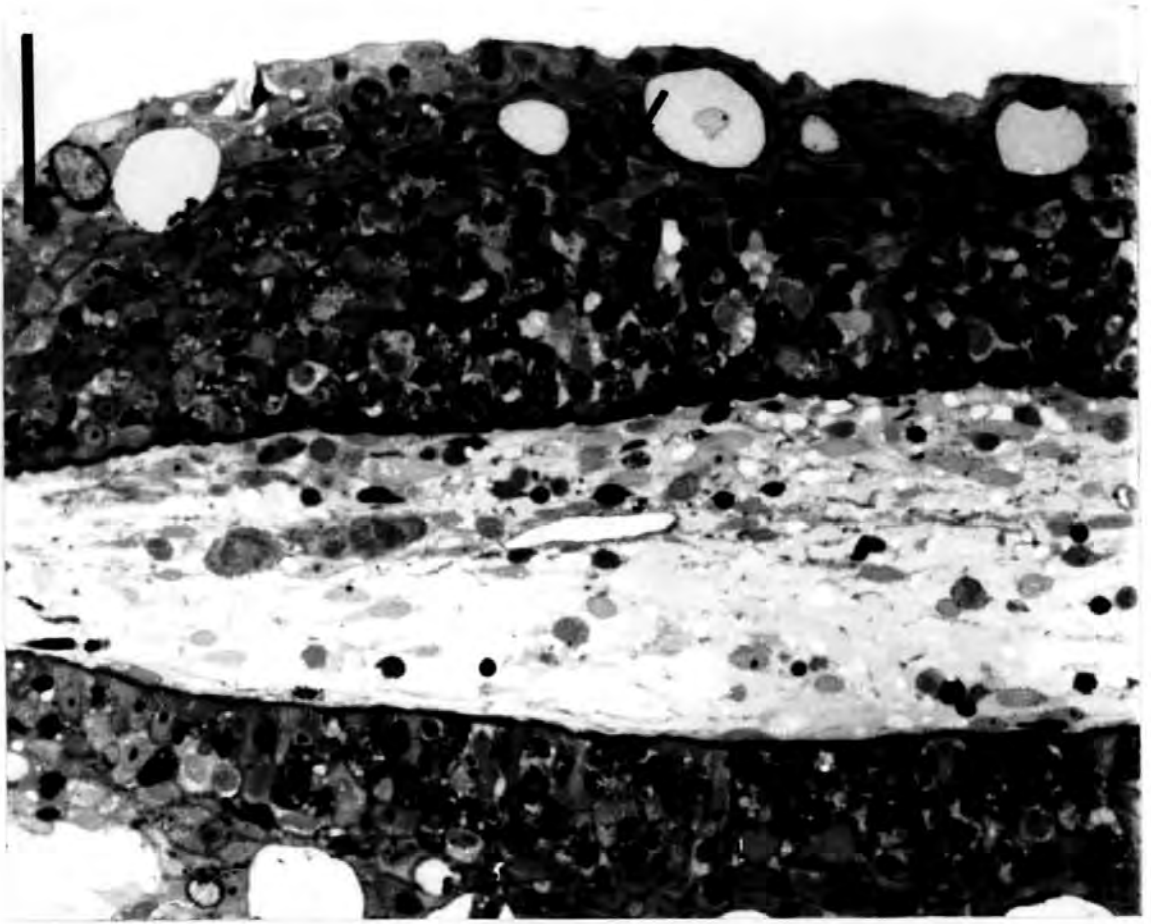


Figure 22. Light micrograph of a mature trophozoite (Tr) in the epidermis of an immunised 'O' group carp 5 days following infection. L = leucocytes. X 200 mag..

Scale bar = 100 μ m.

Figure 23. Electron micrograph enlargement of boxed area from figure 22. Note granules of osmiophilic granular cells (OGC), aggregations of coalescing granules from type III cells (small arrows) and phagocytosis by a filament cell (large arrow). Tr = trophozoite. X 4,000 mag.. Scale bar = 5 μ m.

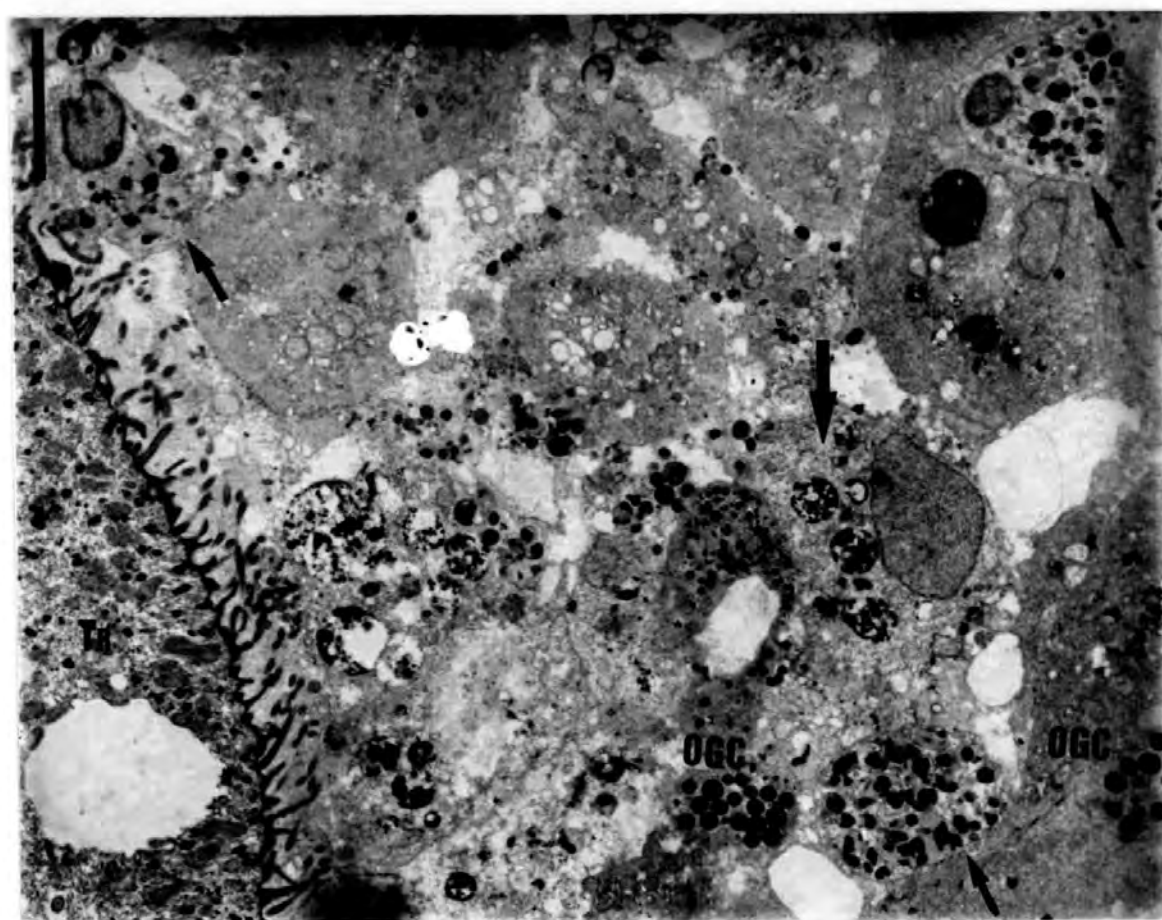
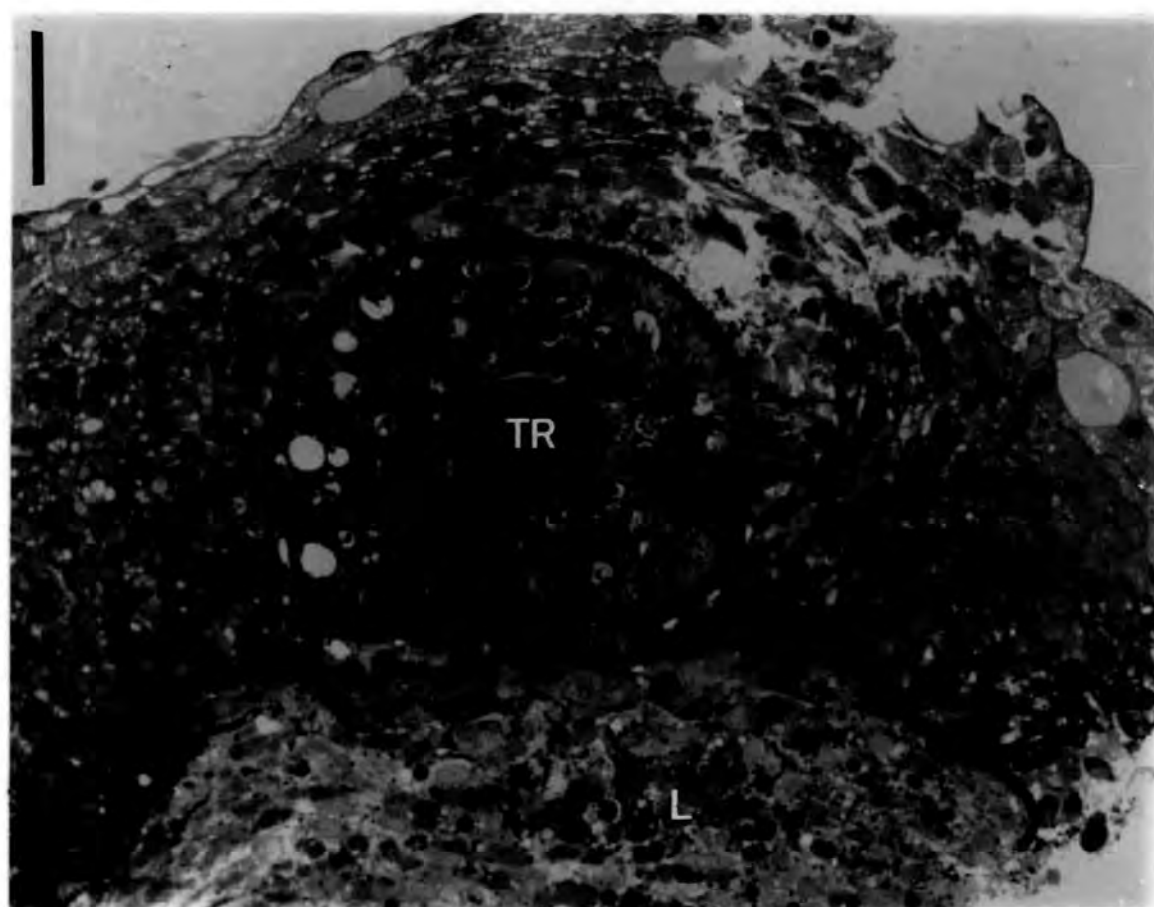
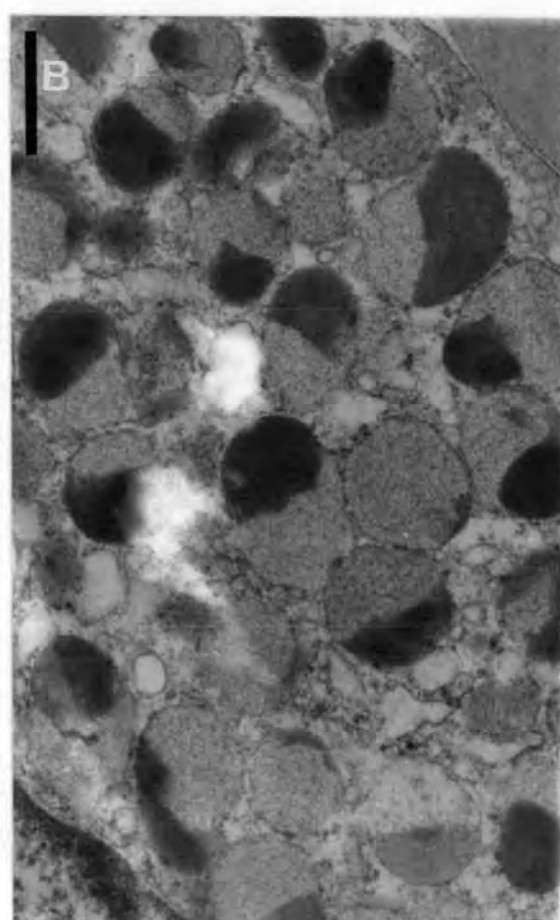
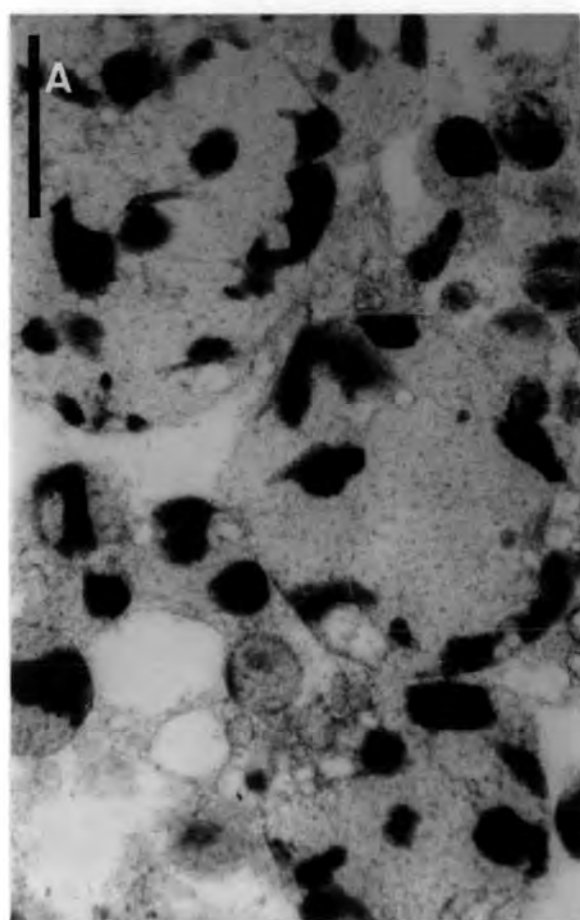
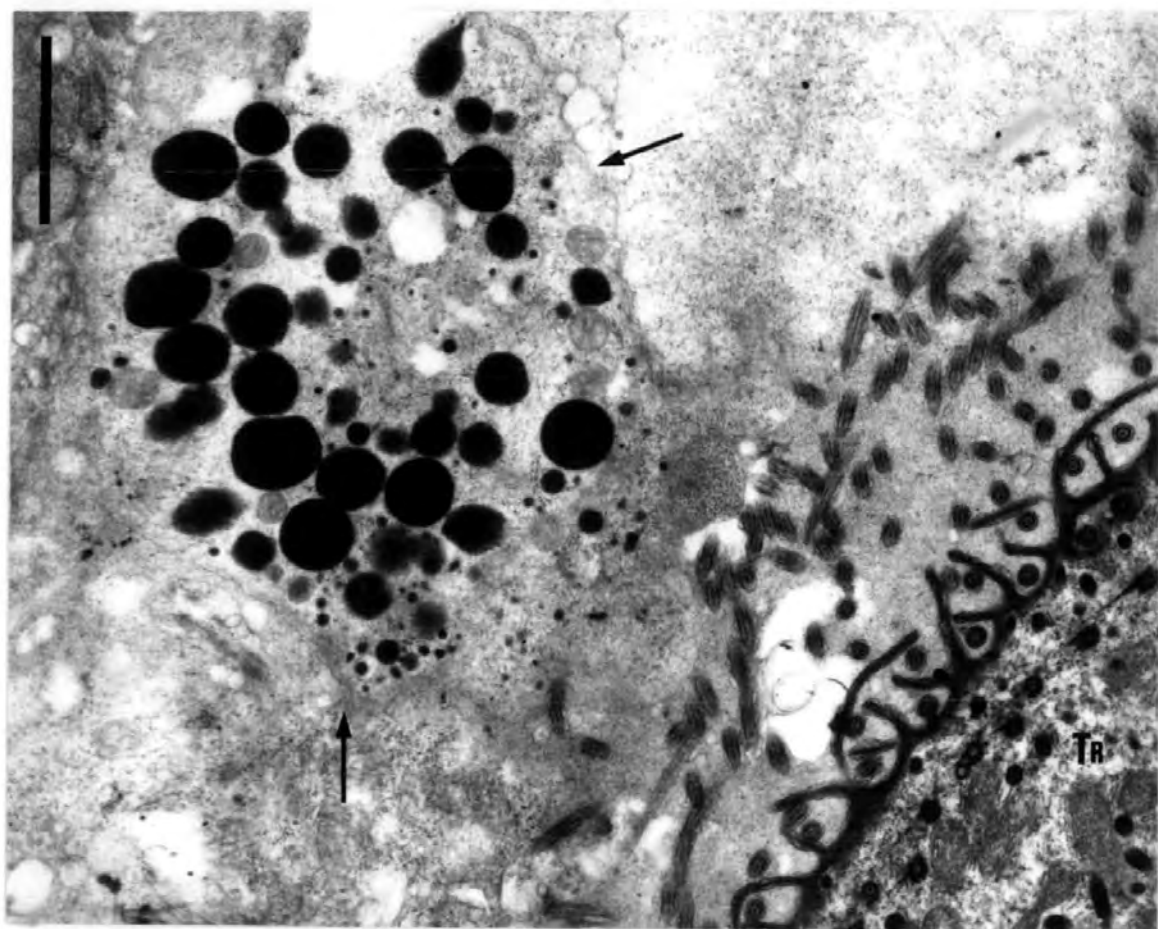


Figure 24. Electron micrograph of an osmiophilic granular cell in close proximity to the surface of a mature trophozoite (Tr) in the epidermis of an immunised 'O' group carp 5 days following infection. Note cell lysis (arrowed). X 12,000 mag.. Scale bar = 2 μ m.

Figure 25. Electron micrograph of granules of type III cells recorded in close proximity to trophozoites following cellular lysis. A = granules from infection site in immunised fish. Note coalescence of granules. B = granules from infection site in primary infection. X 32,000 mag.. Scale bars = 500 nm.



cell; it was not possible to determine whether membranes of these granules were fused (figure 25). Phagocytosis was recorded in the vicinity of the parasite by several host cells, including type I granulocytes (figure 26), macrophages and resident filament cells. There was no evidence of active host cell adherence nor of any localised damage to the parasite.

The vast majority of parasites did not complete development to reach maturity in the epidermis of immunised fish. No identifiable parasite material was detected at sites where parasites had been recorded as present at time 0+ but absent at 2 hours following exposure. At these sites a concentrated cellular response was recorded within a small area of the epidermis (approximately 50 - 100 μm in diameter; figure 27). The cellular infiltration was first recorded at 1 - 2 days and increased up to 5 days following initial exposure to theronts. The response was predominated by macrophages, with intensive phagocytosis by these cells (figures 28 and 29), as well as by type I granulocytes and filament cells. The phagosomes of these cells contained no readily identifiable parasite material.

Figure 26. Electron micrograph of a type I granulocyte observed in close proximity to a mature trophozoite in the epidermis of an immunised 'O' group carp 5 days following infection. Note large phagosome (Ph). X 12,000 mag.. Scale bar = 2 μ m.

Figure 27. Light micrograph of two sites of initial theront penetration where parasites did not complete development in the epidermis of an immunised 'O' group carp, observed 5 days following initial exposure. Note concentrated accumulations of leucocytes at the sites. X 800 mag..Scale bars = 20 μ m.

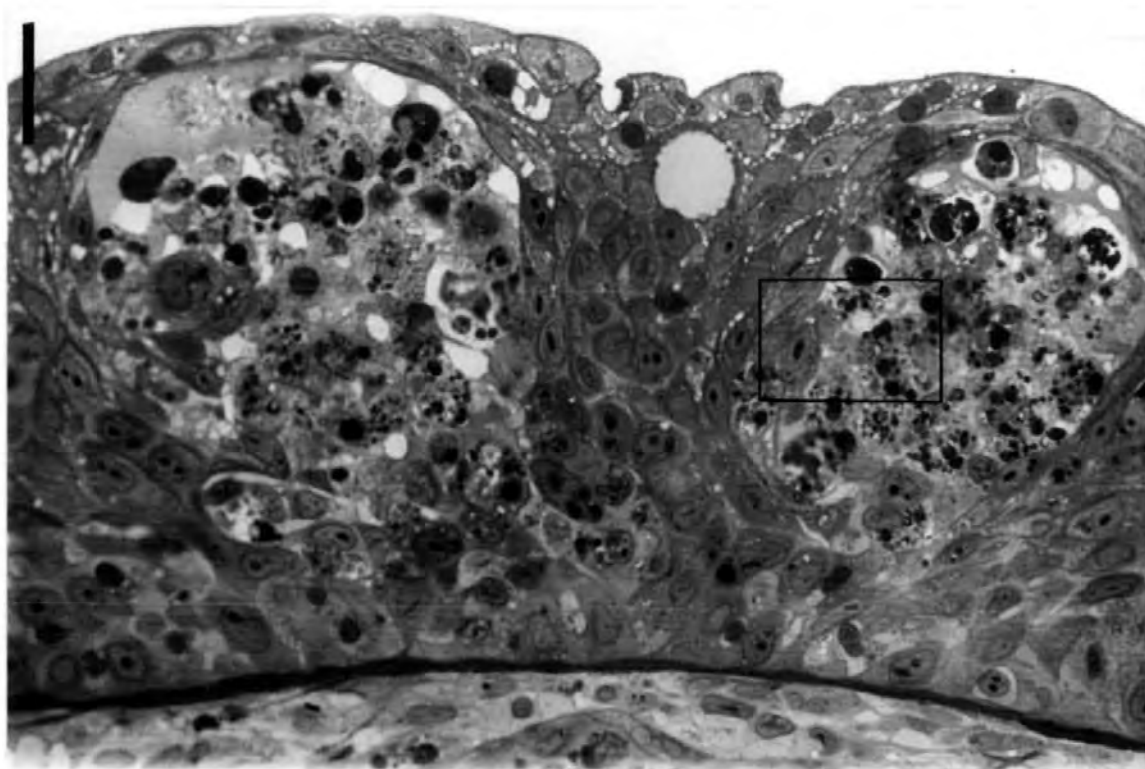
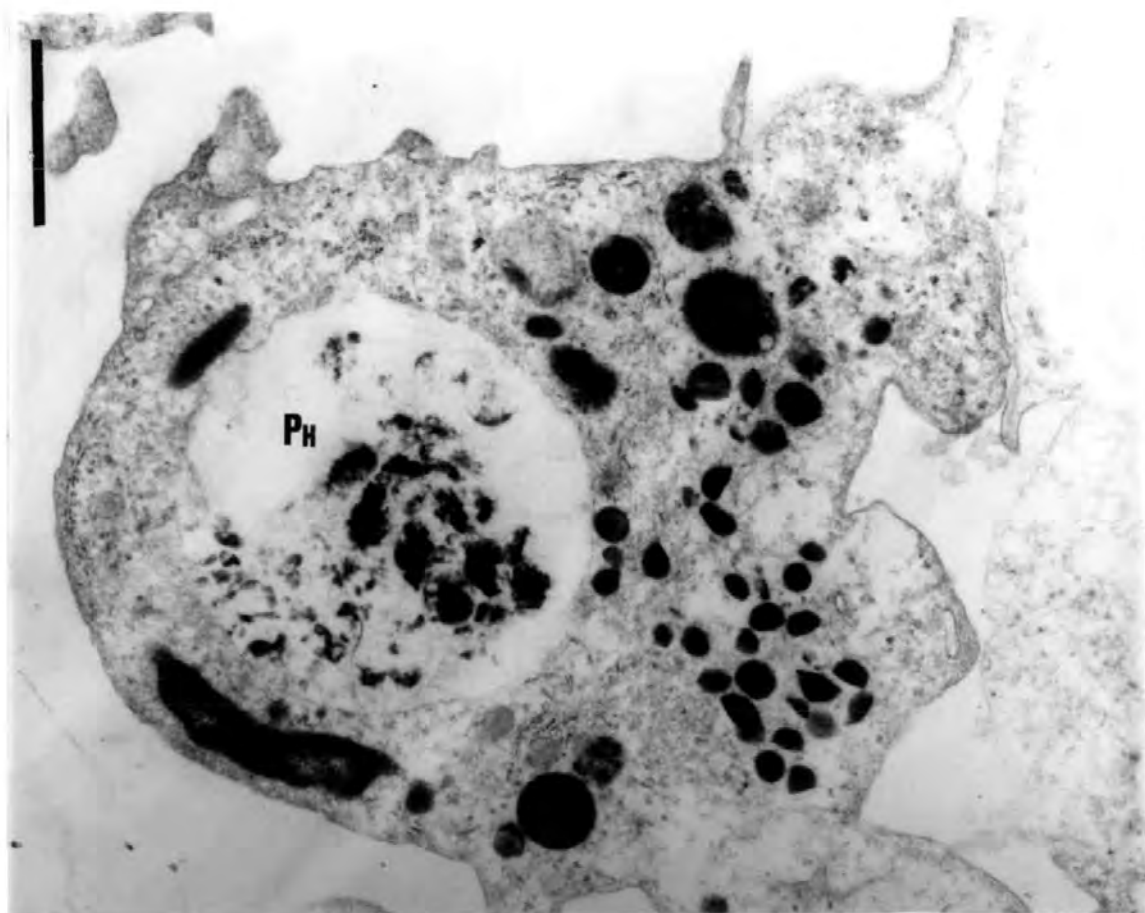
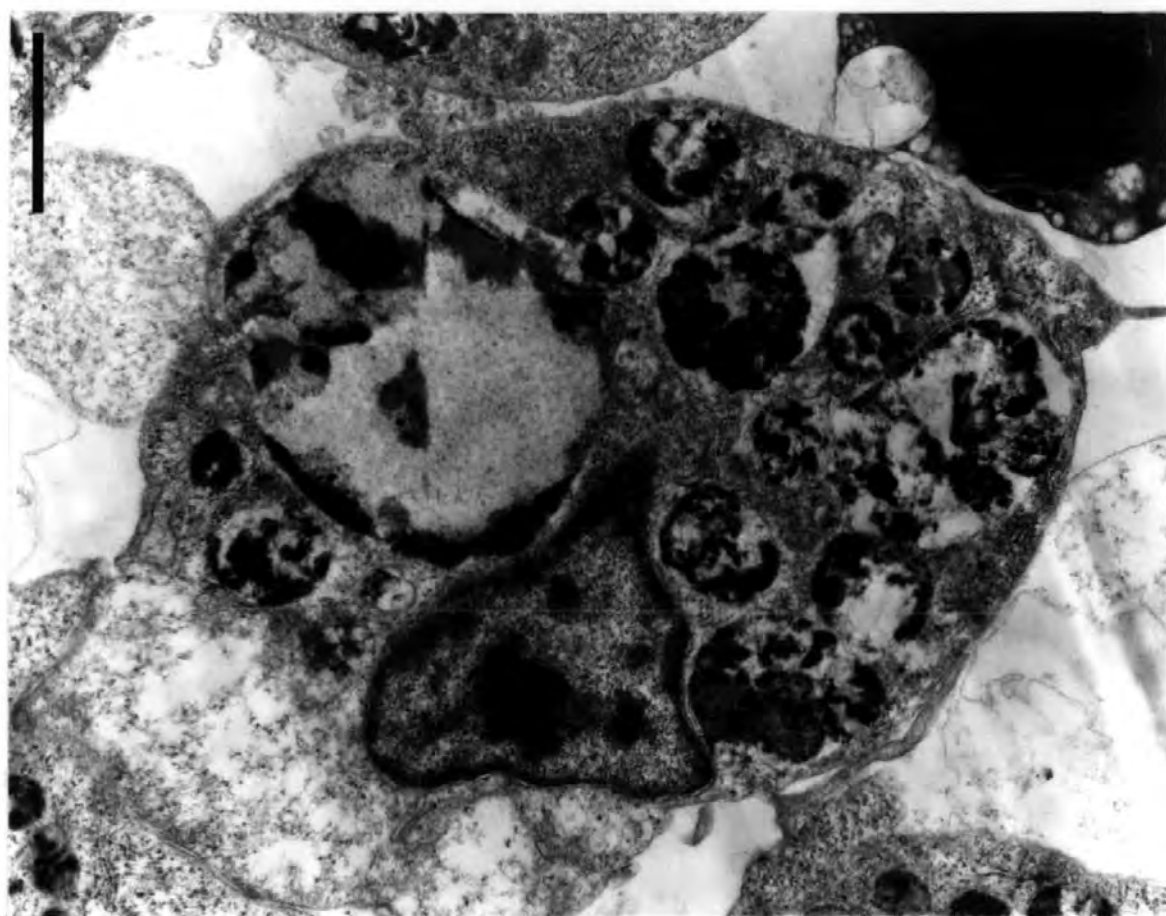
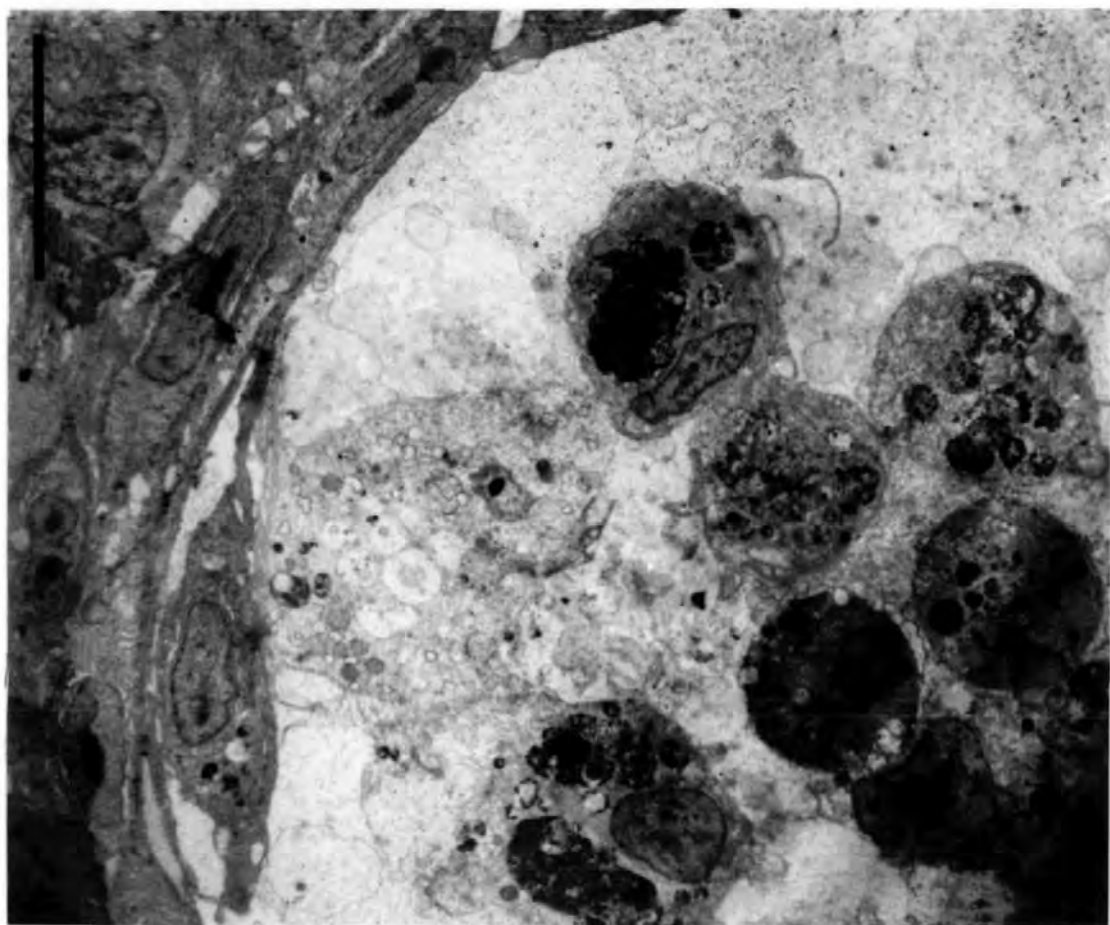


Figure 28. Electron micrograph enlargement of boxed area from figure 27. Note extensive phagocytosis by macrophages. X 3,200 mag.. Scale bar = 10 μ m.

Figure 29. Electron micrograph of a macrophage from a site of initial theront penetration where the parasite did not reach maturity in the epidermis of an immunised 'O' group carp, observed 5 days following initial exposure. Note numerous phagosomes. X 12,000 mag.. Scale bar = 2 μ m.



3. CHARACTERISATION OF ANTISERA

Rabbit anti-carp IgM serum was prepared for use in in vitro immunological studies. Due to initial problems with this antiserum recognising several carp serum proteins (see below) it was considered necessary to purify carp IgM before injection into the rabbits.

3.1. Carp antisera

Anti-SRBC and anti-HRBC sera were raised in four adult 1 Kg.+ carp. Heat inactivated samples of these sera showed specific agglutination titres of 1/262,000 and 1/1,048,000 against sheep and horse RBC targets respectively. Sera from non-immunised adult carp showed no specific agglutination titre against either RBC. Aliquots of carp anti-SRBC whole serum were partially purified by anion exchange and gel filtration chromatography to obtain relatively purified carp IgM samples with specific anti-SRBC activity. The IgM activity of isolated fractions was recorded by direct agglutination of SRBC, peak activity corresponding to a titre of 1/1024 (which was the highest titre tested). The specific anti-SRBC titres obtained following column purification were much lower than the titres obtained for whole serum due to dilution of isolated IgM in the buffer eluent. Following anion exchange, peak specific IgM activity was recorded in fractions 9 and 10 (figure

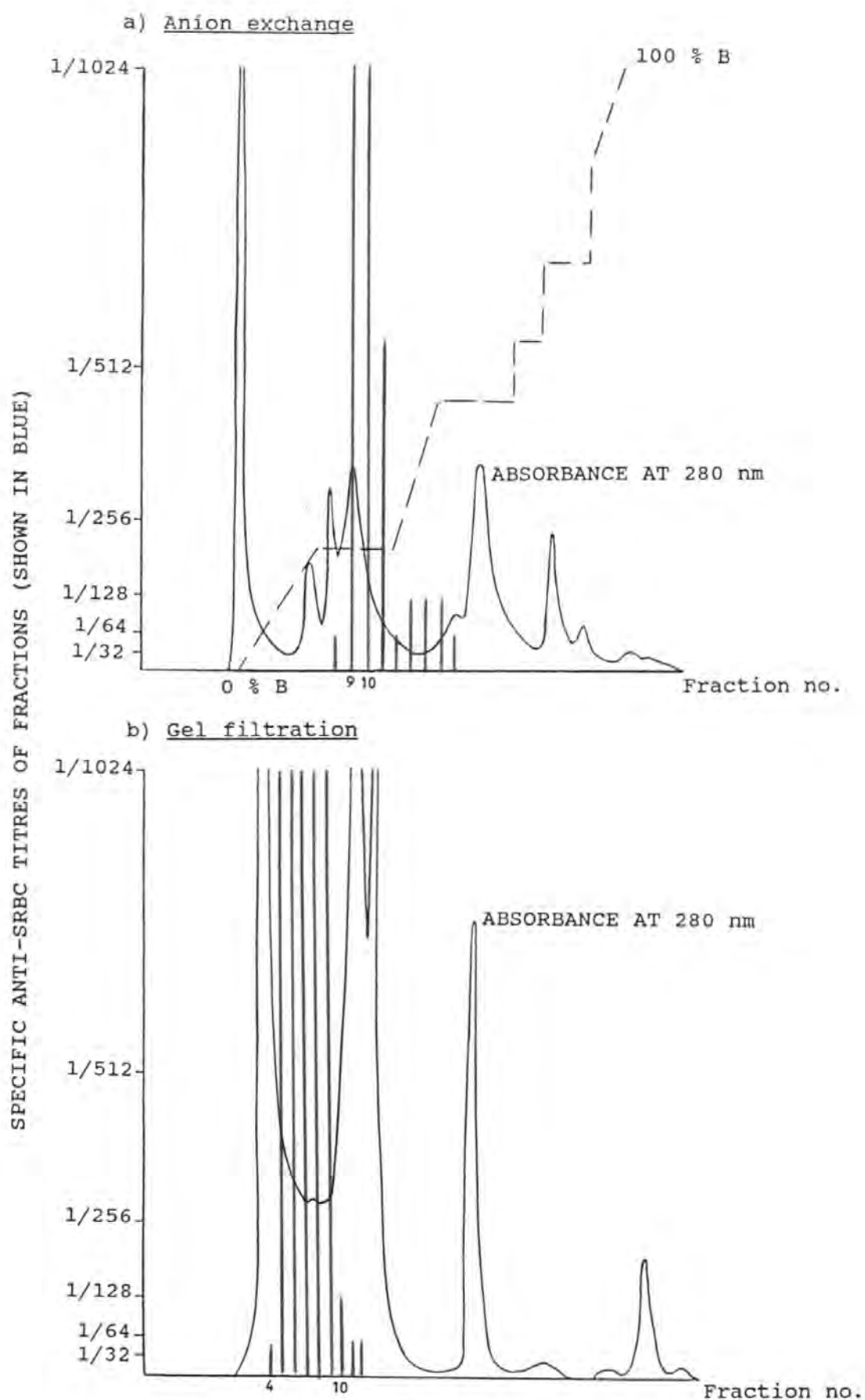
30 a). Following gel filtration, peak specific IgM activity was recorded in a wide band in fractions 4 - 9 (figure 30 b); fractions 3 - 6 and 7 - 10 were pooled and further purified in a second step by anion exchange, and the highest specific IgM activity was recorded as 1/256 and 1/512 in fraction 9 of these eluents (figure 31). This two-step purified IgM was subsequently used as a source of purified carp IgM to immunise rabbits (see below).

3.2. Rabbit antiserum

Two rabbits were initially immunised with SRBC/whole carp serum IgM complex. Serum from the first rabbit produced three distinct precipitin bands against whole carp serum by immunoelectrophoresis, only band 3 of which corresponded to the low mobility position expected of fish IgM (figure 32 a). This rabbit was therefore not involved in subsequent immunisations.

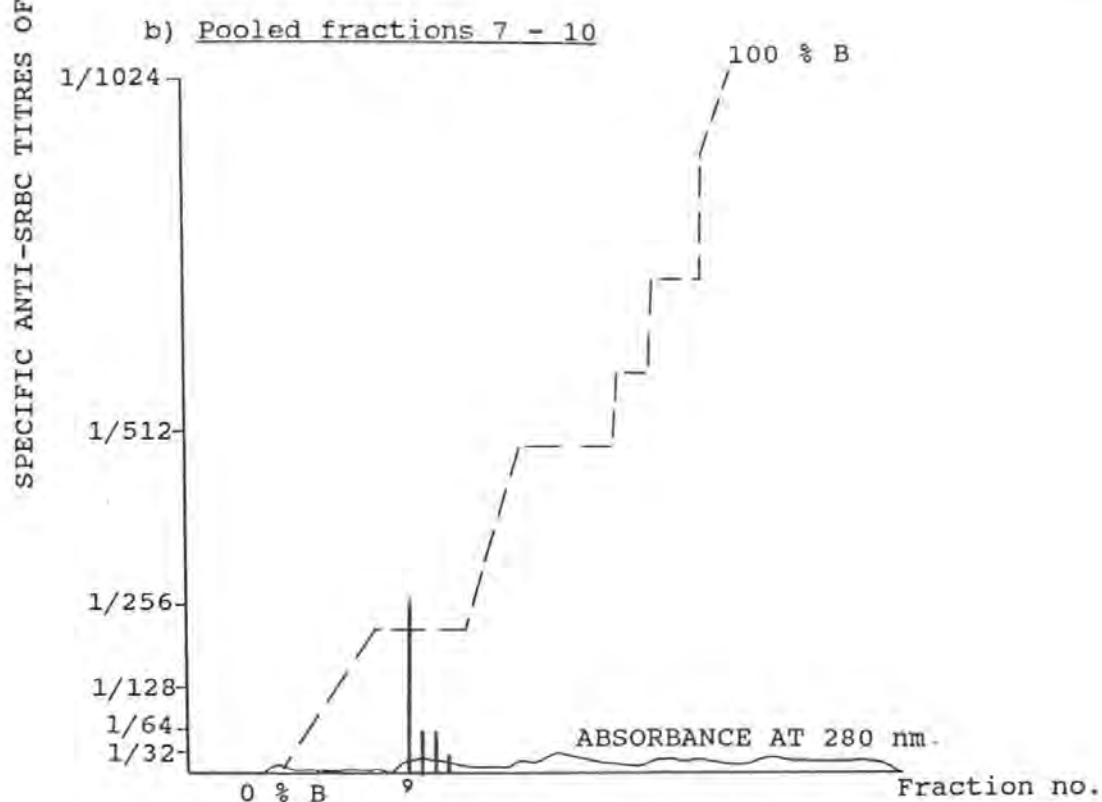
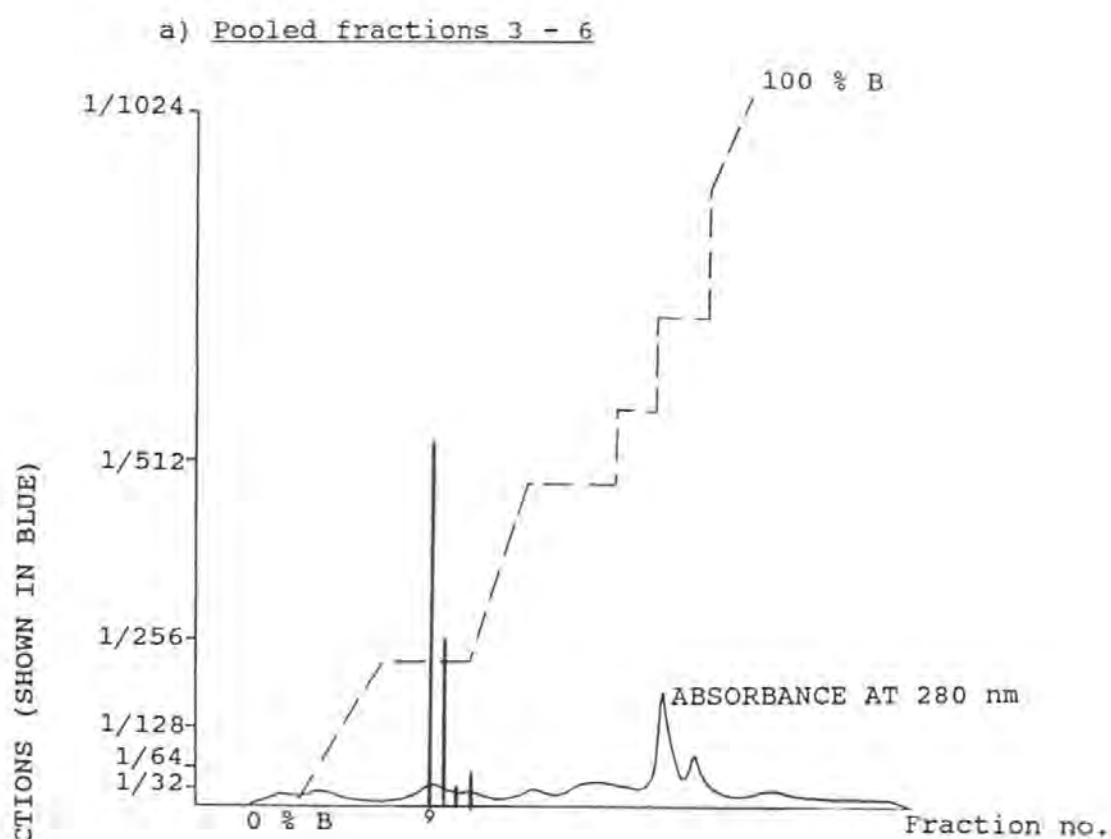
Serum from the second rabbit initially immunised with SRBC/whole carp serum IgM complex produced two distinct precipitin bands against whole carp serum by immunoelectrophoresis (figure 32 b), of which only band 3 corresponded in position to the low mobility band expected for fish IgM. Serum taken from this rabbit produced a single low mobility precipitin band in the

Figure 30. Diagrams showing the elution profiles of carp anti-SRBC serum purified by a) anion exchange and b) gel filtration chromatography. Blue lines represent the specific anti-SRBC titres of isolated IgM fractions; broken line represents the percentage of buffer B in relation to A (see appendix 2).



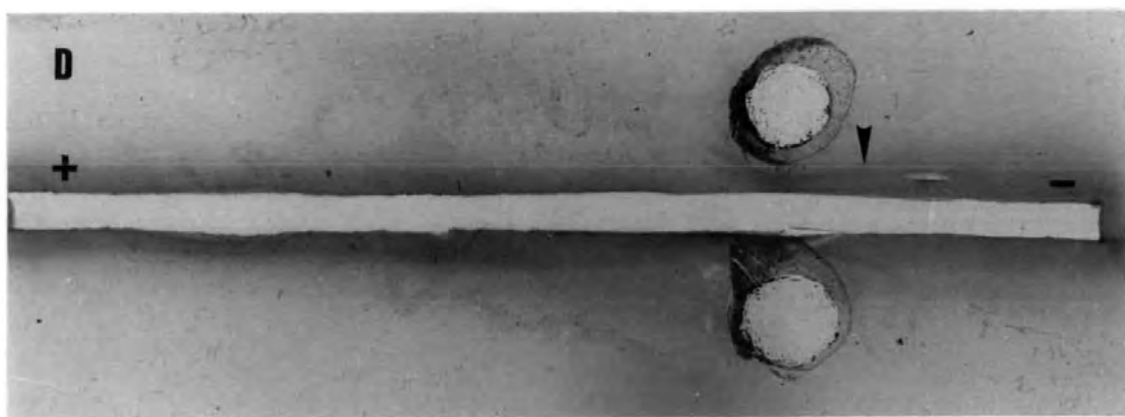
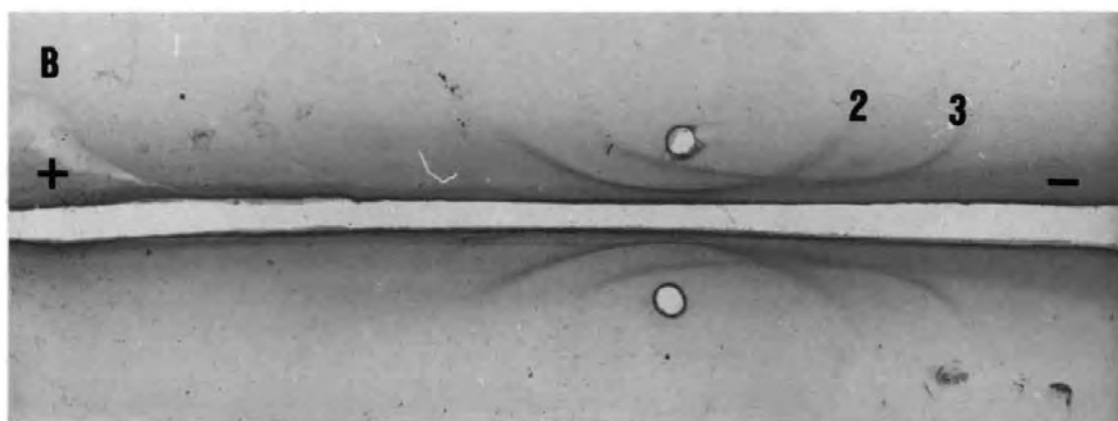
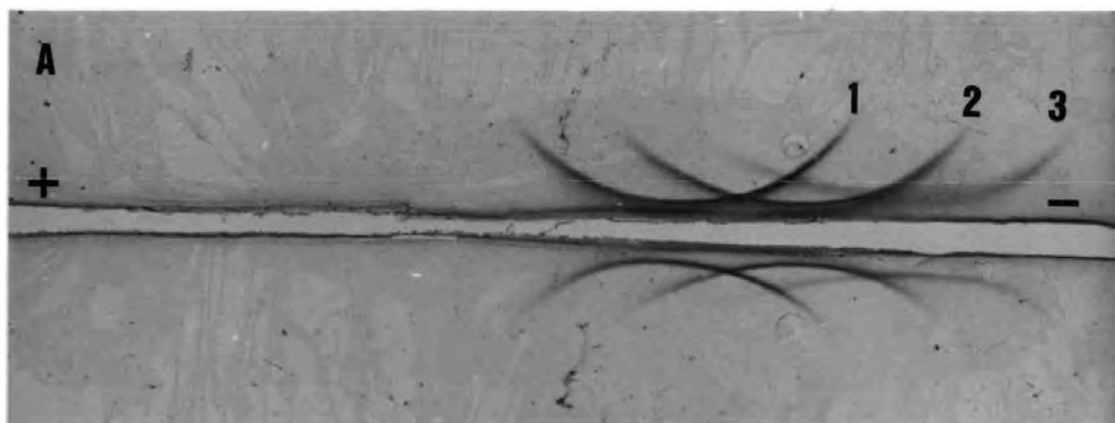
(Broken lines represent the relative % of buffer B compared to A).

Figure 31. Diagrams showing the elution profiles of pooled gel filtration fractions a) 3 - 6 and b) 7 - 10 from fig. 30 b., purified by anion exchange chromatography. Blue lines represent the specific anti-SRBC titres of isolated IgM fractions; broken lines represent the percentage of buffer B in relation to A (see appendix 2).



(Broken lines represent the relative % of buffer B compared to A).

Figure 32. Light micrographs of immunoelectrophoresis slides. Slide A: Antiserum = serum from rabbit initially immunised with whole carp serum IgM/SRBC complex; antigen = whole carp serum. Note three distinct precipitin bands, of which only band 3 corresponds in position to that expected for fish IgM. Slide B: Antiserum = serum from rabbit initially immunised with whole carp serum IgM/SRBC complex; antigen = whole carp serum. Note two distinct precipitin bands, of which only band 3 corresponds in position to that expected for fish IgM. Slide C: Antiserum = serum from rabbit re-immunised with column-purified carp IgM/SRBC complex; antigen = whole carp serum. Note a single distinct precipitin band (arrowed) corresponding in position to that expected for fish IgM. Slide D: Antiserum = serum from rabbit re-immunised with column-purified carp IgM/SRBC complex; antigen = concentrated carp mucus. Note a single distinct precipitin band (arrowed) corresponding in position to that expected for fish IgM.



expected position against chromatography-purified carp IgM. To eliminate reactivity associated with band 2 as far as possible, the anti-carp serum reactivity was allowed to decrease by retaining this rabbit for six months before re-immunisation with SRBC/purified carp IgM complex. Serum then subsequently taken from this rabbit produced only a single distinct precipitin band against whole carp serum (figure 32 c), the position of this band corresponding to the low mobility band characteristic of carp IgM. This rabbit anti-serum also produced a single weak precipitin band against concentrated carp mucus, corresponding in position to that expected for fish IgM (figure 32 d).

The efficacy of this rabbit anti-carp IgM serum in surface immunolabelling was demonstrated by IFAT (using HRBC coated with carp anti-HRBC IgM as the antigen) at dilutions to 1/100. Anti-carp IgM serum from this rabbit was thus subsequently used in all further in vitro immunolabelling studies.

4. CHARACTERISATION OF THE IMMUNE RESPONSES TO I.MULTIFILIIS

4.1. Humoral response

The humoral response to I.multifiliis was characterised

by 1) the detection of anti-parasite antibody activity in vitro and in situ, and 2) the identification of antigenic sites of the parasite recognised by host antibodies, in the sera and mucus of immunised adult carp. For this purpose, sera and mucus samples were used from three 1 Kg.+ adult carp which had been immunised against I.multifiliis by repeated infections, and serum from a single 300 g.+ adult carp which had been immunised by i.p. injection of theront sonicate.

4.1.1.1. In vitro detection of anti-parasite antibodies

The specific anti-parasite activity of sera and mucus from immunised carp against theronts and trophonts was investigated by means of in vitro theront immobilisation assays, IFAT, IGSS, IGL and ELISA.

Pooled sera from carp which had been immunised by repeated infections showed specific immobilising activity against theronts (table 4). Immobilising titres were recorded from 1/16 to 1/32, compared to 0 to 1/2 in control fish naive to I.multifiliis. Pooled concentrated mucus from immunised fish showed immobilising activity against theronts at titres from 1/64 to 1/256; however, this activity was non-specific since similar activity was also shown in mucus from control fish.

Table 4. Anti-theront immobilisation titres observed by serum and mucus from adult carp immunised by repeated infections and fish naive to I.multifiliis as controls.

	SERUM		MUCUS	
	IMMUNE	NAIVE	IMMUNE	NAIVE
Immobilisation titre against theronts	1/16-1/32	0-1/2	1/64-1/256	1/64-1/128

(Figures shown represent the range of titres obtained in five replicate assays.)

Pooled sera from carp which had been immunised by repeated infections contained specific antibodies directed against the theront surface, as demonstrated by IFAT (figure 33). Negligible background fluorescence was obtained in all controls. Pooled concentrated mucus from immunised fish showed no specific fluorescent labelling against theronts compared to controls of mucus from fish naive to I.multifiliis.

Trophonts which had been pre-incubated in vitro with immune carp serum prior to labelling showed weak specific labelling for carp IgM in an amorphous precipitate surrounding the parasite (figures 34 and 35). Trophonts incubated with naive carp serum prior to labelling lacked the amorphous precipitate reaction and its associated gold labelling (figures 34 & 36). Specific gold labelling for carp IgM was also recorded in trophont food vacuoles.

Attempts to develop an ELISA for detecting anti-I.multifiliis antibodies in serum/mucus samples from immunised carp here proved unsuccessful. Although small differences in optical density measurements were observed between test and control samples, the differences were not significant (Student T Test, figure 37). Problems of high non-specific background labelling

Figure 33. Light micrograph of a theront IFAT labelled by a three link system, showing strong fluorescence with 1/5 serum from carp immunised against I.multifiliis by repeated infections as the primary antibody link. Note fluorescence of cilia (C). X 2,000 mag..
Scale bar = 10 μ m.

Figure 34. Light micrographs of the peripheral surface of trophonts labelled by IGSS following in vitro incubation in A. serum from carp immunised against I.multifiliis by repeated infections or B. serum from carp naive to I.multifiliis, viewed under blue light. Note fluorescence of an amorphous precipitate reaction beyond the outer edge of the cilia in A. (arrowed). X 2000 mag.. Scale bars = 10 μ m.

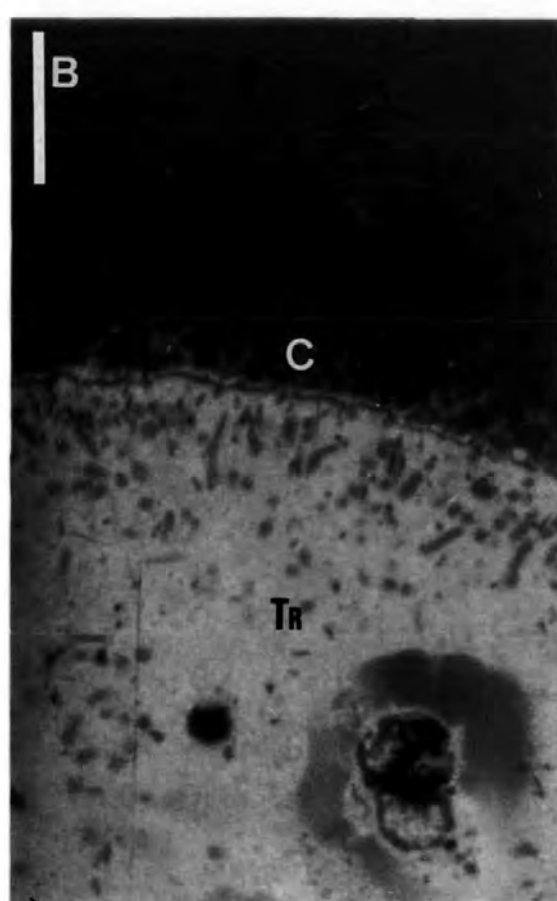
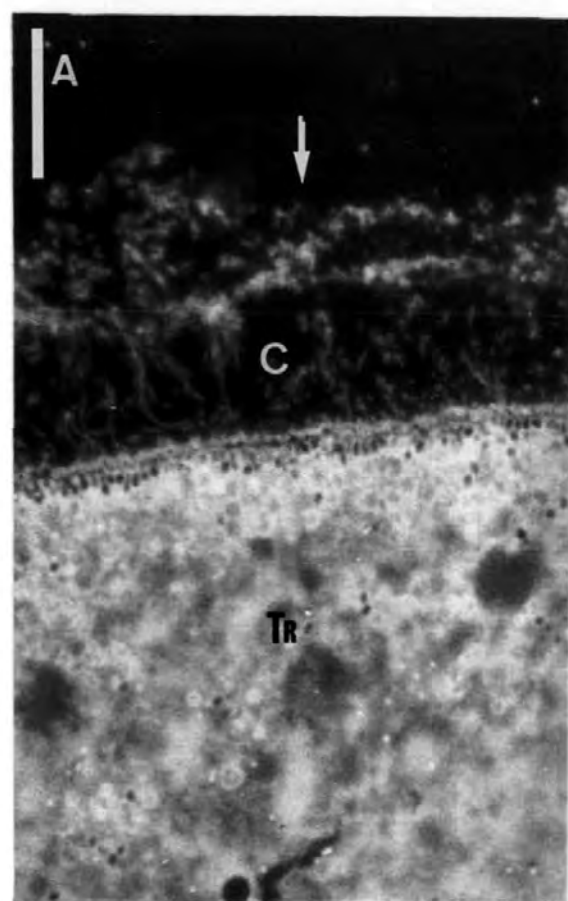
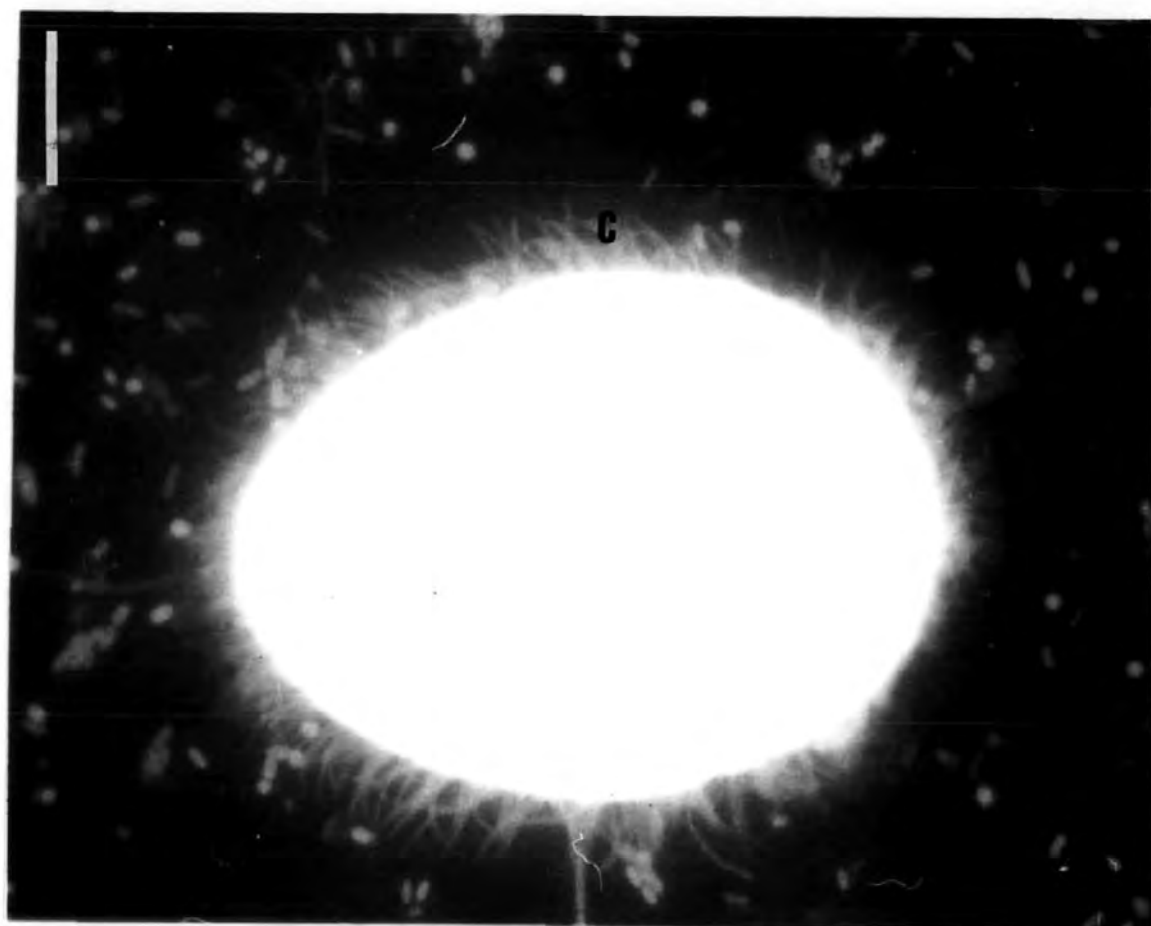


Figure 35. Electron micrograph of a trophont following in vitro incubation in 1/4 serum from carp immunised by repeated infections, showing specific immunogold labelling for carp IgM in an amorphous precipitate (AP) at the periphery of the cilia. X 13,300 mag..

Scale bar = 1 μ m.

Figure 36. Electron micrograph of a trophont, following in vitro incubation in 1/4 serum from carp naive to I.multifiliis, immunogold labelled for in situ carp IgM. Note lack of an amorphous precipitate (as in figure 35), or any specific labelling. X 16,000 mag..

Scale bar = 1 μ m.

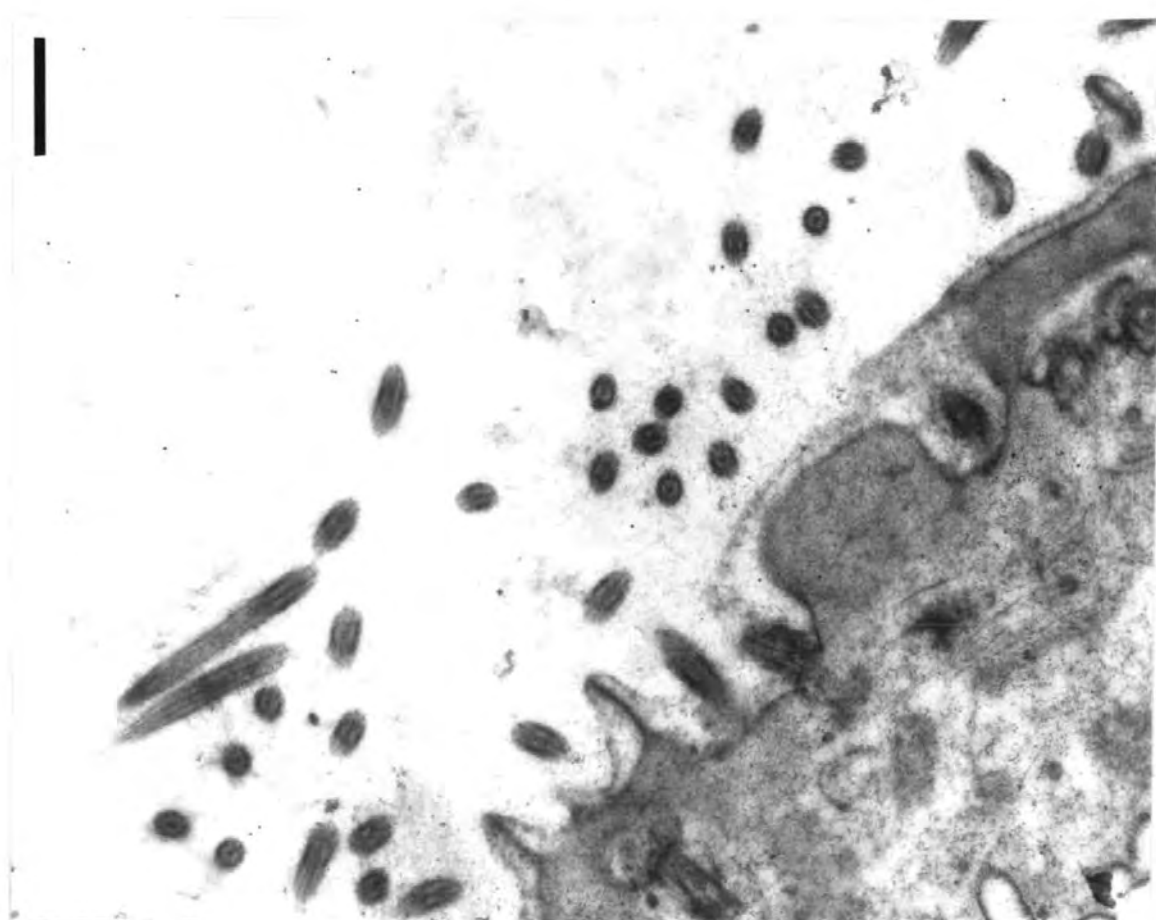
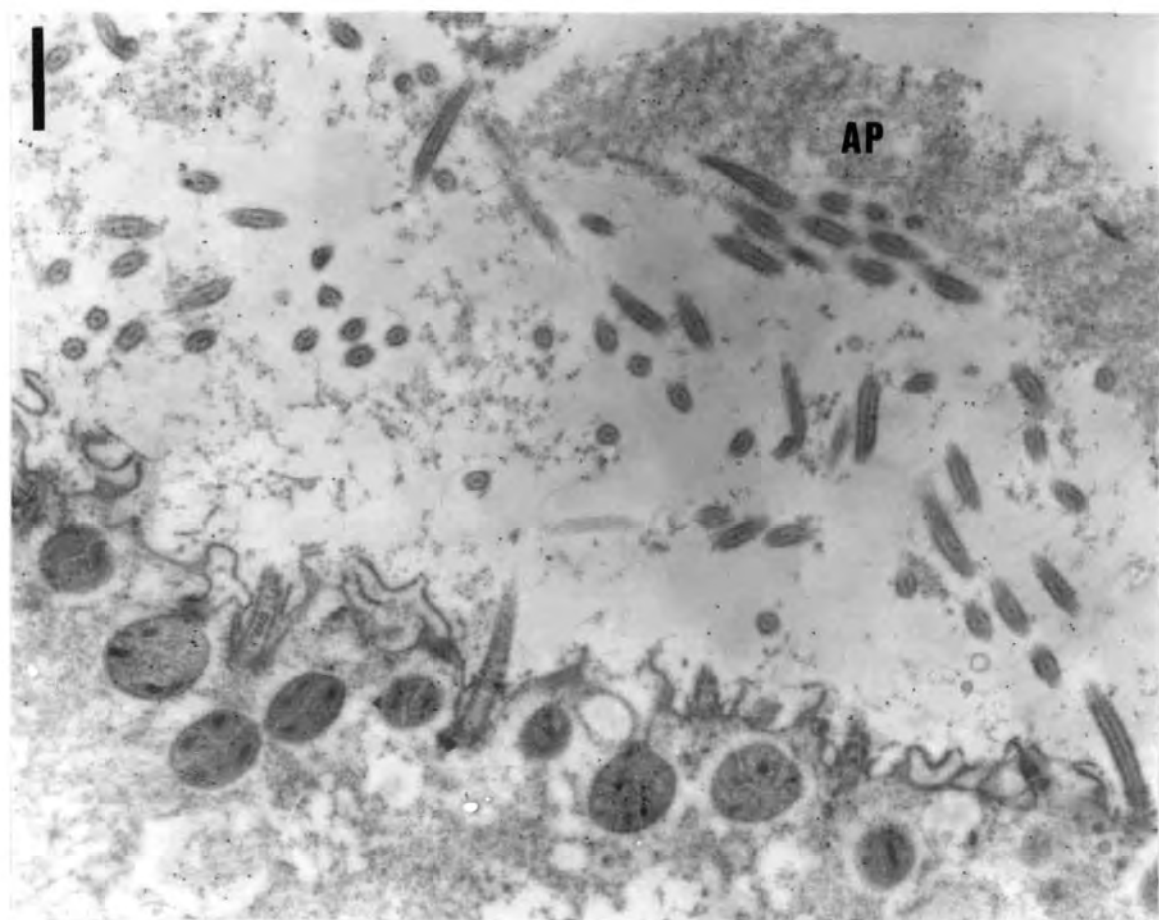
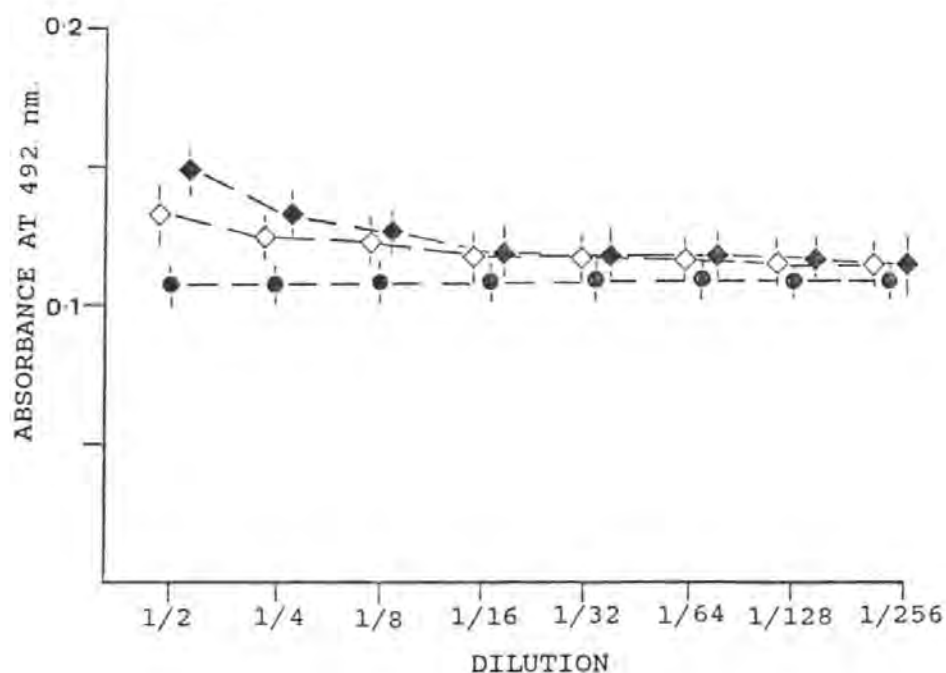
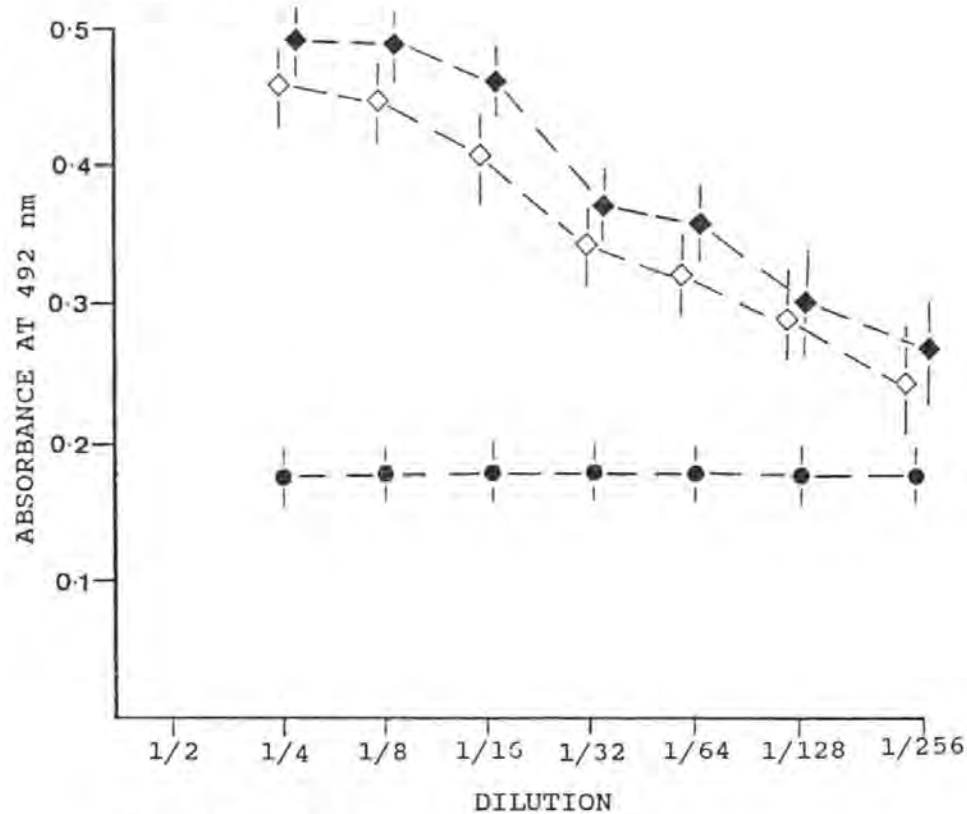


Figure 37. Graphs showing the optical density measurements obtained by ELISA for samples of naive or anti-I.multifiliis carp sera and mucus against theront sonicate.

a) Mucus.



b) Serum.



◇ = NAIVE CARP

◆ = CARP IMMUNISED BY REPEATED INFECTIONS

● = BACKGROUND ABSORBANCE (O.D. OBTAINED BY OMITTING PRIMARY ANTIBODY LINK)

(Figures represent means \pm standard error of means [SEM] for five replicates of each sample.)

signals were observed in assays, which could not be alleviated by the inclusion of BSA blocking steps or partial purification of rabbit anti-carp IgM serum by ammonium sulphate precipitation. The test assays included serum from carp immunised by repeated infections, in which specific antibody activity had been demonstrated by other immunolabelling techniques; in view of this, the ELISA technique was therefore abandoned as a means of detecting anti-parasite antibodies further.

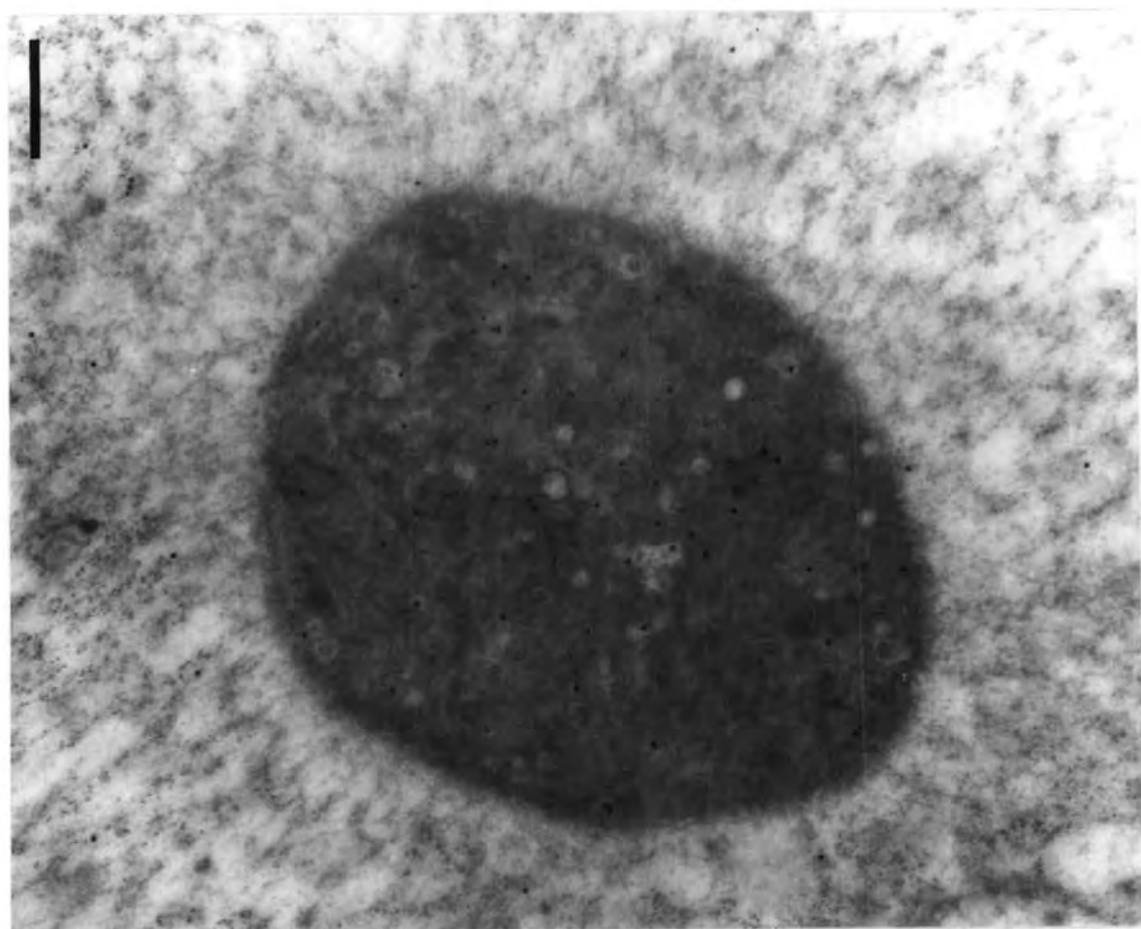
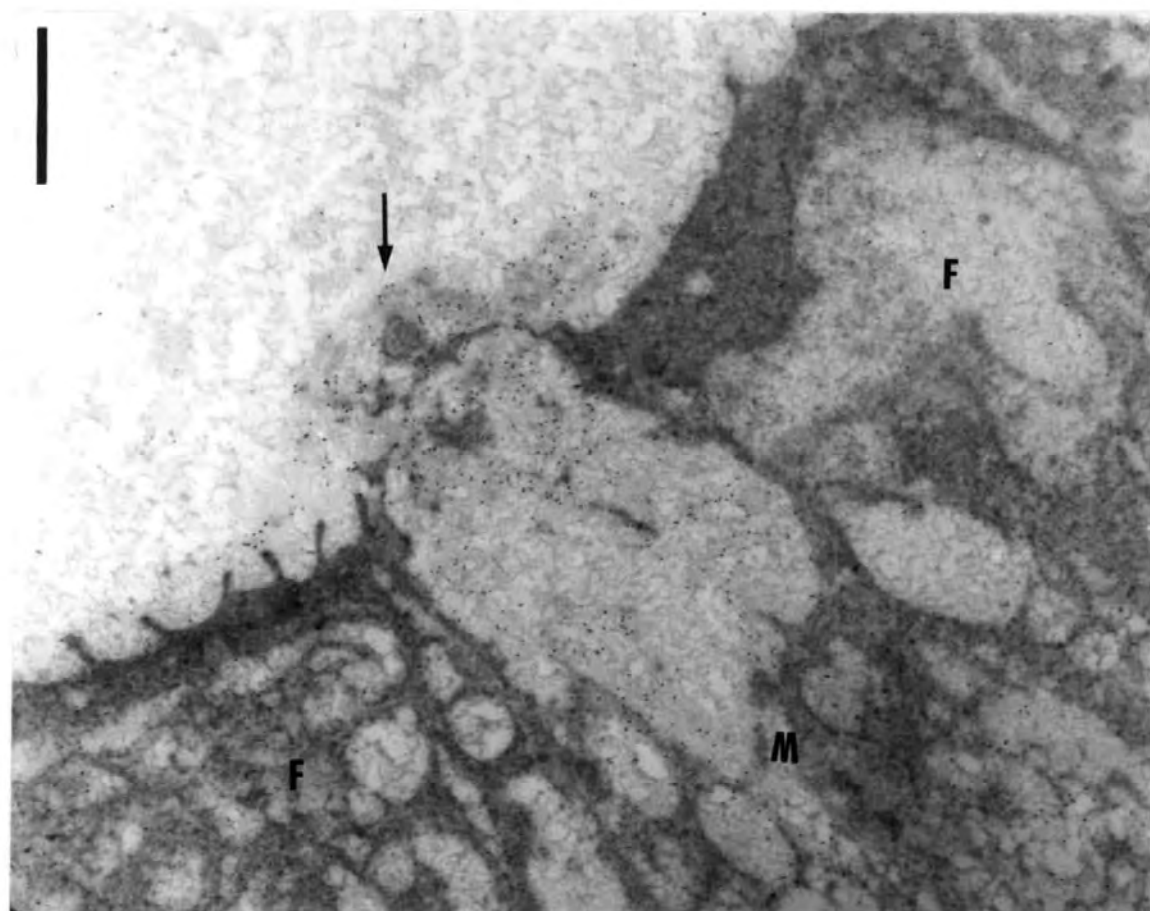
4.1.1.2. In situ detection of anti-parasite antibodies

10 immunised 'O' group carp (average weight $6.84 \text{ g.} \pm 1.62 \text{ g.}$) and 10 previously unexposed fish (average weight $5.96 \text{ g.} \pm 0.67 \text{ g.}$) were infected by droplet infection. The position of approximately 20 parasites in an area of skin chosen at random was then recorded by mapping. At various time intervals over a following 5 day period biopsies of infected sites were taken and the presence of in situ carp IgM investigated by IGSS and IGL techniques.

Intra-epidermal carp IgM was recorded in intercellular spaces throughout the epidermis, although most notably in significant amounts in the vesicles of mucus cells (figure 38). In relation to I.multifiliis in situ in the

Figure 38. Electron micrograph of an epidermal mucus cell (M), showing specific immunogold labelling for in situ carp IgM in the cell vesicles. Note intense labelling of discharged material (arrowed). F = adjacent filament cells. X 20,000 mag.. Scale bar = 1 μ m.

Figure 39. Electron micrograph of a trophont food vacuole, showing specific immunogold labelling for carp IgM. X 16,000 mag.. Scale bar = 1 μ m.



skin, carp IgM could not be detected in significant amounts at the surface of trophozoites or associated with structural parasite material (other than food vacuoles; figure 39) during any stage of development in the epidermis of immunised or previously unexposed carp (figures 40 and 41).

4.1.2. Identification of antigenic sites of

I.multifiliis

The specific antigenic sites of I.multifiliis recognised by the humoral response were determined. Sera and mucus from adult carp immunised by either repeated infections or i.p. injection of theront sonicate were investigated, and the antigenic sites were located using immunogold labelling techniques at both light microscope level (IGSS) and electron microscope level (IGL).

Serum from fish immunised by repeated infections contained antibodies which recognised antigenic sites at the periphery of trophonts, as demonstrated by IGSS (figure 42). This response was predominantly associated with the cilia (figure 43 a), although in addition a strong antibody response to mucocyst organelles was also recorded by means of IGL (figure 44 a). A similar response was recorded against theronts, with antibodies recognising both mucocysts and cilia, the latter

Figure 40. Electron micrograph of a trophozoite (Tr) in the epidermis of an immunised 'O' group carp, 40 minutes following theront penetration, immunogold labelled for in situ carp IgM. Note lack of specific labelling against structural parasite material. X 16,000 mag..

Scale bar = 2 μ m.

Figure 41. Electron micrograph of a trophozoite (Tr) in the epidermis of an immunised 'O' group carp, 5 days following theront penetration, immunogold labelled for in situ carp IgM. Note lack of specific labelling against structural parasite material. X 16,000 mag..

Scale bar = 2 μ m.

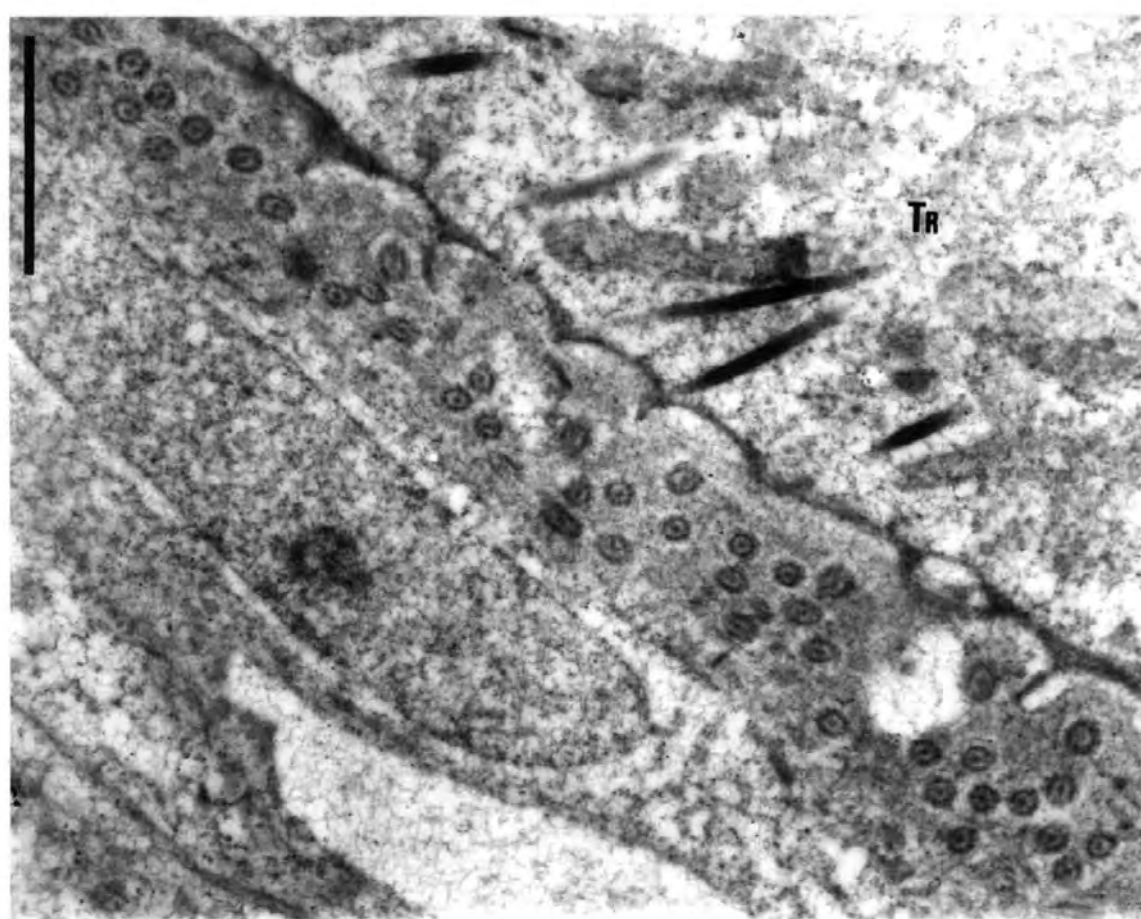
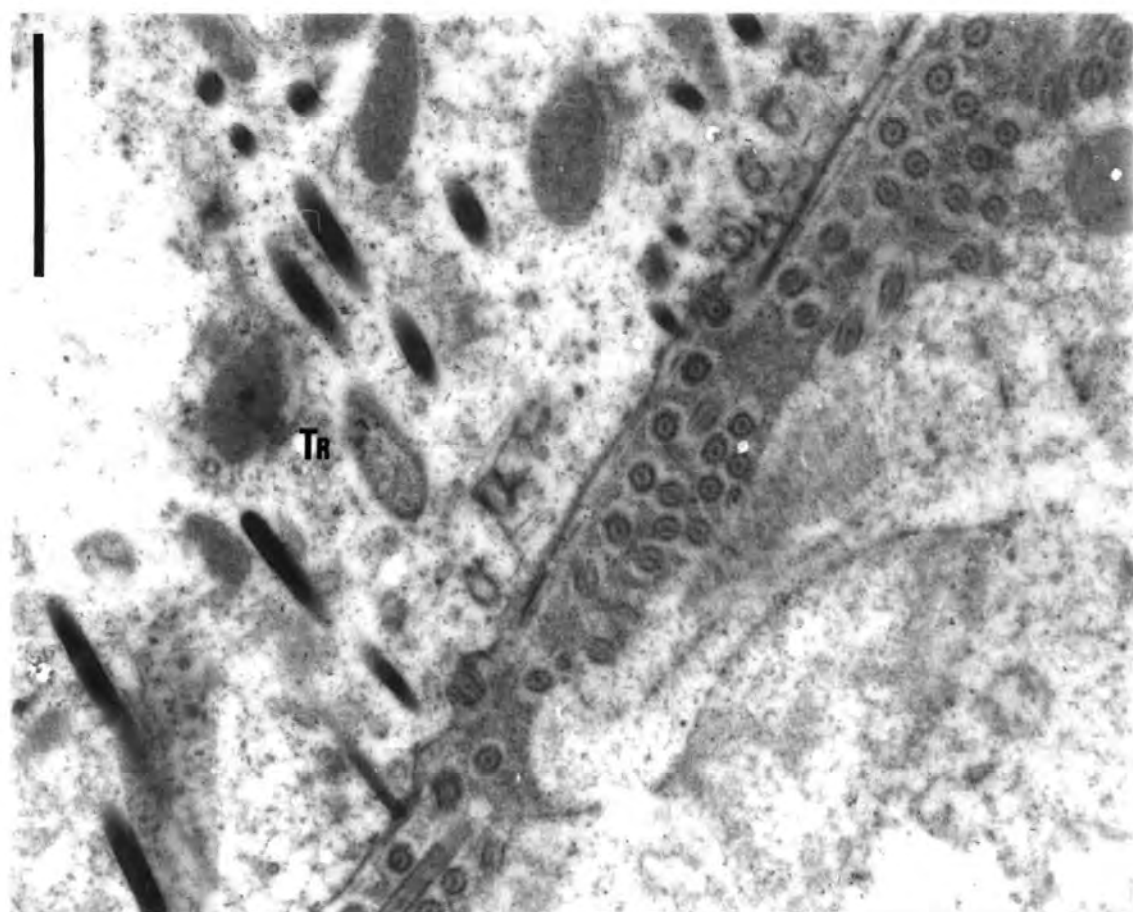


Figure 42. Light micrographs of trophonts (Tr) labelled by IGSS, using 1/5 serum from carp immunised against I.multifiliis by repeated infections as the primary antibody link. A. viewed under normal light; B. same section viewed under epi-polarised light. Note silver reaction product (SRP, arrowed) at the periphery of each parasite. X 320 mag. Scale bars = 50 μ m.

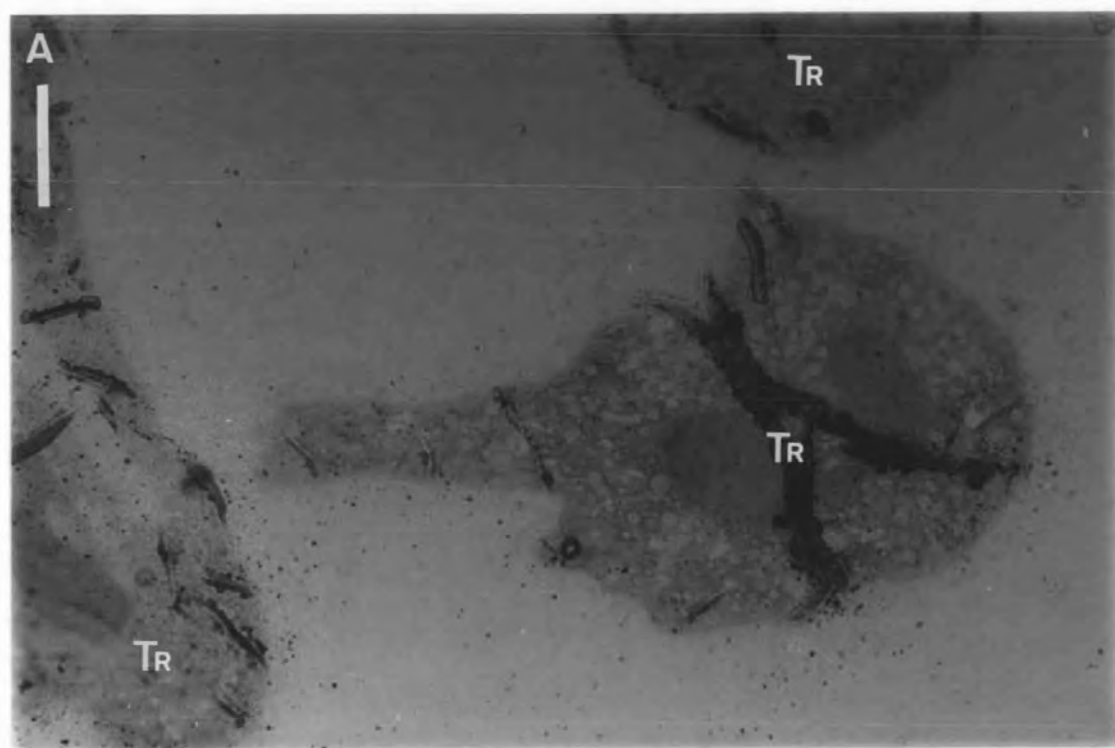
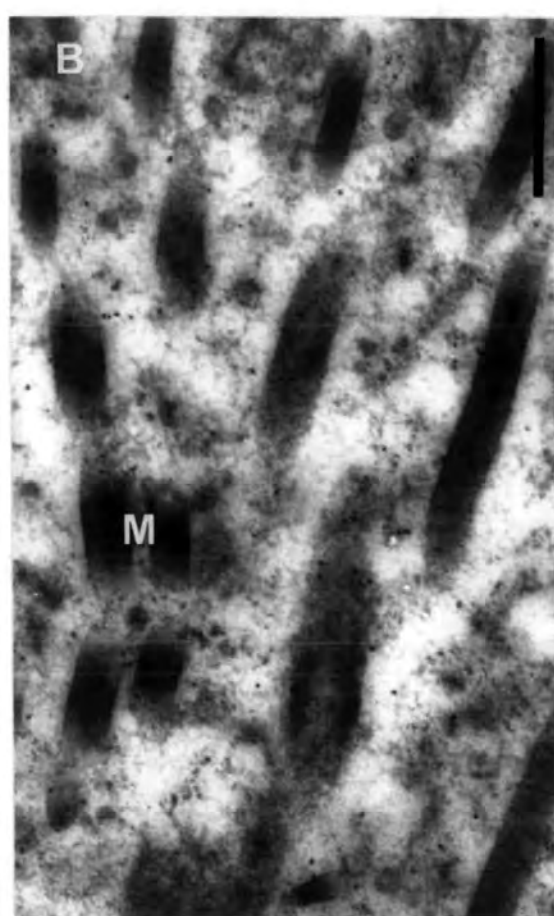
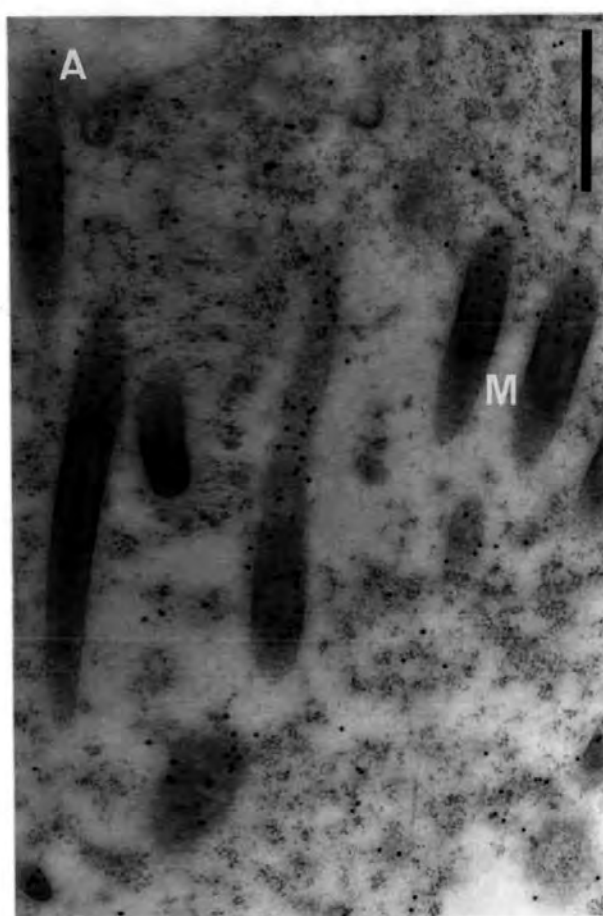
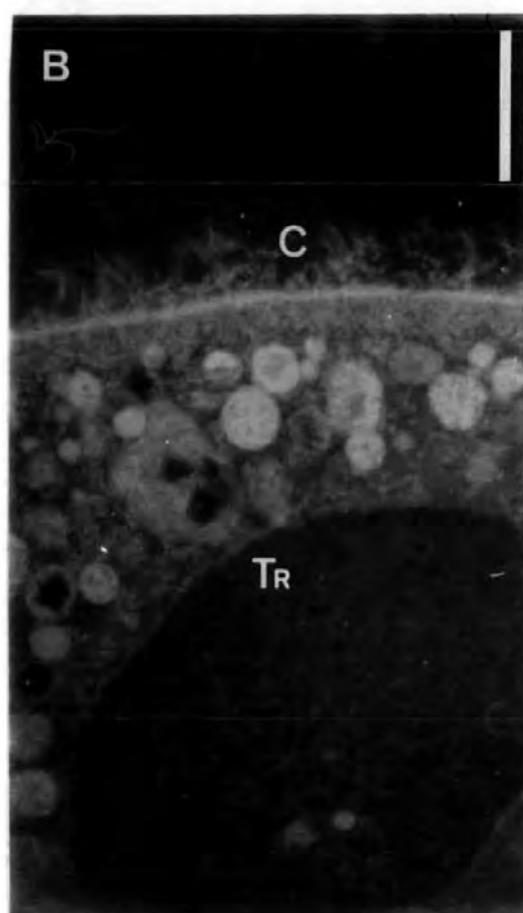
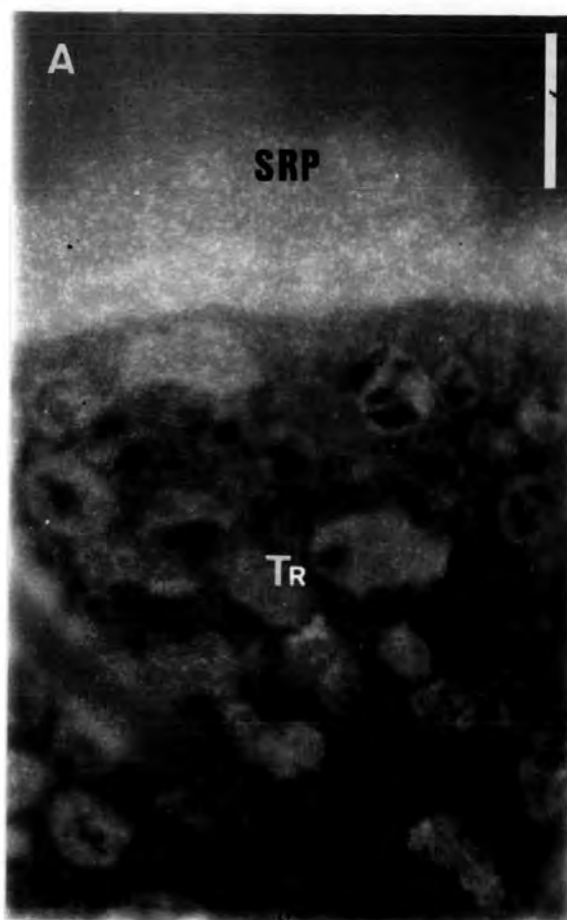


Figure 43. Light micrographs of periphery of trophonts labelled by IGSS, using A. 1/5 serum from carp immunised against I.multifiliis by repeated infections, or B. 1/5 serum from carp naive to I.multifiliis as the primary antibody links, viewed under blue light. Note intense silver reaction product (SRP) in A. X 2,000 mag.. Scale bars = 10 μ m.

Figure 44. Electron micrograph of trophont mucocysts immunogold labelled by a three link system, using A. 1/20 serum from carp immunised against I.multifiliis by repeated infections, or B. 1/20 serum from carp naive to I.multifiliis as the primary antibody links. Note specific labelling of mucocysts in A. only. X 42,000 mag.. Scale bars = 500 nm.



response being directed primarily against the membranes (figure 45). No specific anti-parasite labelling was observed in any of the controls, e.g. using serum from carp naive to I.multifiliis (figures 43 b and 44 b), or a known positive serum sample absorbed of specific anti-parasite antibodies prior to labelling (figure 46). Concentrated mucus from fish immunised by repeated infections did not contain antibodies with specificity for antigenic sites of I.multifiliis (figure 47).

Serum from carp immunised by i.p. injection of theront sonicate predominantly recognised antigenic sites of theronts associated with mucocysts (figure 48), although in this case the gold labelling was more intense than with serum from carp immunised by repeated infections.

4.2. Cellular responses

Chemiluminescence (CL) assays were employed to investigate in vitro phagocytic responses of pronephric cells from both immunised and naive 'O' group carp, by measuring luminol-amplified light emission following production of free radicals from these cells. Assays were conducted to determine the phagocytic response to immunologically relevant material (namely I.multifiliis sonicate/homogenate, pre-incubated with either anti-I.multifiliis serum or serum from non-immunised

Figure 45. Electron micrograph of a theront immunogold labelled by a three link system, using 1/20 serum from carp immunised by repeated infections as the primary antibody link. Note specific labelling of mucocysts (M) and ciliary targets, especially the outer membranes (arrowed). X 12,400 mag.. Scale bar = 1 μ m.

Figure 46. Electron micrograph of a theront immunogold labelled by a three link system (using 1/20 anti-I.multifiliis serum absorbed of specific anti-parasite antibodies as a control) for the primary antibody link. Note lack of specific labelling. X 16,000 mag.. Scale bar = 1 μ m.

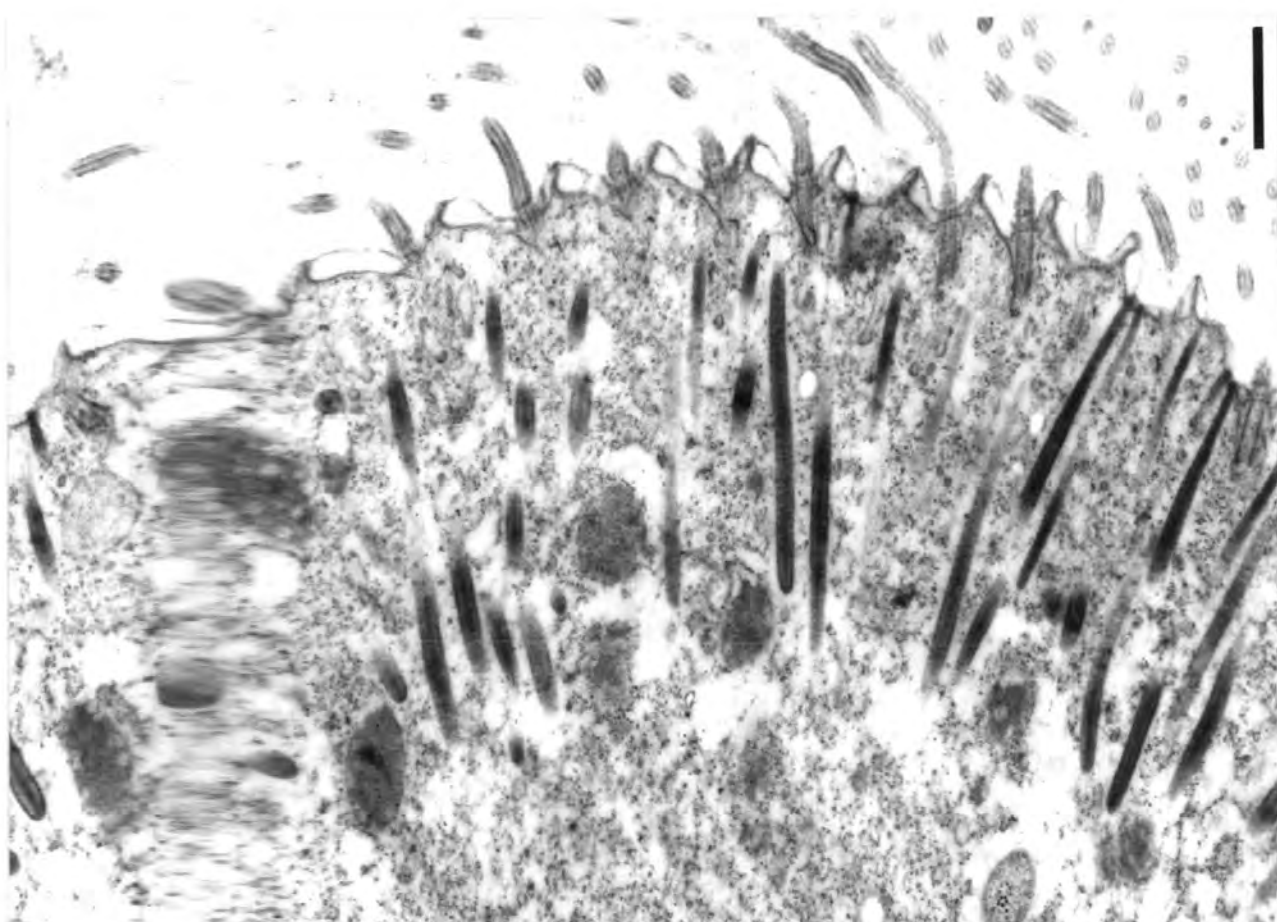
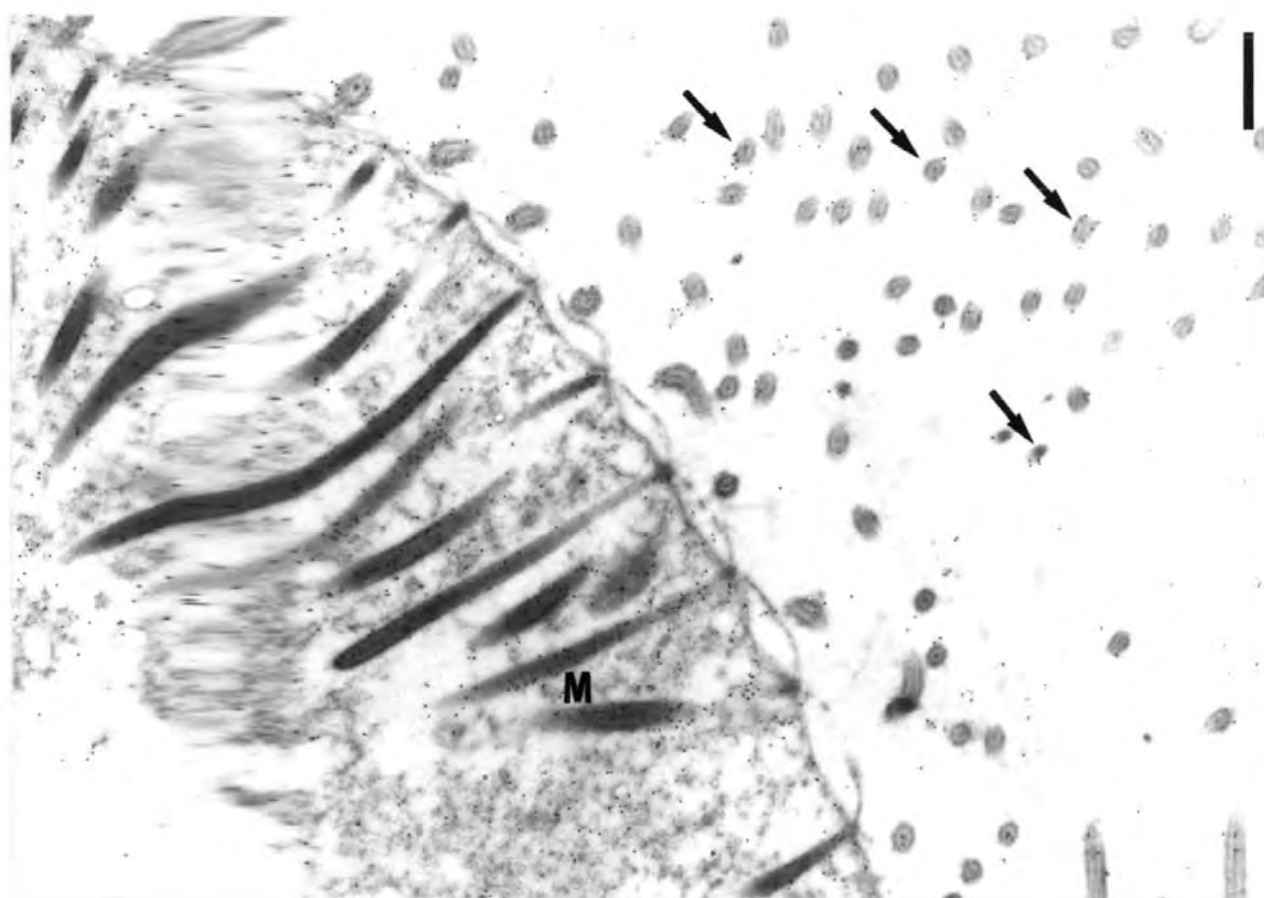
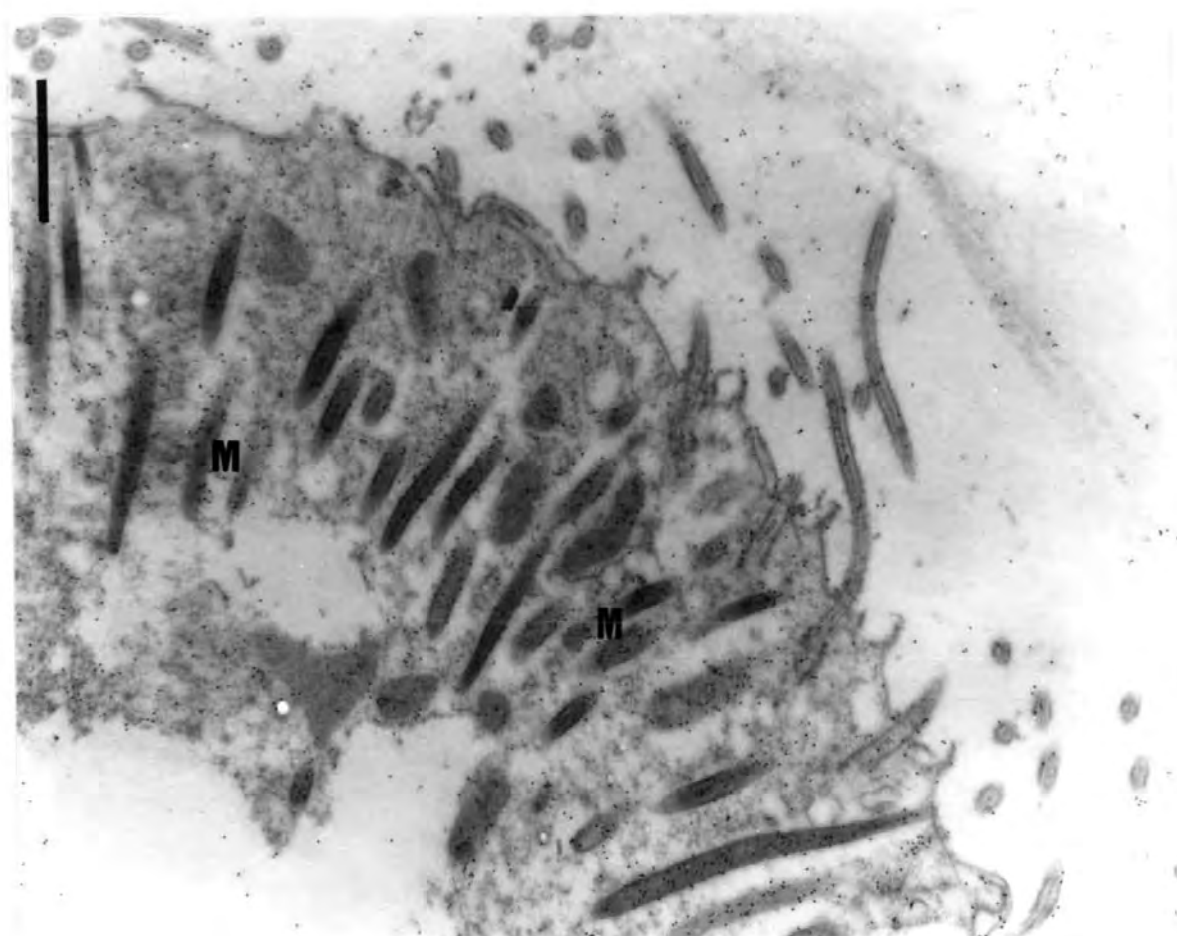
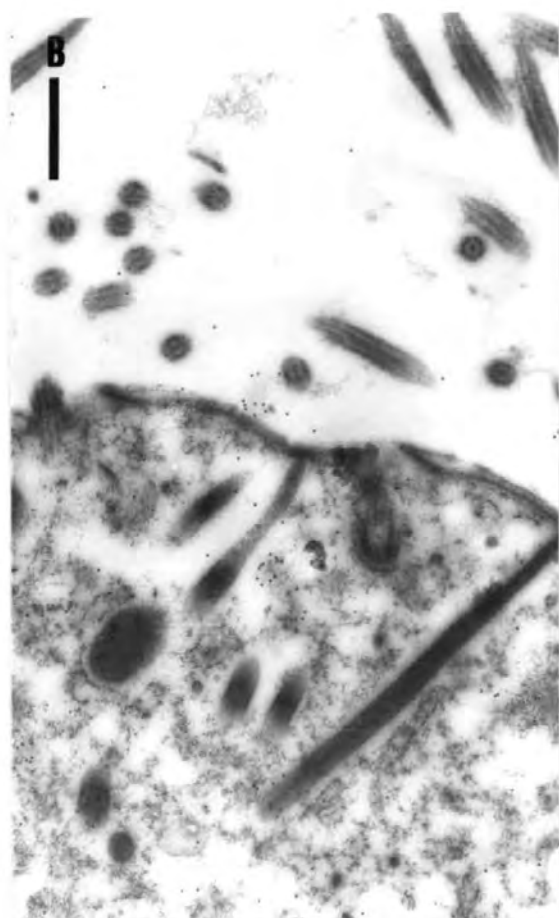
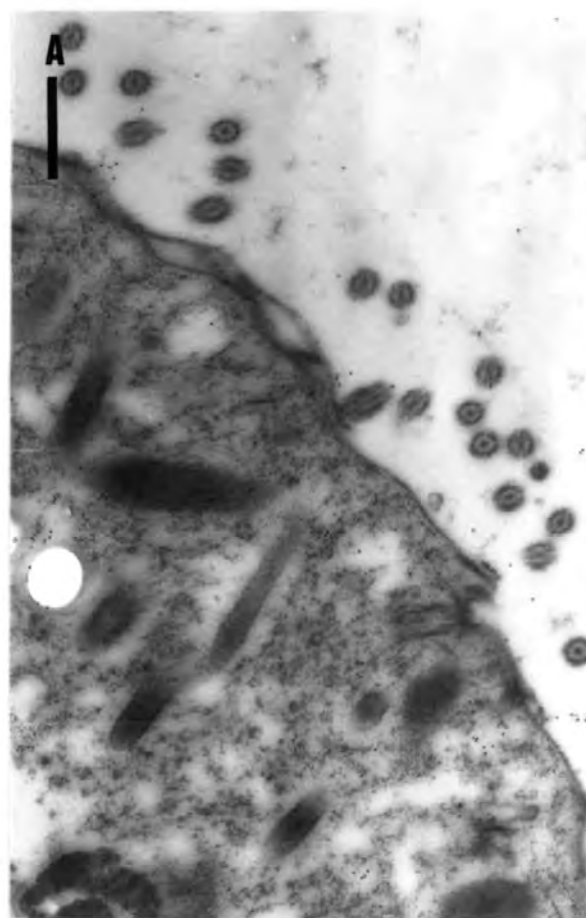


Figure 47. Electron micrographs of the surface of theronts, immunogold labelled by a three link system, using A. 1/5 mucus from carp immunised against I.multifiliis by repeated infections or B. 1/5 mucus from carp naive to I.multifiliis as the primary antibody link. Note lack of specific labelling. X 13,300 mag.. Scale bars = 1 μ m.

Figure 48. Electron micrograph of a theront immunogold labelled by a three link system, using 1/20 serum from carp immunised by i.p. injection of theront sonicate as the primary antibody link. Note intense labelling of mucocysts (M). X 12,400 mag.. Scale bar = 2 μ m.



carp), or to zymosan (opsonised with carp serum or non-opsonised).

5 immunised 'O' group carp (average weight $36\text{ g} \pm 8\text{ g}$) and 5 fish naive to I.multifiliis (average weight $42\text{ g} \pm 13\text{ g}$) were each injected i.p. with $100\text{ }\mu\text{l}$ of theront sonicate ($360\text{ }\mu\text{g}$ protein/ml) 48 hours before each assay. A single cell suspension from the pronephros was prepared for each of these fish in turn. The in vitro phagocytic response of pronephric cells from each of these fish to both I.multifiliis sonicate/homogenate and zymosan was then quantified by means of CL assay.

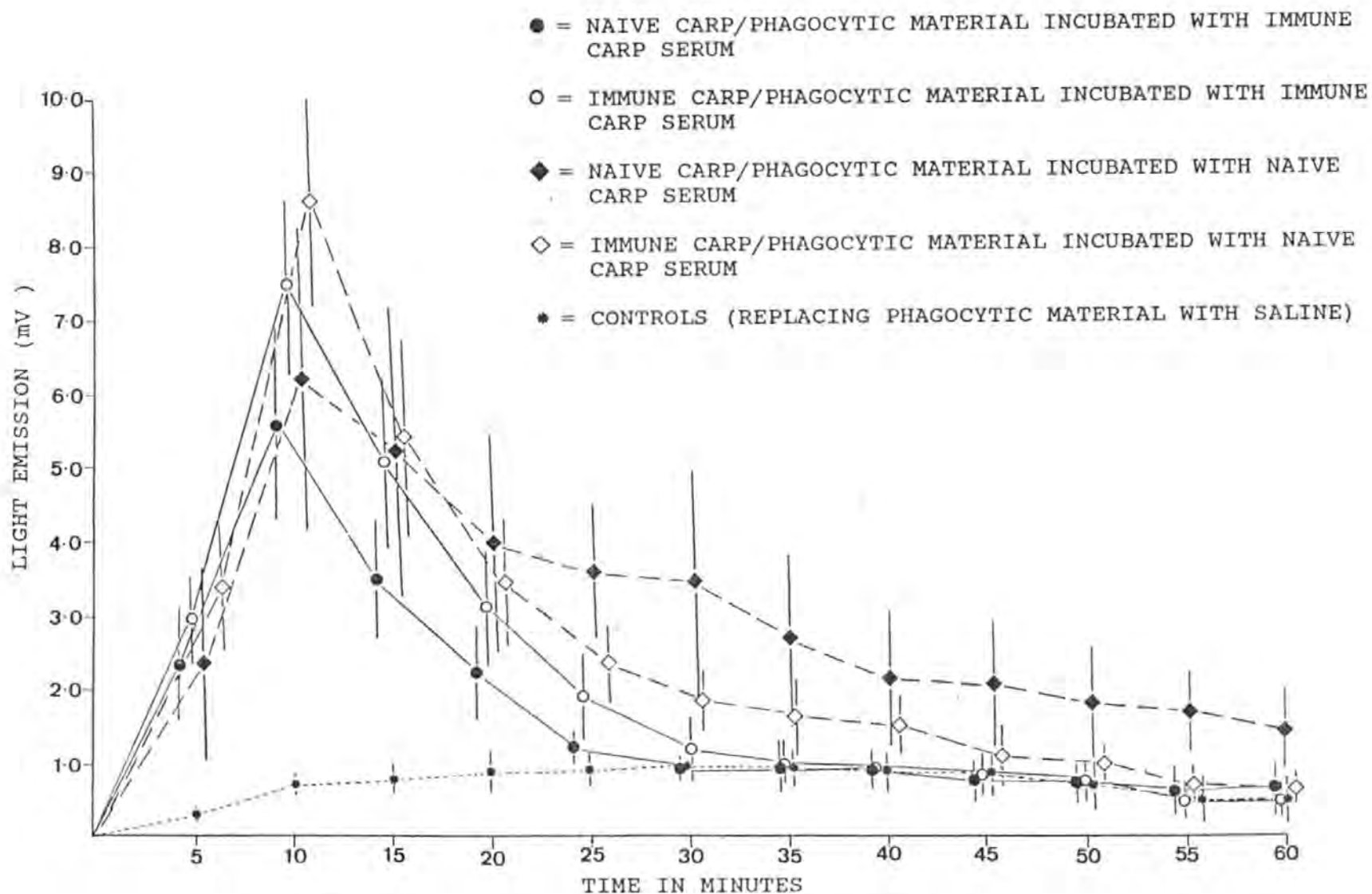
4.2.1. Phagocytic response to sonicate/homogenate of I.multifiliis

'O' group carp pronephric cells displayed only weak phagocytic activity in the presence of theront sonicate/homogenate (figure 49). The phagocytic activity was not significantly enhanced by using either cells or serum from immunologically primed fish (i.e. pronephric cells from immunised carp or homogenate/sonicate which had been pre-incubated with anti-I.multifiliis serum; student T test).

4.2.2. Phagocytic response to zymosan

'O' group carp pronephric cells displayed strong phagocytic activity in the presence of zymosan (figure

Figure 49. Graphs showing the CL response of pronephric cells from naive carp or carp immunised against I.multifiliis by repeated infections to I.multifiliis sonicate/homogenate.

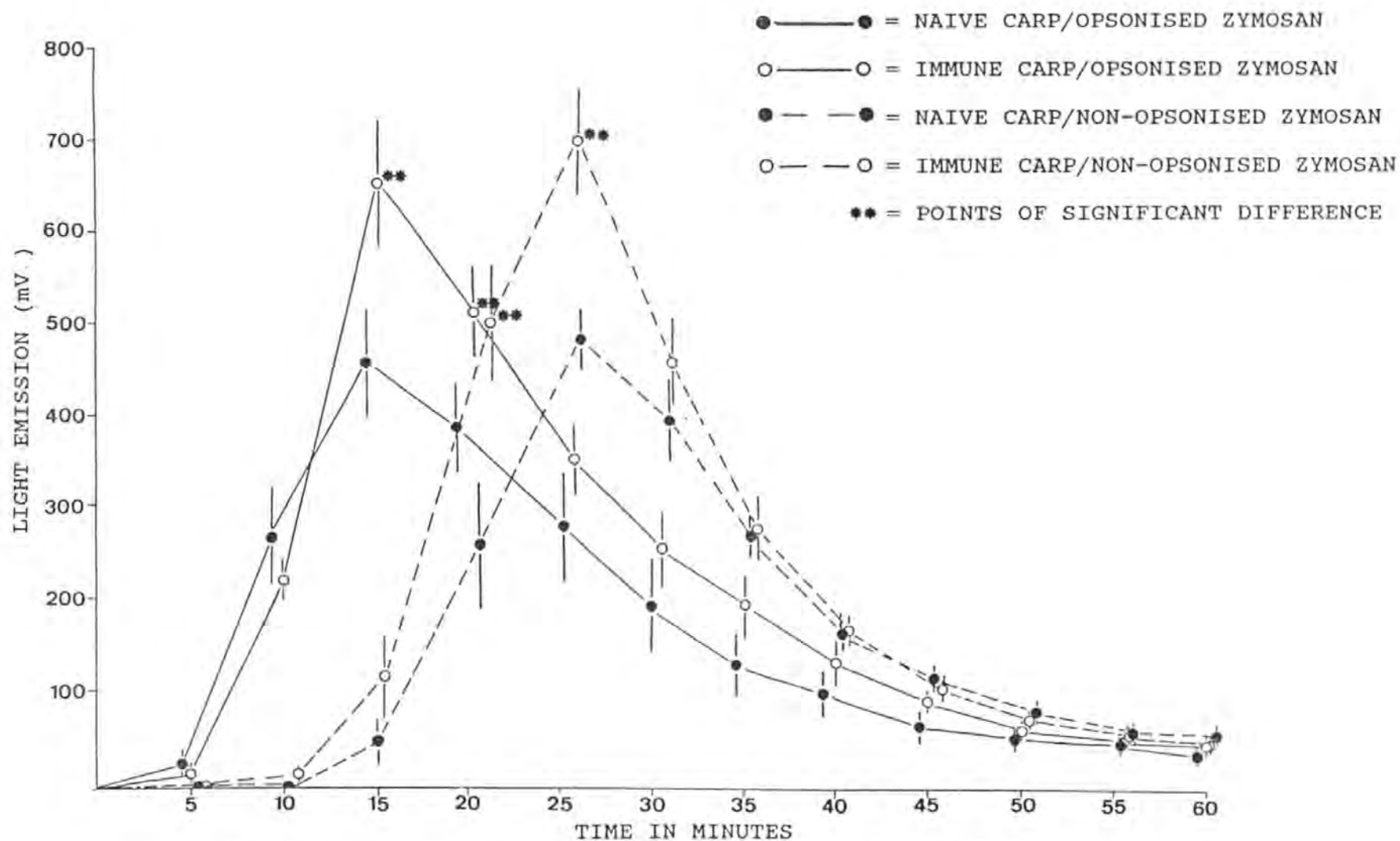


(Figures shown represent means \pm SEM for 5 fish of each group).

51). The peak of this activity occurred more quickly for opsonised zymosan (at 15 minutes following the start of the experiment) than for non-opsonised zymosan (at 25 minutes), although the overall magnitude of the response was similar.

Cells from fish immunised against I.multifiliis showed greater phagocytic activity in the presence of both opsonised zymosan (at 15 and 20 minutes following the start of the experiment) and non-opsonised zymosan (at 20 and 25 minutes) compared to cells from naive carp (P values <0.025 ; student T test).

Figure 50. Graphs showing the CL response of pronephric cells from naive carp or carp immunised against I.multifiliis by repeated infections to zymosan.



(Figures shown represent means \pm SEM for 5 fish of each group).

DISCUSSION

Protective immunity against I.multifiliis was successfully established in 'O' group carp, confirming the previous work of Subasinghe (1982) and Houghton (1987). That a single immunising infection can confer significant protection within two weeks of exposure is in agreement with the observations of McCallum (1986). The time scale of this response is typical of warm water cyprinids, including Cyprinus carpio, to mount a primary humoral or cellular immune response to parenterally administered antigens (Avtalion, 1969; Baba et al., 1988a & 1988b; Lamers, 1985, 1986; Rijkers, 1981; Shelton & Smith, 1970; Trump & Hildemann, 1970). That 'O' group carp are able to mount a humoral immune response to I.multifiliis within this time has been confirmed by Houghton (1987).

Although recognised immunisation procedures were employed here, sterile immunity was not observed in 'O' group carp following exposure to large numbers of theronts (10,000 per fish). This condition was not recorded by Hines & Spira (1974c), who observed that immunity in adult C.carpio kept in a continually infective environment was essentially sterile. Interestingly, Houghton (1987) demonstrated that although immunity in 'O' group carp observed at a challenge level of 8,000 theronts per fish appeared

sterile, a higher challenge level of 16,000 theronts per fish led to the successful establishment of some parasites. Thus, a finely balanced threshold of protection seems to occur in immunised fish, an infection level above which leads to parasite establishment and disease outbreak in enclosed fish populations.

That theronts were observed here to penetrate the epidermis of immunised carp at levels comparable to those in non-immune control fish contradicts suggestions that protective immunity prevents the parasite entering the host (Beckert, 1975; Goven et al., 1980b, 1981a; Hines & Spira, 1974c). Houghton (1987) and Subasinghe (1982) also observed that theronts were capable of penetrating the epidermis of immunised fish, although this did not lead to successful parasite establishment. Protective immunity against I.multifiliis does not, therefore, appear to be operative at the immediate host/environment interface in the same manner as protection against certain bacterial pathogens of fish (Kawai & Kusuda, 1981). The mechanism of protection must therefore be operative following penetration, i.e. in situ within the epidermis.

Of particular interest here was the observation that 79% of parasites which entered immune fish skin could not be re-located in the epidermis within two hours of penetration. The fate of these parasites was uncertain; however, since no identifiable parasite material could be located at sites of initial penetration, it is concluded here that parasites had exited the skin. This is probably an active process, as although I.multifiliis is endoparasitic, its proximity to the external environment during the parasitic stage may facilitate premature exit from immune fish. Premature exit could represent a survival strategy for parasites in the unfavourable environment of an immune host; trophozoites have previously been observed capable of exiting the epidermis of dead fish and re-establishing in a new host without entering the divisional stage (R.A. Matthews, pers. comm.). There appears, therefore, to be a critical period for the establishment of I.multifiliis in the epidermis of immunised fish within the first two hours of penetration, during which time the majority of parasites exit the skin, and following which most of the remaining parasites successfully complete their development. The time scale of premature parasite exit from the skin of immune fish is too rapid to be attributed to a response involving cellular infiltration

into the epidermis, as suggested by Houghton (1987). With respect to this, Iger & Abraham (1990) recorded leucocytes first infiltrating the dermis of carp one hour following non-specific skin injury and reaching the dermal/epidermal junction at a wound site within three hours, whilst Phromsuthirak (1977) demonstrated leucocytes first infiltrating the epidermis itself 12 hours following injury in stickleback (Gasterosteus aculeatus) when fish were held at 20°C. The results here therefore indicate that the protective response to I.multifiliis is humorally based and that protection is characterised by parasite exit from the epidermis, rather than prevention of penetration or in situ parasite killing.

That mucus from carp naive to I.multifiliis was shown to immobilise theronts in vitro at titres as high as 1/128 emphasises the role of mucus as a non-specific protective barrier (Ellis, 1981; Fletcher, 1976 & 1982; Lamers, 1985). Previous reports have suggested that mucus from non-infected fish has no deleterious effect on I.multifiliis (Hines & Spira, 1974c; Subasinghe, 1986; Wahli & Meier, 1985). Nevertheless, a non-specific protective role for mucus could be envisaged to modulate the infection process of I.multifiliis, by deterring if not preventing some theront penetration. In support of

this view, it was noted here that many theronts which did not penetrate immediately on host contact often travelled along the outer face of the epidermis for a few seconds, before returning to the water layer without successful penetration. This phenomenon has previously been recorded by Kozel (1986) in channel catfish Ictalurus punctatus. Further evidence of the in vivo role of mucus in protection against I. multifiliis was provided by Pickering & Christie (1980), who observed that male brown trout Salmo trutta were more susceptible to ichthyophthiriasis following the seasonal skin demucification associated with sexual maturation. Thus, the role of mucus as a non-specific regulatory factor in parasite dynamics cannot be overlooked.

No enhancement of the in vitro theront immobilising activity was recorded in mucus from immunised fish here. Hines & Spira (1974c) demonstrated specific immobilising activity against trophonts in mucus from immune carp to a titre of 1/16, although their mucus samples had been concentrated to 10.3 mg protein/ml compared to 2.0 mg/ml used in the present study. Subasinghe (1986) and Wahli & Meier (1985) also detected parasite immobilising activity in the cutaneous mucus of fish which they considered to be immune, although they presented no data

to indicate the concentration of mucus used. The failure here to detect a theront immobilising response in mucus from immunised fish suggests that the specific activity in cutaneous mucus must be very low, and cannot be detected unless the mucus sample is very concentrated. In common with conclusions here, Houghton (1987) questioned the ability of mucus to immobilise theronts in vivo, since theronts are observed capable of penetrating the epidermis within seconds of host contact. A role for specific factors in mucus actually preventing penetration into the host epidermis therefore seems unlikely.

That specific anti-I.multifiliis antibody could not be detected in cutaneous mucus from immunised carp is surprising, in light of previous reports which have suggested that immunity to I.multifiliis could be characterised by the presence of anti-parasite mucosal antibodies (e.g. Beckert, 1975; Goven et al., 1980b, 1981a; Hines & Spira, 1974c; Subasinghe, 1986). Two hypotheses may be forwarded to explain the failure to detect significant anti-parasite antibody in cutaneous mucus. First, that the degree of concentration of mucus was insufficient to detect antibody activity. This seems unlikely, however, since the 2.0 mg protein/ml concentration used here is within the range quoted from

previous studies concerning general immunoglobulin activity in fish cutaneous mucus (Cipriano & Heartwell, 1986; Diconza & Halliday, 1971; Ourth, 1980). Furthermore, the techniques employed here were sensitive enough to detect immunoglobulin in situ and in vitro in skin and mucus samples. Secondly, the failure to detect anti-parasite antibody or specific theront immobilising activity in cutaneous mucus may reflect that the humoral response to I.multifiliis is purely systemic, such that none or only low levels of parasite-specific antibody present in the blood (viz. a systemic immune response) reach the mucosal layer, as proposed by Ourth (1980) and St. Louis-Cormier et al. (1984). The route of entry of antigens in fish is known to influence the mode of the immune response: In carp, Rombout et al. (1989c) have detected specific cutaneous mucus antibody activity following stimulation of the common mucosal immune system but not following parenteral immunisation. Since I.multifiliis inhabits the skin and gill epithelia, the parasite's proximity to the vascular system favours uptake of material to the blood to stimulate a systemic immune response, and a systemic immune response has clearly been demonstrated in previous immunological studies (Clark et al., 1988; Hines & Spira, 1974c). Furthermore, Pyle & Dawe (1985) failed to detect

specific anti-protozoan activity in the cutaneous mucus of channel catfish Ictalurus punctatus following parenteral immunisation with Tetrahymena pyriformis, although activity was recorded in the serum. The tentative conclusion here is therefore that antigens of I.multifiliis are taken up into the blood during infection, stimulating the systemic immune system but probably not the common mucosal immune system thought necessary to induce a cutaneous mucosal immune response.

In the present study, specific theront immobilising activity was demonstrated in serum from carp immunised by repeated infections, confirming previous reports of immobilising activity against both theronts (Areerat, 1974; Clark et al., 1987 & 1988; Houghton, 1987) and trophonts (Beckert, 1975; Hines & Spira, 1974c; Wahli & Meier, 1985) in serum from immune fish. Parasite surface-binding antibodies, associated primarily with ciliary membrane targets, were also detected in immune carp serum here, thus confirming the results of previous studies on immune channel catfish, Ictalurus punctatus, and rainbow trout, Oncorhynchus mykiss (Clark et al., 1988; Wahli & Meier, 1986). That the in vitro theront immobilisation effect of immune serum is attributable to ciliary-membrane binding antibody has been demonstrated

by Clark et al (1988). However, evidence of strong in vitro immune responses do not necessarily correlate with levels of in vivo protection in host/parasite systems (viz. functional/relevant and non-functional/irrelevant antigens; Anders et al., 1982; Mitchell, 1979; Wakelin, 1984). In the context of I.multifiliis, the demonstration here that parasites are able to enter and then retain sufficient mobility to exit the epidermis of immunised fish provides evidence that the antigens associated with in vitro immobilisation may not be exclusively important in host protection. In support of this hypothesis, Houghton & Matthews (1990) showed that sera from carp immune to I.multifiliis which had been administered with corticosteroids displayed good in vitro theront immobilising activity, although the fish were fully susceptible to parasite establishment on challenge. Furthermore, Goven et al. (1980a) demonstrated that channel catfish parenterally immunised with ciliary preparations of I.multifiliis (presumably containing the membrane-associated "i" antigens) were not completely protected against the parasite, and 42.5 % mortalities were recorded on challenge. Thus whilst the humoral response associated with ciliary antigens undoubtedly affords a degree of protection to the host, more complete resistance to infection may involve other

immune effector mechanisms.

Results here have shown that, as well as ciliary membrane targets, serum from infection-immunised carp also recognised antigens associated with mucocyst organelles. The demonstration that mucocyst material introduced to the host during natural infection is immunogenic is highly relevant to the interpretation of immunological events (R.A. Matthews, pers. comm.). The expulsion of mucocyst material during penetration and, to a lesser extent, during development in the host epithelia (Ewing *et al.*, 1985; Matthews & Matthews, 1984) classifies mucocyst-derived antigens as excretory/secretory ("ES"), although in the strictest sense this classification is reserved for completely soluble material (Mitchell & Anders, 1982). That mucocysts from at least two stages of the parasite's life cycle (namely the theront and trophont) are recognised by the host response suggests that mucocyst material contains antigens which are conserved throughout different stages of the life cycle. These antigens appear to be potent immunogens, since intense immunogold labelling was recorded using serum from carp immunised by either repeated infections or i.p. injection of theront sonicate. However, little information is available concerning the biochemical

composition of mucocyst material or the relevant antigens. The matrix of mucocyst material probably comprises predominantly mucopolysaccharides like the mucocysts of free-living ciliates (Matthews & Matthews, unpublished results; Nilsson, 1979), although other components may well be more functional with respect to the parasitic mode of life. In support of this, Uspenskaya (1963) recorded hyaluronidase activity in water containing theronts, whilst Kozel (1980) has reported acid phosphatase and non-specific esterase activity in theronts. However, there is no conclusive evidence that this enzymatic activity is derived from mucocysts. Further research into the biochemical composition, isolation and purification of mucocyst-derived antigens of I.multifiliis is therefore required. Although there is no evidence here concerning the efficacy of mucocyst material as protective antigens, the possibility of mucocyst material being further investigated with a view to vaccine development cannot be overlooked.

Of particular interest in the present study was the failure to detect surface-binding antibody on trophonts pre-incubated in vitro in immune carp serum. Surprisingly, no significant IgM was detected on the

ciliary membranes, although weak specific labelling was recorded in an amorphous precipitate reaction beyond the outer edge of the cilia. It is proposed that the precipitate is of mucocyst material, which on discharge forms a protective barrier around the parasite that, possibly following complexing with mucus-specific antibodies itself, prevents significant amounts of cilia membrane-specific antibody from binding. Release of mucus following in vitro incubation in specific immune serum has previously been recorded for both I.multifiliis (Clark et al., 1987a; Houghton, 1987) and Tetrahymena pyriformis (Doerder & Berkowitz, 1981; Watson et al., 1964). Nilsson (1979) considered that this response in T.pyriformis formed a mucus "sheath" around the ciliate as a protective response. A similar protective role for secreted mucocyst material of I.multifiliis may therefore be operative in vitro to prevent antibody binding.

In the present study significant quantities of antibody could not be detected in situ at the parasite surface at any stage following penetration into the epidermis of immunised fish. In fact, in relation to the parasite, IgM was only detected in situ in significant amounts in mature trophozoite or trophont food vacuoles, suggesting that the parasite may ingest host antibodies. The

failure to detect an in situ antibody response to ciliary membrane targets in immunised fish skin might be due to the in situ secretion of a protective mucocyst "sheath" which could, initially at least, prevent specific antibody from binding with the ciliary membrane targets in a way similar to the in vitro observations. However, the subsequent humoral response to mucocyst material may then be the cue for premature parasite exit from the epidermis of immunised fish. Possibly, the formation of antibody/antigen complexes soon after penetration in some way affects normal parasite behaviour, such as ease of locomotion or feeding. This reaction may then be detected by the parasite at a sub-lethal level, leading to active exit from the epidermis as a survival strategy. The failure to detect an in situ antibody response to secreted mucocyst material in immunised fish skin may be explained by the self dispersal of material by the parasite's cilia following penetration; the effective removal of antigenic material from the proximal host/parasite interface may therefore partially inactivate any detrimental effect associated with antibody binding in the immediate vicinity of the parasite, and furthermore make detection of this response by immunolabelling techniques difficult. This phenomenon has been suggested

by Wakelin (1984) as presenting a major problem in investigating immune responses to ES antigens.

In the context of the hypotheses above, the development to maturity of a few trophozoites in immunised fish suggests that other factors may influence the survival of the parasite. Possibly, the protection afforded to the parasite by the secreted mucus "sheath" immediately following penetration facilitates the establishment and rapid growth of a few trophozoites, such that the increasing layer of necrotic tissue debris surrounding the growing parasite forms an impenetrable and/or lytic barrier, preventing specific antibody binding to the ciliary membrane targets. Thus the mode of establishment of I.multifiliis in immune fish may represent a fine dynamic balance between the host's humoral response against secreted mucocyst material and parasite's ability to initiate necrosis.

No obvious damage was recorded to trophozoites at any stage of development in the epidermis of either immunised or previously unexposed 'O' group carp. This is contrary to the results of Chapman (1984), who recorded evidence of parasite degradation in situ in the skin of Ictalurus punctatus, i.e. parasite-derived mitochondria and inclusion bodies in the layer of

necrotic tissue debris surrounding an intact parasite, an observation which he attributed to host-mediated damage. It is the conclusion here that trophozoites which remain in immunised skin beyond two hours are able to resist immune mediated damage and complete development as normal.

Cellular infiltration was recorded in response to infection with I.multifiliis, during both the primary infection and infections in immunised fish, and following exit of the parasites from the epidermis. However, one major problem in interpreting the cellular events associated with I.multifiliis here has been the classification of the cell types involved. Carp lymphocytes, monocyte/macrophages and thrombocytes were readily classified on the basis of comparative morphology (Cenini, 1984; Temminck & Bayne, 1987), although in common with many previous studies, classification of the granulocyte series has remained problematic (see Ellis, 1977; Rowley & Ratcliffe, 1987). The three granulocyte types, here termed I, II and III cells, were all involved in cellular responses to I.multifiliis, these cells previously being classified as neutrophils, eosinophils and basophils respectively (Cenini, 1984; Rombout et al., 1989b and Temminck &

Bayne, 1987). However, these authors provided no cytochemical evidence to support their terminology. To confound the matter, comparative studies of the cytochemistry and ultrastructure of leucocytes from related cyprinids have shown that, with the exception of neutrophils, the granulocyte types demonstrated in related cyprinids are ultrastructurally quite distinct from those of C. carpio (Davina et al., 1980; Garavini & Martelli, 1981; Mainwaring, 1984; Temkin et al., 1986). Temminck & Bayne (1987) and Sakai (1984) have reported type II cells to be weakly phagocytic, whilst Beliek (1981) has provided support for their classification as eosinophils by demonstrating endogenous peroxidase within the cytoplasmic granules. In view of the previous reports, the classification here of type I cells as neutrophils and type II cells as eosinophils seems justified, although the classification of type III cells as basophils is regarded with some reserve.

Leucocytes capable of infiltrating the epidermis of C. carpio included lymphocytes, monocyte/macrophages, thrombocytes and granulocytes, all of which have been reported in the epidermis of other teleost species (Archer, 1979; Bullock & Roberts, 1975; Ferri & Macha, 1972; Mittal et al., 1980; Ourth, 1980; Peleteiro & Richards, 1989; Percy, 1970). There was no evidence here

of focal aggregations of lymphoid cells in the epidermis which could form the basis of a localised skin immune system. Of particular interest here was the demonstration of the osmiophilic granular cell (OGC), the origin and phylogeny of which was uncertain. Its large electron dense granules and apparent non-myeloid origin are reminiscent of mammalian mast cells (Lagunoff, 1972; Lagunoff & Chi, 1980; Metcalfe & Kaliner, 1981). There is some evidence of the existence of mast cells in teleosts (Ellis, 1982; Ezeazor & Stokoe, 1980), and immediate hypersensitivity responses typical of allergen-induced mast cell degranulation have been demonstrated (Baldo & Fletcher, 1975; Jurd, 1987). Recently, Vallejo & Ellis (1989) have highlighted important similarities and differences between the functions of presumed mast cells of rainbow trout (Oncorhynchus mykiss) and mammals. In trout, this cell has been implicated in tissue responses to helminth infections (Bullock, 1963; Sharp et al., 1989), which is also a well documented role of mammalian mast cells (Catty & Ross, 1979; Ogilvie & Parrott, 1977). The OGC observed in the epidermis in the present study has previously only been recorded in C. carpio by Rose (1986) at sites of skin damage caused by the feeding activity of fish lice, although Liewes (1977) recorded cells

similar to mast cells in the epidermis of C. carpio at the light microscope level. Shariff & Roberts (1989) recorded large numbers of "eosinophilic granular cells" at sites in the epidermis of the cyprinid Aristichthys nobilis following immunisation and challenge with the lernaeid copepod Lernaea polymorpha, which may be analogous to the OG cell. This type of cell could therefore be important in localised cellular responses to skin parasites, and its classification here as a mast cell seems justified.

The cellular response associated with primary infection in the present study was more extensive than previous reports on I. multifiliis in fish (e.g. Hines & Spira, 1974a; McLay, 1985; Ventura & Paperna, 1985). The early infiltration of neutrophils to the infection site confirms the observations of Hines & Spira (1974a), and is considered to represent a non-specific inflammatory response in teleosts (Finn & Nielson, 1972; Roberts, 1975). Ewing (1981) recorded neutrophils and eosinophils within the layer of necrotic tissue debris surrounding the parasite 2 days following primary infection in carp, whilst Hines & Spira (1974a) and Ventura & Paperna (1985) have recorded neutrophils, eosinophils and lymphocytes, although in the latter two reports there

was no strictly controlled exposure of the fish to a single primary infection. The predominance of type III cells ("basophils") and especially eosinophils in the later stages of the primary infection here suggests a response other than a normal inflammatory reaction, the latter stages of which tend to be predominated by mononuclear cells (Finn & Nielson, 1972; Roberts, 1975). The observations here lend support for chemoattractive cellular migration in response to the parasite. If the granulocytes observed in the present study were in fact responding to exogenous (parasite-derived) or endogenous (e.g. complement-derived) chemoattractive factors, it is possible that under such stimulation they migrated into the epidermis toward the parasite and entered the necrotic tissue debris layer surrounding the parasite, before being lysed. Leucocyte polarisation and migration has been demonstrated in vitro in fish leucocytes to exogenous (Griffen, 1984; MacArthur et al., 1984; Nash et al., 1986; Weeks et al., 1986) and endogenous (St. J. Howell, 1987; Suzuki, 1986a) chemoattractants, and in mammals leucocyte migration in response to complement activation has been implicated as an important step in the initiation of cellular immune responses (McLaren, 1982). That parasite-derived factors can stimulate leucocyte migration in fish was demonstrated by Wood &

Matthews (1987) in mullet (Chelon labrosus) in response to a sonicated preparation of the digenean Cryptocotyle lingua. However, the significance of leucocyte infiltration to host protection here is uncertain, and it is probable that release of granule contents from migrating granulocytes merely exacerbates localised tissue necrosis.

In the cellular response in immunised fish, the predominance of type III cells ("basophils") and mast cells is of particular relevance to the interpretation of immunological events. Basophils are known to play a major role in the immune-mediated rejection of ectoparasitic arthropods in mammals (Askenase, 1980; Brown & Askenase, 1983), whilst in fish mast cell types have been shown to infiltrate the epidermis in response to ectoparasitic crustacea (Rose, 1986; Sharrif & Roberts, 1989). The infiltration of these cells could be in response to localised antibody/antigen complex deposition, as in the response of mammals to skin parasites (Wakelin, 1984), and may play a role in modulating the immune response. In mammalian studies, several biologically active components have been characterised from the amine containing cells (Playfair, 1982; Roitt, 1988). Of relevance to observations here would be the vasoactive substances, which following

release from specific granules could serve to increase vascular permeability in the dermal tissues underlying a site of I.multifiliis infection. This would lead to a greater emigration of parasite-specific antibody (pathotopic transfer) and actively migrating leucocytes from the dermal tissues, which would be of particular relevance in the modulation of immune responses in non-vascularised tissues. The cellular responses reported in immune fish here may therefore be more important in modulating the host's immune responses than being effectors.

There was no evidence here of specific cellular degranulation of the type described for the amine containing cells of mammals (Askenase, 1977; Askenase et al., 1978; Lagunoff, 1973) or such putative cells in fish (Vallejo & Ellis, 1989). Degranulated cells have been recorded in response to parasitic infections in fish (Hawkins et al., 1981; Sharp et al., 1989), although whether this was an active or passive process was not stated by these authors. However, that type III "basophil" granules were observed to coalesce in aggregations only in the skin of immunised fish suggests that this phenomenon may be immune-mediated. Since the mode of basophil degranulation in fish is unknown, the

suggestion here is that the process may involve granule fusion prior to cell lysis, in a way more akin to the degranulation of eosinophils in mammals (McLaren, 1982).

That active phagocytosis by several cell types was recorded in the vicinity of developing trophozoites only in immunised fish suggests that this response may be immune related. It is here considered that phagocytosis was probably in response to localised antigen/antibody complex formation. Phagocytosis of antibody complexed particulate material (bacteria) has previously been demonstrated in fish (Griffen, 1983; Honda et al., 1985 & 1986; Suzuki, 1986b), whilst phagocytic uptake of parasite material has previously been shown in several fish host/parasite systems (e.g. Hoole & Arme, 1982; Matthews & Matthews, 1980; Ralphs & Matthews, 1986; Sypek & Burreson, 1986; Taylor & Hoole, 1989; Woo, 1979). Jones & Woo suggested that antibody-mediated phagocytosis of Cryptobia salmositica may be the major protective response during chronic infections of this parasite in trout. Phagocytosis was recorded here by macrophages, neutrophils and resident filament cells. The former two cell types are well known to display phagocytic activity in fish (Avtalion & Shahrabani, 1975; Braun-Nesje et al., 1982; Finco-Kent & Thune, 1987;

Honda et al., 1986; MacArthur & Fletcher, 1985; McKinney et al., 1977; Siwicki & Studnicka, 1987a & 1987b). Filament cells have also been shown to be capable of phagocytosis (Peleteiro & Richards, 1990; Phromsuthirak, 1977), and may have other immunological functions, since Sigel et al. (1986) detected an IL-1 type substance released by a carp epidermal cell line. As formerly mentioned, recent research suggests that the integument in fish is of less importance as a site of external antigen uptake than the gills, although Peleteiro & Richards (1989) have suggested that actively phagocytosing macrophages in the epidermis of rainbow trout may be involved in localised antigen uptake in a role analogous to the Langerhan's cells of mammalian skin (Bos & Kapsenberg, 1986). With respect to the present study, the intensive phagocytosis observed at sites of premature parasite exit from immunised fish may serve to provide primed fish with fresh antigenic stimulation under conditions of continual exposure. Hines & Spira (1974c) reported that adult carp immune to I.multifiliis retained protection for up to 8 months under such conditions, whilst Houghton (1987) demonstrated that a large proportion of experimentally immunised 'O' group carp had lost protection after 3 months when maintained free from exposure.

During both the primary infection and the development of I.multifiliis in immunised fish, infiltrating leucocytes were observed in close proximity to the parasite surface, although there was no evidence of direct in situ cell adherence. Graves et al. (1985) demonstrated direct in vitro adherence of channel catfish natural cytotoxic cells (NCC) to Tetrahymena pyriformis and (by depletion/binding studies) I.multifiliis. Although cells with NCC-like activity have been recorded in carp (Hinuma et al. 1980), this cell type was not observed at infection sites of I.multifiliis in the present study. Its role in the in vivo cellular response to I.multifiliis therefore seems unlikely. It is the conclusion of the present study that cellular immune reactions play no direct role in the protective response to I.multifiliis in carp, although specific cellular reactions are recorded in immunised fish which may play a role in modulating the immune response.

One particular feature of the cellular response in immunised fish was the concentrated focal infiltration of leucocytes into the epidermis following premature parasite exit. This response was probably initiated by in situ antigen/antibody complex deposition, and that this infiltration increased up to 5 days following parasite exit suggests that the immune complexes were

persistent in the tissue. Cellular infiltration in response to persistent antigenic material in tissues typically leads to the formation of a granulomatous type reaction (Smithers & Doenhoff, 1982; Warren, 1982), and similar reactions have been recorded in fish in response to various persistent protozoan infections (Bartholomew et al 1989; Copland, 1983; Dykova & Lom, 1978b & 1980; Kent & Hedrick, 1985; Halliday, 1976; Matthews & Matthews, 1984; Reimschuessel et al., 1987; Roberts & Elson, 1974; Taylor & Haber, 1974; Stehr & Whitaker, 1986). However, there was no evidence here of the formation of granuloma tissue, and although the sites of premature parasite exit were not examined microscopically beyond 12 days following initial exposure, macroscopically these regions appeared normal. Since the epidermis regenerates rapidly from the basal layers (Bullock et al., 1978; Pickering & Richards, 1980), it is concluded here that a granulomatous-type reaction would not develop in the epidermis.

In the present study isolated cells from the pronephros of immunised carp showed greater in vitro phagocytic activity than cells from fish naive to I.multifiliis. The more intense phagocytosis observed in situ in the epidermis of immunised carp may, therefore, be afforded

by enhanced cellular activity in these fish. Since the predominant phagocytes recorded in the intense in situ response to premature parasite exit were macrophages, the possibility exists that enhanced in vitro phagocytosis was due to macrophage activation. That fish macrophages can display activation has been shown by Chung & Secombes (1987), and this is characterised by enhanced phagocytic ability due to increased oxidative metabolism and prevalence of membrane complement receptors. Oleinic & Ieshko (1987) reported enhanced phagocytosis and higher oxidative enzyme activity in fish following primary infection with I.multifiliis, although their study did not extend to immune fish. Whilst cellular responses do not appear to be directly involved in protection against I.multifiliis, the in vitro demonstration here of enhanced phagocytic ability of pronephric cells from immunised fish may provide a means of improved antigen uptake in these fish to continually stimulate the immune system in an infective environment.

In CL assays conducted here, no differential cell counts were made of the pronephric cell suspensions, hence the enhanced phagocytic response could theoretically be due to quantitative differences in the myeloid cell populations. Changes in the relative leucocyte

populations in peripheral blood is a well documented phenomenon of I.multifiliis infection (Hines & Spira, 1973c; McLay, 1985; Trombitskii, 1984), although quantitative changes in leucocytes from the peripheral blood or lymphomyeloid organs of immunised fish have yet to be investigated.

The weak in vitro CL responses of carp pronephric cells to I.multifiliis material is surprising, in view of the intense phagocytic activity observed in immunised fish in situ. These results may be associated with a number of problems associated with the CL assay, such as absorption of emitted light by the opaque nature of sonicate/homogenate material mixed with serum. However, since no attempt was made to separate the antigen(s) which initiate phagocytosis in vivo, the possibility cannot be ruled out that the crude homogenate/sonicate preparation used here contained parasite-derived factors which actively inhibited cellular function, such as phagocytosis. The interference of parasite-derived factors in cellular effector mechanisms has been shown in several mammalian host/parasite systems (Cohen, 1982; Wakelin, 1984). In fish, Lauden et al. (1986 & 1987) considered that the microsporidian Glugea stephani induced host macrophages to produce lymphocyte-

suppressive prostaglandins, whilst Dykova & Lom (1980) considered that infection with this parasite suppressed inflammatory responses. Alternatively, it is possible that the antigen(s) which promote in situ phagocytosis were in a form inappropriate to promote either antibody binding or cellular recognition when presented to the phagocytes in vitro. The form in which antigen is presented to the immune system in fish has significant bearing on the effectiveness of the host response (Ingram & Alexander, 1976; Lamers, 1985; Pyle & Dawe, 1985; Rombout et al., 1989c; Secombes & Manning, 1980). However, it was not possible to conclude from the present study whether the antigen(s) which promote phagocytosis are the same as those which have previously been shown to be targets of the humoral response (i.e. ciliary membrane targets and mucocyst material).

On the basis of the results and conclusions presented here, a model is proposed to summarise the immune response of carp to I.multifiliis, as depicted in figure 51. The immune response model comprises two components, namely the protective response involving premature parasite exit, and the response to parasites which are able to successfully complete development. Protective immunity appears to be afforded by an in situ antibody response against secreted mucocyst material and also

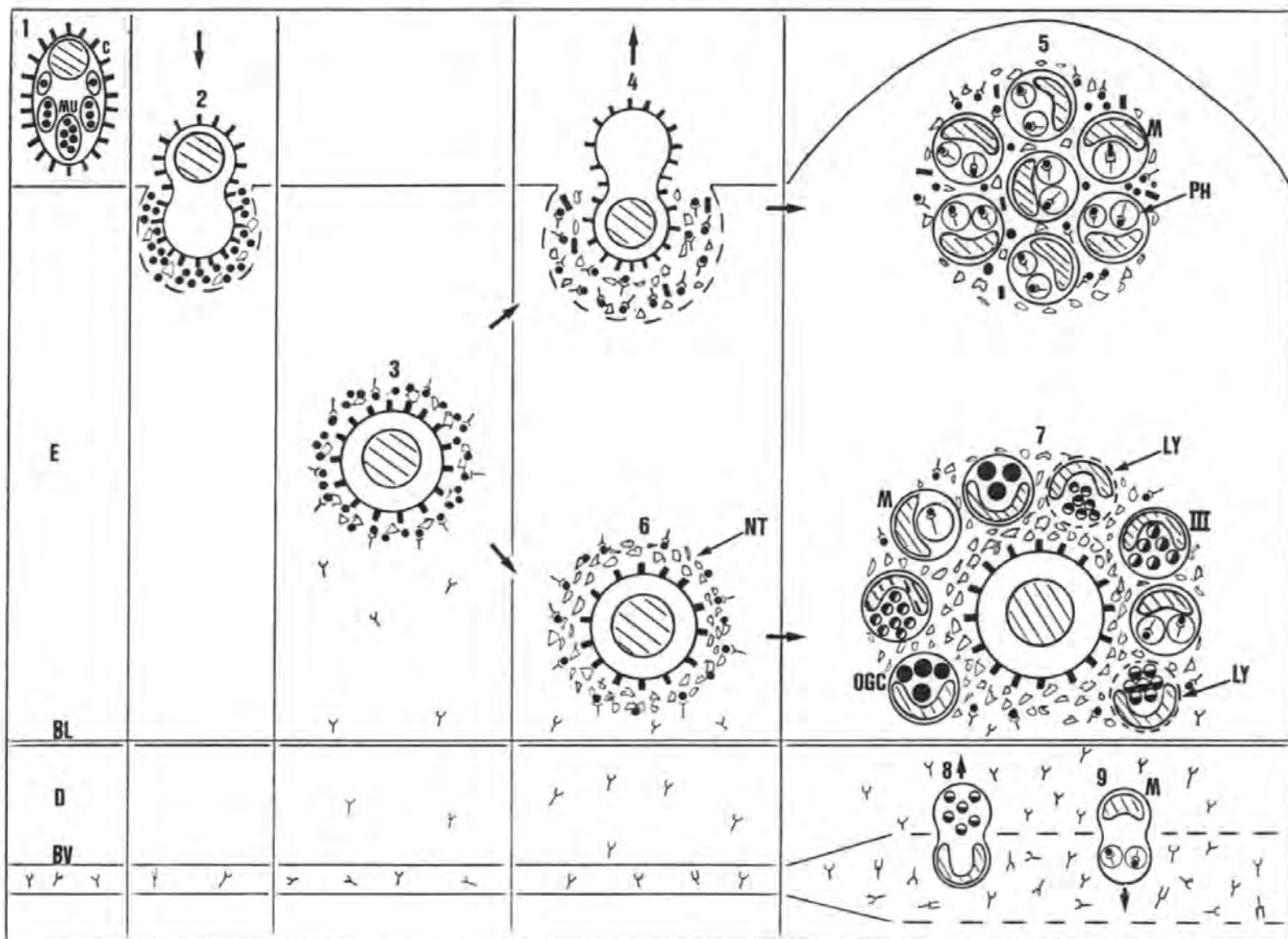
Figure 51. Diagrammatic summary of the proposed model for protection in 'O' group carp against I.multifiliis.

Stages.

1. Theront contacts epithelium of an immune fish.
2. Theront penetrates and secretes mucocyst material.
3. Antibody binds to secreted mucocyst material around the parasite, but cannot reach the ciliary membrane targets due to the formation of a protective mucocyst-derived "sheath".
4. In the majority of cases, antibody binding is detected at a sub-lethal level, leading to premature parasite exit. Antigen/antibody complexes are deposited.
5. Sites of premature parasite exit are infiltrated by actively phagocytic cells, primarily in response to localised antigen/antibody complex deposition.
6. A few parasites are able to induce tissue necrosis swiftly enough to prevent antibody activity at the immediate host/parasite interface, and are able to develop.
7. Parasites surviving to maturity form an impenetrable barrier of necrotic tissue debris which prevents antibody activity at the parasite surface. Released antigens initiate leucocytic infiltration. Mast cells and type III granulocytes (basophils) are lysed to release factors which increase vascular permeability and augment further infiltration and pathologic transfer of antibody (8).
9. Macrophages, possibly in an activated state, phagocytose antigen/antibody complexes, which are then presented to the systemic immune system to further stimulate the immune response.

Key.

- BL = Basal lamina
- BV = Blood vessel
- C = Cilia
- D = Dermis
- E = Epidermis
- III = Type III granulocyte
- LY = Lysis of cells and release of granular contents
- M = Macrophages
- MU = Mucocysts
- NT = Necrotic tissue
- OGC = Osmiophilic granular cell or mast cell
- PH = Phagocytosis of antigen/antibody complexes.
- / = Mucocyst-associated antigens and specific antibody
- / / = Cilia membrane-associated antigens and specific antibody



(possibly) against ciliary membrane targets, which is detected by the parasite at a sub-lethal level soon after penetration, leading to premature exit as a survival strategy. The vacated site of premature parasite exit is infiltrated by phagocytes, including activated macrophages; this may provide an improved means of antigen uptake in primed fish which serves to maintain immunity in a continually infective environment. A few parasites are able to survive to maturity in immune fish by virtue of the rapid formation of a surrounding layer of necrotic tissue which prevents antibody access to the parasite. The formation of diffuse antigen/antibody complexes initiates localised phagocytosis and the infiltration of type III granulocytes ("basophils") and mast cells, released products of which augment further humoral and cellular access to the epidermis. However, the parasite is able to resist host attack and complete normal development.

PROBLEMS IN TECHNIQUE DEVELOPMENT

Research undertaken in the present study required the development of several immunological techniques. Whilst all of the techniques employed here are used routinely in mammalian immunoparasitological research, the adaptation of some of these techniques to the study of fish has proved problematical. It is considered pertinent here to discuss problems associated with two of these techniques, namely raising a rabbit anti-carp IgM antiserum and developing an ELISA to detect anti-parasite antibody activity in serum and cutaneous mucus.

There are currently few commercially available monoclonal antisera for the detection of fish immunoglobulin components. For this reason, most immunological research work has used "home produced" monoclonal or polyclonal antisera for the detection of anti-parasite humoral responses (Bortz et al. 1984; Burresson & Frizzell, 1986; Sharp et al., 1989; Whyte et al., 1987). The rabbit anti-carp IgM serum produced here proved an adequate tool for the detection of antigen-bound and in situ carp IgM. However, a modification of the technique suggested by Ellis (1980) was required to purify carp immunoglobulin before inoculation into the rabbit, since coating SRBC with

immunoglobulin from whole carp serum led to rabbits recognising extraneous fish proteins in their response. This was probably due to non-specific haemabsorbing proteins from carp serum binding to the SRBC membrane. Diconza (1970) and Springer & Desai (1970) have demonstrated the presence of natural haemagglutinins in fish serum, and whilst no agglutination of SRBC was observed in serum from non-immune fish here, it is possible that such proteins could attach to SRBC in sub-agglutinating quantities and initiate a response when injected into rabbits. The rabbit anti-carp IgM serum raised against serum IgM here proved cross-reactive with cutaneous mucus immunoglobulin, confirming the results of Peleteiro & Richards (1985 & 1988) who were able to detect in situ immunoglobulin in the epidermis of rainbow trout Oncorhynchus mykiss using polyclonal rabbit anti-trout IgM serum. Whilst the origin and molecular structure of cutaneous mucus IgM in fish is uncertain (Lobb, 1986), the present study has clearly demonstrated that the molecule shares at least some common epitopes with serum immunoglobulin in carp.

Despite the successful development of immunogold labelling techniques for in situ immunoglobulin and parasite antigen detection here, the ELISA proved

unreliable as a means of detecting anti-parasite antibody, and in test assays significant specific antibody activity could not be demonstrated even when using a known positive serum sample. It was not clear which stage(s) of the ELISA procedure induced the observed high non-specific background reactions: Partial purification of rabbit antiserum by salt precipitation or the inclusion of BSA blocking steps did not alleviate the problem. However, the efficacy of the theront sonicate as an antigen source was clearly demonstrated, by the ability of sonicate-coated ELISA plates to deplete immune carp serum samples of specific anti-parasite activity, prior to use of the absorbed serum as a control in immunogold labelling studies. Although ELISA has previously been used to detect anti-I.multifiliis activity in the serum of immunised rabbits (Pyle, 1983) and mice (Dickerson, 1986), these reports used whole intact theronts as the target to detect only the humoral response to ciliary membrane antigens. With the demonstration here that mucocyst material is an important target of the humoral response, the need exists for the development of antigen detection techniques based on solubilised whole parasite material. Although an enzyme-linked immunoblot assay has been developed to detect antibody responses to

solubilised I.multifiliis ciliary membrane antigens in fish (Clark et al., 1988), this technique has not yet been used to examine the response to somatic antigens.

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APPENDICES

Appendix 1. List of chemotherapies applied to fish from disease-prevalent sources.

A. Treatment of external protozoa and monogenetic trematodes

Fish were treated in well aerated water containing 0.1 ppm malachite green and 1 ppm formalin for 1 hour at room temperature.

B. Treatment of external monogenea and crustacea

Fish were treated in well aerated water containing 1 ppm Dipterex for 1 hour at room temperature.

C. Treatment of systemic bacterial infections

Fish were treated in well aerated water containing 1 ppm Oxolinic acid (Sigma) for 2 - 3 days. Fish were subsequently fed for 1 - 2 weeks on commercial pellets coated with oxolinic acid at approximately 10 mg per Kg of food.

Appendix 2. List of buffers and reagents used in experimental procedures.

A. Buffers used in anion exchange purification of carp IgM

Buffer A: 50 mM Tris HCl containing 0.1 M sodium bicarbonate.

Buffer B: 0.5 M sodium acetate in buffer A.

Buffer gradient profile: Time (minutes) % buffer B

0	0
5	20
10	20
13	45
18	45
18	55
20	55
20	68
23	68
23	85
25	100
31	100

B. Buffers and reagents used in ELISA

Coating buffer: 50 mM carbonate pH 9.6

Incubation buffer: PBS pH 7.6 containing 500 ppm Tween 20.

Peroxidase substrate: Citric acid/sodium phosphate buffer containing 0.1% methanol and 10 ppm orthophenylene diamine.

Wash buffer: 0.85% saline containing 500 ppm Tween 20.

C. Luminol solutions used in CL assays.

Stock luminol solution: 0.78 g KOH, 0.618 g H_2BO_3 , and 100 mg luminol. in 10 ml distilled water.

Working strength luminol: 100 μl stock in 10 ml Hank's balanced salt solution.