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HOUGHTON, GILLIAN

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THE IMMUNE RESPONSE IN CARP, *CYPRINUS CARPIO* L. TO *ICHTHYOPHTHIRIUS MULTIFILIIS*, FOUQUET 1876.

by

GILLIAN HOUGHTON B.Sc., M.Sc.

B.Sc. (Hons.) University of Southampton,

M.Sc. Plymouth Polytechnic

Thesis submitted to the Council for National Academic Awards

part fulfilment of the requirements for the degree of Doctor of Philosophy

Department of Biological Sciences, Plymouth Polytechnic

May 1987

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This is to certify that the work submitted here was carried out by the candidate herself. Due acknowledgment has been given to any assistance received.

Signed ....G. Houghton.....

Candidate

Date ....12th May 1987.....

Signed .....

Supervisor of Studies

Date .....12th May, 1987.....

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ABSTRACT

Gillian Houghton. The immune response in carp Cyprinus carpio L. to Ichthyophthirius multifiliis, Fouquet, 1876.

Protective immunity of carp to ichthyophthiriasis has been confirmed, and demonstrated for the first time in juvenile carp, 10-12 weeks old. Standard immunisation procedures were developed here using the theront in preference to cysts. Immunisation included exposure of fish on 3 separate occasions of 14 day intervals to doses of approximately 2,000 theronts per fish, 80/ cm^{3}. Procedures were controlled so that infections were not allowed to continue beyond the primary stage. pH (7.0-7.2) and temperature (20±2° C) were maintained throughout experimental periods. Four weeks after third immunising dose, fish were exposed to a potentially lethal challenge, approximately 8,000 theronts per fish, 320/cm^{3}. Following immunisation, fish showed total protection up to 1 month and decreasing protection up to 3 months during which period, mortalities were recorded on challenge. Humoral antibody was monitored at specific stages of experimental infections, peak response 6-8 weeks following exposure with detectable levels of antibody persisting for at least 12 weeks. Immunosuppression was demonstrated following intraperitoneal administration of synthetic corticosteroid Triamcinolone acetonide, doses of 200 µg, 100 µg and 10 µg g^{-1} body weight, and corticosteroid Hydrocortisone 21-hemisuccinate, doses of 100 µg and 10 µg g^{-1} body weight, given 14 days after challenge. Immunosuppression was not associated with any significant fall in antibody titre. Studies in cross immunity between Tetrahymena pyriformis (CCAP 1630/W Claff, 1939(w)) and Ichthyophthirius multifiliis showed no evidence of the former conferring protection to ichthyophthiriasis. Methods of administration of T. pyriformis to juvenile carp included intraperitoneal injection of freeze dried cilia and whole, live T. pyriformis. The kinetics of the humoral response were measured over 12 weeks, peak antibody titres occurring 6-8 weeks following antigen administration. Proliferative responses measured by autoradiography were recorded prior to peak antibody production. Overall results are discussed in relation to immunosuppression, mechanisms of immunity and control and treatment of the disease.

INTRODUCTION

Ichthyophthiriasis is a major communicable disease of fresh water fishes accounting for high mortalities and economic loss in aquaculture and is prevalent worldwide (Hoffman and Bauer, 1971, Paperna 1972, Nigrelli et al, 1976). Most fresh water fishes appear susceptible to the disease and treatment, using currently available drugs, has not provided an effective cure, particularly in that none can kill the parasite whilst in the host epidermis. The disease is a major problem in the culture of eel, rainbow trout, salmon, channel catfish, tilapia and carp and used to be considered a disease of extensive fish farming in tropical climates but is now a problem in intensive fish farming in temperate climates. In addition, the disease is also a problem in the aquarist industry, particularly in warm water fish species and valuable koi carp.

The causative agent is the ciliate Ichthyophthirius multifiliis Fouquet, 1876, in the Phylum Ciliophora; class Oligohymenophora; subclass Hymenostomata; order Hymenostomatida; suborder Ophryoglenina (Levine et al, 1980).

Due to the problems associated with treatment of the disease, there is a great need for further immunological studies as a basis for control and treatment. Information regarding acquired immunity in fish to I. multifiliis has been obtained from the work of Beckert and Allison (1964); Hines and Spira (1973 a, b, c; 1974 a, b, c), Beckert (1975), Wahli and Meier (1985) and Wahli (1985). There is a need though for a study of the immune response in relation to the dynamics of infection. Recent work has also involved investigations into a possible vaccine

against the parasite using the related ciliates Tetrahymena pyriformis and Tetrahymena thermophila (Goven et al, 1980 a, b; 1981 a, b; Wolf and Markiw, 1982; Dickerson et al, 1984). All studies in this area still suffer from a lack of immunological work on eukaryotic parasites which are only just receiving attention. There is also a shortage of information on the immune response of juvenile fish to parasites, and selection of this age group was considered relevant as fish are more prone to ichthyophthiriasis when young and thus would gain maximum benefit from protection at this stage.

The main objectives of the present study were to establish a specific "strain" of the parasite from which an experimental system could be set up using juvenile carp, to investigate further the dynamics of infection of the parasite on the host. This would give an increased understanding of the immune response in relation to control and treatment, particularly in the mechanisms of acquired immunity including the humoral response and duration of protection. A further aim was to determine whether the protective immune response could be suppressed following administration of corticosteroids.

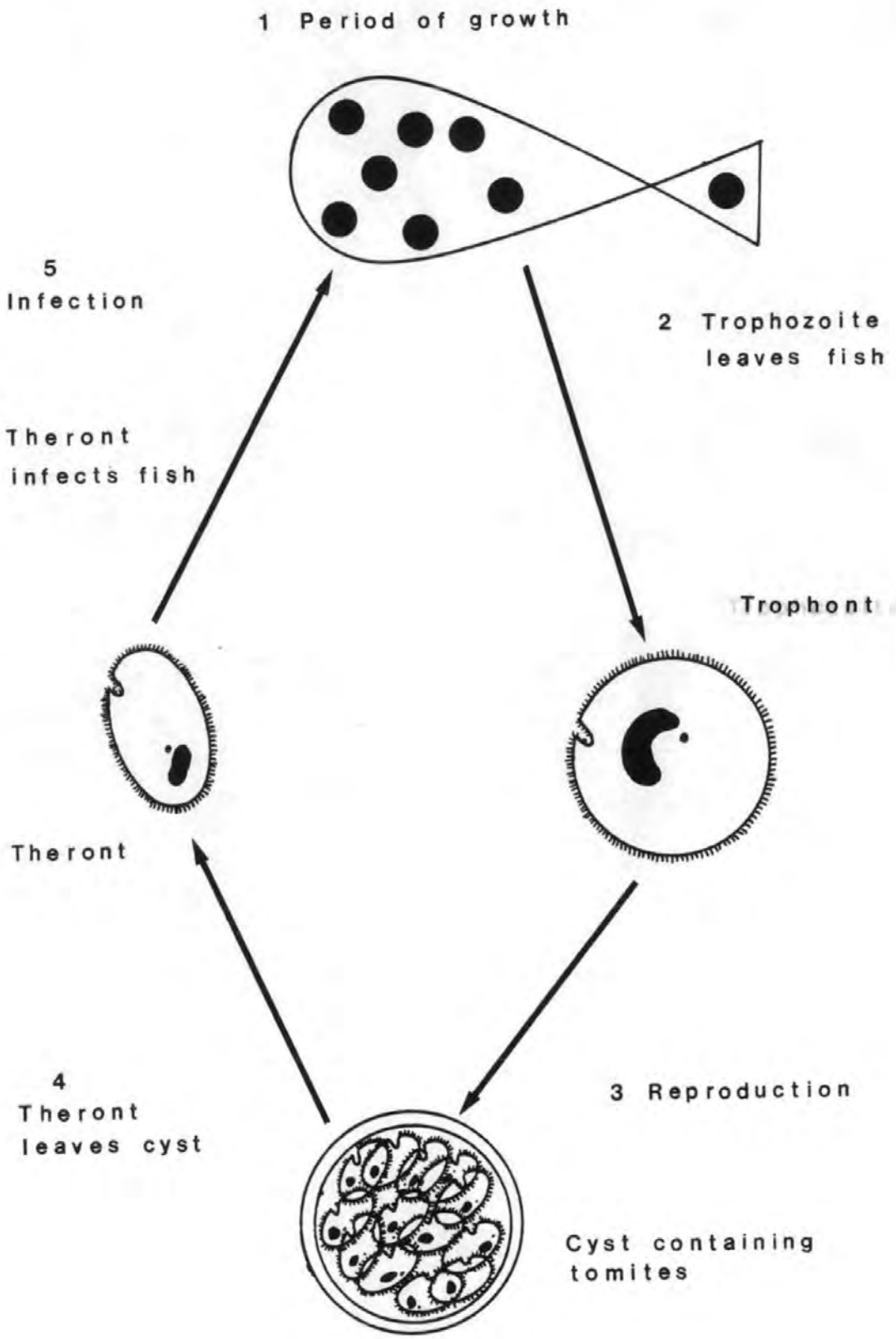
In order to understand the immune response in carp to eukaryotic organisms, particularly in view of the recent interest in vaccines against ichthyophthiriasis, further immunological studies were carried out using Tetrahymena pyriformis. These were also aimed at investigating whether juvenile carp could be protected against the disease.

The life cycle of I. multifiliis is shown in Fig. 1 and consists of a parasitic phase within the epithelium of the skin and gills. The parasite remains on the fish for a period of growth of a duration

dependent upon temperature, taking approximately 7 days at 20±2° C. The site of infection can be seen as 'white spots' (Fig. 2) from which the parasite leaves the fish to become a free swimming trophozoite (Fig. 3). The trophozoite secretes a gelatinious reproductive cyst which attaches to aquaria, weed, stones etc., within which, division takes place leading to the production of several hundred tomites (Fig. 4, A, B). The time of division is temperature dependent taking approximately 15-20 hours at 20° C. Following completion of division, the infective stage, the theront (Fig. 5, A, B) leaves the cyst to infect further susceptible fish. Theronts can survive for up to 3 days following excystment, but infectivity decreases rapidly after 15 hours (McCallum, 1982).

Fig. 1. Life cycle of Ichthyophthirius multifiliis.

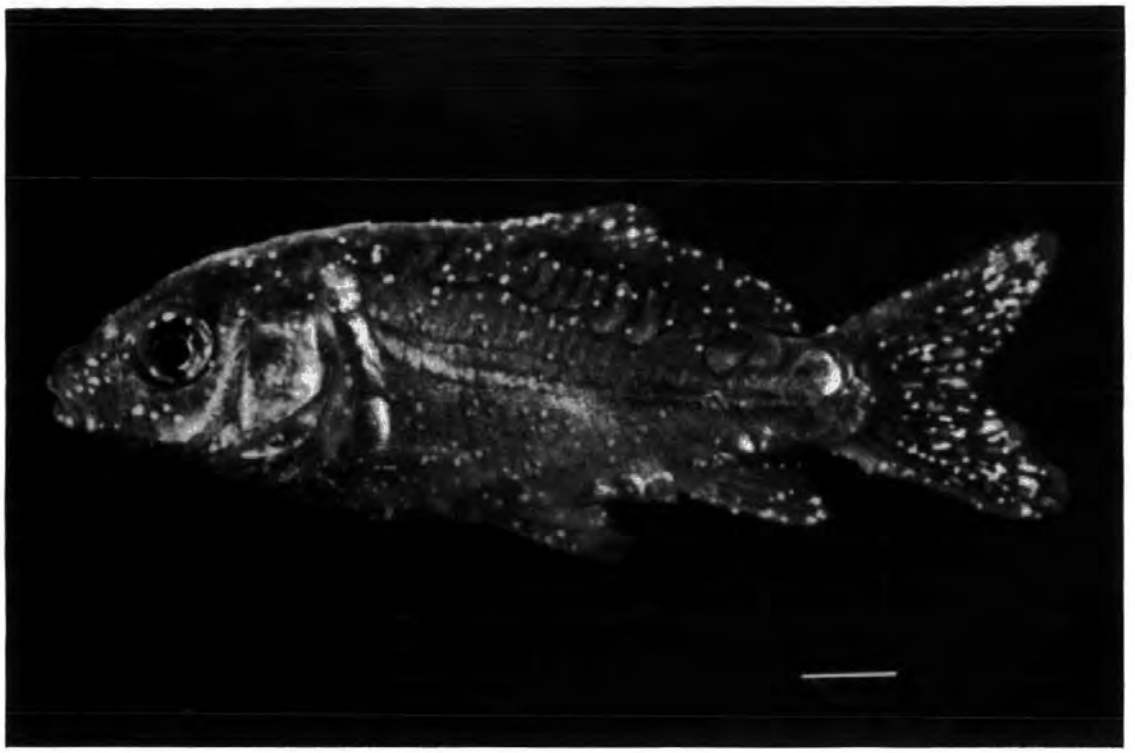
1. Period of growth within the fish epidermis; approximately 7 days at 20° C.
2. Free swimming trophont leaves fish host.
3. Trophont undergoes reproduction within a gelatinous cyst, taking approximately 15-20 hours at 20° C.
4. Theronts leave cyst. Can survive for up to 3 days but infectivity decreases after 15-20 hours.
5. Theront infects susceptible fish.


1 Period of growth
2 Trophozoite leaves fish
Trophont
3 Reproduction
Cyst containing tomites
4 Theront leaves cyst
Theront
5 Infection
Theront infects fish

Figs. 2 and 3 Life cycle of Ichthyophthirius multifiliis

Fig. 2 Five day infection of juvenile carp. Note white spots covering entire body and fins, each spot indicating the site of infection. Scale, 0.5 cm.

Fig. 3 Free swimming trophont. Scale, 40 μm.



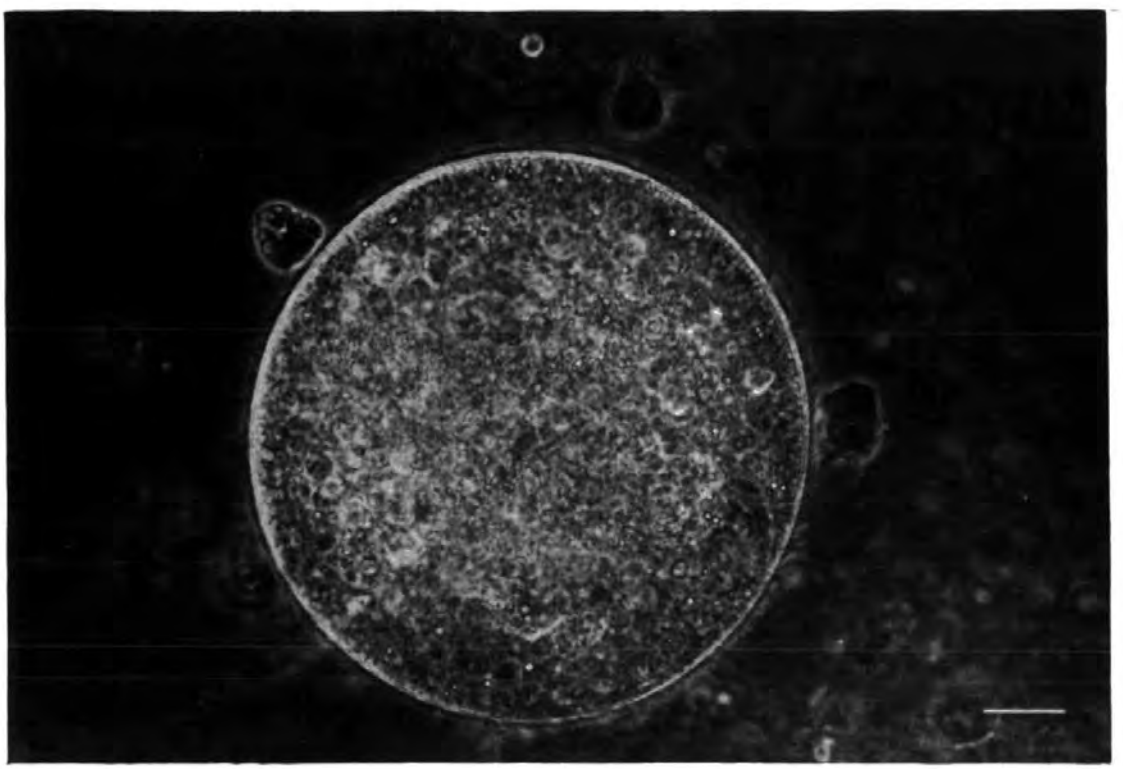


Fig. 4 Reproduction of Ichthyophthirius multifiliis at 20° C.

A. 6 hours. Several cysts are shown. A single cyst is arrowed. Note tomites (t). Scale, 100 µm.

B. 20 hours. Prior to release of theronts (th). Scale, 60 µm.

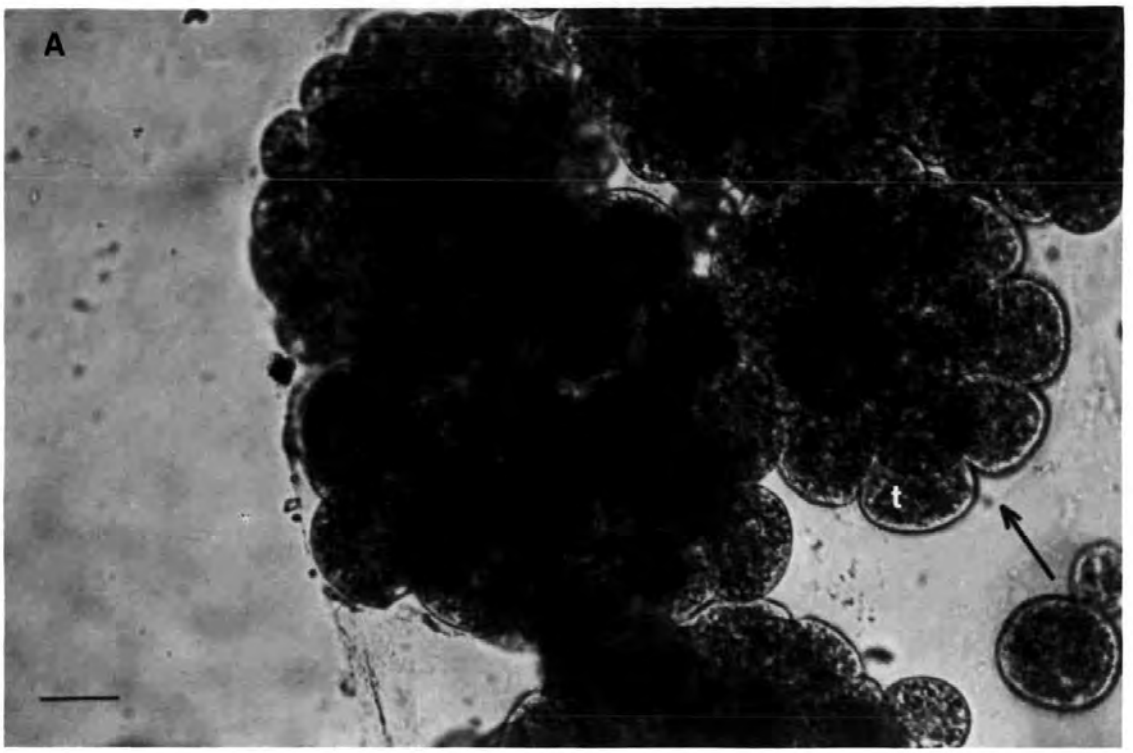

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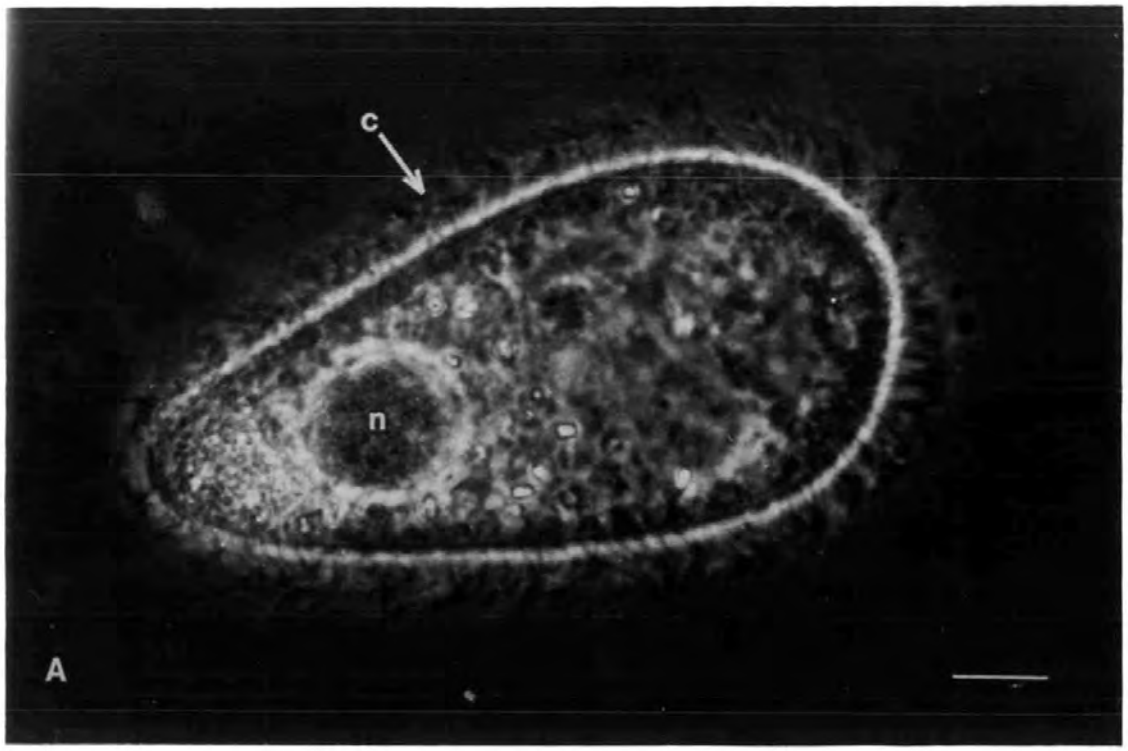
Fig. 5 Life cycle of Ichthyophthirius multifiliis.

A. Formalin fixed theront of I. multifiliis. Note dense ciliature (c) covering the organism. Nucleus (n). Scale, 5 µm.

B. Formalin fixed theront of I. multifiliis. Note organ of Lieberkuhn (ol), cilia (c) and nucleus (n). Scale, 5 µm.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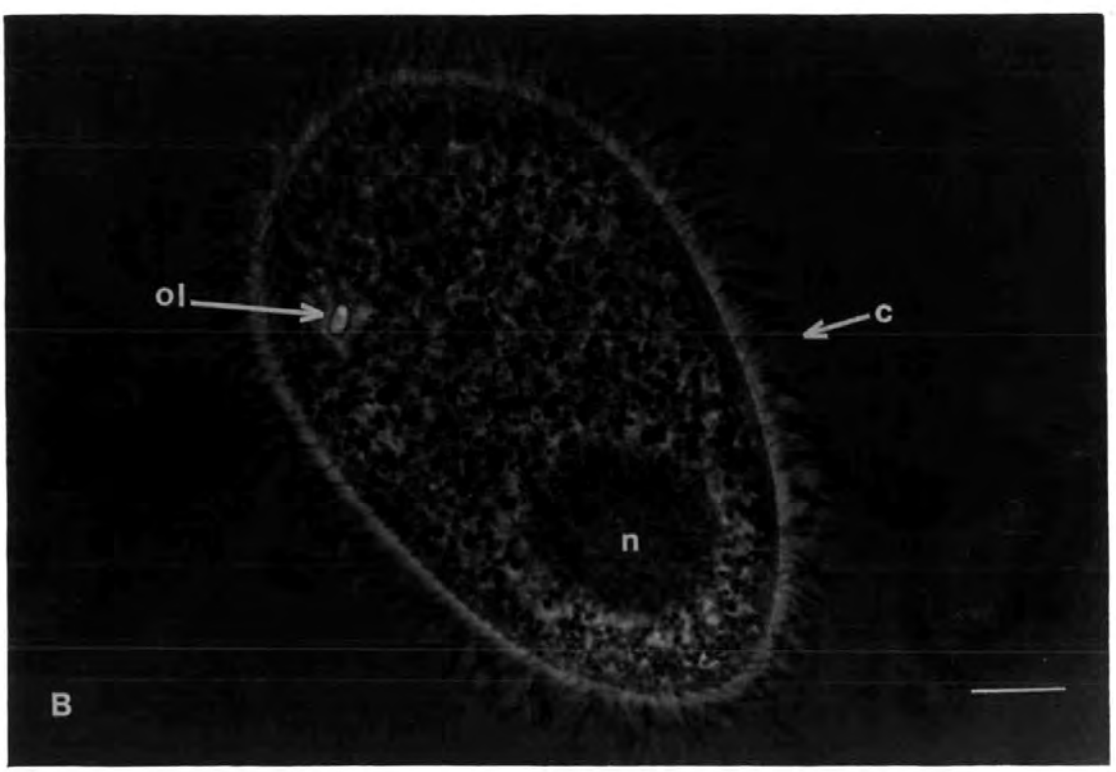
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REVIEW

BIOLOGY OF *ICHTHYOPHTHIRIUS MULTIFILIIS*

The first published observations of *I. multifiliis* were probably those of Hilgendorf and Paulicki (1869). They noticed that it was covered in cilia, possessed a nucleus, contractile vacuole and granules, and that encystment and division took place off the fish. Further, they thought it was related to *Pantotricha*, Ehrenberg. Fouquet (1876) later identified the same parasite on trout and considered the parasite to be representative of a new genus, Heterotrichia, and proposed the name *Ichthyophthirius multifiliis*. He noted additional features including trichocysts, a cytopharynx which he assumed to act as a sucker, and also made observations on the rate of cell division with time. Information on other features of the life cycle were added by Saville-Kent (1880-1882, cited in Stiles, 1893) but there was early disagreement on the status of *Ichthyophthirius multifiliis* designated by Fouquet (1876). Later workers proposed different names for the same parasite from different hosts: *Chromatophagus parasiticus* (Kerbert, 1884, 1885, 1886), *Ichthyophthirius cryptostomus* (Zacharias, 1892 a, b). However, the work of Butschli (1887) and Stiles (1893) reconfirmed that these were synonomous with *I. multifiliis.* Structural aspects of the mouth and function, were described by Stiles (1893). He also noted division of the trophozoite off the fish and that the resulting daughters (theronts) served as the infective stage. Studies on the life cycle have been made notably by Hofer (1904); Neresheimer (1908 a, b); Roth (1908); Bushkiel (1910, 1911, 1936); Plehn (1924); Prytherch (1924). Pearson (1932, 1933) described, in more detail, the life cycle and, in particular, the micronuclear cycle

stating that the 'spores' (theronts) contain a macronucleus and a single micronucleus, although some 'spores' were seen to contain an additional small micronucleur body, the function of which though, was not understood. Once in the fish tissue, the micronucleus was seen to enter the macronucleus.Only after this has happened, were trophozoites observed to undergo successful encystment and division which Pearson (1933) suggested indicated a relationship between the micronucleus and macronucleus, necessary to initiate the process of cell division. More recent studies on the life cycle and division have been made by Haas (1933); Suzuki (1935); Bushkiel (1936); MacLennan (1934, 1935a,b, 1936, 1937, 1939a,b, 1942, 1943); Butcher (1941, 1943, 1947); Mallié (1946); Mugard (1948); Schäperclaus (1954); Dashu and Lien-Siang (1960); Wagner (1960); Roque et al (1966); Hoffman (1978); Lom and Cerkasovova (1974). MacLennan (1935a), in a cytological study, states that nucleur reorganization takes place during division confirming Pearson (1933) and Haas (1933). Work on the neuromotor system has been carried out by Haas (1933) and MacLennan (1934, 1935b, 1936, 1939b, 1943), the latter author stating that there is a de-differentiation stage followed by re-differentiation of the organization of the neuromotor system, during division, in particular in conjunction with the needs of the theront. The mouth area loses its function during division, but, once the theront has entered a fish, the mouth area becomes functional for the feeding stage of the parasite. Both Haas (1933) and MacLennan (1935b) noted a 'knob' devoid of cilia at the anterior end of the theront which Haas (1933) interprets as a perforatorium. This has also been observed by Dashu and Lien-Siang (1960) who also noted a long "caudal bristle", three times longer than the rest of the cilia

A detailed study of the origin and function of the cytoplasmic granules

within the trophozoite and theront have been made by MacLennan (1936, 1939 b). He noted that the number and function altered during the different stages of the life cycle. MacLennan (1937) has also made a fairly detailed description of the formation of the cyst wall, and noted two concentric layers - the ectocyst and the endocyst. The efficiency of encystment was seen to increase as the length of the growth of the trophozoite on the fish increased, along with the ability to encyst being related to the size of the parasite, 100 µm being the minimum size (MacLennan, 1937, 1942). A detailed study and review of various aspects of the biology and structure of I. multifiliis at the light microscopical level has been made by Canella and Rocchi-Canella (1976).

The mid-1960's saw the application of electron microscopical studies on the structure of I. multifiliis. The first examinations were on the contractile vacuole by Mosevitch (1965) and later by Chapman and Kern (1983). Both made similar observations on the system of microtubules adjacent to the contractile vacuole, and noted the discharge canal connecting the contractile vacuole to a pore leading to the external medium. There also appeared to be a number of cristae mitochondria, ribosomes and vesicles in the cytoplasm around the contractile vacuole. The mitochondria have been examined by Mosevitch and Mashansky (1965). The cytological changes within the micronucleus and macronucleus during mitosis at the time of division have been examined by Hauser (1972) and Hauser and van Eys (1973) who noted that the highly electron dense chromosomes of I. multifiliis were very irregularly dispersed within the whole nuclear space. Roque et al (1966) and Canella and Rocchi-Canella (1976) have carried out detailed studies on the ciliature and further examinations of the pellicle have been made by Crilley and

Buckelew (1977) and Chapman and Kern (1983). Chapman and Kern (1983) observed that the membrane system is composed of three unit membranes of which the outer one is continuous with the ciliary membrane. Within the cytoplasm, are numerous electron dense, paracrystalline mucocysts, which have also been observed by Canella and Rocchi-Canella (1976); Ewing et al (1983, 1985); Matthews and Matthews (1984). Secretions from the mucocysts are thought to produce the cyst wall (Ewing et al, 1983) and aid in penetration of the theront into the epidermis of the fish (Matthews and Matthews, 1984; Ewing et al, 1985).

McCallum (1982) has made a study of the dynamics of infection of I. multifiliis, looking in detail at infectivity of the theront, with time from emergence from the reproductive cyst, using mathematical models to analyze results. Further work by McCallum (1985), again using models, examined the regulation of host populations by parasite infections.

Biochemical studies have been made by Uspenskaya (1963), who demonstrated hyaluronidase activity in the trophont immediately after removal from the fish and suggested that it aided in feeding and movement within the epidermis. No hyaluronidase activity was found early in encystment but it was again detected in the ciliospores (theronts) upon leaving the cyst. Further studies were made by Uspenskaya (1966) who examined quantitative changes of DNA and RNA within the macronucleus of theronts and trophozoites. Pyle and Dawe (1985 a) examined the protein patterns of different stages of I. multifiliis and found two distinctly different protein profiles in the theront and in the trophozoite.

GEOGRAPHICAL DISTRIBUTION OF I. MULTIFILIIS

Ichthyophthirius multifiliis has a worldwide distribution within tropical, temperate and sub-arctic regions (Hoffman and Bauer, 1971), for example Australia (Roughley, 1932; Butcher, 1941, 1947; Ashburner, 1976), China (Dashu and Lien-Siang, 1960), Czechoslovakia (Migala 1971 ; Willomitzer, 1980), British Isles (Chappell and Owen, 1969; Chubb, 1970; Kennedy, 1974; Wootten and Smith, 1980), Finland (Calenius, 1980; Valtonen and Keränen, 1981), France (Fouquet, 1876), Greece (Pneumaticatos, 1977; Photis, 1978), India (Tripathi, 1955), Iraq (Khalifa et al, 1983), Israel (Lahav and Sarig, 1973; Hines and Spira, 1973 a), Poland (Antychowicz, 1977), South Africa (Van As et al, 1984), Spain (Pozo and Roman, 1969), Switzerland (Wahli and Meier, 1985; Wahli, 1985), Uganda (Paperna, 1972), U.S.A. (Stiles, 1983; Wolf, 1938; Elser, 1955; Allison and Kelly, 1963; Meyer 1966; Hoffman, 1976; Kozel, 1976; Baker and Crites, 1976; Goven et al, 1980 a) and the USSR (Bauer, 1958).

Most freshwater fish species appear to serve as hosts and serious epizootics have occurred, for example, in barbel (Barbus barbus bacagei) (Pozo and Roman, 1969), carp (Cyprinus carpio) (Lahav and Sarig, 1973; Photis, 1978; Khalifa et al, 1983), channel catfish (Ictalarus punctatus) (Meyer, 1968; Hoffman, 1967), salmon (Salmo salar) (Valtonen and Keränen, 1981), shad (Alosa sapidissima) (Allison and Kelly, 1963; Kozel, 1976), trout (Salmo trutta and S. gairdneri) (Wolf, 1938; Butcher, 1947; Pneumaticatos, 1977), Tilapia spp (Paperna, 1980) and yellow perch (Perca fluvescens) (Elser, 1955). Due to its worldwide distribution, this has led Nigrelli et al (1976) to remark on possible physiological races and species.

Uspenskaya (1963) states that the enzyme hyaluronidase, which was demonstrated to be present in theronts, aids in the entry of the parasite into the host. After entry, histological examination has shown that there is a cavity round the parasite, surrounded by several layers of spindle-shaped epithelial cells (Bauer, 1969; Lucký, 1970 a). Hines and Spira (1974 a), who have carried out an extensive study of the pathology of I. multifiliis in carp, noted that the primary pathology consisted of inflammatory, proliferative and degenerative changes in the epidermis and hyperaemia and oedema of the spongiose dermis. Proliferation also occurred in the gills. Pathological changes were also seen in the liver, kidneys and spleen. Following the pathological changes in the epidermis and gills, physiological reactions also occur, leading to a drop in serum level of sodium and magnesium and a rise in level of potassium (Hines and Spira, 1974 b). Ewing (1981) also noted that the epithelial cells round the parasite were undergoing division. Light microscopical and ultrastructural studies of the host/parasite relationship and pathological changes, have been carried out by Chapman (1984); Ewing et al (1985); McLay (1985); Ventura and Paperna, (1985). Ventura and Paperna (1985) found the greatest proliferation occurring in cyprinids, whereas McLay (1985) and Wahli (personal communication), working on rainbow trout, found no evidence of proliferation of the superficial epidermis. All these workers have noted damage to epidermal cells after a heavy infection and at no time did the parasite penetrate the basement membrane. Matthews and Matthews (1984) and Ewing et al (1985), both state that entry into the host epidermis is probably aided by secretion from the mucocysts.

The haematology of fish during an infection has been examined by Reshetnikova (1962), Hines and Spira (1973 b), Golovina (1976), Magerramova (1977), Ryzhova (1979) and McLay (1985), who also studied the clinical chemistry.

CONTROL AND TREATMENT OF I. MULTIFILIIS

To date, there is no treatment which will kill the parasite, whilst in the epidermis of the fish. There are several reviews on the subject by Meyer (1966, 1974), Herman (1970), Hoffman (1970), Cross (1972) and Hoffman and Meyer (1974). The first chemical used in treatment of fish infected with I. multifiliis was sodium chloride (Stiles, 1893; Wolf, 1938; Butcher, 1941, 1947) although with limited success. Allen and Avault (1970) did find though, that by transferring catfish (I. punctatus) infected with "ich" to brackish water, the disease could be controlled. Stiles (1893), also noted that methylene blue, at concentrations non-toxic to trout, was toxic to the parasite when it left the fish. Other workers have also found methylene blue to be toxic to the parasite at concentrations non-toxic to fish (Johnson, 1949; Ghadially, 1964; Allison, 1968; Cross, 1972; van Duijn, 1973). Allison (1957), found that a single application of 15 ppm formalin controlled "ich", providing that the parasitized fish are treated before they become weakened by the parasite, although this dose was toxic to some species of fish, for example Itiobus bubalus. Davis (1953) and Hoffman (1959) found a dosage of 250 ppm given daily for 1 hour treated the disease and Allison (1968) later reported that 15 ppm daily for 5 - 7 days gave successful treatment, with various doses and time schedules (Clemens and Sneed, 1958; Conrad and Wyatt, 1970; Herman, 1970 (review); Hoffman, 1970; Cross, 1972 (review); Roberts,

1978; Valtonen and Keränen, 1981; van As et al, 1984). All these workers stated that the doses used will kill the free swimming trophozoite or theront although Prost and Studnicka (1972) found negative results using formalin, at a dose of 200 ppm, 1 hour per day for 7 days, or a 25 ppm bath for 4 days. Antychowicz et al (1982) examined doses that were needed to destroy the encysted stage and found that a dose of 400 ppm formalin, 1 hour daily for 6 days was needed. This dose was harmless to juvenile fish but toxic to fry.

Another chemical used with varying success is malachite green. This has treated the disease at doses ranging from 0.05 ppm (for an indefinite time) to 1 ppm as a short bath (Meyer, 1966; Allison, 1968; Herman, 1970; Prost and Studnicka, 1972; van Duijn, 1973; Antychowicz, 1977 and Loyacano and Crane, 1977). van Duijn (1973) does state that it can be toxic to small aquarium fishes. Antychowicz (1977), using a dose of 0.031 ppm on trophozoites, found abnormal development and no infective theronts were produced. At a dose of 1 ppm, it was toxic to cysts but also to fry. In the USA, malachite green has not been approved for use in food fish due to possible carcinogenic properties (cited in Leteux and Meyer, 1972). As well as being a successful treatment on its own, malachite green has also been used in conjunction with other chemicals: malachite green and salt (Johnson, 1961) or with formalin (Leteux and Meyer, 1972; Cross, 1972 (review); Schachte, 1974; Pneumaticatos, 1977; Loyacano and Crane, 1977; Guest, 1983). When mixed with formalin, only a low dose of malachite green is needed, for example 0.05 ppm malachite green plus 15 ppm formalin (Leteux and Meyer, 1972).

Trials with other chemicals have also been carried out, although

commercially they have not been used due to cost, toxicity or ineffectiveness. These are: Mercurochrome (Roughley, 1932; Ghadially, 1964; van Duijn, 1973), Mepacrine hydrochloride (Slater, 1952; van Duijn, 1973), Pyridyl mercuric acetate (Clemens and Sneed, 1958), Quinine (Ghadially, 1964; van Duijn, 1973; Leibovitz, 1980), Acriflavine (Ghadially, 1964), Quicklime (Rychlicki, 1968) - this raised the pH of the water from 7.0/7.5 to 8.5/9.5, Copper sulphate (Hoffman, 1970; Antychowicz, 1977; Antychowicz et al, 1982), Neguvon (van Duijn, 1973), Chloramine T (Schäperclaus, 1954; van Duijn, 1973; Cross and Hursey, 1973), Nifurpirinol (Conroy et al, 1980) and silver nitrate, ronidizole and amprolium (Farley and Heckman, 1980). All these chemicals will only act on the free swimming stages of the parasite. Beckert (1975) found that the anti-oxidant, ethoxyquin, which is often included in fish food, when fed to fish, affected the development of the parasite when it left the host, and, recently, Wahli et al (1985) found an increased survival of fish infected with ich, after being fed a diet with ascorbic acid compared to those fish fed no ascorbic acid.

Other methods, apart from chemicals, have been tried, to prevent re-infection by the parasite, such as an increased water flow (MacLennan, 1935 a; Wolf, 1938; Allison, 1953; Conrad and Wyatt, 1970; Hoffman, 1970), increased temperature (Ghadially, 1964; Meyer, 1966, van Duijn, 1973) - only useful for tropical fish, transfer of fish from contaminated aquaria to clean aquaria (Hoffman, 1970; Subasinghe, 1982), electro therapy - which was unsuccessful (Farley and Heckman, 1980) and ultra-violet light (Gratzek et al, 1982). The possibility of developing a vaccine against ichthyophiriasis has been investigated by Goven et al, (1980 a, b; 1981 a), Wolf and Markiv (1982) and Dickerson et al, (1984) using species of Tetrahymena and more recently, Price and Bone

(1985) have examined genetic resistance in fish to the disease.

IMMUNE RESPONSE TO I. MULTIFILIIS

The first indication of protective immunity of fish to I. multifiliis was by Bushkiel (1910, 1936). Later, Butcher (1941) indicated that immunity might be responsible for a few fish remaining in infected ponds. Bauer (1953, 1956, 1959) also noted that fish recovering from infection with I. multifiliis possessed varying degrees of resistance to subsequent re-infection, and that the level of this immunity, was proportional to the intensity of the primary infection. Reichenbach-Klinke and Elkan (1962) noted that the immunity was short-lived, however, they gave no indication of duration of protection. More quantitative studies of the response have been undertaken by Beckert and Allison (1964); Parker (1965); Hines and Spira (1973 a, b, c; 1974 a, c); Areerat (1974); Subasinghe (1982), McCallum (1986) and in the present work. Beckert and Allison (1964), exposed white catfish to a single dose of 10 trophozoites per fish. Re-infection was prevented by treatment of fish with 0.1 ppm malachite green. Fifteen days after the initial infection, fish were challenged by exposure to varying numbers of trophozoites. A significant degree of protection occurred, but 100% mortality was found in controls. The possible significance of temperature on immunisation was investigated by Beckert and Allison (1964) and Lom (cited in Lom, 1969). Lom (cited in Lom, 1969), found carp infected at 26° C and which recovered from infection were refractory to a second infection whereas those kept at temperatures of 15° C and 20° C could be re-infected. Lahav and Sarig (1973) again indicate that immunity could occur when carp were infected and the infection allowed to continue, fish eventually becoming free of

parasites. Similar observations were made in salmon (Salmo salar) by Valtonen and Keränen (1981).

An extensive study of acquired immunity of carp to I. multifiliis has been made by Hines and Spira (1973 a, b, c; 1974 a, c). Using adult mirror carp (Cyprinus carpio), Hines and Spira (1973 c, 1974 c) were able to induce an immune response by exposing fish to 200 trophozoites per fish and allowing the infection to continue. Fish were then exposed to several different challenges, each resulted with no infection taking place, unlike in previously unexposed controls. Sera from the immune fish were found to immobilize the parasites at titres of 1:65 to 1:1024 whereas mucus/saline washings, immobilized parasites at titres of 1:8 to 1:16. Parasites were seen to occur chiefly on the periphery of fins in fish undergoing an immune response. Immunity was also conferred in carp using a dose of 40 trophozoites per fish (Hines and Spira, 1973 c). Areerat (1974) produced immunity in catfish by one exposure to quantified numbers of trophozoites and found serum immobilization titres of 1:320 (low dose) and 1:5120 (high dose). Protection has also been induced in catfish by intraperitoneal injection of ground trophozoites, with or without the addition of Freund's complete adjuvant, immobilization antibody also being produced (Areerat, 1974; Beckert, 1975). Subasinghe (1982) produced immunity in juvenile carp by exposure to quantified numbers of trophozoites on three separate occasions of 14 day intervals. A lethal challenge was given 28 days later, with all immunised fish surviving.

Wahli and Meyer (1985) investigated the effects of serum and mucus on trophozoites of I. multifiliis, from rainbow trout undergoing an infection of "ich" and from fish having recovered from an infection.

They found that, during a heavy infection, mucus immobilized parasites whereas serum has a low activity. One week after all symptoms had disappeared, a greater degree of immobilization occurred in serum with less in mucus. Seven months after infection, a high serum anti-parasite activity was observed but with a far lower anti-parasite activity in the mucus.

Changes in white blood cells during an infection of "ich" have been recorded by Reshetnikova (1962); Hines and Spira (1973 b); McLay (1985). Reshetnikova (1962) and Hines and Spira (1973 b) both noted an increase in neutrophils and monocytes early during a heavy infection, with a decrease in the number of lymphocytes, although the overall white blood cell count remained the same. McLay (1985) only found changes in lymphocytes and thrombocytes in the first 10 days of infection in rainbow trout, where the number decreased, but found no consistent changes in either the proportion or numbers of neutrophils or monocytes.

The numbers of leukocytes in the epithelial layer during an infection have been observed to increase (Hines and Spira, 1974 a; McLay, 1985; Ventura and Paperna, 1985). Both Hines and Spira (1974 a) and Ventura and Paperna (1985) noted that there was a moderate to heavy infiltration of neutrophils and to a lesser extent lymphocytes. In the gills, the number of neutrophils was also seen to increase (Hines and Spira, 1974 a; McLay, 1985; Ventura and Paperna, 1985) indicating a non specific inflammatory response. An increase in the number of lymphocytes in the spleen, with a decrease in numbers of neutrophils in the kidney of carp during an infection of "ich" was also observed by Hines and Spira (1974 a) but not by McLay (1985) in rainbow trout, who found

that the splenic pulp was greatly reduced, leaving splenic reticular fibres present.

Goven et al (1980 a) have investigated the possibility of developing a vaccine against ichthyophthiriasis. They found that channel catfish, injected with Tetrahymena pyriformis were protected against I. multifiliis. The protective antigen appeared to be of ciliary origin as the greatest protection was obtained from cilia preparations of I. pyriformis and I. multifiliis but not from deciliated cells. Further work (Goven et al, 1980 b, 1981 a), using different doses of I. pyriformis cilia, confirmed their earlier findings. Goven et al (1981 b) demonstrated that an antigenic cross reactivity existed between I. pyriformis and I. multifiliis by using in vitro serologic tests. An indirect immunofluorescent test against both organisms suggested the cross reactivity was localized in the cilia and pellicle. Wolf and Markiw (1982) using I. thermophila were able to induce protection in rainbow trout to Ichthyophthiriasis when administered as a bath immunization. The amount of protection obtained increased, the longer the period of immune induction. Dickerson et al (1984) suggested that there was a difference between different isolates of I. pyriformis in the amount of protection afforded by immunization with cilia from these isolates. Although all fish died, the survival time was extended in those receiving immunization.

More recently, Dickerson et al (1986) have produced murine monoclonal antibodies to Ichthyophthirius multifiliis theronts. Three of the monoclonal antibodies reacted with intact theronts using an enzyme linked immunosorbent assay (ELISA) and all had similar patterns of surface and cytoplasmic staining in indirect immunofluorescent tests.

IMMUNE RESPONSE OF FISH TO TETRAHYMENA

There are numerous references on Tetrahymena spp, reviews of which are covered by Corliss (1954, 1965) and Nanney and McCoy (1976) to name a few. A more detailed study of the biology of Tetrahymena can be found in Elliott (1973) with studies on the biochemistry in Hill (1972). Very little work, though, has been carried out on the immune response of fish to Tetrahymena spp. Most of the work that has been done, has been looking at the possibilities of using Tetrahymena spp as a vaccine against I. multifiliis, with Goven et al (1980 a, b, 1981 a, b) and Dickerson et al (1984) using Tetrahymena pyriformis in channel catfish and Wolf and Markiw (1982) using Tetrahymena thermophila in rainbow trout.

Pyle (1985) and Pyle and Dawe (1985 b) have made further studies on the humoral response of channel catfish following administration of a soluble ciliary preparation of T. pyriformis via the oral route, topical application by applying the antigen over one side of the fish with a canula and by intramuscular injection. A response only occurred following intramuscular injection, with peak response occurring 3 weeks following administration, after which it slowly declined. More recently Graves et al (1985) have demonstrated that non specific cytotoxic cells obtained from channel catfish can kill T. pyriformis.

IMMUNE RESPONSE OF FISH TO PARASITES OTHER THAN CILIATES

Relatively few investigations were undertaken on the immune response of fish to parasites prior to 1970. Up until this time, substantial reviews were produced by Lom (1969) and Sniesko (1969), the former

being specifically concerned with protozoa, the latter with metazoa. Arme and Walkey (1970) further summarized host specificity and immunity to helminths, Kennedy (1975, 1977) discusses the regulation of host-parasite populations by the host immune response and van Muiswinkel and Lagpt (1984) discuss host-parasite relationships. Earlier work by O'Rourke (1961) implied that possible immune responses may explain the specificity of host-parasite relationships in fish.

The following review is an attempt to give an overview of fish immunology related to parasitic diseases, with particular emphasis on acquired immunity, antibody production and cell and tissue responses. In reviewing the literature concerning fish parasites, it was noted that there was a general paucity of data concerning the immune response compared to mammalian systems. In order to gain a better understanding of events in the infection of carp with Ichthyophthirius multifiliis, it seemed appropriate to widen the review to include aspects of immunity to other eukaryotic pathogens, namely metazoa as well as protozoa. Protozoa are considered separate to metazoa.

Protozoa

It is convenient in reviewing the immune response to protozoa to consider humoral response separate to the cellular and tissue reactions

Humoral response

Barrow (1954, 1955) demonstrated that an acquired immunity resulting in the production of trypanocidal antibodies in tench (Tinca tinca) and perch (Perca fluviatilis) occurred in fish infected with trypanosomes,

resulting in *in vitro* lysis of the organisms. Production of antibody was shown to be affected by temperature and social position, and only gave protection of short duration. That fish can become immune to trypanosomes and trypanoplasma has been shown by Lom (1973). Fish surviving infections of *Trypanosoma danilewskyi* and *Trypanoplasma borelli* became resistant to re-infection. Bower and Woo (1977) demonstrated an innate immunity in six freshwater fishes to *Cryptobia catostomi*, associated with a non-specific serum factor. This, they suggested may have been complement by the alternative pathway, leading to resistance in fish. *In vitro* studies by Wehnert and Woo (1980) confirmed this. Woo (1979) and Woo *et al* (1983) suggested that *Trypanoplasma salmositica* in trout (*Salmo gairdneri*) undergoes antigenic variation to evade the host immune response. Similar views were put forward by Lowe-Jinde (1979, 1980) concerning antigenic variation in this host-parasite relationship.

Woo (1981) found that goldfish (*Carassius auratus*), which survived innoculations of either a high or low dose of *T. danilewskyi*, all survived a high challenge infection. A greater percentage of survival occurred after innoculation with a low dose, suggesting the immune response had time to respond and eliminate the parasite. Passive protection was also conferred with immune plasma. Cottrell (1975, 1976, 1977 a) examining *Trypanosoma platessa* in the plaice (*Pleuronectes platessa*) suggested that low parasitemias were possibly attributed to specific acquired immunity, with production of antibody, as electrophoresis of plaice sera indicated levels of immunoglobulin. Seasonal variations in parasitemia were associated to temperature. Burreson and Zwerner (1982) found that juvenile summer flounder (*Paralichthys dentatus*) remained infected with *T. bullocki* over the

winter, followed by a decrease in prevalence in late spring, suggesting a possible activation of the immune response as the temperature increased. Further work (Sypek and Burreson, 1983) found an increase in trypanocidal lysis as water temperature increased. Burreson and Frizzel (1986) immunised summer flounder with formalin killed I. bullocki at 20° C and ambient temperature. Following live challenge, the antibody titre increased with temperature. However, titre was not necessarily related to protection against the parasite, with survival of the parasites occurring. At low temperatures, high titre of antibody was not able to regulate the parasite.

The occurrence of an acquired immunity to the ectoparasitic flagellate, Ichyobodo necator has been demonstrated by Tavolga and Nigrelli (1947) who found that with some species of fish, with time, parasite numbers decreased. Robertson (1979) found a periodicity by I. necator on first feeding salmonid fry, with a rapid increase in numbers of parasites to a peak at 4 weeks, followed by a decline and virtual absence. He suggested this was related to innate or acquired immunity.

The immune response of fish to microsporidiosis has attracted few investigations. Awakuru and Kurahashi (1967) found that fish infected with the microsporidian Pleistophora spp acquired an immunity which gave a resistance to infection for up to a year. Goldfish appeared refractory to natural infection with P. ovariae despite closely related golden shiners and fat head minnows (Cyprinidae) becoming infected (Nagel and Summerfelt, 1977). McVicar (1975) noted that the level of infection of individual plaice (Pleuronectes platessa) infected with Glugea stephani, decreased with time, indicating the possibility of the immune system of the fish contributing to this. In examining the

immune response of the winter flounder (Pseudopleuronectes americanus) to G. stephani, Laudan et al (1986) could find no detectable humoral response to parasitized fish or fish injected with either spores or spore homogenate. Quantification of total immunoglobulin levels showed a decrease rather than an enhancement. This also occurred when fish were simultaneously injected with G. stephani and other antigens, for example, horse red blood cells, leading to lower antibody titres than normal.

Cell and tissue responses

Cellular and tissue responses have also been shown to occur in response to protozoan infections.

Inflammatory responses associated with haemoflagellates have been described by Dykova and Lom (1978 a, b, 1979) and Lowe-Jinde (1979, 1980) and include splenomegaly, an increase in macrophages in both spleen and liver and phagocytosis of organisms. T. salmositica was noted by Woo (1979) to be engulfed by macrophages in ascitic fluid in infected fish.

Dykova and Lom (1978 c) have reviewed inflammatory responses to microsporidian infections and describe a granulomatous inflammatory reaction to Glugea anomala and eventual phagocytosis of spores by macrophages. Similar responses in fish infected with other species of microsporidia have been described by Dykova and Lom (1980); Dykova et al (1980), Matthews and Matthews, (1980); Hauck (1984); Ralphs and Matthews (1986).

An inflammatory reaction has been observed in infections with the parasite Chilodonella hexasticha in cichilids, in particular, in the gills (Paperna and van As, 1983) and in rainbow trout infected with Octomitus salmonis which inhabits the intestine (Miura and Ohshima, 1960).

Coccidia have also been shown to stimulate a host response in turbot (Ferguson, 1976 a) and in killifishes (Solangi and Overstreet, 1980; Hawkins et al, 1981). Hawkins et al (1981)examined the inflammatory response in the liver of the killifishes Fundulus grandis and F. similis to Eimeria funduli and noted an initial infiltration by phagocytic monocytes followed by granular leucocytes - neutrophils, eosinophils and a third unidentified granulocyte. The eosinophils were seen to degranulate near macrogamonts, and both monocytes and eosinophils were seen to contain parasite debris.

Metazoa

As with protozoa, it is again convenient to separate the review into humoral and cell and tissue responses.

Humoral response

Studies on the immune response to myxosporidia are virtually confined to Myxobolus cerebralis and more recently Proliferative Kidney Disease (PKD). Halliday (1974) was unable to detect antibody to Myxobolus cerebralis in rainbow trout using indirect immunofluorescence. Similar results were obtained by Pauley (1974) using ouchterlony immunodiffusion tests. Both workers suggested the involvement of a possible

evasion mechanism, parasite antigen mimicking that of host. Griffin and Davis, (1978), however, were able to detect circulating antibody by indirect immunofluorescence. PKX, the causative agent of Proliferative Kidney Disease, which has recently been attributed to the myxosporidia (Kent and Hedrick, 1985), has been the subject of more recent immunological studies. Ferguson and Ball (1979) and Ellis et al (1982) suggest that fish can acquire immunity to this disease. Klontz et al (1986) found that during the clinical phase of the disease, there was an increase in the beta-globulin portion of serum proteins, suggesting immunoglobulin.

Many early studies on the susceptibility of fish to monogenea infections suggest the possibility of acquired immunity to these parasites (Jahn and Kuhn, 1932; Nigrelli and Breder, 1934; Nigrelli, 1947). Nigrelli (1935 a) undertook studies on mucus of pompano and proposed a possible role in protection against Epidella melleni. Injection of the parasite or sera from immune fish did not transfer protection (Nigrelli 1935 b, 1937). More recently Paperna (1963, 1964) suggested that carp can acquire immunity to both Dactylogyrus vastator and D. extensus. Khalil (1964) found that Polypterus recovering from gyrodactylid infections were not susceptible to a second infestation. Lester (1972) and Lester and Adams (1974) demonstrated a non-specific immunity in sticklebacks (Gasterosteus aculeatus) following repeated infections of Gyrodactylus alexanderi. Protection was short-lived, not more than 4 weeks. Acquired immunity in guppies to Gyrodactylus bullatarudi has been demonstrated by Scott and Robinson (1984); Scott and Anderson (1984); Scott (1985).

Krull (1934) found that sunfish previously infected with Uvulifer

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ambloplitis were refractory to further infection. Ferguson (1943) and Spall and Sumerfelt (1970) were unable to confer immunity in cyprinid fish to Posthodiplostomum minimum. Likewise Hoffman (1956) could not demonstrate acquired immunity to Crassiphiala bulboglossa on repeated exposure of fathead minnow (Pimephales p. promelas) with this parasite. Increased serum globulins were found, though, in Lepomus machrochirus infected with P. minimum (Meade and Harvey, 1969) and a non-specific factor in serum from previously exposed and unexposed fish, toxic to cercariae and metacercariae although encysted metacercariae were not affected. Plaice (Pleuronectes platessa) were found to be refractive to infection with Cryptocodyle lingua following previous repeated exposures (Iddon, 1973). Further work by Cottrell (1976, 1977 b) demonstrated protection following immunization with solubilized C. lingua and also confirmed that exposure to live parasites gave protection. Cottrell (1974, 1976, 1977 b) also demonstrated the presence of precipitating antibodies to C. lingua in infected plaice and to Rhipidocotyle johnstonei in plaice infected with this parasite. Electrophoresis indicated the antibodies were immunoglobulin (IgM).

Humoral antibody has also been found in the serum of the New Zealand eel (Anguilla australis schmidtii) infected with the intestinal trematode Telogaster opisthorchis using passive haemagglutination (McArthur, 1978) and enzyme linked immunosorbent assay (ELISA) techniques (McArthur and Sengupta, 1982). Speed (1976) and Speed and Pauley (1985) found that rainbow trout (S. gairdneri) injected with whole or sonicated metacercariae of Diplostomum spathaceum, had increased survival time over non-immunized controls at challenge, but, serum antibody could not be detected. Using the technique of ELISA, Bortz et al (1984) were able to detect the presence of circulating

antibody in rainbow trout against the metacercariae of D. spathaceum, both in experimental immunization and in fish naturally infected. An enhanced secondary response was found to occur following a booster immunization. Stables (1984) and Stables and Chappell (1986) though, using a variety of techniques, were unable to detect circulating antibody following immunization with killed cercariae or live infections of D. spathaceum, although a lower level of metacercariae became established in immunized fish. A difference in susceptibility of different fish species to D. spathaceum has been found by Betterton (1974), Sweeting (1974) and Speed and Pauley (1984). The latter workers, using four species of fish at two different temperatures, found increased survival time at the higher temperature.

Molnar and Berczi (1965) demonstrated precipitating antibodies in Abramis brama to Ligula intestinalis. However, Sweeting (1977) could find no indication of precipitating antibodies in roach (Rutilus rutilus) infected with L. intestinalis, but did find an increase in the quantity of gamma globulin compared to normal roach. Complement mediated leucocyte adherence has been demonstrated by Hoole and Arme (1986) leading to damage to the pleurocercoid of L. intestinalis. Kennedy (1968, 1969) suggested that a seasonal incidence in infection of the intestinal cestode Caryophyllaeus laticeps in the dace (Leuciscus leuciscus) may be due to increased resistance. In experimental infections of dace with C. laticeps, Kennedy and Walker (1969) found that fish responded to parasites by rejecting them, indicative of an acquired immune response but the presence of cirulating antibodies could not be demonstrated, nor could skin sensitising antibody (Harris, 1973).

A specific serum factor toxic to Acanthobothrium quadripartum in the ray Raja radiata was demonstrated by McVicar and Fletcher (1970), and the presence of C reactive protein has been shown in turbot (Scopthalmus maximus) infected with Bothriocephalus scorpii (Fletcher et al, 1980). Braten (1966) and Orr et al (1969) found a specificity of Schistocephalus solidus in the fish intermediate host. Braten (1966) suggested some fish species had an innate immunity to this parasite whereas Orr et al (1969) propose the production of cytotoxic antibodies as responsible for death of pleurocercoids. Hoffman et al (1986) suggest that immune mechanisms are responsible for rejection of pleurocercoids of Trianophorus nodulosus in burbot (Lota lota) unlike in minnow (Phoxinus phoxinus) and char (Salvelinus alpinus) where infections become established. Similar observations have been made by Powell and Chubb (1966) who found Diphyllobothrium spp survived in char for long periods but possible immune mechanisms led to a decline in infection in brown trout (S. trutta).

Harris (1970, 1972) demonstrated precipitating antibodies in chub (Leuciscus cephalus) to the acanthocephalan Pomphorhynchus laevis in both natural and experimental infections, although no subsequent resistance to infection was evident. Separation of the precipitins gave a peak corresponding to immunoglobulin (IgM). Skin sensitizing antibody could not be demonstrated in fish to antigen from P. laevis (Harris, 1973).

An immunoglobulin was found in plaice (P. platessa) sera to the nematode Proleptus obtusus found in the common dogfish but never in plaice, suggesting a serological cross reaction between helminth antigens (Harris and Cottrell, 1976).

The possibility of an immune response being responsible for rejection of the copepod Lernaea cyprinacea in goldfish was suggested by Shields and Good (1978). Shariff (1981) found indications of an immune response developing in bighead carp (Aristichthys noblis) to L. piscinae, with parasites gradually disappearing three weeks after infection. Shariff (1981) suggested antibodies may have been secreted in the mucus.

Reuling (1919) found that fish developed an immunity to the glochidia from mussels after repeated infections in the gills, and that this was not specific to the species examined. It was suggested that the immunity was due to production of antibodies.

Cell and tissue responses

Cellular and tissue responses are generally a major feature in response to larval stages of helminths, but also play a role in response to copepods and other metazoa.

A less intense inflammatory response has generally been associated with myxosporidian infections (Hahn, 1913, Nigrelli and Smith, 1938; Pulsford and Matthews, 1982), although Dykova and Lom (1978 d) found that with an increase in temperature and, therefore, possible activation of host immune response, rapid destruction of Henneguya cysts in the gills of perch and ruff occurred. In PKD, however, infiltration of the kidney may become a significant feature of the disease process. Mononuclear cell reactions comprising of macrophages, lymphocytes and neutrophils were recorded by Ferguson and Adair (1977); Ferguson and Needham (1978); Hoffman and Lomell (1984);

Clifton-Hadley et al (1985); Klontz et al (1986). Ghittino et al (1977) found increased counts of granulocytes in the blood of PKD infected fish although this was not confirmed by Hoffman and Lomell (1984).

Roubal (1986) found a slight increase in neutrophils in the gills of the sparid Acanthopargus australis infected with the monogenean Lamellodiscus major and Haliotrema spariensis.

An inflammatory response to the metacercariae of Posthodiplostomum cuticola has been recorded by Lucký (1970 b) and to P. minimum by Mitchel et al (1983). Lee and Cheng (1970) noted that the outer layer of encapsulated metacercariae of Stellantchasmus falcatus was of host origin, and leucocytes were situated on the periphery of each cyst. Little inflammatory response was found by McQueen et al (1973) in the skin of plaice (P. platessa) to C. lingua suggesting a long standing host parasite relationship. Chronic inflammation occurs, though in the gill of cutthroat trout (S. clarkii) in tissue surrounding eggs and miracidia of Sanguinicola klamathensis (Evans, 1974), but inflammation in infections of Diplostomum spp in the lens of rainbow trout (S. gairdneri) was only seen when damage and rupture of the lens occurred, leading to an escape of lens material (Shariff et al 1980). An inflammatory response occurred following penetration of Diplostomum cercariae into the fish host but phagocytosis only occurred, of parasites that didn't reach the lens (Ratanarat-Brockleman, 1974). Sommerville (1981) has studied the tissue response to invasion and encystment of Stephanochasmus baccatus in four species of flat fish, noting a chronic granulomatous inflammatory reaction, the intensity and rate of response varying with the species of fish. Further work

at the ultrastructural level has been carried out by Pulsford and Matthews (1984) in plaice, in response to R. johnstonei. The most common cell type found was the macrophage, but, although closely associated with the parasite surface, they were not attached. Occasionally, lymphocytes were found, and early in the infection, plasma cells.

The host response to the pleurocercoids of L. intestinalis has been studied in several species of fish by Arme and Owen (1968, 1970); Sweeting (1977) and Hoole and Arme (1982, 1983 a, b, 1986) and consists of an inflammatory response resulting in infiltration of polymorphonuclear leucocytes and macrophages. Ultrastructural studies have shown macrophages phagocytosing parasite material but penetration of the tegument has not been shown to occur. Blood changes associated with infection of L. intestinalis have shown an increase in monocytes and polymorphonuclear leukocytes (Sadkovskaya, 1958; Shpolyanskaya, 1953). Other cestodes have also been shown to cause inflammatory responses in their fish hosts (Esch and Huffines, 1973; Stromberg and Crites, 1974; Scott and Grizzle, 1979; Hayunga, 1979; Otto and Heckman, 1984; Moser et al, 1984; Rosen and Dick, 1984 a, b; Eiras et al, 1986; Hoffman et al, 1986; Sakanari and Moser, 1986; and Shostak and Dick, 1985).

Acanthocephala have also been shown to induce inflammatory responses of varying degrees in their hosts (Venard and Warfel, 1953; Prakash and Evans, 1960; Bullock, 1963; Chaicharn and Bullock, 1967; Esch and Huffines, 1973 and Hine and Kennedy, 1974). Eosinophilic granulocytes, macrophages and lympocytes were seen to increase in numbers in the area of proboscis attachment in some species (Esch and Huffines,

1973; Hine and Kennedy, 1974). The degree of host response appears to vary with species of fish and parasite. Bullock (1963) found chronic inflammation in trout infected with Acanthocephalus jacksoni, whereas, in other species of fish infected with various species of acanthocephalans, very little host response occurs.

Hauck and May (1977) found an inflammatory response in pacific herring (Clupea harengus pallasi) to Anisakis spp. Larvae were enclosed within a concentrically layered capsule which was closely associated with macrophages and lympocytes. Further work by Elarifi (1982) examining anisakid infections in the liver of whiting (Merlangius merlangus) found a dense neutrophil infiltration as the initial response, followed by macrophages, but few lymphocytes. Inflammatory responses to Spinitectus carolini have also been seen, particularly when third stage larvae penetrate the intestinal wall (Jilek and Crites, 1982).

Shariff (1981), examining the histopathology of the eye with the copepod L. piscinae on its host, the bighead carp, found a severe inflammatory response at the point of attachment of the anchor. Leucocytes were abundant, particularly neutrophils, in the epithelium of the cornea, but, inflammatory cells were also seen in other areas of the eye. Inflammatory responses to other copepods have also been seen to occur (Joy and Jones, 1973; Boxshall, 1977; Kabata and Cousens, 1977 ; Radhakrishan and Nair, 1981; Roubal, 1986).

Crustacea also have an effect on their fish hosts. Nair and Nair (1983), in studying the isopod Alitrophus typhus on the fish host Channa striatus, found that the total count of white blood cells

decreased as an infestation progressed, with leucopenia followed by lymphocytosis. A cellular response was also noted by Rand (1986) in response to infestation of Paranthias furcifer by the isopod Nerocila acuminata.

THE EFFECTS OF STRESS AND EXOGENOUS CORTICOSTEROIDS ON THE IMMUNE RESPONSE OF FISH AND SUSCEPTIBILITY TO DISEASE

Stress has been indicated as an important factor in predisposing fish to disease (Wedemeyer, 1970; Wedemeyer and Wood, 1974; Sniesko, 1974; Wedemeyer et al, 1976), and having effects on defence mechanisms in fish (Ellis, 1981). Mazeud et al (1977) have reviewed the primary and secondary effects of stress in fish, and, the production, quantification and identification of corticosteroids with factors that affect them have been examined by Idler and Truscott (1972) and in Pickering (1981).

Brown et al (1979) found there was a relationship between the level of pollution and frequency of disease. Fish from a heavily polluted river had a far higher prevalence of infection to bacterial, viral and fungal infections. Further effects of pollution as a cause of fish disease has been reviewed by Möller (1985). Several chemicals have been shown to have stressful effects on fish leading to increased susceptibility of rainbow trout (S. gairdneri) to infectious haemopoietic necrosis virus. Similar results were found with copper by Knittel (1981) with Yersinia ruckeri and Baker et al (1983) with Vibrio anguillarum. Ewing et al (1982) found exposure of channel catfish to sublethal copper stress increased the susceptibility of fish to experimental infections of I. multifiliis, although not as

much as they anticipated. Physical factors have also been shown to increase susceptibility of fish to disease. Pippy and Hare (1969) found a combination of high water temperature with zinc and copper pollution, led to an epizootic of a bacterial infection (Aeromonas liquefaciens). Exposure to mild environmental stress led to the isolation of infectious pancreatic necrosis virus (IPN) from carrier fish (Frantsi and Savan, 1971). Roberts and McKnight (1976) found husbandry stress, for example transportation and a sudden temperature rise, led to outbreaks of IPN in apparently healthy fish, indicating carriers. Husbandry stress, for example, handling, has also been implicated in several other disease outbreaks (Bejerano and Sarig, 1979; Walters and Plumb, 1980; Kumar et al, 1986). Hunter et al (1980) found heat stress led to release of Y. ruckeri from carrier fish resulting in infection of recipient controls. Stressful effects of elevated water temperatures have also been implicated in several other outbreaks of bacterial disease (Esch and Hazen, 1978, 1980; Hazen et al, 1978; Sarig and Bejerano, 1980).

Social stress, starvation and oxygen depletion have all been shown to lead to increased parasitemias in fish infected with trypanosomes (Barrow, 1954, 1955; Lom, 1979). Meyer (1970) suggested handling stress may play a part in outbreaks of I. multifiliis in the spring and Ferguson and Needham (1978) and Seagrave et al (1981) indicate adverse water quality, low dissolved oxygen levels and grading and transportation as factors predisposing fish to proliferative kidney disease. Observations have also been made by Lom (1969) concerning stress and protozoal diseases. Stress has also been implicated in Saprolegniasis in sockeye salmon (Oncorhynchus nerka) and other species of fish (Neish, 1977; Pickering and Willoughby, 1982).

Pickering and Christie (1980, 1981) found sexually mature male brown trout were frequently infested by several species of ectoparasites and the fungus Saprolegnia, although the levels of circulating cortisol were not as elevated as in the females which had decreased infections. A high prevalence of Ichtyobodo necator was also found on rainbow trout just starting to mature (Robertson, 1979).

The administration of corticosteroids has also led to the detection of disease states and increased susceptibility to disease. Bullock and Stuckey (1975) and McCarthy (1978) have used corticosteroids to detect asymptomatic carriers of Aeromonas salmonicida, indicating that severe stress may be the reason for outbreaks of the disease among apparently healthy fish. Following administration of cortisol, Robertson et al (1963) found that rainbow trout rapidly developed infections of fungus and Ichthyophthiriasis. Other workers have also found an increased susceptibility of fish to fungal infections after administration of cortisol (Roth, 1972; Pickering and Duston, 1983; Pickering and Pottinger, 1985).

The immune response of fish has also been shown to be affected by several factors. Leucopaenia can occur in fish exposed to malachite green (Bills and Hunn, 1976; Grizzle, 1977; Hlavek and Bulkey, 1980). Pulpmill effluent, zinc, methyl mercury, copper, cadmium and several other chemicals have also been shown to have an effect on the immune system (Anderson et al, 1984 a), either on leucocyte counts (McLeay, 1973 a, 1975 a; Mishra and Spivastara, 1979; Dick and Dixon, 1985; Iwana et al, 1986), antibody levels (Sarot and Perlmutter, 1976; Roales and Perlmutter, 1977; O'Neill, 1978, 1981 a, b; Anderson et al, 1984 b; Viale and Calamari, 1984; Robohm, 1986), cellular responses

(Robohm and Nitkowski, 1974; Spitsbergen et al, 1986), phagocytic activity of macrophages (Weeks and Warinner, 1984; Stave et al, 1984; Elsasser et al, 1986) and chemotactic responses of macrophages (Weeks et al, 1986). Antibiotics have also been shown to have an immunosuppressive effect on the humoral response of carp (Cyprinus carpio) (Rijkers et al, 1980 a; Rijkers et al, 1981; van Muiswinkel et al, 1981) and on leucocyte stimulation by mitogens (Grondel and Boersten, 1982; Grondel et al, 1985). Thermal stress (Weatherley, 1963) and cold shock (Slicher and Ball, 1962; Pickford et al, 1971 a) have been shown to affect numbers of leucocytes. Pickford et al (1971 a) observed a sequence of leucopenia followed by leucocytosis when killifish (Fundulus heteroclitus) were subjected to cold shock. Crowding can also lead to decreased leucocyte numbers (Klinger et al, 1983), and antibody levels (Perlmutter et al, 1973). Decreased numbers of thrombocytes and lympocytes also occurred following handling stress (Casillas and Smith, 1977; Pickering et al, 1982; Tomasso et al, 1983; Peters and Schwarzer, 1985; Ellsaesser & Clem, 1986 a, b). Ellsaesser & Clem (1986 a, b) found a reduction in number of both T and B lymphocytes and a decreased response of cells to mitogens. It was often observed though, that an increase in neutrophils and granulocytes would occur (Peters and Schwarzer, 1985; Ellsaesser & Clem, 1986 b). Captivity was also demonstrated by Miller and Tripp (1981, 1982 a, b) to lead to a decreased immunocytoadherence response. Aggressive behaviour among eels (Anguilla anguilla) led to stressful effects on subordinates resulting in decreased leucocyte counts and destruction of macrophages (Peters et al, 1980 a, b; Peters and Schwarzer, 1985). Refstie (1982) observed a genetic effect on susceptibility to stress and subsequent effect on antibody production, some families of trout being more resistant than others.

Increased levels of cortisol in spawning salmon (Oncorhynchus spp) and rainbow trout (S. gairdneri) have been shown to have an effect on lymphoid organs resulting in a decrease in lymphocytes in the spleen and thymocytes in the thymus (Robertson and Wexler, 1960; Robertson et al, 1961). McLeay (1975 b) found a decrease in lymphocytes and an increase in neutrophils in smoltifying coho salmon. Administration of corticosteroids also affects lymphoid organs, in particular thymus and spleen, again leading to a depletion in thymocytes and lymphocytes (Robertson et al, 1963; van Muiswinkel and van Ginkel, 1981; Chilmonczyk, 1982; Ghoneum et al, 1986). Lymphopenia and thrombocytopenia also occur in the circulatory system following corticosteroid or adrenocorticotrophic hormone (ACTH) administration (Weinreb, 1958; Pickford et al, 1971 b, c; McLeay, 1973 a, b, c; Pickering, 1984). Cellular responses have also been affected leading to prolonged scale allograft survival (Stutzman, 1967), and a decrease in cellular inflammation (MacArthur et al, 1984), in vitro phagocytic activity (Stave and Roberson, 1985) and in vitro mitogen induced proliferation of blood leucocytes (Grimm, 1985). Anderson et al, (1982) found that, following immunization with Y. ruckeri, corticosteroid administration led to reduced numbers of splenic antibody producing cells, splenic lymphocytes and decreased circulating antibody compared to untreated controls. Corticosteroids have also led to decreased antibody titres to A. salmoncida (Chen et al, 1983) and infectious pancreatic necrosis virus (Wechsler et al, 1986) but in young carp, cortisol suppressed tolerance leading to antibody production (Ruglys, 1985 a, b).

MATERIALS AND METHODS

FISH

Collection, maintenance and handling

Although carp, Cyprinus carpio, were used for immunological studies of ichthyophthiriasis, several other species of fishes, namely mullet (Chelon labrosus), black mollies (Poecilia latipinna) and tilapia (Oreochromis mossambicus) were investigated for routine and long term maintenance of the parasite. Details below refer only to carp unless otherwise stated.

The various sources of experimental and stock fish are listed in Table 1. On collection, fry and juveniles up to 8 cm were transported to the laboratory in polythene sacks (150 x 60 cm) with a maximum density of approximately 2000 fry per sack or 200 juveniles containing approximately 50 dm^3 of oxygenated water. Adult carp (1.0-2.2 kg) were caught by rod and line from Drake's reservoir, Plymouth, Devon.

All fish were kept in quarantine for 6 weeks prior to use, and were treated with 'protozin' (Waterlife Research, UK) for 10 days as a prophylaxis against I. multifiliis. Adult carp were treated with 'Sterazin' (Waterlife Research, UK) for the first 10 days of quarantine to remove the parasite Argulus foliaceus which is endemic in Drake's reservoir. Prophylactic treatment was generally found to be effective following quarantine, with only one exception - fry obtained from Humberside fisheries in 1985, which were found to have an active eukaryotic infection of Dactylogyrus spp. Treatment was a daily bath

Table 1. Sources of fish and dates of collection or spawning.

Species	Source	Year	Dates Spawned	Collected or caught	Mean weight (g)	Mean length (cm)
Cyprinius carpio fry	Humberside Fisheries, Driffield	1984 1985	18.4.84 17.4.85	N/A N/A	[a]0.890 1.06	3.40 3.55
	Plymouth Polytechnic	1984 1985	15.11.84 26.4.85	N/A N/A	0.630 1.28	2.80 3.50
	Hampshire College of Agriculture, Sparsholt	1985	30.1.85	N/A	0.796	3.10
	Munton & Fisons, Stowmarket	1984 1985 1986	18.9.84 20.9.85 8.2.86	N/A N/A N/A	0.950 1.02 0.960	3.40 3.25 3.30
Cyprinius carpio juveniles	Bourton-on-the-Water, Glos	1984	N/A	24.10.84	21.2	6.0-8.0
	Fishers Pond, Twyford, Hants	1985	N/A	20.2.85	18.3	6.0-8.0
	Munton & Fisons, Stowmarket	1985 1986	N/A N/A	11.10.85 10.3.86	24.5 22.8	6.0-8.0 6.0-8.0
Cyprinius carpio adults	Drake's reservoir, Plymouth	1984 1985	N/A N/A	July August	1 000-2 000 1 000-2 000	N/A
Chelon labrosus juveniles	St John's Lake, Cornwall	1984	N/A	November	5.6	3.0-5.0

a age 10-12 weeks N/A Not available

in 10 ppm 'Dipterex' for 30 minutes with aeration, for 7 days. 'Sterazin' was also added to the stock tank for 10 days.

Fish were kept at a variety of temperatures depending upon age, maturity and projected usage as follows: fry 22° C, juveniles 13-15°C, production of antisera in adults 20±2° C and broodstock for spawning, 23° C. All experiments were undertaken at 20±2° C. Fish were held in aquaria of different capacity which were related to fish size, fry being kept in glass aquaria (100 dm^3 capacity) and polyethylene tanks (600 dm^3), the latter also being used for adults utilised for raising antisera. Stock adults and juveniles were held in 800 dm^3 polyethylene tanks incorporated into the main aquarium system. Juvenile carp, 10-12 weeks old, used in experiments, were maintained in perspex aquaria of either 12 dm^3 or 20 dm^3 capacity, with stocking densities of 5-10 and 10-15 fish respectively. For some experiments, juveniles were kept in polyethylene tanks of 100 dm^3 capacity.

Water quality was maintained by biological filtration of recirculated water. Subgravel filtration was utilised for experimental perspex tanks and 100 dm^3 glass aquaria holding fry, water being circulated by means of an airlift pump. Larger aquaria, 600 dm^3 and 800 dm^3 tanks were connected to Eheim biological filtration pump units or connected to the main aquarium system where water quality was maintained by passage through a biological filter bed. The capacity of the aquarium was 15,000 dm^3 with a recirculation time of 43 minutes. Oxygenation in all systems was maintained by air stones. The following pH and nitrogen levels were considered acceptable in achieving optimal water quality: pH 7.0-7.5; ammonia 0.096 mg/dm^3; nitrate 21 mg/dm^3; nitrite 0.07 mg/dm^3. Carp and tropical freshwater fishes, where

used, were maintained in mains water, after this had been allowed to stand for 24 hours over shell gravel, to remove chlorine and raise the pH from 6.5 to 7.5-8.0. Sea water used for maintenance of mullet was obtained from the research system at the Marine Biological Association. The quality of this water was carefully monitored prior to pumping to ensure a salinity of 35%. Mullet for experiments were kept in perspex tanks of 12 dm^3 capacity, 5-10 per tank. Stock fish were maintained in the marine system of the main aquarium in 800 dm^3 tanks.

Recirculating aquarium systems, with the exception of the main aquarium, received partial water changes at no more than 3 week intervals. Complete cleaning of these systems was undertaken every 3-4 months when they were sterilised with Hydaine C (Thompson Ltd, UK), a biological disinfectant/detergent.

Feeding regimes for carp were as follows. Fry up to the age of 3 weeks received 4 daily feeds of freshly hatched Artemia salina Willowbank, Warrington, UK), then introduced to dry food (trout pellets, size 00, 01 and 02, Ewos Baker, UK), supplemented with once daily artemia feeds. After approximately 8 weeks, fish were fed twice daily a mix of size 02 trout pellets and tetramin tropical fish flake (Tetra, UK) or goldfish flake (King British, UK). Juvenile carp (6-8 cm), were fed twice daily on size 04 trout pellets, and adult carp, size 05 and 06 trout pellets.

All aquaria were checked daily and dead fish or uneaten food removed as necessary. Separate hand nets were used in each aquarium. These were kept in a 5% solution of sodium hypochlorite (BDH Chemicals, UK) for 24 hours prior to use and rinsed in fresh water.

Anaesthetics

Fry and juvenile fish were anaesthetised with MS222 (Ethyl-m-amino-benzoate; 3-aminobenzoic acid, ethyl ester. Sigma, UK), freshly prepared at a concentration of 1:1000 (w/v), fish being removed immediately upon cessation of movement. Following handling procedures under anaesthetics, they were allowed to recover in well aerated water. Benzocaine (Ethyl-p-aminobenzoate; 4-aminobenzoic acid ethyl ester. BDH Chemicals, UK), was the preferred anaesthetic for adult fish for the procedures of injecting, tagging or spawning, being administered at a concentration of 40 mg/dm^{-3}, previously dissolved in 50 cm^3 of absolute alcohol. Anaesthetics were also used for killing fish; MS222 at a concentration of 1:500 (w/v) and Benzocaine at a concentration of 50-100 mg/dm^{-3}. These solutions could be kept for several weeks in a dark, stoppered bottle, without loss of activitiy.

Collection of blood and preparation of serum

Blood was collected from the caudal vein by one of two methods, depending upon size of fish. For fish up to 10 cm, the caudal vein was directly exposed by severing the caudal peduncle with a sharp scalpel, at right angles, midway between the anal fin and caudal peduncle. The collection of blood from the severed body was by means of capillary action, using a non-heparinised haematocrit tube (Billbate, UK). This was satisfactory for the smallest fish (up to 5 cm) when blood was pooled (up to 300µl blood from 10 fish). For juvenile fish (6-8 cm), larger volumes (up to 700µl per fish) of blood could be obtained and this was collected using a long pasteur pipette. Removal of blood from larger fish (0.5 - 2.2 kg) was carried out by

caudal vein puncture using a 2 cm^3 or 5 cm^3 syringe fitted to a 21 gauge needle. The vein was located just ventral to the vertebral column and posterior to the anal fin. Up to 2-5 cm^3 of blood could usually be collected per fish.

Volumes of blood up to 1 cm^3 were transferred to 1.5 cm^3 microcapped centrifuge tubes (Hughes and Hughes Ltd, UK), larger volumes being placed in glass bijoux or universals. Whole fish blood was left to clot at room temperature for 1 hour and then left overnight at 4° C for clot retraction. Serum was prepared by centrifugation either at 500 g for 5 minutes, when large volumes were required, or, for 2 minutes in a fixed speed micro centrifuge (J.A.Jobling, Stafford, UK). Freshly obtained serum from experimental fish was aliquoted into 60 µl or 120 µl volumes in 1.5 cm^3 microcapped centrifuge tubes, whereas normal pooled carp serum was aliquoted in larger volumes (up to 1 cm^3) Sera were stored at -20° C for short periods (up to 3 months) or -70° C for longer periods. Sera was heat inactivated at 47° C for 20 minutes, when necessary, before use. Serum samples were used once only on thawing as refreezing is claimed to reduce antibody activitiy (Rio and Recco, 1971).

Administration of antigens

Administration of antigens in fish of all sizes was by intraperitoneal injection. This introduction of material into the small fry (4-6 weeks of age) was undertaken making use of an agla glass syringe (Agla, Wellcome, Beckenham, UK) attached to a micrometer. In these cases, a glass needle drawn from a 25 µl glass capillary, was inserted into the peritoneal cavity in a ventro-lateral position, and the dose

administered until a slight expansion of the peritoneal cavity could be seen under a stereo microscope 150-200 x magnification. This approximated to a dose of 1 µl 100 mg $^{-1}$ body weight (10 µl g^{-1} body weight). For fish up to 2.0 g, a 27 g needle was fitted to an agla glass syringe and micrometer, and for larger fish, a 1 cm^3 plastic syringe was used with a 27 g or 25 g needle. The volume injected into fish was 10 µl g^{-1} body weight.

When antigens were administered in Freunds Complete Adjuvant (Difco, UK) to produce a potentially immunogenic dose, particularly with challenge injections, antigen would be suspended in half the volume of saline, the remaining volume being FCA. The antigen plus adjuvant would then be emulsified by being passed several times through a 19 g needle fitted to a 5 cm^3 syringe.

Breeding of carp

On 2 separate occasions, carp from own stock within the Polytechnic were spawned artificially using the method of hypophysation as follows:

Fish to be used for spawning were maintained at 23° C on a high protein diet for 4-6 weeks to bring them into spawning condition; females being ripe with eggs at Stage 7 and males just starting to run milt. The minimum period to achieve spawning condition was 6 weeks for females, 2-3 weeks for males. Females were then injected with carp pituitary extract (Sigma, UK), which was mixed in solvent (0.65% saline diluted 7:3 with glycerol) immediately before use in view of its relatively short shelf life. Administration was by intramuscular

injection just below the dorsal fin, splitting the dose between both sides of the fish. A total volume of 0.5 cm^3 was injected and the sites massaged carefully. Two doses of pituitary were given: a low preparatory dose of 0.3 mg kg^{-1} body weight followed 24 hours later by a higher decisive dose of 3 mg kg^{-1} body weight. Males were only given the decisive dose. It was essential, following the decisive dose to keep the sexes separate to prevent premature spawning. Eggs ripened within 12-15 hours at 23° C. It was necessary, therefore, to test strip at hourly intervals from 12 hours onwards as over-ripening, which can occur within 2 hours, would render the eggs useless. Table 2 shows the record of spawnings.

Fertilization was carried out in vitro, the eggs being stripped into a clean dry bowl followed by addition of milt and mixing dry with a feather. As a precaution to increase the chance of fertilization, milt from 2 males was used. After stirring, the mix was allowed to settle for 30 seconds, after which an equal volume of urea solution (at 23° C) was added (Appendix I). The fertilized eggs briefly re- - stirred and left for 5-10 minutes. As the eggs began to swell, further urea solution was added, with gentle stirring. Swelling was generally complete within 2 hours. Urea was used in preference to water as this ensured the removal of the sticky outer layer of the egg so that they could be more conveniently handled. The eggs were then rinsed of milt with urea solution and tannin solution (23° C, Appendix I) was added for 1-2 seconds to harden the eggs. This was rinsed with running tap water (23° C) and repeated 2-3 times, after which the eggs were washed well with running tap water.

Table 2. Results of spawning of adult carp Cyprinus carpio.

Date of spawning	Sex	Weight (kg)	Stripping success	Hatching date	Hatching success (%)
15.11.84	F	1.6	Good at 11 h	17.11.84	60
		1.6	Good at 15 h	Eggs not used	
	M	2.0	Good at 11 h		
		1.1	Good at 11 h		
26.4.85	F	2.1	Good at 13 h Overripe eggs	Eggs not used	
		1.1	Good at 13 h Good eggs	28.4.85	80
	M	2.4	Not very good		
		1.4	Good		

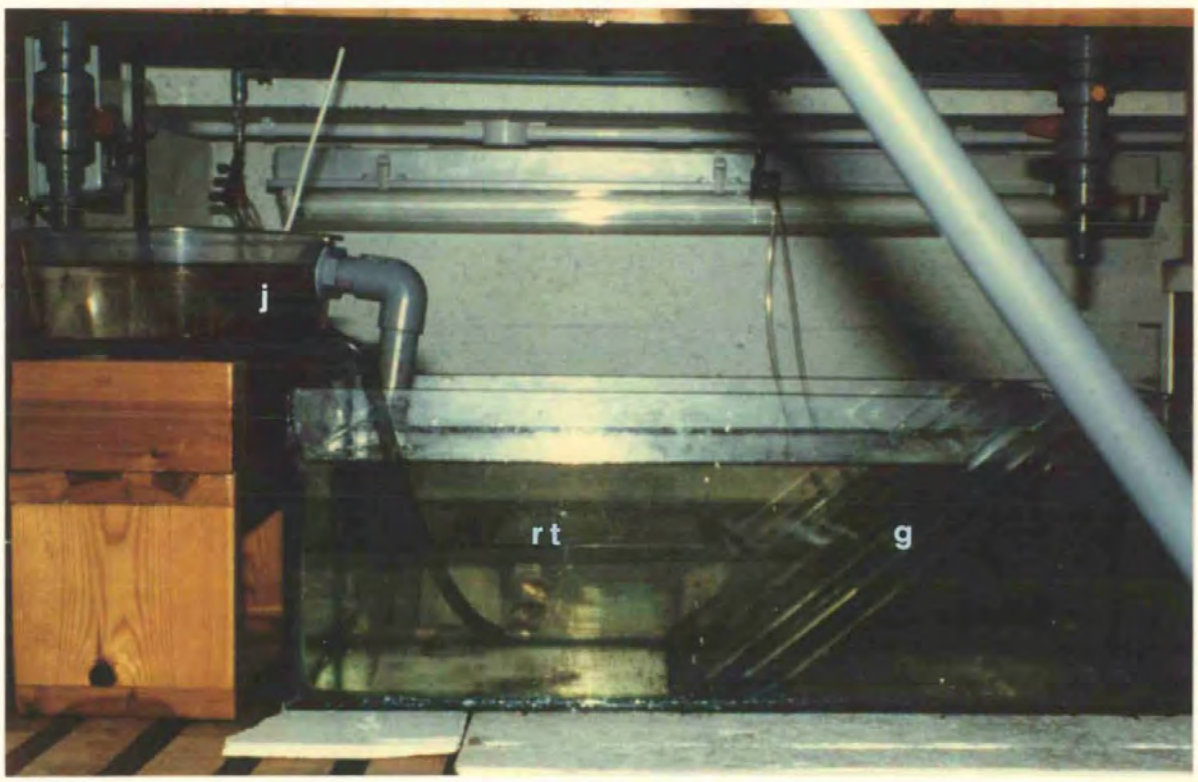
F female, M male

Rearing of fry

Fertile eggs were transferred to a hatching tank connected to a rearing aquarium (Fig. 6), water temperature being maintained at 23°C. As the eggs hatched, which took 2 days at 23° C, the larvae clung to the sides of the jar absorbing the yolk sac. After 1-2 days, the fry swam up to fill the swim bladder, and were then carried with the flow of the water into the glass rearing tank. At this stage, the first feed was taken which consisted of very finely sieved, hard boiled egg yolk. This was fed for 2 days, with freshly hatched Artemia salina being added from the second day onwards (4-5 feeds per day). From day 7-10, finely ground trout food (size 00) was gradually phased in, although Artemia was still fed four times a day up till weeks 3-4, but at a lower density. It was important initially, when the fry were very small, that the Artemia nauplii were separated from the shells. During this time, the fry were thinned out and maintained in glass tanks of 100 dm^3 capacity, utilising undergravel filtration. Care was taken to ensure good water quality and to prevent fungal growth by siphoning the bottom of the tanks daily to remove any uneaten food.

Artemia eggs (Willowbank, Warrington, UK) were hatched in sea water in 5 dm^3 conical flasks maintained at 25-28° C. Aeration was supplied to keep the eggs in constant circulation and the flasks placed in a well-lit position. An approximate volume of 50 cm^3 of eggs was added to each flask. Hatching took place from 24 hours onwards and nauplii were harvested from 36 hours onwards. Two flasks (Total of 10 dm^3) were set-up each day to ensure a constant supply of food. For harvesting, the air supply was turned off and the solution allowed to

Fig 6. Hatching jar and rearing tank for carp fry. Eggs are put in jar (j) through which water flows. Following hatching (2 days at 23° C), fry swim over into the rearing tank (rt). The grid (g) used to prevent escape of fish is changed for a different mesh size, with fish growth.


j
rt
g

settle for 10 minutes, to allow separation of the egg shells (which float) from the nauplii, which collect near the bottom of the flask. The nauplii were then siphoned into a separating funnel (3 dm^3) and again allowed to settle to separate any remaining shells from nauplii. The nauplii were then collected in a fine meshed sieve, rinsed with fresh water, and fed to the fry.

ICHTHYOPHTHIRIUS MULTIFILIIS

Isolation

I. multifillis was isolated from a variety of sources (table 3), including fish from aquarists and fish farms. The procedure for establishment of the parasite and for routine maintenance was as follows:

Infected fish, on collection, were quarantined in perspex aquaria of 2 dm^3 capacity to which some gravel and weed were added to afford some protection for free swimming trophozoites from fish predation. Isolation aquaria were maintained at 20° C or 24° C, depending upon fish species, cold water or tropical. A standard isolation procedure was carried out: juvenile carp (3-5 cm), and occasionally other species which were available for routine maintenance - Tilapia (O. mossambicus), were added to the tank as recipient hosts, to allow infection to occur. These were naïve hosts, having no previous records of exposure to the parasite.

Table 3. Source of Ichthyophthirius multifiliis.

Host species[a]	Source	Date of isolation	Allocated code	Survival (days)[b]
Neon tetra (T)	Aquarist	24.1.84	NT/84	245
Rainbow trout fry (C)	Fish farm	13.3.84	RT/84	217
Tropical fish (unknown species) (T)	Aquarist	12.4.84	AF/84	119
		26.5.84	Mixed AF/84 & RT/84	121
		30.5.84	Mixed NT/84 & AF/84	119
Rainbow trout fry (C)	Fish farm	9.8.84	RT/W	28
Goldfish (from Japan) (C)	Importers	26.10.84	J/84	301
Shubunkin (C)	Aquarist	17.7.85	S/85	287
Platies (T)	Aquarist	13.1.86	P/86	Terminated at 294

a C = coldwater fish T = tropical fish

b survival period under laboratory conditions

Methods for infection

The procedures used for transmitting the parasite varied depending on whether large or small numbers of theronts were required. For small numbers of theronts, one of the following methods was used: tail dipping, body dipping, continuous infection. Prior to the routine use of tail and body dipping, it was ascertained that the anaesthetics used did not impair infectivity of the parasites.

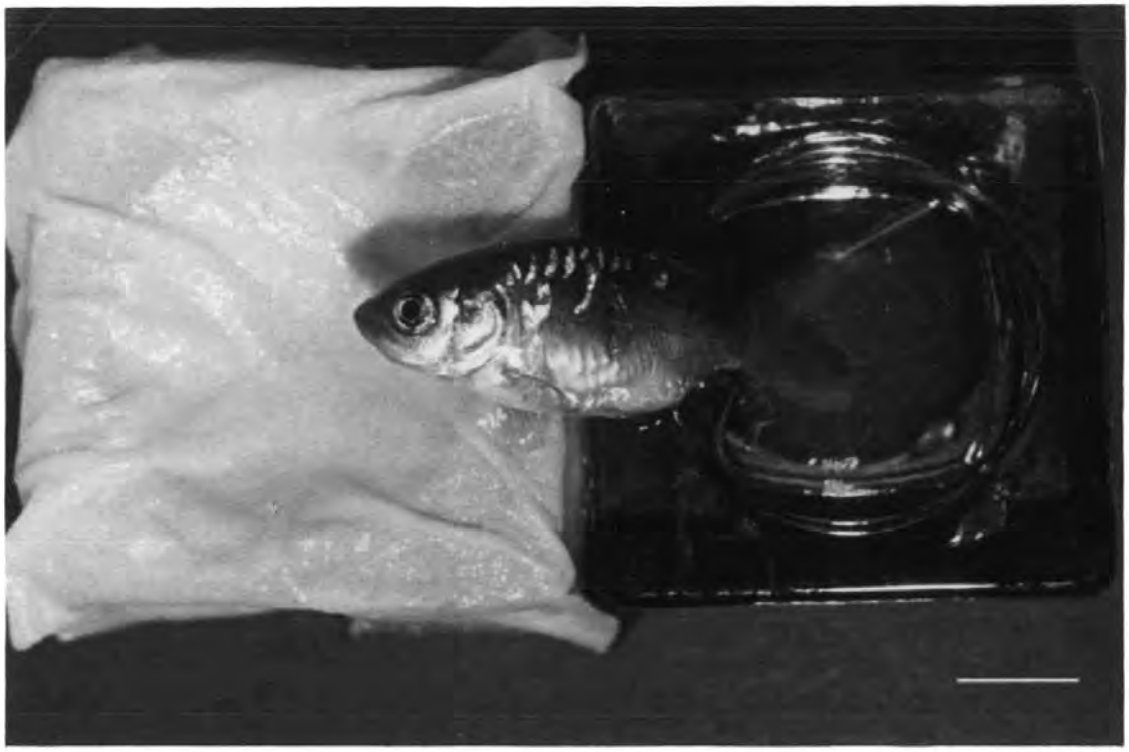
Tail dipping

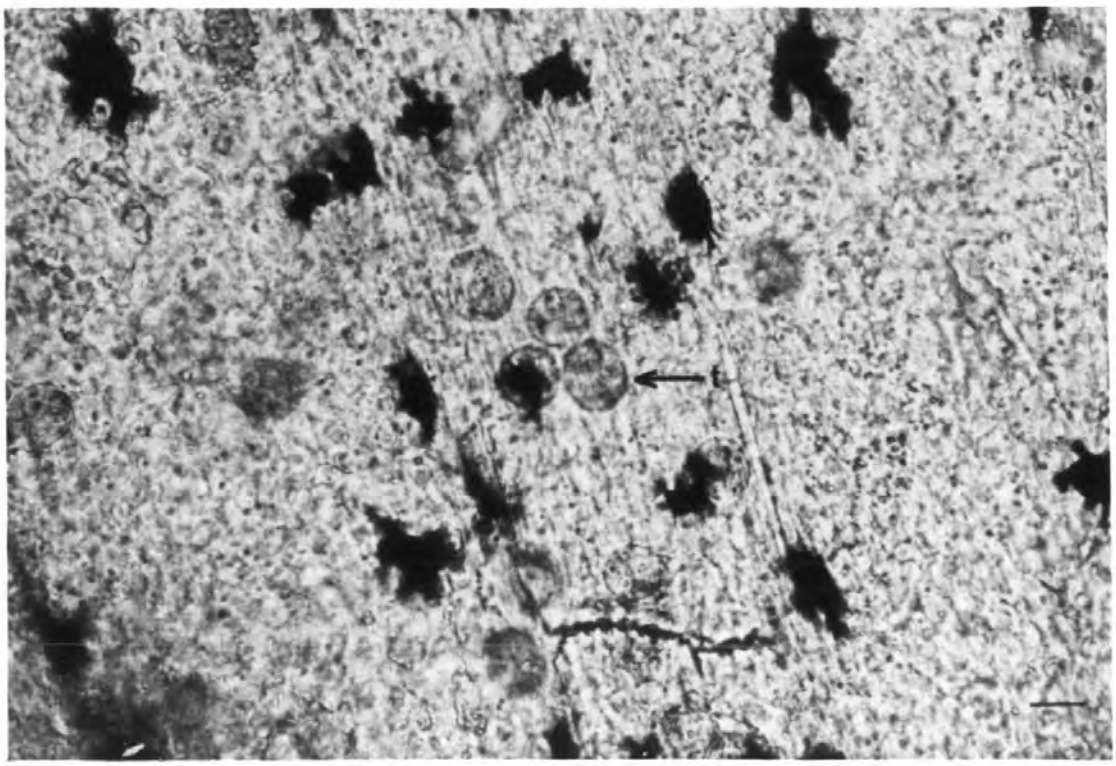
Carp or tilapia (3-5 cm) were lightly anaesthetized in MS222 and placed so that the caudal fin was resting in a glass watchglass measuring 3.5 x 3.5 cm with the head region resting in an adjacent watchglass kept moist with a damp tissue (Fig. 7; method of R. A. Matthews, personal communication). This enabled the caudal fin to be exposed to theronts. A volume of 0.3 - 0.5 cm^3 of theronts (concentration of approximately 2,000/cm^3), was added, and penetration into the fin could be clearly seen using a stereomicroscope, (Kyowa) 450x magnification (see Fig. 8). The exposure time was carefully adjusted to control a reasonable level of infection as it was noted in some cases that the speed of contact and penetration of the host was very rapid (<10 seconds), particularly if the time from excystment was short. A time of 2-3 minutes was usually sufficient for infection to occur with a reasonably uniform level and also was the length of time that fish could be kept anaesthetized satisfactorily. The disadvantages of tail dipping were that only relatively small numbers of parasites could be kept, restricted to one area. It was, therefore, necessary to expose the whole fish by body dipping in order to

Figs 7 and 8. Experimental infection of Ichthyophthirius multifiliis by tail dipping.

Fig 7. Arrangement for tail dipping of juvenile carp (R. A. Matthews, pers. comm) for concentration of trophozoites of I. multifiliis. Scale, 1 cm.

Fig 8. Photomicrograph of caudal fin showing I. multifiliis trophozoite (t) immediately following penetration by the theront. Scale, 30 µm.





establish immunisation levels of infection.

Body dipping

a) In confined volumes for small fish - similar procedures were used as for tail dipping. After anaesthetization, the entire fish was placed in a watchglass and a volume of 0.5 cm^3 of theronts was added (approximate concentration of 2,000/cm^3). Exposure was for a controlled period of time depending on the viability of the theronts, previously determined by tail dipping. If fish had not been anaesthetized for a sufficient length of time, host contact would be impaired due to rapid opercular movement, wafting the theronts away. The advantages of tail dipping and body dipping in a confined volume were that they ensured a high density of infection using small volumes of theronts.

b) In addition to exposing fish to the parasite in a concentrated suspension of theronts in watchglasses, it was also suitable to place the fish in larger volumes in crystalising dishes. This method did not necessitate the use of anaesthetic. Fish (2-3 cm) were placed in a crystalising dish (9.5 x 4.0 cm) containing 200 cm^3 of water, pH 7.2-7.5, with a concentration of approximately 600 theronts per fish, 125 theronts/cm^3, 5 fish per bowl, for 5-10 minutes. This procedure was carried out in the dark to reduce fish movement.

Infected fish following all these procedures, were kept in perspex aquaria (2 dm^3 capacity) without biofiltration, water quality being maintained by complete daily changes. Aeration was provided via a

1 cm^3 syringe barrel attached to an air pump. The temperature was adjusted to the host, 20±2° C for carp, 24° C for tropical.

Continuous infection

Fish which had been given a primary infection by either tail or body dipping, were kept in perspex aquaria (2 dm^3 capacity), 10 fish per tank. The infection was then allowed to continue, re-infection taking place in the tanks. Death of the fish usually took place 5-7 days into the secondary infection when fish were heavily infected. Larger numbers of trophozoites could then be collected.

The time taken to complete development on the fish host is temperature dependent, taking approximately 7 days at 20° C and 5 days at 24° C. When the infection became clearly visible to the naked eye, fish were transferred to a crystalising dish and trophozoites collected as they left the fish during the following few days. Trophozoites were removed immediately and placed in a watchglass or a 12 well flat bottomed microtitre plate and allowed to encyst. These were then incubated in a humidification chamber at room temperature, until division had been completed with the production of theronts, which were then available for collection. Alternatively, when excess trophozoites were available, they were transferred to a clean crystalising bowl and allowed to encyst in the minimum amount of water to ensure good aeration and concentration of theronts.

Large scale production of I. multifiliis

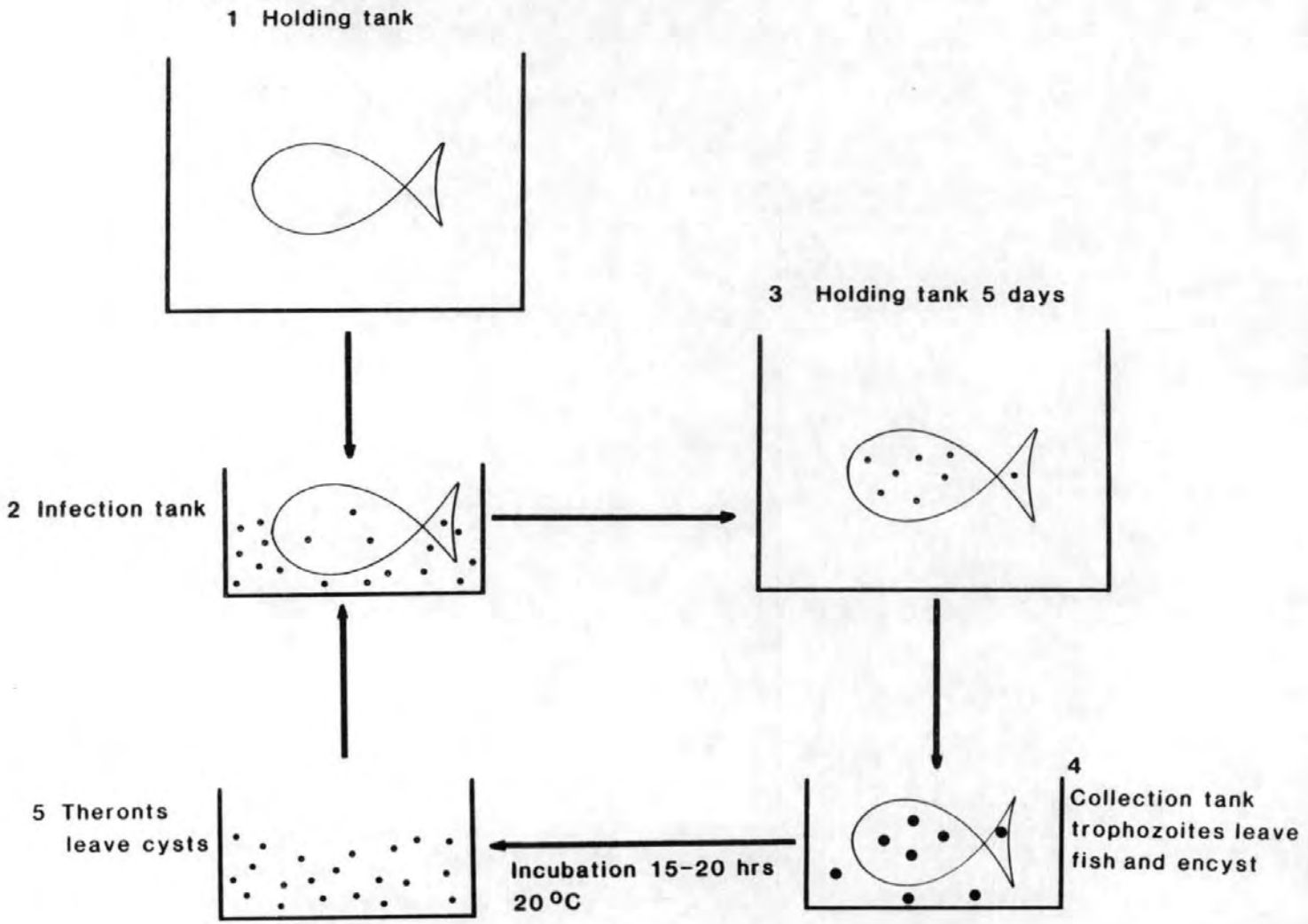
For immunological experiments, it was necessary to obtain high yields

of theronts for immunisation and challenge. The following method was, therefore, developed to meet these requirements. Only carp were used and these were selected in the size range 6-8 cm which were convenient for management. As can be seen summarised in Fig. 9, fish were transferred to perspex infection tanks of 2 dm^3 capacity, containing 1 dm^3 of standing water (pH 7.2) previously aerated. One to four fish were placed in each tank and a suspension of theronts added at a dose of approximately 4,000 - 6,000 per fish. Constant conditions were maintained for this procedure including temperature, 20±2° C, no aeration, pH 7.2, and placed in the dark. Fish were exposed for periods of time dependent on viability of the parasite. Checks on the level of infection were made by removal of fish at 5 minute intervals and the level of parasite invasion checked by examining the caudal fin under a stereomicroscope, 450x magnification. As can be seen in Fig. 8, parasites can be clearly seen in the fin. Fish were removed from exposure to the parasite when it was estimated that a suitable level was obtained. Following infection, fish were transferred to a larger holding tank of 12 dm^3 capacity, subgravel filtration, for 5--7 days, 20±2° C, 5 fish per tank.

Collection of trophozoites

When the parasite was visible to the naked eye, the fish were transferred to a perspex aquaria (2 dm^3 capacity), 1-2 fish per tank (see Fig. 10). Trophozoites leaving the fish could then be collected. Trophozoites were either placed in a glass crystalising bowl and left to encyst in a small volume of water, or, if large numbers, were left in the collection tank, with the fish being transferred to a fresh tank daily. After encystment, the water was changed to remove any

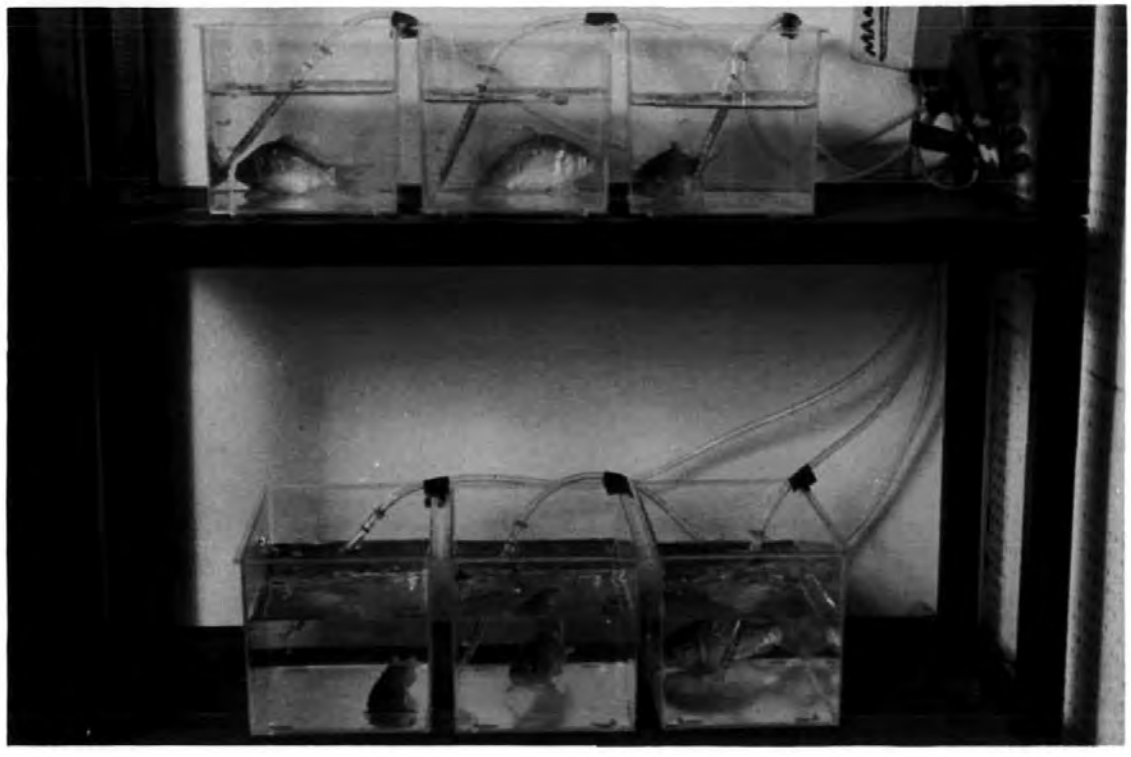
Fig. 9. To show procedures for routine maintenance of Ichthyophthirius multifiliis in the laboratory.

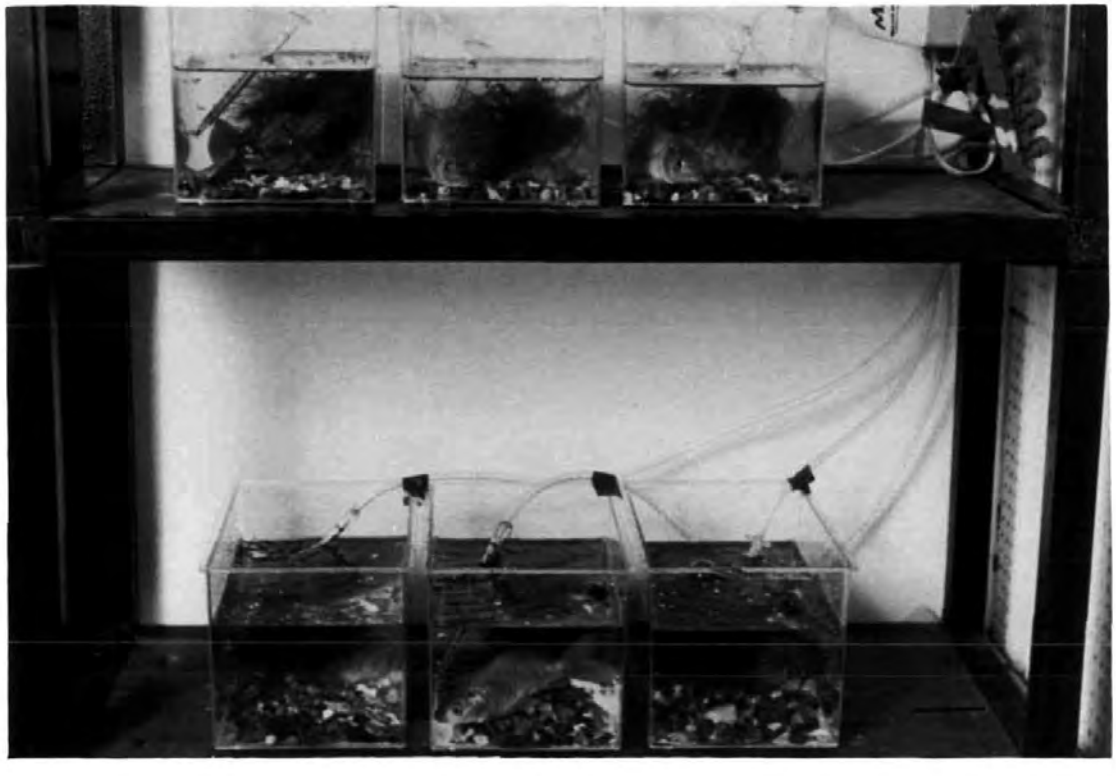


Figs. 10 and 11. Maintenance of Ichthyophthirius multifiliis in carp. Scale, 2 cm.

Fig 10. Arrangement of aquaria (2 dm^3 capacity) used for infection of fish with theronts and collection of trophozoites. Note control of aeration using perspex 1 cm^3 syringes.

Fig 11. Continuous infection. Note addition of gravel and weed which reduces predation of pre-encysted trophozoites.





toxic waste products. With heavily infected fish, death often occurred by day 7. The large numbers of trophozoites leaving these fish were allowed to encyst in a small volume (100-200 cm^3) of water in the collection tank. After most of the trophozoites had left the fish and encysted, the fish was removed to prevent deterioration of water quality, and fresh water (pH 7.2-7.5) added. The cysts were left to divide and the emerging theronts were used to infect more fish for maintenance and/or for immunisation experiments. Large numbers of theronts could be obtained by these methods (up to 1×10^6).

In order to ensure adequate numbers of theronts on appropriate days of experiments, it was necessary to maintain staggered infections enabling harvesting of theronts over a period of consecutive days.

In the event of primary infections being low, the parasite was allowed to recycle and harvesting was done at the secondary infection stage when numbers would normally be greatly increased. Weed and gravel were added to the tank to protect trophozoites from predation by host fish (see Fig. 11).

Long term maintenance of I.multifiliis

The long term maintenance of I. multifiliis was investigated following the technique of intraperitoneal infection (Dickerson et al, 1985).

Theronts (1-5 hours following encystment), were concentrated by centrifugation at 600 g for 15 minutes and resuspended in sterile double distilled water to give a concentration of approximately 2,000/ cm^3. Juvenile mullet of mean weight 5.60 g were injected

intraperitoneally with 100-300 µl of theront suspension (approximately 200-600 theronts per fish). As a control for viability of theronts, juvenile carp were exposed to theronts by tail dipping. Fish were examined at weekly intervals following injection of theronts by killing the fish and opening the peritoneal cavity. The body wall and internal organs were examined by placing the fish in a petri dish of distilled water and with the aid of a stereomicroscope. Trophozoites were transformed to a square watchglass containing distilled water, allowed to incubate and resulting theronts tested for viability by tail dipping of juvenile carp. The results for successive trials are shown in table 4. Trophozoites, where found, were in the body wall, intestine and liver. Due to the small size of the fish and the subsequent low numbers of trophozoites found, this method was not used for routine maintenance.

EXPERIMENTAL TECHNIQUES FOR I. MULTIFILIIS

The following procedures were used to establish experimental infections in juvenile carp. Any differences from these standard procedures will be included under the appropriate experiment in Experiments and Results. The number of fish selected for experiments was always over-estimated in order to make up losses due to mortalities.

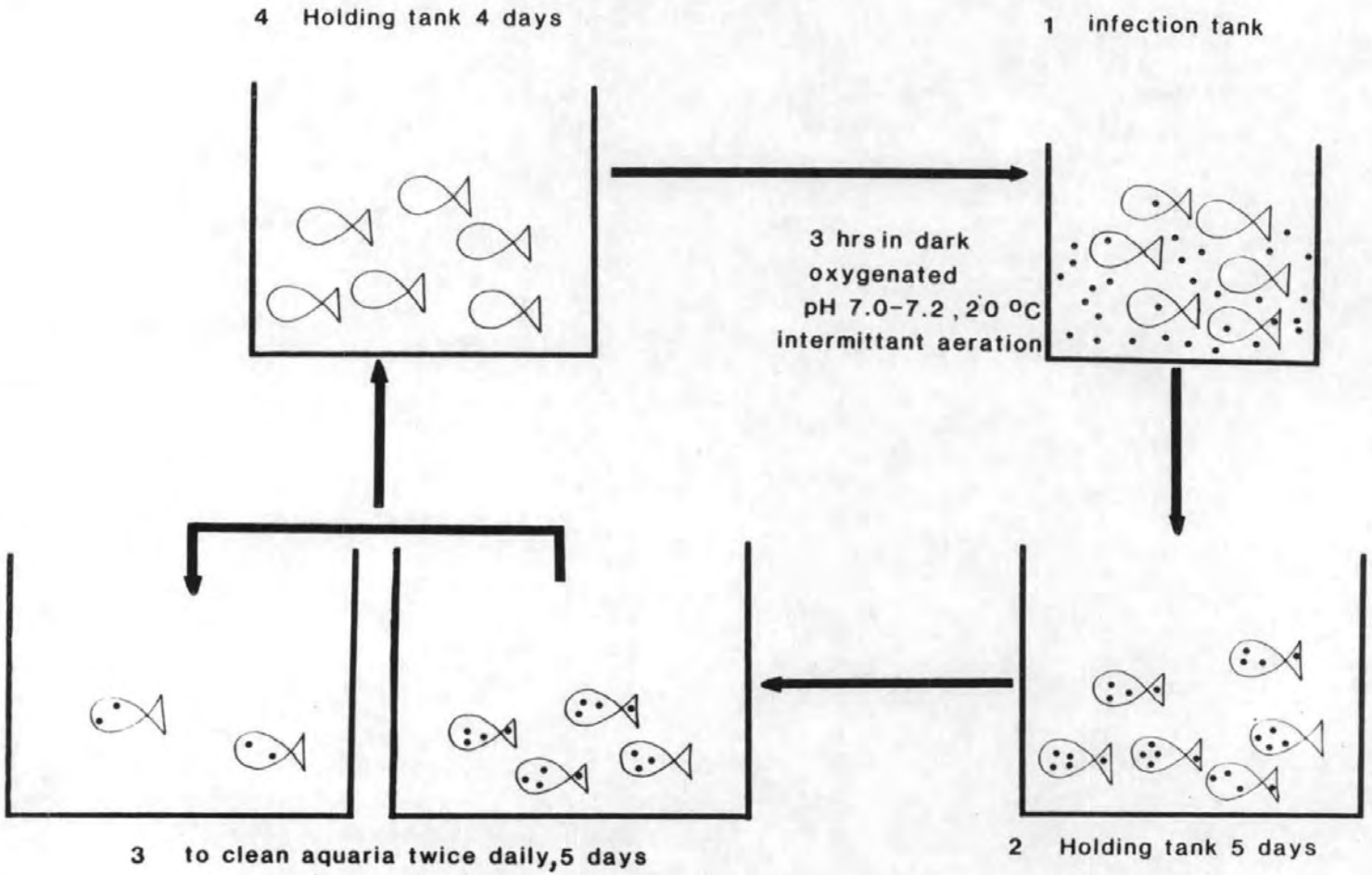
Infection procedures

Experimental infection (standard infection procedure) of fish (Fig.12) was undertaken by transferring carp to small perspex tanks (2 dm^3 capacity) containing an estimated dose of theronts; the latter being

Table 4. Intraperitoneal injection of theronts of *I. multifiliis* into juvenile mullet. Number of fish yielding trophozoites and number of trophozoites recovered. Temperature 20±2 °C.

Group	Time (Days) following injection of theronts	No. of fish	No. of fish with trophozoites	No. of trophozoites
A	8	7	2	9
	15	10	3	12
	21	12	0	0
B	7	10	3	13

Fig. 12. Flow diagram to show procedure used for immunisation of carp with Ichthyophthirius multifiliis.



collected within 5-10 hours of emergence from reproductive cysts. In order to ensure consistency of the procedure, a strict experimental regime was maintained. This included exposure for 3 hours in the dark at a temperature of 20° C, careful maintenance of pH between 7.0-7.2 as relatively small fluctuations from these values was known to effect the viability of theronts, and controlled aeration for 2 hours, starting 30 minutes after introduction of the fish to ensure non-turbulent periods for the theronts. For quantitative studies, numbers of theronts were estimated/cm^{-3} with the aid of a Sedgewick-Rafter counting cell (Dickerson et al, 1981).

Immunisation procedures

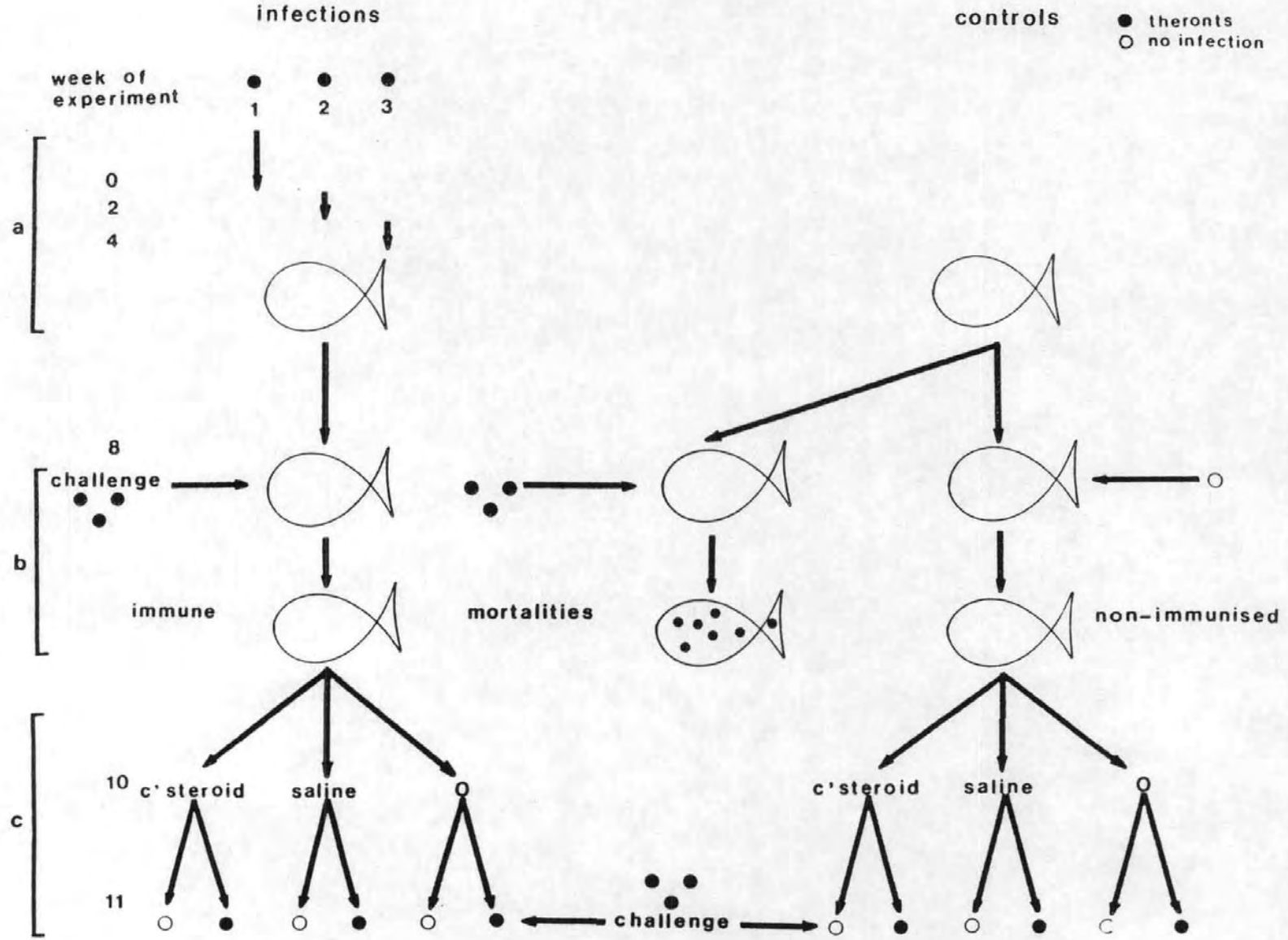
Fish were immunised (standard immunisation procedure) by exposure to theronts at approximate doses of 80/cm^{-3}, 2,000 per fish on 3 separate occasions at 14 day intervals. From days 5-10 following each exposure, re-infection was prevented by transferring fish to clean aquaria at 10-14 hour intervals (as summarised in Fig. 13). By day 10, all trophozoites would have completed their development and therefore, were left behind as free swimming or encysted stages on transfer of fish to aquaria previously disinfected with Hydaine C. Twenty-eight days after the final antigen exposure, fish were challenged (standard challenge procedure) with a potentially lethal dose of approximately 8,000 theronts per fish at a concentration of approximately 320/cm^{-3}. Following challenge, in contrast to immunisation procedures, fish were returned to holding tanks and any infection allowed to continue. Uninfected controls were used to confirm lethal dose on challenge and to check viability of theronts during immunisation.

Fig 13. Flow diagram summarising the procedures used for the immunosuppression of carp immunised with Ichthyophthirius multifiliis.

a) Represents immunisation procedure of exposure to 3 controlled concurrent infections.

b) Tests immunity by challenge

c) Indicates the experimental approach to the establishment of immunosuppression.


infections
controls
theronts
no infection
week of experiment
1
2
3
a
0
2
4
8
challenge
b
immune
mortalities
non-immunised
c
10
c' steroid
saline
0
11
challenge

Immunosuppression

Immunosuppression of Ichthyophthiriasis (standard immunosuppression procedure) was investigated following the administration of the corticosteroid Hydrocortisone 21-hemisuccinate (Cortisol 21-hemisuccinate; 11β,17a,21-Trihydroxy-4-pregnene-3,20-dione 21-hemisuccinate; Sigma UK) or the synthetic corticosteroid Triamcinolone acetonide (9a-Fluoro-16a-hydroxyprednisolone 16a,17a -acetonide; 9a--Fluoro-11β,16a,17a,21-Tetrahydroxy-1,4-pregnadiene-3,20-dione 16,17-acetonide; 1,4-Pregnadiene-9a-fluoro-3,20-dione-11β,16a,17a,21-tetrol 16,17-acetonide; Sigma, UK). Fourteen days after challenge, fish (immunised or control) were administered with one of the above corticosteroids (doses given under appropriate experiments) either as a solution in 0.85% saline (Hudson and Hay, 1980) for Hydrocortisone 21-hemisuccinate or as a suspension in 0.85% saline for Triamcinolone acetonide, by intraperitoneal injection. Additional fish were injected with 0.85% saline or left untreated. Six days after administration of the corticosteroid, half of the immunised fish and half of the non-immunised controls, with representatives from each treatment, were challenged using the procedure previously described. A summary of immunisation and immunosuppression procedures can be seen in Fig. 13.

ANTIGENS OTHER THAN I. MULTIFILIIS

In addition to I. multifiliis, a selection of other antigens were investigated, these were: Tetrahymena pyriformis - both live organisms and extracts, dead bacteria and macromolecules. Further details of these are as follows:

Tetrahymena pyriformis

An axenic culture of T. pyriformis strain CCAP 1630 W Claff, 1939 (w) USA, was obtained in November 1983, from the Cambridge Collection of Algae and Protozoa (Cambridge, UK), and maintained throughout the length of the project. The growth medium consisted of 1% proteose peptone and 0.25% yeast extract (Difco, UK) pH 7.2 (Appendix II). Cultures were grown in 250 cm^3 flasks (100 cm^3 of medium) or 500 cm^3 flasks (200 cm^3 of medium) and kept at 20±2° C, without aeration. The medium was incubated for 24 hours prior to innoculation, to check sterility. The T. pyriformis was subcultured every 7 days, 5 cm^3 into 100 cm^3 or 10 cm^3 into 200 cm^3 of medium. Flasks were incubated at 20±° C, in the dark. Cell density was estimated daily over a period of 18 days on two separate occasions, in order to identify growth phases (Fig. 14). Cell counts were carried out on formalin killed T. pyriformis with the aid of a haemocytometer (improved neubauer).

Freeze dried ciliary antigen (modification of Rosenbaum and Carlson, 1969)

Antigen production always commenced with 7 day cultures of T. pyriformis in the stationary phase of growth (Fig. 14) and adjusted to a concentration of 1×10^6 cells/cm^3. The approximate amount of ciliary antigen obtained from varying volumes of cells is shown in table 5.

Cells were harvested at room temperature by centrifugation at 500 g for 8 minutes in 50 cm^3 conical centrifuge tubes (Sterilin, UK). They were washed twice in sterile PBS pH 7.2 (0.15 M) (Hudson and Hay,1980)

Fig. 14. Growth of T. pyriformis (log cell number per cm^3) with age of culture, showing accelerated increase in number (a), decelerated increase in number (b), stationary phase (c) and decrease in number (d). Temperature $20 \pm 2^{\circ}$ C.

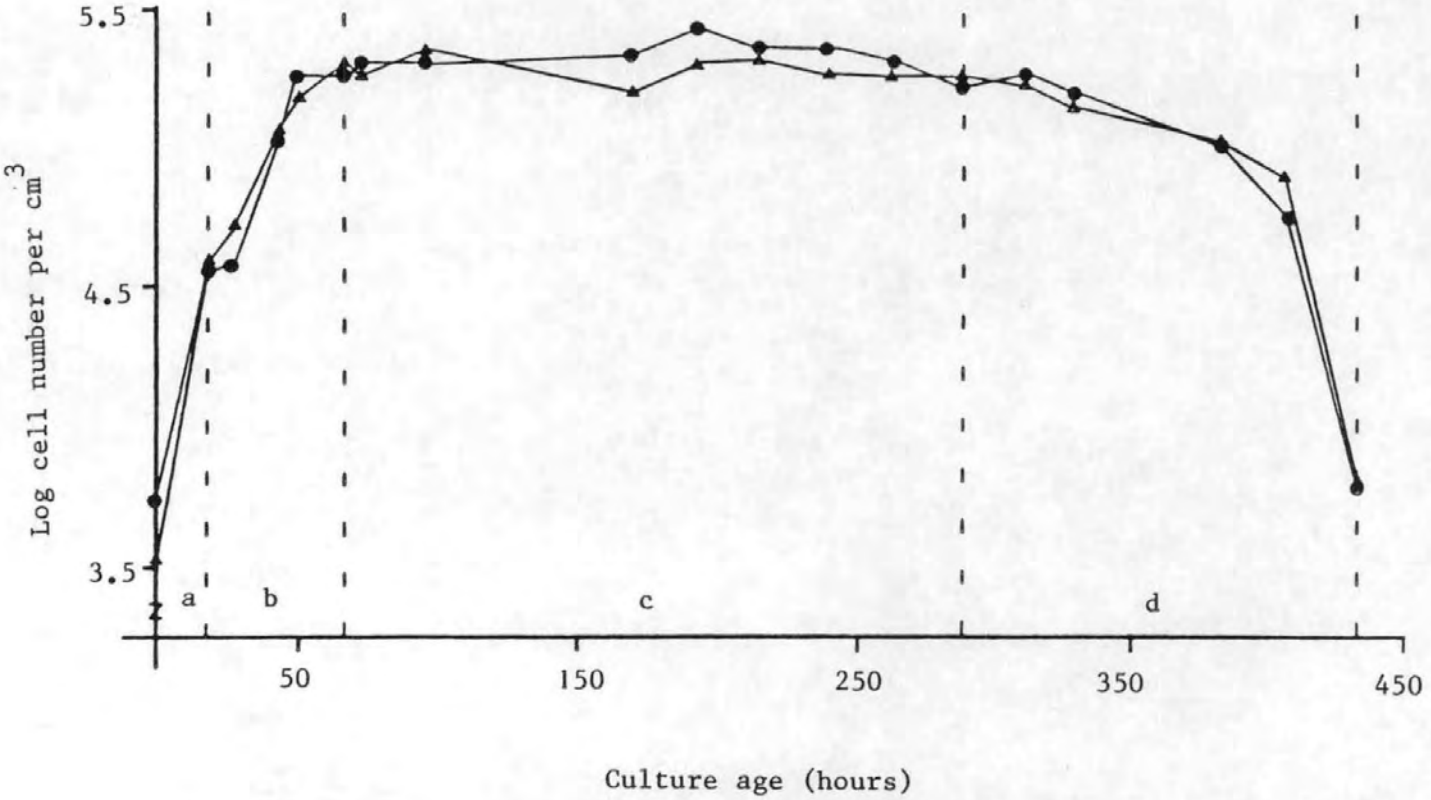


Table 5. Average yield of ciliary antigen of T. pyriformis prepared by modification of the method of Rosenbaum and Carlson (1969). Protein measured by modification of the method of Lowry (Sigma Tech bull, No 690).

Volume of cells cm^{-3}	Mean cell concentration $/cm^3$	Mean ciliary protein concentration μg
400	2.28×10^5	1041.8
500	2.0×10^5	2520.0
600	1.86×10^5	4800.0

resuspended in 5 cm^3 PBS 7.2 and dispensed into aliquots of 2.5 cm^3 at a concentration of approximately 1.5 x $10^6/cm^3$. Deciliation was carried out on ice at approximately 4° C using previously cooled (4°C) sterile solutions. At zero time, 2.5 cm^3 of concentrated cells were added to 5.0 cm^3 of a solution of 10 mM EDTA 0.2 Na (Ethylene diaminetetra-acetic acid, disodium salt; BDH Chemicals, UK) and 50 mM sodium acetate (trihydrate; BDH Chemicals, UK) pH 6.0, in a 50 cm^3 plastic conical centrifuge tube, and mixed by swirling. At 90 seconds, 0.25 cm^3 of 0.2 M calcium chloride (BDH Chemcials, UK) was added and the suspension mixed by inverting the tube several times. At 3.30 minutes, the suspension of cells was subjected to 5 shearings through a 19 g needle fitted to a 5.0 cm^3 plastic syringe. The cells were then concentrated by centrifugation at 1,000 g for 10 minutes in a cooled centrifuge at 4° C (MSE mistral). Cilia were collected in the supernatant without disturbing the pellet of deciliated cells. The procedure was repeated to ensure the removal of any remaining cells. In order to obtain a final product of pure cilia, it was necessary to ensure that all cells had been removed from the final suspension. Direct observations were undertaken on the supernatant under phase contrast microscopy (x 400 and x 1,000 oil immersion) to check that no cells remained in suspension. Similarly, the pellet was examined to ensure adequate or total deciliation of the cells, and that cells were, therefore, non-motile.

The presence of unwanted cells was further examined as follows: the cilia and cell suspension obtained was centrifuged for 10 minutes at 1,000 g. After each centrifugation, 0.1 cm^3 of the supernatant containing cilia was added to 10 cm^3 of growth medium. Under these conditions, deciliated cells, if present, will regrow cilia and multi-

ply. The presence of cells would be detected within 3-5 days by microscopic examination, as significant growth would have occurred within this period. As a control, 0.1 cm^3 of deciliated cells from the centrifuged pellet were also added to 10 cm^3 of growth medium to check that growth had not been impaired by the separation methods.

None of these methods will give 100% assurance that no cells or fractions of cells are present in the cilia suspension. Gibbons (1963) noted that however many times the supernatant was centrifuged, some cells would always be present, but, it does ensure that a reasonable degree of pure cilia are obtained.

Cilia concentration

The supernatant containing the cilia was centrifuged at either 35,000g for 10 minutes, or 14,000 g for 20 minutes, in an MSE 18 high speed centrifuge, at 4° C, to obtain a pellet of cilia. The supernatant was removed and the pellet resuspended and washed twice in sterile distilled water. After the final wash, the cilia were resuspended in 4-6 cm^3 of sterile distilled water, aliquoted into 2 cm^3 quantities and frozen at -20° C. Lyophilization of the cilia was carried out for 12 hours. The lyophilized cilia were stored at -20° C for short-term storage or -70° C for long-term storage. Reconstitution was with sterile 0.85% saline and protein concentration estimated using a sigma total protein concentration kit (No 690-A) (Sigma, UK), based on a modification of the method of Lowry (Sigma Technical Bulletin No 690). All procedures were carried out under asceptic conditions.

Whole T. pyriformis antigen

T. pyriformis strain CCAP 1630/1W Claff (1939) (w) USA, from 7 day cultures in the stationary phase of growth (Fig. 14), were harvested at room temperature by centrifugation at 500 g for 8 minutes in a sterile 10 cm^3 conical centrifuge tube (Sterilin, UK). The concentrated cells were washed twice in sterile PBS pH 7.2 and resuspended in sterile 0.85% saline. The protein concentration was estimated using a sigma total protein concentration kit (No 690-A; Sigma, UK).

Aeromonas salmonocida antigen

A. salmonocida bacteria, Strain 25/77, was grown for 24 hours at 25° C on tryptone soy agar (Oxoid, UK), then innoculated into 10 cm^3 of tryptone soy broth (Oxoid, UK) and incubated for 18 hours at 24° C. 10 cm^3 of the 18 hour culture was then added to 1 dm^3 of tryptone soy broth (10 g/dm^{-3}, autoclaved at 15 psi for 15 minutes), and incubated for 24 hours at 25° C. The cells were then killed with 0.6% formalin (final concentration) solution and incubated at 4° C for 24 hours. Cells were concentrated by centrifugation at 1,000 g for 5 minutes, and washed three times in 0.85% saline. The pellet was resuspended in 0.85% saline to a concentration of 10^{10} cells/cm^{-3} for injecting or 10^{9} cells/cm^{-3} for direct bacterial agglutination, and stored at -20° C.

IMMUNOLOGICAL TECHNIQUES

Raising antisera

Antisera was prepared in rats, rabbits and fish. Three antigens,

namely I. multifiliis, T. pyriformis and rabbit red blood cells were used. All animals were bled before immunisation, to test for any background agglutinating activity.

Carp anti-I. multifiliis

Thirty carp (mean weight 21 g) were maintained at 20±2° C and infected with approximately 4,000 theronts per fish, at a concentration of approximately 20/cm ^{3}, following the standard infection and immunisation procedure described earlier, on three separate occasions of 14 day intervals. The only variation from this procedure concerned the infection and challenge doses. The challenge dose here was increased to approximately 16,000 theronts per fish at a concentration of approximately 80/cm ^{3}. Five weeks later, fish were exsanquinated, serum pooled, and tested (non-heat inactivated) for antibody against I. multifiliis by direct immobilization/agglutination of theronts. The mean titre obtained was $-\log_2$ 10.5.

Rat anti-T. pyriformis antisera

T. pyriformis was harvested from a 7 day culture by centrifugation at 500 g for 8 minutes. Cells were washed twice in PBS pH 7.2 and resuspended in 0.85% saline to give a concentration of 5 mg/cm ^{3} of protein. The cells were ruptured by repeated flushing through a 19 gauge needle fitted to a 5 cm^3 syringe. Four male wistar rats, bred within the Polytechnic, were injected at two weekly intervals for 6 weeks with 0.5 cm^3 of the T. pyriformis suspension. Two of the rats were injected intraperitoneally and two subcutaneously near the ingual lymph node. One rat from each mode of injection was exsanquinated by

cardiac puncture 17 days after the last immunisation; and serum (non-heat inactivated) tested for antibody by direct immobilization/ agglutination of live T. pyriformis. Titres obtained via the intraperitoneal route were $-\log_2$ 10 and by the subcutaneous route, $-\log_2$ 8. Investigation of the 2 remaining rats at 27 days showed little change in antibody level with titres of $-\log_2$ 8 ($-\log_2$ 7 heat inactivated, 56° C 30 minutes) and $-\log_2$ 8 heat inactivated) respectively. The sera was stored at -20° C in 200µl aliquots.

Rabbit anti-T. pyriformis antisera

Cells were prepared as for the rat anti-T. pyriformis antisera. One Dutch rabbit was injected at 2 subcutaneous sites along the dorsal flank with an emulsion of T. pyriformis in Freunds Complete Adjuvant (FCA), 0.5 cm^3 in each site (concentration of T. pyriformis protein, 100 mg/cm^{-3}). Three booster injections in saline were given at 2 weekly intervals. After the second booster, the rabbit was bled from the ear and serum (non-heat inactivated) tested for anti-T. pyriformis antibody by direct immobilization/agglutination of live T. pyriformis. The titre obtained was $-\log_2$ 8 ($-\log_2$ 7 heat inactivated). One week after the third booster, the rabbit was again bled from the ear and serum tested. The titre obtained was $-\log_2$ 9. A week later, the rabbit was exsanquinated, and the titre obtained was $-\log_2$ 9-10. Sera was stored at -20° C in 500 µl aliquots.

Carp anti-T. pyriformis antisera

Four carp (mean weight 300 g) were maintained at 22° C, and injected intraperitoneally with 0.5 cm^3 of a suspension of T. pyriformis

emulsified in FCA at a protein concentration of 2.5 mg/cm^3. Two booster injections in saline were given at 2 weekly intervals. Two weeks after the last booster, 3 of the carp were bled and tested for anti-*T. pyriformis* antibody by direct immobilization/agglutination of live *T. pyriformis*. These all gave titres of $-\log_2$ 6. All fish were given a further booster (concentration 5 mg protein/cm^3) 4 weeks after the last and bled 2 weeks later. Titres obtained were $-\log_2$ 7 for all 4 carp.

Rabbit anti-carp IgM antisera

A modification of the method by Ellis (1980) was selected, using rabbit red blood cells as the antigen.

Three carp (mean weight 2.1 kg), maintained at 20±2° C, were immunised by intraperitoneal injection with 0.5 cm^3 of a 20% concentration of thrice washed rabbit red blood cells (RRBC) in saline (600 g for 5 minutes). The following schedule of immunisation was given: the first injection was given with FCA at week 1, followed by intervals of 3 weeks and 1 week, when injections were given in saline. Fish were challenged 5 weeks after the last booster with a 20% suspension of red blood cells (RBC) in FCA. At week 16, they were bled, serum pooled and heat inactivated (47° C, 20 minutes) and anti-rabbit rbc antibody tested by haemagglutination. The titre obtained was $-\log_2$ 17. Further booster immunisations of 20% RRBC in saline were given after the challenge, at intervals of 1, 3 and 1 months. Haemagglutination titres were raised to $-\log_2$ 22 and 25, 1 month after the last booster.

For immunisation in rabbits, 0.5 cm^3 of heat inactivated carp anti-RRBC

serum was incubated with 10 cm^3 of a 20% suspension of thrice washed RRBC in PBS pH 7.2. Incubation time was 30-60 minutes. The cells agglutinated rapidly and were washed 3 times in PBS 7.2. Some haemolysis did occur despite heat inactivation. Initially, one dutch rabbit was injected at 2 subcutaneous sites along the dorsal flank, with a 50% suspension of RRBC coated with carp IgM, 0.5 cm^3 in each dose, over a period of 14 weeks at 2 weekly intervals. Eleven days after the last injection, the rabbit was bled from the ear and serum tested for specificity against carp IgM by immunoelectrophoresis against whole carp serum. No precipitation due to cross reaction occurred. A second dutch rabbit was then immunised at two weekly intervals for 12 weeks with the same dose previously described. Eleven days after the last injection, the rabbit was bled from the ear and serum tested by immunoelectrophoresis against normal carp serum. Again, no precipitation due to cross reaction occurred. This method was, therefore, abandoned.

Immunoelectrophoresis

Wells were cut in agarose plates (1% w/v agarose in sodium barbitone buffer, pH 8.2, Hudson and Hay, 1980) and 2-4 µl of normal carp serum (antigen) placed in the well. Electrophoresis, in barbitone buffer, pH 8.2 was carried out for 3 hours at 100 volts using a Shandon Southern 600 x 100 electrophoresis unit. Troughs in the agarose plates were filled with rabbit anti-carp IgM (antisera) and allowed to diffuse against the electrophoresed serum overnight. The gels were dried by pressing beneath 5 filter papers, covered by a glass plate with a weight on top. Four pressings were given of 10 minute intervals followed by a wash in 0.1 M sodium chloride and 3 further

pressings. The gels were then dried in a stream of cold air and stained for 5 minutes with coomassie brilliant blue staining solution (Appendix III), destaining was carried out when necessary.

Detection and quantification of antibody activitiy

Three approaches were taken in the detection of antibody activity in fish serum following administration of antigens. These were: direct immobilization/agglutination of whole, live organisms of I. multifiliis and I. pyriformis; direct agglutination of killed A. salmoncida bacterin and haemagglutination of rabbit red blood cells and passive haemagglutination of I. pyriformis.

Direct immobilization/agglutination of I. multifiliis and I.pyriformis

This method has previously been employed for I. multifiliis by Hines and Spira (1973 c, 1974 c), Beckert (1975), Goven et al (1981 b) and Wahli and Meier (1985), and for I. pyriformis by Loeffer and Owen (1958); Margolin et al (1959); Lykakis and Cox (1968); Goven et al (1981 b) and Pyle and Dawe (1985 b). A modified method adapted here was essentially similar for both organisms; theronts being used for I. multifiliis and trophozoites for I. pyriformis. Serial two fold dilutions of non-heat inactivated serum (50 µl), were made along 2 rows (24 wells) in 96 well flat bottomed microtitre plates (Sterilin, UK), using water, pH 7.2, as the diluent for I. multifiliis (50 µl) or PBS pH 7.2 for I. pyriformis. Theronts (50 µl, concentration approximately 2,000 / cm^3) or I. pyriformis (50 µl, concentration approximately 4,000/ cm^3), were added to each well, the plates gently agitated, covered and left at room temperature 20±2° C for 2 hours

before being read. Plates were then left for a further 2 hours before a repeat reading. The *T. pyriformis* was added in proteose peptone medium since previous trials found some natural agglutination occurred in Ringers solution (Appendix III), 0.85% saline and PBS pH 7.2 along with some lysis. As the swimming activity of *T. pyriformis* is often impaired following extended periods of centrifugation, this method also had the advantage in omitting several washings. Controls were also set-up in a similar manner using serum from normal (non-immunised) fish, diluent alone and in addition for the *T. pyriformis*, serum from control fish injected with 0.85% saline. The plates were read with the aid of a stereo microscope at 450x magnification. A positive reaction was indicated by immobilization/agglutination of more than 50% of *I. multifiliis* theronts, or *T. pyriformis* on the bottom of the well. A negative reaction was indicated by less than 50% of organisms being immobilized/agglutinated or by organisms being free swimming. The amount of antibody activity was graded as follows:

Positive	+++	100% immobilization/agglutintion
	++	75% immobilization/agglutination
	+	50% immobilization/agglutination
Negative	±	<50% immobilization/agglutination
	0	0% immobilization/agglutination

Direct agglutination of killed A. salmonocida bacterin and Rabbit red blood cells

Antibody against A. salmonocida or Rabbit red blood cells, (RRBC) was detected by direct agglutination of formalin killed cells of the former and whole red blood cells of the latter. Serial two fold dilutions (50 µl) along 2 rows (24 wells) of heat inactivated serum (47° C, 20 minutes) were made in 96 well, U /well microtitre plates (Sterilin, UK). Diluents used were 0.85% saline as the diluent for A. salmonocida (50 µl) and, initially, carp serum diluent (1% heat inactivated, absorbed with packed RRBC, 1 hour, pooled normal carp serum in 0.85% saline) for the RRBC, but this resulted in a large amount of non-specific agglutination, so this was replaced with rabbit serum diluent (1% heat inactivated (56° C; 30 minutes), absorbed, rabbit serum in saline). For the A. salmonocida, cells (50 µl) at a concentration of 1 x 10^9 in saline, were added to each well, the plates gently agitated, covered and kept at room temperature (20±2° C) for 3 hours before being put at 4° C overnight. For the Rabbit red blood cells, rbc's were washed 3 times in PBS pH 7.2 and used at a concentration of 0.25% in serum diluent, 50 µl being added to each well. Plates were gently agitated, covered and kept at room temperature for 3 hours and then 4° C overnight. For both antigens, controls were set up in a similar manner and included serum from normal (non-immunised) fish, control fish injected with 0.85% saline and diluent. The plates were read the following day using a stereomicroscope at 450x magnification or by eye. The end point was the last well to show full agglutination, indicated by a layer of cells in the well. A negative result was seen as a button of cells in the bottom of the well. Originally the Rabbit red blood cells were kept

in heparin in PBS pH 7.2, but, a large amount of non-specific agglutination occurred. Cells were, therefore, kept in Alsevers solution (Gibco, UK) which was found to reduce non-specific agglutination.

Passive haemagglutination of T. pyriformis

Several methods were examined to absorb antigen from T. pyriformis onto sheep red blood cells (SRBC) to use in a passive haemagglutination test to test serum for antibody against T. pyriformis. The following method, which was a modification of the methods used by Boyden (1951); Stavitsky (1954) and Sailendri and Muthukkaruppan (1975), proved to be successful.

Sheep rbc's (Tissue Culture Services, UK) in Alsevers solution, were washed 3 times with saline and resuspended to 8% (v/v) in PBS pH 7.2. An equal volume of cells were then added to an equal volume of 1.2% formaldehyde in PBS pH 7.2, adjusted with 0.1 N NaOH, mixed thoroughly in a 50 cm^3 centrifuge tube and incubated at 37° C for 24 hours, with intermittent mixing. The formalinized cells were washed 3 times in PBS pH 6.4 (Appendix III), resuspended in PBS pH 7.2 to 2% and treated with tannic acid by mixing with an equal volume of 0.005% (w/v) tannic acid in saline for 10 minutes at 37° C. Cells were then washed 4 times in PBS pH 7.2, and resuspended to 3%.

T. pyriformis (400 cm^3, 7 day culture) were harvested by centrifugation at 500 g for 8 minutes, washed twice in PBS pH 7.2 and resuspended to a volume of 26 cm^3. The organisms were then sonicated (3 x 2 seconds) on ice, and centrifuged at 500 g for 8 minutes to

separate the supernatant. The supernatant and the particulate matter were both tested, but the supernatant produced less non-specific agglutination so was chosen in preference.

The SRBC (3%) were incubated with the supernatant antigen in the following ratios: 4 volumes PBS pH 6.4 : 1 volume antigen: 1 volume treated SRBC. Control treated SRBC were incubated with saline in place of the antigen. Incubation was for 2 hours at 37° C, after which, the cells were washed 3 times in PBS pH 7.2, with a final wash in serum diluent. The serum diluent comprised pooled normal carp serum, heat inactivated at 47° C for 20 minutes, and absorbed with an equal volume of packed SRBC for 60 minutes to remove any non-specific haemagglutinating activity. The serum was then diluted to 1% (v/v) with saline. The coated SRBC were resuspended in serum diluent to a concentration of 0.3%. Test and control sera were previously heat inactivated and absorbed with packed SRBC to remove complement and any non-specific haemagglutinating activity. Serial two-fold dilutions of serum (50 µl) were made along 2 rows (24 wells) in 96 well, U-well microtitre plates (Sterilin, Teddington, UK), using serum diluent as the diluent (50 µl). Coated SRBC (50 µl) were then added to each well, the plates gently agitated and left at room temperature for 3 hours before being placed at 4° C overnight. Controls were also set-up in a similar manner, using uncoated SRBC, and also serum from normal (non-immunised) fish, and serum diluent alone. When tested against rabbit anti-T.pyriformis antisera, the 1% carp serum diluent was replaced with 1% rabbit serum diluent, prepared in the same way as for the carp, but the rabbit serum was heat inactivated at 56° C for 30 minutes.

The activity of the coated cells was investigated with time following

different methods of storage. The cells were either: kept at 4° C in preservative and tested 2 weeks after coating with T. pyriformis antigen, or frozen at -20° C in preservative and tested 12 weeks after coating with T. pyriformis antigen. Tests following both methods of treatment and storage indicated no reduction in activitiy. The preservative consisted of: PBS pH 7.2 containing 5% sucrose, 1.5% normal rabbit serum and 0.1% sodium azide.

Autoradiography

The isotope tritium labelled thymidine was selected in view of its uptake by DNA in dividing cells, thus indicating lymphoid cell proliferation. The isotope was obtained from the Radiochemical centre, Amersham, Bucks, having a specific activitiy of 2Ci/mmol.

Preparation of autoradiographs

Autoradiographs were prepared by dipping sections in liquid emulsion following the method of Rogers (1967) and the modifications for use with fish tissue recommended by Secombes (1981), Grace (1981) and Botham (1982). Using emulsion enables large batches of slides (200-400) to be dipped in one session, thus avoiding variability amongst batches. Fish were injected intraperitoneally, using a 25 g needle with tritium labelled thymidine (Methyl 3H thymidine) in 0.85% saline. The activity injected into each fish was approximately 1-2 $\mu Ci g^{-1}$ body weight. Controls for background activity were injected with saline. All were killed 18 hours later and the lymphoid organs removed:- spleen, pronephros, opisthonephros and, in some cases, liver as a control to check for somatic growth. Organs were initially

fixed in Carnoy's fixative (Bancroft and Cook, 1984), this being substituted later with 10% neutral buffered formal-saline (Drury and Wallington, 1980) as this reduced tissue shrinkage. Fixation in formal--saline was for 3 days, followed by dehydration through an ascending series of alcohols, cleared - initially in xylene, but this was later changed to chloroform, and embedded in fibrowax. Sections were cut at a thickness of 5 µm as recommended by Rogers (1967), Bancroft and Stevens (1982) and D. Bucke (personal communication), and mounted on chrome-gelatin subbed slides. Subbed slides were prepared by cleaning slides in concentrated Hydrochloric acid (BDH Chemicals, UK) overnight followed by several rinses in tap water then distilled water. Slides were then dipped in chrome-gelatin solution containing 5 g gelatin and 0.5 g chrome alum dissolved in 1 dm^3 of distilled water. Slides were air dried and kept at 4° C. Processing of sections before dipping in emulsion involved dewaxing in two changes of xylene, 5 minutes in each and taken to water through a descending series of alcohols, 3 minutes in each. Slides were transported to the darkroom in distilled water. Safe lighting using an Ilford 5902 light, was used throughout the following procedure. The amount of emulsion needed for up to 250 slides was prepared as follows. Using a plastic spoon, Ilford emulsion in gel form, K5 (Ilford Nuclear Research, UK) was put in a measuring cylinder in a water bath (43° C) to give a melted volume of 24 cm^3. The melted emulsion was put in a coplin jar to which was added a 1:50 dilution of glycerol at a volume of 24 cm^3 (23.5 cm^3 distilled water, 0.5 cm^3 glycerol). The diluted emulsion was stirred gently with a glass rod for 5 minutes, ensuring a thorough mixing. Care was taken to avoid too many bubbles. A clean test slide (no section), was dipped into the emulsion to check for bubbles and an even coating. The emulsion was left until no bubbles were present. Experimental slides

were dipped into the emulsion twice, the backs of the slides wiped with a tissue and the slides then dried on a cold surface (section side uppermost), prepared by placing aluminium trays over ice. Drying time was between 3-5 hours; drying was aided by use of cold air from a fan. Slides were put on slide trays, 5 per tray, and packed in boxes. Three layers of silica gel were placed on slide trays in the boxes and the boxes were then wrapped in a double layer of black polythene to exclude light from the slides, and placed at 4° C. Sample slides were packed separately and initially, developed at weekly intervals to assess the degree of development of the silver grains. It was found that 4 weeks gave good autoradiography and this period was, therefore, adopted as standard.

Development

Slides were unpacked in the darkroom using a safelight and placed in a slide rack in tap water. Slides were developed in D-19 developer (Kodak UK) for 4 minutes, rinsed in 2 changes of tap water to remove excess D-19 and then placed in fixer for 5 minutes at a dilution of 1:6 AMFIX (May and Baker, UK) to which 'S' type hardener (May and Baker,UK) was added at a dilution of 1:40. After fixing, slides were placed in running tap water for 15 minutes before exposing to the light. Sections were stained with methyl green pyronin (R. Lamb, UK) for 30-- 60 minutes, and dehydrated in tertiary Butyl Alcohol (2-Methyl propan 2-ol; BDH Chemicals, UK).

Photography

Live fish

Individual fish were lightly anaesthetised and placed in a small glass aquarium, care taken to avoid scratches to the glass. The aquarium was placed on a black background and illuminated with one electronic flash-gun held directly over the top of the aquarium. Photographs were taken with a Pentax 35 mm camera fitted with a micro-takumar 50 mm lens, images were recorded on Pan F film (50 ASA, Ilford, UK) and developed in Microphen developer, 4½ minutes at 20° C, and printed on grade 3 Ilford Ilfospeed paper.

Photomicrography

Photographs of live I. multifiliis in the caudal fin, and live or formalin fixed I. multifiliis or I. pyriformis were taken on a Zeiss standard microscope fitted with a Zeiss MC63 automatic camera. Bright field and phase contrast images were recorded on Pan F film (50 ASA).

Statistical analysis

Statistical analysis using two sample analysis was used ($P<0.05$). In immunisation studies with I. pyriformis, results were analysed by Relative Percent Survival (RPS) as used by Amend (1981) and Ward (1982).

$$RPS = 1 - \frac{\%\ \text{mortality of vaccinated fish}}{\%\ \text{mortality of controls}} \times 100$$

Vaccinated fish should show a RPS of >60% (Amend, 1981) or >70% (Ward, 1982) for a vaccine to have been successful.

EXPERIMENTS AND RESULTS

Results are covered under three main headings; Ichthyophthirius multifiliis, Tetrahymena pyriformis and the effect of immune serum on I. multifiliis and T. pyriformis.

ICHTHYOPHTHIRIUS MULTIFILIIS

A total of 33 experiments have been undertaken in the investigation of basic aspects of the immune response. These are considered under growth, infection, immunisation, immunosuppression and latancy.

Growth and infection

EXPERIMENT 1. In vivo growth of Ichthyophthirius multifiliis in the caudal fin of carp at 20° C.

Forty juvenile carp, age 10 weeks, obtained from Sparsholt (spawned 30.1.85), were allowed to acclimate in aquaria for 2 weeks at 20° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 12 weeks, the mean weight of the fish was 1.08 g.

All fish were exposed to a dose of approximately 2,000 theronts per fish at a concentration of approximately 80/cm^{3}, for 30 minutes in the dark without aeration.

Following exposure to theronts, fish were returned to holding tanks and 5 fish, immediately following infection and at daily intervals up to 6

days, were anaesthetised and the caudal fin examined. Trophozoites were measured with the aid of a Zeiss microscope, x160 magnification. Ten trophozoites on each fish were measured, and the mean diameter calculated.

The results can be seen in Table 6 and Fig. 15. Parasite growth was linear up to day 6, at which stage the parasites began to leave the fish host. The size of parasites was from 31.8 µm immediately following infection with growth up to 397 µm by day 6, with a range in size from 286-522 µm. Fig. 16 shows the size of parasites within the caudal fin.

EXPERIMENT 2. Development of trophozoites in juvenile carp exposed to different doses of theronts.

Forty juvenile carp age 9 weeks of similar size (mean 3.1 cm) obtained from Driffield (spawned 18.4.84), were separated into 4 groups, A, B, C and D, each containing 10 fish. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment at 11 weeks, the mean weight of the fish was 880 mg.

Fish in all groups were exposed to the following doses of theronts of I. multifiliis following the standard infection procedure as described in the Materials and Methods section:-

Group A approximately 1,000 theronts per fish at a concentration of approximately $40/cm^{3}$.

Table 6. *In vivo* growth of *Ichthyophthirius multifiliis* at 20 °C. Dimensions of parasites with time.

Time (days) post exposure to theronts	Diameter µm mean ±SD	Range µm
0	31.8±3.427	24-36
1	46.5±1.11	36-54
2	69.7±7.93	54-78
3	106.5±14.28	72-132
4	234.9±21.08	198-282
5	326.1±69.48	240-456
6	397±41.59	286-522

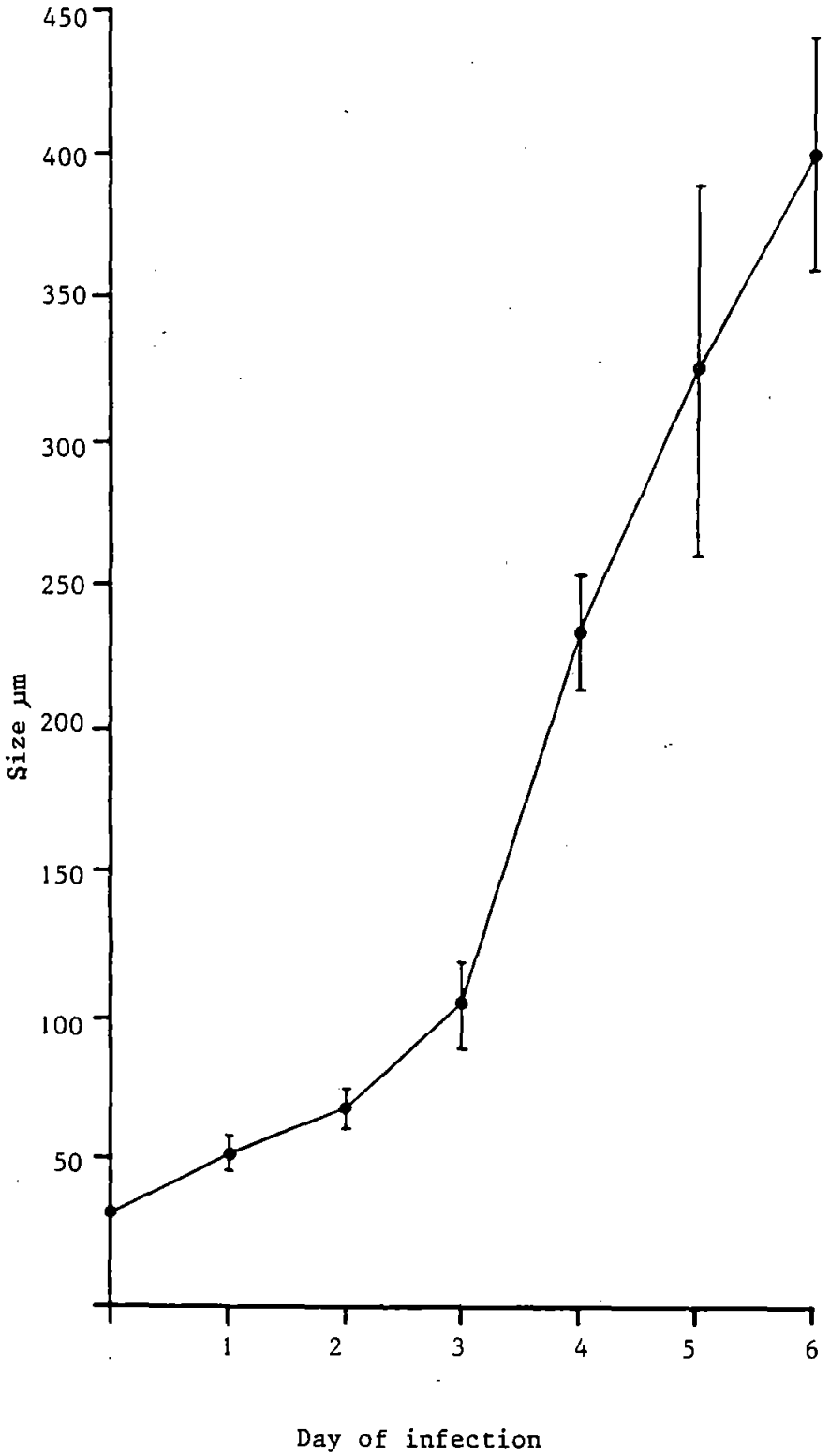


Fig. 15. In vivo growth of trophozoites of I. multifiliis with time, temperature 20 °C.

Fig. 16. Showing *in situ* growth of trophozoites of *Ichthyophthirius multifiliis* within the caudal fin of juvenile carp following an experimental infection. All to the same magnification, scale, 30 µm.

A Trophozoite immediately following penetration.

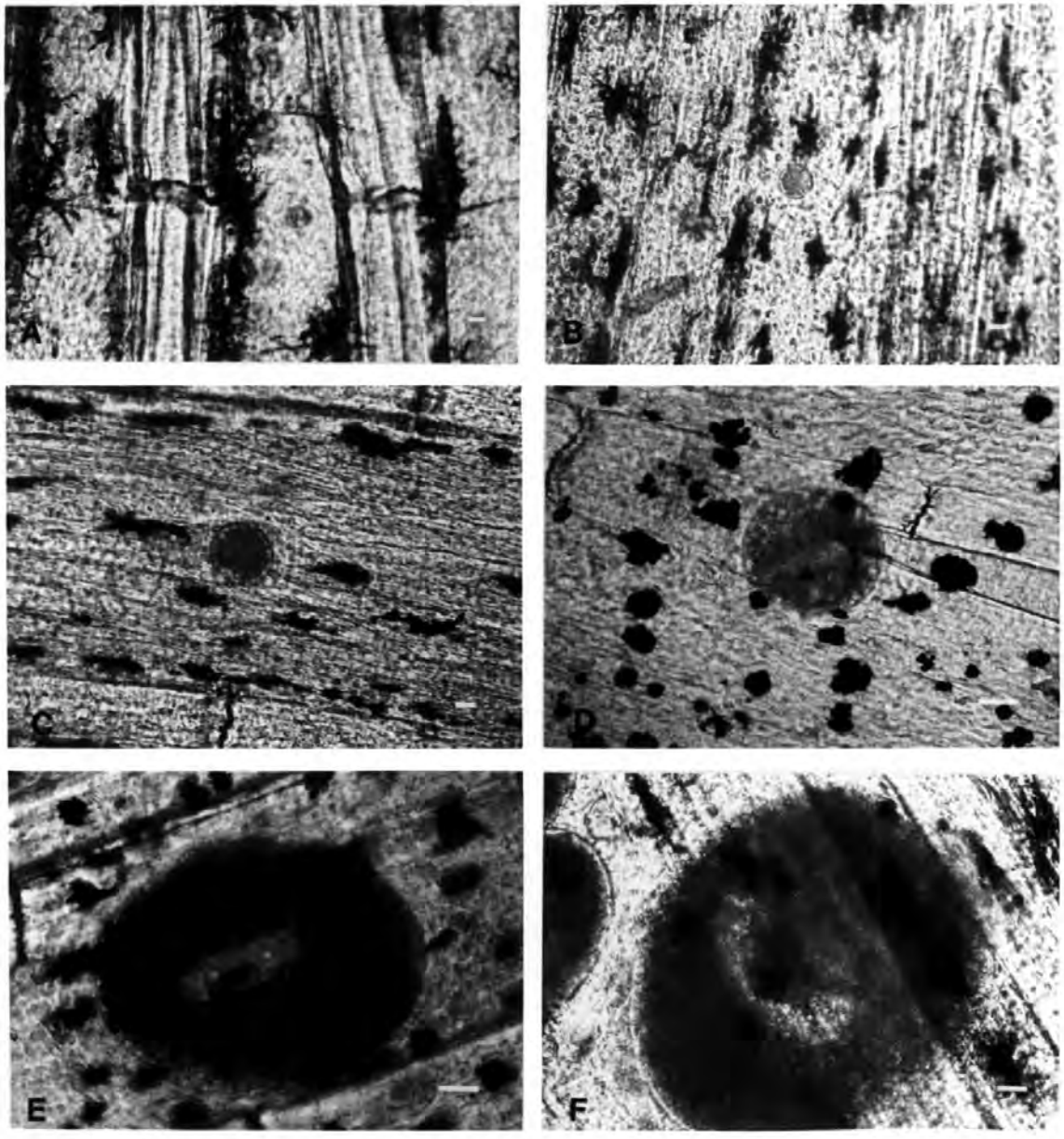
B 24 hours

C 48 hours

D 72 hours

E 96 hours

F 120 hours


A
B
C
D
E
F

Group B approximately 2,000 theronts per fish at a concentration of approximately 80/cm^{3}.

Group C approximately 4,000 theronts per fish at a concentration of approximately 160/cm^{3}.

Group D approximately 8,000 theronts per fish at a concentration of approximately 320/cm^{3}.

Following exposure, fish were transferred to aquaria until day 4, when they were killed and developing parasites counted with the aid of a stereomicroscope. Only those parasites present in the fins and body were counted, not those in the gills or buccal cavity.

The results can be seen in tables 7, 8 and 9. A heavy infection occurred in Group D, which were infected with 8,000 theronts per fish, with the parasites being too numerous to count. In Group A, (table 7) exposed to approximately 1,000 theronts per fish, the mean number of parasites developing in the body was 72.75 and in the fins was 51.75. The overall mean for the body and fins was 124.5 that is 12.5%. For Group B, (table 8) exposed to approximately 2,000 theronts per fish, the mean number of parasites developing in the body was 130.7 and in the fins was 58.7. The overall mean for the body and fins was 189.4, that is 9.4%. For Group C, (table 9) exposed to approximately 4,000 theronts per fish, the mean number of parasites developing in the body was 641.3 and in the fins was 379.2. The overall mean for the body and fins was 1020.0, that is 25.5%.

Table 7. Number of trophozoites of *I. multifiliis* developing on the body and fins of juvenile carp at day 4 following exposure to approximately 1 000 theronts per fish, 40/cm ^{3}, temperature 20±2 °C.

Fish No	Area of fish. No of parasites							
	Body		Fins					
	Left	Right	Caudal	Anal	Dorsal	Pectoral	Pelvic	Total
1	54	56	14	4	14	20	0	162
2	47	27	73	1	17	10	5	180
3	31	27	18	2	8	18	5	119
4	71	66	31	6	25	19	4	222
5	29	23	3	0	4	19	1	69
6	24	36	11	7	17	13	5	113
7	23	37	17	1	13	2	1	94
8	9	22	0	1	4	1	0	37
Mean ±SD	36.7 ±19.9	36.7 ±16.1	20.8 ±23.1	2.8 ±2.6	12.8 ±7.20	13.0 ±6.5	2.6 ±2.3	124.5 ±60.7

Mean number in body = 72.75.
Mean number in fins = 51.75

Table 8 . Number of trophozoites of *I. multifiliis* developing on the body and fins of juvenile carp at day 4 following exposure to approximately 2 000 theronts per fish, 80/ cm^{-3}. Temperature 20±2 °C.

Fish No	Body		Area of body. No of parasites. Fins					
	Left	Right	Caudal	Anal	Dorsal	Pectoral	Pelvic	Total
1	102	91	57	2	2	26	6	286
2	56	53	19	8	8	3	1	148
3	26	16	9	2	16	3	3	75
4	79	82	27	2	16	33	3	242
5	51	59	29	9	3	8	8	167
6	141	152	41	10	48	8	3	403
7	34	61	25	6	2	16	2	146
8	81	159	60	3	30	3	4	340
9	18	26	3	0	0	1	0	48
10	5	15	16	0	1	0	2	39
Mean ±SD	59.2 ±41.9	71.5 ±51.4	28.6 ±19.0	4.2 ±3.7	12.6 ±15.7	11.2 ±11.42	3.2 ±2.3	189.4 ±124.8

Mean no in body = 130.7
Mean no in fins = 58.7

Table 9 . Number of trophozoites of I. multifiliis developing in the body and fins of juvenile carp at day 4 following exposure to approximately 4 000 theronts per fish, 160/ cm^{-3}. Temperature 20±2 °C.

Fish No	Area of body. No of parasites. Body		Fins					
	Left	Right	Caudal	Anal	Dorsal	Pectoral	Pelvic	Total
1	553	436	272	28	96	28	16	1429
2	431	430	222	22	95	52	43	1295
3	264	320	267	21	47	50	15	984
4	271	196	89	19	22	16	0	613
5	318	360	276	45	87	105	55	1246
6	225	213	155	26	72	36	4	731
7	242	263	221	9	29	31	12	807
8	405	381	312	51	88	37	23	1297
9	255	209	172	25	66	25	31	783
Mean ±SD	329.3 ±110.6	312 ±95.3	220.6 ±70.9	27.3 ±13.0	63.5 ±26.2	42.2 ±26.1	22.1 ±18.0	1020.0 ±300.5

Mean no in body = 641.3
Mean no in fins = 379.2

EXPERIMENT 3. Immunisation of juvenile carp with Ichthyophthirius multifiliis. Induction of immunity following 3 serial infections and a potentially lethal challenge.

One hundred juvenile carp age 8 weeks obtained from Driffield (spawned 18.4.84) were separated into 2 groups, A and B. Group A contained 50 fish and Group B, 50. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks, the mean weight of the fish was 950 mg.

Fish in Group A were exposed to 3 infections of I. multifiliis following the standard procedures of infection and immunisation. Twenty-eight days after the last immunisation, fish were exposed to a potentially lethal challenge dose following the standard challenge procedure. At each immunisation 10 fish from Group B and at challenge, 20 fish from Group B were also exposed to theronts to test for viability of theronts.

It can be seen from the results shown in table 10, that, after immunisation with controlled infections of I. multifiliis, all immunised fish (Group A) survived following challenge. Only 10% of the immunised fish developed infections. These were very light and restricted to the periphery of fins and no mortalities occurred. All non-immunised controls (Group B) subjected to the challenge dose, became infected with a medium infection, and suffered 100% mortalities.

Table 10. The effect of a potentially lethal challenge of *I. multifiliis* on juvenile carp following immunisation with 3 serial infections of the parasite. Temperature 20 ±2 °C.

Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality
A	Immunised	40	10	very light - periphery of fins	0
B	Control	20	100	medium - entire body	100

EXPERIMENT 4. The humoral response of juvenile carp following one exposure to Ichthyophthirius multifiliis, and after a potentially lethal challenge following immunisation with I. multifiliis.

Five hundred and eighty juvenile carp age 8 weeks obtained from Stowmarket (spawned 8.2.86), were separated into 4 groups A, B, C and D. Group A contained 140 fish, Group B, 70 and C and D each contained 180 fish. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks, the mean weight of the fish was 765 mg.

Fish in Group A were exposed to one infection of I. multifiliis following the standard infection procedure and immunisation dose as described in the Materials and Methods section. Fish in Group B were maintained as uninfected controls. At weeks 2, 3, 4, 6, 8, 10 and 12 after infection, 20 fish from Group A and 10 from Group B were exsanquinated. Serum was pooled from groups of 10 fish within each group and tested (non-heat inactivated) for antibody against I. multifiliis using direct immobilization/agglutination of live theronts.

Fish in Group C were exposed to 3 infections of I. multifiliis following the standard procedures of infection and immunisation, with the exception that the 3rd immunising dose consisted of approximately 4,000 theronts per fish at a concentration of approximately 160/ cm^3

This increased dose was to compensate for a drop in infectivity of the theronts. Twenty-eight days after the last immunisation, fish were

exposed to a potentially lethal challenge dose following the standard challenge procedure. At each immunisation and challenge, 20 fish from Group D were also exposed to theronts. At weeks 0, 1, 2, 3, 4, 6, 8, 10 and 12 after challenge, 20 fish from Group C and 10 from Group D were exsanquinated, with the exception of weeks 0, 1 and 3 when only 10 fish were used from Group C. Serum was pooled from groups of 10 fish within each group and tested (non-heat inactivated) for antibody against I. multifiliis using direct immobilization/agglutination of theronts.

It can be seen from the results (Table 11) that, after immunisation with controlled infections of I. multifiliis, all immunised fish (Group C) survived following challenge. Only 2% of the immunised fish developed infections. These were very light and restricted to the periphery of fins and no mortalities occurred. All non-immunised controls (Group D) subjected to the challenge dose, became infected with a medium to heavy infection, and 100% mortalities. Cumulative mortality (%) is shown in Fig. 17 . Deaths occurred between days 6 and 13, following termination of primary infection and secondary re--invasion, and during the secondary infection.

The mean antibody titres of carp after one infection of I. multifiliis (Group A) are shown in Fig. 18 . The kinetics of the response were studied over a period of 12 weeks. Results indicate a gradual increase in titre with antibody first detected at week 2 ($-\log_2$ 1.5) with the peak response being observed at week 6 ($-\log_2$ 7.5).

After this, antibody titre began to decrease to $-\log_2$ 4.5 by week 12, when the experiment was terminated. Sera from untreated controls

Table II. The effect of a potentially lethal challenge of *I. multifiliis* on juvenile carp following immunisation with three serial infections of the parasite. Temperature 20±2 °C.

Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality	Mean days to death (±SD)
A	Immunised	140	2	very light - periphery of fins	0	N/A
B	Control	40	100	Medium-heavy entire body	90	10.6±2.2

Fig. 17. Cumulative mortality (%) for non-immunised controls following a potentially lethal dose of I. multifiliis (8,000 theronts/fish, 320/cm ^{3}). Temperature 20±2°C.

P primary, S secondary infections of I. multifiliis.

Fig. 18. Kinetics of the humoral antibody response of juvenile carp to I. multifiliis following a single exposure to the parasite and following immunisation and exposure to a potentially lethal challenge dose. Temperature 20±2°C.

● single exposure to I. multifiliis.

▲ immunisation and challenge exposure to I. multifiliis.

Fig. 17.

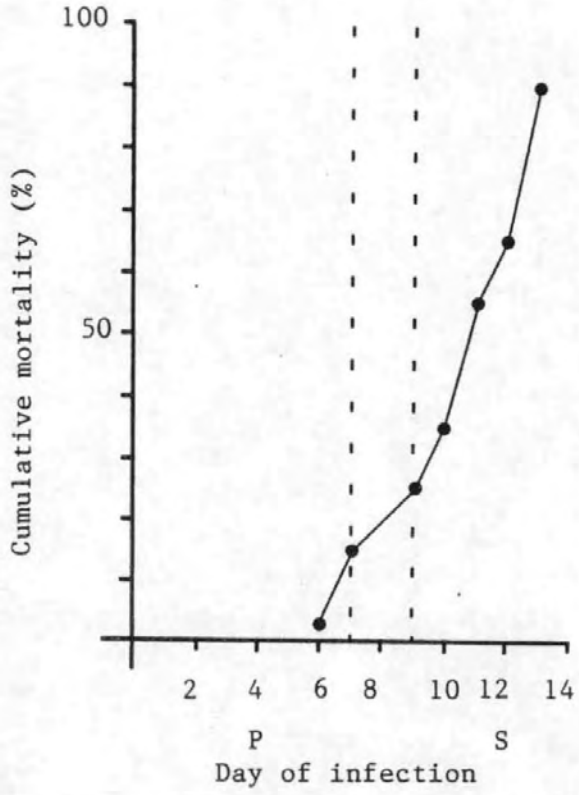
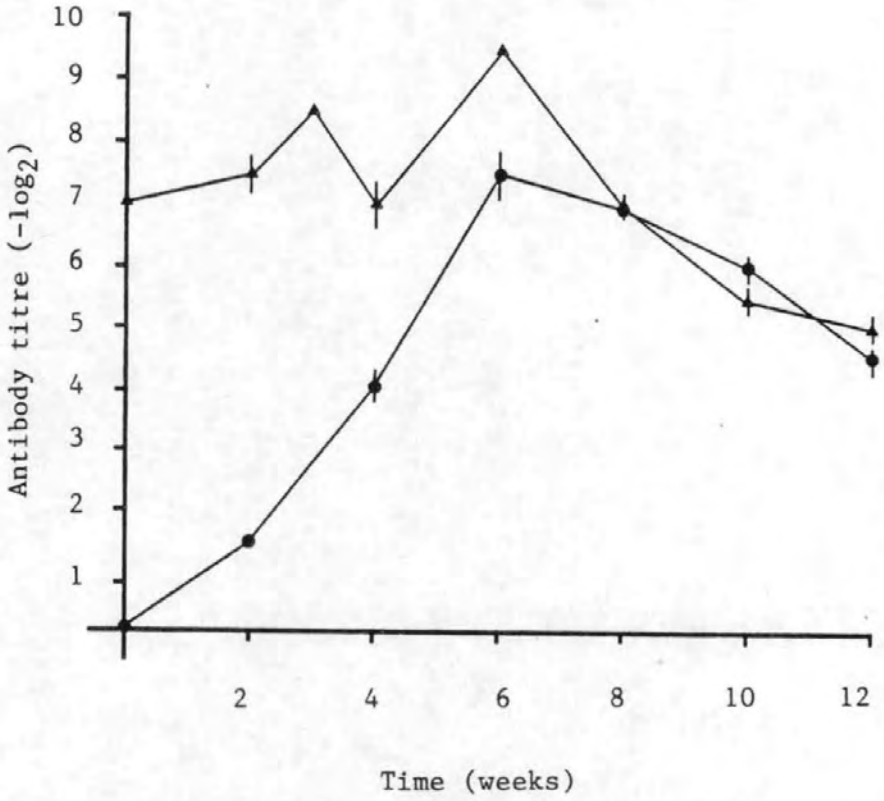

100
50
Cumulative mortality (%)
2
4
6
8
10
12
14
P
S
Day of infection

Fig. 18.


10
9
8
7
6
5
4
3
2
1
Antibody titre (-log2)
2
4
6
8
10
12
Time (weeks)

(Group B) were negative to I. multifiliis and showed no natural agglutinins.

Following immunisation and exposure to a potentially lethal challenge infection of I. multifiliis (Group C), antibody titre (see Fig. 18) at the time of challenge was already high ($-\log_2$ 7). This titre increased to a peak response at week 6 ($-\log_2$ 9.5) following challenge, after which the titre began to decrease to a titre of $-\log_2$ 5.5 at week 12, when the experiment was terminated. Sera from non-immunised controls (Group D) were negative to I. multifiliis and showned no natural agglutinins.

EXPERIMENTS 5 AND 6 The humoral immune response of juvenile carp to Ichthyophthirius multifiliis following immunisation with theronts.

A further 2 experiments were performed to measure the humoral response following immunisation and challenge exposure to theronts. Information concerning the source of fish, age and weight at the start of the experiments can be seen in table 12.

All fish were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. The standard procedures of infection, immunisation and challenge as described in the Materials and Methods section were used with the exception that in experiment 5, the challenge dose consisted of approximately 8,000 theronts per fish, but at a concentration of approximately $160/cm^{-3}$ to compensate for the increased size of the fish.

Table 12. Number, size and source of fish used in investigations on the humoral antibody response of juvenile carp to I. multifiliis.

Experiment	No. of fish		Source of fish	Date spawned	Start of experiment	
	Immunised	Non-immunised controls			Age (weeks)	Mean weight (mg)
5	40	70	Driffield	17.4.85	14	1254
6	60	80	Stowmarket	20.9.85	11	800

In experiment 5, at weeks 1, 2, 3, 4 and 6 following challenge exposure, 5 immunised fish at each time were exsanquinated and pooled sera (non-heat inactivated), tested for antibody to I. multifiliis by direct immobilization/agglutination of live theronts.

In experiment 6 at weeks 4, 8, 12 and 16 following challenge exposure, 10 immunised fish at each time were exsanquinated and pooled sera (non-heat inactivated) tested for antibody to I. multifiliis by direct immobilization/agglutination of live theronts.

At each immunisation and challenge, 10 non-immunised controls in each experiment were also exposed to theronts. Also, at the same time that immunised fish were exsanquinated, 5 (experiment 5) or 10 (experiment 6) non-immunised fish were also exsanquinated and pooled sera (non--heat inactivated) tested for antibody to I. multifiliis.

Mean antibody titres for experiment 5 can be seen in Fig. 19. At week 1, enhanced antibody titres were present of $-\log_2$ 8, which, after an initial decrease, rose to $-\log_2$ 11.5 at week 6.

The mean antibody titres of carp following immunisation and challenge with I. multifiliis for experiment 6 can be seen in Fig. 20. At week 4, antibody titres were already enhanced but rose to a peak of $-\log_2$ 7.5 at week 8 after which, levels began to decrease to a titre of $-\log_2$ 3 at week 16. In both experiments, non-immunised controls showed no antibody to I. multifiliis.

Fig. 19.

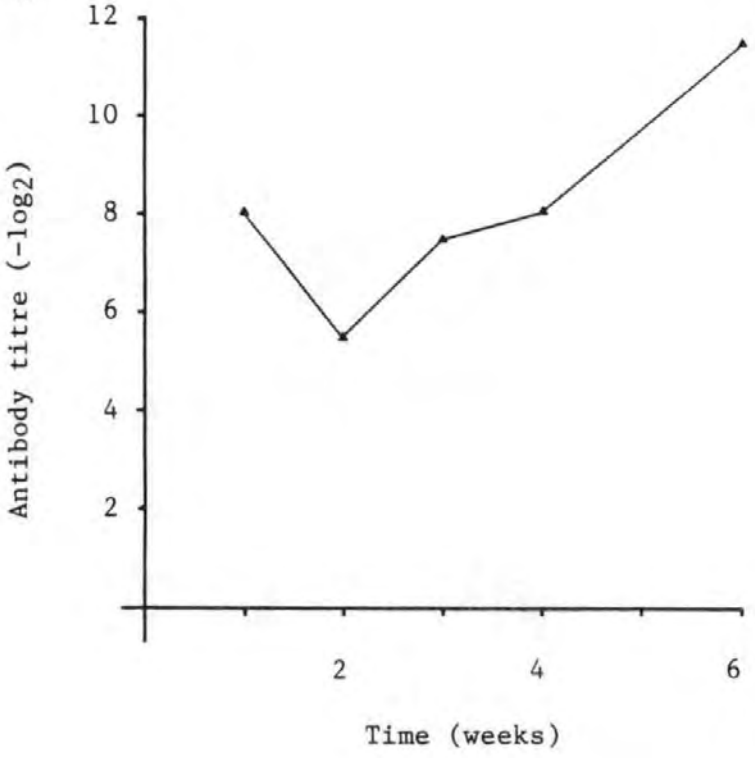
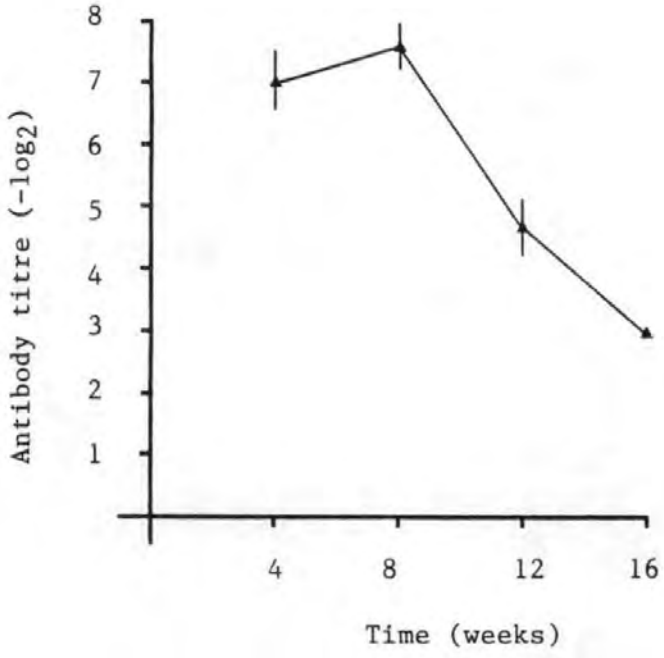


Fig. 20.



Figs. 19 and 20. Kinetics of the humoral response of juvenile carp to I. multifiliis following immunisation and exposure to a potentially lethal challenge dose. Temperature 20±2°C.

EXPERIMENT 7. The humoral immune response of 9 month old carp to Ichthyophthirius multifiliis following immunisation with theronts.

Eighty carp, age 9 months, were obtained from Winchester and separated into 2 groups, A and B. Group A contained 30 fish and Group B, 50. These were allowed to acclimate in polypropylene tanks (100 dm^3 capacity) for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the start of the experiment, the mean weight of the fish was 22.3 g.

Fish in Group A were immunised using the standard procedure of infection and immunisation but, due to the increased size of the fish, the immunising dose consisted of approximately 8,000 theronts per fish, 40/cm^3. Thirty days after the last exposure to theronts, fish were exposed to a potentially lethal dose of approximately 20,000 theronts per fish, 100/cm^3. At weeks 1, 2, 4, 6, 8, 10, 12, 14 and 16 after challenge, 3 fish in Group A at each time were exsanquinated and individual sera (non-heat inactivated) were tested for antibody to I. multifiliis by direct immobilization/agglutination of live theronts. At each immunisation and at challenge, 5 non-immunised controls (Group B) were also exposed to theronts. Also, at the same time that Group A were exsanquinated, 3 fish from Group B were also exsanquinated and individual sera tested for antibody to I. multifiliis.

The mean antibody titres of carp following immunisation and challenge with I. multifiliis are shown in Fig. 21. The kinetics of the response were studied over a period of 16 weeks. There was already an enhanced level of antibody at the time of challenge which increased to a peak

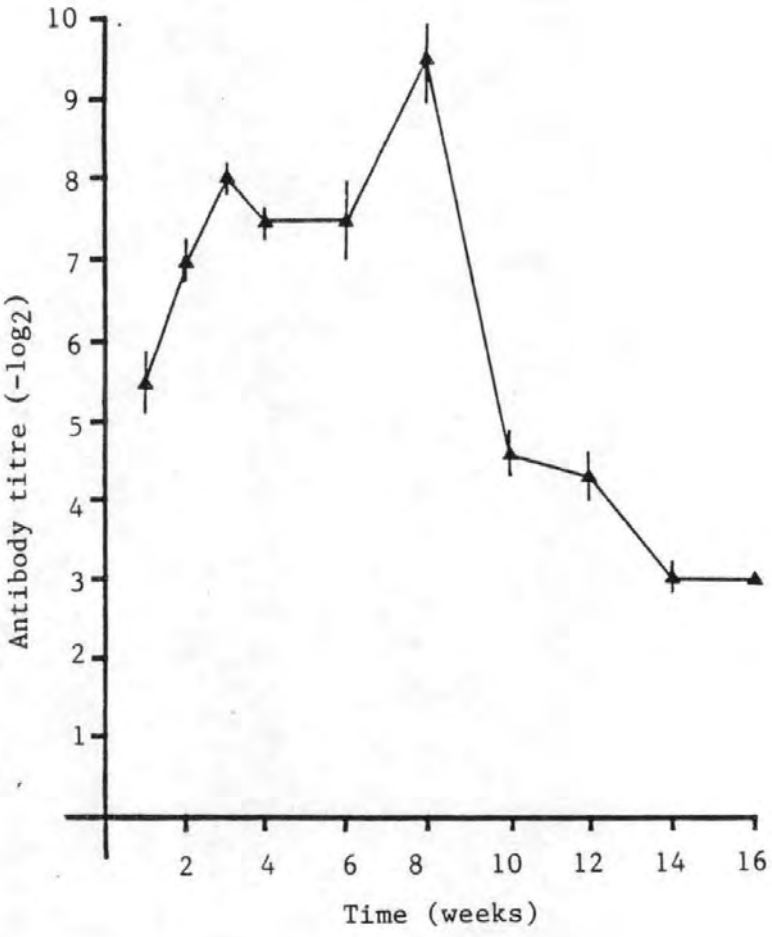


Fig. 21. Kinetics of the humoral antibody response of 9 month old carp to I. multifiliis following immunisation and exposure to a potentially lethal challenge dose. (20,000 theronts/fish, 100/cm ^{3}). 3 fish per group, temperature 20±2°C.

response at week 8 of $-\log_2$ 9.5, after which, levels began to decrease to a titre of $-\log_2$ 3 at week 16. Non-immunised controls showed no antibody or natural agglutinins to I. multifiliis.

Autoradiography

EXPERIMENT 8. The cellular proliferative response and humoral response of juvenile carp to I. multifiliis after 4 exposures to the parasite.

Three hundred and ninety juvenile carp, age 8 weeks, obtained from Stowmarket (spawned 8.1.86), were separated into 2 groups, A and B, of 170 and 220 fish respectively. These were allowed to acclimate for two weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Following acclimation and immediately prior to the experiment, the mean weight of the fish at 10 weeks, was 767 mg.

Fish in Group A were exposed to 3 immunising doses of I. multifiliis on 3 separate occasions of 14 day intervals. After each exposure and following a potentially lethal challenge 28 days after the last immunising dose, 40 fish were removed for investigations of cell proliferation by autoradiography. These fish received the following treatment at each stage. They were injected with methyl ^{3}H thymidine (1 µCi g^{-1} body weight) in 4 batches of equal number at intervals of 1, 2, 3 and 4 weeks after the appropriate exposure, and exsanquinated 18 hours after the injection. The following organs were fixed in 10% buffered formal saline: spleen, pronephros, opisthonephros, and fibrowax sections at 5 µm were processed for autoradiography. Serum was pooled within each group of 10 fish and tested (non-heat in-

activated) for antibody against I. multifiliis using direct immobilization/agglutination of theronts. Fish were taken from control Group B at each immunisation stage to test for viability of theronts and at challenge to confirm protective immunity. In addition, uninfected controls (Group B) were injected with methyl ^{3}H thymidine, at each stage as for Group A, as controls for interpretation of autoradiography and serum also tested by direct immobilization/agglutination of theronts of I. multifiliis.

It can be seen from the results (table 13) that, after immunisation with controlled infections of I. multifiliis, all immunised fish survived with no infections developing. Non-immunised controls subjected to challenge infections suffered 88% mortality. Cumulative mortality (%) with time, for the controls, can be seen in Fig. 22 . First deaths occurred 6 days after challenge, with all mortalities within 13 days. The deaths were chiefly between days 9 to 13, that is, during the secondary infection.

Results of antibody tests are shown in table 14. Following one exposure to I. multifiliis, (Group A) antibody was not detected until week 3 being $-\log_2$ 3 and at week 4, $-\log_2$ 4. This was further elevated following 2 exposures to I. multifiliis being $-\log_2$ 4, 1 and 2 weeks following exposure, increasing to $-\log_2$ 5, 3 weeks after exposure and $-\log_2$ 7, 4 weeks after exposure. Further elevation of antibody occurred following 3 exposures to the parasite, with the titre at week 1 being $-\log_2$ 5, at week 2, $-\log_2$ 8, and at week 3, $-\log_2$ 9. A slight increase in titre occurred following the challenge exposure to I. multifiliis with titre at week 1 being $-\log_2$ 7, week 2, $-\log_2$ 8, week 3, $-\log_2$ 9 and week 4, $-\log_2$ 10. Sera from untreated controls (Group B) were

Table 13. The effect of a potentially lethal challenge of *I. multifiliis* on juvenile carp following immunisation with 3 serial infections of the parasite. Temperature 20 ±2 °C.

Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality
A	Immunised	40	0	None	0
B	Control	40	100	Moderate to heavy, entire body	88

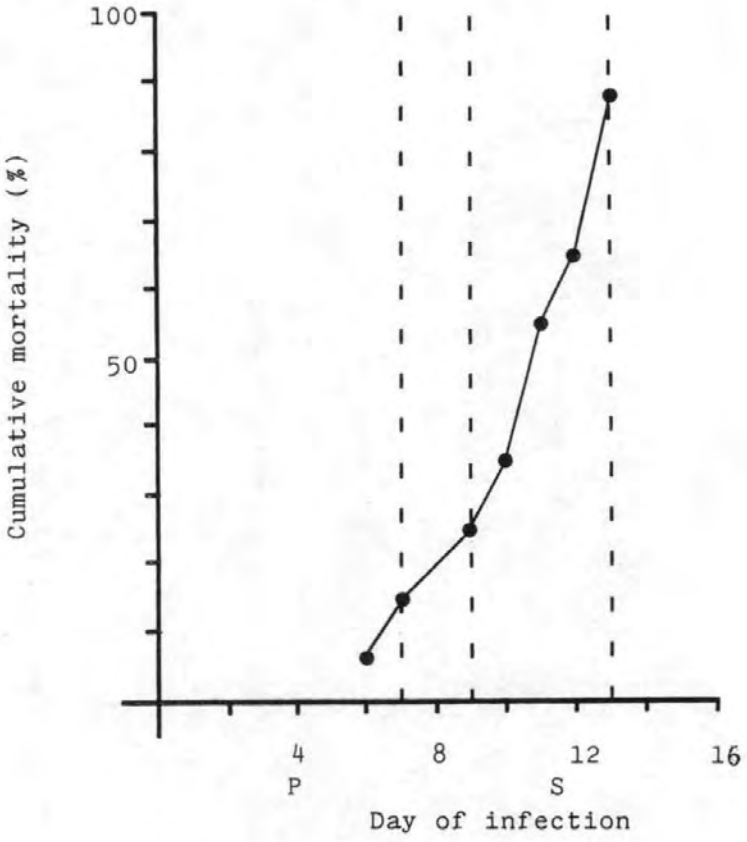


Fig 22. Cumulative mortality (%) for non-immunised controls following a potentially lethal dose of I multifiliis (8 000 theronts/fish, 320/cm ^{3}). Temperature 20±2 °C.

P primary, S secondary infections of I multifiliis

Table 14. Humoral response of juvenile carp to I. multifiliis with time following 1, 2, 3 or 4 exposures to the parasite. Mean titre from groups of 10 fish following direct immobilization/agglutination of theronts after 2 and 4 hours. Temperature 20±2 °C.

Group	Procedure No of exposures to I. multifiliis	No. of fish per group	Mean antibody titre ($-\log_2$) serum pooled from groups of 10 fish. Time (weeks) from exposure.				
			0	1	2	3	4
A	1	50	0	NT	0	3	4
B	0					0	0
A_1	2	50	0	4	4	5	7
B_1	0			0	0	0	0
A_2	3	40	4	5	8	9	[a]NT
B_2	0		0	0	0	0	NT
A_3	challenge	50	7	7	8	9	10
B_3	0		0	0	0	0	0

[a]NT Not tested.

Table 15. Mean autoradiography counts in juvenile carp following 1 and 2 exposures to I. multifiliis. Temperature 20±2°C.

Time after exposure to I.multifiliis (weeks)	Organ	Mean autoradiography counts/ 0.1mm^2 (± standard error) Treatment		No. of fish/ group
		1 exposure to I.multifiliis	None	
1		N/A	45.07±3.50	
2	Opistho-	65.60±5.88	68.20±4.16	10
3	nephros	19.90±3.79	35.86±1.87	9
4		N/A	43.76±2.57	10
2		N/A	34.00±2.70	10
3	Spleen	N/A	37.90±2.37	9
4		31.13±2.72	14.78±1.34	10
		2 exposures to I.multifiliis	None	
1		96.32±4.80	N/A	8
2	Opistho-	97.30±3.21	N/A	10
3	nephros	112.38±3.10	68.18±2.40	9
4		85.37±2.54	94.82±3.23	
1		N/A	8.30±1.08	8
2		51.35±4.01	47.26±6.97	10
3	Spleen	N/A	5.43±0.68	9
4		24.22±2.40	61.40±2.97	

N/A not available

Table 16. Mean autoradiography counts in juvenile carp following 3 and 4 exposures to I. multifiliis. Temperature 20±2°C.

Time after exposure to I.multifiliis (weeks)	Organ	Mean autoradiography counts/ 0.1mm^{-2} (± standard error) Treatment		No. of fish/ group
		3 exposures to I.multifiliis	None	
1	Opistho-	72.87±2.05	47.52±2.27	10
2	nephros	98.48±3.40	83.75±5.66	9
1		11.63±1.56	49.40±3.01	10
2	Spleen	20.00±0.85	29.84±1.52	9
3		N/A	33.74±1.10	10
		4 exposures to I.multifiliis	None	
1		78.02±1.97	47.52±2.27	10
2	Opistho-	59.97±1.90	83.75±5.67	10
3	nephros	66.77±2.11	N/A	8
4		44.01±1.69	N/A	9
1		N/A	11.63±1.56	10
2	Spleen	38.37±1.68	20.00±0.85	10

N/A not available

negative to I. multifiliis and showed no natural agglutinins.

The results for the autoradiography can be seen in tables 15 and 16. Gaps in the data relate to poor sections not suitable for counting. The results indicate that there are no clear cut differences between fish exposed to immunising infections of I. multifiliis, and challenge doses and non-immunised controls.

Duration of the immune response

EXPERIMENTS 9-14. Duration of the protective immune response of juvenile carp to Ichthyophthirius multifiliis

Six experiments were performed to investigate the duration of the protective immune response of carp to I. multifiliis. General information concerning number of fish, source and size for each experiment is shown in table 17. Experimental results are shown separately for each of the experiments 9, 10, 11, 12, 13, 14 in tables 18, 19, 20, 21, 22 and 23 and Figs. 25-27 respectively.

All fish were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. The standard procedure of infection, immunisation and challenge are described in the materials and methods. Following exposure to challenge infections, fish were returned to holding tanks. Groups of immunised fish within each experiment, were then re-exposed to a 2nd challenge at either 1, 2, 3 or 4 months following the original challenge infection. The exception to this was in experiments 13 and 14, where fish were challenged as normal, but given a 2nd challenge

Table 17 . Number, size and source of fish used for investigation of the duration of the protective immune response of juvenile carp to I. multifiliis (experiments 9 to 14).

Experiment	No. of fish		Source of fish	Date spawned	Start of experiment	
	Immunised	Non-immunised controls			Age (weeks)	Mean weight (mg)
9	30	60	Sparsholt	30.1.85	13	1040
10	40	55			12	702
11	50	30	Plymouth Polytechnic	26.4.85	9	640
12	140	110	Stowmarket	8.2.86	10	735
13	80	90			10	742
14	60	80			10	970

exposure to I. multifiliis one month following the first and then a 3rd challenge 1 and/or 3 months following the 2nd challenge. Non-immunised control fish were also exposed to the challenge infections. Any infections which developed were allowed to continue. In experiments 12, 13 and 14, at the time of each re-exposure to challenge infections, 10 fish from each group were exsanquinated, serum pooled from within each group of 10 fish, and tested for antibody to I. multifiliis by direct immobilization/agglutination of live theronts of I. multifiliis.

In experiments 9, 11 and 12 (tables 18, 20, 21) it can be seen that when immunised fish were re-exposed to a challenge of I. multifiliis, 2 months following the first challenge, light infections of either the body (experiments 9 and 12) or periphery of fins (experiment 11) developed, although no mortalities occurred, with the exception of 20% in experiment 9. In non-immunised controls exposed to challenge at this time, 100% infections ranging from medium to heavy, and covering the entire body developed with 100% mortality. When immunised fish were re-exposed to I. multifiliis, 3 months following the initial challenge, 100% infection occurred in all experiments. Infections were from light to heavy, covering the entire body, and mortalities of 100% occurred. In experiments 12 and 13, cumulative mortalities (%) with time can be seen in Figs. 23-26 . In experiment 12, first mortalities of immunised fish occurred on day 15 during termination of secondary infections and tertiary re-invasion, with all mortalities occurring by day 22, during the tertiary infection. The mean days to death was 18.55 (±2.44 SD) which was significantly different ($P<0.05$) from that of the non-immunised controls exposed to the challenge infection. These fish had a mean day of death of 12.70 (±2.25 SD) with 1st deaths occurring on day 10 during the secondary infection, with all

mortalities occurring by day 16, on termination of the secondary infection. Similar results were found in experiment 13, (table 22), in that mortalities of the immunised fish occurred later than in the non-immunised control fish, occurring between days 5-9 for the former on termination of the primary infection and secondary re-invasion, and between days 3-5 for the latter, during the primary infection. The mean days to death for the immunised fish were 6.70 (±1.5), which was significantly different ($P<0.05$) from 4.09 (±0.83) recorded for the non-immunised controls. In experiments 9 and 10 (tables 18 and 19) again, when immunised fish were re-challenged 4 months following the first challenge exposure, 100% infections leading to 100% mortalities occurred. Similar results were recorded for the non-immunised controls.

In experiment 14 (table 23), where fish were exposed to 2 challenge infections 1 month apart, followed by another challenge exposure 3 months later, all immunised fish developed light to medium primary and secondary infections, with 20% mortality occurring. Non-immunised controls developed 100% infection which was medium to heavy and 100% mortality occurred. Cumulative mortality (%) can be seen in Fig. 27 . With 1st deaths for non-immunised controls (Group B) occurring between days 9 to 13, during the secondary infection, with the mean day of death being 10.8 (±1.4). Mortalities of the immunised fish (Group A) occurred between days 10 to 16, with the mean day of death being 13.25 (±2.5).

In experiments 12 and 13, the antibody titre to I. multifiliis was measured at the time of each re-exposure to the parasite. The results can be seen in tables 21 and 22. At the time of the first challenge,

Table 18. Duration of the protective response of juvenile carp to I. multifiliis following immunisation with 3 serial infections followed by challenge doses. Susceptibility to re-infection at 2, 3 and 4 months. Temperature 20±2°C.

Time after challenge (months)	Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality
0	A	Immunised	15	0	None	0
	B	Control	10	100	medium, entire body	100
2	C	Immunised	5	100	light, body	20
	D	Control	5	100	medium-heavy, entire body	100
3	E	Immunised	3	100	medium, entire body	100
	F	Control	3	100	medium, entire body	100
4	G	Immunised	5	100	medium, entire body	100
	H	Control	5	100	medium, entire body	100

Table 19. Duration of the protective response of juvenile carp to *I. multifiliis* following immunisation with 3 serial infections followed by challenge doses. Susceptibility to re-infection at 3 and 4 months. Temperature 20±2 °C.

Time after challenge (months)	Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality
0	A	Immunised	10	10	light, periphery of fins	0
	B	Control	10	100	Heavy, entire body	100
3	C	Immunised	3	100	light-medium, entire body	100
	D	Control	3	100	medium, entire body	100
4	E	Immunised	6	100	medium, entire body	100
	F	Control	6	100	medium, entire body	100

(Experiment 10)

Table 20. Duration of the protective response of juvenile carp to I. multifiliis following immunisation with 3 serial infections followed by challenge. Susceptibility to re-infection at 2 and 3 months. Temperature 20±2 °C.

Time after challenge (months)	Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality	Mean days to death ±SD
0	A	Immunised	25	20	very light, periphery of fins	0	N/A
	B	Control	20	100	Heavy, entire body	100	6.75±1.62
2	C	Immunised	5	20	light, periphery of fins	0	N/A
	D	Control	5	100	Heavy, entire body	100	-
3	E	Immunised	5	100	light-medium, entire body	100	8.8±0.44
	F	Control	5	100	medium, entire body	100	5.6±1.14

(Experiment 11)

Table 21. Duration of the protective response of juvenile carp to I multifiliis following immunisation with 3 serial infections followed by challenge. Susceptibility to re-infection at 1, 2 and 3 months. Mean antibody titre ($-\log_2$) at time of initial challenge following direct immobilization/agglutination of theronts. Pooled sera from groups of 10 fish. Temperature 20±2 °C.

Time following challenge (months)	Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality	Mean days to death ±SD	$-\log_2$ antibody titre
0	A	Immunised	90	0	None	0	N/A	7
	B	Control	10	100	Medium, entire body.	100	-	0
1	C	Immunised	20	0	None	0	N/A	10
	D	Control	11	100	medium, entire body	100		0
2	E	Immunised	20	65	light	0	N/A	7
	F	Controls	20	100	medium, entire body	100	11.9±1.17	0
3	G	Immunised	20	100	light-medium, entire body	100	18.55±2.44*	4
	H	Control	20	100	medium, entire body	100	12.70±2.25*	0

*Significantly different ($P < 0.05$) DF 37 (Experiment 12)

Table 22. Duration of the protective immune response of juvenile carp to I. multifiliis following immunisation wiyh 3 serial infections followed by challenge. Fish were re-challenged 1 month later. Susceptibility to re-infection 3 months from the 2nd challenge. Mean antibody titre at time of challenge from pooled sera from groups of 10 fish following direct immobilization/agglutination of theronts. Temperature 20±2°C.

Time after challenge (months)	No. of fish	Treatment	$-\log_2$ antibody	Percentage infected	Degree of infection	Percentage mortality	Mean days to death ±SD
0	50	Immunised	7	0	None	0	N/A
	20	Control	0	100	Medium-heavy, entire body.	100	10.65±2.4
Time after 2nd challenge. 0	10	Immunised	10	30	Light, body.	0	N/A
	10	Control	0	100	Medium, entire body.	100	9.9±1.10
3	10	Immunised	6	100	Light-medium, entire body.	100	6.7±1.57+
	11	Control	0	100	Medium-heavy, entire body.	100	4.09±0.83+

+ significantly different $P < 0.05$ (Experiment 13)

Table 23. Duration of the protective immune response of juvenile carp to I. multifiliis following immunisation with 3 serial infections followed by challenge. Fish were re-challenged 1 month later. Susceptibility to re-infection 3 months from the 2nd challenge. Mean antibody titre at time of challenge from pooled sera from groups of 10 fish following direct immobilization/agglutination of theronts. Temperature 20±2°C.

Time after challenge (months)	No. of fish.	Treatment	$-\log_2$ antibody	Percentage infected	Degree of infection	Percentage mortality	Mean days to death ±SD
0	90	Immunised	7	0	None	0	N/A
	10	Control	0	100	Medium, entire body.	90	-
Time after 2nd challenge. 0	40	Immunised	8	7.5	V.light, periphery of fins.	0	N/A
	10	Control	0	100	Medium-heavy, entire body.	100	-
3	20	Immunised	4	100	Light-medium, entire body.	20	$13.25 \pm 2.5^{+}$
	10	Control	0	100	Medium-heavy, entire body.	100	$10.8 \pm 1.4^{+}$

$^{+}$ significantly different $P < 0.05$ (Experiment 14)

Fig. 23. Cumulative mortality (%) for non-immunised controls following a potentially lethal dose of I. multifiliis (8,000 theronts/fish, 320/cm ^{3}). Temperature 20±2°C.

P primary, S secondary infections of I. multifiliis.

Fig. 24. Cumulative mortality (%) for non-immunised controls and immunised fish following a potentially lethal dose of I. multifiliis (8,000 theronts/fish, 320/cm ^{3}) given 3 months following the first challenge. Temperature 20±2°C.

▲ immunised fish, ● non-immunised controls.

P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 23

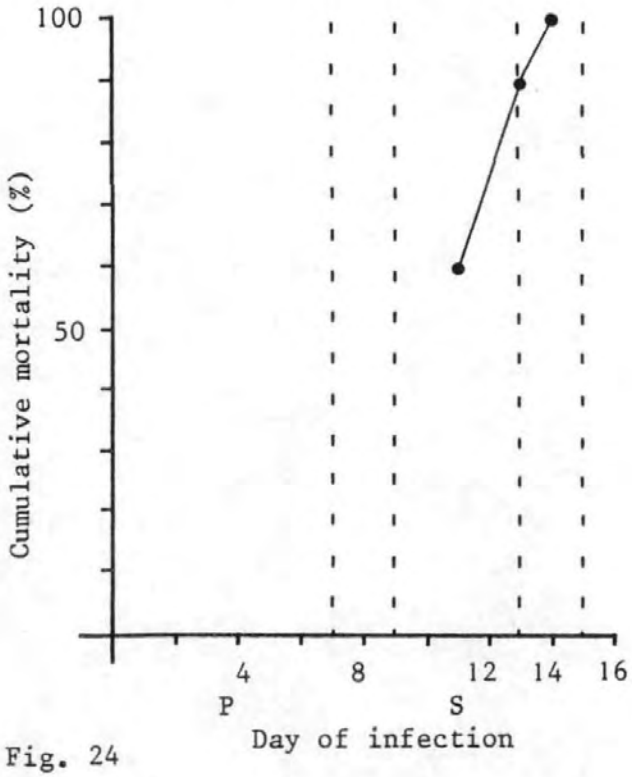

100
50
Cumulative mortality (%)
4
8
12
14
16
P
S
Day of infection

Fig. 24

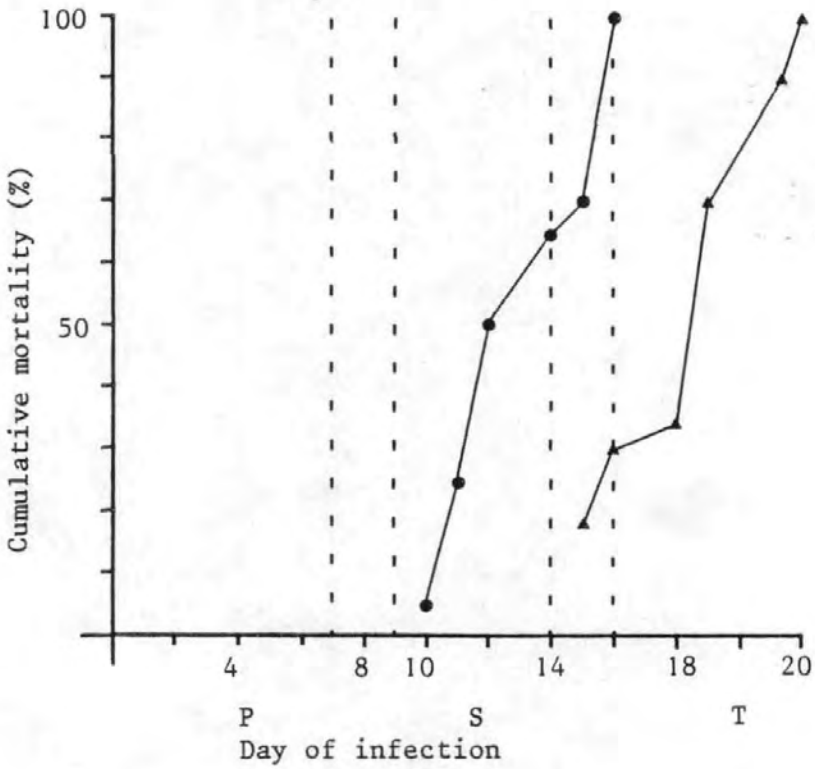

100
50
Cumulative mortality (%)
4
8
10
14
18
20
P
S
T
Day of infection

Fig. 25. Cumulative mortality (%) for non-immunised controls following a potentially lethal dose of I. multifiliis given as a second challenge. Temperature 20±2°C.

P primary, S secondary infections of I. multifiliis.

Fig. 26. Cumulative mortality (%) of immunised fish following a potentially lethal dose of I. multifiliis 3 months after a second challenge. Temperature 20±2°C.

▲ immunised fish, ● non-immunised controls.

P primary infection of I. multifiliis.

Fig. 25

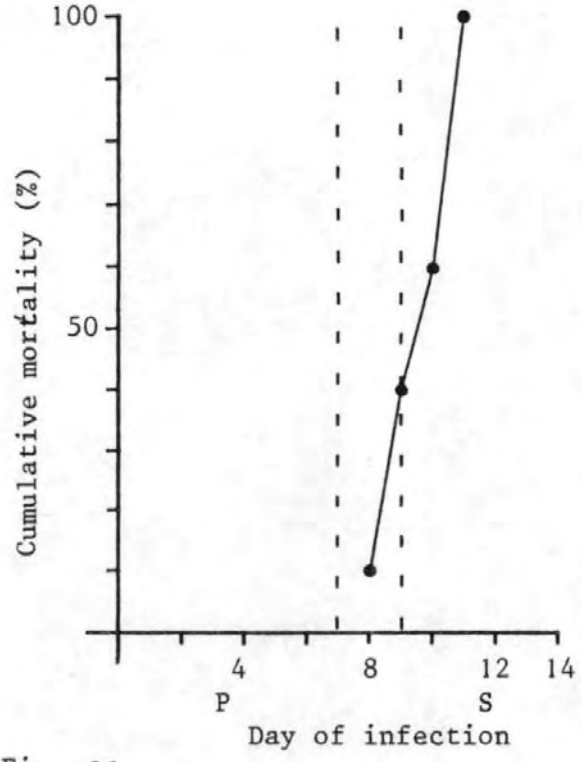
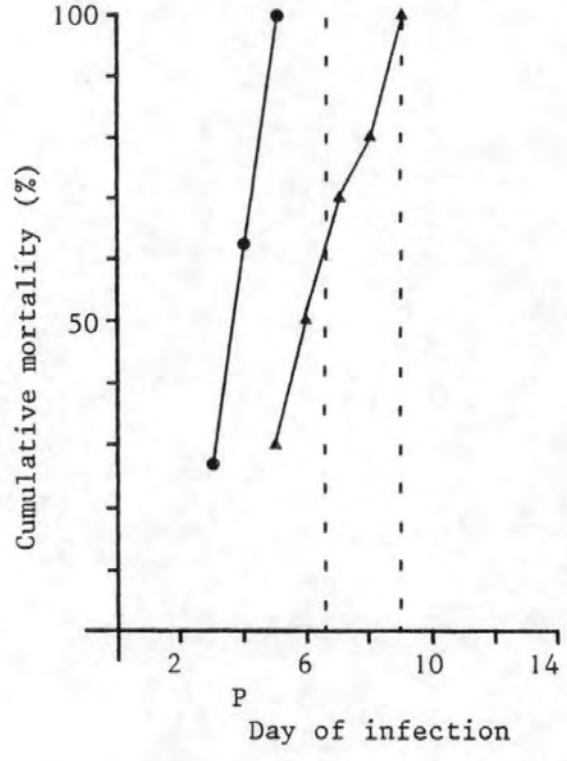

100
50
Cumulative mortality (%)
4
8
12
14
P
S
Day of infection

Fig. 26


100
50
Cumulative mortality (%)
2
6
10
14
P
Day of infection

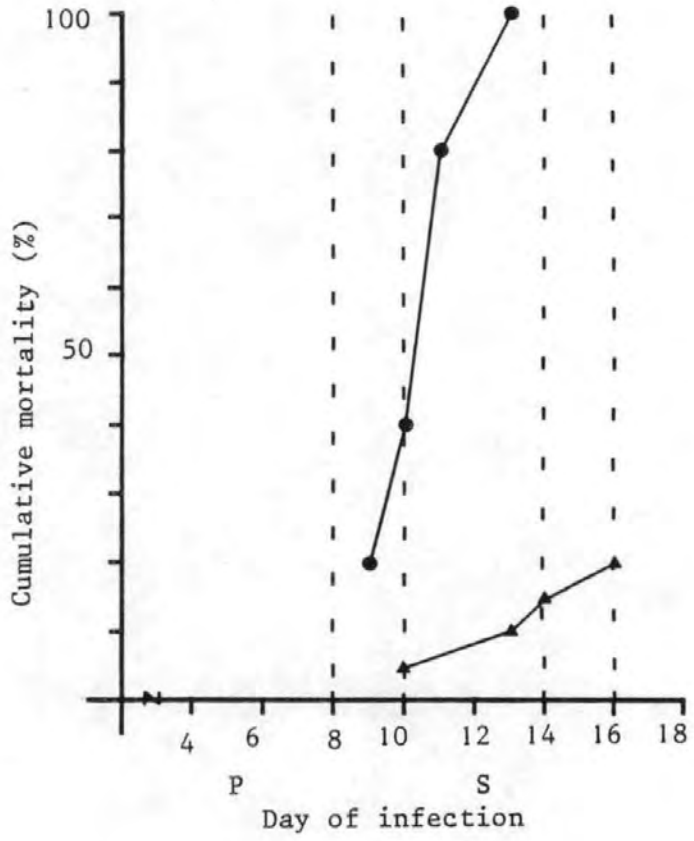


Fig. 27. Cumulative mortality (%) of immunised fish following a potentially lethal dose of I. multifiliis 3 months after a second challenge. Temperature 20±2°C. ▲ immunised fish, ● non-immunised controls. P primary S secondary infections of I. multifiliis.

both groups of immunised fish had titres of $-\log_2$ 7. One month following challenge when fish were re-exposed to infections, antibody titres again for both groups were $-\log_2$ 10. In experiment 12, where antibody was measured for the 2 month re-exposure, the titre was $-\log_2$ 7. This had decreased by the 3 month re-exposure to $-\log_2$ 4 (experiment 12) and $-\log_2$ 6 (experiment 13). Non-immunised controls showed no antibody to I. multifiliis.

In experiment 14, (table 23), the antibody titre at the time of the 2nd challenge exposure was $-\log_2$ 7, non-immunised controls showed no antibody to I. multifiliis.

Immunosuppression

EXPERIMENTS 15-18. The effects of the corticosteroids Triamcinolone acetonide and Hydrocortisone 21-hemisuccinate on the humoral antibody response of juvenile carp to Ichthyophthirius multifiliis.

Four experiments were performed to investigate the effects of the corticosteroids Triamcinolone acetonide and Hydrocortisone 21-hemisuccinate on the humoral antibody response of carp to I. multifiliis. General information concerning number of fish, source and size for each experiment, and dose of corticosteroids used can be seen in table 24. Experimental results for each experiment are treated separately.

All fish were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. The standard procedure of infection, immunisation, challenge and immunosuppression as described in the Materials and Methods

Table 24. Number, size and source of fish and dose of corticosteroid used in investigations on the effects of corticosteroids on the humoral antibody response of juvenile carp to I. multifiliis (experiments 15 to 18).

Experiment	No of fish		Source of fish	Date spawned	Start of experiment		Corticosteroid examined	Dose $\mu g\ g^{-1}$ body wt
	Immunised	Non-immunised Controls			Age (wks)	Mean weight (mg)		
15	80	100	Sparsholt	30.1.85	13.5	1020	Hydrocortisone 21-hemisucciante Triamcinolone acetonide	100 10
16	100	130	Stowmarket	8.2.86	10	740	Hydrocortisone-21-hemisuccinate (cortisol)	100
17	120	140			11	790	Triamcinolone acetonide	200 100 10
18	80	100			12	985	Triamcinolone acetonide	100 10

section, was used. At intervals of either 1 week (experiments 15, 16, 18), or 12 hours and 1 week (experiment 17) following administration of corticosteroids, 10 fish within each treatment including non-immunised controls subjected to the same treatments, were exsanquinated and serum pooled within each group of 10 fish. Sera (non-heat inactivated) was tested for antibody against I. multifiliis by direct immobilization/-agglutination of live theronts.

At the same time that fish were exposed to immunising and challenge infections, 10-20 fish from the non-immunised control groups were also exposed to these infections in order to check for viability of theronts.

The results (table 25) first indicate that protective immunity was established in the carp to I. multifiliis. Following immunisation with controlled infections of I. multifiliis, all fish survived. Light infections, restricted to the periphery of fins occurred in experiment 16 (3%) and in experiment 17 (20%).

Mortalities of 100% were recorded in non-immunised control fish exposed to the challenge dose, with medium to heavy infections occurring. Cumulative mortalities (%) with time are shown for experiment 16 in Fig. 28 and experiment 17 in Fig. 29 . Mortalities occurred over a period of 14 days in experiment 16, first deaths following day 6 with the majority occurring during the secondary infection. In experiment 17 deaths occurred within 8 days, the majority occurring on termination of primary infection.

The results for antibody tests are shown in tables 26, 27, 28 and 29.

Table 25. The effects of a potentially lethal challenge of I. multifiliis (8 000 theronts/fish, 320/cm ^{3}) on juvenile carp following immunisation with 3 controlled infections of the parasite (2 000 theronts/fish, 80 cm^{-3}). Temperature 20±2 °C.

Experiment	Treatment	No. of fish/group	Percentage infected	Degree of infection	Percentage mortality
15	Immunised	60	0	None	0
	Control	10	100	medium-heavy, entire body	100
16	Immunised	74	3	v light, periphery of fins	0
	Control	20	100	medium-heavy, entire body	100
17	Immunised	100	20	light, periphery of fins	0
	Control	10	100	medium-heavy, entire body	100
18	Immunised	65	0	None	0
	Control	10	100	medium, entire body	100

Fig 28.

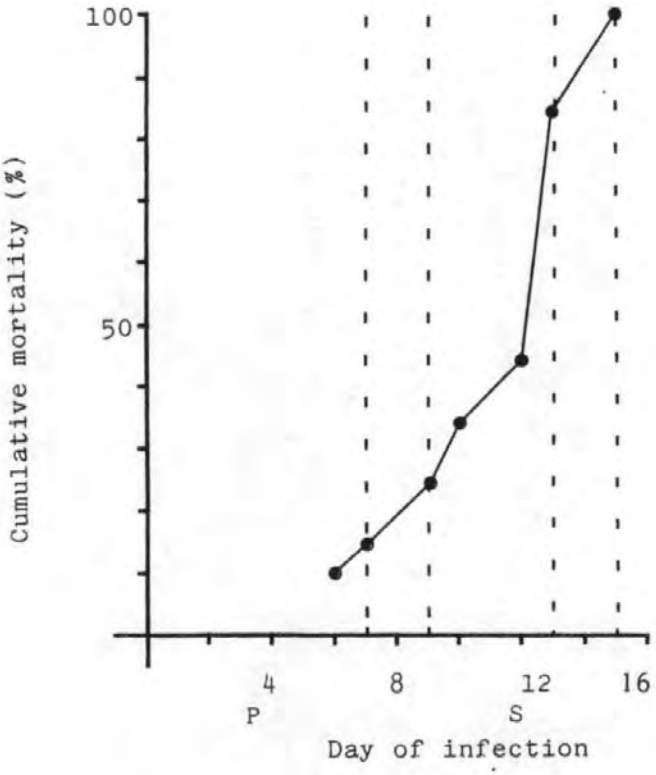
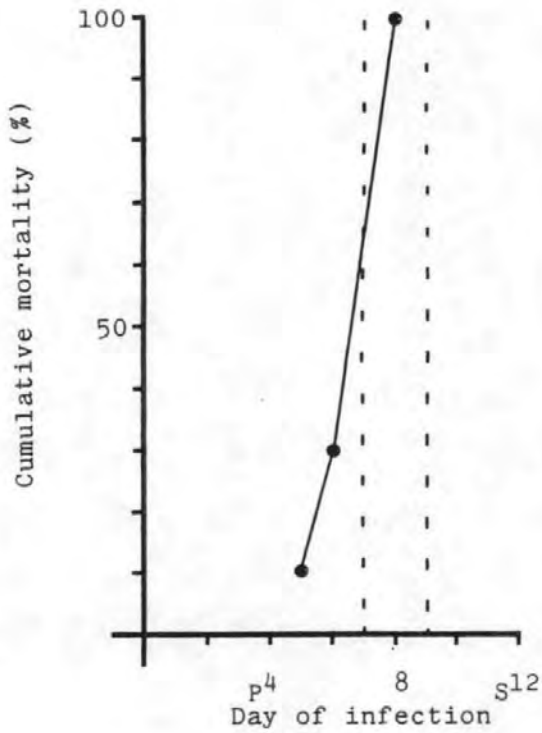


Fig 29.



Figs 28 and 29. Cumulative mortality (%) for non-immunised control fish following a potentially lethal dose of *I multifiliis* (8 000 theronts/fish, 320/cm ^{3}). Temperature 20±2 °C. P primary S secondary infections of *I. multifiliis*.

In experiment 15 (table 26), immunised fish, either untreated, had a titre of $-\log_2$ 11; injected with saline or cortisol (10 μg g^{-1} body weight), $-\log_2$ 9, injected with Triamcinolone acetonide (10 μg g^{-1} body weight), or cortisol (100 μg g^{-1} body weight), $-\log_2$ 8, or injected with Triamcinolone acetonide (100 μg g^{-1} body weight), $-\log_2$ 7. Non-immunised control fish either untreated, treated with saline or with corticosteroid, had either no antibody or some lysis occurring at titres of $-\log_2$ 1.

In experiment 16 (table 27), immunised fish, either untreated or treated with cortisol (100 μg g^{-1} body weight) had titres of $-\log_2$ 7: those injected with saline had a titre of $-\log_2$ 8. Non-immunised control fish either untreated, treated with saline, or with cortisol, had either no antibody or some lysis occurring at titres of $-\log_2$ 1.

In experiment 17 (table 28), 12 hours following administration of saline or Triamcinolone acetonide at all doses, immunised fish had antibody titres of $-\log_2$ 10. Untreated immunised fish had a mean titre of $-\log_2$ 9.5. One week following administration of saline or Triamcinolone acetonide, immunised fish, either untreated or treated with corticosteroid (200 μg, 100μg or 10 μg g^{-1} body weight) all had antibody titres of $-\log_2$ 10, and immunised treated with saline had a titre of $-\log_2$ 11. Non-immunised control fish either untreated or treated with saline or Triamcinolone acetonide, either at 12 hours or on week following administration had either no antibody or some lysis at titres of $-\log_2$ 1.

In experiment 18 (table 29), immunised fish either treated with saline or Triamcinolone acetonide (10 μg g^{-1} body weight) had mean antibody titres of $-\log_2$ 9.5; those untreated had a titre of $-\log_2$ 9 and those

Table 26. The effects of the corticosteroids Triamcinolone acetonide and Hydrocortisone 21-hemisuccinate on the humoral response of juvenile carp to I. multifiliis: direct immobilization/agglutination of theronts of I. multifiliis. Results are mean titre of duplicate tests, obtained after 2 hours and re-examined after 4 hours, temperature 20±2 °C, 10 fish in each group.

Group	1st treatment	2nd treatment	Antibody titre $-\log_2$	No. of fish/group
A	immunised	0	11	10
		saline	9	
		Ta 100	7	
		Ta 10	8	
		cortisol 100	8	
		cortisol 10	9	
B	control	0	0	
		saline	0	
		Ta 100	1	
		Ta 10	0	
		cortisol 100	0	
		cortisol 10	0	

Ta Triamcinolone acetonide No. refers to dose $\mu g\ g^{-1}$ body weight.
Cortisol Hydrocortisone 21-hemisuccinate

Table 27. The effect of Hydrocortisone 21-hemisuccinate (100 $\mu g\ g^{-1}$ body weight) on the humoral response of juvenile carp to I. multifiliis: Direct immobilization/agglutination of theronts of I. multifiliis. Results are mean titre of duplicate tests, obtained after 2 hours and re-examined after 4 hours, temperature 20±2 °C.

Group	1st treatment	2nd treatment	Mean antibody titre $-\log_2$	No. of fish/group
A	Immunised	0	7	10
		saline	8	
		cort 100	7	
B	Control	0	0	
		saline	0	
		cort 100	1	

cort 100 Hydrocortisone 21-hemisuccinate 100 $\mu g\ g^{-1}$ body weight.

Table 28 . The effect of Triamcinolone acetonide (200 µg, 100 µg or 10 µg g^{-1} body weight) on the humoral response of juvenile carp to I. multifiliis: Direct immobilizaiton/ agglutination of theronts of I. multifiliis. Results are mean titre of triplicate tests obtained after 2 hours and re-examined after 4 hours, temperature 20 ±2 °C.

Group			Mean antibody titre $-\log_2$ 12 hours	1 week	No. of fish/group
A	Immunised	0	9.5	10	10
		saline	10	11	
		Ta 200	10	10	
		Ta 100	10	10	
		Ta 10	10	10	
B	Control	0	1	0	
		saline	0	0	
		Ta 200		1	
		Ta 100		0	
		Ta 10		0	

Ta 200 Triamcinolone acetonide 200 µg g^{-1} body weight

Table 29. The effect of Triamcinolone acetonide (100 µg or 10 µg g^{-1} body weight) on the humoral response of juvenile carp to I. multifiliis: Direct immobilization/agglutination of theronts of I. multifiliis. Results are mean titre of duplicate tests obtained after 2 hours and re-examined after 4 hours, temperature 20±2 °C.

Group	1st treatment	2nd treatment	Mean antibody titre $-\log_2$	No. of fish/group
A	Immunised	0	9	10
		saline	9.5	
		Ta 100	8.5	
		Ta 10	9.5	
B	Control	0	0	
		saline		
		Ta 100		
		Ta 10		

Ta 100 Ta 10 Triamcinolone acetonide 100 µg or 10 µg g^{-1} body weight

treated with Triamcinolone acetonide (100 μg g^{-1} body weight) had a titre of $-\log_2$ 8.5. Non-immunised control fish had no antibody titres to I. multifiliis.

Autoradiography and humoral response

EXPERIMENT 19. The effects of corticosteroids on the proliferative and humoral response of juvenile carp to I. multifiliis

One hundred and ninety juvenile carp age 8 weeks, obtained from Stowmarket (spawned 8.1.86), were separated into 2 groups, A and B. Group A contained 80 fish and Group B, 110. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks the mean weight of the fish was 950 mg.

Fish in Group A were exposed to 3 immunising doses of I. multifiliis at 14 day intervals, the third dose consisted of approximately 4,000 theronts per fish at a concentration of approximately 160/cm^3. This increased dose was to compensate for a decrease in infectivity of the theronts. Twenty-eight days after the last immunisation, fish were exposed to a potentially lethal challenge dose. At each immunisation, 10 fish from Group B were also exposed to theronts and at challenge, 20 fish.

Fourteen days after challenge, immunised fish (Group A) and control fish (Group B) were administered either Triamcinolone acetonide suspension in saline at a dose of 100 μg g^{-1} body weight or 10 μg g^{-1}

body weight; Hydrocortisone 21-hemisuccinate (cortisol) in saline at a dose of 100 µg g^{-1} body weight or 10 µg g^{-1} body weight, or 0.85% saline. Fish were also left untreated. Administration was by intraperitoneal injection, 10 µl g^{-1} body weight, 10 fish in each group.

Six days after administration of corticosteroid, fish were injected with methyl ^{3}H thymidine at a rate of 1 µCig^{-1} body weight. Eighteen hours later, fish were exsanquinated and the following organs were fixed in 10% buffered formal saline: spleen, pronephros, opisthonephros and liver. Fibrowax sections at 5 µm were processed for autoradiography. Serum was pooled within each group of 10 fish and tested (non-heat inactivated) for antibody against I. multifiliis using direct immobilization/agglutination of theronts.

It can be seen from the results (table 30) that, after immunisation with contolled infections of I. multifiliis, all immunised fish survived. Infections did develop on 11% of the challenged, immunised fish but these were light and restricted to the periphery of fins. Non-immunised controls subjected to challenge infections, suffered 80% mortality. Cumulative mortality (%) with time for the controls can be seen in Fig. 30 . First deaths occurred 6 days after challenge, with all mortalities within 17 days. The deaths were chiefly between days 7 and 15 following termination of primary infection, during secondary infection and termination of secondary infection.

The results for the antibody tests are shown in table 31. Non-immunised control fish, either untreated, treated with saline or with corticosteroid had no antibody titre to I. multifiliis. Immunised fish,

Table 30. The effect of a potentially lethal challenge of *I. multifiliis* on juvenile carp following immunisation with 3 serial infections of the parasite. Temperature 20±2 °C.

Group	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality	Mean days to death (±SD)
A	Immunised	60	11	v. light, periphery of fins	0	N/A
B	Control	20	100	heavy, entire body	80	11.3±4.52

N/A Not applicable

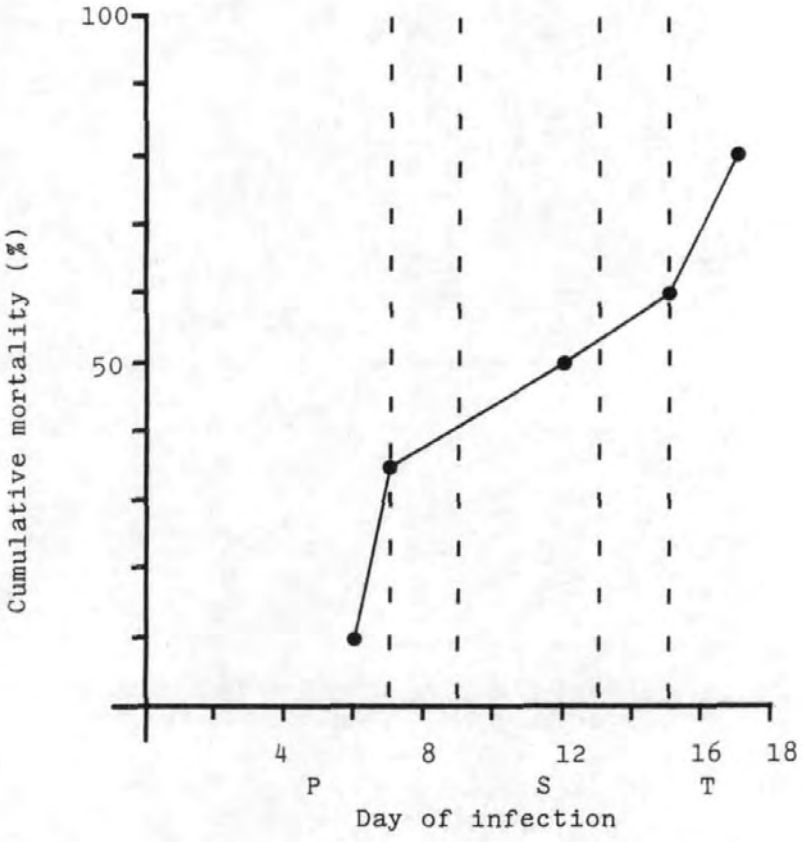


Fig 30 . Cumulative mortality (%) for non-immunised controls following a potentially lethal dose of I. multifiliis (8 000 theronts/fish, 320/cm ^{3}). Temperature 20$\pm$2 °C. P primary, S secondary, T tertiary infections of I. multifiliis.

Table 31. The effects of the corticosteroids Triamcinolone acetonide and Hydrocortisone 21-hemisuccinate on the humoral response of juvenile carp to *I. multifiliis*: direct immobilization/agglutination of theronts of *I. multifiliis*. Results are mean titre of duplicate tests, obtained after 2 hours and re-examined after 4 hours, temperature 20±2 °C, 10 fish in each group.

Group	1st treatment	2nd treatment	Antibody titre $-\log_2$	No. of fish/group
A	immunised	0	8	10
		saline	8	
		Ta 100	8	
		Ta 10	7	
		cortisol 100	10	
		cortisol 10	8	
B	control	0 saline Ta 100 Ta 10 cortisol 100 cortisol 10	0	

Ta Triaminolone acetonide No. refers to dose µg g^{-1} body weight.
Cortisol Hydrocortisone 21-hemisuccinate

Table 32. Mean autoradiography counts in juvenile carp following immunisation with 3 serial infections of I. multifiliis and challenge: Effects of the corticosteroids Triamcinolone acetonide and Hydrocortisone 21-hemisuccinate on the cellular proliferative response. Temperature 20±2°C.

Treatment	Organ	Mean autoradiography counts/ 0.1mm^{-2} (± standard error) Treatment		No. of fish/ group
		Immunised	Non-immunised	
None	Pro-nephros	289.82±2.70	208.00±9.62	10
Saline		N/A	161.90±10.9	
Ta 100		N/A	N/A	
Ta 10		380.70±11.32	180.16±7.44	
Cort 100		N/A	253.84±11.72	
Cort 10		332.00±19.8	N/A	
None	Opistho-nephros	190.70±3.90	124.90±3.78	
Saline		107.80±16.10	186.50±8.43	
Ta 100		102.70±6.10	123.6±6.81	
Ta 10		145.6±6.72	127.16±6.30	
Cort 100		194.90±10.91	158.23±5.15	
Cort 10		177.50±9.18	141.40±7.84	
None	Spleen	119.25±4.58	83.43±3.34	
Saline		138.65±12.61	68.03±7.62	
Ta 100		169.4±11.98	103.23±5.84	
Ta 10		142.90±7.80	130.40±4.63	
Cort 100		N/A	N/A	
Cort 10		131.94±6.13	N/A	

N/A not available
Ta 100 Ta 10 Triamcinolone acetonide 100 µg or 10 µg g^{-1} body weight
Cort 100 cort 10 Hydrocortisone 100 µg or 10 µg g^{-1} body weight.

untreated or administered saline or cortisol (10 μg g^{-1} body weight) had titres of $-\log_2$ 8; immunised fish administered Triamcinolone acetonide (100 μg or 10 μg g^{-1} body weight) had titres of $-\log_2$ 7, and immunised fish administered cortisol (100 μg g^{-1} body weight) had a titre of $-\log_2$ 10.

The results for the autoradiography are shown in table 32; gaps in the data relate to poor sections not suitable for counting. From the results, it can be seen that there is no clear cut difference between fish exposed to immunising infections of I. multifiliis, and challenge doses and non-immunised controls. Corticosteroid treatment appears to have no effect.

EXPERIMENTS 20-25. The effects of the corticosteroids Triamcinolone acetonide and Hydrocortisone 21-hemisuccinate on the protective immune response of carp to Ichthyophthirius multifiliis

Six experiments were performed to investigate the effects of the corticosteroids Triamcinolone acetonide and Hydrocortisone 21-hemi-succinate on the protective immune response of carp to I. multifiliis. General information concerning number of fish, source and size for each experiment and dose of corticosteroids used can be seen in table 33. Experimental results are shown separately for each of 6 experiments (20, 21, 22, 23, 24, 25) in Tables 34-41. The results are summarised under Triamcinolone acetonide and Hydrocortisone.

All fish were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. The standard procedure of infection, immunisation

Table 33. Number, size and source of fish and doses of corticosteroid used in investigations on the effects of corticosteroids on the protective immune response of juvenile carp to I. multifiliis (experiments 20-25).

experiment	No. of fish		source of fish	date spawned	Start of experiment		corticosteroid examined	dose μg g^{-1} body wt
	immunised	non-immunised controls			age (wks)	mean weight (mg)		
20	80	120	Plymouth Polytechnic	15.11.84	10	630	Triamcinolone acetonide	200
21	90	110	Sparsholt	30.1.85	12	710		
22	110	130	Driffield	17.4.85	14	1060	Triamcinolone acetonide	100 and 10
23	160	150	Stowmarket	8.2.86	10	950		
24	160	150	Stowmarket	8.2.86	10	1087	Hydrocortisone 21-hemisuccinate (cortisol)	100 and 10
25	120	170	Stowmarket	8.2.86	10	743		

challenge and immunosuppression as described in the Materials and Methods section, was used.

Triamcinolone acetonide - The results (Tables 34) first indicate that protective immunity was established in the carp to I. multifiliis. Following immunisation with controlled infections of I. multifiliis, all fish survived, with the exception of experiment 22, where 10% mortality occurred, this, however, being confined to undersized fish. Light infections, restricted to periphery of fins also occurred in a small percentage of the groups of immunised fish: 19% (experiment 20), 36% (experiment 22) and 10% (experiment 23). Mortalities of 100% were recorded in non-immunised control fish exposed to the challenge dose, with the exception that in experiment 23, 80% mortality occurred. Cumulative mortalities (%) (Figs 31-34) over a period of 17 days indicate first deaths following day 1, with the majority from day 7, coinciding with secondary infection.

Following administration of Triamcinolone acetonide (Tables 35-38) all immunised and non-immunised fish developed medium to heavy infections of I. multifiliis on further exposure to a challenge dose of the parasite, with 100% mortalities. 100% infections also occurred in these latter fish, with the exception of experiment 21 (Table 36) where only 50% died in both saline and untreated controls. No mortalities occurred in immunised fish, including those injected with saline and those left untreated. However, light infections confined to the periphery of fins occurred in a small percentage of fish in experiment 20 (Table 35) and 22 (Table 37) at levels of 30% and 10% respectively. Cumulative mortality (%) for immunised fish treated with corticosteroid and for non-immunised controls injected with

corticosteroid, saline, or left untreated, are shown in Figs. 35-38. The results for mortalities are considered separately under each experiment. The results for experiments 20 and 21 are similar. The effects of a single dose of Triamcinolone acetonide (200 μg g^{-1} body weight) on mortality as investigated in experiment 20 (Fig. 35) and repeat experiment 21 (Fig. 36) show that deaths occurred in experiment 20, 6 days after challenge, on termination of primary infection for immunised fish injected with Triamcinolone acetonide and for control fish injected with Triamcinolone acetonide, or untreated. Mortalities for controls injected with saline occurred from days 8 to 12 during the secondary infection. Likewise in experiment 21, mortalities were chiefly 7 days after challenge following termination of primary infection and commencement of secondary invasion. All mortalities occurred within 8 days.

For experiment 22 (Fig. 37) mortalities for control fish injected with corticosteroid (Ta 100 and Ta 10), occurred chiefly on termination of primary infection and commencement of secondary invasion. Mortalities for all other groups occurred chiefly on commencement of secondary invasion and during the secondary infection. All mortalities occurred within 18 days.

For experiment 23, (Fig. 38) all mortalities for immunised fish injected with Triamcinolone acetonide (100 μg) occurred between days 6 to 8 at termination of the primary infection and commencement of secondary invasion. In all other groups, mortalities occurred chiefly during the secondary infection and at the termination of the secondary infection and commencement of tertiary invasion. All mortalities occurred within 17 days.

Table 34. The effects of a potentially lethal challenge of I.multifiliis (8,000 theronts per fish, 320/cm^{-3}) following immunisation with 3 controlled infections of the parasite (2,000 theronts per fish, 80/cm^{-3}). Temperature 20±2°C.

Experiment	Treatment	No of fish/ group	Percentage infected	Degree of infection	Percentage mortality	Mean days to death (± SD)
20	immunised	60	19	very light, periphery of fins	0	N/A
	control	20	100	very heavy, entire body	100	6.75±1.62
21	immunised	60	0	none	0	N/A
	control	10	100	very heavy, entire body	100	8.4±2.2
22	immunised	80	36	light, periphery of fins	10	-
	control	10	100	heavy, entire body	100	7.6±2.83
23	immunised	160	10	very light, periphery of fins	0	N/A
	control	20	100	heavy, entire body	80	10.83±4.5

SD standard deviation N/A not applicable

Fig 31

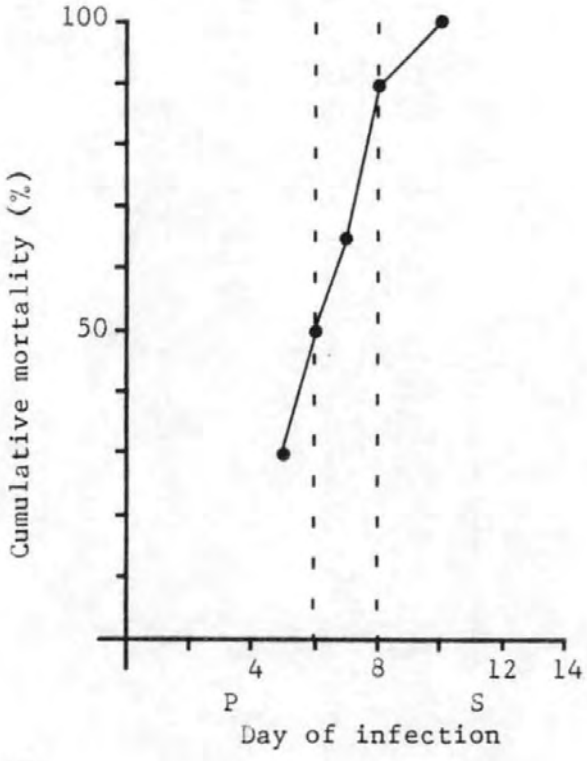
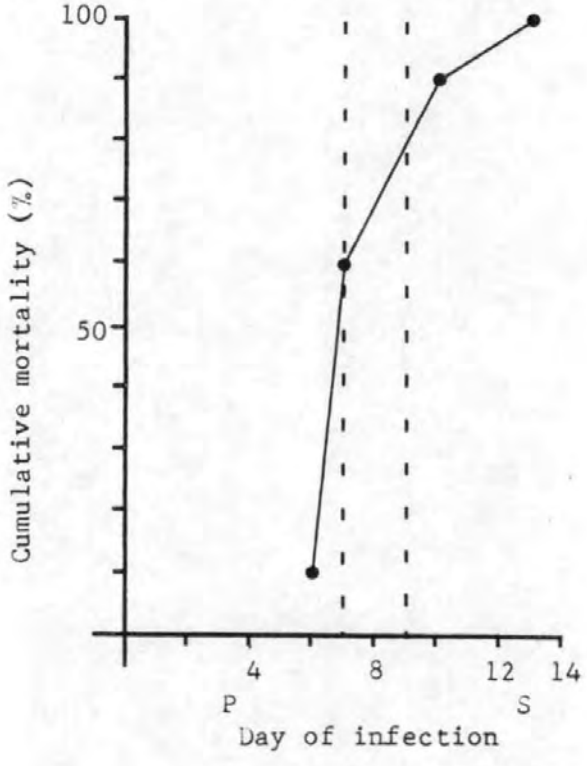


Fig 32



Figs 31 and 32 . Cumulative mortality (%) for non-immunised control fish following a potentially lethal dose of I. multifiliis (8 000 theronts/fish, 320/cm ^{3}). Temperature 20±2 °C.

P primary, S secondary infections of I. multifiliis.

Fig. 33.

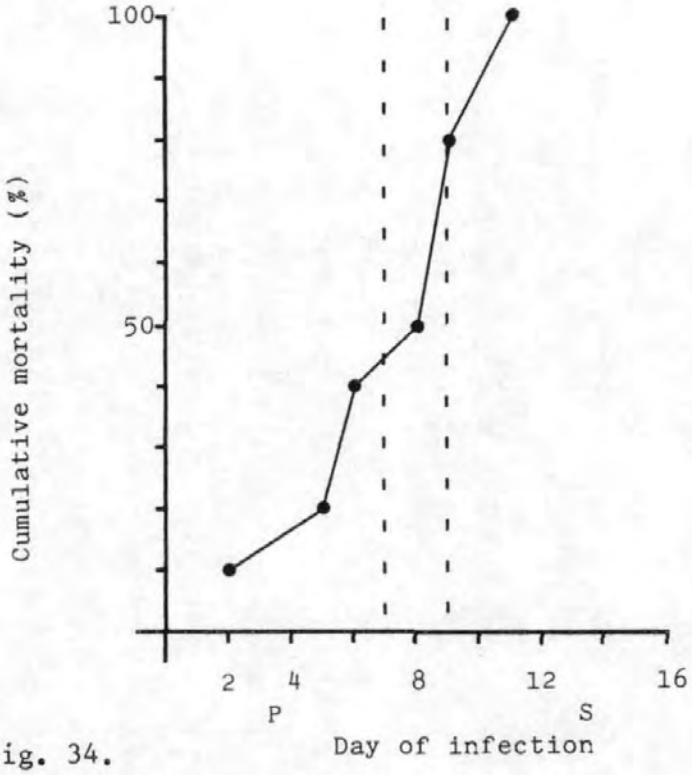
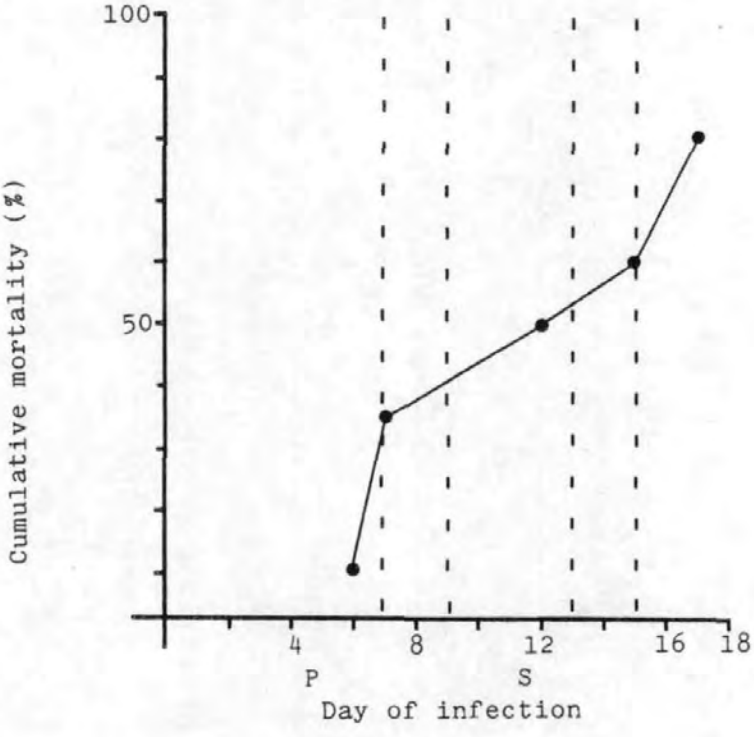


Fig. 34.



Figs 33 and 34. Cumulative mortality (%) for non-immunised control fish following a potentially lethal dose of I. multifiliis (8 000 theronts/fish, 320/cm ^{3}). Temperature 20±2 °C.

P primary, S secondary infections of I. multifiliis.

Table 35. The effect of the corticosteroid Triamcinolone acetonide (200 μg g^{-1} body weight) on the immune response of 10 week old juvenile carp to I. multifiliis. Each group was comprised of 10 fish. Temperature 20±2°C.

Group	Treatment	Re-challenge	% infected	Degree of infection Dose and area	% mortality	Mean days to death (±SD)
Immunised	0 Saline Ta 200[a]	0	0	None	0	N/A
Control	0 Saline Ta 200					
Immunised	0 Saline	ich	30	very light, periphery of fins	0	N/A
	Ta 200		100	very heavy, entire body	100	6.0±0.0
Control	0					6.0±0.0
	Saline					8.90±1.20
	Ta 200					6.0±0.0

a Ta 200 Triamcinolone acetonide 200 μg g^{-1} body weight

Table 36. The effect of the corticosteroid Triamcinolone acetonide (200 μg g^{-1} body weight) on the immune response of 12 week old juvenile carp to I. multifiliis. Each group was comprised of 10 fish. Temperature 20±2°C.

Group	Treatment	Re-challenge	% infected	Degree of infection Dose and area	% mortality	Mean days to death (±SD)
Immunised	0 saline Ta 200[a]	0	0	None	0	N/A
Control	0 saline Ta 200					
Immunised	0 saline	ich				
	Ta 200		100	Heavy, entire body	100	6.80±0.79
Control	0			very heavy, entire body	50	5.80±1.10
	saline					6.80±0.84
	Ta 200				100	7.30±0.95

a Ta 200 Triamcinolone acetonide 200 μg g^{-1} body weight

Table 37. The effect of the corticosteroid Triamcinolone acetonide (100µg or 10 µg g^{-1} body weight) on the immune response of 14 week old juvenile carp to I.multifiliis. Each group was comprised of 10 fish.

Group	Treatment	Re-challenge	% infected	Degree of infection Dose and area	% mortality	Mean days to death (±SD)
Immunised	0 saline Ta 100[a] Ta 10[b]	0	0	None	0	N/A
Control	0 saline Ta 100 Ta 10					
Immunised	0	ich	10	very light, periphery of fins		
	saline		0	None		
	Ta 100		100	moderate to heavy, entire body	100	7.20±4.87
	Ta 10					10.70±2.50
Control	0					11.40±1.26
	saline					11.80±5.79
	Ta 100					6.50±1.78
	Ta 10					8.60±1.26

a Ta 100 Triamcinolone acetonide 100 µg g^{-1} body weight
b Ta 10 Triamcinolone acetonide 10 µg g^{-1} body weight. Temperature 20±2°C.

Table 38. The effect of the corticosteroid Triamcinolone acetonide (100µg or 10µg g^{-1} body weight) on the immune response of 10 week old juvenile carp to I.multifiliis. Each group was comprised of 10 fish. Temperature 20±2°C.

Group	Treatment	Re-challenge	% infected	Degree of infection Dose and area	% mortality	Mean days to death (±SD)
Immunised	0 saline Ta 100 Ta 10	0	0	None	0	N/A
Control	0 saline Ta 100 Ta 10					
Immunised	0	ich				
	saline					
	Ta 100		100	Moderate, entire body	100	7.40±0.70
	Ta 10					10.70±1.83
Control	0			light to moderate, entire body		13.30±2.79
	saline					15.30±1.89
	Ta 100					10.30±2.41
	Ta 10					9.80±2.66

N/A not applicable

Figs. 35 and 36. Cumulative mortality (%) for immunised and non-immunised control fish following administration of Triamcinolone acetonide (200 µg g^{-} body weight) 6 days before a potentially lethal dose of I. multifillis. 10 fish per group, temperature 20±2°C.

▽ immunised treated with Triamcinolone acetonide, ● non-immunised controls treated with Triamcinolone acetonide, O non-immunised controls untreated, ▲ non-immunised controls treated with saline.

P primary, S secondary infections of I. multifiliis.

Fig. 35

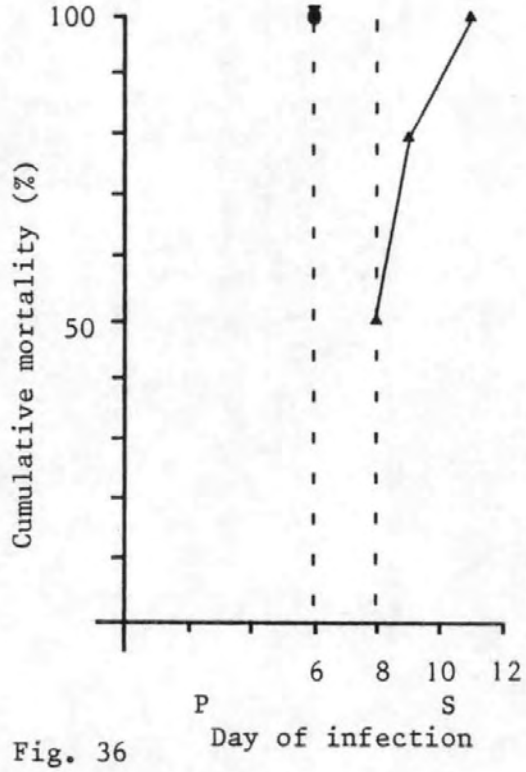
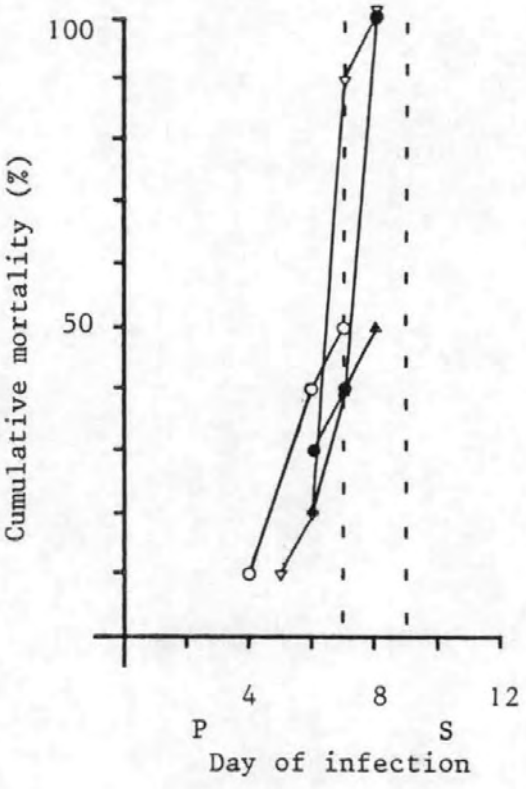

100
50
Cumulative mortality (%)
6
8
10
12
P
S
Day of infection

Fig. 36


100
50
Cumulative mortality (%)
4
8
12
P
S
Day of infection

Figs. 37 and 38. Cumulative mortality (%) for immunised and non-immunised control fish following administration of Triamcinolone acetonide (100 µg or 10 µg g^{-1} body weight) 6 days before a potentially lethal dose of I. multifiliis. 10 fish per group, temperature 20±2°C.

▽ immunised treated with Ta 100, ■ immunised treated with Ta 10, ○ non-immunised controls untreated, ▲ non-immunised controls treated with saline, ● non-immunised controls treated with Ta 100, □ non-immunised controls treated with Ta 10.

Ta 100, Ta 10 Triamcinolone acetonide 100 or 10 µg g^{-1} body weight.

P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 37

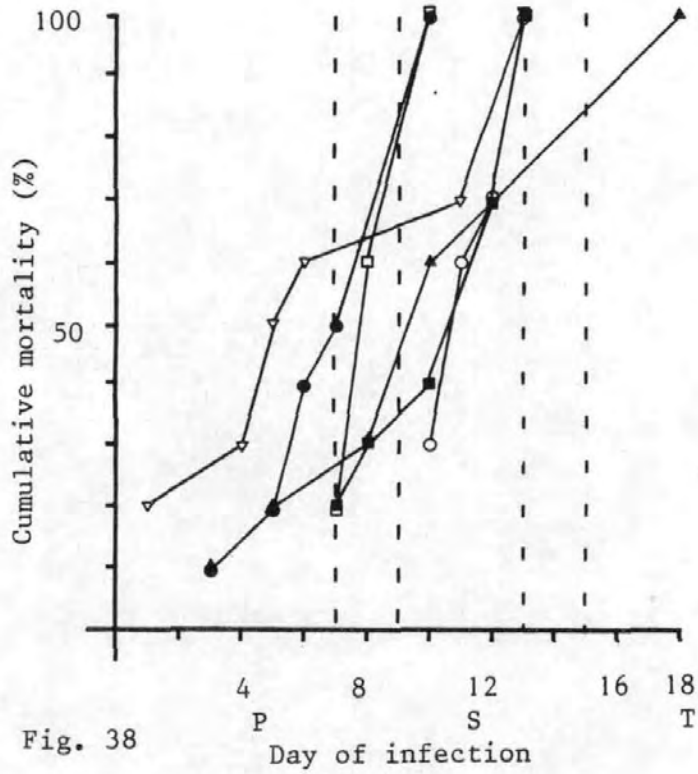
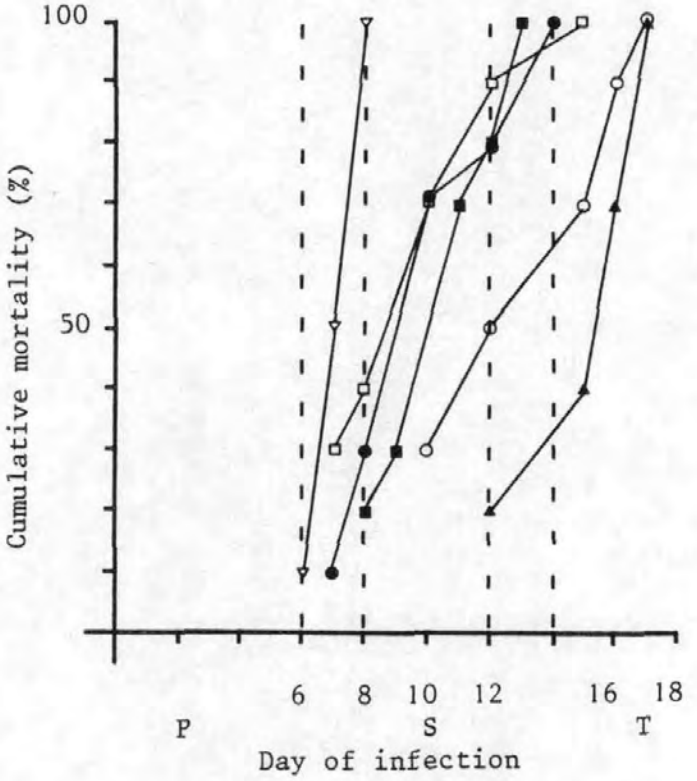

100
50
Cumulative mortality (%)
4
8
12
16
18
P
S
T
Day of infection

Fig. 38


100
50
Cumulative mortality (%)
6
8
10
12
16
18
P
S
T
Day of infection

Hydrocortisone 21-Hemisuccinate - The results first indicate (Table 39) that protective immunity was established in the carp to I. multifiliis. Following immunisation with controlled infections of I. multifiliis, all fish survived. Light infections, restricted to the periphery of fins also occurred in a small percentage of the immunised fish. 10% (experiment 24) and 2% (experiment 25). Mortalities in uninfected control fish exposed to the challenge dose were recorded with 80% in experiment 24 and 100% in experiment 25. Cumulative mortalities (%) (Figs 39, 40) over a period of 17 days indicate first deaths following day 6 with all mortalities occurring within 17 days (experiment 24, Fig. 39) and 15 days (experiment 25, Fig. 40). The mortalities were chiefly from day 7 after challenge, following termination of primary infections and during the secondary infection.

Following administration of cortisol (Hydrocortisone 21-hemisuccinate) (Tables 40, 41) all immunised and non-immunised fish developed medium to heavy infections of I. multifiliis on further exposure to a challenge dose of the parasite, with 100% mortality occurring in experiment 24, (Table 40) and non-immunised controls for experiment 25 (Table 41). 90% mortality occurred in immunised fish injected with 100 µg g^{-1} body weight cortisol and 80% mortality in immunised fish injected with 10 µg g^{-1} body weight in experiment 25 (Table 41). No mortalities occurred in immunised fish, including those injected with saline and those left untreated, with the exception of immunised fish injected with saline and exposed to a challenge infection in experiment 25 (Table 41) where 50% mortality occurred. In experiment 24, (Table 40) no infection occurred in immunised fish injected with saline or left untreated, however, light infections occurred in 90% of immunised fish injected with saline and in 30% of immunised fish left

untreated in experiment 25. Cumulative mortality (%) for immunised fish treated with corticosteroid and saline (experiment 25 only) and for non-immunised controls injected with corticosteroid, saline or left untreated, are shown in Fig. 41, 42. The results for mortalities are considered separately under each experiment. For experiment 24, (Fig. 41) mortalities for immunised fish and non-immunised controls treated with cortisol (100 μg g^{-1} body weight) occurred chiefly between days 7 to 10 on termination of primary infection and commencement of secondary invasion. For all other groups, mortalities occurred chiefly during the secondary infection and on commencement of tertiary invasion. All mortalities occurred within 17 days.

For experiment 25, (Fig. 42) mortalities occurred chiefly between days 5 to 9 for immunised fish injected with saline or cortisol (10 μg g^{-1} body weight) and non-immunised controls injected with saline, cortisol (100 μg g^{-1} body weight) or left untreated, on termination of primary infection and commencement of secondary invasion. For immunised fish treated with cortisol (100 μg g^{-1} body weight), mortalities occurred during the secondary infection and for non-immunised controls, during the primary and secondary infections. All mortalities occurred within 14 days.

EXPERIMENTS 26 - 28. The effect of *Ichthyophthirius multifiliis* on the humoral response of juvenile carp to *Aeromonas salmonocida*

Three experiments were undertaken to investigate the possible immunodepressant effect of *I. multifiliis*. These varied in the timing and sequences of antigen administration of *A. salmonocida* in respect to a single infection of *I. multifiliis*, as shown in Fig. 43.

Table 39 . The effects of a potentially lethal challenge of *I. multifiliis* (8 000 theronts per fish, 320/cm^{-3}) following immunisation with 3 controlled infections of the parasite (2 000 theronts per fish, 80/cm^{-3}). Temperature 20±2 °C.

Experiment	Treatment	No. of fish	Percentage infected	Degree of infection	Percentage mortality	Mean days to death (±SD)
24	Immunised	153	10	very light, periphery of fins	0	N/A
	Control	20	100	heavy, entire body	80	11.5±4.6
25	Immunised	84	2	very light, periphery of fins	0	N/A
	Control	40	100	medium-heavy, entire body	100	11.12±2.50

N/A not applicable

Fig 39

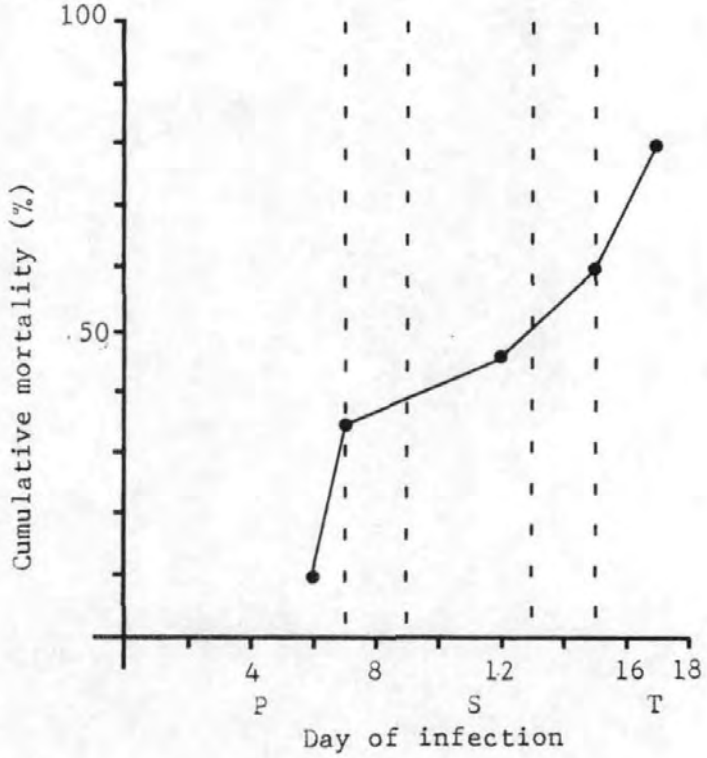
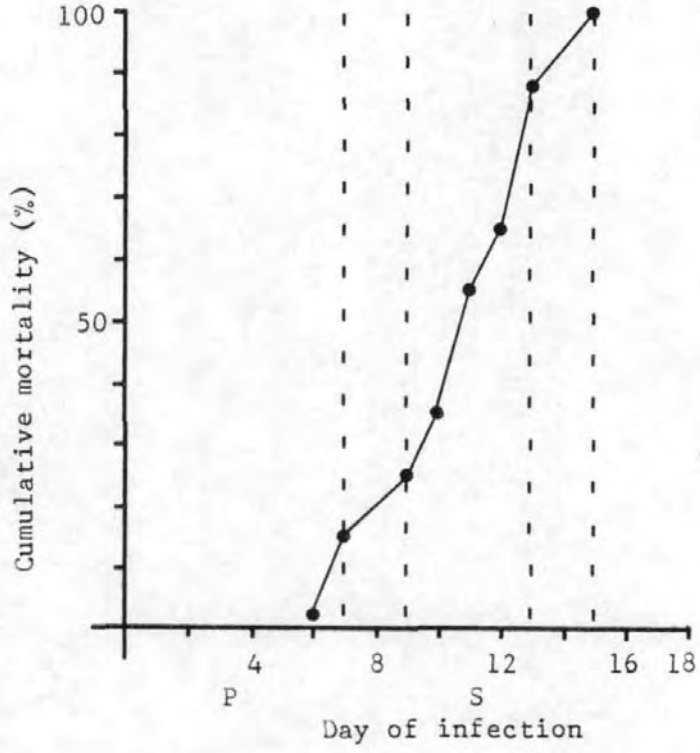


Fig 40



Figs 39 and 40 . Cumulative mortality (%) for non-immunised control fish following a potentially lethal dose of I. multifiliis (8 000 theronts/fish, 320/cm ^{3}). Temperature 20±2 °C.

P primary, S secondary infections of I. multifiliis.

Table 40. The effect of the corticosteroid Hydrocortisone 21-hemisuccinate(100µg g^{-1} body weight) on the immune response of 10 week old juvenile carp to I. multifiliis. Each group was comprised of 10 fish. Temperature 20 ±2°C.

Group	Treatment	Re-challenge	% infected	Degree of infection Dose and area	% mortality	Mean days to death (±SD)
Immunised	0 saline cortisol 100[a] cortisol 10[b]	0	0	None	0	N/A
Control	0 saline cortisol 100 cortisol 10					
Immunised	0 saline	ich				
	cortisol 100		90	light-medium, entire body	100	9.0±0.50
	cortisol 10		80			14.20±2.44
Control	0		100			13.30±2.79
	saline		90			15.30±1.89
	cortisol 100		100			9.10±1.20
	cortisol 10		90			12.30±3.65

a 100 ug b 10 ug Hydrocortisone 21-hemisuccinate g^{-1} body weight.

Table 41. The effect of the corticosteroid Hydrocortisone 21-hemisuccinate (100 μg g^{-1} body weight) on the immune response of 10 week old juvenile carp to I. multifiliis. Each group was comprised of 10 fish.

Group	Treatment	Re-challenge	% infected	Degree of infection, Dose and area	% mortality	Mean days to death (±SD)
Immunised	0 saline cortisol 100[a] cortisol 10[b]	0	0	None	0	N/A
Control	0 saline cortisol 100 cortisol 10					
Immunised	0	ich	30	light, periphery of fins	0	N/A
	saline		90		50	6.60±0.55
	cortisol 100		100	medium, entire body	90	10.56±1.59
	cortisol 10				80	6.38±1.06
Control	0			heavy, entire body	100	6.60±0.70
	saline					7.90±0.74
	cortisol 100					6.50±0.71
	cortisol 10					5.60±5.54

a 100 μg b 10 μg Hydrocortisone 21-hemisuccinate g^{-1} body weight

Figs. 41 and 42. Cumulative mortality (%) for immunised and non-immunised control fish following administration of Hydrocortisone 21-hemisuccinate (100 µg or 10 µg g^{-1} body weight) 6 days before a potentially lethal dose of I. multifiliis. 10 fish per group, temperature 20±2°C.

▽ immunised treated with cort 100, ■ immunised treated with cort 10, ✱ immunised treated with saline, ○ non-immunised controls untreated, ▲ non-immunised controls treated with saline, ● non-immunised controls treated with cort 100, □ non-immunised controls treated with cort 10.

Cort 100, cort 10 Hydrocortisone 21-hemisuccinate. P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 41

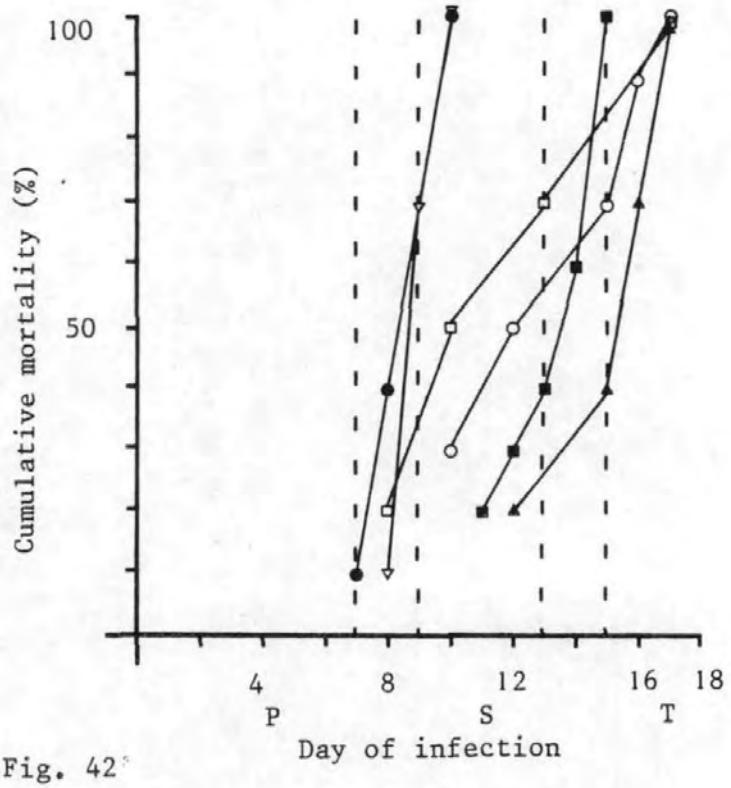

100
50
Cumulative mortality (%)
4
8
12
16
18
P
S
T
Day of infection

Fig. 42

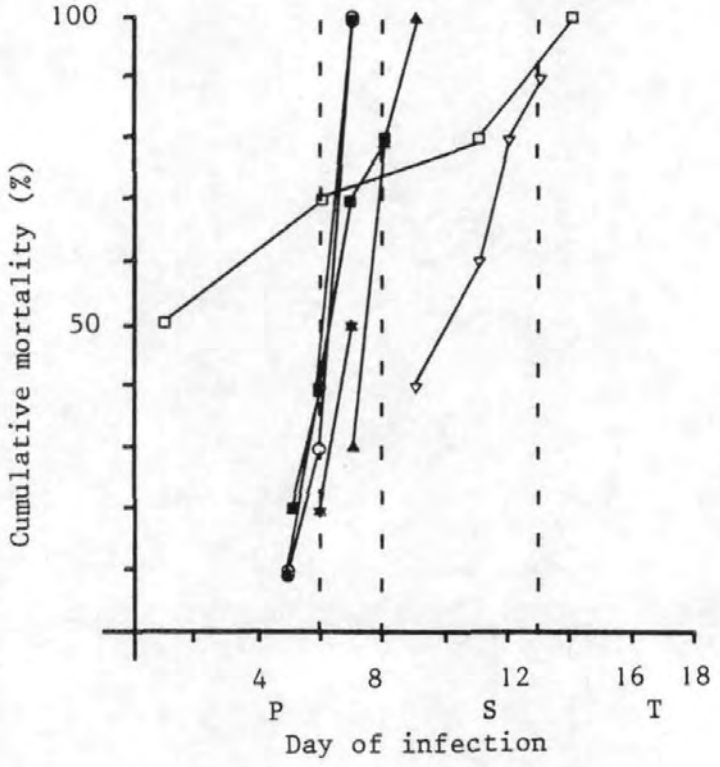
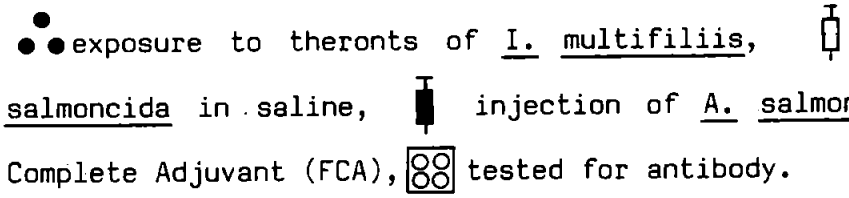

100
50
Cumulative mortality (%)
4
8
12
16
18
P
S
T
Day of infection

Fig. 43. Timing and sequences of administration of formalin killed Aeromonas salmonocida in respect to a single infection of Ichthyophthirius multifiliis.

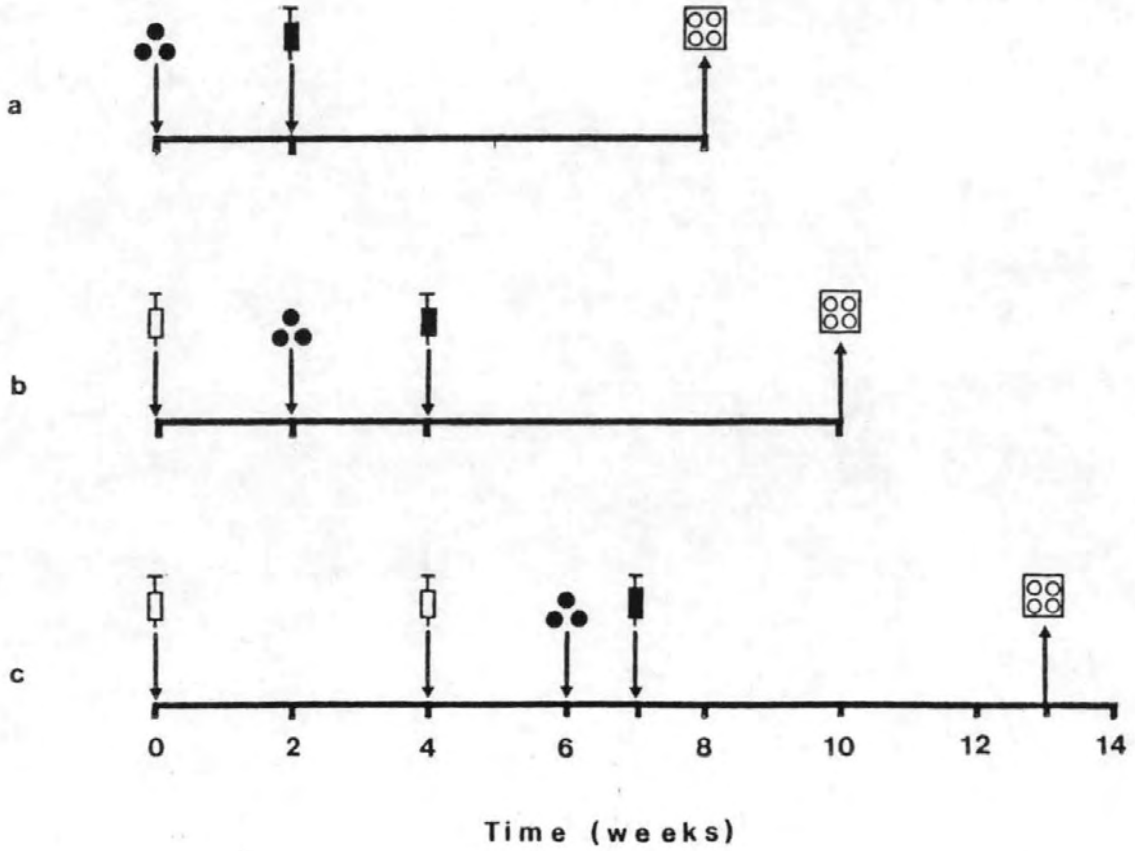

exposure to theronts of I. multifiliis, injection of A. salmoncida in saline, injection of A. salmonocida in Freund's Complete Adjuvant (FCA), tested for antibody.

a. Fish received a single injection of A. salmonocida in FCA following a primary infection of I. multifiliis. (Experiment 26).

b. Fish received an injection of A. salmonocida in saline 2 weeks prior to a primary infection of I. multifiliis followed by a challenge injection of A. salmonocida in FCA. (Experiment 27).

c. Fish received an injection of A. salmonocida in saline at 6 weeks and 2 weeks prior to a primary infection of I. multifiliis followed by a challenge injection of A. salmonocida in FCA. (Experiment 28).

Fig. 43.



EXPERIMENT 26. The immune response of juvenile carp to A. salmonocida administered as a single dose in Freund's complete adjuvant, following a primary infection of I. multifiliis.

One hundred and ten juvenile carp age 7½ weeks obtained from Plymouth Polytechnic's own stock (spawned 26.4.85.), were separated into 6 groups, A, B, C, D, E and F, each containing 20 fish with the exception of Group F, which contained 10. These were allowed to acclimate in aquaria for 2 weeks at 22±1° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 9½ weeks, the mean weight of the fish was 1.41 g.

Fish in Groups B and E only, were exposed to I. multifiliis given as a single exposure of approximately 2,000 theronts per fish (standard procedure), exposure being restricted to to a single, primary infection. Group C were sham controls receiving identical treatment as B and E including transference to fresh aquaria twice daily from days 5 - 10 of the experiment, as would be used for the prevention of secondary infection. Groups A, D and F received neither of these treatments. Two weeks later, fish in Groups A, B and C were injected with formalin killed Aeromonas salmonocida in Freund's complete adjuvant (FCA) at a dose of 1×10^{10} cells g^{-1} body weight by intraperitoneal injection, 10 µl g^{-1} body weight. Groups D and E being injected with 0.85% saline in FCA, and Group F were left untreated. Six weeks later, all fish were exsanquinated, serum being pooled from batches of 10 within each group of fish. Heat inactivated serum was tested by direct agglutination of formalin killed A. salmonocida.

It can be seen from the results (Table 42) that fish which had no prior treatment before challenge with A. salmoncida plus FCA (Group A) had a mean antibody titre of $-\log_2$ 11. Although the titres were lower for fish receiving a primary infection of I. multifiliis (Group B), the mean titre of $-\log_2$ 8 was not significantly different from fish receiving a sham infection (Group C), $-\log_2$ 9. Non-immunised control carp (Groups D, E and F) showed no background agglutination of A. salmonocida.

EXPERIMENT 27. The immune response of juvenile carp to A. salmonocida administered 2 weeks prior to a primary infection of I. multifiliis followed by a challenge dose in Freund's complete adjuvant

One hundred and ten juvenile carp age 8 weeks, obtained from Plymouth Polytechnic's own stock (spawned 26.4.85.), were separated into 6 groups A, B, C, D, E and F, each containing 20 fish with the exception of Group F, which contained 10. These were allowed to acclimate in aquaria for 2 weeks at 22±1° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks, the mean weight of the fish was 1.43 g.

Fish in Groups A, B and C were given a priming injection of formalin killed Aeromonas salmonocida in saline at a dose of 1×10^{10} cells g^{-1} body weight by intraperitoneal injection, 10 µl g^{-1} body weight. Fish in Groups D and E were injected with 0.85% saline and Group F were left untreated. Two weeks later, fish in Groups B and E were exposed to a single controlled primary infection (standard procedure), Group C, as in experiment 26 were given a sham exposure and the remaining groups (A, D, F) received no further treatment at this stage. Two

weeks later, fish in Groups A, B and C received a second injection of formalin killed A. salmonocida (1 x 10^{10} cells g^{-1} body weight), on this occasion, in FCA rather than saline. Groups D and E were injected with 0.85% saline in FCA and Group F were left untreated. Six weeks later, fish were exsanquinated, serum being pooled from batches of 10 within each group of fish. Heat inactivated serum was tested by direct agglutination of formalin killed A. salmonocida.

It can be seen from the results (Table 43) that fish which received no prior treatment before challenge with A. salmonocida plus FCA (Group A) or which received a primary infection of I. multifiliis (Group B), had antibody titres of $-\log_2$ 14. Those which received a sham infection (Group C) had an antibody titre of $-\log_2$ 15. Thus there was no significant difference in antibody titres to A. salmonocida following immunisation procedures. Non-immunised control carp (Groups D, E and F) showed no background agglutination of A. salmonocida.

EXPERIMENT 28. The immune response of juvenile carp to A. salmonocida administered at 6 weeks and 2 weeks prior to a primary infection of I. multifiliis followed by a challenge in Freund's complete adjuvant

One hundred and thirty juvenile carp age 6 weeks obtained from Plymouth Polytechnic's own stock (spawned 26.4.85.) were separated into 7 groups, A, B, C, D, E, F and G, each containing 20 fish with the exception of Group G, which contained 10. These were allowed to acclimate in aquaria for 2 weeks at 22±1° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 8 weeks, the mean weight of the fish was 1.33 g.

Fish in Groups A, B and C were all given a priming injection of formalin killed A. salmonocida in saline (1 x 10^{10} cells g^{-1} body weight) by intraperitoneal injection, 10 µl g^{-1} body weight, this was followed by a booster injection administered 4 weeks later. Those in groups D, E and F were injected with 0.85% saline following the same regime. Group G were kept as uninjected controls. Two weeks later, fish in Groups B and E were exposed to a single controlled primary infection (standard procedure) of I. multifiliis, Groups C and F were given a sham exposure, and the remaining groups (A, D and G) received no further treatment at this stage. One week later, fish in Groups A, B and C received a third injection of formalin killed A. salmonocida, on this occasion, in FCA rather than saline. Groups D, E and F were injected with 0.85% saline plus FCA and Group G was left untreated. Six weeks later, fish were exsanquinated, serum being pooled from batches of 10 within each group of fish. Heat-inactivated serum was tested by direct agglutination of formalin killed A. salmonocida.

It can be seen from the results (Table 44) that fish which received 3 injections of A. salmonocida but no exposure to I. multifiliis had an antibody titre of $-\log_2$ 13. Those which received a primary infection of I. multifiliis as well as 3 injections of A. salmonocida had antibody titres of $-\log_2$ 14 and those which received a sham infection (Group C) had an antibody titre of -log 12. Thus there was no significant difference in antibody titres to A. salmonocida following immunisation procedures. Non-immunised control carp (Groups D, E, F and G) showed no background agglutination of A. salmonocida.

Table 42. The effect of I. multifiliis, administered as a controlled primary infection, on the humoral response of juvenile carp to A. salmonocida . Fish were exposed to the parasite prior to the A. salmonocida. Results are mean titre from batches of up to 10 fish, following direct agglutination of the bacterin. Temperature 22± 1° C.

Group	No. of fish group	1st treatment	2nd treatment	Antibody titre -$\log_2$	No. of fish/group
A	20	0	A. salmonocida + FCA	11, 11	20
B		ich		8, 8	
C		Sham ich		9, 9	18
D		0	saline + FCA	0	10
E		ich			
F	10	0	0		

Table 43. The effect of I. multifiliis, administered as a controlled primary infection, on the humoral response of juvenile carp to A. salmonocida. Fish were exposed to the parasite following a single immunising dose of the bacterin, prior to a challenge injection. Results are mean titre from batches of up to 10 fish, following direct agglutination of the bacterin. Temperature 22± 1° C.

Group	No. of fish/group	Priming	1st treatment	2nd treatment	Antibody titre -$\log_2$	No. of fish/group
A	20	A. salmonocida	0	A. salmonocida + FCA	14, 14	20
B			ich		14, 14	15
C			sham ich		15, 15	20
D		saline	0	saline plus FCA	0, 0	16
E			ich			11
F	10	0	0	0		10

Table 44. The effect of I. multifiliis, administered as a controlled primary infection, on the humoral response of juvenile carp to A. salmonocida. Fish were exposed to the parasite following 2 immunising doses of the bacterin, prior to a challenge injection. Results are mean titre from batches of up to 10 fish, following direct agglutination of the bacterin. Temperature 22± 1° C.

Group	No. of fish/group	Primings	1st treatment	2nd treatment	Antibody titre $-\log_2$	No of fish/group
A	20	2 x A. salmonocida	0	A. salmonocida plus FCA	13, 13	13
B			ich		14, 14	19
C			sham ich		12, 12	20
D		2 x saline	0	saline plus FCA	0, 0	
E			ich			
F			sham ich			
G	10	0	0	0		10

EXPERIMENTS 29-32. Fate of theronts in the immune host

A series of 4 investigations were undertaken to determine the behaviour and fate of theronts contacting immune hosts. As a similar approach was adopted for each investigation, a summary of the procedures common to all is given below; general information being presented in table 45.

All fish were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. The standard procedures of infection and immunisation are described in the Materials and Methods, with the exception that in experiment 29 the third immunising dose of theronts was increased to approximately 4,000 theronts per fish at a concentration of approximately 160/cm^{3} to compensate for a decrease in infectivity of the theronts.

At each immunisation and at challenge between 10-40 non-immunised controls, (depending on the experiment), were exposed to theronts, to test for viability of theronts.

Twenty-eight days following the last immunisation dose, fish were challenged following the standard procedure. Immediately following challenge, immunised and non-immunised fish were treated with protozin (Waterlife Research, UK) in order to kill off any parasites that had not entered the epidermis. The treatment undertaken was first to rinse the fish 3 times in water and then to place them in protozin for

Table 45. Number and size of fish used for investigations on the fate of theronts in the immune host (experiments 29-32). All fish were obtained from Stowmarket (spawned 8.2.86).

experiment	No. of fish		Start of experiment	
	immunised	non-immunised controls	age (weeks)	mean weight (mg)
29	70	100	11	950
30	110	110	10	743
31	160	160	10	960
32	100	110	10	965

5 minutes at a dose which killed theronts in 90 seconds. Following exposure, immunised and non-immunised control fish were each divided into 3 groups of 10 and given one of the following treatments: injected intraperitoneally with 200 µg g^{-1} body weight of Triamcinolone acetonide suspension in saline, or injected intraperitoneally with saline, or left untreated. Fish were then maintained free from exposure to I. multifiliis. The same procedure was carried out with other batches of 30 immunised and 30 control fish, at varying intervals following challenge of either 1, 2 or 3 days. Five days following challenge, fish were examined for the presence of I. multifiliis.

Exceptions to the above procedure were as follows: in experiment 29, treatment was only given on the day of challenge, and a further group of fish were included which were administered Hydrocortisone-21-hemisuccinate at a dose of 100 µg g^{-1} body weight. In experiment 32, fish were exposed to challenge infections 28 days after the last immunising dose, and re-exposed to challenge infections a further 28 days later, after which the regime of administration of corticosteroids was followed. In addition, in experiments 31 and 32, at the time of the last challenge infection, 10 immunised and 10 non-immunised control fish were exsanquinated and serum pooled within each group of fish. Serum was tested (non-heat inactivated) for antibody against I. multifiliis by direct immobilization/agglutination of live theronts of I. multifiliis.

Any infections that developed were allowed to continue.

Results (tables 46, 47, 49, 53) indicate that protective immunity was

established in all the carp to I. multifiliis, and that fish survived challenges with controlled levels of theronts, although a light infection confined to the periphery of the fins occurred in a small percentage of fish in experiment 32 (10%). Mortalities of 90-100% occurred in control fish following challenge at similar doses. Cumulative mortalities (%) for non-immunised controls, experiments 29-32, Figs. 44-47 respectively, over a period of 18 days indicate first deaths following day 6 with the majority during the secondary infection.

The results indicate that some theronts have entered and survived in the immune host.

Following administration of Triamcinolone acetonide and Hydrocortisone 21-hemisuccinate in experiment 29, (Table 46) immediately following exposure to challenge infections of I. multifiliis, immunised fish did develop infections of I. multifiliis. A greater degree of infection developed in those fish administered Triamcinolone acetonide than Hydrocortisone. A small percentage (20%) of immunised fish injected with saline developed an infection of I. multifiliis, but this was restricted to the periphery of fins. Non-immunised control fish not exposed to challenge infections and administered saline or Hydrocortisone did not develop any infection of I. multifiliis.

In experiment 30, (tables 47, 48, Fig. 45), when immunised fish were administered Triamcinolone acetonide immediately following an exposure to challenge infections (Table 47) and one day following exposure to challenge infections of I. multifiliis, infections of the parasite developed in both groups of fish, although were heavier covering the

entire body, in the group administered corticosteroid immediately after challenge with lighter infections developing in those fish administered corticosteroid one day after challenge infections (Table 48). Here, only 60% of the fish developed an infection over the entire body whereas the remaining 40% had infections restricted to the fins. In the immunised fish administered saline immediately following challenge, 10% of the fish showed an infection but this was restricted to the periphery of the fins. Mortalities of 100% occurred in both groups of immunised fish administered corticosteroid, (Fig. 45) and a combined cumulative mortality (%) for day 0 and day 1 indicated that deaths occurred chiefly during and on termination of the secondary infection. All mortalities occurred within 15 days. No infections or mortalities occurred in non-immunised control fish administered Triamcinolone acetonide or saline and not exposed to challenge infections.

In experiment 31 (Tables 49, 50, 51, 52, Figs. 46, 48) the results show that immunised fish administered Triamcinolone acetonide, immediately after exposure to challenge infections, and in different groups on day 1, 2 and 3 following challenge, infections of I. multifiliis did develop. The greatest degree of infection occurred on day 0 (Table 49), with infections being heavy and covering the entire body. From day 1 to day 3 (Tables 50, 51, 52) the infections developing on the body became lighter, with a greater percentage of the infections being restricted to the fins (Fig. 48). Mortalities of up to 100% resulted from these infections. Cumulative mortality (%) can be seen in Fig. 46 with mortalities chiefly occurring during the secondary infection, with the exception of immunised fish administered corticosteroid on day 3 following challenge, where mortalities

occurred during tertiary reinvasion. No infections or mortalities occurred in non-immunised controls not subject to challenge infections of I. multifiliis, but administered corticosteroid or saline. The antibody titre of the immunised fish at the time of challenge was $-\log_2$ 7. Non-immunised controls had no antibody to I. multifiliis.

Similar results were recorded in experiment 32 (Tables 53, 54, 55, Figs. 47, 49) where immunised fish were rechallenged with I. multifiliis and then administered Triamcinolone acetonide immediately following the challenge exposure to I. multifiliis and on days 1 and 2 following challenge. Heavy infections covering the entire body occurred in the group administered corticosteroid immediately following challenge (Table 53), but on day 1 (Table 54) and day 2 (Table 55) after challenge, lighter infections occurred on the body, with a higher percentage of infected fish having infections restricted to the fins (Fig. 49). Mortalities of 100% occurred in all immunised fish administered corticosteroid resulting from these infections. Cumulative mortality (%) can be seen in Fig.47. Mortalities chiefly occurred during secondary infection with the exception of those immunised fish administered corticosteroid on day 2 following challenge, where mortalities occurred during the tertiary infection. All mortalities had occurred by day 20. No infections or mortalities occurred in non-immunised controls not subject to challenge infections of I. multifiliis but administered corticosteroid or saline. The antibody titre of the immunised fish at the time of re-challenge was $-\log_2$ 8. Non-immunised controls had no antibody to I. multifiliis.

Table 46. The effect of the corticosteroids Triamcinolone acetonide (100 µg g^{-1} body weight) and Hydrocortisone 21-hemisuccinate (100 µg g^{-1} body weight) on carp previously immunised with I. multifiliis, administered immediately following challenge. Temperature 20± 2° C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity		% mortality	Mean days to death ±SD
				Fins	Body		
immunised	10	0	0	None	None	0	N/A
		saline	20	20 very light, periphery	0		
		Ta 100	90	20 heavy	70 medium to heavy	N/A	N/A
		cortisol 100	40	30 light, periphery	10 heavy		
control		0	0	None	None	0	N/A
		saline					
		Ta 100					
		cortisol 100					
control	20	challenged	90	heavy	heavy, entire body	90	12.56±4.56

N/A Not applicable

Table 47. The effect of the corticosteroid Triamcinolone acetonide (200 $\mu g\ g^{-1}$ body weight) on carp previously immunised with I. multifiliis, administered immediately following challenge . Temperature 20 ± 2^{o} C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity		% mortality	Mean days to death ±SD
				Fins	Body		
Immunised	10	0	0	None	None	0	N/A
		saline	10	10 very light, periphery	0	0	N/A
		Ta 200	100	Heavy	100 heavy	100	13.26±1.48
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					
Control	40	challenged	100	Heavy	100 medium-heavy	100	11.12±2.5

N/A Not applicable

Table 48. The effect of the corticosteroid Triamcinolone acetonide (200 μg g^{-1} body weight) on carp previously immunised with I. multifiliis, administered one day following challenge. Temperature 20± 2° C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death ±SD
Immunised	10	0	0	None	None	0	N/A
		saline					
		Ta 200	100	40 heavy	60 light	100	13.26±1.48
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					

N/A Not applicable

Table 49. The effect of the corticosteroid Triamcinolone acetonide (200 μg g^{-1} body weight) on carp previously immunised with I. multifiliis, administered immediately following challenge. Temperature 20± 2° C.

Group	No.of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death ±SD
Immunised	10	0	0	None	None	0	N/A
		saline					
		Ta 200	100	Heavy	100 Heavy	100	10.9±1.10
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					
Control	40	challenged	100	medium-heavy	100 medium-heavy	90	11.33±2.40

N/A Not applicable

Table 50. The effect of the corticosteroid Triamcinolone acetonide (200 μg g^{-1} body weight) on carp previously immunised with I. multifiliis, administered one day following challenge. Temperature 20± 2° C.

Group	No.of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death ±SD
Immunised	10	0	0	None	None	0	N/A
		saline					
		Ta 200	90	60 heavy	30 medium	100	11.7±1.25
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					

N/A Not applicable

Table 51. The effect of the corticosteroid Triamcinolone acetonide (200 $\mu g\ g^{-1}$ body weight) on carp previously immunised with I. multifiliis, administered 2 days following challenge. Temperature 20± 2° C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death ±SD
Immunised	10	0	0	None	None	0	N/A
		saline	20	20 very light, periphery	None		
		Ta 200	80	60 light	20 light	100	15.0±0
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					

N/A Not applicable

Table 52. The effect of the corticosteroid Triamcinolone acetonide (200 µg g^{-1} body weight) on carp previously immunised with I. multifiliis, administered 3 days following challenge. Temperature 20± 2° C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death ±SD
Immunised	10	0	0	None	None	0	N/A
		saline					
		Ta 200	80	60 light,	20 very light	100	15.6±0.52
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					

N/A Not applicable

Table 53. The effect of the corticosteroid Triamcinolone acetonide (200 μg g^{-1} body weight) on carp previously immunised with I. multifiliis, administered immediately following a second re-challenge. Temperature 20± 2° C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death ±SD
Immunised	10	0	10	10 very light	None	0	N/A
		saline	0	None	None		
		Ta 200	100	medium	100 medium to heavy	100	12.2±0.79
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					
Control	10	challenged	100	medium	100 heavy	100	11.7±1.70

N/A Not applicable

Table 54. The effect of the corticosteroid Triamcinolone acetonide (200 $\mu g\ g^{-1}$ body weight) on carp previously immunised with I. multifiliis, administered one day following a second challenge. Temperature 20± 2° C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death (±SD)
Immunised	10	0	0	None	None	0	N/A
		saline	10	10 very light, periphery	None		
		Ta 200	100	50 heavy	50 medium	100	12.2±0.91
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					

N/A Not applicable

Table 55. The effect of the corticosteroid Triamcinolone acetonide (200 µg g^{-1} body weight) on carp previously immunised with I. multifiliis, administered two days following a second challenge. Temperature 20± 2° C.

Group	No. of fish/group	Treatment	Total % infected	% of infections covering 2 separate regions, fins and body, together with severity. Fins	Body	% mortality	Mean days to death ±SD
Immunised	10	0	0	None	None	0	N/A
		saline	10	10 very light, periphery	None		
		Ta 200	90	70 medium-light	20 medium-light	100	18.6±0.70
Control		0	0	None	None	0	N/A
		saline					
		Ta 200					

N/A Not applicable

Fig. 44. Cumulative mortality (%) for non-immunised control fish following a potentially lethal dose of I. multifiliis. (8,000 theronts per fish, 320/cm ^{3}). Temperature 20±2°C. P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 45. Cumulative mortality (%) of immunised fish following administration of Triamcinolone acetonide (200 µg g^{-1} body weight) on day 0 and day 1 ▲ (pooled results) following a potentially lethal dose of I. multifiliis. (8,000 theronts/fish, 320/cm ^{3}). Mortalities for non-immunised control fish exposed to a challenge infection are also shown ● . 10 fish per group. Temperature 20±2°C.
P primary , S secondary, T tertiary infections of I.multifiliis.

Fig. 44

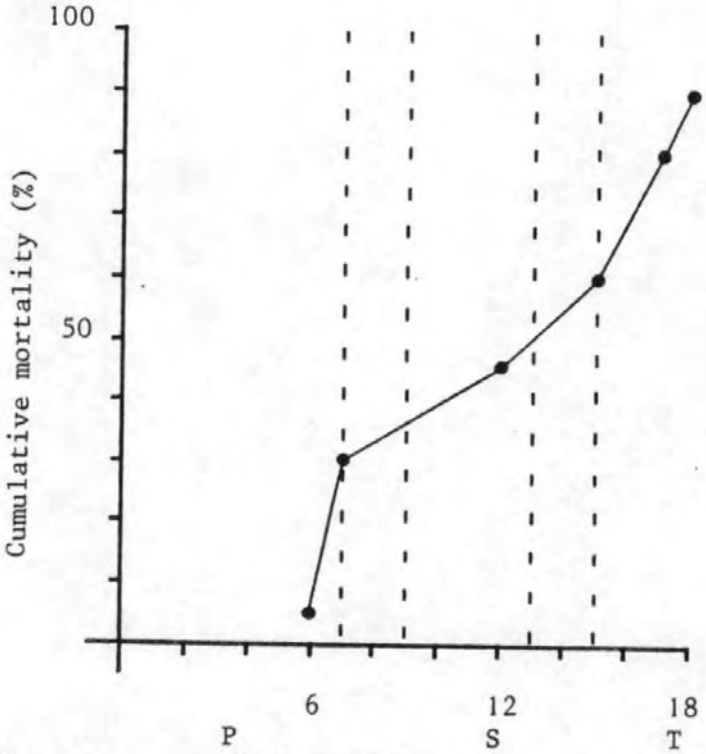

100
50
Cumulative mortality (%)
6
12
18
P
S
T
Day of infection

Fig. 45

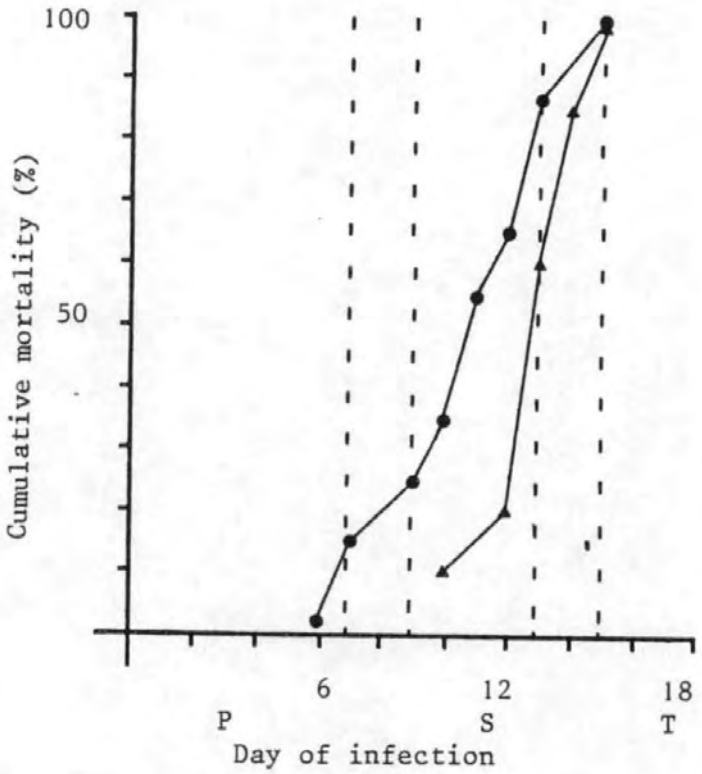

100
50
Cumulative mortality (%)
6
12
18
P
S
T
Day of infection

Fig. 46. Cumulative mortality (%) of immunised fish following administration of Triamcinolone acetonide (200 μg g^{-1} body weight) on days 0, 1, 2 and 3 following a potentially lethal dose of I. multifiliis (8,000 theronts/fish, 320/cm^3). Mortalities for non-immunised control fish exposed to a challenge infection are also shown. 10 fish per group, temperature 20±2°C. Immunised fish day 0 ▲ , day 1 ▽ , day 2 □ , day 3 ■ . Non-immunised controls ● .

P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 47. Cumulative mortality (%) of immunised fish following administration of Triamcinolone acetonide (200 μg g^{-1} body weight) on days 0, 1 and 2 following a potentially lethal dose of I. multifiliis. (8,000 theronts/fish, 320/cm^3), Mortalities for non-immunised control fish exposed to a challenge infection are also shown. 10 fish per group, temperature 20±2°C. Immunised fish day 0 ▲ , day 1 ▽ , day 2 □ . Non-immunised controls ● .

P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 46

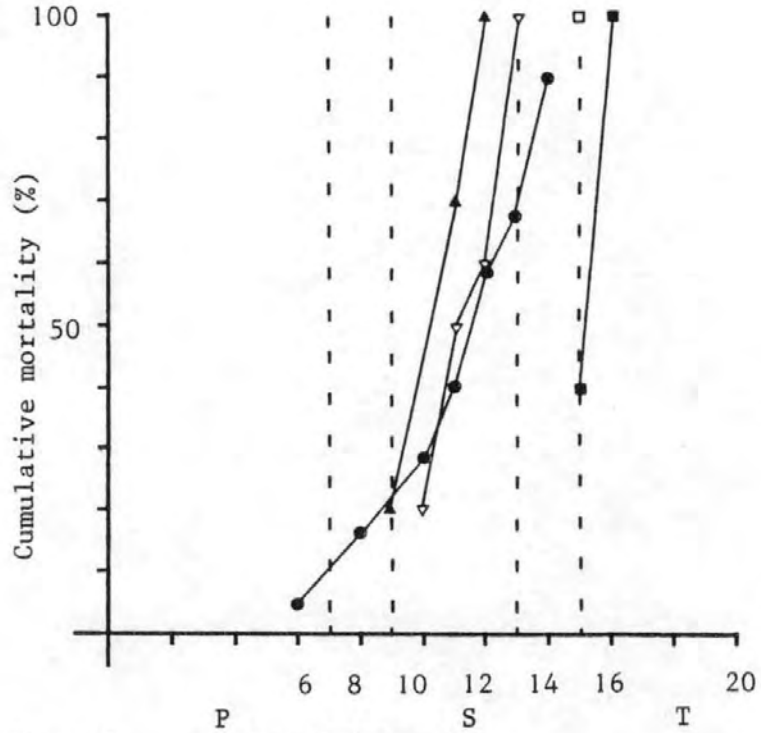

100
50
Cumulative mortality (%)
6
8
10
12
14
16
20
P
S
T
Day of infection

Fig. 47

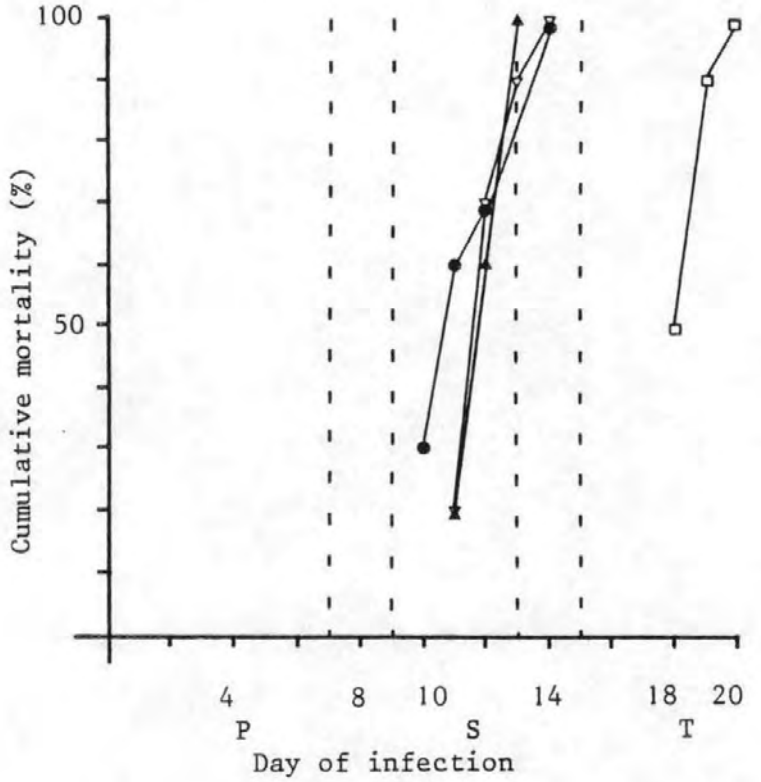

100
50
Cumulative mortality (%)
4
8
10
14
18
20
P
S
T
Day of infection

Fig. 48.

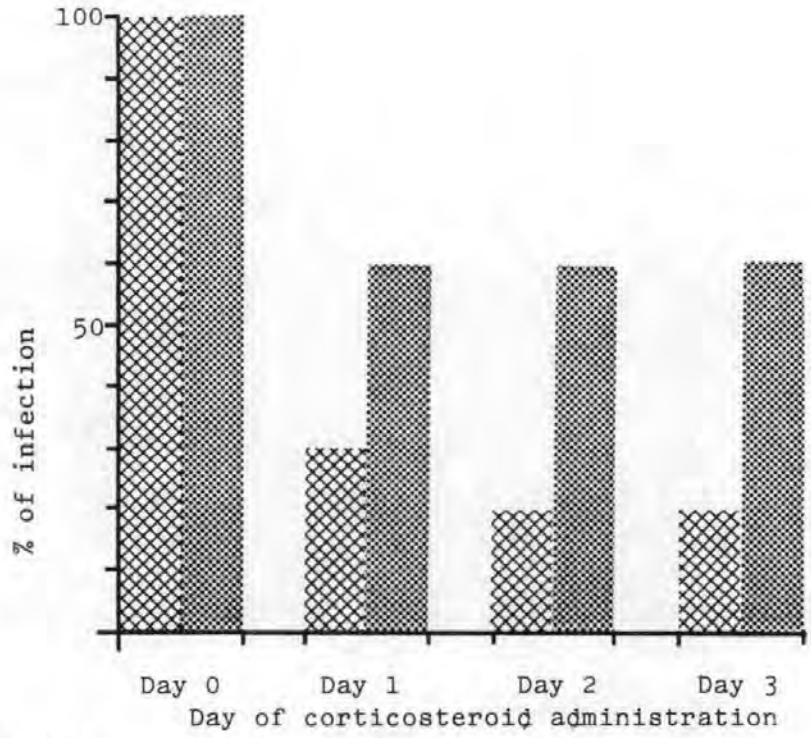
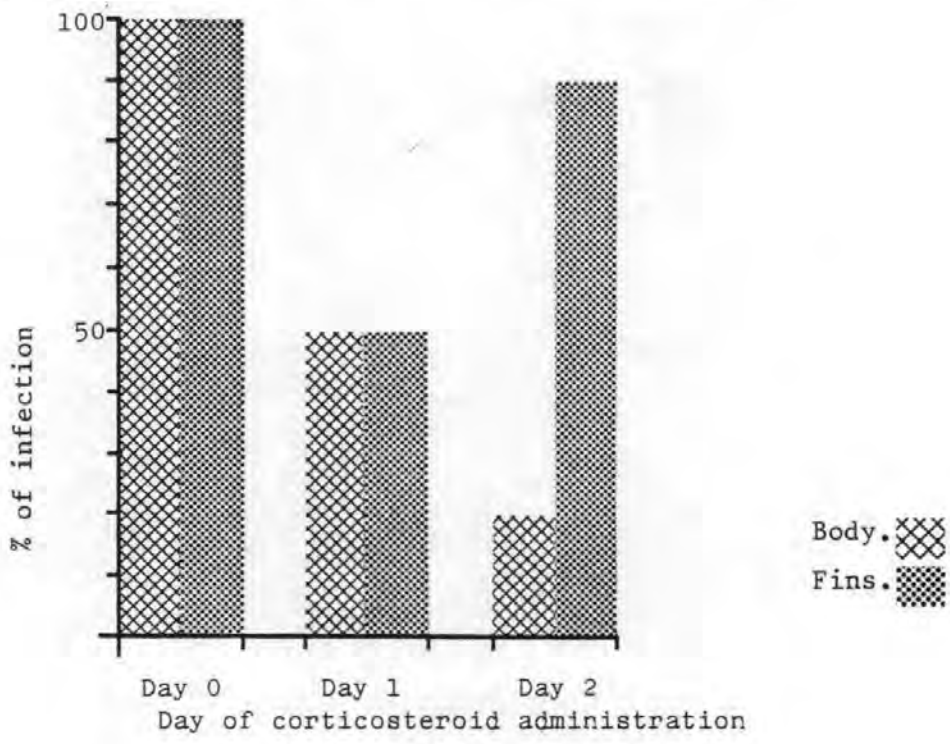


Fig. 49.



Figs 48 and 49 . Area and percentage of fish developing an infection of I. multifiliis following administration of Triamcinolone acetonide (200 µg g^{-1} body weight) on days 0, 1, 2 or 3 following a potentially lethal challenge (8 000 theronts/fish, 320/ cm^{3}) of I. multifiliis.

EXPERIMENT 33. Possible survival of Ichthyophthirius multifiliis in immunised juvenile carp

Three hundred and fifty juvenile carp age 7 weeks obtained from Plymouth Polytechnic stock (spawned 26.4.85.) were separated into 2 groups A and B, containing 180 and 170 fish respectively. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 9 weeks, the mean weight of the fish was 1.06 g.

Fish in Group A were exposed to 3 immunising doses of I. multifiliis on 3 separate occasions of 14 day intervals. Fourteeen days after each exposure and following a potentially lethal challenge 28 days after the last immunising dose, 30 fish were removed for investigation of possible survival of I. multifiliis. Ten fish were administered Triamcinolone acetonide suspension in saline at a dose of 200 µg g^{-1} body weight, 10 were administered saline and 10 were untreated. Administrations were by intraperitoneal injections, 10 µl g^{-1} body weight. Fish were taken from control Group B (10) at each immunisation stage to test for viability of theronts and at challenge to confirm protective immunity. In addition, uninfected controls (Group B) were administered the same treatments as for Group A. Ten days after treatment, fish were exsanquinated. Serum was pooled within each group of 10 fish and tested (non-heat inactivated) for antibody against I. multifiliis using direct immobilization/agglutination of theronts. During the 10 days between treatment and when fish were exsanquinated, observations were made to see whether any signs of the development of latent infections of I. multifiliis occurred.

It can be seen from the results (Table 56) that all immunised fish survived a potentially lethal challenge of I. multifiliis, although light infections on the periphery of fins were present in 25% of these. Non-immunised controls subjected to challenge infections suffered 100% mortality. Cumulative mortality (%) with time, for the controls, can be seen in Fig. 50. First deaths occurred at 4 days, with all mortalities within 13 days. The deaths were chiefly from day 8 coinciding with the secondary infection. Mean days to death were 9.6.

Table 57 shows the results of antibody titre and whether infections of I. multifiliis developed following corticosteroid treatment. From the results, there is no evidence under the treatments and exposure regimes investigated that I. multifiliis extends its survival within the fish host beyond its expected duration of 8 days at 20±2° C. Antibody titres following the various procedures were: after one exposure to I. multifiliis immunised fish, (Group A) either untreated or treated with saline had antibody titres of $-\log_2$ 2. Those treated with corticosteroid had an antibody titre of $-\log_2$ 1. After 2 exposures to I. multifiliis, immunised fish (Group A1) either untreated or treated with corticosteroid had antibody titres of $-\log_2$ 3. Those treated with saline had an antibody titre of $-\log_2$ 2. After 3 exposure to I. multifiliis, immunised fish (Group A2) untreated, had an antibody titre of $-\log_2$ 7; saline $-\log_2$6, and those treated with corticosteroid, $-\log_2$ 4. Following challenge with I. multifiliis, immunised fish (Group A3) untreated, had an antibody titre of $-\log_2$ 8; saline, $-\log_2$ 9 and those treated with corticosteroid, $-\log_2$ 6.

Investigations of antibody titre, therefore, show no significant change following administration of corticosteroids, although a slight

Table 56. The effect of a potentially lethal challenge of I. multifiliis following immunisation with 3 serial infections of the parasite. Temperature, 20 ±2 °C.

Group	Treatment	No. of fish	percentage infected	Degree of infection	Percentage mortality	Mean days to death (±SD)
A	Immunised	40	25	light-medium, periphery of fins	0	N/A
B	Control	10	100	heavy, entire body	100	9.6±2.83

N/A not applicable

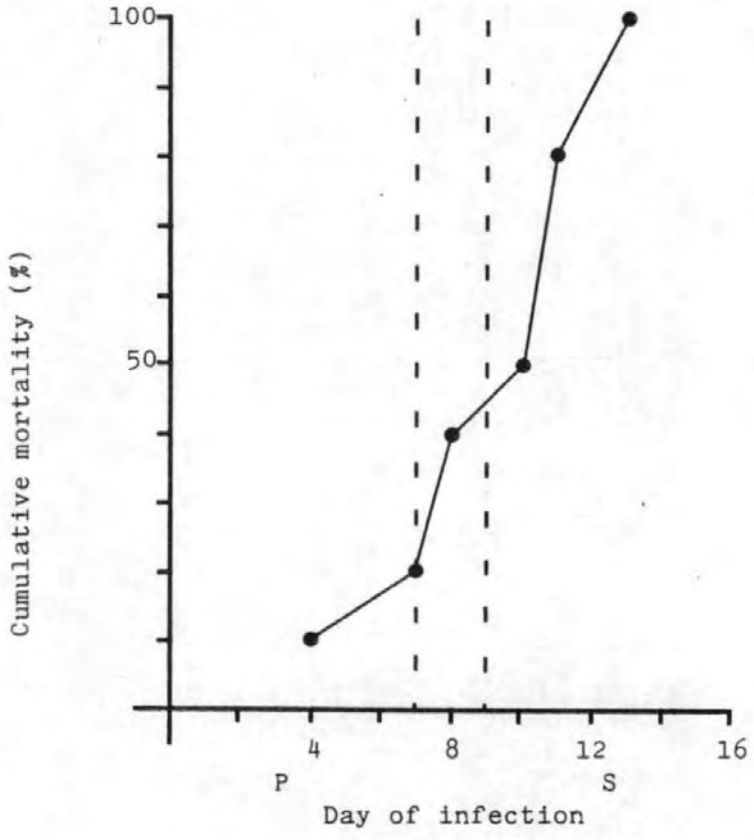


Fig 50. Cumulative mortality (%) for non-immunised control fish following a potentially lethal dose of I. multifiliis (8 000 theronts/fish, 320/cm ^{3}).

P primary, S secondary infections of I. multifiliis.

Table 57. The use of the corticosteroid Triamanolone acetonide (200 μg g^{-1} body weight) to examine whether I. multifiliis survives in the host following 1, 2, 3 and challenge exposures to the parasite, with effects on the humoral response. Temperature 20±2 °C, serum pooled from 10 fish.

Group	Treatment I	II	No. of fish/ group	Presence of ich	Mean antibody titre $-\log_2$ [b]
A	One exposure to I. multifiliis	0 saline Ta 200[a]	10	0	2 2 1
B	controls	0 saline Ta 200			0
A_1	Two exposures to I. multifiliis	0 saline Ta 200	10	0	3 2 3
B_1	controls	0 saline Ta 200			0
A_2	Three exposures to I. multifiliis	0 saline Ta 200	10	0	7 6 4
B_2	controls	0 saline Ta 200			0
A_3	Three exposures plus challenge to I. multifiliis	0 saline Ta 200	10	0	8 9 6
B_3	controls	0 saline Ta 200			0

a Ta 200, Triamcinolone acetonide 200 μg g^{-1} body weight
b Duplicate tests

reduction occurred in A3. Non-immunised controls (Groups B, B1, B2 and B3) either untreated, or treated with saline or corticosteroid, at no time showed any background antibody to I. multifiliis.

TETRAHYMENA PYRIFORMIS

Basic aspects of the immune response are covered under humoral antibody response, cellular proliferation, immunisation against I. multifiliis and cross reactivitiy between I. pyriformis and I. multifiliis. This has involved a total of 11 experiments.

Humoral response

EXPERIMENT 34. The humoral response of juvenile carp to Tetrahymena pyriformis.

460 juvenile carp age 8 weeks obtained from Stowmarket (spawned 8.2.86.) were separated into four groups, A, B, C and D. Groups A and B contained 150 fish and Groups C and D contained 80 fish. These were allowed to acclimate in tanks (600 dm^3 capacity) for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks, the mean weight of the fish was 951 mg.

Fish in Groups A and B were injected intraperitoneally with approximately 25 µg of protein g^{-1} bodyweight of whole, live, T. pyriformis. Groups C and D were injected with 0.85% saline. Group A with saline controls C, were used to investigate the primary immune response and Group B, with saline controls D, were used to investigate the secondary immune response. The secondary response was begun 4 weeks after the first injection, by giving the fish a booster injection of the same dose of T. pyriformis.

For the primary response, 20 fish from Group A and 10 fish from Group C were exsanquinated at the following weeks after the injection: 1, 2, 3, 4, 6, 8, 10 and 12. For the secondary response, 20 fish from Group B and 10 fish from Group D were exsanquinated at the following weeks after the booster injection: 1, 2, 3, 4, 6, 8, 10, 12, 14 and 16. Serum was pooled from Groups of 10 fish and tested (non-heat inactivated) for antibody against T. pyriformis using direct immobiliz-

ation/agglutination of live organisms.

The mean antibody titres of carp after one injection of T. pyriformis are shown in Fig. 51. The kinetics of the primary antibody response were studied over a period of 12 weeks. Results indicate a gradual increase in antibody titre following primary injection of T. pyriformis with antibody first detected at week 2 ($-\log_2$ 2.5) with the peak response being observed at week 6 ($-\log_2$ 6.5) after which, antibody titre began to decrease to $-\log_2$ 4 by week 12 when the experiment was terminated. Following a second injection of T. pyriformis, the antibody titre was further elevated, with peak antibody titre ($-\log_2$ 11.5) being observed at week 6. Antibody titre began to decrease but at a slower rate than for the primary response, relatively high levels still being maintained at week 16 ($-\log_2$ 8.5) when the experiment was terminated. Serum from saline and untreated controls were negative to T. pyriformis and showed no natural agglutinis.

Autoradiography

EXPERIMENT 35. The ceullular proliferative response and humoral response of juvenile carp to Tetrahymena pyriformis.

100 juvenile carp age 8 weeks obtained from Sparsholt (spawned 30.1.85.) were separated into 7 groups, A, B, C, D, E, F and G. Groups A, B and C contained 20 fish, the remaining groups each containing 10 fish. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment,

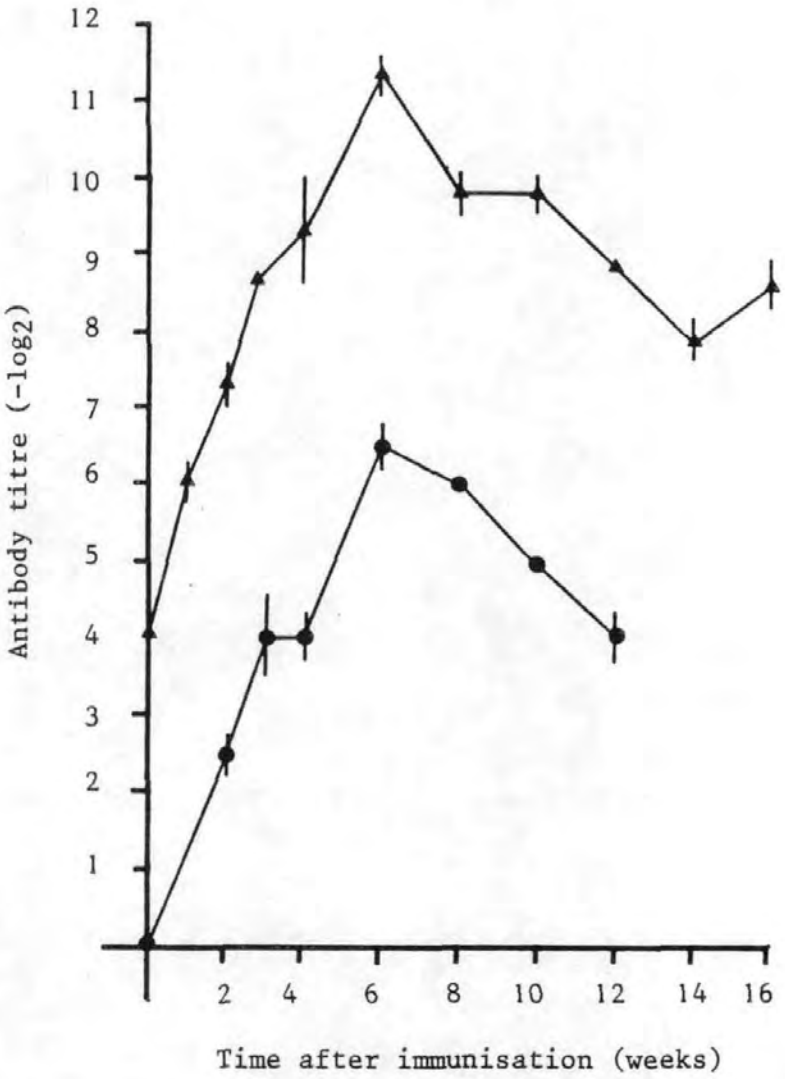


Fig. 51. Kinetics of primary and secondary responses of juvenile carp to Tetrahymena pyriformis. Temperature 20± 2°C, 10 fish per group.

● primary response after a single injection.
▲ secondary response after two injections.
The second injection was administered 1 month after the first.

at 10 weeks, the mean weight of the fish was 690 mg.

Fish in Groups A, E and F were injected intraperitoneally with approximately 25 µg protein g^{-1} body weight of whole, live, T. pyriformis antigen in 0.85% saline at a volume of 10 µl g^{-1} body weight. This was followed by two booster injections administered at 2 weekly intervals. Those in Groups B, D and G were injected with 0.85% saline, following the same regime. Group C were kept as uninjected controls. Four weeks after the last injection, fish were administered the following challenge injections: Groups A and D, T. pyriformis in Freund's complete adjuvant (F.C.A.); Groups B and E, saline in F.C.A. and Groups C, F and G remaining untreated. Three weeks following challenge injections, all fish in Groups D, E, F and G and 10 fish from each of Groups A, B and C were injected with Methyl-^{3}H thymidine, and exsanquinated 18 hours after the injection. The following organs were fixed in 10% buffered formal saline: spleen, propephros, opisthonephros and fibrowax sections at 5 µm were processed for autoradiography. Serum was pooled within each group of 10 fish and tested (non-heat inactivated) for antibody against T. pyriformis using direct immobilization/agglutination of live organisms. Three weeks later, the remaining fish in Groups A, B and C were exsanquinated. Serum from fish within each group was pooled and tested for antibody against T. pyriformis using direct immobilization/agglutination of live organisms.

The results for antibody tests are shown in table 58. Three weeks after the challenge injections, immunised fish in Group A had an antibody titre of $-\log_2 10$. Fish in Groups E and F which received the three priming injections of T. pyriformis in saline, but no challenge

Table 58. Immunisation of juvenile carp with 25 μg g^{-1} body weight of T. pyriformis: direct immobilization/agglutination of live T. pyriformis. Temperature 20 ±2°C. Results are mean titre, obtained from triplicate tests of pooled sera from up to 10 fish per group, obtained after 2 hours and re-examined after 4 hours.

Group	No. of fish	Procedure 3 injections	Final injection + FCA	No. of fish/group	Antibody titre -$\log_2$ 3 weeks	6 weeks
A	20	T. pyriformis	T. pyriformis	20	10	11
B		saline	saline		0	0
C		0	0			
D	10	saline	T. pyriformis	10	6	NT
E		T. pyriformis	saline		9	
F		T. pyriformis	0	6	10	
G		saline	0	10	0	

NT not tested

Table 59. Mean autoradiography counts in juvenile carp 3 weeks following immunisation with 25 µg protein g^{-1} body weight of T. pyriformis. Temperature 20±2°C.

1st treatment	2nd treatment	Organ	Mean autoradiography counts/0.1mm ^{2}. (± standard error)	No.of fish/ group
T. pyriformis	T. pyriformis	Pro-nephros	16.38±2.60	10
T. pyriformis	Saline		30.60±2.75	
T. pyriformis	None		N/A	
Saline	T. pyriformis		64.78±2.99	
Saline	Saline		12.0±1.15	
None	None		38.4±1.60	
T. pyriformis	T. pyriformis	Opistho-nephros	29.97±2.60	
T. pyriformis	Saline		31.60±2.80	
T. pyriformis	None		32.91±1.00	
Saline	T. pyriformis		41.74±1.23	
Saline	Saline		34.07±1.47	
None	None		36.57±1.53	
T. pyriformis	T. pyriformis	Spleen	7.13±1.31	
T. pyriformis	Saline		6.30±2.00	
T. pyriformis	None		14.94±1.30	
Saline	T. pyriformis		10.44±0.86	
Saline	Saline		11.87±0.82	
None	None		16.09±2.01	

N/A not available

injection had titres of $-\log_2$ 9 and 10 respectively. Group D which were primed with saline but challenged with T. pyriformis had a lower titre of $-\log_2$ 6 All non-immunised saline and untreated controls (Groups B, C, G) showed no background antibody to T. pyriformis. When sera were tested six weeks after challenge (Groups A, B and C), fish immunised with T. pyriformis (Group A) still had a high titre of $-\log_2$ 11 whereas control groups B and C still remained negative.

The results for autoradiography can be seen in table 59, and show that there is little difference between fish immunised with T. pyriformis and saline injected or untreated controls. Fish which received priming injections of saline followed by a final challenge with T. pyriformis, have slightly higher autoradiography counts in the pronephros compared to immunised and saline injected or untreated controls.

EXPERIMENT 36. The cellular proliferative response and humoral response of juvenile carp to Tetrahymena pyriformis.

240 juvenile carp age 8 weeks obtained from Stowmarket (spawned 8.2.86.) were separated into 4 groups, A, B, C and D, each group containing 70 fish with the exception of Group D which contained 30 fish. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks, the mean weight of the fish was 925 mg.

Fish in Group A were injected intraperitoneally with approximately 25 µg protein g^{-1} bodyweight of whole, live T. pyriformis antigen in

0.85% saline at a volume of 10 µl g^{-1} bodyweight. This was followed by two booster injections administered at 2 weekly intervals. Those in groups B and D were injected with 0.85% saline, following the same regime. Group C were kept as uninjected controls. Four weeks after the last injection, fish were administered the following challenge injections: Group A and D, T. pyriformis in F.C.A.; Group B saline in F.C.A. and Group C remaining untreated. At the time of challenge injections, 10 fish from each group were exsanquinated. Serum from fish within each group was pooled and tested for antibody against T. pyriformis by direct immobilization/agglutination of live organisms.

At weekly intervals following challenge injections, up to 3 weeks, 10 fish from Groups A, B and C were injected with Methyl ^{3}H thymidine and exsanquinated 18 hours after the injection. The following organs were fixed in 10% buffered formal saline: spleen, pronephros, opisthonephros and fibrowax sections at 5 µm were processed for autoradiography. Group D were killed at 3 weeks and the same procedure carried out. Serum was pooled within each group of 10 fish and tested (non-heat inactivated) for antibody against T. pyriformis using direct immobilization/agglutination of live organisms. Three weeks and 5 weeks later, the remaining fish in Groups A, B, C and D were exsanquinated. Serum from fish within each group was pooled and tested for antibody against T. pyriformis.

The results for antibody tests are shown in table 60. At the time of the challenge injection, immunised fish in Group A had an antibody titre of $-\log_2 10$ Fish of all other groups had no antibody titre to T. pyriformis. Antibody titre in immunised fish (Group A) increased from weeks 1 to 8 following challenge injection, up to a titre of

Table 60. Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of T. pyriformis: direct immobilization/agglutination of live T. pyriformis. Temperature 20±2 °C. Results are mean titre obtained from duplicate tests of pooled serum from up to 10 fish per group, obtained after 2 and 4 hours.

Group	No. of fish/ group	Procedure		Antibody titre $-\log_2$, 10 fish per group Time (weeks) after challenge					
		3 injections	Final injection + FCA	0	1	2	3	6	8
A	70	T. pyriformis	T. pyriformis + FCA	10	11	12.5	12.5	13.5	15.5
B		saline	saline + FCA	1	1	0	1	0	0
C		0	0	0	0	0	0	0	0
D	10	saline	T. pyriformis + FCA	0	NT	NT	12.5	12	NT

NT not tested

Table 61. Mean autoradiography counts in juvenile carp 1, 2 and 3 weeks following immunisation with 25 µg protein g^{-1} body weight of T. pyriformis. Temperature 20±2°C.

Time after treatment. (weeks)	Organ	Mean autoradiography counts/ 0.1 mm ^{2} (± standard error) Treatment			No. of fish/ group.
		T. pyriformis + FCA	Saline + FCA	None	
1	pro-nephros	142.80±4.24	90.00±2.90	N/A	10
2		132.20±2.77	82.10±2.28		
3		127.46±3.60	75.44±2.14		
1	opistho-nephros	88.80±2.60	99.20±2.65	45.07±1.46	
2		89.40±2.44	52.43±1.51	43.79±1.46	
3		132.20±2.68	48.30±1.40	64.56±2.00	
1	spleen	88.30±2.60	26.25±0.84	47.26±4.97	
2		51.43±2.10	29.32±0.86	34.00±2.70	
3		38.08±1.31	27.08±0.52	21.05±0.85	

N/A not available

$-\log_2$ 15.5. Fish in Group D which only received a challenge injection of T. pyriformis had antibody titres of $-\log_2$ 12.5 at 3 weeks and $-\log_2$ 12 at 6 weeks following challenge. Control groups B and C remained negative.

The results for autoradiography can be seen on table 61. From the results, it can be seen that high counts were obtained in fish immunised with T. pyriformis, when examined one week following the last injection of the organism in F.C.A. These counts began to decrease when fish were examined two and three weeks following the last injection of T. pyriformis, but in the pronephros and opisthonephros, they still remained higher than non-immunised controls. The pronephros gave the highest counts compared to spleen and opisthonephros, whereas the spleen had much lower counts. There was very little difference in counts in the opisthonephros between the controls and immunised fish.

EXPERIMENT 37. The cellular proliferative response and humoral response of juvenile carp to T. pyriformis administered intraperitoneally as live, whole cells.

270 juvenile carp age 9 weeks obtained from Stowmarket (spawned 8.2.86), were separated into three equal groups of 90, namely A, B and C. These were allowed to acclimate in tanks (600 dm^3 capacity) for two weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Following acclimation and immediately prior to the experiment, the mean weight of these fish was 972 mg.

Fish in Group A were administered intraperitoneally approximately

25 μg of protein g^{-1} bodyweight of whole, live T. pyriformis in saline on two separate occasions with an interval of four weeks between the two injections. After each administration of T. pyriformis, 40 fish were removed for investigations of cell proliferation by autoradiography. Ten of each group of 40 were then injected with methyl ^3H thymidine (1 μCi g^{-1} bodyweight) at either 1, 2, 3, or 4 weeks after the administration of T. pyriformis. Fish were exsanquinated 18 hours after the injection and the following organs were fixed in 10% buffered formal saline: spleen, pronephros and opisthonephros and fibrowax sections at 5-7 μm were processed for autoradiography. Serum was pooled within each group of 10 fish and tested (non-heat inactivated) for antibody against T. pyriformis using direct immobilization/agglutination of live T. pyriformis. In addition, fish in Group B were administered intraperitoneally 0.85% saline following the same regime and fish in Group C were uninjected controls. Fish in both groups were injected with methyl ^3H thymidine, at each stage as for Group A, as controls for interpretation of autoradiography, and serum also tested by direct immobilization/agglutination of live T. pyriformis.

Results of antibody tests (tables 62, 63) indicate a gradual increase in antibody titre following primary injection of T. pyriformis (table 62) at two weeks being $-\log_2$ 2, at three weeks $-\log_2$ 4 and at four weeks $-\log_2$ 4. This was further elevated following a second injection of T. pyriformis (table 63), four weeks after the first, with antibody titre at one week being $-\log_2$ 6, at two weeks $-\log_2$ 6.5, at three weeks $-\log_2$ 8 and at four weeks, $-\log_2$ 9. Sera from saline and untreated controls were negative to T. pyriformis and showed no natural agglutinins.

Table 62. Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of T. pyriformis. Humoral antibody response with time following one injection. Mean titre from groups of 10 fish following direct immobilization/ agglutination of live organisms after 2 and 4 hours. Temperature 20±2 °C.

Group	Procedure	No. of fish per group	Mean antibody titre ($-\log_2$) for serum pooled from groups of 10 fish. Time (weeks) from administration				
	1st injection		0	1	2	3	4
A	T. pyriformis	30	0	[a]NT	2	4	4
B	saline				0	0	0
C	0						

[a]NT Not tested.

Table 63. Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of T. pyriformis. Humoral antibody response with time following 2 injections, 4 weeks apart. Mean titre from groups of 10 fish following direct immobilization/agglutination of live organisms after 2 and 4 hours. Temperature 20±2 °C.

Group	Procedure	No. of fish per group	Mean antibody titre ($-\log_2$). Serum pooled from groups of 10 fish. Time (weeks) from administration.				
	2nd injection		0	1	2	3	4
A	T. pyriformis	55	4	6	6.5	8	9
B	saline	58	0	0	0	0	0
C	0	60					

Table 64. Mean autoradiography counts in juvenile carp 2, 3 and 4 weeks following one immunisation with 25 µg protein g^{-1} body weight of T. pyriformis. Temperature 20±2°C.

Time after treatment (weeks)	Organ	Mean autoradiography counts/0.1 mm ^{2} (± standard error) Treatment			No. of fish/ group
		1 injection of T. pyriformis	1 injection of saline	None	
2	pronephros	N/A	85.10±3.37	N/A	10
3		189.60±12.44	53.38±3.52		
4		158.20±7.43	63.10±3.04		
2	opistho-nephros	N/A	46.00±8.66	45.07±3.50	
3		191.47±5.63	52.70±7.93	58.20±4.16	
4		63.70±1.60	43.15±1.34	35.86±1.87	
2	spleen	91.30±2.70	33.46±5.47	34.00±2.70	
3		95.50±2.82	54.00±1.90	37.90±2.37	
4		56.41±4.90	31.52±1.35	32.15±1.87	

N/A not available

Table 65. Mean autoradiography conts in juvenile carp 1, 2, 3 and 4 weeks following two immunisations with 25 µg protein g^{-1} body weight of T. pyriformis 4 weeks apart. Temperature 20±2°C.

Time after treatment (weeks)	Organ	Mean autoradiography counts/0.1 mm ^{2} (± standard error) Treatment			No. of fish/ group
		1 injection of T. pyriformis	1 injection of saline	None	
1	pro-nephros	180.71±5.44	53.00±3.50	N/A	10
2		214.80±11.76	52.48±3.38		
3		191.70±5.36	84.10±3.14		
4		59.10±3.76	72.00±0.77		
1	opisth-nephros	103.57±2.24	42.15±1.26	47.52±2.27	
2		128.47±2.50	52.70±2.04	36.47±1.04	
3		96.50±2.06	46.00±1.20	35.67±1.86	
4		37.45±1.28	27.00±1.04	N/A	
1	spleen	74.06±2.32	52.00±1.88	56.04±2.05	
2		53.98±2.28	30.52±1.42	31.54±1.32	
3		55.36±1.92	32.46±1.74	36.74±1.65	
4		58.64±2.46	23.64±1.25	16.05±2.01	

N/A not available

The results for autoradiography can be seen in tables 64, 65. Gaps in the data relate to poor sections not suitable to counting. From the results it can be seen that following one injection of T. pyriformis, immunised fish had higher counts than non-immunised controls. The greatest counts were at two and three weeks following administration of organisms, with a decrease at four weeks. following two injections of T. pyriformis, immunised fish again had higher counts than non-immunised controls, with the highest counts occurring one and two weeks following the second injection. The pronephros and opisthonephros gave higher counts than the spleen.

Immunisation against I. multifiliis

EXPERIMENT 38. Immunisation of 5 week old carp fry with freeze dried ciliary antigen of Tetrahymena pyriformis against Ichthyophthirius multifiliis.

100 carp fry age 3 weeks, obtained from Stowmarket (spawned 21.1.84) were separated into 5 groups, A, B, C, D and E each containing 20 fish. These were allowed to acclimate in aquaria (10 fish per tank), for two weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment at 5 weeks, the mean weight of the fish was 120 mg.

Fish in groups A and B were injected intraperitoneally using an agla syringe and micrometer, with approximately 25 µg of protein g^{-1} of freeze dried ciliary antigen of T. pyriformis in 0.85% saline at a volume of 10 µl g^{-1} body weight. This was followed by two further booster injections administered at 2 weekly intervals. Those in groups C and D were injected with 0.85% saline, following the same regime. Group E were maintained as uninjected controls. Fish in

groups A, C and E were exposed to I. multifiliis by adding one infected fish into each aquaria, 4 weeks after the last injection. Fish in groups B and D were exposed to theronts of I. multifiliis as a 'body dip' of approximately 600 theronts per fish at a concentration of approximately 600/cm ^{3} for 2 minutes. Fish were lightly anaesthetised and placed in a watchglass containing the theronts.

It can be seen from the results (table 66) that, following challenge, 100% mortality occurred in group A, 70% in group B, 80% in group C, 60% in group D and 90% in group E. The cumulative mortality (%) with time for groups A and C can be seen in Fig. 52. The mortalities chiefly occurred 8 days after challenge, following termination of primary infections and commencement of secondary invasion. By day 9, skin began to slough off, consistent with intense re-infection by theronts during the secondary phase. All fish in groups A and C had a heavy infection 6 days after challenge (Primary infection) and then 9 days after challenge (2° infection). Deaths had all occurred within 10 days.

EXPERIMENT 39. Immunisation of 4½ week old carp fry with freeze dried ciliary antigen of Tetrahymena pyriformis against Ichthyophthirius multifiliis.

This experiment was basically a repeat of the previous experiment and as such, all procedures were similar with the exception that fish were obtained from Driffield (spawned 18.4.84) with a mean weight at 4½ weeks of 160 mg. Fish were separated into 3 groups, namely A, B and C, each containing 20 fish.

Table 66 . Immunisation of carp fry with 25 µg protein g^{-1} body weight of freeze dried ciliary antigen of T. pyriformis: percentage mortality following challenge infections with I. multifiliis. Temperature 20±2 °C, 20 fish per group.

Group	Treatment	No. of fish/ group	Percentage mortality	Mortality/ No tested	RPS
A	Immunised T. pyriformis cilia	20	100	20/20	0
B			70	14/20	-16
C	saline controls		80	16/20	N/A
D			60	12/20	
E	untreated controls		90	18/20	

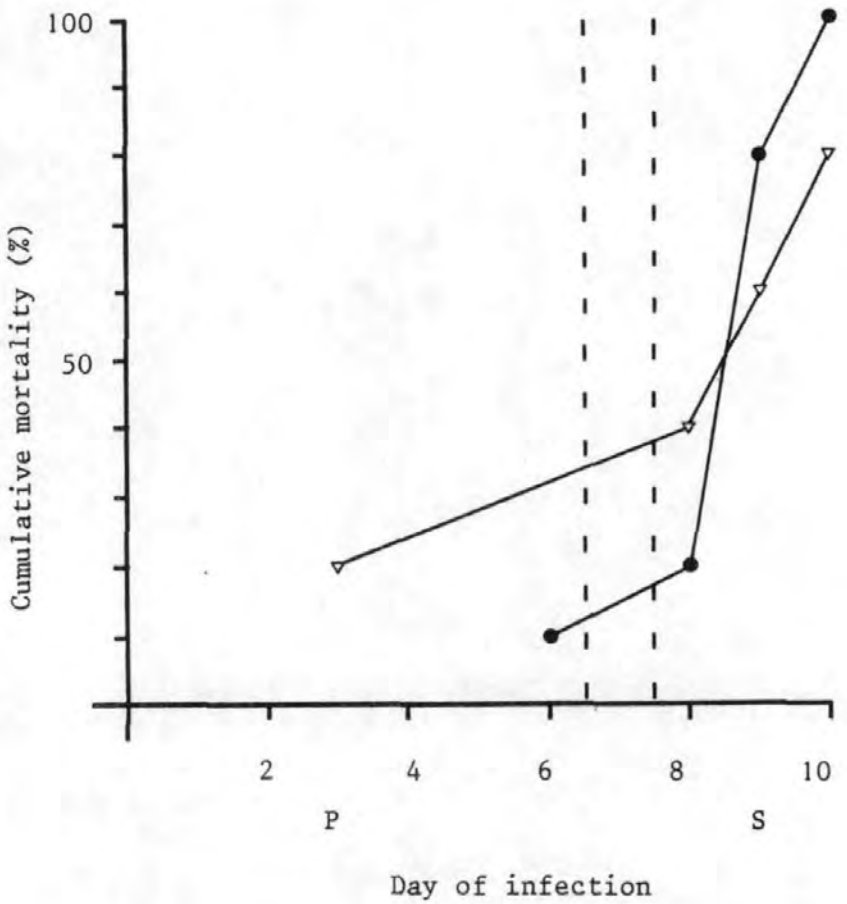


Fig. 52. Immunisation of carp fry with 25µg protein g^{-1} body weight of freeze dried ciliary antigen of T. pyriformis : Cumulative mortality with time after exposure to I. multifiliis 4 weeks after the last injection of T. pyriformis. Temperature 20± 2°C.

P primary, S secondary infections of I. multifiliis.

● immunised with T. pyriformis, ▽ saline controls.

Fish in group A were administered intraperitoneally, freeze-dried ciliary antigen of T. pyriformis (25 µg protein. g^{-1} body weight). Those in group B were administered 0.85% saline intraperitoneally and fish in group C were untreated controls. Twenty-eight days following the last injection, fish in all groups were exposed to theronts of I. multifiliis at a dose of approximately 4,000 theronts per fish at a concentration of approximately 400/cm^3, for 60 minutes, continuous aeration and in the dark.

It can be seen from the results (table 67) that fish in all groups became infected following exposure to I. multifiliis. The primary infection was visible 3 days after challenge infections with the secondary infection being visible 9 days after challenge. Both primary and secondary infections were moderate but no mortalities occurred in any of the groups.

EXPERIMENT 40. Immunisation of juvenile carp with freeze-dried ciliary antigen of Tetrahymena pyriformis against Ichthyophthirius multifiliis.

Forty juvenile carp age 6 months obtained from Driffield were separated into 2 groups, A and B, each containing 20 fish. These were allowed to acclimate in aquaria for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, the mean weight of the fish was 8.5 g.

Fish in group A were administered intraperitoneally, approximately 25 µg protein g^{-1} body weight of freeze-dried ciliary antigen of

Table 67. Immunisation of carp fry with 25 µg protein g^{-1} body weight of freeze dried ciliary antigen of T. pyriformis: percentage mortality following challenge infections (4 000 theronts per fish, 400 / cm^{-3}) with I. multifiliis. Temperature 20±2 °C, 20 fish per group.

Group	Treatment	No. of fish/group	Percentage infected	
			Primary infection	Secondary infection
A	T. pyriformis cilia	20	100	100
B	saline			
C	0			

I. pyriformis in 0.85% saline at a volume of 10 µl g^{-1} body weight. This was followed by two further booster injections administered at 2 weekly intervals. Those in group B were injected with 0.85% saline, following the same regime. Twenty-eight days following the last injection fish in both groups were exposed to a dose of approximately 8,000 theronts per fish at a concentration of approximately 80 cm^{-3} for 6 hours in the dark, with constant aeration.

It can be seen from the results shown in table 68 that fish in both groups became infected following exposure to I. multifiliis. Both immunised and saline injected fish developed 100% primary infections but only 86% of immunised (Group A) fish developed a secondary infection. No mortalities occurred in either group.

EXPERIMENT 41. Immunisation of juvenile carp with a low immunising dose of 25 µg protein. g^{-1} body weight of live Tetrahymena pyriformis against Ichthyophthirius multifiliis.

100 juvenile carp age 8 weeks, obtained from Sparsholt (spawned 30.1.85), were separated into 5 groups, A, B, C, D, E, each containing 20 fish. These were allowed to acclimate in aquaria (10 fish per tank), for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks, the mean weight of the fish was 676 mg.

Fish in groups A and B were injected intraperitoneally with approximately 25 µg of protein g^{-1} body weight of whole, live I. pyriformis

Table 68. Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of freeze dried ciliary antigen to *T. pyriformis*: percentage mortality following challenge infections with *I. multifiliis* (8 000 theronts per fish, 80/ cm^3). Temperature 20±2 °C.

Group	Treatment	No. of fish/group	Percentage infected	
			Primary infection	Secondary infection
A	*T. pyriformis* cilia	15	100	86
B	saline	20	100	100

antigen in 0.85% saline at a volume of 10 µl g^{-1} body weight. This was followed by two further booster injections administered at 2 weekly intervals. Those in groups C and D were injected with 0.85% saline, following the same regime. Group E were maintained as uninjected controls. Fish in all groups were then exposed to a potentially lethal dose of approximately 8,000 theronts of I. multifiliis per fish at a concentration of approximately 320 theronts cm^{-3} following the standard challenge procedure. Groups A and C were exposed 2 weeks after the final I. pyriformis injection and B and D after 4 weeks. Group E were also exposed as untreated controls at the same time as B and D.

It can be seen from the results (table 69 fig. 53) that, following challenge, 90% mortality occurred in group A and 100% in group C. The mortalities were chiefly 8 days after challenge, following termination of primary infections and commencement of secondary invasion. All mortalities occurred within 23 days, and there was no significant difference between the mean days to death for group A (9.28) and group C (9.40). Observation on the condition of challenged fish indicated that saline controls suffered more skin damage after invasion of theronts.

Similar results were obtained in groups B, D and E, as can be seen in table 69 and fig. 54 with 100% mortality in saline and non-injected controls. Immunised fish in group B developed both primary and secondary infections with 65% mortality. The deaths were chiefly 8 days after challenge following termination of primary infections and commencement of secondary invasion for groups B and D and between days 13 to 15, following termination of secondary infections and commence-

Table69 . Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of T. pyriformis: percentage mortality following a potentially lethal dose (8 000 theronts per fish, concentration of 320 theronts/cm^3) of I. multifiliis. Temperature 20±2 °C, 20 fish per group.

Group	Treatment	No of fish/ group	Percentage mortality	Mortality/ No tested	Relative % survival	Mean days to death (± SD)
A	Immunised T. pyriformis	20	90	18/20	10	9.28±4.57
B			65	13/20	35	8.62±1.26
C	saline controls		100	20/20	N/A	9.40±3.52
D			100	20/20		9.80±1.82
E	untreated		100	20/20		16.20±1.36

Groups A and C were exposed to I. multifiliis 2 weeks following the last T. pyriformis injection, and groups B, D and E after 4 weeks.

Figs. 53 and 54. Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of live T. pyriformis: Cumulative mortality (%) after a potentially lethal dose of I. multifiliis (8,000 theronts per fish, 320/cm^3). Temperature 20±2°C.

Fig. 53. Two weeks after the last injection of T. pyriformis.

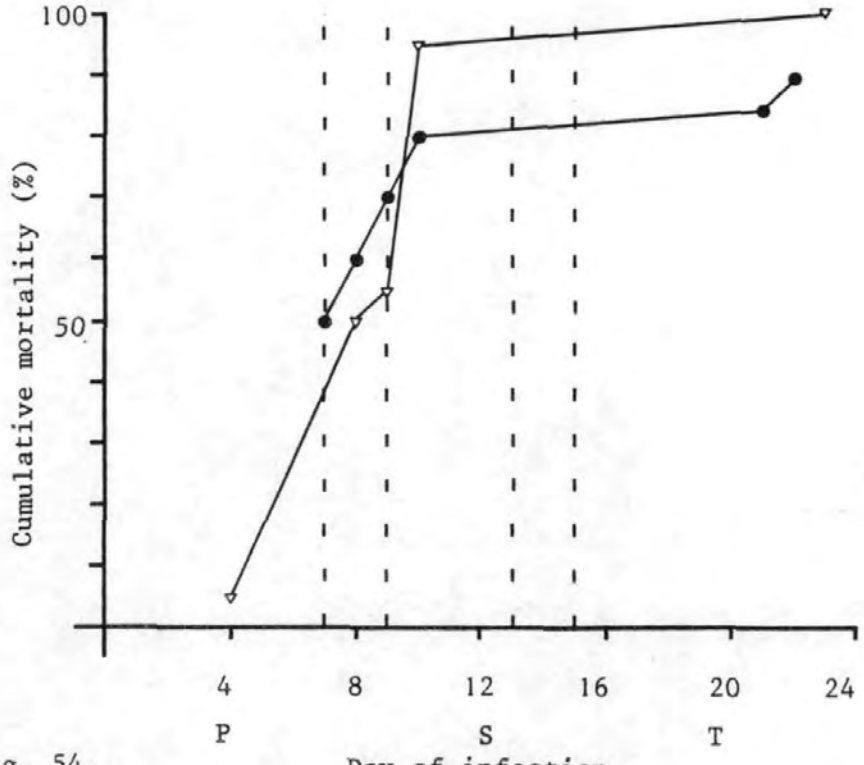
Fig. 54. Four weeks after the last injection of T. pyriformis.

● immunised T. pyriformis, ▽ saline controls,

▲ untreated controls.

P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 53.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Cumulative mortality (%)
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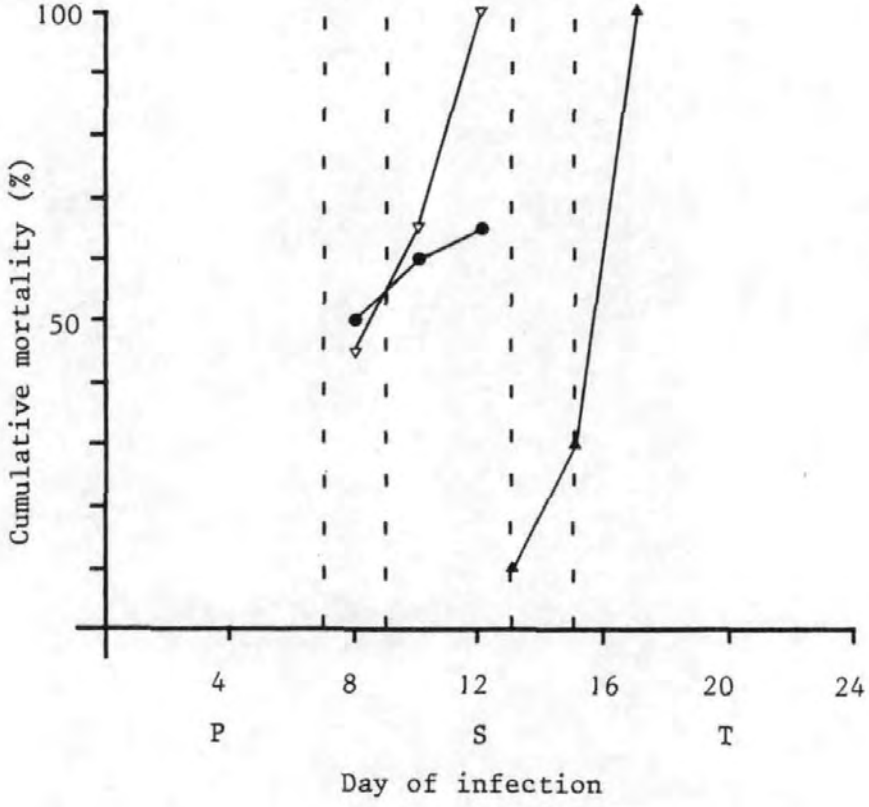
Day of infection

Fig. 54.


100
50
Cumulative mortality (%)
4
8
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24
P
S
T
Day of infection

ment of tertiary invasion for group E. All mortalities occurred within 17 days. There is a significant difference between the mean days to death for group B (8.62) and group D (9.80) and also for group E (16.20) with groups B and D, in that group B died earlier than groups D and E ($P<0.05$) and group E died later than groups B and D ($P<0.05$). The Relative Percent Survival (RPS) for group A is 10% and for group B, 35%.

EXPERIMENT 42. Immunisation of juvenile carp with a low immunising dose of 25 µg protein g^{-1} body weight of live *Tetrahymena pyriformis* against *Ichthyophthirius multifiliis*.

This experiment was basically a repeat of the previous experiment and as such all procedures were similar with the exception that fish were obtained from Stowmarket (spawned 8.2.86.) with a mean weight at 10 weeks of 1.04 g. In addition, all groups contained 40 fish, and a further control group was included (Group F), these remaining untreated and serving to confirm susceptibility on exposure to theronts on week 6 of the experiment.

At the same time fish were exposed to theronts of *I. multifiliis*, the additional 20 fish in each group were exsanquinated, serum being pooled from 10 fish. Sera (non-heat inactivated) were tested for antibody against *I. pyriformis* and *I. multifiliis* using direct immbolization/agglutination of organisms.

It can be seen from the results (Table 70, Fig. 55) that, following exposure to theronts 2 weeks after the last injection, 100% mortality occurred in group A (immunised), 90% in group C (saline) and 80% in

group F (untreated). The mortalities were chiefly 8 days after challenge for group A, following termination of primary infections and commencement of secondary invasion. For groups C and F, mortalities were chiefly 12-15 days, following termination of secondary infections and commencement of tertiary invasion. All mortalities occurred within 22 days. There was a significant difference ($P<0.05$) between the mean days to death for group A (8.75) and groups C (13.28) and F (14.38) with the latter two groups surviving for a greater length of time than group A, there was no significant difference between groups C and F. Similar results were obtained in groups B, D and E, following exposure to theronts 4 weeks after the last injection, as can be seen in table 70, fig. 56, with 100% mortality in group B (immunised) and group D (saline) and 90% mortality in group E (untreated). The mortalities were chiefly 8 days after challenge for groups B and D following termination of primary infections and commencement of secondary invasion, and between days 8 to 13 for group E, during secondary infection. All mortalities occurred within 13 days. There was a significant differenc ($P<0.05$) between the mean days to death for group E (9.61) and groups B (7.95) and D (7.70) with the latter two groups surviving for a shorter time than group E. There was no significant difference between groups B and D.

No significant difference ($P<0.05$) was found between the mean days to death for those fish immunised and exposed to theronts of I. multifiliis either 2 weeks (Group A) or 4 weeks (Group B) after the last injection. The Relative Percent Survival (RPS) for both groups A and B is 0.

The results for the antibody tests can be seen in table 71. It can be

Table 70. Immunisation of juvenile carp with 25µg protein g^{-1} body weight of T. pyriformis: percentage mortality following a potentially lethal dose (8,000 theronts/fish, 320/cm^3) of I. multifiliis. Temperature 20±2°C, 20 fish per group.

Group	Treatment	No of fish/ group	Percentage mortality	Mortality/ No tested	Relative % survival	Mean days to death (± SD)
A	Immunised T. pyriformis	20	100	20/20	0	8.75±2.34
B			100	20/20	0	7.95±0.22
C	saline controls		90	18/20	N/A	13.28±2.87
D			100	20/20		7.70±1.03
E	untreated controls		90	18/20		9.61±1.58
F			80	16/20		14.38±3.77

Groups A, C and F were exposed to I. multifiliis 2 weeks after the last T. pyriformis injection, and groups B, D and E, after 4 weeks. N/A not applicable. SD standard deviation.

Figs. 55 and 56. Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of live T. pyriformis: Cumulative mortality (%) after a potentially lethal dose of I. multifiliis (8,000 theronts per fish, 320/cm^3). Temperature 20±2°C.

Fig. 55. Two weeks after the last injection of T. pyriformis

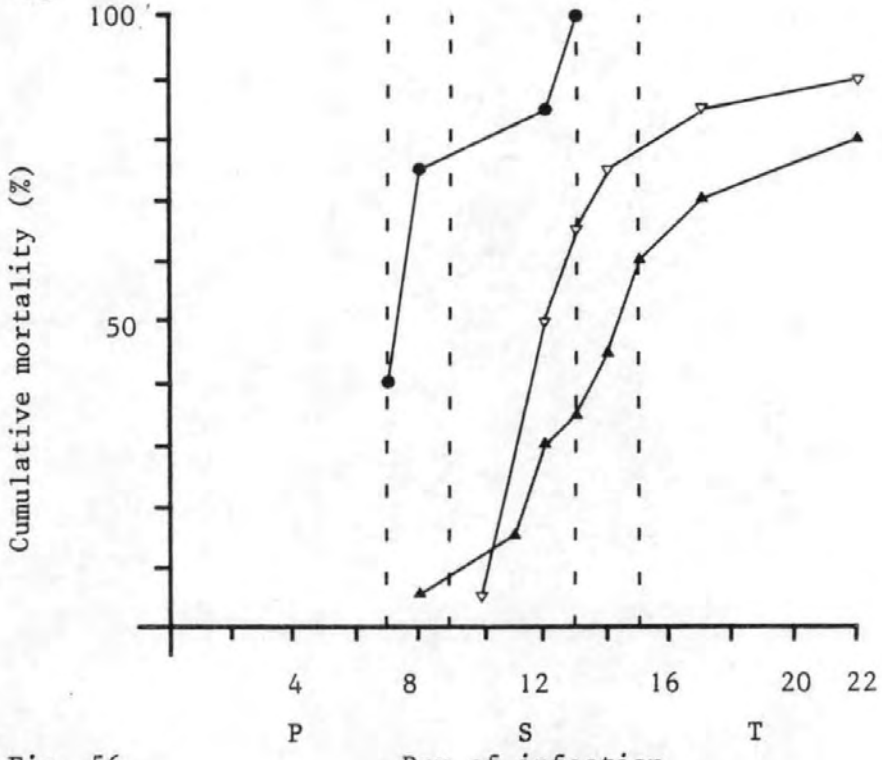
Fig. 56. Four weeks after the last injection of T. pyriformis

● immunised T. pyriformis, ▽ saline controls,

▲ untreated controls.

P primary, S secondary, T tertiary infections of I. multifiliis.

Fig. 55.


100
50
Cumulative mortality (%)
4
8
12
16
20
22
P
S
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Day of infection

Fig. 56.

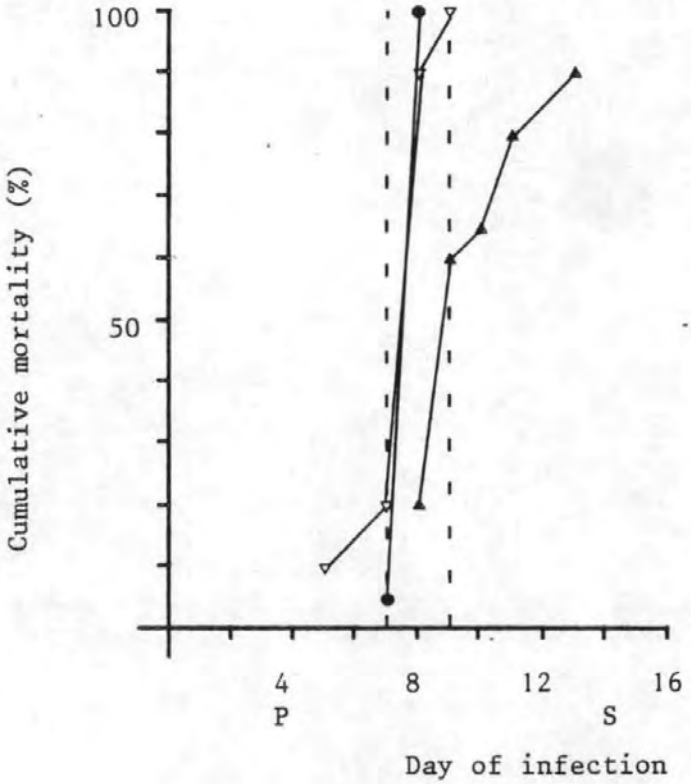

100
50
Cumulative mortality (%)
4
8
12
16
P
S
Day of infection

Table 71. Immunisation of juvenile carp with 25 µg protein g^{-1} body weight of T. pyriformis. Direct immobilization/agglutination of live T. pyriformis and theronts of I. multifiliis. Results obtained after 2, and re-checked after 4 hours, temperature 20±2 °C.

Group	Treatment	Antibody titre -$\log_2$ against[a]: T. pyriformis	I. multifiliis	No. of fish/group
A	Immunised T. pyriformis	10,10	0,0	10,10
B		10,10	0,0	
C	saline controls	0,0	0,0	
D		0,0	0,0	
E	untreated controls	0,0	0,0	
F		0,0	0,0	
	Diluent	0,0	0,0	N/A

a Results indicate mean titre for triplicate tests from pooled serum from groups of 10 fish.

Groups A, C and F were tested 2 weeks after the last injection and groups B, D and E after 4 weeks. N/A = not applicable.

seen that for both the 2 week and 4 week challenge groups, immunised fish (Groups A and B) had antibody titres against T. pyriformis of $-\log_2$ 10. Lysis occurred at low serum dilutions, with immobilization and agglutination at high serum dilutions. Saline and untreated fish (Groups C, D, E) had negative titres to T. pyriformis. When tested against theronts of I. multifiliis, no antibody titre was obtained in any of the groups.

EXPERIMENT 43. Immunisation of juvenile carp with a high immunising dose of 50 µg protein g^{-1} bodyweight of live Tetrahymena pyriformis against Ichthyophthirius multifiliis.

110 juvenile carp age 8 weeks obtained from Stowmarket (spawned 8.2.86) were separated into three groups, A, B and C. Group A contained 50 fish and groups B and C 30 fish each. These were allowed to acclimate in aquaria (10 fish per tank) for 2 weeks at 20±2° C, this temperature being maintained throughout the following experimental period. Immediately prior to the experiment, at 10 weeks, the mean weight of the fish was 1.08 g. Fish in group A were injected intraperitoneally with approximately 50 µg of protein g^{-1} bodyweight of whole, live, T. pyriformis antigen in 0.85% saline at a volume of 10 µl g^{-1} bodyweight. This was followed by two further booster injections administered at 2 weekly intervals. Those in group B, were injected with 0.85% saline following the same regime. Group C were kept as untreated controls. Forty fish from group A and 20 each from groups B and C were then exposed to a potentially lethal dose of approximately 8,000 theronts of I. multifiliis per fish at a concentration of approximately 320 theronts/cm^3 four weeks after the last injection using the standard challenge procedure. At the same

time that fish were exposed to theronts of I. multifiliis, the remaining 10 fish in each group were exsanquinated, Serum (non-heat inactivated) was pooled for each group and tested for antibody against I. pyriformis using direct immobilization/agglutination of organisms.

It can be seen from the results (Table 72, Fig. 57) that following exposure to theronts, 100% mortality occurred in group B (saline controls) with 90% mortality in group C (untreated) and 87.5% in group A (immunised). For groups B and C, the mortalities were chiefly 8-10 days after challenge following termination of primary infections and commencement of secondary invasion. For group A, the mortalities were chiefly during the secondary infection and commencement of tertiary invasion (days 13-15). All mortalities occurred within 19 days. The immunised fish were seen to have a lighter primary infection than controls. There is a significant difference between the mean days to death for group A (11.06) with groups B (7.70) and C (9.61) in that the immunised fish (Group A) survived for longer than groups B and C ($P<0.05$). The Relative Percent Survival (RPS) of the immunised group (Group A) was 12.5%.

The results for the antibody tests are shown in Table 73. It can be seen that at the time of exposure to theronts, immunised fish (Group A) had an antibody titre against I. pyriformis of $-\log_2$ 14. Lysis occurred at low serum dilutions with immobilization and agglutination at higher serum dilutions. Negative titres were obtained with saline and untreated controls.

Table 72 . Immunisation of juvenile carp with 50 µg protein g^{-1} body weight of T. pyriformis: percentage mortality after a potentially lethal dose (8 000 theronts per fish, 320/ cm^{3}) of I. multifiliis, 4 weeks after the last injection of T. pyriformis. Temperature 20±2 °C.

Group	Treatment	No. of fish/ group	Percentage mortality	Mortality/ No tested	Relative % survival	Mean days to death (± SD)
A	Immunised T. pyriformis	40	87.5	35/40	12.5	11.06±3.08
B	saline controls	20	100	20/20	N/A	7.70±1.03
C	untreated		90	18/20		9.61±1.58

SD Standard deviation
N/A Not applicable

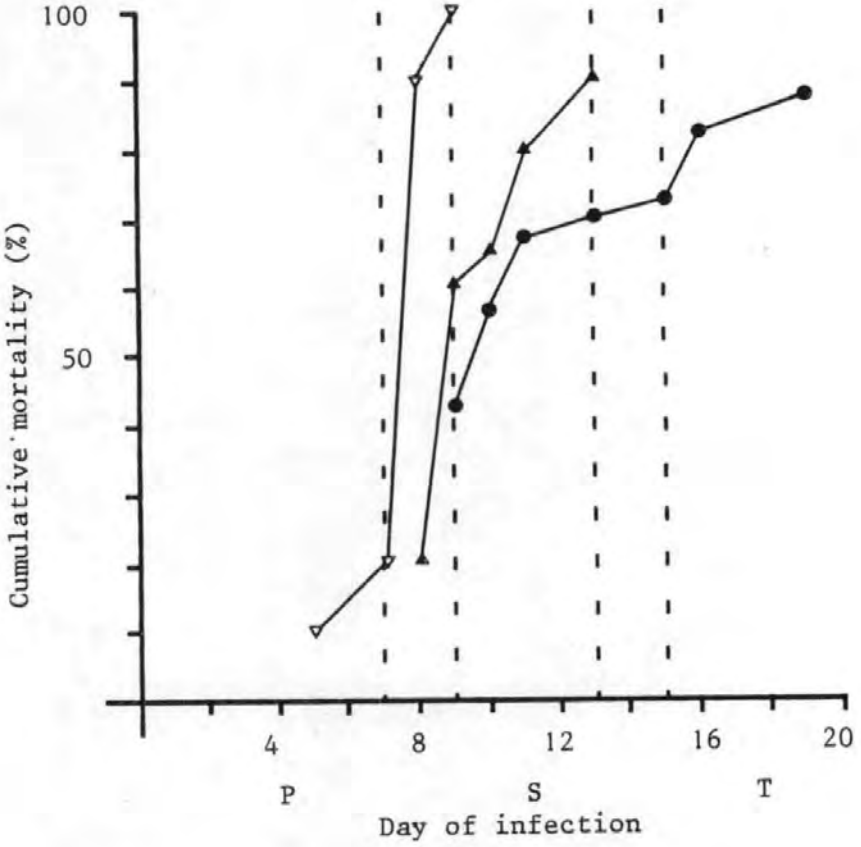


Fig. 57. Immunisation of juvenile carp with 50ug g^{-1} body weight of live T. pyriformis : Cumulative mortality (%) after a potentially lethal dose of I. multifiliis (8,000 theronts/fish 320/cm^3) 4 weeks after the last injection of T. pyriformis. Temperature 20± 2°C.

P primary, S secondary, T tertiary infections of I. multifiliis.
● immunised with T. pyriformis, ▽ saline contols, ▲ untreated.

Table 73. Immunisation of juvenile carp with 50 µg protein g^{-1} body weight of T. pyriformis: Direct immobilization/agglutination of live T. pyriformis, four weeks after the last injection. Results obtained after 2 hours and re-checked after 4 hours, temperature 20±2 °C.

Group	Treatment	Antibody titre $-\log_2$* T. pyriformis	No. of fish/ group
A	Immunised T. pyriformis	14	10
B	Saline controls	0	
C	Untreated controls	0	
	Diluent	0	N/A

* Results indicate mean titre for duplicate tests from pooled serum from groups of 10 fish.
N/A = Not applicable.

EXPERIMENT 44. To investigate cross reactivity of Tetrahymena pyriformis with Ichthyophthirius multifiliis in antisera to the former raised in carp, rats and rabbits.

Antisera to I. pyriformis raised in carp, rats and rabbits, was tested against theronts of I. multifiliis in order to investigate common antigenicity between these two organisms. The antigen/antibody response was measured using direct immobilization/agglutination of live organisms. The results indicate (Table 74) that live I. pyriformis are immobilized and agglutinate when exposed to heat inactivated and non-heat inactivated antisera prepared in carp, rats and rabbits. Serum titres obtained were of $-\log_2$ 8-9 with carp (mean 8.18), $-\log_2$ 8-10 with rats (mean 9.16) and $-\log_2$ 8-9 with rabbit antiserum (mean 8.2). In non-heat inactivated serum, lysis of organisms occurred in dilutions lower than $-\log_2$ 3. In contrast, there was no response to the sera to theronts of I. multifiliis.

All animals were pre-bled and tested before immunisation to ensure there was no background antibody to I. pyriformis and I. multifiliis.

EXPERIMENT 45. To investigate cross reactivity of Ichthyophthirius multifiliis with Tetrahymena pyriformis in antisera to the former raised in carp.

Antisera to I. multifiliis raised in carp was tested against I. pyriformis in order to investigate common antigenicity between these two organisms. The antigen/antibody response was measured using direct immobilization/agglutination of live organisms.

Table 74. Direct immobilization/agglutination of live T. pyriformis and theronts of I. multifiliis to anti-T. pyriformis serum. The results are mean titres of successive tests, obtained after 2 hours and re-checked after 4 hours, temperature 20 2 °C. Heat inactivated titres are shown in parenthesis.

Antiserum	Antibody titre $-\log_2$: T. pyriformis	I. multifiliis
Carp anti-T. pyriformis		
Fish no. 1	8.2	0
2	8	0
3	8	0
4	8.5	0
Overall mean	8.1	
Normal carp serum	0	0.0
Rat anti-T. pyriformis		
Rat no. 1	9.3 (8)	0 (0)
2	8.6 (9)	0 (0)
3	8.3 (8.3)	0 (0)
4	10.3 (9)	0 (0)
Overall mean	9.16 (8.5)	0 (0)
Normal rat serum	0 (0)	0 (0)
Rabbit anti-T. pyriformis	8.2 (7.75)	0.2 (0)
Overall mean	8.2 (7.75)	0.2 (0)
Normal rabbit serum	0.4 (0)	0.1 (0)

Table 75 . Direct immobilization/agglutination of theronts of I. multifiliis and T. pyriformis to carp anti-I. multifiliis serum. The results are mean titres of successive tests obtained after 2 hours and rechecked after 4 hours, temperature 20±2 °C.

Antiserum	Antibody titre $-\log_2$:	
	I. multifiliis	T. pyriformis
Carp anti-I. multifiliis	8	0
Normal carp serum	0	0

The results indicate (Table 75) that theronts of I. multifiliis are immobilized and agglutinate when exposed to non-heat inactivated antisera prepared in carp. Serum titres of $-\log_2$ 8-9 were obtained (mean 8). Lysis of organisms occurred at low serum dilutions of $-\log_2$ 2-3. In contrast, there was no response of the sera to T. pyriformis.

THE EFFECT OF IMMUNE SERUM ON I. MULTIFILIIS AND T. PYRIFORMIS.

EXPERIMENT 46. The effect of immune serum on theronts of I. multifiliis.

The effects of non-heat inactivated carp anti-I. multifiliis antisera on theronts can be seen in Figs. 58-63. Incubation in low serum concentration resulted in agglutination of theronts, agglutination appearing to take place at the posterior end of the organism (Fig.58). The time taken for this to occur varied from 10 minutes to over 2 hours. Following incubation in high serum concentrations, immobilization and agglutination also occurred but the theronts became rounded (Fig. 59). Immobilization of theronts appeared to be due to agglutination of the cilia as can be seen in Fig. 60.

At high serum concentrations, theronts became rounded (Fig. 61) and, with time, dilation of the parasite membrane occurred, being evident after 30 minutes (Fig. 62) with extensive dilation after 60 minutes (Fig. 63). Rupture of the parasite occurred by 80 minutes.

Theronts in non-immune serum were actively swimming.

EXPERIMENT 47. The effect of immune serum on trophozoites of T. pyriformis.

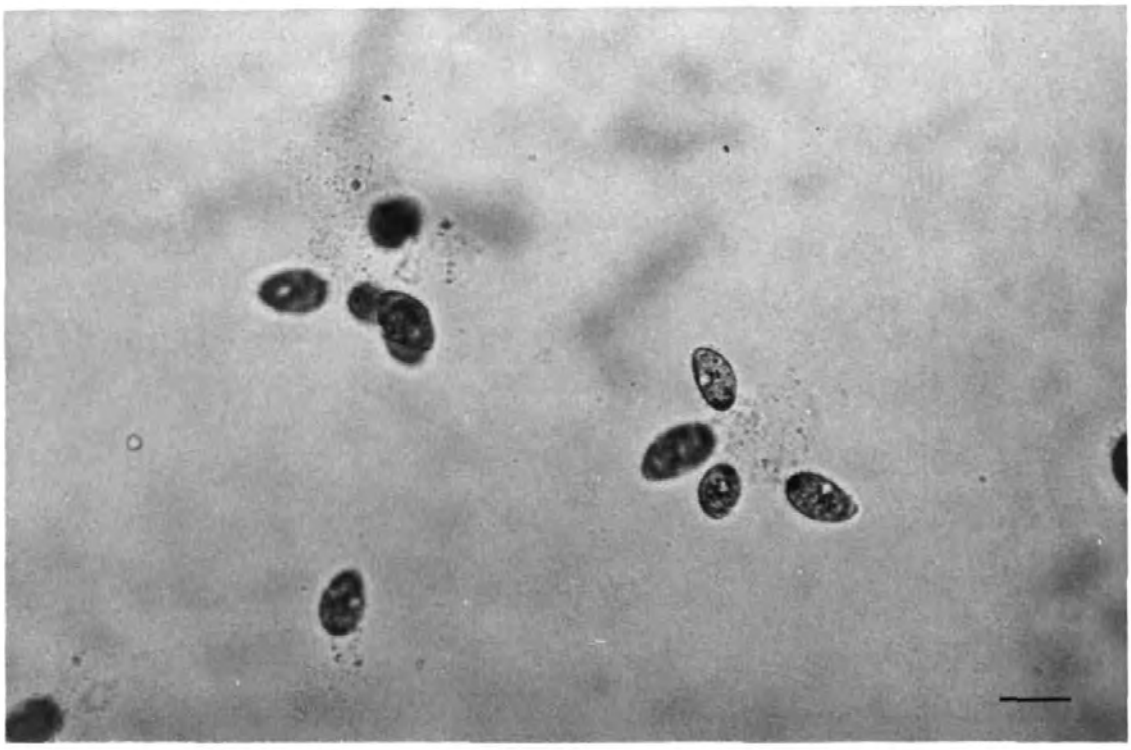
The effect of non-heat inactivated carp anti-T. pyriformis serum can be seen in Figs. 65-69. Fig. 64 shows a formalin fixed trophozoite of T. pyriformis showing general features of the organism, notably cilia and membranelles surrounding the cytopharynx. Trophozoites in non-immune serum were actively swimming. Following incubation in low serum concentrations of antisera, trophozoites became immobilized and agglutinated (Figs. 65, 66, 67). Immobilization appeared to be due to immobilization and agglutination of the cilia (Figs. 65 and 67).

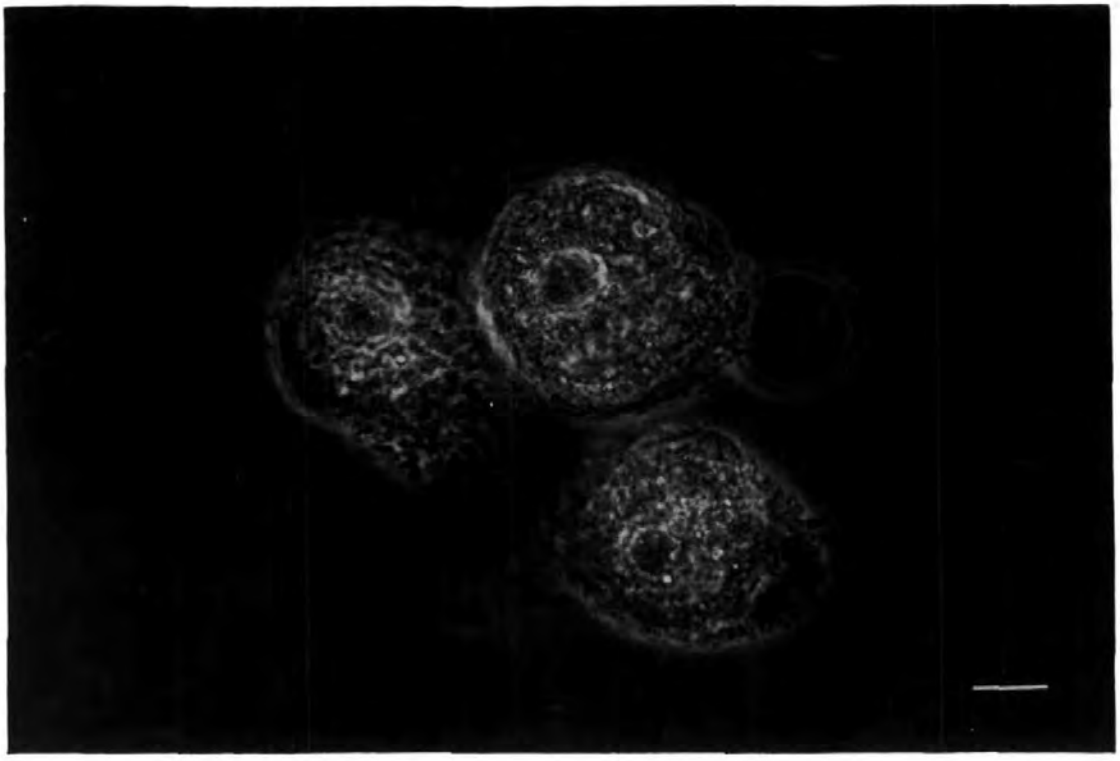
At high serum concentration, cilia were immobilized and dilation of the trophozoite membrane occurred after 15 minutes (Fig. 68). Dilation of the membrane continued with lysis of the organism after 30 minutes (Fig. 69).

Figs 58 and 59 The effects of carp anti-Ichthyophthirius multifiliis antisera on theronts of I. multifiliis.

Fig 58 Agglutinated theronts after 30 minutes. Antibody titre $-\log_2 4$. Note, agglutination at posterior of theronts. Scale 35 μm.

Fig 59 Agglutinated theronts after 30 minutes. Antibody titre $-\log_2 2$. Scale, 10 μm.

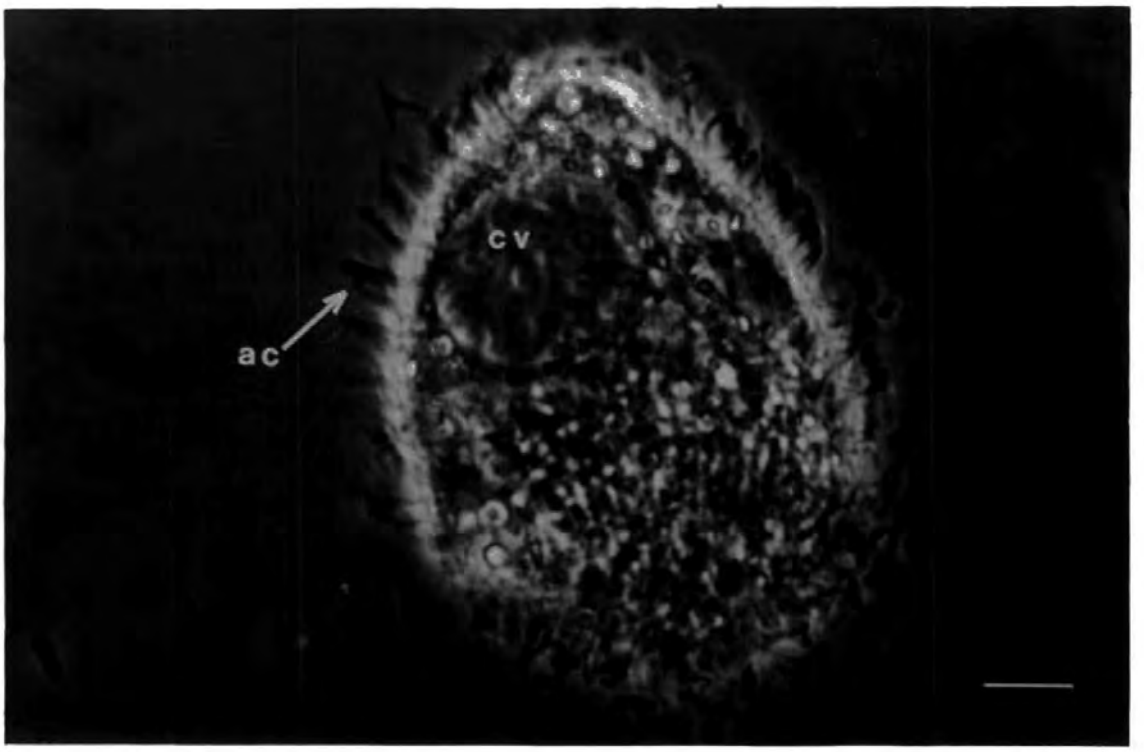


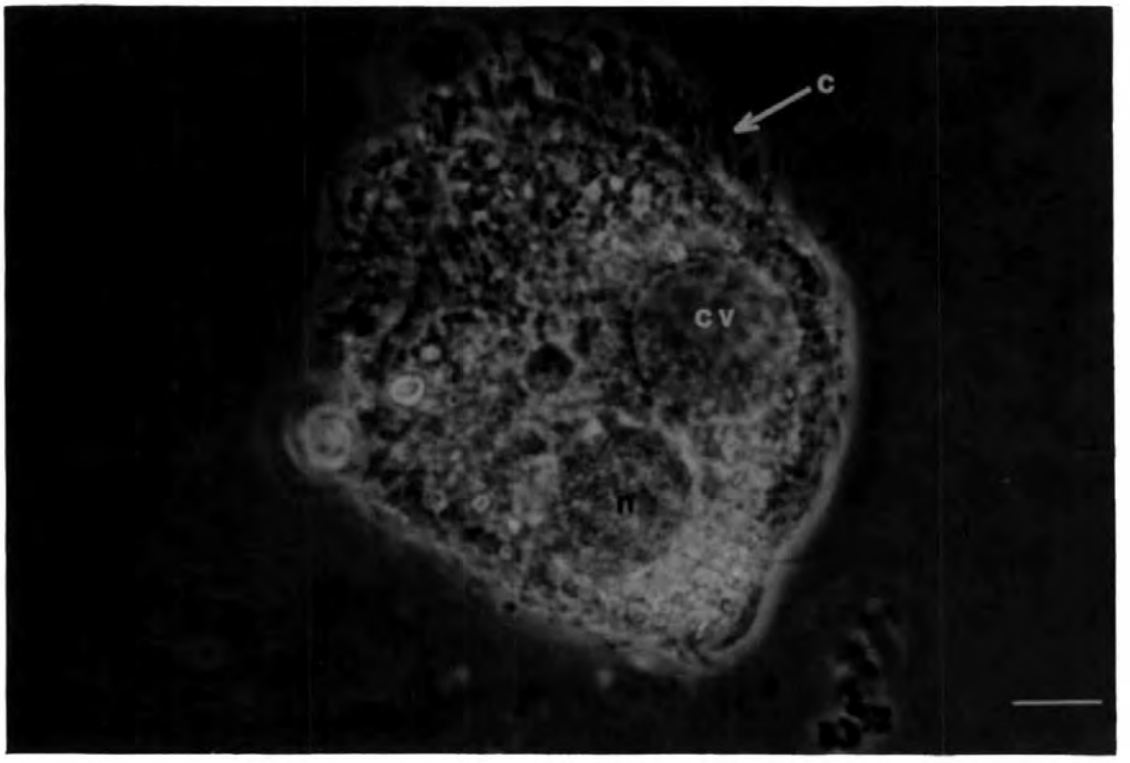


Figs 60 and 61 The effects of carp anti-*Ichthyophthirius multifiliis* antisera on theronts of *I. multifiliis*.

Fig 60 Agglutinated cilia (ac) after 30 minutes. Antibody titre $-\log_2$ 4. Contractile vacuole (cv). Scale 10 μm.

Fig 61 Incubation of theront in carp anti-*I. multifiliis* antisera after 5 minutes. Antibody titre $-\log_2$ 1. Cilia (c), nucleus (n), contractile vacuole (cv). Scale, 10 μm.

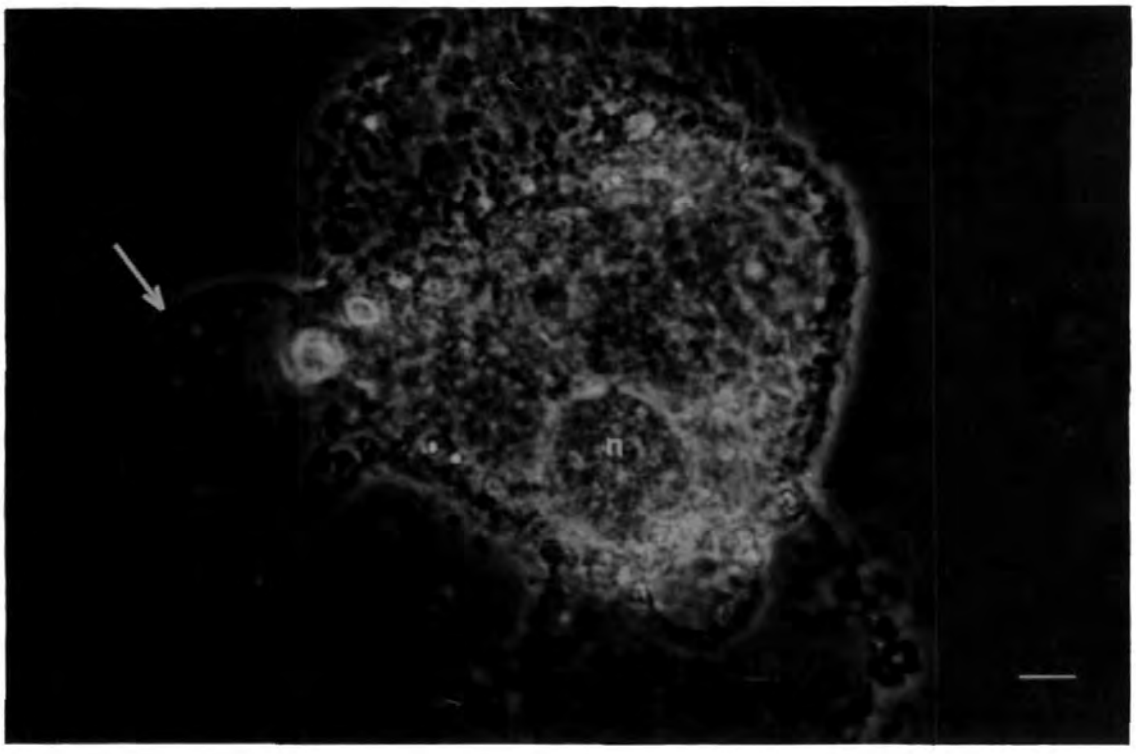

cv
ac

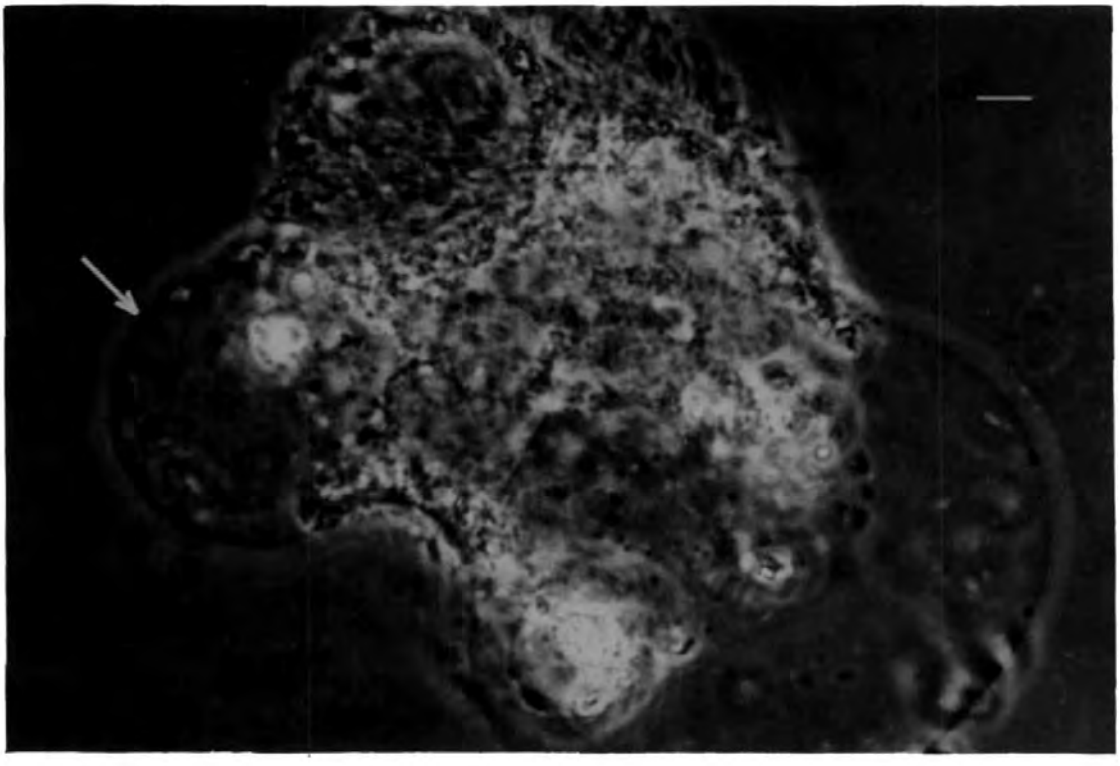

c
cv
n

Figs 62 and 63 The effects of carp anti-*Ichthyophthirius multifiliis* antisera on theronts of *I. multifiliis*.

Fig 62 Incubation of theront in carp anti-*I. multifiliis* antisera after 30 minutes. Antibody titre $-\log_2$ 1. Note dilation of membrane (arrowed). Nucleus (n). Scale, 5 µm.

Fig 63 Incubation of theront in carp anti-*I. multifiliis* antisera after 60 minutes. Antibody titre $-\log_2$ 1. Note extensive dilation of membrane (arrowed). Scale, 5 µm.



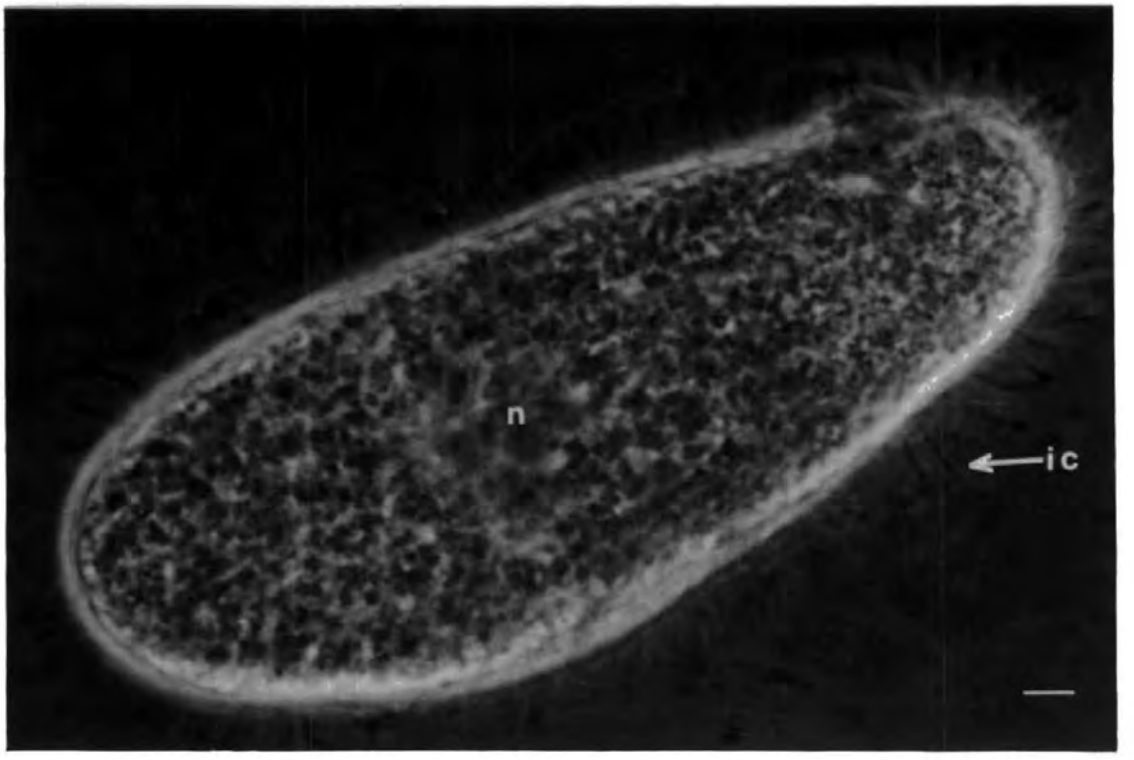


Figs 64 and 65 The effects of carp anti-Tetrahymena pyriformis antisera on trophozoite of T. pyriformis.

Fig 64 Formalin fixed specimen to show appearance of control and general features. Note one of three membranelles (m) surrounding the cytopharynx, cilia (c) and nucleus (n). Scale 5 μm.

Fig 65 The effect of carp anti-T. pyriformis antisera on a live specimen. Note immobilised cilia (ic) and nucleus (n). Antibody titre $-\log_2$ 6. Scale, 5 μm.

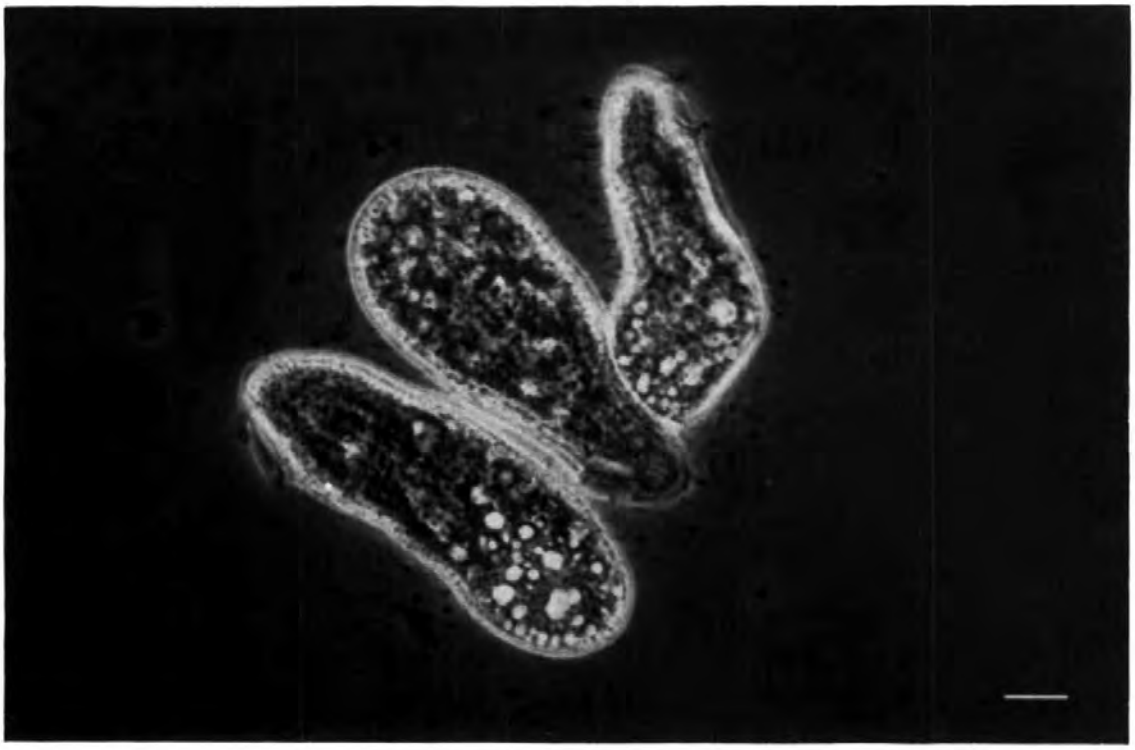
n
c
m

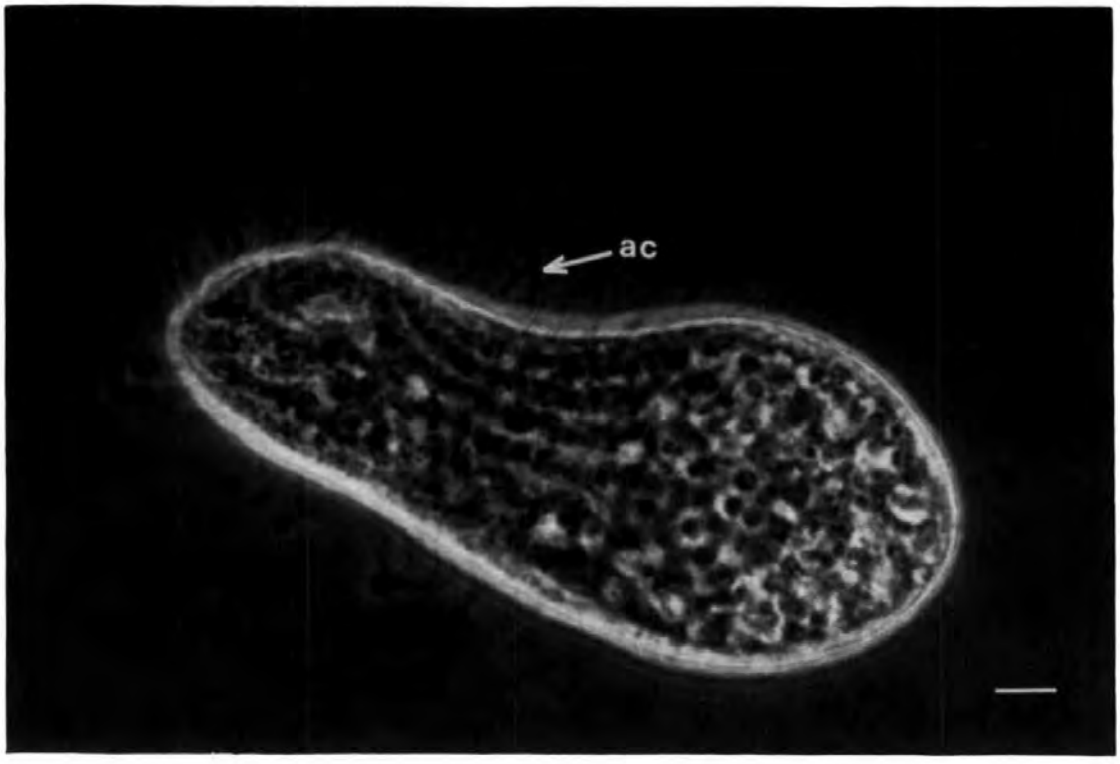

n
ic

Figs 66 and 67 The effects of carp anti-Tetrahymena pyriformis antisera on trophozoites of T. pyriformis.

Fig 66 Showing a group of agglutinated trophozoites in sera. Antibody titre $-\log_2$ 6. Scale, 10 μm.

Fig 67 Single specimen to show agglutinated cilia (ac). Antibody titre $-\log_2$ 6. Scale, 5 μm.

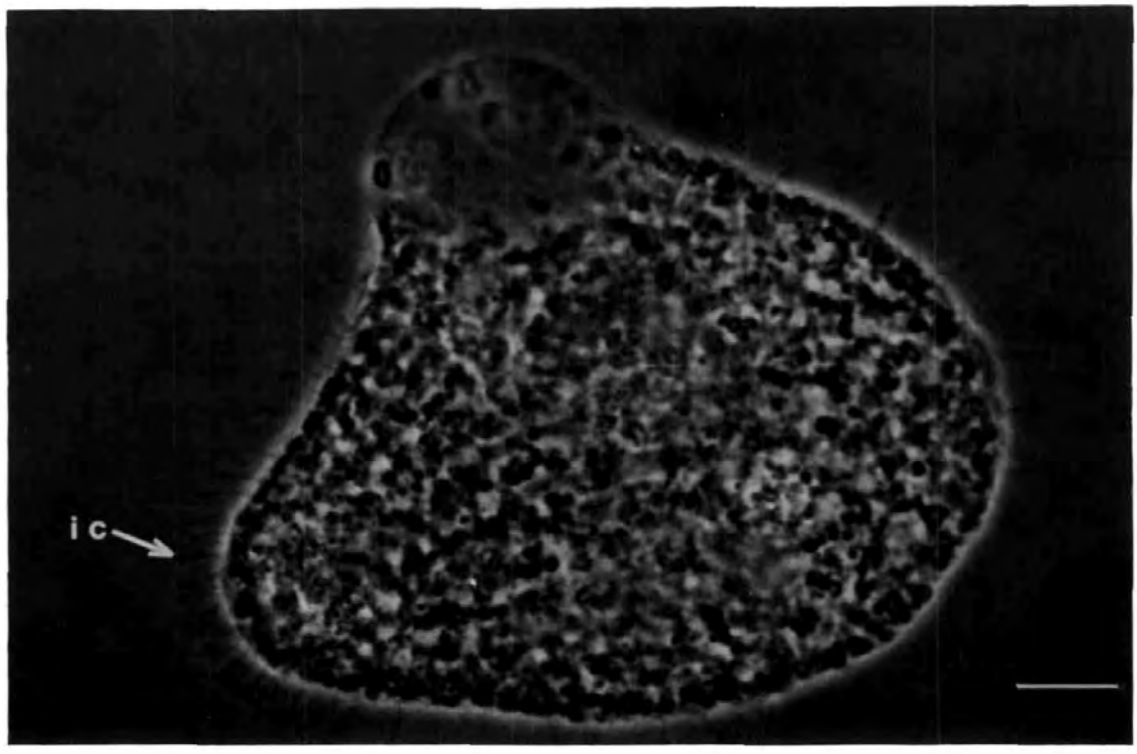


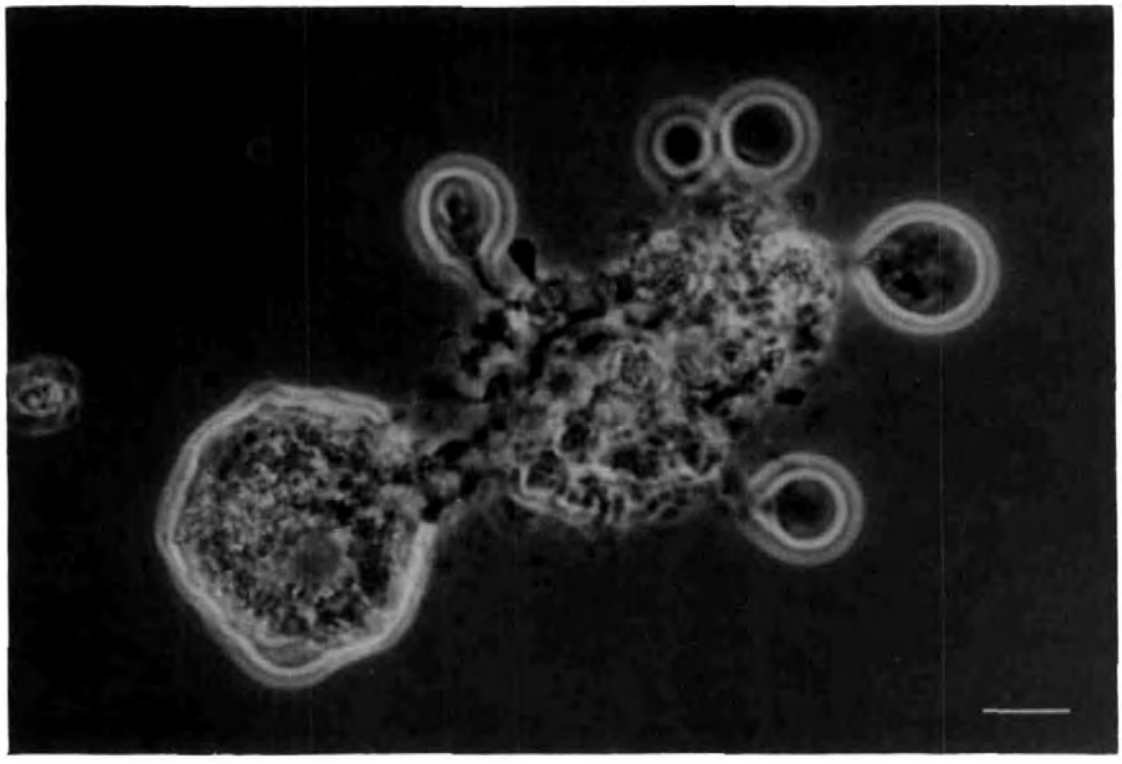

ac

Figs 68 and 69 The effects of carp anti-*Tetrahymena pyriformis* antisera on trophozoites of *T. pyriformis*.

Fig 68 Straightened immobilised cilia (ic) and dilation of membrane after 15 minutes. Antibody titre $-\log_2$ 2. Scale, 10 μm.

Fig 69 Lysis after 30 minutes. Antibody titre $-\log_2$ 2. Scale, 10 μm.


ic



DISCUSSION

INFECTION AND IMMUNISATION.

One of the main objectives of the present work was to investigate the immune response of carp to *I. multifiliis* in respect to infection dynamics. In undertaking this, it was necessary to standardise both parasite, host and infection regimes.

Ichthyophthirius multifiliis poses a problem in that no one to date has successfully passaged the parasite indefinitely and there are no methods of *in vitro* culture or freeze storage despite attempts having been made (Hlond, 1966; Beckert, 1975; Beeler, 1980). In the present study, it was possible to maintain the parasite for up to 301 days but, during the course of the work, it was necessary to re-establish new infections from a total of 7 isolates from a wide variety of fish species including temperate and tropical. Despite this, the course of infection did not vary within each infection nor with those of other workers (Hines and Spira, 1973a). The possibility of physiological strains existing as suggested by Nigrelli *et al* (1976), which might vary in pathogenicity, cannot be ruled out, although there was no evidence here.

Carp were selected as hosts, not only in their major importance in warmer areas where *I. multifiliis* is endemic, but also as there is more background knowledge on the immune response than in any other fish. Further, juveniles were selected as fish are more prone to damage by the disease when young so would benefit from protection.

In standardising an immunisation procedure, it was essential to devise a system which gave consistency, ensured maximum host contact and which lended itself to an assessment of levels of infection. For this reason, carp were therefore exposed to the infective agent, the theront, in preference to the introduction of the reproductive cyst as reported by other workers (Beckert and Allison, 1964; Parker, 1965; Hines and Spira, 1973c, 1974c; Subasinghe, 1982). Several problems arise in adding reproductive cysts, including vulnerability to predation by fish and unknown reproductive capacity and viability of emerging theronts. One of the major advantages in using the theront was that it permitted viability tests prior to fish exposure permitting adjustment of the immunising dose. In establishing viability with time, theronts were found to be most infective immediately on leaving the cyst but, it was more difficult to control the threshold between sublethal and lethal infections. Therefore theronts were used here, 5-10 hours following excystment when, viability was still sustained but at a more controllable level. After 15-20 hours, viability did decrease although the theronts were capable of swimming for up to 3 days at 20°C. This agrees with observations by McCallum (1982), and might be as expected since, as far as is known, the theront is a non-feeding stage and is therefore expending energy so would need to exploit the first opportunity of host contact. Since the organism is dependent on nutrients stored from the previous parasitic phase, energy sources would be expected to decrease rapidly with active swimming behaviour.

In assessing viability, the assumption was made that each theront will enter a susceptible host on contact, but, infection levels of up to 25.5% only were recorded for development of trophozoites following

exposure to theronts. Maximum host contact was enhanced by reducing the turbulence of the water, by keeping the fish in the dark and reducing aeration. To what extent the percentage of infection is related to theront viability is open to question. It seems likely that the theronts that enter are those that contact the fish first, after which, others may not contact the fish, possibly due to any loss of mucus confusing the theronts if host contact is a chance encounter. Viability might also change during infection due to small changes in pH, for example if the fish defecated, since pH can affect the parasite with levels above pH 8.0 leading to parasite death (Rychlicki, 1968; Cross 1972).

Following the establishment of an immunisation regime, it was of particular interest that occasional low level infections become established in supposedly immunised fish, confined to the periphery of fins. It is interesting that Hines and Spira (1974c) also found this, and it is possible that establishment in these sites is related to a restricted blood supply to these regions. These areas may also lack the cells necessary for local defence via the mucosal immune system suggesting an inability to respond. This might be significant in maintaining low levels of infection in the wild and in fish farming. Such an occurrence was found in the tertiary and/or challenge infections.

The production of humoral antibody to I. multifiliis in juvenile carp age 10-12 weeks in the present investigation is shown for the first time although Hines and Spira (1974c) have demonstrated an antibody response in 1 year old carp to the parasite. Antibody levels were shown to be sustained for at least 12 weeks here. However to what

extent this response can be related to protective immunity is discussed later. A more detailed analysis of the humoral response of juvenile carp was confined to Tetrahymena pyriformis, a closely related ciliate (Levine et al, 1980) and is also discussed later.

Acquired protective immunity to ichthyophthiriasis can be extended for at least 3 months in juvenile carp following experimental immunisation. The degree of immunity, however, was shown to decrease during this period. This was evident by skin damage and developing infections becoming evident at about 2 months with a large proportion of immunised fish dying on challenge at 3 months although surviving longer than non-immunised. That protection to I. multifiliis is lasting has been shown by Hines and Spira (1974c) using 1 year old carp, however, they did not investigate immunity beyond 35 days.

Following immunisation, the degree of protection could depend on the levels of re-exposure with a minimum infection enhancing the response but a maximum infection killing the host. One interesting observation here indicated that a threshold level of infection at challenge may be important in the extent of protection as levels of 16,000 theronts at challenge one month following expression of full immunity led to fish becoming infected whereas those challenged with the standard challenge dose of 8,000 theronts per fish did not become infected. Possibly, if too high a level enters the fish, there is so much disruption of the epidermis that the fish cannot cope with the parasite load. Also, the immune system may be overwhelmed. Infection levels could build up rapidly in a fish farm where, particularly with ponds, water flow generally tends to be slower than in rivers. In the wild, densities of fish are at a lower level so generally, epizootics of the disease are

rare although a severe outbreak of the disease in a river fish population has been reported by Allison and Kelly (1963).

The route of presentation of antigen is important in whether a good response occurs or not. Work on I. pyriformis here, showed greater antibody levels of longer duration following intraperitoneal administration compared to exposure to the live parasite of I. multifiliis, where relatively low levels of antibody occurred by 12 weeks. Similarly, the ability of rainbow trout to develop long term protection to Vibrio anguillarum was found to be decreased in fish vaccinated by direct immersion (47% protection) compared to those vaccinated by intraperitoneal injection (97% protection). Duration of the immune state is also related to method of antigen presentation with high levels of antibody to A. salmonocida bacterin being detected for up to a year following intraperitoneal injection in F.C.A. This may have been due to long term persistence of F.C.A. with slow release of antigen allowing the antigen to complex with serum antibody as it is produced (Paterson and Fryer, 1974b). When aluminium hydroxide was substituted for F.C.A., a significant decrease in antibody to A. salmonocida within only 6 weeks after injection occurred.

In considering other factors which might effect duration of protection to I. multifiliis, age and size of the fish must be taken into account. In the present study, the average weight of fish at the start of experiments was 700-1050 mg. These fish were found to be able to produce a protective response to I. multifiliis, but Johnson et al (1982b) have found the size of rainbow trout to be important in the development of a strong protective immunity following direct immersion vaccination with V. anguillarum or Y. ruckeri bacterins.

Fish less than 1g had protection for 90-120 days whereas those of 4g were protected for over a year. It would be interesting to see whether carp of a comparable size would have increased length of protection to ichthyophthiriasis.

To what extent premunition plays a role in maintaining protective immunity remains unknown. In experimental systems investigated here, immunity appears to be sterile in that no latant stages were detected. However, even in fully protected carp, theronts were observed to penetrate the epidermis on contact, thus affording a mechanism of continual antigenic stimulation in infected waters. Hines and Spira (1974c) found fish were refractory to I. multifiliis for up to 8 months when maintained under conditions of continuous exposure to the parasite, although recovered fish, maintained in tanks free of the parasite were found not to harbour any parasites, and thus latant infections.

There appears to be a paucity of literature on duration of the immune response of carp to parasite infections although there have been some studies in other fish species. From these studies, it appears that duration of the immune response appears to depend on whether a true sterile immunity occurs or not, with there being a tendency for increased duration of protection corresponding to non-sterile immunity. For example, Khalil (1964) found that following acquired immunity in Polypterus senegalis to Macrogyrodactylus polypteri, fish were refractory to reinfection if a few parasites remained on the fish (cf non-sterile) whereas those fish free of parasites (cf sterile) could be reinfected on re-exposure to the parasite after a "short time". Where protection has occurred for a longer duration, appears to have been

related to a non-sterile immunity or a state of premunition, with the development of mild chronic infections. This has been well illustrated in infections with haemoflagellates. Woo (1981) found that goldfish, immunised with Trypanosoma danilewskyi were immune to homologous challenge for at least 120 days after the disappearance of trypanosomes from the circulation. He suggested that the immunity was non-sterile, with small populations of trypanosomes and/or antigen occurring in internal organs. Similar suggestions have been made by Burreson and Zwerner (1982) as a cause for the seasonality of infections of summer flounder with Trypanoplasma bullocki where low or no infections occur in the summer but high in the winter. This may act in conjunction with increased levels of antibody during the summer, but low in the winter when parasitemias are greatest (Burreson and Frizzel, 1986). Sypek and Burreson (1983) though, suggest that immunity is sterile to T. bullocki unlike the situation proposed by Woo (1981) that T. danilewskyki in goldfish is non-sterile.

IMMUNOSUPPRESSION

Immunosuppression of carp to I. multifiliis using the synthetic corticosteroid Triamcinolone acetonide and the corticosteroid Hydrocortisone 21-hemisuccinate (cortisol) is demonstrated here for the first time. There are no previous records of this phenomenon in any fish to ichthyophthiriasis although immunosuppression is not unknown in fish immunised with other antigens, for example to the o-antigen from the bacterium Yersinia ruckeri (Anderson et al, 1982).

There is some evidence from the results that the suppression due to the Hydrocortisone (cortisol) was reduced compared to that from Triam-

cinolone acetonide. This would agree with evidence by Dale (1984) and Bach and Strom (1985) that the synthetic corticosteroid Triamcinolone acetonide is a stronger corticosteroid than Hydrocortisone, from it's action in mammals.

The application of the corticosteroids did not appear to affect humoral antibody to I. multifiliis already in circulation as antibody titres remained unaffected or were only slightly reduced when corticosteroids were administered fourteen days after protective immunity had been established. Similarly, Chen et al (1983) found no reduction in antibody titres to Aeromonas salmonocida bacterin when Triamcinolone acetonide was injected six days following antigen administration. Although antibody in circulation appears to remain relatively unaffected, Anderson et al (1982) have shown a reduction in antibody producing cells, splenic lymphocytes and circulating antibody in rainbow trout when administered Triamcinolone acetonide 24 hours following flush exposure to Y. ruckeri O-antigen. Similar effects on circulating antibody have been found by Wechsler et al (1986) who injected striped bass with Triamcinolone acetonide 24 hours before intraperitoneal injection of infectious pancreatic necrosis virus, where consistently lower titres were obtained. Since in both examples, administration of corticosteroid occurred close to exposure to antigen, it would appear that immunosuppression affects production of or secretion from antibody producing cells.

One of the problems encountered in investigating immunosuppression to active infections of I. multifiliis, was that the corticosteroids had to be administered after clearance of the parasite; failure to do so would have resulted in death from an overwhelming infection. The

origin of high antibody levels recorded to I. multifiliis following immunosuppression here would therefore be expected to represent either levels already in circulation, although additional secretion from committed plasma cells could not be ruled out.

Immunosuppression is known to be an important aspect of protozoa infections in mammals leading to increased parasitemias and host susceptibility (Cox, 1968, 1974; Young and Cox, 1971; Seed et al, 1972; Balber, 1974; Hussein, 1985). The view that corticosteroids are unlikely to affect antibody in circulation is supported by Young and Cox (1971) who found that following administration of the corticosteroid betamethasone 3-7 or 10-14 days after infection of mice with Babesia microti, no difference in antibody levels occurred early in the infection and only began to decrease late in the infection. Likewise, Seed et al (1972) found very little difference in agglutinating titre in rabbits infected with Trypanosoma gambiense when administered Hydrocortisone prior to or during an infection, although parasitemias increased and a decrease in the cellular response occurred. Further, it is well known that corticosteroids affect the humoral response in mammals only when administered before the antigen or at the same time as the antigen (Berglund, 1956 a, b; Craddock et al, 1967; Elliott and Sinclair, 1968; Ferreira et al, 1973; Hersch, 1974; Dracott and Smith, 1979). There was less effect when corticosteroids were administered after the antigen although according to Bjornboe et al (1950), administration after the antigen could result in suppression of the humoral response. Whether suppression of the humoral response is a direct result of the action of glucocorticoids on B cells is not known although according to Miller (1983), the greatest effects are on T cells. Ultimately, this may affect antibody due to an interaction of

cell types.

The results in the present investigation suggest that the mechanisms of protection in carp to I. multifiliis are more complex than that suggested by Hines and Spira (1974c) based on humoral antibody alone. According to these workers, theronts would be immobilised at the host surface by antibody in the mucus. Contrary to the views of Hines and Spira (1974c), theronts were here recorded to penetrate the epidermis of immune, juvenile fish. In addition, following immunosuppression when humoral antibody was sustained at high titres, active infections were established, with even death of the host.

Nevertheless, that humoral factors can exert an effect on ectoparsites at the surface affords the best explanation to acquired immunity to Ichtyobodo necator, Trichodina spp, Gyrodactylus spp. (Robertson, 1979; Scott, 1985), although as yet there is no firm evidence. The role of mucus in defence has been discussed by several people as it is the first point of contact between pathogens and the fish, thus forming a potentially protective barrier (Pickering and Richards, 1980; Roberts and Bullock, 1980). Mucus has also been implicated, to some extent, to have a fungicidal and bacteriacidal activity as demonstrated by the presence of lysozyme (Fletcher and White, 1973; Ourth, 1980) and protease (Hjelmeland et al, 1983).

Wahli and Meier (1985) have detected immobilization antibody to I. multifiliis in the mucus of rainbow trout but with substantially lower titres than in the serum. Interestingly, anti-parasite activity of the mucus was greatest during an infection whereas serum activitiy was low. One week following the disappearance of all symptoms of I. multifiliis,

the anti-parasite activitiy in the serum had increased while less efficacy was detectable using mucus. Seven months following an infection, antibody was still detectable in the serum but there was only a very low anti-parasite activitiy in the mucus. Thus in immune fish, one may not necessarily expect high antibody titres in the mucus. As to why different levels of antibody occur in the mucus and serum is not certain but it may be due to different groups of cells being stimulated by local amounts of antigen.

It is possible that those theronts successfully entering the epidermis avoid mucus antibody activitiy due to speed of entry and possibly also as a result of secretion from the mucocysts. The theront has been observed by Ewing et al, (1985) and R. A. Matthews (personal communication) to be enclosed/coated by a gelatinous material released from the mucocysts at contact and penetration of the epidermis. Similar observations have been made with Tetrahymena spp. When Tetryhymena were placed in immune serum, a gelatinous 'sheath' appeared to be secreted over the organism and protected it from the harmful effects of the immune serum. This 'sheath' also appeared to be secreted from the mucocysts (Robertson, 1939 a, b; Harrison and Fowler, 1945; Doerder, 1981). According to Wahli and Meier (1985) full activity of the mucus leading to 75% of the theronts being immobilized, took up to 2 hours. In the present study, immobilization/agglutination activitiy of serum could also take up to 2 hours. In some instances, full activitiy of the serum was not observed till up to 4 hours when theronts were used immediately following excystment as opposed to ones 5-10 hours old. In contrast, entry into the epidermis of juvenile carp by the theront was achieved within 10 seconds as recorded here. In this respect, it would be expected that surface antibody would be a significant factor in the

control of ectoparasites which are continually in contact, for example, Epibdella melleni. Nigrelli (1935 a) found mucus from fish immune to Epibdella melleni did not reach full activity on the parasites till after 4 hours.

Nevertheless, mucus cannot be ruled out as a contributory factor in controlling ichthyophthiriasis and antibody is certainly detected (Hines and Spira, 1974c; Wahli and Meier, 1985). Interestingly, Paterson et al (1981) demonstrated agglutinin activitiy in cutaneous mucus to bacterial kidney disease following immunisation but, it was not effective in preventing infection by this bacterium. The possibility of antibody from this source being a contributory factor in immunity could operate in one of two ways; by having a delayed effect of surface antibody after the theront has entered the epidermis and, in addition, it might be assumed that even low levels of surface antibody might be sufficient to control infections of theronts at a threshold of infectivity, particularly older and less viable theronts (McCallum, 1982). This may occur, particularly in the wild where theronts may not have immediate contact with the host. In experiments here, theronts were taken during maximum viability.

In the present study, immune serum was seen to have an effect on theronts of I. multifiliis leading to immobilization and agglutination of cilia and, at high serum concentrations, dilation of the parasite membrane occurred. Antibody on the surface of the theronts/trophozoites may lead to lysis of the organism by opsonisation. Whether opsonisation may occur is unknown since there are only a few reports of this mechanism occurring in fish (Griffin, 1983; Johnson and Smith, 1984; Sakai, 1984). Other workers have not been able to demonstrate

opsonic activity of fish antibody and complement of fish macrophages, which may be due to the absence of Fc and C receptors on the phagocytic cells (Wrathmell and Parish, 1980).

The fact that antibody is secreted in the mucus of skin and also in the instestine (Fletcher and Grant, 1969; Bradshaw et al, 1971; Di Conza and Halliday, 1971; Harris, 1972; Harrell et al, 1976; Ourth, 1980; St. Louis-Cormier et al, (1984) indicates that it has some role to play in control of fish diseases, probably on the entry of pathogens by the epithelial layers. The most probable explanation for the origin of antibody within the mucus is the localised production within antibody producing cells within the epidermis. The apparent absence of proliferative activitiy within the lymphoid organs here supports the view that antibody secretion may be a localised response unlike when I. pyriformis was injected into carp, leading to lymphoid organ proliferation. In support of this view, is the work by Georgopoulou and Vernier (1986) and Rombout et al (1986). Immunoglobulin producing cells have been demonstrated in the epidermis of rainbow trout (St. Louis-Cormier et al, 1984, Pelletero and Richards, 1985) although it was not possible to identify whether the Ig was on the cell membrane or cytoplasmic. Lymphocytes have been identified in the skin of carp infected with I. multifiliis (Hines and Spira, 1974a; Ventura and Paperna, 1985) and in the skin of other fish species, for example rainbow trout (Pickering and Richards, 1980), three spined stickleback (Phromsuthirak 1977), channel catfish (Ourth, 1980) and in several other species (Roberts and Bullock, 1976, 1980; Mittal et al, 1980).

Localised production of antibody could explain the results of Wahli and Meier (1985) of high levels of antibody during an active infection

since during these periods, considerable damage to the surface with a great deal of cell disruption will occur which could lead to antibody seepage.

Results here lend more support to the view that cellular aspects of immunity play more of a role than hitherto thought in protection against ichthyophthiriasis. This is supported by the re-establishment of I. multifiliis following immunosuppression of immunised hosts which retain high antibody titres. However, such a view would be supported by the location of relevant blood cell types or leucocytes, associated with the parasite, leading to a possible granulomatous response.

A major factor in immunosuppression in fish is an impaired cellular response, as illustrated by a depletion in lymphocytes in the lymphoid organs (Robertson et al, 1963; van Muiswinkel and van Ginkel, 1981; Chilmonczyk, 1982; Ghoneum et al, 1986), increased survival of allografts (Stutzman, 1967), a reduction in peritoneal leucocyte accumulation (MacArthur et al, 1984) and, in vitro, a reduction in phagocytic activity of phagocytes as measured by chemiluminescence (Stave and Roberson, 1985). It would appear therefore, that the effects of corticosteroids on the immune response of fish is not dissimilar to that described for mammals as far as is known. It seems reasonable in examining the mechanisms, that some parallels will be found. In mammals, corticosteroids have generally been considered as anti-inflammatory drugs due to their effects on the cellular response, for example, in a reduction in function and activity of phagocytic cells such as the inhibition of superoxide and hydrogen peroxide production (Levine et al, 1981; Welch and Devlin, 1983). Effects on phagocytosis are varied with Nicoll (1956) finding a depression unlike

Claman (1975), Sneiderman and Wilson (1975) and Dannenburg (1979), although there does appear to be a reduction in numbers of cells at the site of tissue damage (Sneiderman and Wilson, 1975). Further effects have shown a reduction in lymphocytes in lymphoid organs and the circulation (Claman, 1975; Fauci, 1975; Pearson et al, 1978; Miller, 1983) along with reduced in vitro function (Tormey et al, 1987; Pearson et al, 1978; Fauci, 1978-1979).

Further evidence in agreement that cellular responses may be important in controlling ichthyophthiriasis is the recent work by Graves et al (1985) who have demonstrated that non-specific cytotoxic cells (NCC) from channel catfish are capable of killing I. pyriformis, providing evidence that fish may respond to parasitic protozoa infections by production of cellular cytotoxic responses. The lytic ability of the NCC occurred greatest in immobilized I. pyriformis since cell to cell contact was necessary for cytolysis. They suggested that a similar response may occur in fish immune to I. multifiliis in that theronts may be able to invade the mucus and epithelium after which they are immobilized by antibody allowing the NCC's to function cytolytically. In view of the evidence in the present study that theronts do penetrate the immune host, this hypothesis by Graves et al (1985) may well be a mechanism in protection. Non specific cytotoxic cells have been found in several species of fish in the head kidney, spleen and peripheral blood, such as in carp (Hinuma et al, 1980), channel catfish (Evans et al, 1984 a, b, c; Graves et al, 1984), atlantic salmon, rainbow trout and golden shiners (Moody et al, 1985), and have been shown to have a similar function to natural killer cells in mammals, lysing target cells. Graves et al (1985) found the time for cytolysis of I. pyriformis in the presence of NCC's exceeded 10 hours for optimal

killing in co-cultivation of effectors and target cells, with some organisms still being viable after 18 hours. Observations in the present investigation on parasite development in situ within immune fish, indicates that their destruction is gradual within the first few days, those at the periphery of fins surviving longest. It seems probable therefore, that NCC depletion could explain immunosuppression in fish to ichthyophthiriasis.

Recent work on other eukaryotic parasites of fish supports the role that in general, cellular responses are important in the control of tissue parasites. This has been shown in the response of fish to Ligula intestinalis where an infiltration of polymorphonuclear leucocytes and macrophages occurs round the parasite (Hoole and Arme, 1982, 1983 a, b, 1986). Similar responses have been seen in other fish hosts to cestodes (Scott and Grizzle, 1979; Moser et al, 1984; Shostak and Dick, 1985), and to microsporidia infections (Dykova et al, 1980; Ralphs and Matthews, 1986).

In response to I. multifiliis infections, other cell types as well as NCC's may be important. That leucocytic infiltration is a characteristic of infections has been shown by Hines and Spira (1974a) and Ventura and Paperna (1985). According to these workers, the predominant cells are lymphocytes and 'neutrophils' although 'neutrophils' are associated more with the humoral response in mammalian systems. It must be emphasised however that specific roles of fish leucocytes are unresolved (Ellis, 1977 b) and speculation as to function has been based chiefly upon morphological and more recently, histochemical and functional studies (Barber and Mills Westerman, 1978; Bayne, 1986; Suzuki, 1986). Some workers suggest that 'neutrophils' or granulocytes

have none or only a weak phagocytic activity *in vitro* or *in vivo*, in plaice (Ellis, 1977 b; Ferguson, 1976 b) and in gar (McKinney *et al*, 1977) whereas others have reported active phagocytosis of 'neutrophils' and granulocytes, in carp (Bayne, 1986; Suzuki, 1986) rainbow trout (Finn and Nielson, 1971), plaice (MacArthur, *et al*, 1984) and tilapia (Suzuki, 1986).

Further work is needed at the electron microscopical level to fully identify cell types involved in the response to *I. multifiliis*, and particularly to find the involvement of macrophages and subsequently whether these cells are affected by immunosuppression.

STRESS

That fish can produce cortisol has been well reviewed by Idler and Truscott (1972) with an increase being associated with physiological stress (Mazeud *et al*, 1977; Donaldson, 1981; Wedemeyer and McCleay, 1981). Various factors leading to this condition such as handling (Wedemeyer, 1976; Barton *et al*, 1980; Pickering *et al*, 1982), confinement and over-crowding (Wedemeyer, 1977; Strange *et al*, 1978; Barton *et al*, 1980; Davis and Parker, 1983), transport (Barton and Peter, 1982), oxygen depletion (Tomasso *et al*, 1981 a), exposure to formalin (Wedemeyer and Yasutake, 1974), copper sulphate (Donaldson and Dye, 1975), ammonia and nitrite (Tomasso *et al*, 1981 b) and anaethetisation (Wedemeyer, 1970; Smith *et al*, 1979 a, b; Davis *et al*, 1982). These procedures are frequently used in fish farming and stress is known to render fish more susceptible to disease (Wedemeyer, 1970; Sniesko, 1974; Wedemeyer and Wood, 1974; Pickering and Duston, 1983), with cortisol affecting the immune response and defence of fish to disease

(Pickering and Duston, 1983; Pickering and Pottinger, 1985; Ellis, 1981), although many reports on susceptibility of fish to disease following physiological stress are based largely on circumstantial evidence.

It would be interesting to speculate that stress in the fish farming situation could lead to outbreaks of ichthyophthiriasis due to any immunological control on the parasite being relinquished. However, interpretation of the results in the present study must be viewed with some degree of caution in view of the use of the synthetic corticosteroid Triamcinolone acetonide. Although cortisol was also used, it is not known whether the doses were pharmacological or physiological. Nevertheless, possible effects of stress should be considered in the development of vaccine programmes for fish against ichthyophthiriasis.

LATENCY

Although there have been many claims of a carrier state existing for I. multifiliis and the marine species, Cryptocaryon irritans (Reichanbach-Klinke and Elkan, 1963; Nigrelli and Ruggieri, 1966; Hoffman, 1976; Curry, 1977), there has been no convincing evidence in support of these views. Studies in the present work here failed to establish a carrier state following different regimes of immunisation and challenge in an attempt to establish different degrees of immunity. Likewise, Hines and Spira (1974 c) were unable to detect trophozoites in mucus scrapings of the skin epithelium or gills, 21 or 25 days following recovery of carp from infections of the parasite. Bauer (1953) was also unable to find any evidence of I. multifiliis in carp recovering from infection.

The use of immunosuppressants was considered appropriate to test for latency in that they would relinquish immunological control on 'latent' parasites and thus lead to expression of the disease. The possibility still cannot be overlooked however, since many factors may be involved in whether 'latent' infections may become established or not such as the immune state of the host and on host genetic status to resistance. That a carrier state can be established in fish to viral and bacterial infections has already been documented, in particular for infectious pancreatic necrosis virus (IPNV) (Wolf et al, 1968; Yamamoto, 1975 a,b; Roberts and McKnight, 1976; Yamamoto and Kilistroff, 1979; Bootland et al, 1986) and for the bacterin Aeromonas salmonocida (Bullock and Stuckley, 1975; McCarthy, 1978) and Yersinia ruckeri (Hunter et al, 1980). Frequently it has been observed that low antibody is correlated with an increased length of time of the carrier state (Yamamoto, 1975 b). Stress has also been related to IPNV outbreaks in chronic carriers (Roberts and McKnight, 1976) although administration of Triamcinolone acetonide did not give the same results (Wechsler et al, 1986).

Latent infections of both viral, bacterial and eukaryotic parasites are well documented in the higher vertebrates including toxoplasma and Strongyloides stercoralis (Cox, 1982; Schad et al, 1985), with administration of corticosteroids leading to expression of the infections.

TETRAHYMENA PYRIFORMIS: HUMORAL RESPONSE, IMMUNISATION AGAINST ICHTHYOPHTHIRIASIS AND APPLIED ASPECTS OF VACCINATION.

That fish are able to respond immunologically to live T. pyriformis has been confirmed here using juvenile carp. Further, the results are in agreement with Pyle and Dawe (1985 b) in that antibody was detected following a single administration of antigen, reaching a peak at 6-8 weeks and persisting for at least 12 weeks. Although intraperitoneal injection gave a response, other routes, including oral administration, were not investigated here in view of the work of Pyle and Dawe (1985 b) who recorded no response to cilia administered to channel catfish via this route, only by the intramuscular route.

The present study represents the first covering Tetrahymena pyriformis in juvenile carp. Few quantitative assessments of the kinetics of a primary response in fish have been undertaken to eukaryotic parasites, although Bortz et al (1984) have used an Enzyme linked immunosorbent assay (ELISA) to detect antibody to Diplostomum spp. in rainbow trout. In contrast, however, there are many assessments of the humoral response to other antigens including bacteria (Anderson et al, 1979 a, b; Lamers and Pilarczyk, 1982; Kaattari and Irwin, 1985; Cossarini-Dunier, 1986), immune complexes (Secombes and Resink, 1984) and bacteriophage (O'Neill, 1979). There appears to be little variation in the response of fish to all of these antigens including eukaryotes.

Antibody titres were maintained for up to 3 months, the duration of monitoring. That significant levels of antibody can be sustained for up to 12 months in fish to other antigens is indicated by Lamers (1985, 1986). Lamers (1985, 1986) and Lamers and De Haas (1985 a), attributed this long term response to persistence of antigen in the lymphoid organs in the form of antigen/antibody complexes. This supports the work of Ellis (1980) who also followed the process of antigen trapping

in the lymphoid organs of plaice (Pleuronectes platessa) using the soluble antigen bovine serum albumin (BSA), and suggested that antigen is probably held as immune complexes.

Cell proliferation was recorded here within the lymphoid organs, notably the pronephros using autoradiography, although it was not possible to attribute division to specific cell types. The peak response for proliferation proceeded that of humoral antibody, suggesting that division may have been occurring in the antibody producing cells (PFC). At the time of peak proliferation, humoral antibody was barely detectable, but, as antibody titres increased, proliferation had decreased. Anderson et al (1979a, b) have followed the kinetics of the primary response in trout to Yersinia ruckeri o-antigen, measuring the number of PFC's and humoral antibody titre. It is interesting to note that the PFC's followed a similar trend to that of antibody titre, but occurring 10 days earlier. As the PFC's began to decrease, antibody titres started to rise. Although time did not permit the measuring of PFC's, investigations by other workers using mainly bacteria and red blood cells, have indicated populations of PFC's within the pronephros (Smith et al, 1967; Chiller et al, 1969; Rijkers et al, 1980b; Lamers and Pilarzyk, 1982), spleen (Smith et al, 1963; Chiller et al, 1969; Anderson et al, 1979 a, b) and thymus (Sailendri and Muthukkaruppan, 1975; Kaattari and Irwin, 1985). This has still to be confirmed here, but, it would be expected that PFC's to I. pyriformis would be found in similar areas. Kaatari and Irwin (1985), have demonstrated that the PFC response in coho salmon is immunoglobulin (Ig) mediated, due to the inhibition of the response using specific anti-coho Ig antisera.

Although a single administration of T. pyriformis has been shown here to produce a marked antibody response with the possibility of long term persistence (Lamers, 1985), a second exposure to the antigen resulted in an enhancement of antibody titre. Such a response of fish to a wide range of antigen has been recorded by many other researchers. This is generally accepted as homologous to mammals, bearing in mind that in fish, there is only one class of immunoglobulin (Marchalonis, 1971; Dorson, 1972; Corbel, 1975). Thus fish may be considered to represent an earlier stage of phylogeny. In addition, there are some inconsistencies in the timing of the peak of secondary response. Unfortunately, the work in the present study throws no further light on the situation as the secondary level was measured at the same time interval as the primary. Although this was higher, it may represent a plateau or drop from an earlier level. Whether an increased rate of antibody production does occur in a secondary response of fish has not been resolved by other workers, and there is some contradiction. Rijkers and van Muiswinkel (1977) and Lamers et al (1985a), agree that peak response of either PFC's or antibody levels, occur earlier during the secondary than primary response, whereas Sailendri and Muthukkaruppan (1975) and O'Neill (1979), found peak secondary response occurred at the same time as for the primary. Some of these inconsistencies might be explained on the basis of temperature due to fish being poikilotherms.

Although in the results here, a heightened response to a second administration of antigen occurred, 1 month is too early to say definitely that memory has developed to T. pyriformis. Ideally, the fish need to be given a second exposure to the antigen at 3 months or longer from the first. Lamers et al (1985a, b) detected a weak memory

level at one month, but this was shown to rise to an optimal level when fish were given a second exposure to antigen 3 - 8 months following the first.

There appears to be a paucity of information on the secondary response in carp to protozoa but there have been studies on the secondary response of carp to other pathogens and antigens (Avtalion, 1969; Rijkers et al, 1980 c, d; Secombes and Resink, 1984; Lamers and De Haas, 1985 b; Lamers, 1985) and in other species of fish, for example rainbow trout (S. gairdneri) (Anderson and Dixon, 1980; Blazer et al, 1984; Dunier, 1985), brown trout (S. trutta) (O'Neill, 1979), rosy barb (Barbus conchonius) (Rijkers and van Muiswinkel, 1977) and tilapia (Tilapia mossambica) (Sailendri and Muthukkaruppan, 1975). Burreson and Frizzell (1986), found a rise in antibody titre after a challenge infection with I. bullocki following injection of formalin killed flagellates to this parasite into juvenile summer flounder (Paralichthys dentatus) indicating a possible secondary response.

The use of eukaryotic organisms requires careful evaluation of dose as, in the development of vaccines, the significant antigens might only represent a small component of the whole organism. Thus immunisation requires a different approach to that for single molecules and bacterial antigens. In retrospect, it would probably have been advisable to have used a higher dose here to ensure an adequate response to the component antigens. Nevertheless, a good response of carp has been achieved to I. pyriformis with a standard dose of 25 μg g^{-1} body weight, comparable to that of other workers, and corresponding to previous doses of pure antigen in immunising carp in the laboratory. This was enhanced when administered as a dose in Freund's complete

Adjuvant (FCA). Further work should be directed more to isolates of cilia and electrophoretic separation of antigens. The use of whole T. pyriformis in the present study has made it difficult to relate results to those of other workers. O'Neill (1979), Rijkers et al (1980b) and Lamers et al (1985a), have found that secondary enhancement is dependent on the level of the dose, and can be depressed by too high or too low a dose. In administering whole T. pyriformis, the fish were effectively being exposed to both soluble and particulate antigen. Bradshaw et al (1969), found that a good secondary response of the gar, only occurred when a particulate antigen was used, although Avtalion (1969) found a secondary response following administration of a soluble immunogen, bovine serum albumin.

The control and treatment of I. multifiliis remains almost totally dependent on the addition of chemicals to the water, including formalin, malachite green, methylene blue and chloramine T, all of which are stressful in themselves and are ineffective in killing the parasite in situ. The development of a vaccine therefore, might be considered as a matter of urgency in combatting the disease. That fish can acquire protective immunity to I. multifiliis has been shown in the present study and by several others (Hines and Spira, 1973c, 1974c; Beckert and Allison, 1964). However, one of the major problems in producing a vaccine to any eukaryotic parasite is that of obtaining sufficient antigen for immunisation. The advent of biotechnology now offers the possibility in the future of a more productive means of isolating and producing antigen in bulk. However, at present, although this approach is well advanced in medical and veterinary parasitology, to date, not a single specific antigen has been recognized or isolated from I. multifiliis. An alternative strategy has been the invest-

igationof antigenic cross reactivity between more convenient organisms which can be cultured in large numbers. Of particular significance in this respect has been the work of Goven et al (1980a,b 1981a), in which channel catfish were immunized with a strain of Tetrahymena pyriformis. The investigations of these workers prompted further studies here on I. pyriformis in carp. Results however, show little evidence in support of protection to Ichthyophthiriasis, in carp using I. pyriformis 1630/1W Claff 1939(W), USA obtained from the Cambridge collection of Algae and Protozoa (CCAP), despite the use of carp of all ages and a range of antigenic preparations including live I. pyriformis and freeze-dried ciliary preparations.

Although the majority of results were consistent with no protection to the disease, two occasions occurred in which an extended survival was seen. One, where survival time was slightly extended compared to control fish, and another where 35% survived challenge. This level of 35% is well below the Relative Percent Survival (RPS) of a vaccine as defined by Amend (1981) (60%) and Ward (1982) (70%). That this was not a protective response was further confirmed by 100% mortality in a repeat experiment, and humoral antibody to I. multifiliis was not detectable in any of the experiments where measured. Although, as mentioned earlier, cellular factors may be important.

Nevertheless, although antibody to I. multifiliis was not found, there were high antibody levels to I. pyriformis. Further investigations failed to show any cross antigenicity between the two protozoa used here although specific anti-I. pyriformis antisera did cause immobilization and agglutination with damage to I. pyriformis trophozoites at high serum concentrations. These interpretations were based on

antisera raised in rabbits, rats and carp and suggest that the strain of T. pyriformis or the parasite, were not compatible with those used by Goven et al (1980a,b 1981a,b). That these latter workers demonstrated cross reactivity by passive haemagglutination and indirect immunofluorescence supports these views. Although the possibility of cross reactivity between different species of Tetrahymena must be considered in affording protection to I. multifiliis as has been reported in rainbow trout by Wolf and Markiw (1982), a recent extensive study by Dickerson et al (1984) suggest that strains of T. pyriformis vary in their antigenicity. They assessed the protectiveness of different strains of T. pyriformis and detected considerable variation in isolates between the American Type Culture Collection and the Midwest Culture Service, several of these affording no protection.

As a basis for the use of T. pyriformis as a protective vaccine for Ichthyophthiriasis, a more rigorous assessment of the strains already in culture collections throughout the world is urgently required on the basis of both immunological and isozyme techniques. Some of these problems have been identified by Borden et al (1973) and Nielsen and Andronis (1975), who found differences in electrophoretic patterns of selected enzymes between designated strains from different laboratories. However, they suggest caution in interpreting these results due to local factors such as environment, temperature, salt concentrations, nutritional status, handling and number of divisional cycles. Temperature and salt concentration have previously been shown to determine which immobilization antigen is expressed. Changes in these factors can bring about transformations to other types within the genetic potential of the organism. This has been shown for temperature by Margolin et al (1959); Loeffer and Owen, (1961); Nanney (1963);

Phillips, (1967); Juergensmeyer (1969); Bruns (1971); Doerder and Berkowitz (1986). High salt concentration can also lead to a transformation of serotype as shown by Grass (1972) and protein synthesis, van Bell and Williams (1982).

Differences in cross antigenicity have been found between and within strains and even clones of Tetrahymena spp indicating that the immobilization antigen is very specific (Kidder et al, 1945; Loeffer and Owen, 1958; Margolin et al, 1959; Nanney and Dubert, 1960; Nanney, 1960; Phillips, 1967; Juergensmeyer, 1969). For example, Loeffer and Owen (1958) found that when 31 strains of I. pyriformis were tested against unabsorbed, inactivated antisera raised in rabbits to these strains, 14 serological groups were identified. Antisera to strains within these groups would only react by immobilization with organisms of these groups, with little or no cross reaction occurring in the other serological groups. In retrospect, it is not surprising therefore that the results obtained here have not been comparable to those of Goven et al (1980a, b, 1981a, b), considering the different strain used, obtained from the Cambridge Collection of Algae and Protozoa where as Goven et al (1980a,b, 1981a,b) obtained theirs from the Mid-West Culture Service. Also, different host species were used. However, it does emphasise a need for careful retyping and electrophoretic comparisons of the species used, with an immunogenetic strain.

Similarly, differences may exist between I. multifiliis collected from different sources. Nigrelli et al (1976) suggested that different "races" of I. multifiliis exist, although so far, no serological or biochemical tests have been performed. Recently, Dickerson et al (1986), have developed monoclonal antibodies to I. multifiliis which

will prove a valuable immunological tool in identifying different strains or species of the parasite. These potential problems of strain variations reinforce the view here that a series of experiments must be undertaken using a single isolate of I. multifiliis to ensure genetic uniformity. Further work must be directed towards detailed biochemical investigations of the strains of I. multifiliis and T. pyriformis used here. There have, however, been studies by other workers indicating that differences exist between structural proteins among different strains of T. pyriformis. Vaudaux et al (1977), following SDS-PAGE analysis of pellicles from different strains of T. pyriformis, found a pronounced variation in the epiplasmic proteins, although not between the kinetodesmal proteins. Further biochemical differences have been found between and within strains of Tetrahymena spp such as membranes (Williams et al, 1980; Buhse and Williams, 1982). Generally though immobilization has been used as the main criterion in assessing Tetrahymena spp by serological methods.

Watson et al, (1964) and Bruns (1971) have suggested that the cilia carry the immobilization antigen/s. Some support for this view has been provided by Goven et al (1980a,b, 1981a), who showed that cilia extracts from T. pyriformis afforded protection against I. multifiliis. More recently, Pyle and Dawe (1985b) proposed that the T. pyriformis antigen was soluble. In view of this, it seems that the method of concentration and freeze-drying of ciliary preparations in the present study, may not in fact have been a suitable method of storing the antigen, as it would only have contained particulate material, rather than soluble. Cilia though, have also been found by Seyfert and Willis (1981) to vary in biochemical structure between various species of Tetrahymena. Analysis by SDS-PAGE indicated that of at least 32

polypeptides, only 2 were found with the same molecular weight and no immunological cross reactions occurred between cilia from different species.

In the development of any vaccine, it is essential to sort out the antigens, but also to reclassify the present strains of Tetrahymena spp. and identify any strong immunogens. Strains should be constantly updated as in the medical field, where antigenic variation can occur.

In considering a programme for vaccination of fish against Ichthyophthiriasis, the following factors should be considered: age and size of fish, mode of antigen administration. That fish become immunocompetant at this early age is of considerable practical advantage in affording protection by immunisation at a more manageable age for stocking ponds. Also, less antigen is required if the fish are small. In modern fish farming practice, there is a need for a fast turnover from the hatchery, therefore, the younger the age that the fish can be vaccinated, the better, particularly since I. multifiliis can be more devastating in fry. It is already known that by 4 weeks of age, fish have well developed lymphoid organs as has been shown for salmon (Ellis, 1977a), trout (Grace and Manning, 1980) and carp (Botham and Manning, 1981). With specific and non-specific cell mediated responses already occurring in trout as young as 4 days (Grace et al, 1981; Tatner and Manning, 1985) and in carp as young as 2 weeks (Grace et al, 1981; Botham, 1982), with graft rejection occurring by 16 days post hatch (Manning et al, 1982; Botham et al, 1980; Botham and Manning, 1981).

One of the problems that might arise from a vaccine against Ichthyo-

phthiriasis based on Tetrahymena might relate to the nature of the protective antigen as van Loon et al (1981), Mughal (1984) and Mughal and Manning (1985), have found immunological tolerance developing in carp fry when injected with a thymus dependent antigen. The T independent antigen, Aeromonas salmonocida however did not produce tolerance in 4 week old fry (Mughal, 1984; Mughal and Manning, 1985; Mughal et al, 1986). At present, it is not known whether I. multifiliis and I. pyriformis behave as T dependent or T independent antigens (in mammals) and therefore whether they are likely to induce tolerance or not. This could be partly resolved by investigating the response of fry to these organisms which would be an important step in developing a vaccine.

Although the experimental results here indicate that intraperitoneal administration using whole I. pyriformis gave a good response, this method may not be practical in a farming situation. Nevertheless, the method of injection (both intraperitoneal and intramuscular) was one of the earliest methods in immunising fish against bacteria, (Patterson and Fryer, 1974; Michel, 1979; Cipriano, 1982; Horne et al, 1982, 1984; Sakai, 1985, Shieh, 1985; Cossarini-Dunier, 1986), with Pyle and Dawe (1985 b) finding an antibody response in channel catfish injected with I. pyriformis by the intramuscular route. These routes of administration have the advantage of giving a readily quantified dose of antigen to the fish but can be time consuming and produce physiological stress and, by removing mucus, leave the fish more prone to infection. Injection however, remains a valuable method of immunising brood stock fish and generally large fish, employing automated techniques. Where smaller fish or large numbers require protection then the current method of choice is direct immersion. This method was

first developed for bacterial antigens (Anderson et al, 1979 c, d; Smith, 1982; Johnson et al, 1982; Johnson and Amend, 1984; Tatner and Horne, 1985), and has more recently been used successfully by Wolf and Markiw (1982) in immunising rainbow trout fry with I. thermophila against Ichthyophthiriasis. Although oral vaccines have been developed, these have not generally found widespread use as it is impossible to control the dose of antigen taken in by fish and little is known about uptake of antigen by Gut associated lymphoid tissue (GALT).

There is no evidence in the present study as previously mentioned, to suggest or support that latant infections or carrier states occur in carp to I. multifiliis, nevertheless the possibility of vaccination programmes leading to carrier states cannot be ignored.

In developing any effective agent against the disease, a knowledge of the etiology of the parasite or disease is needed. This will ensure, in temperate regions, that vaccination will take place in anticipation of the disease risk, or could be designed to afford protection over the season. Using I. multifiliis exposure here, it has been shown that acquired immunity appears to decrease after 3 months at 20±2° C. Whether this period is extended using an intraperitoneal Tetrahymena vaccine has still to be determined.

Experiments were designed in the present study to investigate the possible immunodepressant effect of I. multifiliis of the host response to killed Aeromonas salmonocida. The results proved inconclusive, although further work is necessary before firm conclusions can be drawn, notably in the timing of infections before

antigen administration. In this respect, it is interesting that Jones et al (1986) have recently shown that a concurrent infection of Cryptobia salmositica has an immunodepressive effect on protection to Yersinia ruckeri and on the humoral antibody response to Y. ruckeri and sheep red blood cells. The possibility of vaccines being rendered ineffective cannot be ruled out where fish are already infected with I. multifiliis.

The need to vaccinate against an endemic disease will ultimately depend upon the standard of husbandry. In situations where adequate facilities for careful disease monitoring are available, a decision might be possible to allow low infections to persist to allow natural immunity to occur.

To date, although Ichthyophthiriasis remains a major disease of freshwater fish, there is no commercially available vaccine. Doubt still remains concerning effectiveness of Tetrahymena and much needs to be done to test strains and to present the antigen in a readily useable form. Although such a vaccine would have high advantages in the intensive farming situation of channel catfish and salmonids and for exotic species such as koi carp, it is unlikely that it could be used for developing countries where carp and tilapia are farmed.

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APPENDIX I

SOLUTIONS USED FOR BREEDING OF CARP

Fertilisation solution

Urea	30.0 g
Sodium chloride NaCl	40.0 g
Tap water	10.0 dm^3

This solution reduces stickiness of the eggs and aids fertilisation.

Hardner solution

Tannin	7.0 - 10.0 g
Tap water	10.0 dm^3

This solution helps to harden the eggs.

APPENDIX II

CULTURE MEDIUMS

Growth medium for Tetrahymena pyriformis

Proteose peptone (Difco)	10.0 g
Yeast extract (Difco)	1.0 g
Double distilled water	1 dm^3

Adjust to pH 7.2 - 7.4

Autoclave 15 p.s.i. for 15 minutes

APPENDIX III

STAINING SOLUTIONS, BUFFERS, SALINES

Electrophoresis staining solution

Coomassie Brilliant Blue R-250	1.0 g
Ethanol	90.0 cm^3
Acetic acid	20.0 cm^3
Double distilled water	90.0 cm^3

Electrophoresis destain

Same as for staining solution but omit coomassie brilliant blue.

Ringers Solution

Sodium chloride NaCl	3.75 g
Potassium chloride KCl	0.10 g
Sodium hydrogen carbonate $NaHCO_3$	0.10 g
Calcium chloride $CaCl_2$	0.10 g
Double distilled water	1.0 dm^3

Phosphate Buffered Saline pH 6.4

Sodium chloride NaCl	4.24 g
Disodium hydrogen phosphate Na_2HPO4	3.42 g
Potassium dihydrogen phosphate KH_2PO4	5.90 g
Double distilled water	1 dm^3

Check pH before use, adjust if necessary.

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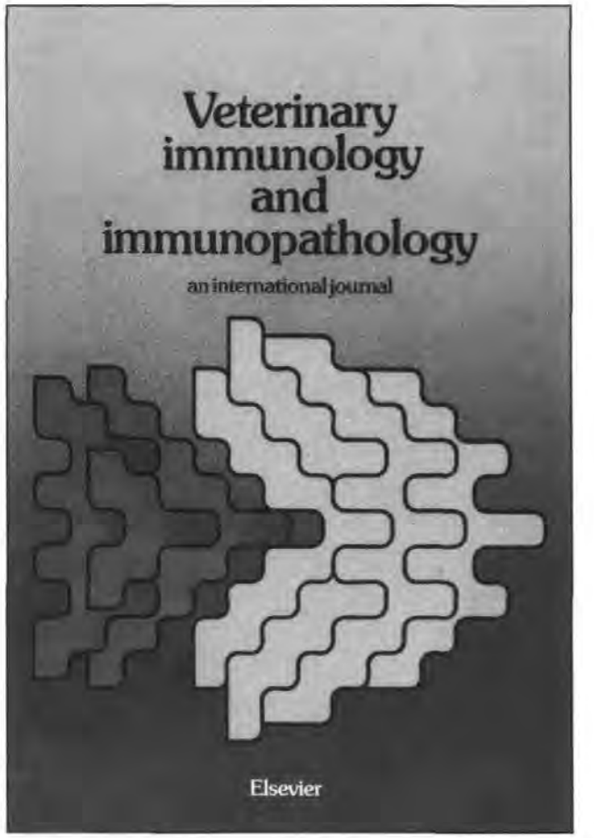

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Veterinary immunology and immunopathology

An International Journal

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IMMUNOSUPPRESSION OF CARP (*CYPRINUS CARPIO* L.) TO ICHTHYOPHTHIRIASIS USING THE CORTICOSTEROID TRIAMCINOLONE ACETONIDE

G. HOUGHTON and R.A. MATTHEWS

Dept. of Biological Sciences, Plymouth Polytechnic, Plymouth, Devon, PL4 8AA, England

ABSTRACT

Houghton, G. and Matthews, R.A., 1986. Immunosuppression of carp (*Cyprinus carpio* L.) to ichthyophthiriasis using the corticosteroid triamcinolone acetonide. Vet. Immunol. Immunopathol., 12: 413-419.

The immune response of juvenile mirror carp (*Cyprinus carpio* L.) has been investigated in relation to protective immunity and immunosuppression to the disease ichthyophthiriasis. Protective immunity was induced by exposing juvenile carp to approximately 2,000 theronts per fish for 3 hours at a concentration of approximately 80 theronts cm^{-3} in dechlorinated water pH 7.0 - 7.2, 20 ± 2°C in the dark, on three separate occasions of 14 day intervals. Following each exposure, re-infection was prevented by transferring fish to clean aquaria twice daily from day 5-10. Fish were challenged 4 weeks following exposure to the third immunising infection with a potentially lethal dose of approximately 8,000 theronts per fish for 3 hours at a concentration of approximately 320 theronts cm^{-3}. All immunised fish survived although 19% showed initial signs of invasion, the parasites in these instances being confined to periphery of fins. Mortalities of 100% were recorded in non-immunised controls, first deaths occurring 5 days after challenge. Fourteen days following challenge, immune fish received one of the following treatments; intraperitoneal injection of the corticosteroid triamcinolone acetonide at a dose of 200 $\mu g\ g^{-1}$ body weight, intraperitoneal injection of 0.85% saline or left untreated; non-immunised fish were subjected to similar procedures. On challenge 6 days later, all fish developed heavy infection with up to 100% mortalities with the exception of immunised fish administered saline or left untreated. Repeat experiments gave comparable results. The results indicate that immunosuppression can be induced by administration of high levels of synthetic corticosteroid. The possibility of elevated levels of adrenocorticotrophic hormones occurring in fish populations subjected to stress could be significant in lowering resistance to disease even following programmes of vaccination.

INTRODUCTION

Ichthyophthiriasis or 'white spot' is a major disease of freshwater fish and occurs worldwide leading to great economic loss (Paperna, 1972; Hines and Spira, 1973; Nigrelli, Pokorny and Ruggieri, 1976). The causative agent, *Ichthyophthirius multifiliis* Fouquet, 1876 (Protozoa: Ciliophora) 'Ich',

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invades the epithelium of skin and gills, where after a period of growth, of a duration dependant upon temperature, it enters the aquatic environment undergoing a cycle of multiplication with the production of new infective stages, the theronts

That fish can acquire protective immunity to 'Ich' has been shown by Bauer (1953), Beckert and Allison (1964) and Hines and Spira (1974). Goven, Dawe and Gratzek (1980) have successfully protected channel catfish against the disease by immunisation with cilia isolated from Ichthyophthirius multifiliis and a related species Tetrahymena pyriformis. It is possible that other materials of parasitic origin in addition to cilia and including secretory products, may serve also as protective antigen.

The aim of the present study was to determine whether the protective immune response could be suppressed following administration of a corticosteroid. Such investigations of immunosuppression are considered highly pertinent to the development of effective programmes of vaccination in fish farms (documented in the discussion section). Selection of juvenile carp (Cyprinus carpio L.) 10-12 weeks of age for experiments was considered relevant as fish are more prone to damage by the disease when young and thus would gain maximum benefit from protection at this stage. Previous immunological studies have been chiefly confined to the response of adult fish (Beckert and Allison, 1964; Hines and Spira, 1974).

MATERIALS AND METHODS

Fish

Juvenile C. carpio were used in all experiments and for the routine maintenance of I. multifiliis. Those used for experiments were bred from stock at Plymouth Polytechnic or were obtained as fry from The Hampshire College of Agriculture, Sparsholt, Hants. Prior to experiments, fish from both sources were maintained free from exposure to I. multifiliis.

Fish were maintained in perspex aquaria of 12 litre capacity. Aquaria were aerated and kept at a temperature of 20 $\pm$ 2°C with pH range of 7.0 - 7.5. In addition, water quality was maintained by biological filtration using sub gravel filters; partial changes of water being undertaken at 2-3 week intervals. Stocking densities in experimental aquaria were established at 5-10 fish of 10-12 weeks of age (mean weight 650 mg). Fish were fed twice daily a mix of No. 2 trout pellets (Ewos Baker, Bathgate, West Lothian) and goldfish flake (King British, Bradford, West Yorkshire).

Parasite

I. multifiliis was established from 2 trophonts isolated from a single goldfish imported from Japan. This parasite strain was maintained for up to 9 months after which period there was a loss of viability although there was no change in infection procedures.

Experimental procedures

Experimental infection of fish was undertaken by transferring susceptible carp to small perspex tanks (capacity of 2 litres) and containing theronts. Theronts were used 5-10 hours following emergence from the reproductive cysts. For immunisation experiments, fish were exposed to theronts for 3 hours in the dark. Particular care was taken to ensure a pH of 7.0 - 7.2 as relatively small fluctuations from these values was known to effect the viability of theronts. Aeration was restricted to a period of 2 hours, starting 30 minutes after introduction of the fish to ensure non turbulent periods for the theronts. For quantitative studies, numbers of theronts were estimated cm^{-3} with the aid of a Sedgewick Rafter Counting Cell (Dickerson et al., 1981).

Immunisation

Fish were immunised by exposure to theronts at approximate doses of 80 cm^{-3}, 2,000 per fish on 3 separate occasions at 14 day intervals. From days 5-10 following each exposure, reinfection was prevented by transferring fish to clean aquaria at 10-14 hour intervals. By day 10 all trophozoites had completed their development and were removed from aquaria during fish transfer and before further infective stages were produced. Twenty eight days after the final antigen exposure fish were challenged with a potentially lethal dose of approximately 8,000 theronts per fish at a concentration of approximately 320 cm^{-3}. Following challenge, in contrast to immunisation procedures, fish were returned to holding tanks and any infection allowed to continue. Uninfected controls were used to confirm lethal dose on challege and to check viability of theronts during immunisation. In addition some were maintained free from 'Ich' until the termination of the experiment.

Immunosuppression

Immunosuppression of ichthyophthiriasis was investigated following stress induced by the synthetic corticosteroid, Triamcinolone acetonide (Sigma, U.K.). Fourteen days after challenge, immunised fish were administered approximately 200 $\mu g\ g^{-1}$ body weight of Triamcinolone acetonide suspension in 0.85% saline by intraperitoneal injection. Additional fish were injected with 0.85% saline or left untreated. Non-immunised control fish were also subjected to the same procedures. Six days after administration of the corticosteroid, half of the immunised fish and half of the non-immunised controls, with representatives from each treatment, were challenged using the procedure previously described.

RESULTS

Immunisation

The results (Table 1) show that after immunisation with controlled infections of 'Ich', all immunised fish survived on challenge. Although infections developed in 19% of the challenged, immunised fish, these were light, restricted to periphery of fins, and caused no deaths. Non-immunised controls subjected to challenge infections, suffered 100% mortalities, first deaths occurred after 5 days.

Immunosuppression

After injection of corticosteroid (Table 2), all immunised and non-immunised fish developed heavy infections of 'Ich' with 100% mortalities. Deaths also occurred in saline and untreated controls, but not in immunised fish injected with saline or left untreated.

TABLE 1

The effect of a potentially lethal challenge of *I. multifiliis* after immunisation with three serial infections of the parasite.
Data represent pooled results from two separate experiments.

Group and no. of fish	% Infected	Degree of Infection	% Mortality
immunised (120)	19	very light, periphery of fins	0
control (30)	100	very heavy, entire body	100

DISCUSSION

The results indicate first that juvenile carp less than 12 weeks of age can respond to antigenic stimulus from the parasite *I. multifiliis* by eliciting an immune response which protects against lethal challenge. Secondly, protection thus elicited can be suppressed when a high dose of a synthetic corticosteroid is administered.

Triamcinolone acetonide has previously been used to investigate the immune response of fish to other pathogens. Anderson *et al.* (1982) have found that it can affect the immune response in fish immunised to *Yersinia ruckeri*; this leads to a reduction in splenic antibody producing cells, circulating antibody titres, and numbers of splenic lymphocytes. Although the mechanisms

TABLE 2

The effect of the corticosteroid Triamcinolone acetonide on the immune response of 10-12 week old carp to I. multifiliis. Each group was comprised of 10 fish. Results of a repeat experiment are shown in parenthesis.

Group	Treatment	Re-challenged	% Infected	Degree of Infection dose and area	% Mortality *
Immunised	0	0	0 (0)		0 (0)
Immunised	Saline	0	0 (0)		0 (0)
Immunised	Corticosteroid	0	0 (0)	None	0 (0)
Control	0	0	0 (0)		0 (0)
Control	Saline	0	0 (0)		0 (0)
Control	Corticosteroid	0	0 (0)		0 (0)
Immunised	0	'Ich'	30 (0)	None or very light,	0 (0)
Immunised	Saline	'Ich'	30 (0)	periphery of fins	0 (0)
Immunised	Corticosteroid	'Ich'	100 (100)		100 (100)
Control	0	'Ich'	100 (100)	Very heavy,	100 (50)
Control	Saline	'Ich'	100 (100)	entire body	100 (50)
Control	Corticosteroid	'Ich'	100 (100)		100 (100)

* All mortalities occurred within 6 days of experiment 1 and 11 days in experiment 2 (results shown in parenthesis).

of suppression have not been examined here, the results do suggest that immune protection against I. multifiliis might not necessarily be maintained following administration of immunosuppressant drugs or exposure of the host to any other factors inducing physiological stress.

The effects of stress on fish defence mechanisms has been extensively reviewed by Ellis (1981). That stress can lower resistance of fish to disease has already been established (Wedemeyer and Wood, 1974; Pickering and Duston, 1983). Donaldson (1981) described how the physiological response to stress leads to increase in circulating corticosteroids. However, interpretation of the results in the present study must be viewed with some degree of caution in view of the use of the synthetic product triamcinolone acetonide and the high dose used. Nevertheless, it might be concluded that any factors in a fish farming situation leading to elevated corticosteroid levels could interfere with protective immunity to ichthyophthiriasis. Such factors include netting, transportation, water quality and crowding. Consideration of these should be taken into account in the development of vaccine programmes for fish.

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