1999

Ichthyophthirius multifiliis Fouquet: development and assessment of in vitro systems for long term maintenance

Hurley, Louise Margaret

http://hdl.handle.net/10026.1/2526

http://dx.doi.org/10.24382/4165
University of Plymouth

Ichthyophthirius multifiliis Fouquet: development and assessment of *in vitro* systems for long term maintenance

by

Louise Margaret Hurley, BSc.(Hons.), MSc

A thesis submitted to the University of Plymouth in partial fulfillment of the degree of

DOCTOR OF PHILOSOPHY

Fish Research Unit

Faculty of Science

January 1999

Louise Margaret Hurley,
***Ichthyophthirus multifiliis*: development and assessment of an *in vitro* culture system.**

ABSTRACT

Twelve isolates of *Ichthyophthirius multifiliis* were successfully established and maintained by serial passage through naïve carp, for a maximum of 39 laboratory cycles. The management system employed was such that large numbers of the parasite were available for all investigations.

The ability to induce exit of immature trophonts through media incubation was used to confirm events in the initial stages of host colonisation. The normal course of primary infection was also established providing useful criteria for assessing success of the *in vitro* systems tested. Survival of both theronts and tomonts within selected monophasic media was investigated. Theronts in Eagles Minimum Essential medium (EMEM), survived and were viable for 120 hours, 72 hours longer than water controls. No further development of the theronts was observed. Tomonts also demonstrated an increased survival time in comparison to the controls with tomites surviving within the cyst for 22 days within EMEM-S media diluted 50:50 with sterile distilled water. Division of tomonts was identified as being precystic, post divisional cystic or cystic, and the frequency of such divisions was dependent upon dilution of media. Sterile viable theronts were recovered at 168h from tomonts that had been incubated within EMEM diluted 30:70 with distilled water. Delayed encystment was achieved by incubation in concentrated media, theront production being delayed for 96h, 72h later than seen in the aquatic environment.

Cultured cell monolayers were used as associates within culture systems. Behaviour of theronts on introduction into the culture systems indicated recognition of the cultured tissue as potential host material, sustained contact of up to 120hours was observed between the introduced parasite and cells. However, no developmental markers were identified within the cultured parasite and no significant growth was achieved. Attempts to simulate the situation *in vivo* by use of multilayered systems and crude cell explants were also unsuccessful. Transmission electron microscopy of the parasite within a cell aggregate system was undertaken at daily intervals up to 120h providing evidence that the parasite was attempting to gain nutrients by phagocytosis. However, increased vacuolation of the parasite during the period of culture was clearly evident leading eventually to parasite death.

The significance of the results is discussed in relation to the normal course of infection and the future promise of a long term culture method for this important pathogen.

LIST OF CONTENTS

LIST OF CONTENTS

PAGE NUMBER

LIST OF CONTENTS

LIST OF TABLES	PAGE NUMBER

LIST OF TABLES	PAGE NUMBER

| | |

ACKNOWLEDGEMENTS

Grateful thanks to Tony Matthews my supervisor for his sustained enthusiasm and faith throughout the whole of this project, without Tony I could not have continued. For invaluable advice on parasitology, parasite staining and EM techniques I am exceedingly grateful to Ben Matthews whose enthusiasm for parasites and EM is rivaled only by Tony's. Thanks also to Pete Burgess for his advice on fish and parasite husbandry and all people in the laboratory who helped look after the fish and parasites during holidays, namely Mike Field, Richard Ticehurst and Ann Torr. For training and advice on fish cell culture I would like to thank Sara McMahon and my second supervisor Christine King. For help in electron microscopy, specifically the development of electron micrographs, I would like to thank Jane Green and Roy Moate. Special thanks must also go to my parents for all of their love and faith over the years, to the best of all friends Tracy and Gary for computing and scanning advice, (and all the ink and paper) and especially to my husband Peter for his unfailing love and support.

DECLARATION

This is to certify that the work submitted here was carried out by the candidate,

Candidate's Signature.....

Date....12-5-99....

Supervisor's Signature.....

Date.....

The study was supported with the aid of a studentship from the Science and Engineering Council.

All experimental work was carried out under Home Office License No. PIL: 30/02519 granted to the author.

CHAPTER 1

INTRODUCTION

Ichthyophthiriasis is a major disease of freshwater fish accounting for economic losses in both food and ornamental species (Bauer, 1973; Hines and Spira, 1973a; Goven *et al.*,1980). It is recorded in freshwater teleosts worldwide, in both tropical and temperate regions, extending north to sub arctic zones (Nigrelli, Pokorny and Ruggieri, 1976). The causative agent, the ciliate *Ichthyophthirius multifiliis* (Fouquet, 1876) appears to have little impact on wild fish populations although epizootics are occasionally reported (Allison and Kelly, 1963; Elser, 1955; Kozel, 1976; Wurtsbaugh and Tapia, 1988).

Until recently, methods for the control and treatment of this disease have placed reliance on the use of chemicals, notably malachite green. However, the effectiveness and safety of such treatments for food fish is questionable (Wahli, Meier and Pfister, 1986; Bernoth, 1991). Alternative approaches are likely to place more emphasis on the development of a vaccine.

Although the life cycle of *I. multifiliis* has been widely described in the past (MacLennan, 1935; Butcher, 1943; Hines and Spira, 1973a; Hoffman, 1978; Lom and Dykova, 1992; Nie-Dashu and Lie-Siang, 1960; Wagner, 1960) more recent work, (Matthews, Matthews and Ekless, 1996) suggests a more complicated series of events, including the possible involvement of a sexual phase within the fish host.

Despite the appearance of a simple direct lifecycle one of the major problems encountered with *I. multifiliis* is that of long term maintenance within laboratory systems. No laboratory has been able to maintain a single isolate population of the parasite for periods of longer than 12 months (Matthews, 1994). Attempts at *in vitro* culture and cryopreservation of the parasites have had little success (Hlond, 1966; Beeler, 1980). This is a major drawback for research on *I. multifiliis* and has denied the establishment of known strains and the production of parasite material for vaccine production. Investigations into molecular approaches for development of sub-unit vaccines against *I. multifiliis* based on surface antigens have as yet provided no protection against the disease (Dickerson, Clarke and Findly 1989; Lin and Dickerson, 1992).

The present study aimed to develop a method for the *in vitro* culture of *I. multifiliis* using monophasic media and cultured cell associates. This could lead to an alternative approach to vaccine development using whole organisms and provide a basis for more fundamental studies of the parasite including food uptake and metabolism.

CHAPTER 2

LITERATURE REVIEW

ICHTHYOPHTHIRIUS MULTIFILIIS

The first description of the causative agent of white spot disease was by Hilgendorf and Paulicki in 1869 (see Stiles, 1894). In 1876 Fouquet published a detailed description of the organism and its life cycle placing the organism in a new genus and proposing the name *Ichthyophthirius multifiliis*. *Ichthyophthirius multifiliis* is at present placed in the class Oligohymenophora, sub class Hymenostomata, order Hymenostomatida, suborder Ophryoglenina and the family Ichthyophthiridae.

LIFE CYCLE

Major events in the life cycle of I. multifiliis are well documented by MacLennan (1935, 1936,1937, 1942), Wagner (1960) and more recently by Ewing and Kocan, (1992) and Dickerson and Dawe (1995). The main features of the life cycle are shown in Figure 1. The terminology adopted is that of Roque, Puytorac and Lom (1967).

Theront.

The theront is the infective stage of the parasite and is fusiform to pyriform in shape with a tapered posterior end. It measures approximately 30 to 50μm in size, the actual size being dependent upon the initial size of the tomont prior to

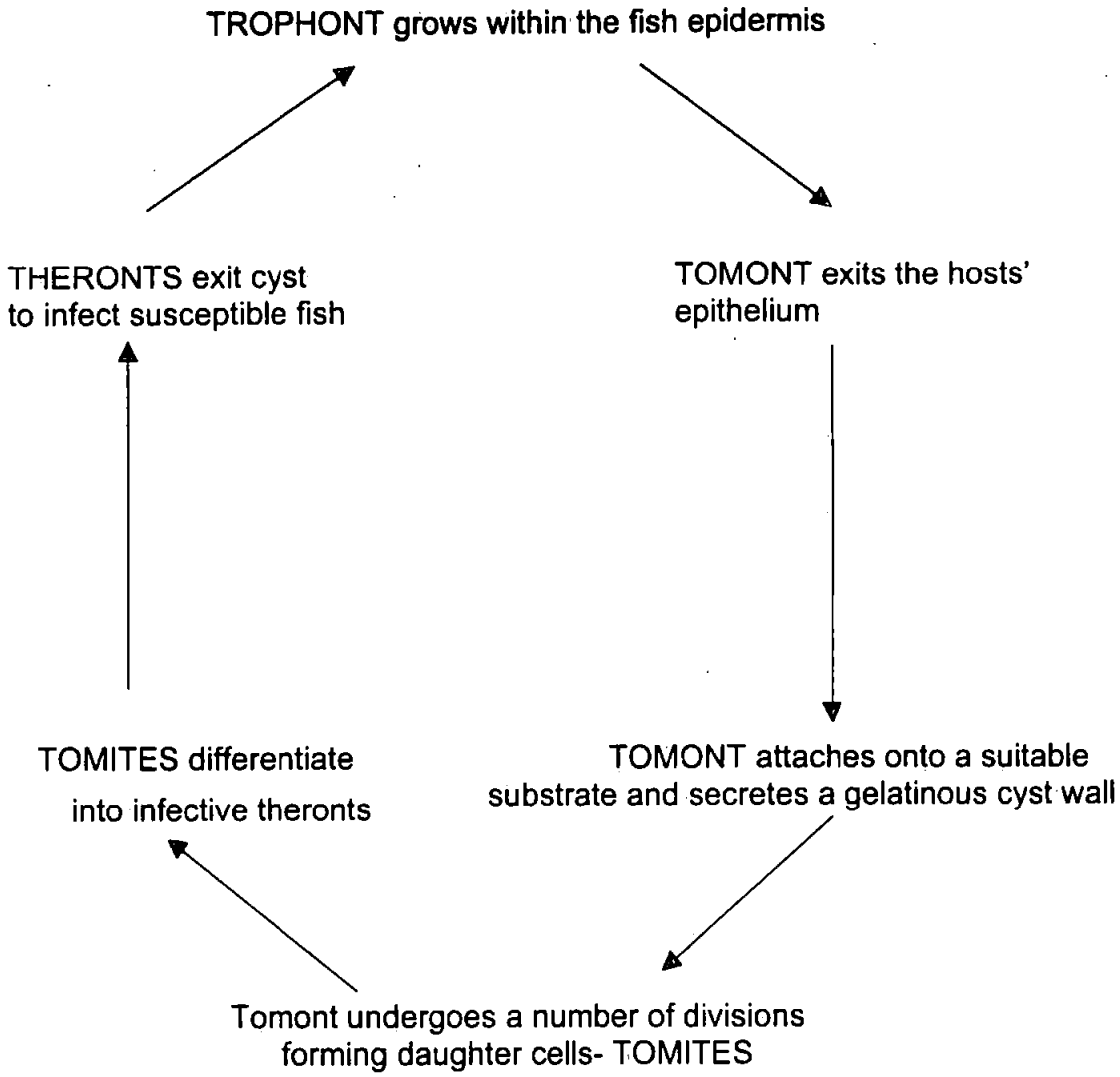


FIGURE 1: Life cycle of *I. multifiliis*

division and differentiation (MacLennan, 1942; Cannella and Rocchi-Canella, 1976; Kozel, 1986; Geisslinger 1987). The theront is completely covered with cilia possessing a longer cilium at the posterior end of the cell, which is termed the caudal cilium (Canella and Rocchi-Canella, 1976; Geisslinger, 1987). Following excystment the theront demonstrates a characteristic swimming pattern, slow cruising alternating with spontaneous bursts of speed during which the theront wildly gyrates and frequently changes course, host contact usually occurs during these fast bouts of swimming (Matthews 1994). Host location could be as a result of the reported ability of theronts to show positive chemotaxis (Lom and Cerkasova, 1960) and phototaxis (Wahli, Meier and Schmitt, 1991) when examined *in vitro.*

On contacting the host epithelium penetration is accomplished within 10 to 30 seconds (Matthews, 1994) and the parasite is located next to the basal lamina within 5 mins (Ewing, Kocan and Ewing, 1985). Initial attachment of the theront to the host could be aided by the discharge of secretory mucocysts which produce a mucoid envelope around the theront as it approaches the host (Ewing and Kocan, 1992). Geisslinger (1987), postulated that specialised cilia, thigmotactic cilia were responsible for initial host attachment and found no evidence at scanning electron microscope level for mucocyst involvement. Possibly the most important structure associated with penetration is the perforatorium located at the anterior end of the theront. This structure consists of a group of apical ectoplasmic ridges arising between ciliary rows that converge to form a 1.5 to 2μm protrusion (Canella and Rocchi- Canella, 1976). This perforatorium is thought to act as a pivot during the invasion process, the cilia providing the driving force for entry. Considerable disruption of the host epithelium occurs on penetration and may be due to

mechanical activity of the parasite; however, invasion may be aided by the release of histolytic enzymes (Uspenskaya, 1963; Kozel, 1980; Lobo da Cuhna and Azevedo, 1988a, 1990, 1993). The exact role of the organelle of Lieberkühn which is found only within the theront and for a matter of hours within the trophont following host colonisation is not clear (Ewing and Kocan, 1992; Matthews, 1994).

Trophont

The trophont is located within the host epidermis adjacent to the basal lamina (Ventura and Paperna, 1985) were it actively feeds upon host cells (Lobo da Cuhna, 1988a). All trophonts must attain a critical size and stage of differentiation prior to leaving the host epithelium if they are to successfully complete their life cycle (MacLennan, 1942; Ewing, Lynn and Ewing, 1986). Therefore, during the period of host residence a number of characteristic ultrastructural changes are observed, such as increase in numbers of lipid bodies, contractile vacuoles and secretory mucocysts, all of which play a crucial role in parasite survival and further development on leaving the fish host (Ewing and Kocan, 1986). The duration of the parasitic phase is temperature dependent (MacLennan, 1942; Noe and Dickerson, 1995).

Tomont

Having achieved the minimum size and level of development the trophont leaves the host epithelium (MacLennan, 1942; Ewing, Lynn and Ewing, 1986), possibly aided by the discharge of contractile vacuoles (Ewing and Kocan, 1987) and becomes a tomont. Once free of the host the tomont swims for a period of time until it locates a suitable surface, possibly under the influence of light intensity (Nickell and Ewing 1989). It then becomes attached by the secretion of a

gelatinous cyst which is formed by the discharge of secretory mucocysts. The cyst has been examined at light level by MacLennan (1937) and at electron microscope level by Ewing, Kocan and Ewing (1983). Both studies indicate that the cyst is made up of two layers the innermost layer bearing a resemblance to the content of mucocysts whilst the outermost layer is less dense in structure and commonly covered with bacteria and other debris. Shortly after encystment the tomont divides by binary fission, the number of divisions being dependent upon the initial size of the tomont prior to encystment and temperature (MacLennan, 1937; Ewing, Kocan and Ewing, 1986). These daughter cells or tomites differentiate into the infective theront and once development is complete the theronts actively leave the cyst. The whole process of division and differentiation usually takes 18-24h at 20°C.

PATHOLOGY

The parasite invades the epithelium over the whole body surface of the fish, affecting the gills, skin, eyes and fins impairing the respiratory, excretory and protective function of these organs. Pathogenesis of the disease has been well documented by Hines and Spira (1973a,b; 1974a,b,c) and Ventura and Paperna (1985) and recently reviewed by Dickerson and Dawe (1995). It will therefore not be reviewed here.

CONTROL

The wide environmental tolerances and low host specificity displayed *by I. multifiliis* mean that it poses considerable problems in fish culture systems worldwide (Nigrelli et al., 1976; Valtonen and Keranen, 1981). Control of such a prevalent disease is therefore of paramount importance and a number of methods are currently available.

Chemotherapy

Commonly used methods for the treatment and control *of I. multifiliis* infections make use of a number of chemicals to kill one or more of the lifecycle stages (Farley and Heckmann, 1980; Scmahl, Taraschewski and Melhorn, 1989a). Sodium chloride was one of the earliest chemicals to be used, Stiles (1894) reporting that saturated salt solutions killed theronts instantly. Methods for its application in both aquaria and ponds have since been devised (Kabata, 1985; Selosse and Rowland, 1990). Chemicals most commonly used today include formalin and malachite green applied to the water either individually or in combination (Brown and Gratzek, 1980). Other chemicals used include potassium permanganate (Brown and Gratzek, 1980), copper sulphate (Straus, 1993), and chloramine –T (Cross and Hursey, 1973). The chemicals listed above are usually active against the free-living stages of the lifecycle, predominantly the theront, the cyst presumably providing some degree of resistance to chemical activity. However, the immersion of infected fish in 10μg/ml of toltrazuril for 4hours killed trophonts whilst within the host epidermis but had no effect on theronts (Schmahl, Melhorn and Taraschewski, 1989b). The addition of malachite green to food pellets has also been effective in killing trophonts *in situ* following a 4 day course of treatment (Schmahl *et al.*, 1992). As mentioned previously in Chapter 1, the effectiveness and safety of such chemical treatments is questionable (Bernoth, 1991), and alternative methods can be employed such as the use of ultraviolet light (Gratzek *et al.*1983), electrotherapy (Farley and Heckmann, 1980) or the twice daily removal of fish into fresh aquaria (Houghton and Matthews, 1990). However, these methods are of use only within closed systems or when small numbers of fish are being handled. For the large-scale control of disease the development of a vaccine provides the most promising approach.

Vaccine development

Protection against ichthyophthiriasis by acquired immunity has been demonstrated in carp (Hines and Spira 1974c; Houghton and Matthews, 1986) and also in other fish species (Dickerson *et al.*, 1984; Wahli and Meier, 1985; Subasinghe and Sommerville,1986). This protection appears to be sustained for approximately 8 months under conditions of continual exposure, however, in the absence of exposure protection is lost within 3 months (Houghton, 1987). The immune response of fish to *I. multifiliis* has recently been reviewed in some depth and so will not be covered here (Matthews, 1994; Dickerson and Dawe, 1995).

Procedures for immunisation have been standardised (Dickerson *et al.,* 1981; Houghton and Matthews, 1990) by exposing the fish to known numbers of theronts or by intraperitoneal injection with whole parasites or cilia isolates. A variety of components of *I. multifiliis* have been used to experimentally induce immunity, however, because of the difficulty in maintaining parasite populations within the laboratory, large scale production of antigenic material for immunisation studies and vaccine development is difficult. *Tetrahymena pyriformis,* a related ciliate protozoan, is in contrast easily established and maintained within the laboratory and has been investigated for its ability to induce immunity to ichthyophthiriasis (Goven, Dawe and Gratzek, 1981; Wolf and Markiw, 1982; Dickerson *et al.*, 1984; Houghton, Healey and Matthews, 1992) although its effectiveness is questionable (Matthews, 1994; Dickerson and Dawe, 1995). The application of molecular biology by the cloning of potential immunogens and their expression in vectors appears to be the most promising way forward. However, at present no recombinant vaccine has yet been released for general use in either veterinary or

medical fields, areas that are usually considerably more advanced than those in aquaculture (Willetts and Cobon, 1993).

Genetic susceptibility

Research has shown that there is significant interspecific and intraspecific variation in susceptibility to infection *by I. multifiliis* amongst fish species (Clayton and Price 1988,1992,1994) with hybrid vigour or heterosis within fish being a significant factor contributing to resistance to infection. Selective breeding may therefore be an option for disease control.

IN VITRO CULTURE

In medical and veterinary fields *in vitro* culture of important protozoan parasites is more advanced than aquacultural culture methods (Taylor and Baker, 1987; Zaman, 1978). Culture systems used are classified in various ways: xenic cultures where the protozoan is grown in the presence of several unidentified associates usually bacteria; monoxenic where one known associate is present and axenic where no associate is present. Media used can be defined as diphasic where both liquid and solid phase are present or monophasic where one phase is involved.

One of the most widely cultured protozoan species is *Tetrahymena* a free living holotrichous ciliate that has been extensively cultured for many years within the laboratory. Kidder and Dewey (1951) were the first to develop a defined medium, which was later revised by among many Holz (1973) and Sripati (1987).

A number of flagellated fish parasites have been successfully cultured (Becker, 1977; Qadri, 1955; Putz, 1972). Uzmann and Haydak (1963), developed a method

for the *in vitro* culture of the flagellate protozoan *Hexamita salmonis* (Moore, 1923). Success was achieved using an axenic monophasic medium in a nitrogen atmosphere. Under these conditions parasite isolates were cultured for up to 98 days. Fish endoparasites such as trypanosomes have lent themselves more easily to the process of *in vitro* culture (Lom, 1979) methods initially using monophasic or diphasic media frequently containing blood, subcultures taking place at appropriate intervals (Lom and Suchanokva, 1974). Although *T. danilewskyi* can be propagated *in vitro* for long periods of time with little loss of infectivity (Smolikova *et al.*,1977). *Cryptobia salmositica* is attenuated by continuous culture within minimum essential medium. Following culture, on inoculation into a susceptible host only low levels of infection were achieved and no disease was caused. Challenging these fish with the virulent form of the parasite demonstrated that these previously naïve fish had gained immunity (Woo and Sen Li, 1990). The attenuation of parasitic Protozoa through *in vitro* culture could provide a valuable source of live vaccine material.

The dinoflagellate *Amyloodinium ocellatum* an ectoparasite of marine fish has successfully been cultured *in vitro* axenically initially by use of fish cell monolayers (Noga, 1987; Noga, 1989) and organ cultures (Noga and Bower, 1987) and more recently by use of cell aggregates (Oestmann and Lewis, 1996). Walking catfish gill cells (G1B) (Noga and Hartmann, 1981) and organ cultures produced from explants growing from killed infected gnotobiotic guppy fry, were successfully used to maintain the entire life cycle of the parasite *in vitro*. Using a medium made from an artificial sea water solution mixed in equal parts with Hanks balanced salt solution. Cell and organ cultures were seeded using the *A. ocellatum* equivalent to theronts, the dinospores, which attached to the cell layer within minutes, this was incubated until 90% of the trophonts had detached and encysted within the surrounding medium.

These were then harvested and the dinospores subsequently released were used to seed following cultures. This culture system provided sufficiently large numbers of parasite for immunisation studies, also allowing quantification of the effect of immune serum on the infectivity and growth of the parasite *in vitro* (Smith, Noga and Gerig, 1993).

More recently, however, it was discovered that the culture requirements of *Amyloodinium* differ depending on the isolate. All initial culture systems were tested using isolate DC-1 which was obtained from an aquarium reared clown fish (*Amphiprion ocellaris*). More recent isolates obtained from red drum (*Sciaenops ocellatus*) on the Texas gulf coast demonstrated poor levels of differentiation from dinospore to trophont and little growth when cultured within a G1B monolayer system or a red drum dorsal fin (RDFC) monolayer system (Oestmann and Lewis, 1996). The strain appeared to tolerate cell culture media or foetal calf serum. When placed within a culture system containing RDFC cell aggregates in a 10ppt saltwater medium the gulf coast strain thrived with no decrease in viability being observed.

FISH CELL CULTURE

The culture of fish cells provides an invaluable tool when investigating problems relating to fish health such as toxicology (Babich and Borenfreund, 1991), parasitology (Oestmann and Lewis, 1996), and virology (Kamei, Yoshimizu, Ezura and Kimura, 1989).

One of the first fish cell lines to be established originated from rainbow trout gonadal cells and was coded RTG-2 (Wolf and Quimby 1962). Since this time many more

fish cell lines have been established, and the first comprehensive list of poikilotherm cell lines was published by Wolf and Mann in 1980, listing 61 lines of fish cells representing 17 families and 36 donor species.

Techniques used for the development of fish cell lines have developed from early work by Fryer (1965) and Wolf and Quimby (1962,1969). Wolf and Quimby (1969) and Wolf (1973) provide clear information on the initiation and maintenance of fish cell lines within the laboratory. Methods used for initiating primary cell cultures make use of cold trypsinisation or direct planting of minced tissues; tissues used can be internal or external in origin. The first trypsinisation of fish tissues with the aim of starting a primary culture was by Grutzner (1958), this procedure was undertaken at 20°C. Following this a method of cold trypsinisation was developed (Wolf, Quimby, Pyle and Dexter, 1960), fish tissue being minced slightly then undergoing 24h of trypsinisation at 4°C. After this period of time the cell suspension is harvested by centrifugation at 200*g*, re-suspended in the appropriate growth medium and seeded into a culture flask. Trypsinisation is now the most commonly used method for the initiation of fish primary cell cultures (Fernandez, Yoshimuzu, Kimura and Ezra, 1993). The method of direct planting is not commonly used, monolayers obtained from this method taking considerably longer to achieve confluency when compared to trypsinised cultures. Primary cultures from direct planting do, however, retain more characteristics of the originator tissue during the initial stages of culture, which could have applications for the *in vitro* culture of fish parasites.

CHAPTER 3

IN VIVO MAINTENANCE AND COURSE OF INFECTION OF *ICHTHYOPHTHIRIUS MULTIFILIIS*

INTRODUCTION

Standard procedures are now available for the routine *in vivo* maintenance of *I. multilfiliis* under laboratory conditions (Houghton and Matthews, 1986; Cross and Matthews, 1992). These involve serial passage of the parasite through susceptible hosts, methods ranging from simple co-habitation of infected with non-infected fish (Pyle and Dawe, 1985) to the exposure of fish to known numbers of theronts under controlled environmental conditions (Dickerson *et al.*, 1981). Hines and Spira (1973a) established laboratory infections by the addition of known numbers of mature tomonts to aquaria, allowing the parasite to develop through general, subliminal generations within the fish host. However, the need to quantify exposure levels for immunological studies has lead to most current methods of continuing infection using theronts released following incubation of mature trophonts. In this way infection levels can be more closely gauged (Houghton and Matthews, 1990; Cross and Matthews, 1992).

In vivo maintenance under laboratory conditions has so far failed to provide an approach to the long-term maintenance of *I. multifiliis*. The eventual loss of isolates being attributed to a wide range of factors, including water quality (Wagner, 1960; Dickerson *et al.* 1985; Subasinghe and Sommerville, 1989; Ewing,*et al.*,1991), the

development of immunity (Hines and Spira, 1974c; McCallum, 1986; Houghton and Matthews,1990) and problems ascribed to parasite senescence (Houghton and Matthews, 1986; Burkart *et al.*, 1990; Matthews, 1994).

The aims of this study were to provide a laboratory source of the parasite for *in vitro* studies and also to confirm events in the life cycle, to establish specific stages in development with temperature and time which could be used as criteria for assessing the success of *in vitro* culture.

FISH

Source.

Carp *Cyprinus carpio* (L.) 5-10cm in length and tilapia *Oreochromis mossambicus* (Peters) approximately 7cm in length were used for the routine transmission of *Ichthyophthirius multifiliis* within the laboratory. Carp were obtained from Munton and Fisons plc where they had been reared in conditions free from exposure to the parasite. On arrival in the laboratory, 2 or 3 from each batch were tested for susceptibility to infection before being incorporated within the laboratory maintenance system. Tilapia were used only occasionally for *in vitro* maintenance and were obtained from the university aquarium as required.

In addition, however, tilapia of approximately 2-3cm in length were also used for theront viability tests, the small size allowing ease of handling and clear visibility of sometimes low level infections.

All infected fish collected as potential initiators of infection were held in isolation in 12*l* glass aquaria prior to passage.

Maintenance.

Stock carp prior to infection were held in 30-40l circular tanks within the laboratory. Fish on infection were held in glass tanks of 12l volume and transferred to 2l perspex tanks at the end of infection facilitating the collection of emerging tomonts.

Tap water was used in all aquaria following standing over shell gravel, with aeration, for a minimum period of 72 h, in order to bring the pH to between 6.8 and 7.5, whilst also removing any chlorine present. Water quality was maintained within the larger aquaria by recirculation through an Eheim biological filtration unit and in the smaller aquaria of volumes 20l or below by air powered sponge filters. Stock fish were held at ambient temperatures of 16-24°C. Experimental fish infected with *I. multifiliis* were held at 20°C, temperature was maintained within these aquaria by means of thermostatically controlled 100W submersible heaters. In order to arrest or slow the development of the parasite, aquarium temperatures could be reduced to 10°C by the use of a dip cooler.

Fish were handled using soft nylon mesh nets that were kept in a solution of biocide and rinsed thoroughly before use. The rule of separate nets for each aquaria and each isolate of the parasite was strictly adhered to in order to prevent premature infection of stock fish and cross contamination of isolates. All aquaria were checked daily and the pH and temperature monitored. Particulate matter and dead fish were removed and partial water changes were undertaken when necessary.

Large carp were fed once daily with TetraPond Floating Koi sticks (Tetra, Germany), smaller carp being fed with Promin (Winchester, UK) medium grain fish food and tilapia once daily with Flaked Goldfish Food, (Aquarian).

Anaesthetisation.

Benzocaine (Ethyl-*p*- amino-benzoate, SIGMA) rather than MS222 was selected as the anaesthetic of choice within this study in view of its reported less harmful effects

on the fish (Ross and Geddes, 1979). A stock solution of 20mg of benzocaine/ml in 90% ethanol was kept within the fridge and immediately prior to use was diluted 1:100 with aquarium water to provide the working concentration. Fish once placed in the anaesthetic were closely observed and following cessation of swimming and slowing of opercular movement were removed. Following anaesthetisation if fish were slow to recover, aid was given by gently passing water over the gills by means of a pasteur pipette until self sustaining opercular movement returned.

Control of secondary infection in aquaria.

Health status of fish was monitored each day and the following measures taken to control bacterial and fungal infections. Either two 24h immersions in a 1% NaCl solution or by addition of Aquarian Fungus remedy (AQUARIAN,® UK). Bacterial infections were treated with Pond Doctor (Technical Aquatic Products, England) as recommended by the manufacturers. Systemic or chronic bacterial infections were treated by chemotherapeutic baths combined with feeding with oxolinic coated pellets. Use of chemotherapeutics was restricted to non-experimental tanks in order to prevent any possible deleterious effects to the parasite.

ICHTHYOPHTHIRIUS MULTIFILIIS

Isolation

A total of 12 isolates were established throughout the 3 year period of this study from 1990 to 1993, 3 were from local pet shops, 7 were obtained from tributaries of the river Erme downstream from Ludbrooke trout farm and 2 were from outbreaks in the University nutrition aquarium. Each isolate was attributed a code according to the following format: the initials of the genus and species of the donor fish / year of

isolation/ isolate number. All isolates were initially established as primary infections using stock carp following cohabitation and the first generation of tomonts collected for further transmission within the laboratory as follows.

Collection of tomonts

Infected fish were held at 20°C until white spots were clearly visible indicative of imminent tomont emergence. At this stage fish were removed to 2l aquaria and free swimming tomonts harvested by means of a pasteur pipette; viewing the aquaria against a black background greatly enhanced the location of the parasites. Emergence was stimulated by placing the infected fish in a small volume of water for a short period of time, the aggravated movements of the fish under such conditions aiding tomont emergence and following removal of the fish, actively swimming parasites were easily harvested. After collection tomonts were pipetted through 2-3 water washes, and allowed to encyst in a watch glass within a humidity chamber at 20°C. Theronts were produced between 18-24h at this temperature. To prevent reinfection fish were removed to fresh aquaria twice daily.

Enumeration of theronts

A suspension of theronts was gently agitated to ensure uniform distribution and 100µl sample immediately withdrawn and transferred to a Sedgewick-Rafter counting chamber. Theronts were inactivated by addition of an equal volume of formalin. After a 2 minute time interval which allowed dead theronts to settle, the chamber was examined under x10 objective and the number of theronts recorded by means of a tally counter. The number within the total sample was then estimated.

Experimental infection of fish

Primary infections in fish were established following exposures to known numbers of theronts (Fig 2). Each fish was exposed to 2000 theronts and maintained under conditions of darkness and gentle aeration for 2h. The total number of fish infected at any one time varied depending upon the numbers of theronts available. However, during periods of optimum parasite infection approximately 20 fish were infected per week at staggered intervals. Care was taken in all cases to ensure the establishment of sub lethal infections in which the disruption of the epithelium was minimised, reducing opportunities for secondary infections to occur. As a consequence of this, tomonts on emergence were less likely to be contaminated with cellular debris, assisting with the axenic isolation of the parasite.

In addition to controlled infections, the parasite was also maintained by cohabitation and low temperature incubation. Transmission by cohabitation was undertaken during periods of low parasite numbers. Allowing infections to proceed within the aquaria with new hosts being added as required, minimised handling of the parasite and enabled high infection levels to be achieved. The duration of the parasitic phase could be extended from 5 days to 3 weeks, by reducing the temperature of the aquaria to 10°C. Under these conditions controlled infections or the continuation of infection by the addition of new hosts could be undertaken less frequently. When required the temperature could be increased to 20°C and the infection proceed as normal.

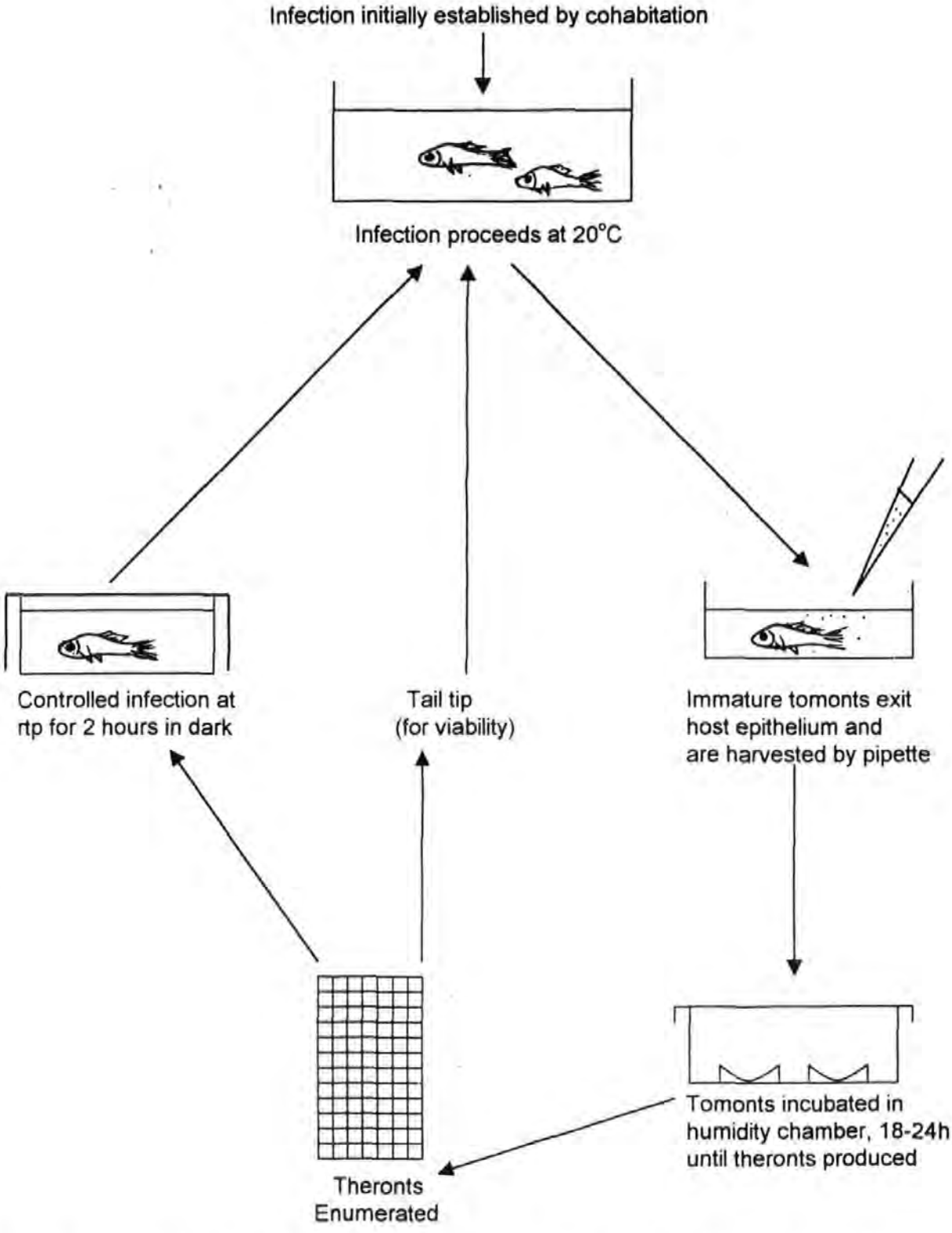


FIGURE 2. Procedures for the standard laboratory maintenance of *I. multifiliis* Infection at 20°C

Examination of trophont development

Studies of parasite growth and development were undertaken on parasite isolate GA/92/12 between laboratory cycles 26-35. Twenty carp naive to *I. multifiliis* were infected by exposing the tail to a suspension of theronts for 5 minutes. Following infection the fish were held at 20°C within a 12l aquarium. Half of the infected fish were used to study the parasite from 0-9 h post infection (PI) and the other half were used to evaluate trophont growth rate *in situ* between 24-96h.

For the later stages of development 24-96h parasites were examined *in situ* within the caudal fin of the anaesthetised live fish. Forty parasites were measured at each time interval. Theronts were measured following formalin fixation (TABLE 2).

For the study of early development one fish was removed at hourly intervals PI, anaesthetised and a portion of the caudal fin containing the parasite removed and incubated within Eagles Minimum Essential medium (see Chapter 3). Parasites emerged within minutes under these conditions and were harvested by pipette with the aid of a binocular microscope.

Staining methods for trophonts

In order that the characteristic features of early development of the parasite were more easily observed simple staining techniques were applied. Therefore, immediately following collection the parasites were stained by either the application of an equal volume of methyl green acetic which stains the nuclei bright green, or parasite suspensions were added to slides which had been precoated by an alcohol suspension of the *intra vitam* stain brilliant cresyl blue that had been allowed to dry;

this clearly stained food vacuoles and the organelle of Lieberkühn (Pantin, 1962). A minimum of 10 parasites were examined at each time interval.

Photomicroscopy

Photomicrographs were taken with a Zeiss MC63 automatic camera fitted to a Zeiss 16 standard microscope. Films used were Fujichrome 100ASA colour and TMAX 100ASA black and white (KODAK).

RESULTS

ISOLATE SURVIVAL

Of 12 isolates of *I. multifiliis* obtained in the present study (Table 1) those from the stickleback, collected from the River Erme survived longest under *in vivo* conditions within the laboratory. Isolate GA/92/12 was the most successful, being maintained for 10 months and undergoing 39 successive cycles of infection. Isolate GA/91/5, the second best completed only 16 cycles. Those obtained from ornamental species of fish bought through retail outlets were generally short lived, RB/91/4 failing to produce viable theronts after a single cycle within a donor fish. The successful establishment of an isolate did not appear to be influenced by season. Loss of viability of the isolate within the laboratory was usually heralded by low numbers of theronts being produced per cyst, low infection levels being achieved from controlled infections and following incubation of cysts many round tomites being visible within the watch glass. The appearance of isolated accumulations of black pigment within emerging tomonts provided a good indication of imminent loss of viability within an isolate.

COURSE OF 1° INFECTION OF GA/92/12

Growth and development

During the first 48h of host residence significant changes in the shape of the trophont was observed. At 24h the trophont was shorter in length (45µm) than the theront (54µm) but measured twice the width (Table 2). The trophont appeared to round up within 24h of entering the epithelium. After 48h length and width appears to increase in a linear fashion the increasing variation in size.

TABLE 1: *Icthyophthirius multifiliis*: source of isolates and duration of maintenance

DATE OF ISOLATION		DONOR FISH	CODE	NO. OF CYCLES
MONTH	YEAR			
01/10	90	*Cyprinus carpio* (L.)[a]	CC/90/1	8
26/10		*Carassius auratus*(L.)[a]	CA/90/2	3
12/02	91	*Oncorhynchus mykiss*[b] (Walbaum)	OM/91/3	11
05/06		*Botia modesta*[a]	BM/91/4	1
10/07		*Gasterosteus aculateus* (L)[c]	GA/91/5	16
16/07		*Onchorynchus mykiss*[b] (Walbaum)	OM/91/6	15
18/08		*Gasterosteus aculateus* (L)[c]	GA/91/7	3
02/12			GA/91/8	2
13/01	92		GA/92/9	10
22/04			GA/92/10	8
03/07			GA/92/11	3
21/09			GA/92/12	39

Source of parasite

a: Donor fish obtained from local retail outlet

b: Donor fish obtained from the University of Plymouth aquarium

c: Donor fish caught in tributaries of the R. Erme situated downstream of Ludbrook trout farm

TABLE 2: Measurements of *I. multifiliis* trophonts taken *in situ* within the caudal fin of carp. (n=40).

PARASITE AGE (Hours)		LENGTH (μm)		WIDTH (μm)	
		x	σ^{n-1}	x	σ^{n-1}
THERONT		54.166	6.8	22.08	3 .5
TROPHONT	24	45.64	7.3	45.64	7.3
	48	88	16.45	70	17.72
	72	238.9	41.85	176.95	53.35
	96	464.5	90	333.9	69.3

TABLE 3: Comparison of average trophont diameters in μm recorded by different authors at 24h intervals.

PARASITE AGE (hours)	Ekless, GA/92/12 1993 x	Ewing & Kocan, 1986 x	Hines & Spira 1973(a) x	Houghton, 1987 x
0	22.08	-	-	31.8
24	45.64	48	66.8	46.5
48	70	78	84.0	69.7
73	176.5	102	186.7	106.5
96	333.9	175	317.9	234.9

corresponding to the staggered emergence of the tomonts from the epithelium, such that harvesting of the parasite takes place over a 2-3 day period. At 20°C the course of primary infection in carp was completed within 5 days, the duration of encystment, division and excystment was 18-24h. On comparing the widths measured within this study with the diameter of trophonts recorded by other workers, (Table 3) the measurements are comparable for 48h (Ewing and Kocan, 1986; Hines and Spira 1973; Houghton, 1987). After this the growth rate of GA/92/12 appears to accelerate, diameter increasing by up to 100µm during the following 24h period as compared to a 34-36µm increase recorded by other workers.

The presence of the organelle of Lieberkühn was detected in 100% of sampled trophonts for up to 2h-post infection (PI) and can be seen in Fig. 3. However, at 3h PI only 50% of trophonts examined possessed the organelle (Table 4) and at 4h PI no organelle was observed in any of the parasites although a doubling in the number of micronuclei occurred at this time. At 4h PI 5/15 (33%) of trophonts possessed 2 micronuclei (Fig. 4). At 7h PI 2 and 4 micronuclei were observed within 2 trophonts and at 8h PI one individual was observed to have 7 clearly stained micronuclei. On one occasion trophonts containing 8 micronuclei with an apparently disintegrating macronucleus were observed.

At all examined time intervals macronuclear shape was recorded (Table 4). There appeared to be no synchronised development of the characteristic macronucleus of the trophont. Both oval and curved macronuclei were observed in all early life cycle stages post infection.

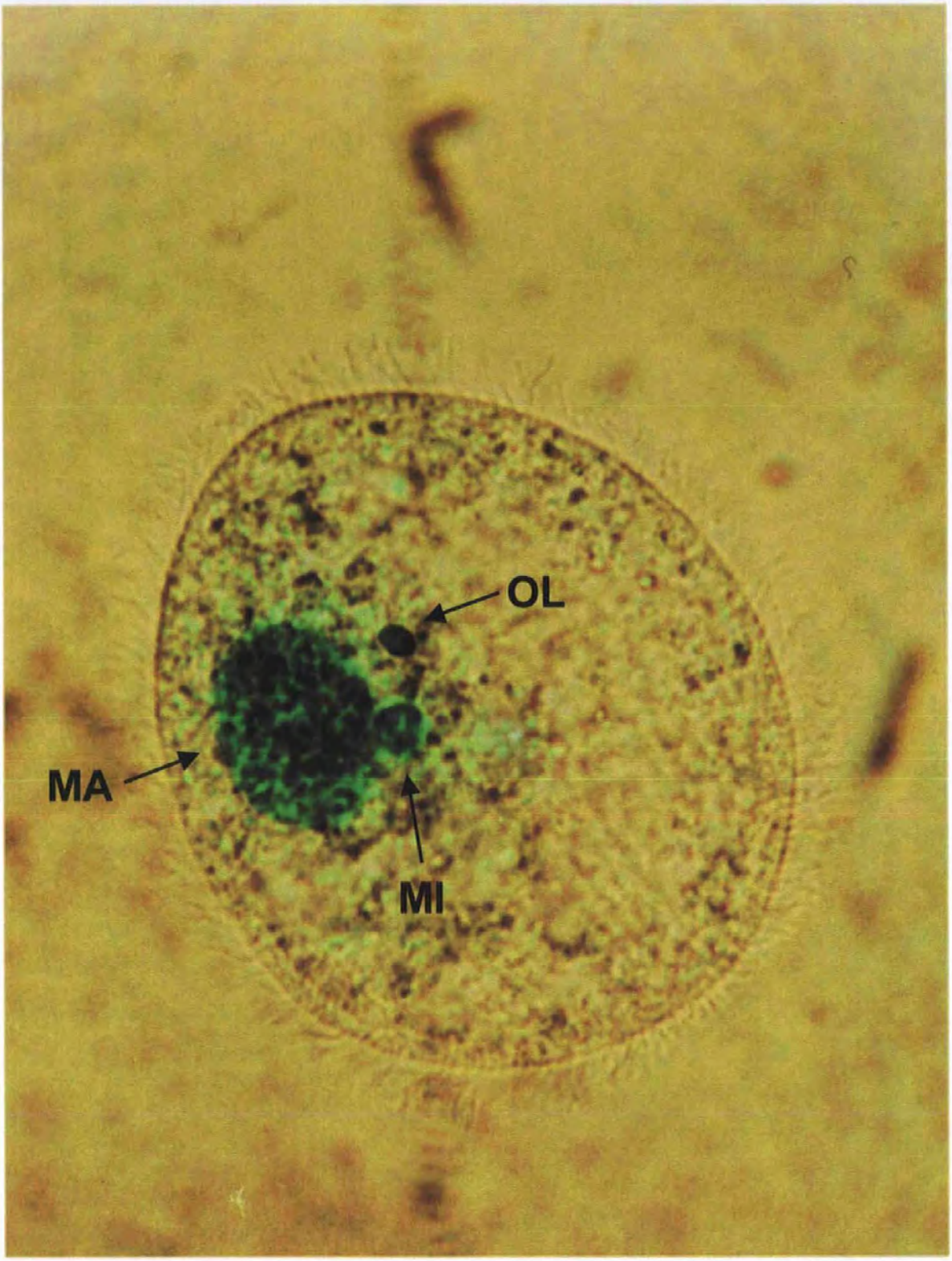


FIGURE 3: Trophont 2h PI, macronucleus (MA), micronucleus (MI) and organelle of Lieberkuhn (OL) are indicated.

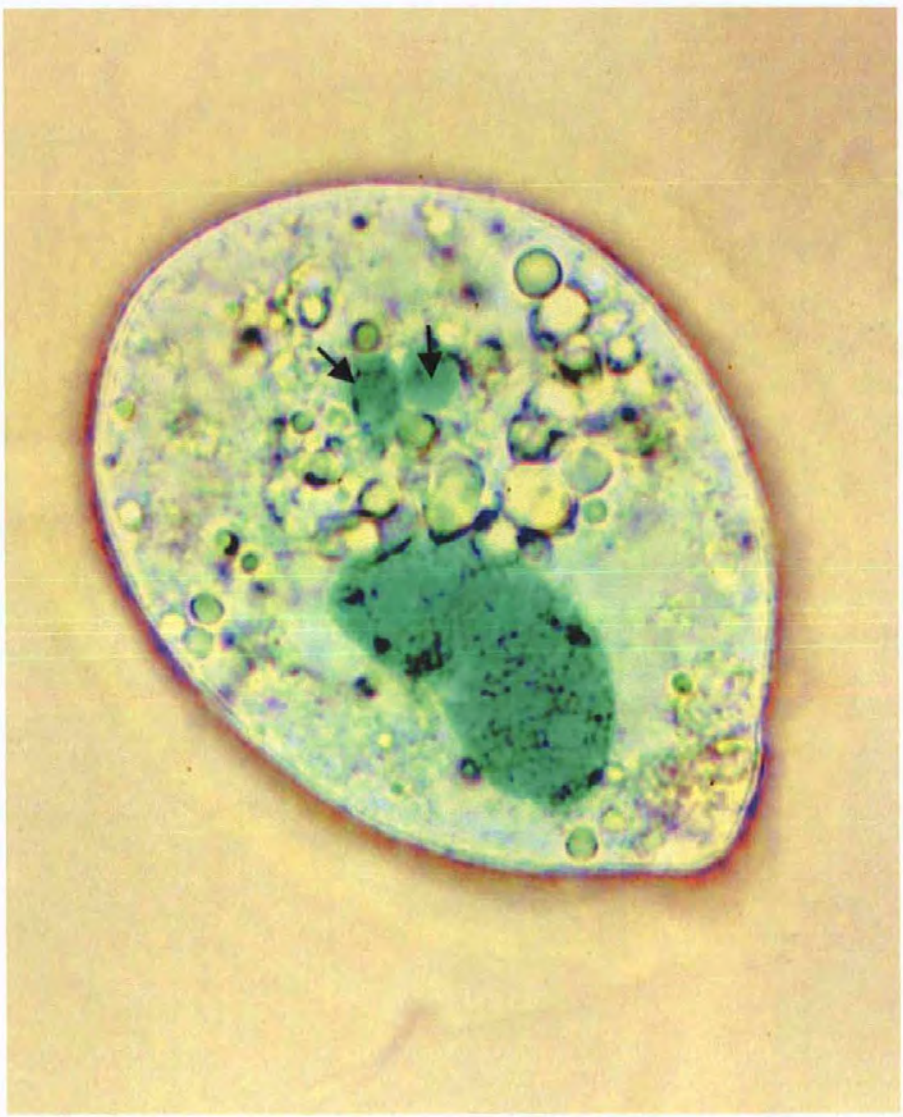


FIGURE 4: *I. multifiliis* trophont 4h PI. Two micronuclei are clearly visible. Curvature of the macronucleus is also evident

TABLE 4: Characteristic features of trophont development from 0- 8 hours of primary infection in carp.

HOURS PI	NO. EXAMINED	% TROPHONTS WITH ORGANELLE OF LIEBERKÜHN	SHAPE OF MACRONUCLEUS	
				
0	15	100	15	0
1	8	100	6	2
2	10	100	6	4
3	8	50	4	4
4	15	7	10	5
5	14	0	8	6
6	5	0	4	1
7	18	0	11	7
8	7	0	4	3

DISCUSSION

All isolates used here were obtained from cold water sources, the most successful of which were established from sticklebacks caught locally from the river Erme. On all occasions infections were established within days following the co-habitation of sticklebacks with carp. Previous authors have successfully utilised wild stock populations for the purpose of initiating laboratory infections of *I. multifiliis* (Kozel and Whittaker, 1982). Wagner (1960) also made use of sticklebacks as a source of parasite, in one catch from the Schonerlinde ponds Germany 60% of sticklebacks caught were recorded as being infected by *I. multifiliis*.. That *I. multifiliis* has been isolated from the stickleback population of the River Erme over several years suggests a stable relationship between fish and host, with low level infections being present at all sampling times providing an excellent and exploitable reservoir of parasite possibly from a single gene pool.

Once established within the laboratory survival of each isolate was variable, the longest (GA/92/12) being approximately 10 months and the shortest 7 days (BM/91/4), the duration of the primary infection. BM/91/4 was obtained from a retail outlet and as such the immediate history of the fish as regards the use of chemotherapeutics or chemoprophylactics was unknown and application of such treatments could possibly be the cause of transmission failure. It could also be considered that use of chemotherapeutics at a fish farm situated upstream from where the sticklebacks were caught could be a factor involved in the failure of three isolates GA/91/7, GA/91/8 and GA/92/11 obtained from this source in the autumn of

'91 and the summer of '92. All three of these failed to complete more than 3 cycles of infection within the laboratory.

To date no-one has reported the ability to maintain a single isolate of the parasite under laboratory conditions for more than 2 years, although some workers have maintained mixed populations of the parasite within natural ponds for up to 4 years (Hines and Spira, 1973a) The failure of sustained *I. multifiliis* transmission observed within this study and others is suggestive of parasite senescence. Burgess (1992) noted a similar loss of infectivity over time of *Cryptocaryon irritans* a ciliate parasite of marine fish with a similar life cycle to *I. multifiliis*. That sexual reproduction by either conjugation or autogamy has not been observed within the laboratory for either *Cryptocaryon* or *Ichthyophthirius* may be responsible for parasite loss under such conditions. Ageing within populations of ciliates in the absence of genetic exchange is well recognised (Nanney, 1980; Lynn and Corliss, 1992). A possible lack of complementary mating types within the isolate could account for the eventual parasite loss, a mixing of mating types having been observed to initiate conjugation for other holotrichous ciliates such as *Tetrahymena* and *Balantidium coli* (Svensson, 1955; Miyake, 1981) as well as other ciliate species (Miyake, 1981). However, even with occurrence of sexual reproduction a loss in the ability to produce viable progeny can be observed, and could be due to mutational overload or the accumulation of toxins Nanney (1974). It could therefore be considered that senescence is necessary as a method for disposing of individuals that become hazardous to the species through the accumulation of deleterious genetic mutations.

Of particular interest within this study was the increase in micronuclei numbers of trophonts during the first 8h of host colonisation. However, it should be noted that nuclear material from ingested host tissue held within food vacuoles, can hamper the accurate enumeration of micronuclei after 7.5h PI (Matthews, Matthews and Ekless, 1996). Ingested host tissue could therefore account for the increase of micronuclei numbers recorded within this study at 8h PI. However, the increase in nuclear material recorded here between 0-8h PI is suggestive of a sexual process within the life cycle, possibly being indicative of nuclear changes occurring prior to autogamy or to the behaviour of exconjugants. That a form of sexual reproduction occurs within the epithelium of the host is feasible considering that opportunities for sexual reproduction by conjugation would necessitate close proximity of individuals, these conditions being found within the epithelium where parasites are observed to gather in "corridors" within the host epithelium (Ewing, *et al.*, 1988). One advantage of intra epithelial reproduction would be the occurrence of close contact between daughters from different cysts, thereby encouraging an increase in nuclear variation. However, Matthews, Matthews and Ekless (1996) recorded that 32% of theronts emerging from cysts had 2-4 micronuclei per cells and Matthews (1994) previously recorded the presence of 4 micronuclei within the infective theront. This could indicate that autogamy or conjugation occurs prior to host colonisation, possibly occurring within the cyst where again close contact between individuals is assured.

The presence of the organelle of Lieberkühn within the established trophont for up to 5h PI observed within this study contrasts with observations of Ewing and Kocan (1992), where the organelle was reported to disappear within minutes of host

colonisation and was therefore implicated in the invasion process. That the organelle remains for a period of time PI indicates that its role could be one of establishment of the parasite, a period which is considered to be critical in the parasite's life cycle, over 50% of parasite mortalities occurring during this process (Ewing, Lynn and Ewing, 1986). The function of the organelle could be in aiding the initial uptake, digestion and assimilation of fish cells thereby replenishing the reserves used up during host invasion. Uptake of cells damaged by colonisation is known to take place during the first 40 minutes of colonisation (Ewing, Kocan and Ewing, 1985). However, that the disappearance of the organelle appears to be followed by increasing micronuclei numbers could implicate involvement of the organelle in nuclear reorganisation. However, Matthews (pers. comm.) on application of Feulgen-silver stain at EM level found no indication of nuclear material within the organelle.

The longest surviving laboratory isolate GA/92/12 was observed to achieve maturity and leave the fish host within a 5 day period at 20°C, 2 days shorter than the cycle described by Suzuki (1935). That such rapid growth was recorded within this laboratory for a single isolate indicates that differences in growth rate could occur, either due to physiological differences (Nigrelli *et al.*, 1976) or possibly to the cell status of the parasite life (Maupas, 1889).

Results here show that by lowering the water temperature in which infected fish were maintained extended the duration of the life cycle. This is as would be expected in view of the reported ability of the fish to overwinter on the fish host (Bauer *et al.*, 1973). Nevertheless this provides a convenient approach to

managing the *in vivo* maintenance of *I. multifiliis* providing some flexibility in parasite management. Other laboratories have subsequently successfully maintained I. multifiliis infections at lower temperatures. Noe and Dickerson (1995) maintained a clone of *I. multifiliis* for over a year at 9°C. The parasite remained on the fish host for an average of 20.4 days greatly reducing the number of passages and the number of fish required during the year, providing a more flexible method of managing laboratory infections.

CHAPTER 4

AXENIC ISOLATION AND MAINTENANCE OF LIFE CYCLE STAGES IN MONOPHASIC CULTURE

INTRODUCTION

There has previously been a number of attempts to culture *I . multifiliis in vitro* (Hlond 1966; Beckert, 1975; Kozel, 1979) all meeting with limited success. All of these early attempts stated that the presence of bacterial contamination introduced to the culture system along with the parasite was a major restriction in the successful development of an *in vitro* culture system. Both Beckert (1975) and Kozel (1979) stated that bacterial contamination of the culture media was the most significant cause of parasite death and Noga and Bower (1987) noted that bacteria markedly reduced the survival and subsequent development of *Amyloodinium ocellatum* in culture.

Several methods have been used through the years for the removal of bacteria from ciliates prior *to in vitro* culture, namely: washing individual cells in successive dishes of sterile water, Parpart (1928); migration of motile ciliates through sterile media (Glaser and Coria, 1930; Hetherington, 1934; van Wagtendonk and Soldo, 1970); chemical treatment of cysts (Luck and Sheets, 1931), the addition of antibiotics to culture media (Seaman, 1947) or a combination of the above (Lyman Allen and Nerard, 1978). More recently Protozoa have been axenically prepared

by using density gradient centrifugation in Percoll® in the presence of penicillin and streptomycin (Oestmann and Lewis, 1996). The aim of this study was to develop a method to remove bacterial contamination from the collected parasite stages and axenically maintain the parasite within a variety of monophasic media.

All parasite procedures, including axenic isolation and maintenance in selected media were undertaken at 20°C. Standard aseptic technique was used to maintain parasite sterility throughout the investigations, handling and media changes being undertaken within a positive pressure laminar flow hood.

AXENIC ISOLATION OF *I. MULTIFILIIS*

Tomonts collected immediately on exit from the fish host were rapidly transferred by pipette through 3 washes in sterile distilled water to remove any associated host debris and reduce any bacterial flora present by dilution. The procedures that followed differed depending upon which stage of the parasite was required and are illustrated in Figure 5.

Tomonts

Active tomonts prepared as above were subjected to 3 further washes each of 5 minutes duration in $10cm^3$ of the appropriate culture medium containing 1-% penicillin and streptomycin (FLOW) at a working concentration of 100IU/ml and 100µg/ml.

Theronts

Tomonts removed from the host and prepared as described earlier were transferred into a 96 well plate, one per well, and were incubated for 5 hours. Parasites which had encysted after this period and were showing signs of division were surface sterilised by repeated washing using a 1% solution of penicillin:streptomycin in

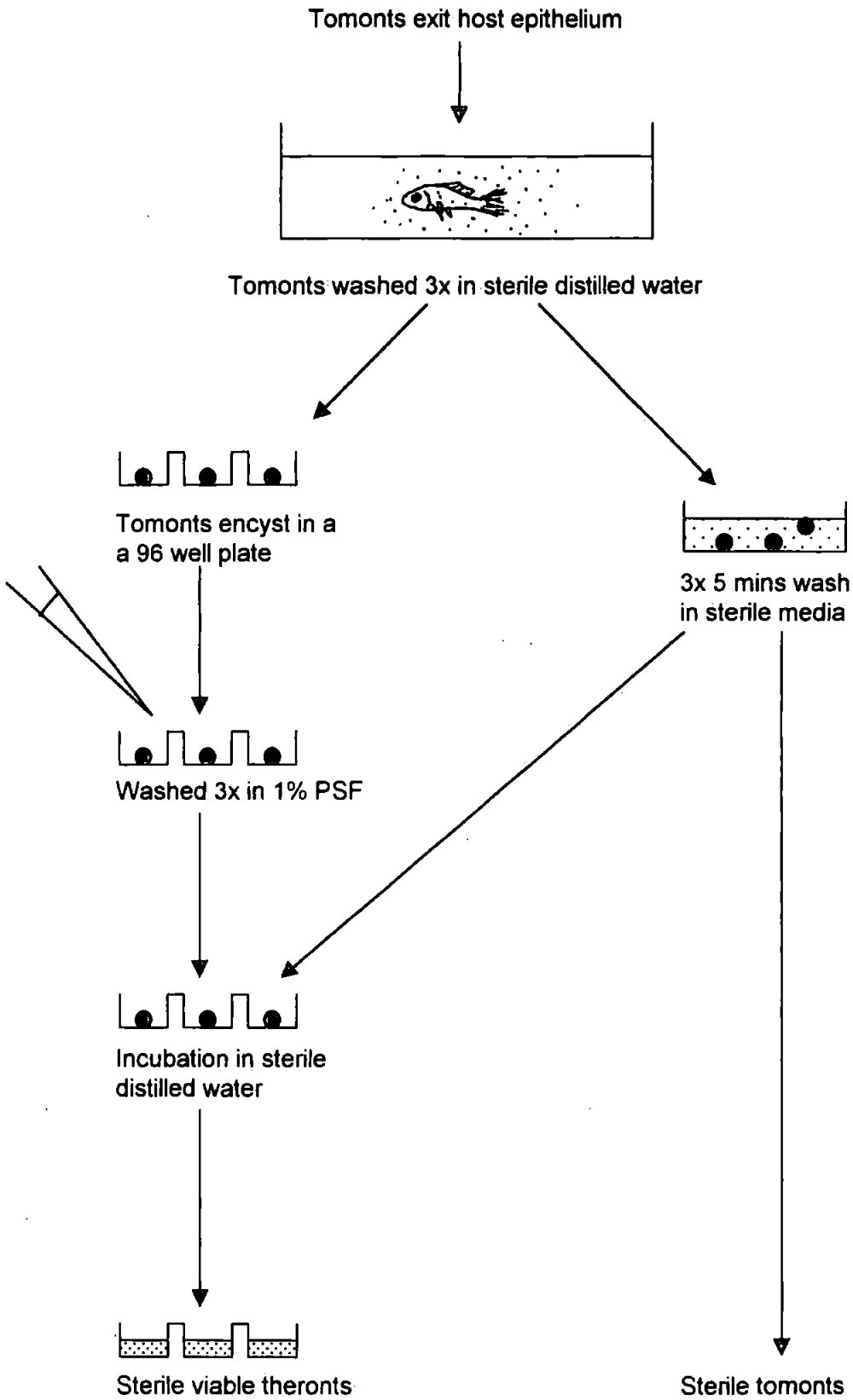


FIGURE 5. Axenic isolation of viable theronts and trophonts for *in vitro* culture

distilled water. The tomont was then allowed to complete its division within a fresh solution of distilled water without antibiotics. Alternatively tomonts following 3 sterile DW washes were transferred aseptically through 3 EMEM washes of 5 minute duration and afterwards allowed to encyst and divide within a fresh solution of sterile DW. Theronts following the treatments listed above were tested for viability and sterility as described below.

Viability of theronts

Oreochromis mossambicus (Peters) naive to *I. multifiliis* were anaesthetised and exposed to a suspension of theronts by application to the caudal fin. After 3 minutes the fish and parasite suspension were placed in a small beaker containing 200ml of previously aerated standing water for a further 3h before transfer to a 2l tank. Fish were held in isolation at room temperature and monitored over a period of 8 days, the presence of white spots after 5 days indicating active infection and tomont exit at 7 to 8 days confirming parasite viability.

Sterility of theronts

Microbial contamination was detected by pipetting 100µl of theront suspension onto a nutrient agar plate and incubating for 7 days at 20°C, after which time the plate was examined for the presence of colonies.

CULTURE MEDIA

A total of 16 media were used within this study. (Table 5)

Commercial preparations, obtained already sterile were as follows.

EMEM, Eagles Minimum Essential Medium (Modified) (FLOW) with Earles salts and

2% bicarbonate, and added immediately prior to use 1% penicillin:streptomycin (100IU/ml:100µg/ml), 1% L-glutamine (200mM) (FLOW) and 10% foetal calf serum (IMPERIAL). EMEM-S, as above excepting presence of foetal calf serum. ET, Glasgow Modification of Eagles Medium (GMEM) (FLOW) with 2.75g/L sodium bicarbonate and added immediately prior to use 10% tryptose phosphate broth, 1% L-glutamine and 1% penicillin:streptomycin solution. ETC, ET with added 10% foetal calf serum. HAMS F10 (FLOW) and HAMS F12 (FLOW) with sodium bicarbonate and added immediately prior to use 1% glutamine (200mM), 1% penicillin:streptomycin and 10% foetal calf serum. HAMS F10-S and HAMS F12-S as above but without serum. MEDIUM 199 (GIBCO) with Earles salts, sodium bicarbonate and L-glutamine 1% and added prior to use 1% penicillin:streptomycin, and 10% foetal calf serum. 199-S as above excepting the presence of serum.

Those listed below, including salines and distilled water (DW) were sterilised by autoclaving at 121°C for 15 minutes.

- Nutrient broth, (NB) 1.3g of nutrient broth (OXOID) in 100 ml of DW.
- Enriched proteose peptone (EPP), 2.5g proteose peptone and 0.625g of yeast extract (OXOID) in 250ml of DW.
- Glucose broth (GB), nutrient broth was made up as previously described and sterilised after which 1% of sterilised glucose solution was added.
- Tryptone soy broth (LABM), (TSB), 3g of tryptone soy broth in 100 ml of DW.
- Young's teleost saline, (YTS) (Young, 1933).
- Dulbecco A phosphate buffered saline (PBS).
- Aquarium water AW used as a control was passed through a 0.22µm Millipore filter before use.

TABLE 5: Preparation of culture media and saline solutions used in the maintenance of *I. multifiliis*

Media and stock solutions	Preparation	
	Constituents	Sterilisation
P:S	Penicillin:Streptomycin (10,000IU/cm^3:10,000µgcm^3) (Flow laboratories)	Commercial preparations supplied sterile
FCS	Foetal calf serum (Imperial Laboratories)	
EMEM	Eagles minimum essential medium (modified) with Earle's Salts and 0.85g sodium bicarbonate: 1% P:S, 1% L-glutamine (200mM) (Flow Laboratories) and 10% FCS added immediately before use	
EMEM-S	As above but without FCS	
ET	Glasgow modification of Eagle's medium with 2.75gl^{-1} sodium bicarbonate and 10% tryptose phosphate broth,1% P:S, 1% L-glutamine (200mM) (Flow Laboratories) added immediately before use	
ETC	ET with added 10% FCS	
MEDIUM 199	With Earle's salts, L-glutamine. and 1.25g sodium bicarbonate. 1% P:S, 1% L-glutamine (200mM) (Flow Laboratories) and 10% FCS added immediately before use.	
199-S	As above but without FCS	
HAMS F10	With 1% L-glutamine added immediately prior to use. (200mM).	
HAMS F10-S	As above but without FCS	
HAMS F12	With 1% L-glutamine added immediately prior to use (200mM)	
HAMS F12-S	As above but without FCS	
NB	1.3% nutrient broth (Oxoid) in DW	AUTOCLAVE
EPP	2.5g proteose peptone and 0.625g of yeast extract (Oxoid) in 250cm^3 DW	
GB	Nutrient broth with 1% glucose	
TSB	3% Tryptone soy broth (LabM) in DW	
YTS	Young's teleost saline (freshwater) (Young, 1933)	
PBS	Dulbecco A phosphate buffered saline	
DW	Distilled water (pH 7.0)	
AW	Aquarium water (de-chlorinated tap water buffered to pH 7.4)	Millipore filter (0.22µm)

INTRA VITUM STAINING OF FOOD VACUOLES USING CARMINE RED

A carmine red suspension was prepared by the addition of 4mg of carmine (SIGMA) to 10ml of EMEM. (Chapman-Andresen and Nilsson, 1968; Nilsson, 1972). The solution was then agitated and filtered through Whatman No.1 paper. A fresh suspension was prepared for each experiment and immediately prior to use, diluted 50:50 with EMEM. Vacuoles containing carmine red markers were photographed with the aid of a Zeiss standard microscope fitted with MC63 camera attachment.

CRITERIA FOR SUCCESS

Success of the culture was ascertained on the basis of cell survival, behaviour and development: including, encystment, division and production of infective stages. The activity of cilia present in all stages of the parasite life cycle, provided a simple approach in assessing cell survival and in the absence of obvious motility cells were scored as alive if ciliary beat was detectable.

Priorities here were to ascertain the changes in behaviour between life cycle stages. Swimming behaviour in the theront is characterised by anticlockwise spinning on the longitudinal axis, slow cruising alternating with spontaneous bursts of speed during which the theront widely gyrates and may alter course. Trophonts usually rotate in a fixed position within the host epithelia, and when having left the host swim freely without sudden changes in direction until a suitable surface is located for encystment.

It was necessary, in this study, to clearly define the terms used here as regards division and encystment observed within the cultures. Division was identified as

either occurring before encystment (precystic divisions) or following encystment (cystic divisions). Encystment that occurred following a precystic division was termed post divisional encystment, if no preceding divisions occurred it was simply termed encystment.

A total of 5 investigations were undertaken in order to assess the survival, behaviour and development of both theronts and tomonts within selected monophasic media culture systems and water controls.

EXPERIMENT 1: Survival of theronts in selected culture media.

100µl aliquots of a theront suspension collected within 2h of excystment were dispensed into each of 24 wells of a cell culture plate containing 1cm^3 of one of the following media:DW; AW; YTS; EMEM; EMEM:DW (50:50 and 30:70); ET; ET:DW (50:50 and 30:70); F10; F10:DW (50:50 and 30:70); F10-S; F10-S:DW (50:50 and 30:70); F12; F12:DW (50:50 and 30:70); F12-S; F12-S:DW (50;50 and 30:70); 199; 199:DW (50:50 and 30:70); 199-S; 199-S (50:50 and 30:70); EPP; EPP:DW (50:50 and 30:70); GB; GB:DW (50:50); NB; NB:DW (50:50); TSB; TSB:DW (50:50). Plates were incubated at 20°C for a minimum of 144h. Theronts were monitored daily for survival, behaviour and development and samples taken for viability testing.

The longest survival (Table 6a and 6b) was recorded in media containing EMEM, with a maximum of 120h when undiluted and a minimum of 96h at dilutions of 30:70 distilled water. Survival of up to 96h was also recorded in undiluted Hams F10, F12 and Medium 199 all containing serum and also in NB:DW 50:50. Theronts survived for 72h in diluted F10 and F12, and all media containing Hams F10-S, F12-S whether neat or diluted, exceeding survival within water controls by 24h. Survival within dilutions of Medium 199, was comparable to that observed within AW and DW controls. All other media, however, appeared to have a detrimental effect on the theronts, survival being less than that recorded within both water controls.

TABLE 6a: Survival of *I. multifiliis* theronts in monophasic media

MEDIA	TIME							
	MINS		HOURS					
	0	30	24	48	72	96	120	144
AW	+++		++	+	-			
EMEM	++	+++			++	++	++	-
EMEM:DW 50:50	++	+++			++	++		
EMEM:DW 30:70	+++				++	+		
ET	++	+++	++	+	-			
ET:DW 50:50	++	+++		++	-			
ET:DW 30:70	+++			++	-			
EPP	++	++	+	-				
EPP:DW 50:50	++	+++	++	-				
EPP:DW 30:70	++	+++	++	-				
GB	++	+	+	-				
GB:DW 50:50	++	+++	++	-				
TSB	++	++	-					
TSB:DW 50:50	++	+++	+	-				
NB	++	+	-					
NB:DW 50:50	++	+++					-	
F10	+++				++	+		
F10:DW 50:50	+++				++	-		
F10:DW 30:70	+++				++	-		

+++ parasite normal ++ parasite alive but slowing down + parasite alive but immotile
- parasite dead

TABLE 6b: Survival of *I. multifiliis* theronts in monophasic media

MEDIA	TIME							
	MINS		HOURS					
	0	30	24	48	72	96	120	144
YTS	++		+	-				
DW	+++			++	-			
F10-S	++	+++			++	-		
F10-S:DW 50:50	++	+++			+	-		
F10-S:DW 30:70	+++	+++			+	-		
F12	++	+++			++	+	-	
F12:DW 50:50	++	+++			+	-		
F12:DW 30:70	+++	+++			+	-		
F12-S	++	+++			++	-		
F12-S:DW 50:50	++	+++		++	+	-		
F12-S:DW 30:70	+++	+++		++	+	-		
199	++	+++			++	+	-	
199:DW 50:50	++	+++			-			
199:DW 30:70	+++	+++		++	-			
199-S	++			-				
199-S:DW 50:50	++			-				
199-S:DW 30:70	+++	++		-				

- Media abbreviations as in Table 5

+++ parasite normal ++ parasite alive but slowing down
+ parasite alive but immotile - parasite dead

Normal theront swimming behaviour, as described above, was observed for up to 48h within many of the nutrient media, twice as long as those maintained within DW controls. Slowing down of the theront appeared to signal cell death rather than any stage of development and no indication that transformation to trophont had occurred was observed.

Theront viability was significantly extended within EMEM, the parasite successfully establishing infections in carp 120h post excystment, 3 days longer than controls. Theronts maintained in dilutions of EMEM also retained viability for periods of 96 (30:70)and 72h (50:50). All other media did not extend theront viability.

EXPERIMENT 2: Survival and subsequent development of tomonts within selected culture media.

Surface sterilised tomonts were transferred individually in 10µl of appropriate culture medium into the wells of a 96 well plate containing 90µl of fresh incubating medium. Their survival was investigated in each of the following 30 media: EMEM; EMEM:DW (50:50 and 30:70); EMEM-S; EMEM-S:DW (50:50 and 30:70); ET, ET:DW (50:50 and 30:70); ETC, ETC:DW (50:50 and 30:70);F10; F10:DW (50:50 and 30:70); F10-S; F10-S:DW (50:50 and 30:70); F12; F12:DW (50:50 and 30:70); F12-S; F12-S:DW (50:50 and 30:70); 199; 199:DW (50:50 and 30:70); 199-S and 199-S (50:50 and 30:70). In addition to the above media, parasites were similarly maintained in DW controls. 8 tomonts were incubated in each medium, 1 tomont per well. Tomonts were examined at regular intervals, and the details concerning their survival and further development recorded in Table 7a and 7b.

All test media supported parasite survival beyond that of DW, with a maximum of 22 days in EMEM-S:DW 50:50. Parasites survived for at least 14 days in the following 5 media, EMEM:DW 30:70, EMEM-S, EMEM-S:DW 30:70, ET:DW 50:50, ETC and F10:DW 50:50. ETC:DW and ET:DW both diluted 30:70 supported survival for the shortest period. In all media tested survival was extended over that seen in water controls.

Precystic division occurred in 21 out of the 31 test media, frequency in most cases increasing with decreasing water dilution, 25% dividing in EMEM:DW 30:70 compared to 50% in undiluted EMEM. The products of precystic divisions were often atypical in shape having large projections, which disrupted their swimming patterns. These atypical individuals would not go on to post divisionally encyst. There was no precystic division of tomonts in Medium 199, 199-S, Hams F10, F10-S, F12, and F12-S at full concentration. Division did occur on media dilution, these being more frequent in tomonts within a 50:50 dilution than a 30:70 dilution. Precystic divisions did not occur within DW and 199:DW (30:70) and all media containing ETC.

Encystment and subsequent reproduction occurred only within DW and media diluted with water with the exception of media containing ETC where no division or encystment was recorded. Division when occurring within cysts usually proceeded through the 32 cell stage although no more than 2 divisions were observed in ET:DW 30:70. The percentage of tomonts encysting increased with media dilution and always occurred within the first 24h of incubation. In contrast post divisional

TABLE 7a: Survival and development of *I. multifiliis* tomonts in monophasic media

MEDIA	SURVIVAL (DAYS)	NO FURTHER DEVELOPMENT	NO.S OUT OF 8 DIVIDING			THERONT EMERGENCE (h)
			PRECYSTIC	PDC *	CYSTIC	
DW	2	0	0	0	6	48
EMEM	6	3	4	0	0	-
EMEM:DW 50:50	7	3	3	1	2	72-168
EMEM:DW 30:70	14	2	2	2	2	-
EMEM-S	14	5	3	0	0	-
EMEM-S:DW 50:50	22	1	3	3	4	48
EMEM-S:DW 30:70	14	0	1	0	6	-
ET	12	5	1	0	1	-
ET:DW 50:50	15	1	2	1	4	-
ET:DW 30:70	3	2	1	1	5	-
ETC	16	8	0	0	0	-
ETC:DW 50:50	9	8	0	0	0	-
ETC:DW 30:70	4	8	0	0	0	-
199	5	8	0	0	0	-
199:DW 50:50	9	3	4	3	1	24
199:DW 30:70	7	0	0	0	8	24-48

* PDC= post divisional cystic

- Media abbreviations as in Table 5

TABLE 7b: Survival and development of *I. multifiliis* tomonts in monophasic media ·

MEDIA	SURVIVAL (DAYS)	NO FURTHER DEVELOPMENT	NO.S OUT OF 8 DIVIDING			THERONT EMERGENCE (h)
			PRECYSTIC	PDC*	CYSTIC	
DW	2	0	0	0	7	24
199-S	6	8	0	0	0	-
199-S:DW 50:50	11	0	5	5	3	-
199-S:DW 30:70	9	0	1	1	7	24
F10	6	8	0	0	0	-
F10:DW 50:50	14	1	3	3	4	24
F10:DW 30:70	9	0	2	2	5	24-48
F10-S	4	7	1	0	0	-
F10-S:DW 50:50	10	2	5	1	1	-
F10-S:DW 30:70	6	0	3	3	5	-
F12	4	8	0	0	0	-
F12:DW 50:50	10	4	2	2	2	-
F12:DW 30:70	10	1	1	1	6	24
F12-S	7	8	0	0	0	-
F12-S:DW 50:50	11	0	4	4	4	24
F12-S:DW 30:70	7	1	1	0	6	24

* PDC= post divisional cystic

• Media abbreviations as in Table 5

encystments decreased with increased water dilutions and were usually observed from 24 to 48h.

The parasite excysted within EMEM:DW, EMEM-S:DW, 199, F10, F10-S F12 and F12-S all diluted 50:50 and 199, 199-S, F10, F10-S, F12 and F12-S at 30:70 dilutions. Timing of theront release was between 24- 72h and therefore comparable to controls. Excystment appeared to be impeded in certain diluted media even though active theronts were detected within the cyst. However, failure to excyst resulted in a steady decline in the number of live parasites present within the cyst, and by day 12 these no longer exhibited normal behaviour of the encysted theront, assuming a more rounded appearance with behaviour resembling the preceding tomite stage. Excysted theronts survived for up to 6 days within EMEM-S:DW (50:50) 3 times longer than those released from the water controls.

EXPERIMENT 3: Tomont encystment and subsequent development in diluted media

Tomonts were dispensed, 1 per per well, into a 96 well plate. 24 wells were assigned to one of the following media, selected from the previous experiment for their ability to support encystment and advanced divisions. These were EMEM, EMEM-S, 199, 199-S, F10, F12-S, 199 and 199-S diluted 50:50 and 30:70 with DW. Parasites were monitored at daily intervals and theronts on emergence tested for sterility and viability. Results are shown in Table 8 and 9.

The parasite survived longest in EMEM-S media, live theronts being detected within the cyst for up to 16 days. The total number of precystic divisions within diluted

TABLE 8: Survival and subsequent development of *I. multifiliis* tomonts within monophasic media

MEDIA	SURVIVAL (DAYS)	DIVISION				NUMBER PRODUCING THERONTS
		NO DIVISION	PRECYSTIC	PDC*	CYSTIC	
EMEM:DW 50:50	11	3	8	7	9	7
EMEM:DW 30:70	13	0	8	4	14	4
EMEM-S:DW 50:50	16	0	3	2	16	7
EMEM-S:DW 30:70	16	0	0	0	23	11
199:DW 50:50	10	10	9	2	5	3
199:DW 30:70	9	4	7	0	13	13
199-S:DW 50:50	9	6	9	3	9	1
199-S:DW 30:70	9	0	5	4	15	7
F10:DW 50:50	14	5	5	3	13	1
F10:DW 30:70	10	3	4	4	17	4
F10-S:DW 50:50	9	4	5	5	15	3
F10-S:DW 30:70	7	0	2	1	22	3
F12:DW 50:50	10	8	8	4	18	7
F12:DW 30:70	12	3	2	2	19	3
F12-S:DW 50:50	13	4	11	7	9	2
F12-S:DW 30:70	7	1	3	3	20	3

- Media abbreviations as in Table 5

TABLE 9: The proportion of *I. multifiliis* theronts produced from post divisional cysts and cysts

MEDIA	NO. OF CYSTS PRODUCING THERONTS		THERONTS		
	PDC	CYST	TIME OF RELEASE (HOURS)	STERILE	VIABLE
EMEM:DW 50:50	5	2	24-48	YES	YES
EMEM:DW 30:70	2	2	24-168		
EMEM-S:DW 50:50	2	5	24-48		
EMEM-S:DW 30:70	0	11	24-72	YES AT 24+48 HRS NO AT 72	
199:DW 50:50	2	1	24-48	YES	YES
199:DW 30:70	0	3	24-48		
199-S:DW 50:50	0	1	24-72		
199-S:DW 30:70	1	6	24-72		
F10:DW 50:50	0	1	48		
F10:DW 30:70	0	4	24-72		
F10-S:DW 50:50	0	3	24-48		
F10-S:DW 30:70	0	3	24-48		
F12:DW 50:50	2	5	24-96		
F12:DW 30:70	1	2	24-48		
F12-S:DW 50:50	0	2	24		
F12-S:DW 30:70	0	3	24		

- Media abbreviations as in Table 5

EMEM-S media was less than that observed in all other media, the highest number of precystic divisions being recorded within F12-S:DW 50:50 where 11 out of 24 parasites divided precystically. Within 199:DW, 199-S:DW and F12:DW all diluted 50:50 and EMEM diluted both 50:50 and 30:70 over 1/3 of tomonts underwent precystic divisions. Within EMEM:DW 50:50, however, a high percentage of the daughters from precystic divisions underwent the normal process of encystment. Nevertheless the overall numbers of encystments were greatest for EMEM-S media with the maximum occurring within EMEM-S:DW 30:70. All test media supported the encystment and reproductive phase of *I. multifiliis* with production of theronts within 24h in all cases. Theronts continued to be released for up to 48h in media diluted 50:50 the time being increased with further dilution up to 168h in the case of EMEM:DW 30:70. 199:DW 30:70 was the most successful media used here in terms of the greatest number of tomonts undergoing this stage of development with 13 out of 24 completing encystment and excystment.

Theront release occurred mostly by the direct route following tomont encystment. (Table 8). However, in media EMEM:DW 50:50 and 199:DW diluted 50:50 tomonts were more likely to encyst and produce theronts following a precystic division. In EMEM:DW 30:70 theront release was equal for both cyst types. Unsuccessful attempts were made to induce excystment either by the replacement of media with water or by puncturing the cyst wall *in vitro* with a sterile needle. Removal of media and addition of water appeared to have a deleterious effect causing death of the theronts within the cyst.

Viability and sterility was confirmed for all theronts tested at the time of their release.

EXPERIMENT 4: Development of tomonts in water following delayed encystment by undiluted media.

240 surface sterilised tomonts were transferred individually in 10µl of culture medium into the wells of a 96 well plate each containing a further 90µl of the fresh medium. 24 wells were assigned to each of the following media previously found to permit development but inhibit encystment: EMEM, ET, ETC, EMEM-S, F10, F10-S, F12, F12-S, 199 and 199-S undiluted. The number of parasites surviving was recorded each day and 5 living individuals removed from each of the 4 different media. These were washed three times in sterile DW, placed into 100µl of DW and observed for further development. Theronts produced were tested for sterility and viability.

Tomonts incubated within all media excepting ET produced theronts when transferred to sterile DW (Table 10). In each case, however, the number of tomonts completing development with the subsequent production of theronts decreased with media incubation time. Theronts produced at 96h following a 72h incubation within EMEM-S media and those produced at 72h following a 48h incubation within ETC, 199, F10, F10-S, F12, F12-S were both sterile and viable. In EMEM viable theronts were obtained from tomonts following a 24, 48 and 96h incubation, however, in the latter case theronts were not produced until 48h after removal to water (144h post incubation and were not sterile or viable). One tomont that had been incubated within EMEM for 24h was still encysted and alive after 120h in water although no further development was recorded.

TABLE 10: Delay of encystment by incubation within monophasic media

MEDIA	MAXIMUM DELAYMENT (Hs)			THERONTS	
	E	D	T	STERILE	VIABLE
EMEM	96	96	96 (144)	YES (NO)	YES
EMEM-S	72	72	96	YES	YES
ET	-	-	-	-	-
ETC	48	48	72	YES	YES
F12	72	72	96	YES	YES
F12-S	72	72	96	YES	YES
F10	48	48	48	YES	YES
F10-S	48	48	48	YES	YES
199	72	72	96	YES	NO
199-S	24	24	48	YES	YES

E- encystment D- division T-theronts produced

- Media abbreviations as in Table 5

EXPERIMENT 5: Detection of phagocytic activity in tomonts following incubation within EMEM

20 tomonts were added to a $1cm^3$ suspension of carmine red in EMEM. At 10-minute intervals, individual tomonts were removed from the culture medium and examined for the presence of red stained food vacuoles with the aid of a compound microscope. Vacuoles containing carmine red particles were located within the tomont after 30 minutes within EMEM (Fig 6).

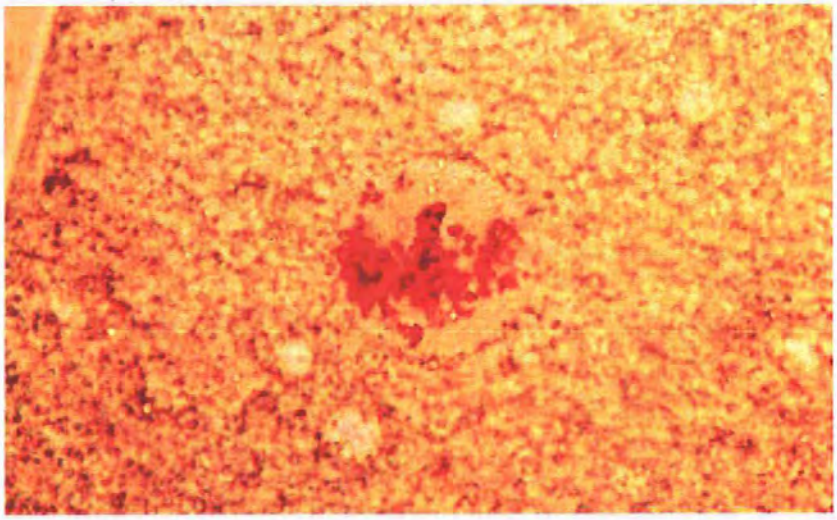


FIGURE 6: Carmine labelled food vacuole of an I. multifiliis tomont following incubation within an EMEM:carmine suspension (x40).

All stages in the life cycle of *I. multifiliis* were axenically isolated in the present study with the aid of penicillin and streptomycin, chemicals which have been used successfully for the surface sterilisation of other species of parasites (Taylor & Baker, 1987). Ekless (1990), however, found concentrations of penicillin and streptomycin as low as 5IU/cm^3 and 5µg/cm^3 respectively to have detrimental effects on free living stages of *I. multifiliis* when included in aquarium water at pH 7.4. The numbers of tomonts completing excystment, theronts produced per cyst and theronts subsequently surviving were reduced, the effects being enhanced with increasing antibiotic concentration. Surprisingly, these stages were unaffected when exposed to equivalent levels of penicillin and streptomycin within media containing serum. It may be that the presence of serum within the medium in some way ameliorates any possible anti-protozoan activity of the antibiotics (Loefer, 1949).

Although free-living ciliates have been isolated without the use of antibiotics, by repeated washing in sterile water (Elliott, 1933; Parpart, 1928), Ekless (1990) did not find this method alone satisfactory for *I. multifiliis*. An alternative approach to obtaining sterile preparations of *I. multifiliis*, described by Dickerson *et al.* (1985), involved the intraperitoneal injection of theronts into channel catfish *Ictalurus punctatus* (Rafinesque) the trophonts then being harvested by dissection under sterile conditions. Employment of such a method is however still dependent upon the supply of naive hosts and is not suitable for long term maintenance of the parasite. Isolation of the tomont and aseptic maintenance of the subsequent stages

of development through to excystation provided the most effective route here for the production of sterile theronts. The simultaneous discharge of mucocyst material during encystment might also be expected to remove surface contaminating bacteria to the exterior of the cyst wall. MacLennan (1937) and Ewing, Kocan and Ewing, (1983) report that bacteria are usually attached to the sticky outer layer indicating that some degree of debris expulsion occurs within the cyst. Although Parpart (1928) attributed contamination of *Paramecium* cultures to defaecated bacterial endospores, there was no evidence for this in *I. multifiliis*. This might be expected as the trophic phase of the latter is located within the fish epidermis and is not normally known to ingest bacteria. Nevertheless xenosomes of unknown function have been detected free within the cytoplasm of an isolate of *I. multifiliis* by Lobo-da-Cunha and Azevedo (1988). These inclusions could be significant in the long-term culture of *I. multifiliis* if proven to have a mutualistic role in cell metabolism.

McCallum (1982) proposes that theront survival in water is limited by depletion of food reserves, and therefore only limited time is available for host location. That the period of theront survival and viability was extended in this study by media incubation provides evidence that the free-living stages of the parasite are capable of replenishing these reserves to some extent without further development. This is as might be expected as the theront would be pre-adapted to exploiting the host's nutrients immediately following entry. On examination at EM level the buccal region of *I. multifiliis* theronts can be seen to be fully complete (Geisslinger,1987) nutrient uptake by phagocytosis is therefore possible. That carmine red particles were observed within tomonts following media incubation indicates that the parasite

retains its ability to take nutrients from the environment and that attempts were made to obtain nutrients from the liquid medium. In addition to phagocytosis nutrient uptake of ciliates can be either passive, occurring by simple diffusion or active involving pinocytosis. Exhaustive studies have been undertaken on nutrient uptake in *Tetrahymena spp.* and it is apparent that the modes and paths of uptake are dependent on the nature of nutrients available, the environmental circumstances and the physiological state of the organism (Holz 1973). The major component of nutrient uptake within ciliates is thought to occur through food vacuole formation, although whether this is sufficient to meet the cell requirement is much deliberated (Nilsson, 1976). Orias and Rasmussen (1977) proposed that *Tetrahymena* has a dual capacity for nutrient uptake using both uptake by food vacuole formation and also a surface uptake system via parasomal sacs (Pitelka, 1961). Although a *Tetrahymena* mutant has been identified that grows without phagocytosis in media supplemented with vitamins and heavy-metal salts (Rasmussen and Orias, 1975), formation of food vacuoles seems essential for growth in the standard *Tetrahymena* laboratory culture media EPP (Rasmussen, 1974). Within this study the finite survival of the tomonts within the culture media, despite attempts at nutrient uptake through phagocytosis, may indicate that insufficient or incorrect nutrients are available, or that not all pathways of nutrient uptake are active under these *in vitro* conditions.

Reproduction in *I. multifiliis* is generally associated with the production of tomites during encystment which differentiate into theronts. The detection of precystic tomont divisions in culture, here, may be indicative of a normal although rarely observed event in the life cycle of this parasite, Ewing, Ewing and Kocan (1988)

provided evidence that division of the trophont takes place within the fish epidermis. Butcher (1943) occasionally recorded a single precystic division of tomonts following exit from the host epidermis, although these forms usually went on to encyst, however, it was reported that it was not uncommon for one of these daughters to possess a nipple-shaped projection at one end, corresponding to observations here. Multiple precystic divisions by repeated binary fission were also recorded, here, resulting in numerous small individuals many with abnormal swimming patterns, possibly representing the phase of tomite production. According to MacLennan (1937), however, successful reproduction *in vivo* requires the secretion of a cyst wall at least for the initial cycles of division. That tomonts encysted and excysted as normal in all controls and media diluted with water, except ETC, provides convincing evidence that further development is triggered by reduced osmolarity, as would be experienced by the parasite on entering the aquatic environment. However, replacement of media with water following completion of division in media does not aid excystment, and could indicate that either, structural changes of the cyst wall have occurred, or that failure to differentiate into theronts had occurred.

Although the media investigated failed to support growth and development of *I. multifiliis* comparable to that within the fish host, the extended survival of all stages of the life cycle provides some optimism for the development of an *in vitro* culture method. Of particular interest in this study was the ability of concentrated media to delay encystment and the production of viable, sterile theronts allowing a 4day extension of the life cycle at 20°C providing increased management potential.

CHAPTER 5

IN VITRO CULTURE OF *ICHTHYOPHTHIRIUS MULTIFILIIS* USING CELL ASSOCIATES

INTRODUCTION

As described in the previous chapter *Ichthyophthirius multifiliis* is shown to survive longer within monophasic culture media than in fresh water controls, unfortunately these media failed to provide the necessary requirements for parasite growth and development (Ekless and Matthews, 1993).

The use of fish cells as associates within culture has enabled other protozoan fish parasites to be successfully cultured *in vitro*. *Amyloodinium ocellatum* Brown, 1931 a dinoflagellate ectoparasite of marine fish with a similar life cycle to that of *I. multifiliis* has been successfully cultured *in vitro* in association with both cell and organ associates (Noga, 1987; Noga and Bower, 1987; Noga, 1990). Under such conditions the parasite could be maintained throughout the complete life cycle within a single culture flask including the feeding stage, the free living reproductive cyst and infective stages. The marine holotrichous, hymenostomatous, ciliate *Miamiensis avidus*, isolated from skin tumours of the sea horse *Hippocampus erectus*, thrives within a cultural system containing Grunt Fin (GF) or Rainbow trout gonad (RTG) monolayers, the ciliates feeding so aggressively that the cell monolayer was removed within hours (Moewus-Kobb, 1965). The fresh water trypanosome of cyprinids, *Trypanoplasma danilowski* (Laveran and Mesnil, 1904) has been cultured in association with Fat Head Minnow (FHM) and Black Bullhead (BB) cell lines within EMEM medium with added 10% foetal calf serum, media alone failing to support continuous growth of the parasite (Smolikova, 1977). *Myxobolus exiguus* a

microsporidian parasite of marine fish has also been shown to begin its development in cultures of RTG (Grasse, 1977).

The present investigation aimed to culture *I. multifiliis* in association with cultured fish cells in an attempt to replicate conditions seen within the fish host.

Routine methods for the maintenance of cultured fish cell monolayers are described below, in addition methods are also described for the development of complex culture systems. All procedures were carried out within a laminar flow hood and aseptic technique was used throughout. Culture flasks and Linbro multiwell plates including those that were collagen coated, were obtained sterile from the manufacturers (IMPERIAL). All glass pipettes used for culture were washed overnight in a 5% solution of 7X (FLOW) and then subjected to a 3h tap water wash in a FISONS pipette cleaner. Following 2 overnight-distilled water washes the pipettes were dried, plugged with cotton wool and sterilised overnight in a hot air oven. Pipette canisters that were not used immediately were firmly sealed with tape until use. Gilson pipettes used for *in vitro* studies were periodically sterilised by an alcohol wash and pipette tips sterilised by autoclave prior to use.

Preparation of media and solutions

EMEM, Eagles minimum essential medium (modified), (FLOW) containing Earles salts, non-essential amino acids and 0.85g/L sodium bicarbonate was used for routine maintenance of the cell line and *in vitro* studies. Working media was prepared by adding 1% penicillin (10,000IU/ml):streptomycin (10,000µg/ml) (FLOW), 1% L-glutamine (200mM) (FLOW) and 10% foetal calf serum (IMPERIAL). Distilled water and phosphate buffered saline (PBS), (Oxoid: Dulbecco A) were autoclaved prior to use.

FISH CELL CULTURE

Two cell lines namely Atlantic salmon cells and fat head minnow were used as associates in the *in vitro* culture of *I. multifiliis*. Both are epithelial type cells the former taken from tissue posterior to the anus and coded **AS** (Nicholson and Byrne, 1973); the latter taken from the caudal trunk and coded **FHM**, (Gravell and Malsberger,1965).

Routine subculture of fish cell lines

Standard methods reported in cell culture texts (Wolf and Quimby, 1969; Nicholson, 1985) were used for the routine subculture of **AS** cells. Confluent monolayers of cells were washed free of media with PBS and exposed to 1ml of cold trypsin solution (0.25%, GIBCO). Detachment of cells from the flasks could often be slow and was aided by gentle agitation or short incubations at 37°C. Once cells were visibly suspended within the liquid, a small volume of EMEM was slowly added to the suspension with constant mixing in order to prevent cell clumping. The number of cells/ml was determined using a haemocytometer and new culture vessels were seeded at concentrations of 10^5 cells/ml of medium by further dilution of the suspension within the flask. Seeded flasks and multiwell plates had 5% CO_2 added prior to incubation at 20°C. **AS** monolayers were grown in both 24 and 96 well plates for experimental procedures.

Cryopreservation of fish cell lines.

Cells were trypsinised as for routine subculture and then resuspended within freezing medium (APPENDIX 1). The cryoprotectant used was glycerol (never over 12 months old) at a concentration of 10%. Cells were held at room temperature for

30 minutes prior to freezing to allow permeation of the cryoprotectant and then placed within a lagged polystyrene box filled with cotton wool and placed into a -70°C freezer overnight. The cells were then plunged into liquid nitrogen for long term storage. Recovery of the cells following freezing was accomplished by rapid thawing under a hot tap, the cell suspension immediately on thawing was gently pipetted into a 75cm^2 flask and left undisturbed thereby allowing attachment of the fragile cells to occur. After a 2 hour period the cells were gassed with 5% CO_2 and incubated at 20°C.

Underlay systems

Collagen, gelatin, and gelatin-coated agar were examined for their ability to support both **AS** cell and parasite growth. Two percent gelatin gel (SIGMA) obtained from bovine skin was heated to 37°C and small volumes pipetted over the surface of the culture vessels and allowed to dry at room temperature for 2h after which time the surplus liquid was gently removed. Agar (SIGMA) was made up as a 2% solution with PBS, autoclaved at 110°C for 10 minutes and 500µl pipetted into the wells of a 24 well plate and allowed to gel prior to addition of a gelatin layer. Collagen-coated 24 well plates were obtained sterile form the manufacturers (IMPERIAL). In all cases 50% of the total volume of medium was added 30 minutes prior to the addition of cells. Collagen and gelatin supported excellent growth of monolayers, agar underlay with gelatin coating failed to encourage cell attachment.

Cell aggregates

AS cell aggregates were formed by pipetting a suspension of trypsinised cells onto an agar underlay. The agar provided no opportunity for cell attachment resulting in

cells forming dense aggregates.

Overlay systems

Four methods for developing a monolayer overlay system were established incorporating either agarose, carboxymethyl cellulose, with or without added **FHM** cells, or **FHM** cell suspension and killed **AS** cell suspension. Only wells showing good levels of sustained parasite/cell contact were used for overlay experiments. All experimental conditions were tested using 6 wells of a 24 well plate.

Agarose. **AS** monolayers were exposed to a theront suspension and 24 hours later the media was removed and a layer of agarose or agarose plus cells was pipetted over the cell surface. Agarose (Type VII),(SIGMA), a low temperature gelling agarose with high clarity, was made up as a 2% solution in PBS. Following autoclaving the agarose was held at 35°C and immediately prior to use an equal volume of prewarmed 2x EMEM (FLOW) with or without cells was added to the agarose. **FHM** cells were used within the agarose overlay in preference to **AS** cells because of the ease with which single cell suspensions were formed on trypsinisation. This made cell enumeration by haemocytometer easier and a uniform cell suspension within the agarose possible. Cell concentrations of 5×10^5 and 1×10^6 /cm^3 were used within the EMEM.

Carboxymethyl cellulose is a low viscosity liquid allowing slow sedimentation of cells. As described for agarose, CMC was mixed 50:50 with 2x EMEM with or without cells and pipetted over the surface of a monolayer previously exposed to theronts.

FHM cell suspension. FHM cells were trypsinised, suspended in EMEM at a concentration of (1×10^6 cells /cm^3) and added to the experimental well.

Killed **AS** cell suspension. **AS** cells were killed by undergoing repeated freezing and thawing and were added to an experimental well.

Fin and epithelial explant

Fin cultures were initiated from caudal fin tissue and skin cultures from epithelial tissue obtained from the lateral surface of a scattered scale mirror carp. The method used for establishing cell cultures was a direct planting method (Wolf, 1969). All tissues were removed aseptically from an anaesthetised carp and immediately following removal immersed for 10 seconds in 70% alcohol, rinsed in sterile PBS and incubated in a 2% penicillin:streptomycin solution in PBS for 2h. Following incubation the tissue was finely chopped, within a small volume of PBS, until pieces of approximately 1-2mm were obtained. These pieces were removed individually to a sterile 24 well plate and with the aid of a sterile glass rod, were smeared over the well bottom. The sections were incubated at 20°C in a semi-dry condition for 2h, after which time EMEM was gently pipetted into the well avoiding dislodging the tissue. 50% media changes were undertaken 48h after initiation of the culture and thereafter as required.

PROCEDURE FOR THE CULTURE OF *I. MULTIFILIIS*

Theronts were introduced into all of the culture systems described above, trophonts and tomonts were maintained only with monolayers. Parasites were isolated axenically and enumerated as described earlier (Chapter 4).

Theronts

100µl of theront suspension containing approximately 600-800 theronts was added to each well of a 24 well plate containing each of the test systems described above. The subsequent behaviour and development of the theronts was observed by means of an inverted microscope.

Trophonts

These were collected from fish carrying primary infections of 24, 48, 72, 96 and 120h duration. At each time interval 10 fish were sacrificed by an anaesthetic overdose and fins containing high numbers of parasites removed and washed with 1% penicillin:streptomycin. Trophonts aged 72h and above were harvested by incubating the fin within sterile DW. Tomonts exiting the fin were observed by means of a dissecting microscope and quickly harvested by sterile pipette and subjected to 3 DW washes and 3 EMEM washes of 5-minute duration. Trophonts of less than 48h did not readily leave the fish epidermis following host death, these were effectively collected, however by incubation within EMEM as described previously in Chapter 3.

Photomicroscopy

Cultured cell and live parasite photomicrographs were taken on an inverted microscope using interference filters LB45 and LBD and KODAK E6 film. Cultured parasites were photographed on a Zeiss MC63 automatic camera fitted to a standard Zeiss microscope.

Measurement of parasite within the culture system

Parasites were measured *in situ* with the aid of an inverted microscope fitted with an ocular micrometer under a x4 objective.

CRITERIA FOR SUCCESS

The same criteria as described in Chapter 4 for monophasic media were used here for assessing the survival and behaviour of the parasite. It was necessary, however, to extend the criteria in this present study in order to determine whether growth and development had taken place. These criteria described below were based upon observation of normal development in primary infections within a host fish (Chapter 3).

The presence of growth, change in shape of the macronucleus, and disappearance of the organelle of Lieberkühn are taken as indicators of change. The patterns of behaviour observed within the wells were also noted, behaviour being known to differ between life cycle stages, the free living host seeker changing to sedentary feeder on location and penetration of a suitable host. Parasite/cell contact was therefore interpreted as the number in the well in close contact with cultured cells and this was further identified as being either transient or sustained in nature.

A total of 7 experiments were undertaken in order to assess survival and growth of the parasite within each culture system. These experiments were divided into 2 groups based upon the type of system adopted, monolayer or multilayer. All test systems were tested in triplicate.

MONOLAYER SYTEMS

EXPERIMENT 1: Behaviour of theronts within an **AS** culture system with EMEM (10% FCS)

AS cells were allowed to achieve confluency within the wells of a 24 well plate and immediately prior to theront addition a 50% media change was undertaken within the experimental well. Following addition of a 100µl suspension of theronts to the well the subsequent behaviour and development were observed. Results are recorded in TABLE 11.

Within minutes a small number of introduced theronts were seen to make contact with the fish cell monolayer. In some cases this association appeared to be transient in nature the parasite pausing at the cell surface for short periods 5 seconds to 2 minutes. However, contacts of longer duration were also recorded, the parasite maintaining a fixed position within the monolayer whilst rotating. Contact such as this was in places sustained for periods of up to 120h. After this period parasites died still in position, no ciliary beat being detected. All theront/cell contact both transitory and sustained occurred in thicker areas of the cell layer, (Fig 7).

TABLE 11: Survival and percentage contact of theronts in association with **AS** monolayers in EMEM with added 10% serum.

SURVIVAL (days)		6
TIME (h)	% CONTACT WITH CELLS AT 24h	SIZE(µm)
24	1-21	41.9
48	0-11	46.42
72	0-11	46.5
96	0-6	45.4
120	0-3	52.5
144	0.3	51.63

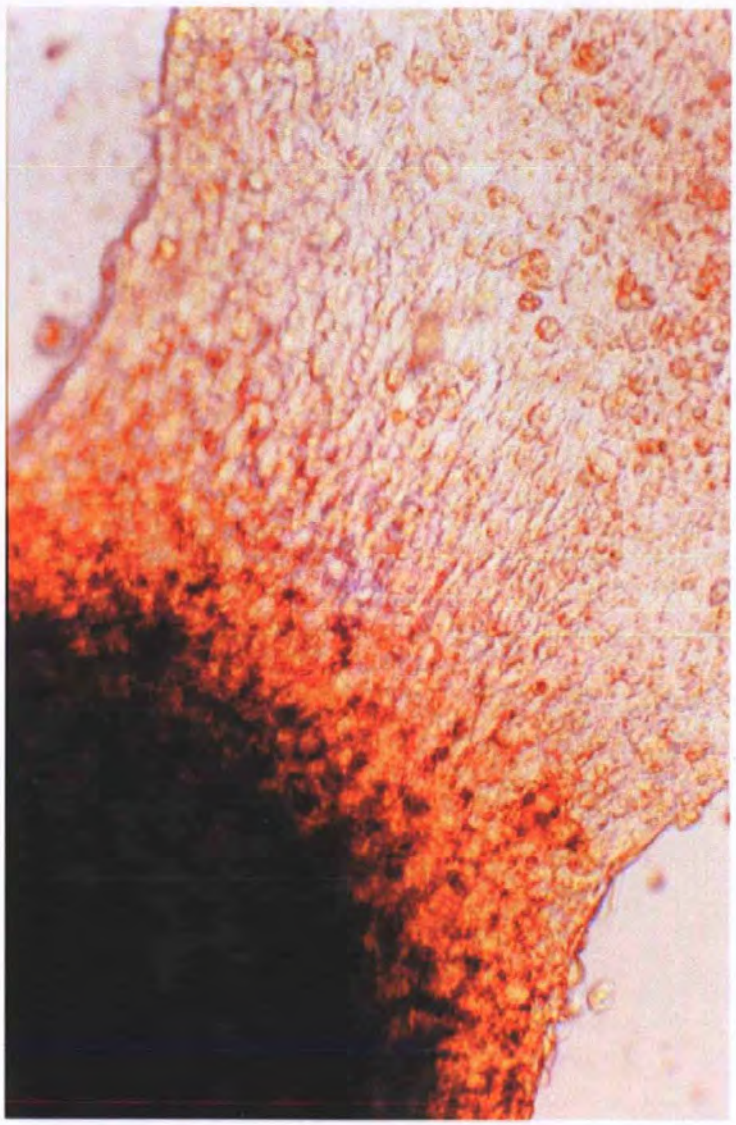


FIGURE 7: **AS** cell monolayer demonstrating areas of cell thickening where parasite/cell contact occurred.

Percentage contact varied considerably between replicate wells (TABLE 11) and the numbers in close contact with the cell layer decreased over time either due to the parasite leaving the cell layer or death. Parasites in contact with the cell layer showed a degree of aggregation and it was rare to see a single parasite in contact with the monolayer. Figure 8 shows a group of 5 introduced parasites in very close proximity to each other whilst in sustained contact with the cultured cells. Following culture for 24h, methyl green acetic stain was applied to introduced parasites and slight curvature of the macronucleus was observed in some individuals (Fig 9). The organelle of Lieberkühn was not observed at this level but its absence was not confirmed. Parasites in contact with the cell layer were measured at each time interval, but no increase in size was recorded. Disruption of the monolayer following water addition lead to immediate death of the parasite.

EXPERIMENT 2: Distribution of free swimming parasites within a monolayer culture system

2 wells of a 24 well plate containing confluent monolayers of **AS** cells in EMEM with 10% added FCS were exposed to a 100µl suspension of theronts and examined after 24h. Each well was divided into 4 distinct areas; thick cells; thin cells; no cells and areas where parasites were in sustained contact with cells. Location of free swimming individuals within the well was observed using the x10 objective of an inverted microscope. Each area type was observed for two 1 minute periods and the number of parasites entering the field of view were counted with a tally counter. After each one-minute period the plate was returned to the dark to compensate for theronts phototactic responses. The results are recorded in Table 12.

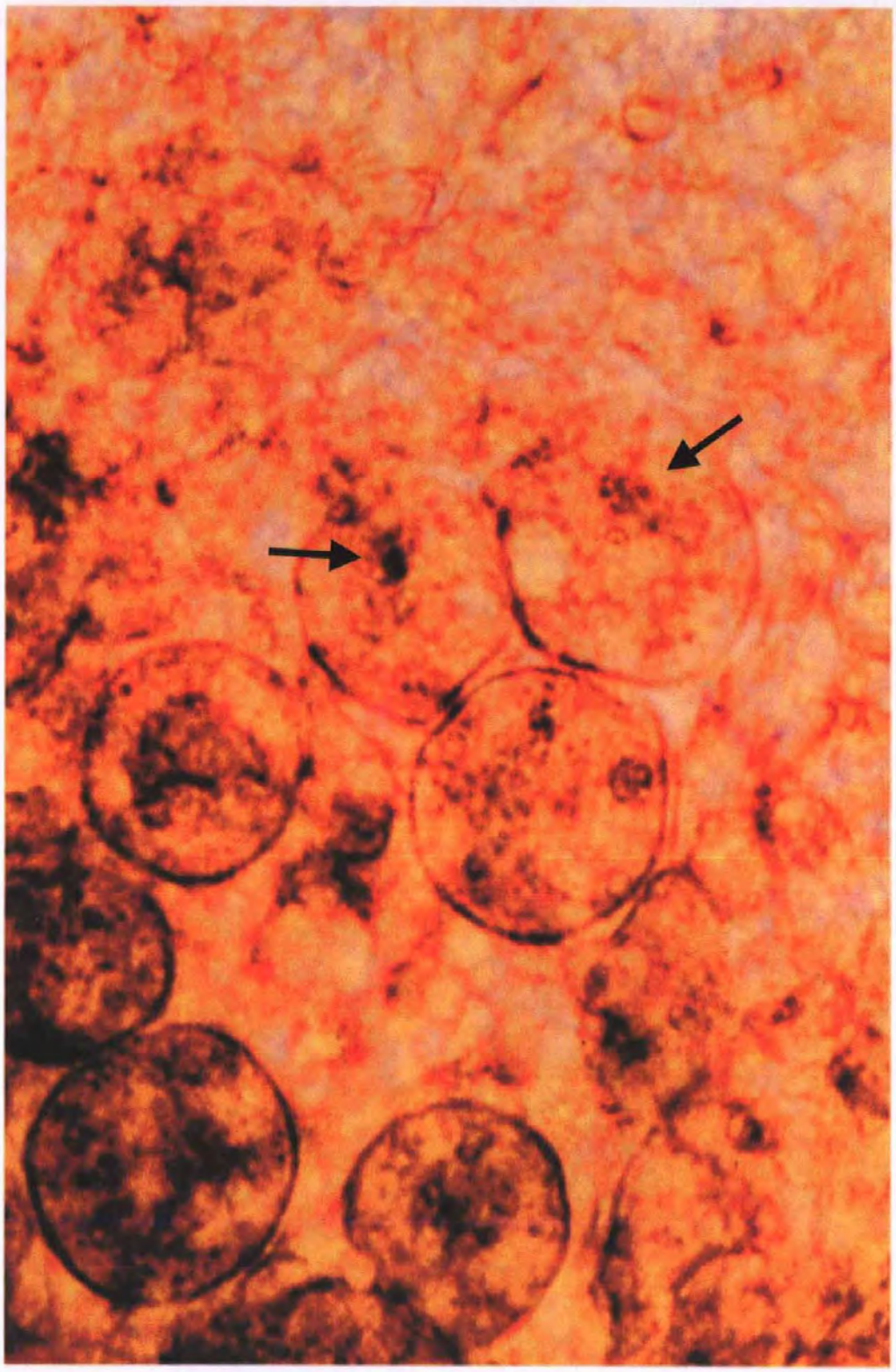


FIGURE 8: *I. multifiliis* in sustained contact with cultured cells 24h after introduction into an **AS** monolayer cell system.

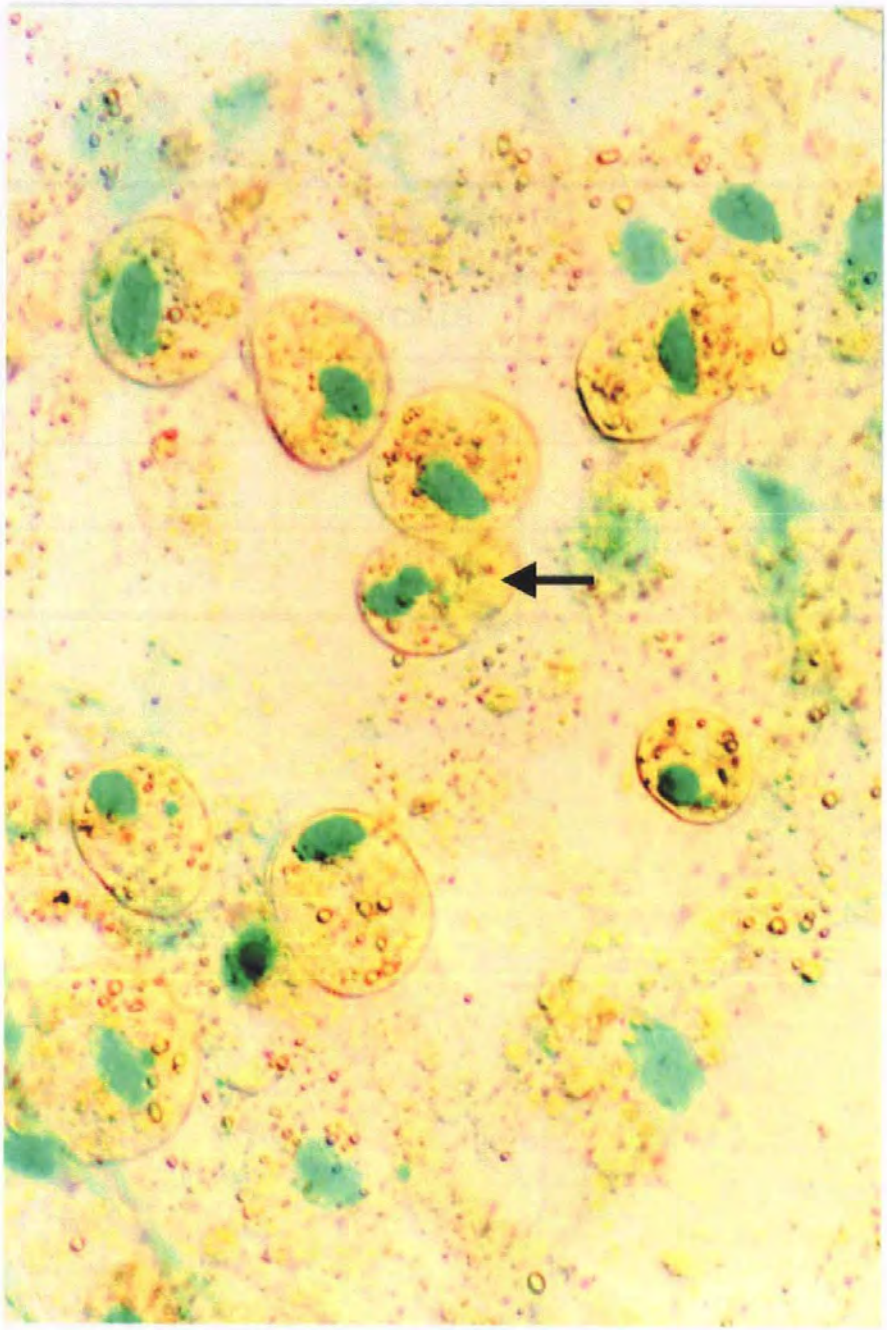


FIGURE 9: Methyl-green acetic stained *I. multifiliis* following 24h incubation in association with cultured **AS** cells. Slight curvature of a macronucleus is indicated (arrow).

TABLE 12: Distribution of free swimming parasites within an **AS** monolayer culture system.

WELL	AREA TYPE			
	THICK CELLS	THIN CELLS	NO CELLS	CELLS/ PARASITE
1	29,30	18,34	24,5	36,35
2	47,17	21,23	25,59	24,23
I	30.75 +/- 11	24.3 +/- 7	28.25 +/- 19.5	29.5 +/- 6

There appears to be no significant difference between parasite distribution in any of the well areas. The greatest variation in parasite numbers entering the field of view was seen in areas where no cells were present these varying from 25 to 59. The smallest variation was seen in areas where parasites were seen in sustained contact with the cell layer.

EXPERIMENT 3: Theront survival in association with **AS** cells using different types and concentrations of sera

Foetal calf (FCS) both heat inactivated (HI) and non-heat inactivated (non HI) and non-heat inactivated carp serum were tested at 0,5,10,20 and 40% concentrations within EMEM. The differing sera were added to confluent **AS** monolayers within a 24 well plate and incubated at 20°C for 3h prior to theront introduction. Survival and size of the parasites was recorded and the motility of the parasites that formed either no or transitory contact with the cell layer was also noted. (Tables 13-18).

Survival in all systems varied between 4-7 days. The longest survival of 7 days was recorded within 10% non HI FCS, a day longer than survival within 10% and 20% HI FCS and 5% carp serum. No significant growth was recorded over the period of observation, however the greatest average size, 62.78µm, was recorded within 5% carp serum at 144h. Carp serum (20%) had the largest maximum size of an individual parasite of 85µm. Whether this was due to swelling of the cell or to growth could not be determined. The smallest individual parasites, 20µm, were recorded at 48h within 20% calf serum (HI) approximately half the average size of other cultured parasites.

TABLE 13: Size of parasites in association with **AS** cell monolayers with added foetal calf serum (heat-inactivated). (The number in brackets is the number of parasites measured at each time interval).

% SERUM	AVERAGE SIZE (µm) AT TIME INTERVALS (h)							MAX SIZE (µm)	SURVIVAL (DAYS)
	24	48	72	96	120	144	168		
0	42.5 (20	43.8 (20)	40 (12)	50 (5)	-	-	-	50	4
5	40 (25) +/- 5.4	44.7 (20) +/-6	46 (8) +/- 7	45.5 (6) +/-5.2	-	-	-	50	4
10	41.9(22 +/- 6.6	46.4 (20) +/- 6.9	46.5 (14) +/-9	45.4 (10) +/- 4	52.5 (8) +/-2.3	51.6 (4) +/- 2.35	-	60	6
20	41.3 (20) +/-2.2	42.5 (20) +/-2.5	45.3 (15) +/-8	45.2 (10) +/- 6	52.1 (9) +/-9.85	60 (2)	-	65	6
40	44.5 (20) +/-4.5	50 (16) +/-9.8	50.8 (16) +/-9.7	54.1 (16) +/-9.5	60 (7) +/-10	-	-	75	5

TABLE 14: Motility of free swimming parasites in the presence of **AS** cell monolayers with added foetal calf serum (heat-inactivated).(600-800 theronts were introduced to each well).

% SERUM	TIME (H)						
	24	48	72	96	120	144	168
0	+++	+++	++	-			
5	+++	++	++	+	-		
10	+++	++	++	+	+	-	
20	+++	+++	++	++	-		
40	+++	++	++	+	+	-	

+++ parasite normal
++ parasite alive but slowing down
+ parasite alive but immotile
- parasite dead

TABLE 15: Size of parasites in association with **AS** cell monolayers with added foetal calf serum (non heat-inactivated). (The number in brackets is the number of parasites measured at each time interval).

% SERUM	AVERAGE SIZE (µm) AT TIME INTERVALS (h)							MAX SIZE (µm)	SURVIVAL
	24	48	72	96	120	144	168		
5	45.4 (25)	42.5 (15)	45.3 (12)	49.8 (10)	50.1 (9)			55	5
10	48.1 (25) +/-8.7	46.8 (25) +/-5.3	46.8 (20) +/-8.98	50.8 (15) +/-3.4	51 (10)	51.6 (4)	50 (2)	55	7
20	46.6 (20) +/-5.5	47.8 (20) +/-5.6	47.5 (18) +/-7.5	52.2 (15) +/-4.32	52 (3)	_	_	60	5
40	47.2 (20) +/-7.9	46.5 (22) +/-6.5	47.5 (24) +/-8.7	52.7 (20) +/-8.1	52.6 (4) +/-8.01	-	-	75	5

TABLE 16 : Motility of free swimming parasites in the presence **AS** cell monolayers with added foetal calf serum (Non heat-inactivated). (600-800 theronts introduced o each well).

% SERUM	TIME (H)						
	24	48	72	96	120	144	168
5	+++	+++	++	++	-		
10	+++	+++	++	+	-		
20	+++	++	+	+	+	-	
40	+++	++	++	+	+	-	

+++ parasite normal
++ parasite alive but slowing down
+ parasite alive but immotile
- parasite dead

TABLE 17:Size of parasites in association with **AS** cell monolayers with added carp serum (non heat-inactivated). (The number in brackets is the number of parasites measured at each time interval).

% SERUM	AVERAGE SIZE (µm) AT TIME INTERVALS (h)						MAX. SIZE (µm)	SURVIVAL (DAYS)
	24	48	72	96	120	144		
5	41.9 (25) +/-5.75	51.2 (22) +/-5.75	47.3 (20) +/-9	51.2 (25) +/-9	55.8 (20) +/-15.2	62.8 (4) +/-2.32	73	6
10	40.2 (15) +/-3.2	46.6 (15) +/-7.7	47.6 (15) +/-9	44.2 (10) +/-6.9	-	-	75	5
20	43.5 (28) +/-6.5	51.4 (25) +/-7.89	48.8 (25) +/-9	59.3 (8) +/-12.7	-	-	85	4
40	45.3 (15) +/-7.1	50.2 (10) +/-8.1	48.2 (8) +/-7.2	51.2 (5) +/-12.1	-	-	75	4

TABLE 18: Motility of free swimming parasites in the presence of **AS** cell monolayers with added carp serum (Non heat-inactivated). (600-800 theronts were introduced to each well)

% SERUM	TIME (H)						
	24	48	72	96	120	144	168
5	+++	+++	+++	++	++	-	-
10	+++	+++	++	++	-	-	-
20	+++	+++	++	++	+	-	-
40	+++	+++	++	++	+	-	-

+++ parasite normal
++ parasite alive but slowing down
+ parasite alive but immotile
- parasite dead

In the all test systems excepting carp serum survival of the free-swimming individuals was either less than or equal to that seen for associated individuals. In 20 and 40% carp serum, however, motile parasites were observed at 120h when no living associated parasites were present. On occasion contamination of the test systems would occur by another holotrichous ciliate, thought to be a T*etrahymena* spp. The swimming behaviour of the ciliates was markedly different and enabled clear differentiation between the two. *Tetrahymena* never displayed sustained contact with the cell layer but thrived within the culture systems always to the detriment of *I. multifiliis*.

EXPERIMENT 4: Behaviour of trophonts within an **AS** culture system

Axenically isolated trophonts of known ages (24-120h) were individually introduced into 24 wells of a 96 well plate containing confluent **AS** cell monolayers and were observed over the following 120h. No signs of growth or further development of immature trophonts was observed, although division of 1 96h trophont was recorded resulting in 2 daughters both of which survived for 120h. Survival of trophonts in this system was directly linked with how long the individual had been within the host epidermis prior to placement in cell culture

MULTILAYER SYSTEMS

Complex multilayered cell systems were selected in attempt to more closely simulate the epidermal layer of the fish host.

EXPERIMENT 5: Theront behaviour within an **AS** culture system combined with different cell underlays

Collagen, gelatin, agar and gelatin coated agar and cell were prepared as described earlier and examined for their ability to support both **AS** cell and parasite growth. Confluent monolayers of **AS** cells were achieved in all systems excepting agar underlay systems. However, theront/cell contact was only observed in the presence of cell aggregates. Cell aggregates were therefore examined in DW, DW:EMEM (50:50) and EMEM. Results are recorded in Tables 19 and 20.

In all aggregate systems contact between cells and parasite was observed, however, determining whether such contact was sustained or transient was difficult due to the density of cells. Parasites were seen to enter and exit the aggregates throughout the period of observation, their aggressive swimming moved the aggregates around within the wells of the culture plate. Survival of the parasite within the aggregate:water system was 48h after this period the cell aggregate had lost its integrity and all parasites in contact were dead; free swimming parasites survived for 96h. Both EMEM:DW (50:50) and EMEM aggregate system supported survival of sustained contact individuals for 120h. No growth was recorded in any test system.

TABLE 19: Size of parasites in association **AS** cell aggregates over time with different media. (The number in brackets is the number of parasites measured at each time interval).

MEDIA	AVERAGE SIZE AT TIME INTERVALS (h)					% CONTACT	SURVIVAL (DAYS)
	24	48	72	96	120		
DW	43 (15)	43.6 (10)	-	-		5-7	2
DW:EMEM 50:50	50.4 (25)	50.4 (25)	50.6 (15)	51.0 (20)	50.6 (15)	2-7	5
EMEM	50.0 (25)	49.6 (20)	50.1 (15)	52.3 (15)	51.0 (10)	0-2	5

TABLE 20: Motility of free swimming parasites in the presence of **AS** cell aggregates over time with different media. (600-800) theronts introduced to each well).

MEDIA	TIME (h)						
	24	48	72	96	120	144	168
DW	+++	++	+	+	-	-	
DW:EMEM	+++	+++	+++	++	++	+	-
EMEM	+++	+++	+++	+	+	-	

+++ parasite normal
++ parasite alive but slowing down
+ parasite alive but immotile
- parasite dead

EXPERIMENT 6: Theront behaviour within an **AS** cell system combined with different cell overlays.

Suspensions of theronts were added to **AS** monolayers as described previously.

After 24h media was carefully withdrawn, removing any free swimming parasites and leaving only individuals in contact with the cell layer, and one of the following overlay systems was then added; agarose; agarose and fish cells; CMC; CMC and fish cells and also cultured fish cells both alive and dead. Results are recorded in Table 21

Survival varied between 3 and 6 days for the overlay systems tested. No significant increase in parasite size was recorded for parasites in sustained contact with the cell layer with agarose or CMC overlay.

EXPERIMENT 7: Theront behaviour and development when exposed to primary explants.

Theront suspensions were added to wells of a 24 well plate that had either a fin or skin explants. 12 wells were used for each tissue type. The results are recorded in Tables 22 and 23

The parasite survived for 5 days in association with both explants. However, free-swimming individuals were alive in the presence of fin explant for 144h, 72h longer than those in the presence of skin. No significant increase in size was seen in either system although parasites in contact with the skin explant achieved a maximum average size of 78.6μm which was the largest average size recorded for any of the culture systems tested.

TABLE 21: Survival of *I. multifiliis* in association with overlay systems.

CULTURE SYSTEM		SURVIVAL (Days)
CELL OVERLAY	Killed AS cells	3
	FHM cells	4
AGAROSE OVERLAY	Without cells	5
	With FHM cells (1×10^6)	6
	With FHM cells (5×10^5)	6
CMC	Without cells	4
	With FHM cells (1×10^6)	4
	With FHM cells (5×10^5)	4

TABLE 22: Size of parasites in association with primary fish cell explant. (The number in brackets is the number of parasites measured at each time interval).

EXPLANT	AVERAGE SIZE AT TIME INTERVALS (h)					SURVIVAL
	24	48	72	96	120	
FIN	42.5 (20)	46.36 (20)	49.2 (10)	46 (10)	47.3 (5)	5
SKIN	45.3 (20)	47.2 (25)	52.1 (12)	78.6 (10)	62.5 (5)	5

TABLE 23: Motility of free swimming parasites in the presence of fish explants. (600-800 theronts introduced to each well).

EXPLANT	TIME (h)					
	24	48	72	96	120	144
FIN	+++	+++	+++	++	++	+
SKIN	++	++	++	-		

+++ parasite normal
++ parasite alive but slowing down
+ parasite alive but immotile
- parasite dead

Of particular interest in this study was the recognition by the introduced parasite of cultured fish cells. That parasites associated chiefly with the thickened areas of the cell layer suggests that this would be more recognisable as representing the multilayered epithelium. Hines and Spira (1973a) suggested that the parasite requires a minimum thickness of epidermis in which to develop as they noted a scarcity of the parasite in heavily infected fish where a large proportion of the epithelium had been sloughed off. Recognition by the theront of a potential host is possibly as a result of short range chemo-attraction, although Wahli, Meier and Schmitt (1991) demonstrated strong phototaxis by the theront, but no chemotaxis when in the presence of live fish. It was therefore stated that location of the host by the parasite was incidental rather than active. However, other studies and observations have recorded positive chemotaxis of the theront, Lom and Cerkasovova (1974) investigated the response of theronts to potential chemo attractants of host origin and recorded positive chemotaxis when theronts were exposed to diluted fish serum. *Tetrahymena* spp are known to be responsive to chemical stimuli associated with the detection of food, mates and unfavourable environments (Levandowsy *et al.,* 1984; Leick and Hellung-Larsen, 1985) as are many other free living ciliates (Van Houten, Hauser and Levandowsky, 1981; Antipa, Martin and Rintz, 1983). It would seem reasonable, therefore to assume that *I. multifiliis* is capable of recognising associated host factors. Further amplification of these signals by an invading theront penetrating the epithelium could be a reason for the aggregated patterns of invasion observed *in vivo* (Buschkiel, 1936: Canella and Rocchi-Canella, 1976; Ewing, Ewing and Kocan, 1988; Matthews, 1994; Matthews, Matthews and Ekless, 1996). Formation of

aggregates of parasites was also observed, within this study, when parasites were in sustained contact with cultured fish cells. It has been suggested that aggregations are formed by the parasite undergoing division whilst in the host epithelium (Stiles, 1893: Ewing, Ewing and Kocan, 1988). That trophonts readily divide within culture media has been previously described (Ekless and Matthews, 1993) indicating that under such conditions division is possible. During this study 48h after addition of theronts to wells, containing **AS** monolayers small individuals of approximately 20µm in size were observed adjacent to larger individuals that were in contact with the cell layer, division, however, was not observed.

Although theronts appeared to be responding to some host factors in cultured fish cells that they failed to develop further indicates that the situation *in vitro* does not mirror closely enough that *in vivo*, where following invasion of the fish host the parasite lies close to or directly upon the basal lamina (Hines and Spira, 1973; Ewing and Kocan, 1986; Chapman, 1984). The effects, therefore, of underlaying the cell monolayer with collagen, was investigated presuming that the collagen or gelatin layer would simulate the basal lamina. The reported ability of collagen enabling cultured cells to resemble more closely the characteristics of the cells *in vivo* was also considered an advantage for this study where cells surrounding the parasite *in situ* are said to retain their differentiated components (Ventura and Paprena, 1987). The use of treated surfaces, in cell culture, such as collagen or gelatin coating, have been reported to have an enhancing effect on cell attachment and growth, also possibly being necessary for the differentiated functions of the cells (Elsdale and Bard, 1972; Gospodarowicz, Delago and Vlodavsky, 1980; Lillie,1980). In this study the time taken for confluency of the cell layer to occur within treated

wells was reduced when compared to that of uncoated wells and cell clumping that was a frequent phenomenon with **AS** cells was avoided. However, the presence of collagen as underlay was considered to be a disadvantage for the purposes of parasite culture, the excellent monolayers formed allowing no areas suitable for parasite/cell contact.

No significant difference in parasite/cell contact for any of the multilayered or monolayer systems was observed. Parasite/ cell contact at 24h in association with cultured **AS** cells varied between 1-21%. Under experimental conditions it is thought that only 20% of the theront population will invade a host and survive to maturity (McCallum, 1982) and of those that successfully invade over 50% die within the period of establishment which is thought to be 45 minutes (Ewing, Lynn and Ewing, 1986). It appears, therefore, that if sustained contact with the cultured cells *in vitro* is comparable in some degree to the position of the invading parasite, the parasite in this study has survived the critical period of establishment, and the death of the parasite *in vitro* appears to be post-establishment.

An increase in parasite size recorded here *in vitro* appeared on comparison with the literature to approximate the 24h stage *in vivo* and not progress beyond, (Ewing, Lynn and Ewing, 1986; Hines and Spira, 1973; Ekless unpublished). Whether this increase in size is due to growth or swelling of the parasite cell due to osmotic imbalance is unclear. However, all parasites alive *in vitro* had toward the end of the culture period greatly vacuolated cytoplasm. The *in vitro* system differs from that *in vivo* as regards number of cells and their position relative to the parasite. *In vivo*, cells are available for phagocytosis immediately following invasion (Reichenbach-

Klinke, 1965; Ewing, Kocan and Ewing, 1985) and within 45 minutes of entry intact epithelium covers the invading theront, providing a specific microenvironment for parasite growth and development (Ventura and Paperna, 1987; Ewing, Kocan and Ewing, 1985). Duplication of this micro environment was attempted in this study by use of more complex 3-dimensional systems such as cellular overlays, cell aggregates and primary explants; however, no further growth over that observed in monolayer systems was observed.

It was considered that the inability of the parasite to grow *in vitro* could be because under these conditions differentiation of theront to trophont had not occurred. It is possible that the stimulus for differentiation maybe absent in the systems tested. However, on addition of immature trophonts to cell monolayers no growth was observed indicating that the lack of parasite growth could be due to a nutrient not differentiation deficit.

Of particular interest in this study was the discovery that immature trophonts could be stimulated to exit the epithelium of a host, an environment that would have presumably fulfilled the developing parasite's nutritional requirements. In all *in vitro* culture systems tested here, the stimulus to cease sustained host contact would have been provided by the presence of culture media and this could have prevented the parasite maintaining contact with the cell layer and therefore opportunities to grow and develop. The trophont appears to demonstrate an ability to sense external changes from its position within the epithelium and exit is obviously an active process, young trophonts exiting within minutes when incubated within EMEM. Host death also appears to be stimulate exit of trophonts, however, under

these conditions mature trophonts exit quite rapidly whilst immature trophonts often die *in situ* (MacLennan, 1937; Ewing, Lynn and Ewing, 1986; Ewing and Kocan, 1987). The presence of an osmotic boundary, may be required by the trophont as a stimulus to remain within the host epithelium. The breakdown of osmoregulation following host death, with the subsequent dissolution of the water:internal environment boundary may be the cue that induces premature exit of the host. However, that trophonts survive and grow within the peritoneal cavity of fish (Dickerson *et al.*, 1985) where the osmotic boundary would be unlike that of the epithelium demonstrates a flexibility by the parasite as regards growth requirements which must be beneficial to the development of an *in vitro* culture system.

CHAPTER 6

ULTRASTRUCTURAL STUDIES OF *I. MULTIFILIIS IN VITRO* IN ASSOCIATION WITH AGAR INDUCED AS CELL AGGREGATES

INTRODUCTION

Ultrastructural studies of *I. multifiliis* have been widely undertaken covering all phases of the parasite's life cycle and particular emphasis has been given to host parasite interaction and a great deal of information is now available on the ultrastructure of the trophont, tomont and theront (Mosevitch, 1965; Chapman, 1984; Chapman and Kern, 1983).

Of particular relevance to this investigation are those studies that examine host invasion (Ewing, Kocan and Ewing, 1986; Ewing and Kocan, 1992); development within the host (Ewing and Kocan, 1986; Chapman, 1984) and the formation and processing of food vacuoles *in vivo* (Lobo da Cuhna and Azevedo, 1988a; Lobo-da-Cuhna and Azevedo, 1993).

That extensive studies have so clearly identified developmental characteristics associated with host residence now allows for comparisons to be made between *in vivo* and *in vitro* situations. As yet the application of electron microscopy to the *in vitro* development of *I. multifiliis* has not been undertaken. The aim of the present study therefore was to use electron microscopy to assess the affect of *in vitro* culture methods on the ultrastructure of *I. multifiliis* when in association with cultured

fish cells. Success would be determined by the presence of developmental markers within the cultured parasite.

Several culture systems were examined for their suitability for transmission electron microscopy.

PREPARATION OF CULTURE SYSTEMS

Host parasite material was prepared in one of the following ways, coverslip, flask or **AS** cell aggregate.

Coverslip. **AS** monolayers were established on melinex film (Agar Scientific) and glass coverslips that were both uncoated and coated with gelatin. Melinex was cut to the required size and sterilised by three ethanol washes before being allowed to air dry within a laminar flow cabinet. Prior to use the pieces of melinex film were incubated with serum containing medium at 37°C overnight followed by 3 fresh media washes, thereby removing any associated toxins. Glass coverslips were washed in ethanol, rinsed in sterile water, air-dried and sterilised overnight in a hot air oven. Coverslips were coated using 2% gelatin solution (Sigma) obtained from bovine skin, which was heated to 37°C, pipetted onto the coverslip, spread over the surface of the coverslip by use of a sterile glass spreader and allowed to dry at room temperature. Monolayers were established by placing the coated or uncoated coverslip within a 3cm petri dish (Cel-Cult) and adding 1.5cm^3 of EMEM and leaving for 1h prior to addition of cells at a concentration of 10^5 cells /cm^3 of medium. The final volume of media within the dishes was 3cm^3.

Flask. 25cm^2 flasks were seeded as previously described in Chapter 5.

AS cell aggregates. Aggregates of cells were formed as described in Chapter 5 and were placed in each well of a 24 well plate containing fresh EMEM immediately before use.

Addition of theronts

100µl of DW containing approximately 750 axenically prepared theronts were added to each well of a 24 well plate. 3cm^2 petri dishes had 200µl of theront suspension added and approximately 1cm^3 of the suspension was added to culture flasks.

PREPARATION FOR ELECTRON MICROSCOPY

All material was fixed for 1h at 20°C in 2.5% glutaraldehyde in 0.1M cacodylate buffer pH 7.2, containing 3mM calcium chloride. Fixation of monolayers took place *in situ* following removal of the medium; all procedures involving cell aggregates, however, took place within specimen vials. Following fixation cells were washed 3 times in cacodylate buffer, post fixed for 1h in 1% osmium tetroxide in 0.1M cacodylate buffer at 4°C, washed again 3 times in cacodylate buffer and then dehydrated through a graded series of ethanol (30%-100%) with 3 incubations within absolute alcohol.

Monolayers both flask and coverslip were embedded within Araldite A2201/Z CY212 (Bio-Rad). All embedding procedures and polymerisation of flask monolayers took place *in situ*. However, for the final embedding and polymerisation of coverslip monolayers the coverslips were removed from the petri dish and inverted over an araldite filled plastic container ensuring close and even contact between resin and cell layer thereby preventing the formation of air bubbles. Following polymerisation

the coverslips were peeled away from the plastic container leaving the monolayer upon the surface of the resin. Once removed from the plastic container, cell areas could be identified for EM preparation. Within culture flasks areas of parasite/ cell association were pin pointed by eye and the relevant areas removed from the flask by hacksaw and shaped by razor blade for sectioning.

Cell aggregates were embedded within Spurr's epoxy resin (Agar Scientific) in 1h stages with the following proportions of 2:1 spurrs: absolute alcohol, 1:1, 2:1, 100% resin x 3. Polymerisation took place at 70°C for 9h.

Sections were cut using a Reichert Ultra-Cut microtome using a glass knife. Semi-thins for light level observations were stained with 1-% methylene blue, thereby allowing precise location of the parasite within the section. Ultra -thins were collected onto copper grids double stained with saturated aqueous uranyl acetate and 1 % lead citrate for 20 minutes each and examined and photographed on a Jeol 1200 transmission electron microscope at 80Kv. All micrographs were developed on Ilfospeed grade 3 paper.

System selection

The use of monolayers in flasks and on both coated and uncoated coverslips provided few areas suitable for parasite/cell association, parasites appearing to prefer the areas of cell thickening occurring where coverslip edges touched the floor of the petri dish. Processing of cell monolayers for EM was expensive in terms of both volumes of fixative and resin and the time involved in preparation. It was therefore decided that cell aggregates were the best system for ultrastructural

studies, showing consistently high levels of parasite/cell contact as well as being easy to handle during preparation for EM.

Cell aggregates were therefore used in order to examine ultrastructural changes occurring over the period of parasite survival under these conditions. Cell aggregates were fixed every 24h up to a maximum of 120h and observations made on cell integrity, food vacuole formation and content, and association between parasite and cell.

Parasites were located successfully within **AS** cell aggregates at each time interval.

24h

Introduced parasites were seen in very close association with the cell aggregates and this is evident when examining semi thin methylene blue sections, 5 parasites are clearly visible within this one section (Fig 10). The parasite appears to be located within a cavity surrounded to a varying degree by cellular debris. The cellular integrity of the aggregate appears to vary within the section. Examination of parasites within the aggregate at this stage demonstrates a resemblance to the theront (Fig. 11), showing an elongated shape, posterior positioning of the micro and macro nucleus, peripheral crystalline mucocysts and the contractile vacuole occupying most of the mid region. Within both parasites in Fig.11 large vacuoles containing diffuse matter and smaller vacuoles containing quite dense matter are visible, suggesting that the parasite is attempting to gain nutrients by phagocytosis within this culture system. Dense glycogen rosettes were visible throughout the cytoplasm in every parasite examined at this stage along with mitochondria, and endoplasmic reticulum (ER) (Fig12). The pellicle of the cell appears to have maintained its integrity thus far.

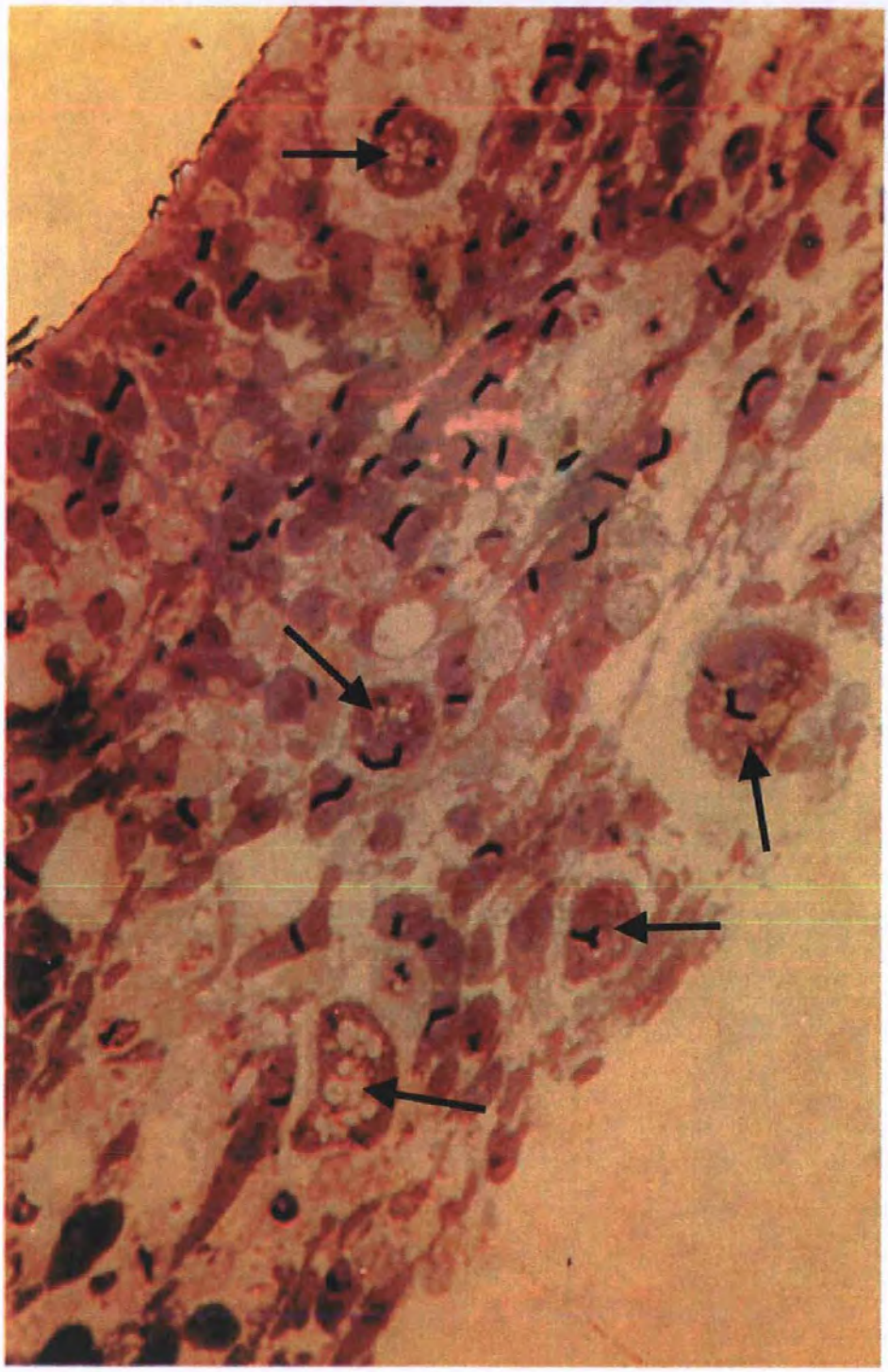


FIGURE 10: Methylene blue section of a cultured **AS** cell aggregate at 24h. Five introduced parasites are clearly visible firmly located within the aggregate (arrows). (x10).

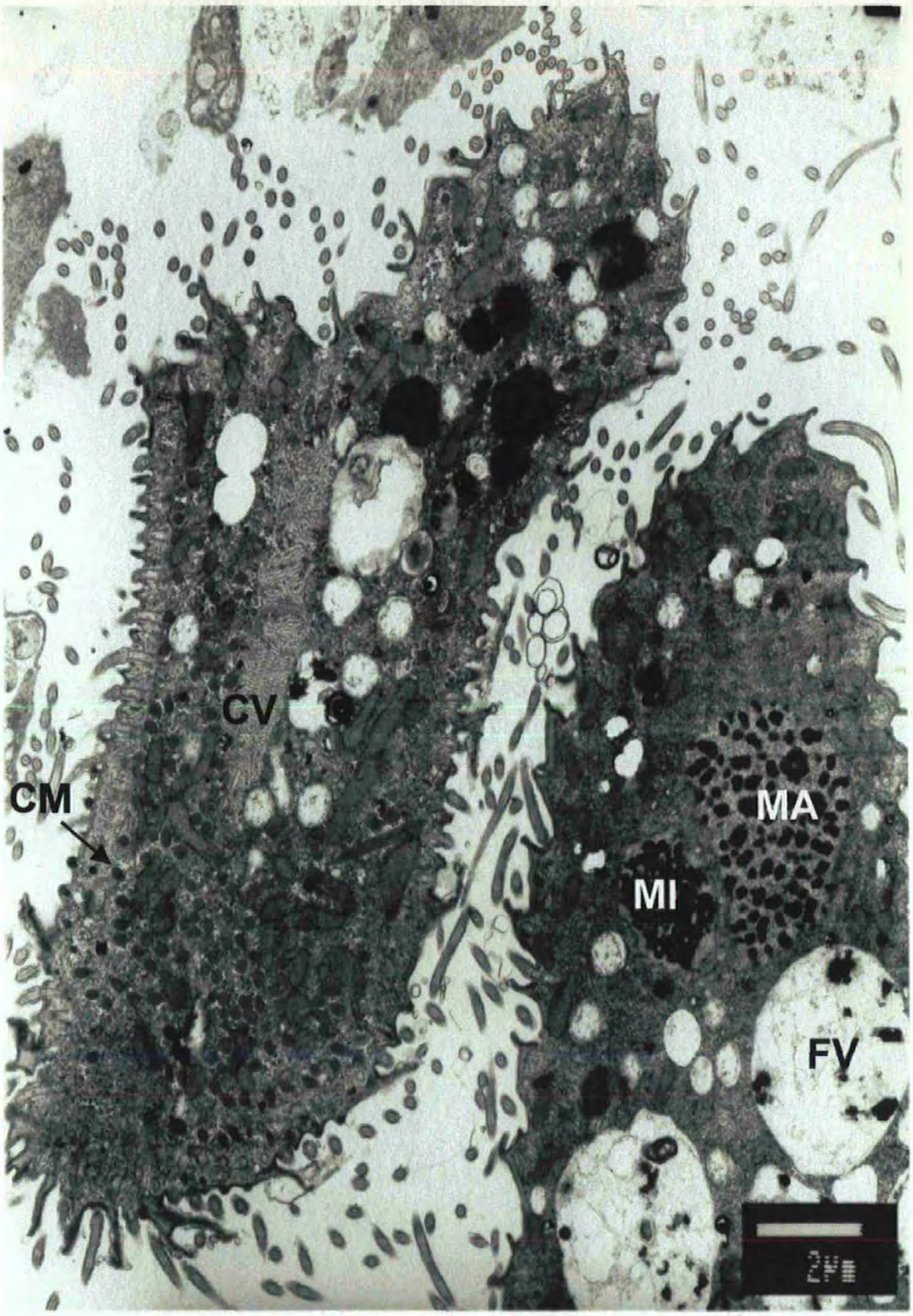


FIGURE 11: Two parasites in association with **AS** cell aggregates at 24h. (x3000). Food vacuoles (FV), macronucleus (MA) and micronucleus (MI), contractile vacuole (CV) and peripheral crystalline mucocysts (CM) are indicated. Scale bar 2µm.

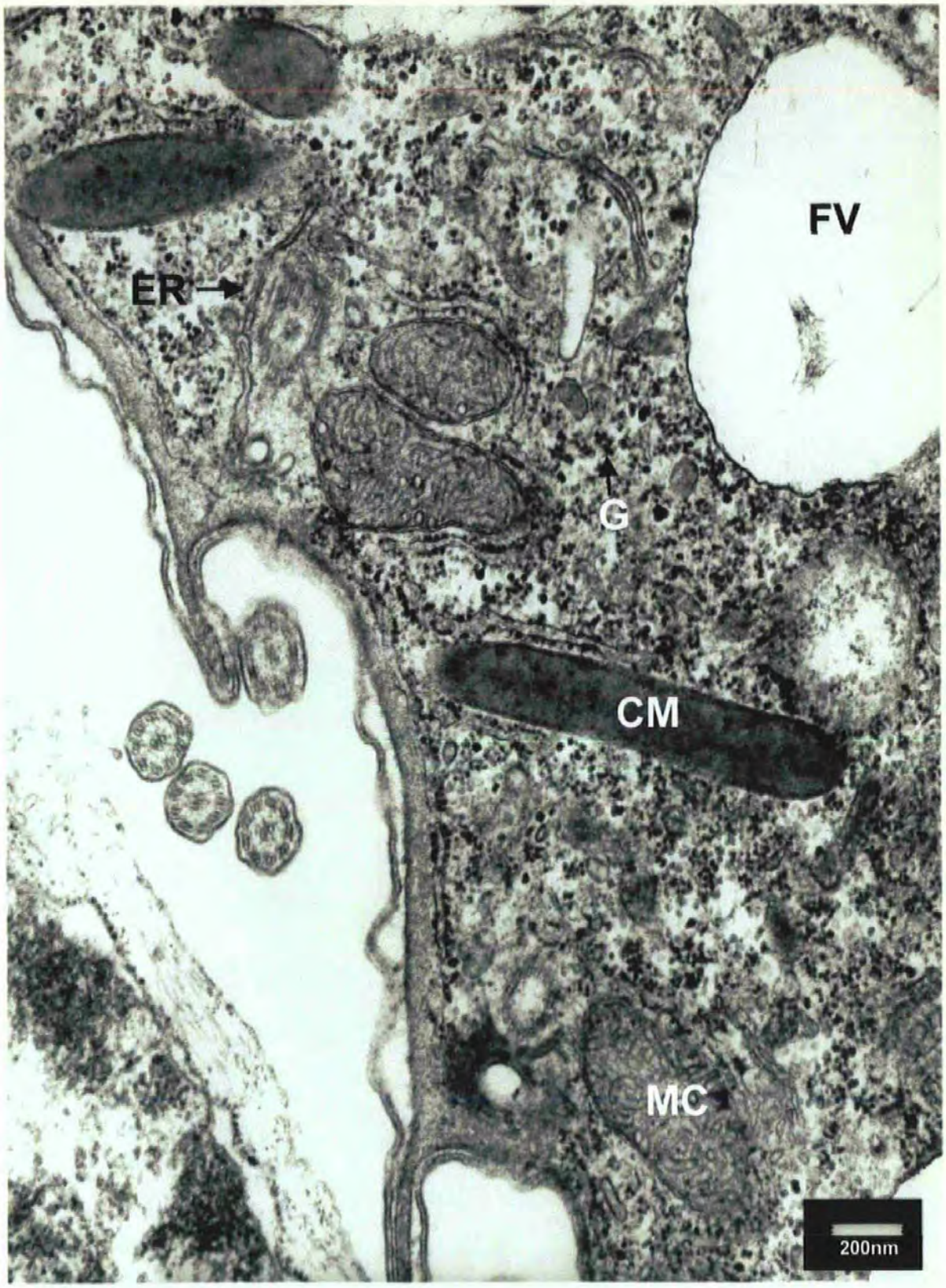


FIGURE 12: Higher magnification of a 24h parasite in association with **AS** cell aggregate. Glycogen rosettes (G), endoplasmic reticulum (ER), food vacuoles (FV), mitochondria (MC) and crystalline mucocysts (CM) are indicated. (x20k). Scale bar 200nm.

48h

As age of the culture increases increased numbers of food vacuoles are present in the cytoplasm of all parasites examined, with a trail of food vacuoles forming almost a ring around the macronucleus (Fig.13 and 14). Food vacuoles at this stage contain predominantly what appears to be partly condensed cellular matter, very few vacuoles with dense contents were observed. Both Figures have indications that fusion of food vacuoles is occurring and in Fig. 14 there is evidence that egestion is occurring. Closer examination of the vacuoles of a 48h parasite demonstrated the presence of protein whorls these could be indicative of autophagocytosis (Fig.15). Cytoplasm integrity appears to still be intact, and mitochondria, glycogen rosettes and ER are still clearly visible. A number of crystalline mucocysts were still observed at the cell periphery.

72h

The parasite is still firmly located within the cell layer with cilia in close contact with the surrounding tissue. However, all parasites observed at this stage are showing increased vacuolation of the cytoplasm. The pellicle including the cilia appear to be intact and cell integrity is still quite good, glycogen rosettes are visible and all cell organellae are present providing some evidence of metabolic activity. Portions of the contractile vacuole system are visible both centrally and at the periphery and injection canals can be clearly seen (Fig.16). Food vacuoles containing disperse material are still evident many of which appear to be fusing.

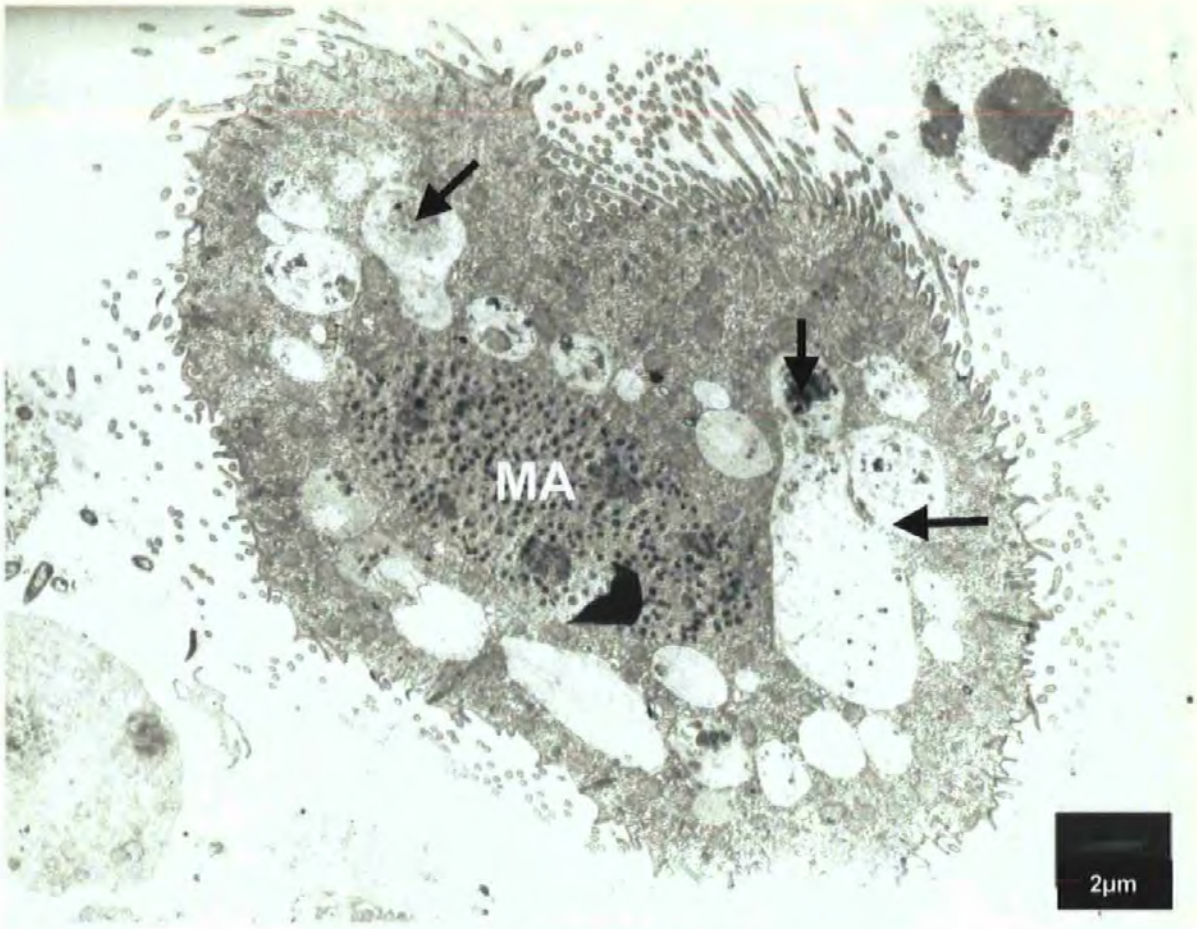


FIGURE 13: A 48h parasite with a number of food vacuoles forming a ring around the nucleus. Two food vacuoles are in the process of fusing (arrows). (x2000). Scale bar 2μm.

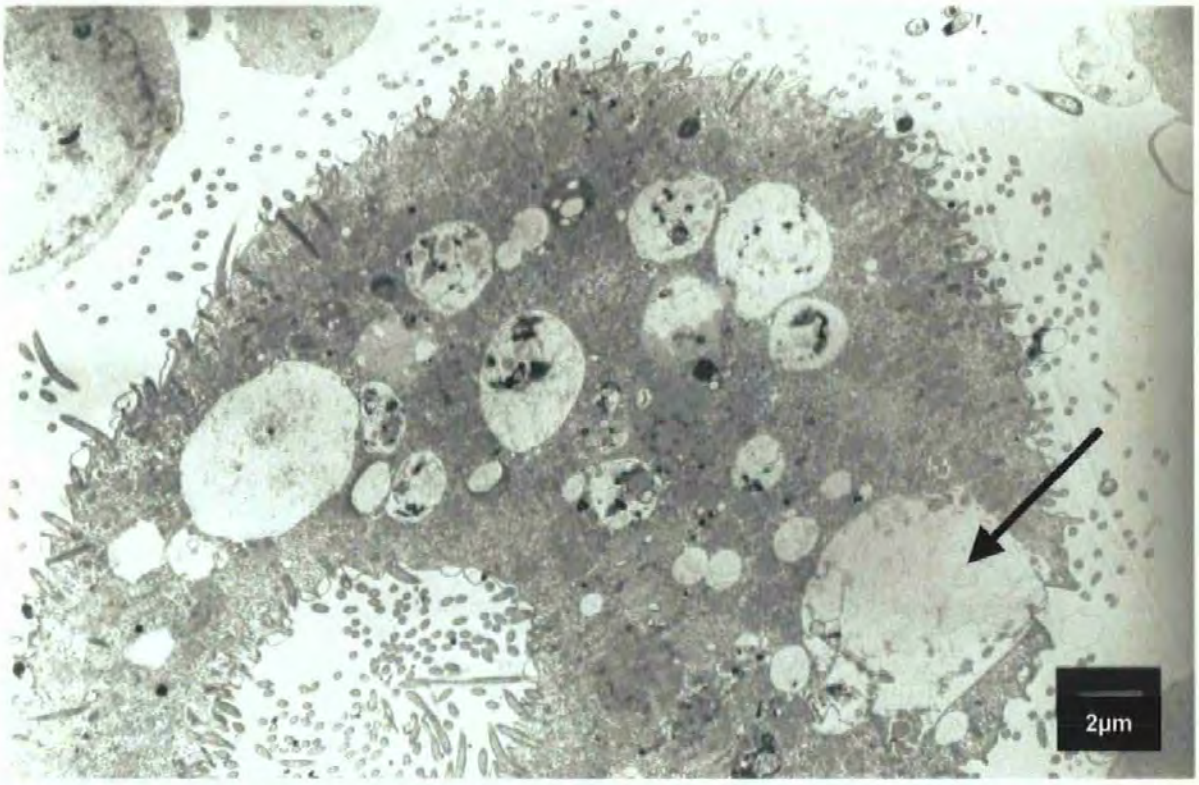


FIGURE 14: A 48h parasite in association with an **AS** cell aggregate with a food vacuole in the process of egesting (arrow). (x2000). Scale bar 2μm.

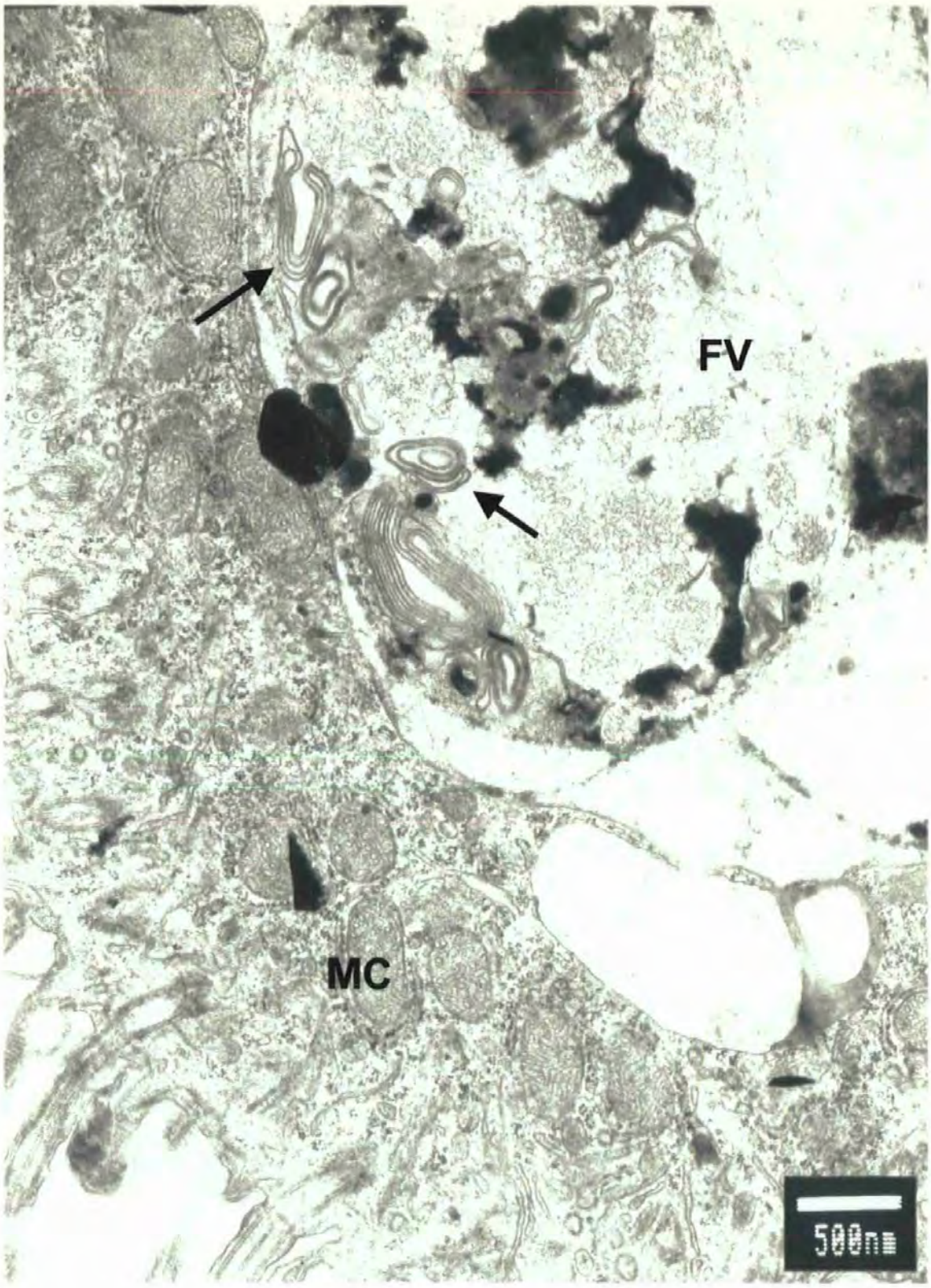


FIGURE 15: Protein whorls (arrows) within a food vacuole (FV) of a 48h parasite. A number of food vacuoles closely adjacent appear to be in the process of fusing. (x12k). Scale bar 500nm.

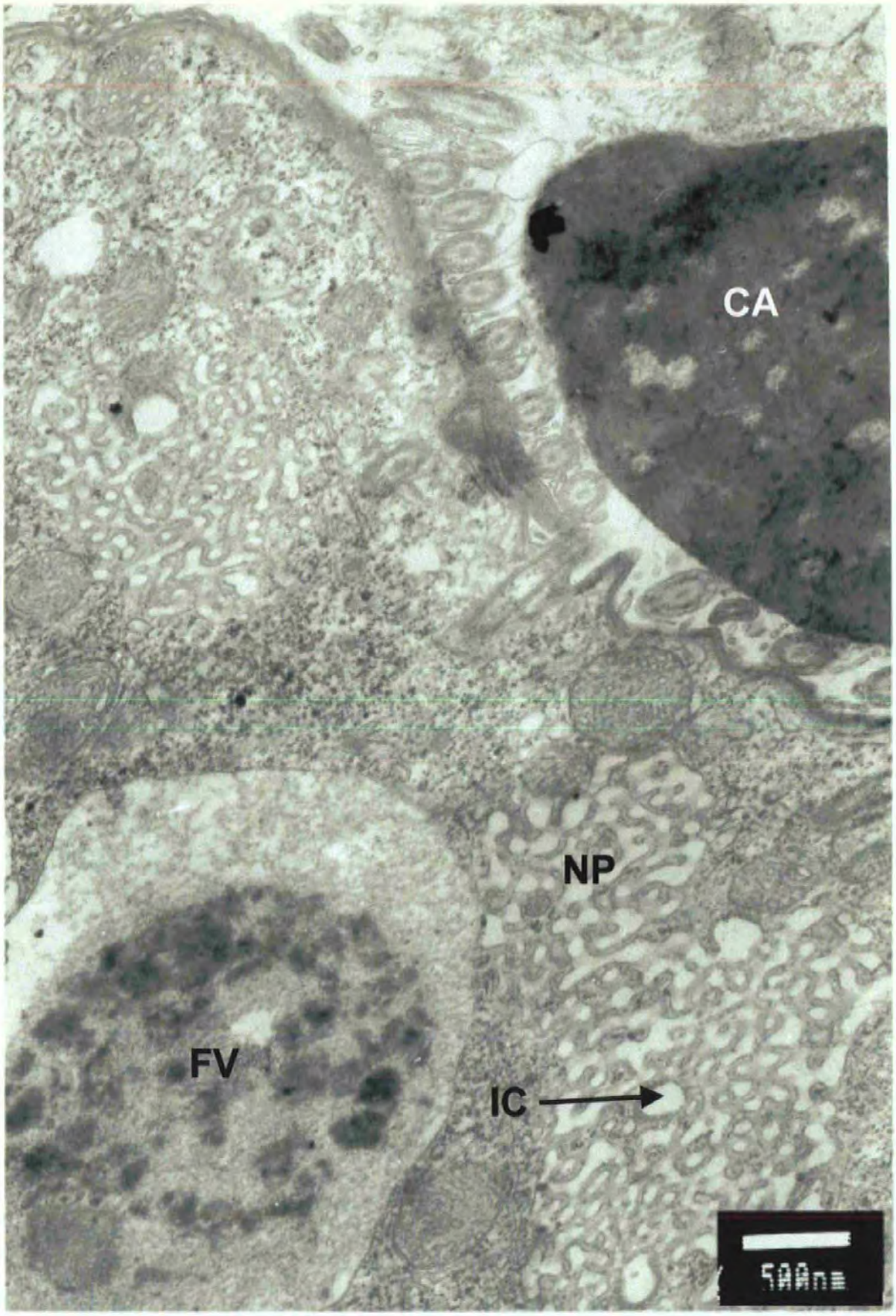


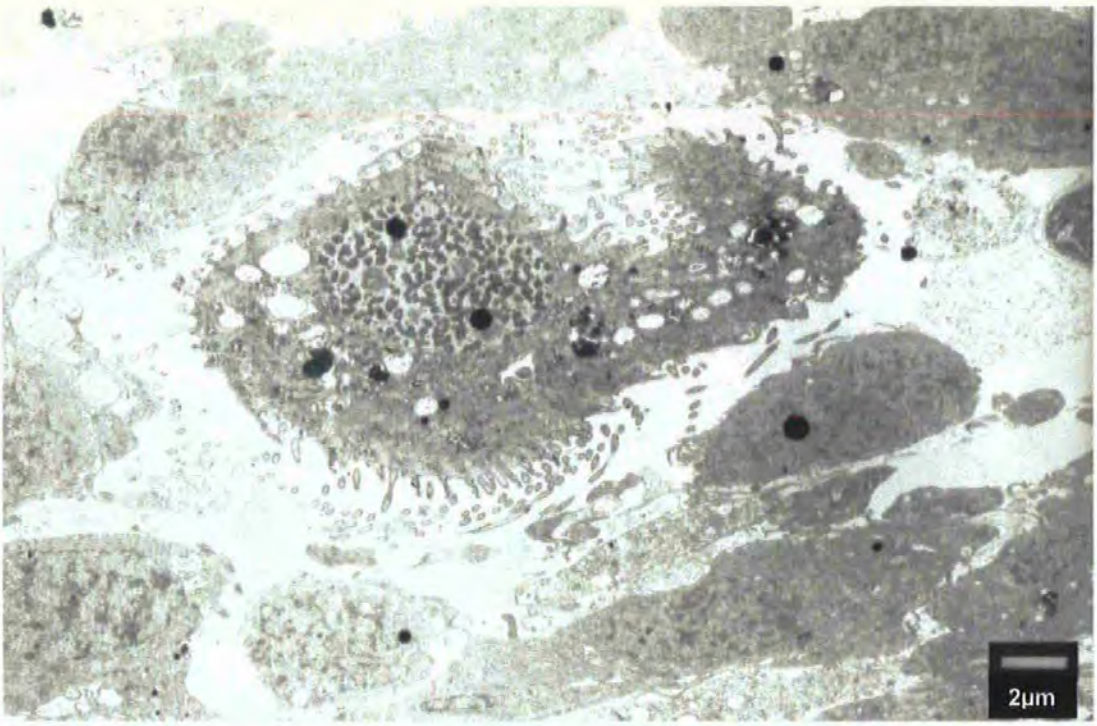
FIGURE 16: 72h parasite demonstrating the close contact between parasite and cell aggregate (CA). The contractile vacuole system is clearly visible at the periphery of the cell, the nephridiaplasma (NP) and injection canals (IC) are indicated. A food vacuole is also indicated with relatively dense contents. Mitochondria, glycogen and ER are still present. (x12k). Scale bar 500nm.

96h

The majority of parasites seen at this stage were showing advanced cellular breakdown, this is clearly visible when comparing parasites at 24 and 96h (Fig.17). Extensive vacuolation of the cytoplasm is visible within the 96h parasite, intact food vacuoles are still present but appear largely empty. Mitochondria appear still intact are visible at the periphery of the cell and a small amount of the contractile vacuole system is also present. Dissolution of the cell appears to occur from the inside out, the cell membrane appearing to be intact. The amount of cytoplasmic vacuolation in the 96h parasite is in direct contrast to the relatively dense cytoplasm of the 24h parasite where only small discrete vacuoles are observed. The presence of xenosomes free within the cytoplasm of the parasite was first recorded at 96h (Fig. 18).

120h

All parasites at this stage were extensively vacuolated although all organellae were still visible. No parasites were alive in contact with cell aggregates after this time.



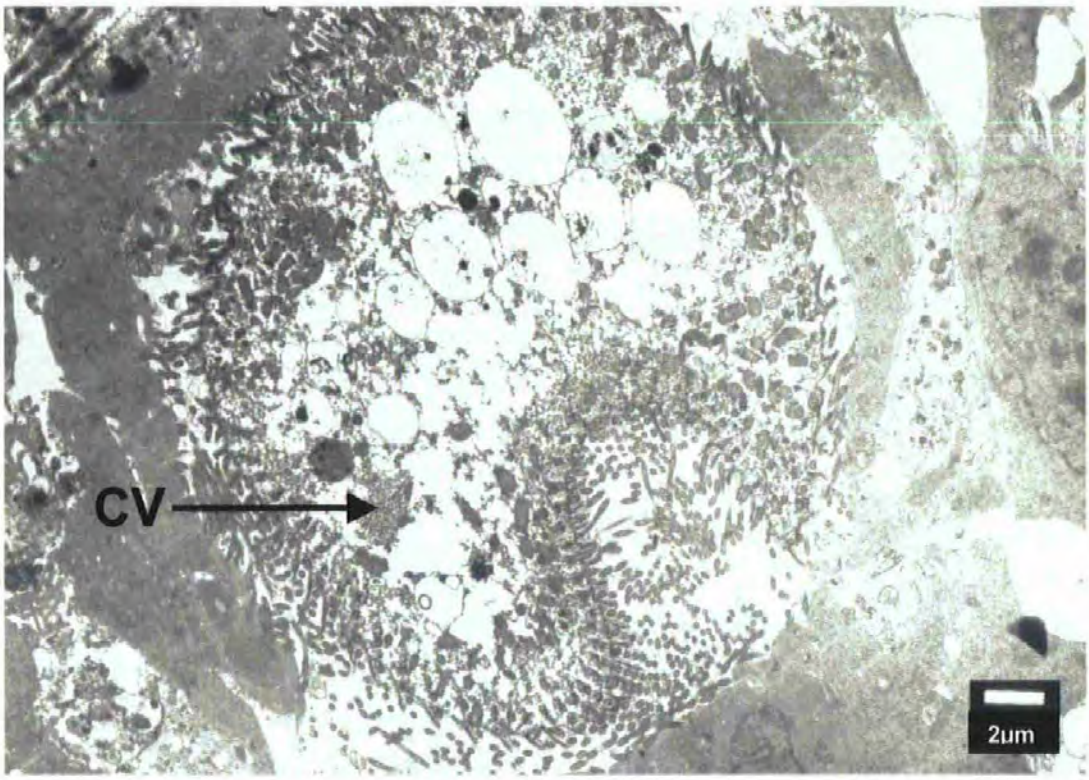


FIGURE 17: A comparison between a 24h and 96h parasite, note the increased vacuolation in the 96h parasite. Mitochondria are still visible at the periphery of the 96h cell and a portion of the contractile vacuole system is indicated (CV). (x2000). Scale bar 2µm.

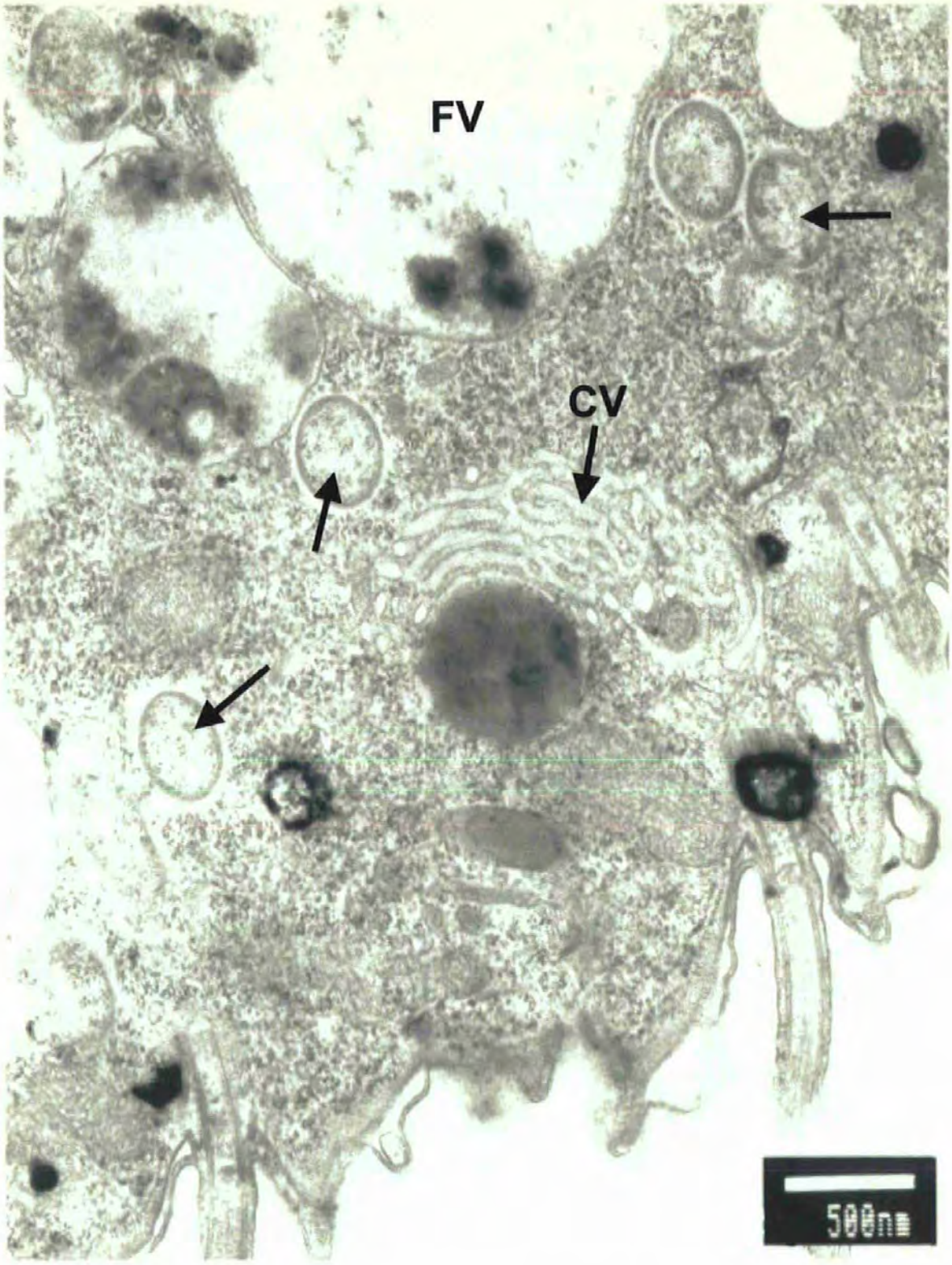


FIGURE 18: A 96h parasite with xenosomes visible free within the cytoplasm of the cell (arrows). Food vacuoles (FV) and part of the contractile vacuole (CV) system are also visible. (x12k). Scale bar 500nm.

It would appear from photomicrographs taken in this study that theronts introduced into culture systems penetrate the cell aggregates and maintain close contact with the cells for up to 120h, providing evidence of recognition by the parasite of the cultured fish tissue as a potential host. This recognition correlates well with observations in Chapter 5 regarding the occurrence of sustained cell/ parasite contact within the culture systems.

The presence of food vacuoles within the cytoplasm of the cultured *I. multifiliis* indicates that attempts were made to gain nutrients from the surrounding environment. The different stages of the digestive cycle of ciliates has been closely examined, focusing predominantly on *Tetrahymena* (Muller and Rohlich, 1961; Elliott and Clemmons, 1966; Mislan and Smith-Somerville, 1986) and *Paramecium* (Fok, Lee and Allen, 1982; Muller and Toro, 1961). The digestive cycle *of I. multifiliis* tomonts following host exit has been described in detail by Lobo da Cuhna and Azevedo (1988a and 1993) who classified food vacuole types as A, B and C. Type A are the youngest food vacuoles within the cytoplasm containing undigested host cells, these condense to form type B vacuoles which expand due to re-hydration to form type C vacuoles which contain a diffuse mass of digestive substances surrounded by an empty space. This particular classification of food vacuole may not be applicable within this study, as a different stage in the life cycle is being examined. It must also be noted that all studies on ciliate digestive cycles have been made using known acceptable food sources, here, however, the parasite was supplied with an atypical food source, cultured fish cells. The types of food

vacuole formed by *Tetrahymena* differ depending upon the available food source (Elliott and Clemmons, 1966).

Vacuoles within *I. multifiliis* containing matter resembling cultured fish cells were observed here *in vitro* and could be classified as type A vacuoles, indicating successful ingestion. Few condensed vacuoles that could equate to type B were observed, although some vacuoles did possess more condensed contents than others (Fig 16). Fusion of large vacuoles (Fig 13) was observed within the cultured parasite and could therefore be type C vacuoles, as fusion of type C vacuoles is common prior to egestion (Mislan and Smith-Somerville, 1986; Lobo da Cuhna and Azevedo, 1993). If this framework of vacuole type can be applied *to I. multifiliis* within the *in vitro* culture system it would appear that all vacuole types are present and with the evidence that egestion has occurred (Fig.14), it would appear that a digestive cycle is present. Lobo da Cuhna and Azevedo (1993), by the application of enzyme cytochemistry detected acid phosphatase activity predominantly within type B vacuoles, indicating that it is here that most digestion occurs, and therefore as regards nutrient gain this is the most important stage. Application of enzyme cytochemistry, here, to these cultured parasites would be extremely useful in detecting the presence of and level of enzymatic activity within parasites under these conditions and would provide a clearer picture of the digestive cycle *in vitro*.

Although the presence of protein whorls within the food vacuoles of cultured *I. multifiliis* could be indicative of autophagocytosis no recognised organellae were seen within vacuoles. In cultures of *Paramecium* and *Tetrahymena* autophagosomes can be formed as a result of clonal age (Fok and Allen, 1981) or

cell starvation (McArdle, Berquist and Ehret, 1980), or as a normal event allowing recycling of organelles. Such vacuoles are usually distinguished by the presence of mitochondria, ribosomes, glycogen granules and whorls of cytoplasmic membranes. It is possible, therefore that the vacuoles observed within this study may not be indicative of cell starvation and could be a normal event or they may contain matter taken in from the surrounding host cells.

Although the location and attempts to feed within the cell layer indicate behavioural changes of the introduced parasite no ultrastructural indicators of development were observed. Peripheral crystalline mucocysts are recorded as abundant within the invading theront, scarce immediately following entry and then significantly increasing over the period of host residence (Ewing and Kocan, 1992). In the 24h cultured parasite large numbers of crystalline mucocysts are clearly visible (Fig11), and close observation provided some evidence of mucocyst discharge, there was, however, no indication that the large scale discharge usually associated with host invasion had occurred (Ewing, Kocan and Ewing, 1985; Matthews, 1994). At all other time intervals observed here, crystalline mucocysts are present but not abundant, this disappearance could be due to the inability of the theront to maintain mucocysts in a ready state for invasion for periods longer than the duration of theront viability seen in normal transmission (Lom and Cerkasova, 1974; Ewing and Kocan, 1992). Secretory mucocysts were not observed at any stage. Other developmental markers such as curvature of the macronucleus and the absence or presence of the organelle of Lieberkühn were not located at EM level. Parts of the contractile vacuole system were however, located at each time interval and could

indicate a possible increase in the osmoregulatory system over time, but further investigations would be necessary to fully qualify this statement.

The demise in internal cell structure clearly visible at 96h indicates a break down in the internal regulation of the parasite cell. Such a breakdown could be due to starvation, the cell culture system not providing specific nutrients either for theront differentiation or for trophont development. *Amyloodinium ocellatum*, an external parasite of marine fish survives in a culture system very similar to the aggregate system tested here, and appears to gain sufficient nutrients from cultured marine fish cells to complete its entire lifecycle (Oestmann and Lewis, 1996). That *Tetrahymena* spp was occasionally present as a contaminant in all systems tested here, and thrived within the presence of cultured cells indicates that the **AS** cell aggregates are a source of nutrients. *Tetrahymena* is however a facultative parasite and therefore as such must be an opportunistic feeder capable of exploiting a variety of nutrient sources. *I. multifiliis*, is an obligate parasite and appears to have more specific nutrient requirements that are not met within the systems tested here.

Of interest within this study was the presence of xenosomes within the cytoplasm of a 96h parasite. Lobo da cuhna and Azevedo (1988b) observed xenosomes within all life cycle stages of one isolate of *I. multifiliis* and in particular a close association between xenosomes and glycogen deposits was observed within the trophont. The exact function, whether it be detrimental or beneficial, of these observed xenosomes is not yet known, however that the numbers of xenosomes increased over culture time maybe of significance.

CHAPTER 7

GENERAL DISCUSSION

The *in vitro* culture of *I. multifiliis* was not achieved during the period of this study. Monophasic media alone and in the presence of cell associates failed to allow full differentiation of theront to trophont, and trophont growth and development was not supported. However, behaviour within the cell culture systems examined here, indicates recognition of the fish cells by the theront as potential host material, with sustained contact between parasite and cell being clearly evident within minutes of introduction into the culture system.

That survival of all stages was extended following media incubation indicates that all stages are capable of a degree of nutrient uptake from the surrounding environment. The free living stages, theront and tomont are usually identified in the literature as non-feeding stages, but it appears that if required, nutrient uptake can occur sufficient to extend the duration of each stage over that seen within the aquatic environment. Modification of media could provide longer maintenance of all life cycle stages providing some possibility of a management system.

The mode of nutrient uptake within culture systems for tomont and trophont appears to be phagocytic as evidenced here by TEM and carmine red tests, this would be as expected as both stages are capable of ingesting host tissues. The state of development of the theront's buccal apparatus on invasion is a more contentious issue, Canella and Rocchi-Canella (1976) state that the cytostome

develops 18-32 hours after invasion at 26°C; MacLennan (1935) believed that the theront is fully able to ingest host tissue immediately following entry. Ewing, *et al.*, (1985) identified the presence of host tissue within a trophont 40 minutes post infection, Matthews *et al.* (1996), however, did not observe phagocytosis of host cells until 7-8h post infection. The actual status of the buccal apparatus of the theront could be a critical factor in the culture of this stage of the life cycle as it would be a limiting factor on nutrient uptake in the form of ingestion of cells. If we presume that no differentiation of theront to trophont occurred in the culture systems tested, would an immature phagostome be capable of exploiting the food source available within the culture systems?

The endurance of the organelle of Lieberkühn for a number of hours post infection indicates a role in the establishment of the parasite. It has been postulated (Matthews, 1994) that the organelle, is involved in the feeding, possibly as a source of enzymes, the release of such enzymes serving to enlarge the cavity and provide cells for ingestion. The organelle is ideally positioned within the oral cavity of the theront to fulfil such a role. It could be that within the culture system the organelle is not discharged following contact of the cultured cells, and therefore the theront is unable to exploit the cells present. However, immature trophonts on introduction to a monolayer system were also unable to gain sufficient nutrients to complete their growth and development, indicating that differentiation of theront to trophont is not a limiting factor within culture systems.

The presence of food vacuoles within the cultured parasite raises queries regarding its terminology. If an introduced theront appears by its behaviour to recognise cultured tissue as a potential host and is attempting to feed off the cell

culture systems it has essentially become parasitic and could therefore be classified as the parasitic phase of the life cycle, the trophont. Does a theront immediately differentiate into a trophont upon entering a host or on penetrating a cell aggregate? It is likely that the change from one to the other is a gradual process fuelled by the uptake of host cells. Attempts to feed could, therefore be, an initial stage in the process of differentiation, all subsequent developments being dependant upon attaining a minimum level of nutrition. It would appear, therefore, that within the culture systems tested here this minimum level of nutrition was not achieved, and this could indicate that a significant factor is either absent or not present at levels required to sustain the culture. However, all culture systems tested provided nutrients sufficient for the contaminant *Tetrahymena* to thrive, whilst even the presence of crude explants of carp fin and skin failed to support growth and development of *I. multifiliis*. Indicating that a combination of factors is likely to be responsible for culture failure. Presuming that nutrients are available for uptake and that the parasite is attempting to continue its lifecycle what prevents it?

Within the host, the presence of an osmotic boundary could be fundamental in the growth and development of the parasite. The exit of immature trophonts following media incubation indicates that the parasite is able to sense changes to the external environment and therefore must have some degree of contact with the aquatic environment throughout its period of residence within the host epithelium. Could this premature exit of immature trophonts on incubation within medium indicate that the trophont is making an active choice and perceives the media as a nutrient source and one that is possibility better than its' present host? This could be possible, trophonts *in situ* are known to demonstrate a degree of site selection,

80% of trophonts moving to a position adjacent to afferent blood vessels during the period of host residence (Ewing and Kocan, 1986). It is likely that the culture systems tested here did not simulate closely enough the conditions *in vivo*, and to achieve success methods will have to be developed to create more realistic 3-dimensional culture systems, possibly incorporating a cell:water interface.

Of interest within this study was the success of an autumn isolate of the parasite which completed 39 cycles within the laboratory. Seasonal fluctuations in infection levels have been reported in the literature (Elser 1955; Valtonen and Keränen, 1988) with outbreaks often occurring in the spring as the water temperature rises. The duration of the lifecycle of the parasite is dependant upon temperature, increasing temperatures leading to a shortened life cycles and increased numbers of theronts per cyst and therefore higher chances of infection (Nigrelli *et al.*, 1976). The isolation of *I. multifiliis* and removal to the laboratory where infections proceeded at 20°C could simulate the conditions in spring, providing an extremely viable source of parasite for laboratory investigations.

That laboratory isolates were chiefly gained from one source, (River Erme sticklebacks), could be of significance when considering the strain specific culture demands of *Amyloodinium ocellatum* (Oestmann and Lewis, 1996). A gulf coast isolate of the parasite failed to differentiate and grow in a culture system that had previously proved successful, therefore the possibility that *I. multifiliis* strains exist must be an issue when considering *in vitro* culture methods. The possibility that strains of *I. multifiliis* exist was initially put forward by Nigrelli *et al*, (1976) and more recently Dickerson, Clark and Leff (1993) and Clarke, Lin and Dickerson,

1996) have identified serotypic variation in particular antigens amongst a number of isolates, providing a method for identifying strains.

The need to develop and preserve strains of *I. multifiliis* is therefore of the utmost importance and cryopreservation of such strains would provide an excellent long term holding method. Techniques for the cryopreservation of certain protozoa such as *Tetrahymena* and *Paramecium* are well established (Daggett and Nerard 1992a,b). Initial studies within this laboratory demonstrated a tolerance by theronts and tomonts for the cryoprotectants glycerol and DMSO but further work is needed to develop a suitable freezing program.

I.multifiliis is frequently described in the literature as a cosmopolitan parasite of fresh water fish, infecting fish in nearly all regions of the world. Even with the existence of strains, *I. multifiliis* appears to have considerable flexibility as regards its ability to exploit many different types of fish hosts and therefore cell types as a food source. The reported ability of the parasite to successfully complete its development within the peritoneal cavity of fish either experimentally (Dickerson, *et al.*, 1985) or naturally (Hoffman 1967) also indicates adaptability by the parasite as regards host material. The presence of such flexibility and the observed attempts of introduced theronts to exploit the culture systems tested here must provide optimism that an *in vitro* culture system can be developed within the near future.

REFERENCES

Allison, R. and Kelly, H.D. (1963). An epizootic of *Ichthyophthirius multifiliis* in a river fish population. *Progressive Fish Culturist.* **25**, 149-150.

Antipa, G.A., Martin, K. and Rintz, M.T. (1983). A note on the possible ecological significance of chemotaxis in certain ciliated protozoa. *Journal of Protozoology.* **30**, 55-57.

Babich, H. and Borenfreund, E. (1991). Cytotoxicity and genotoxicity assays with cultured fish cells: a review. *Toxicology In Vitro.* **5**, 91-100.

Bauer, O.N., Musselius, U.A., Strelkov, Y.A. (1973). *Diseases of Pond Fishes.* Keter Press. Jerusalem.

Beckert, H. (1975). Observations on the biology of *Ichthyophthirius multifiliis* (Fouquet), its susceptibility to Ethoxyquin, and some immunological responses of channel catfish *Ictalurus punctatus* to this parasite. Ph.D Dissertation. The University of Southwestern Louisiana, Louisiana.

Beeler, C.R. (1980). Fish Parasites: The cryopreservation of *I. multifiliis.* *Fish Health News.* **9**, i

Bernoth, E.M. (1991). Possible hazards due to fish drugs. *Bulletin of the European Asociation of Fish Pathologists.* **11**,17-21

Brown, E.M. and Gratzek, J.B. (1980). *Fish Farming Handbook. Food, Bait, Tropicals and Goldfish.* AVI Publishing Co., Westport, Connecticut.

Burgess, P. (1992). *Crytocaryon irritans* Brown, 1951, ciliophora: transmission and immune response in the mullet *Chelon labrosus* (Risso, 1826). PhD Thesis University of Plymouth.

Burkart M.A., Clark T.G. and Dickerson H.W. (1990). Immunization of channel catfish *Ictalurus punctatus* Rafinesque, against *Ichthyophthirius multifiliis* Fouquet killed versus live vaccines. *Journal of Fish Biology.* **13**, 401-410.

Buschkiel, A.L. (1910). Beitrage zur Kenntnis des *Ichthyophthirius multifiliis* Fouquet. *Archiv fur Protistenkunde.* **21**, 61-102.

Butcher A.D. (1943). Observations on some phases of the life cycle of *Ichthyophthirius multifiliis* (Fouquet 1876) a ciliate protozoan parasite of freshwater fish. *Australian Zoologist.* **10**, 125-139.

Canella, M.F. and Rocchi-Canella, I. (1976). Contributions a la connaissance des Cilies. VII Biologie des Ophryoglenina (Cilies Hymentostoes Histphages). L epoque des ultrastructures' de la ciliotologie: pourra-t-elle aboutir a une systematique

phylogenetique ou s'agit-il d'une flatteuse illusion? *Ann. University Ferrera Seg. II Biology Anim.* **3**, 1-510.

Chapman, G.B. and Kern, R.C. (1983). Ultrastructural aspects of the somatic cortex and contractile vacuole of the ciliate *Ichthyophthirius multifiliis*, Fouquet. *Journal of Protozoology*: **30**, 481-490.

Chapman, G.B. (1984). Ultrastructural aspects of the host-parasite relationship in ichthyophthiriasis. *Transactions of the American Microscopical Society.* **103**, 364-375.

Chapman-Andresen, C. and Nilsson, J.R. (1968). On vacuole formation in *Tetrahymena pyriformis* GL. *Comptes Rendus des Travaux du Laboratoire Carlsberg.* **36**, 405-432.

Clark T.G., Lin, T-L, and Dickerson, H.W. (1996). Surface antigen cross-linking triggers forced exit of a protozoan parasite from its host. *Proceedings of the National Academy of Sciences USA.* **93**, 6825-6829

Clayton, G.M. and Price, D.J. (1988). Pleiotropic effect on scale pattern genes in common carp: susceptibility to *Ichthyophthirius multifiliis* infection. *Heredity.* **60**, 312 (Abstract).

Clayton, G.M. and Price, D.J. (1992). Interspecific and intraspecific variation in resistance to ichthyophthiriasis among poeciliid and goodeid fishes. *Journal of Fish Biology*. **40**, 445-453.

Clayton, G.M. and Price, D.J. (1994). Heterosis in response to *Ichthyophthirius multifiliis* infection in poeciliid fish. *Journal of Fish Biology*. **44**, 59-66.

Cross, D.G. and Hursey, P.A. (1973). Chloramine T for the control of *Ichthyophthirius multifiliis*. *Journal of Fish Biology*. **5**, 789-798.

Cross, M.L. and Mathews, R.A. (1992). Ichthyophthiriasis in carp, *Cyprinus carpio L.*: fate of parasites in immunized fish. *Journal of Fish Diseases*. **15**, 497-507.

Daggett, P-M. and Nerard, T.A. (1992a). *Cryopreservation of Paramecium*. In: *Protocols in Protozoology*. (Ed.s J.J. Lee and A.T. Soldo), Society of Protozoologists, A-90.1- A-90.3.

Daggett, P-M. and Nerard, T.A. (1992b). Cryopreservation of *Tetrahymena*. In: *Protocols in Protozoology*. (Ed.s J.J. Lee and A.T. Soldo), Society of Protozoologists, A-91.1- A-91.4.

Davis, H.S. (1926). *Octomitus salmonis* a parasitic flagellate of trout. U.S. Dept. Commerce Bull. Fisheries Document no.988: W.T. Yabutake, D.R., Buhler, W.E., Shanks. *Journal of Parasitology*. **41**, (1961).

Dickerson, H.W., Brown, J., Dawe, D.L., Graztek, J.B., (1984). *Tetrahymena pyriformis* as a protective antigen against *Ichthyophthirius multifiliis* infection: comparisons between isolates and ciliary preparations. *Journal of Fish Diseases.* **24**, 523-528.

Dickerson, H.W., Dawe, D.L., Graztek, J.B., Brown, J. and Pyle, S.W. (1981). Induction of *Ichthyophthirius multifiliis* Fouquet infections in channel catfish *Ictalurus punctatus* Rafinesque: standardisation of the procedure. *Developments in Biological Standardization.* **49**, 331-336.

Dickerson, H.W., Lohr, A.L. and Gratzek, J.B. (1985). Experimental intraperitoneal infection of channel catfish, *Ictalurus punctatus* (Rafinesque), with *Ichthyophthirius multifiliis* (Fouquet). *Journal of Fish Disease.* **8,** 139-142.

Dickerson, H.W., Evans, D.L. and Gratzek, J.B. (1986). Production and preliminary characterisation of murine monoclonal antibodies to *Ichthyophthirius multifiliis* a protozoan parasite of fish. *American Journal of Veterinary Research.* **47**, 2400-2404

Dickerson, H.W., Clarke, T.G. and Findly, R.C. (1989). *Ichthyophthirius multifiliis* has membrane-associated immobilisation antigens. *Journal of Eukaryotic Macrobiology.* **40**, 816-820.

Dickerson, H.W. and Dawe, D.L. (1995). In *Fish Diseases and Disorders* (Woo, P.T.K., eds.) pp181-227, CAB International.

Ekless, L.M. (1990). *In vitro* culture of *Ichthyophthirius multifiliis* (Fouquet). MSc. Thesis, Polytechnic South West, 61pp.

Ekless, L.M. and Matthews, .R.A.,(1993). *Ichthyophthirius multifiliis*: axenic isolation and short-term maintenance in selected monophasic media. *Journal of Fish Diseases.* **16,** 437-447.

Elliott, A.M. (1933). Isolation of *Colpidium striatum* Stokes in bacteria free cultures and the relation of growth to pH of the medium. *Biological Bulletin.* **65,** 45-56.

Elliott, A.M. and Clemmons, G.L. (1966). An ultrastructural study of ingestion and digestion in *Tetrahymena pyriformis. Journal of Protozoology.* **13**, 311-323.

Elsdale, T. and Bard, J. (1972). Collagen substrata for studies on cell behaviour. *Journal of Cell Biology.* **54**, 626-637.

Elser, H.J. (1955). An epizootic of Ichthyophthiriasis amongst fish in a large reservoir. *Progresssive Fish Culturist.* **17,** 132-133

Ewing, M.S., Kocan, K.M. and Ewing, S.A. (1983). *Ichthyophthirius multifiliis*: morphology of the cyst wall. *Transactions of the American Microscopical Society.* **102**, 122-128.

Ewing, M.S., Kocan, K.M. and Ewing, S.A. (1985). *Ichthyophthirius multifiliis*, (ciliophora). Invasion of gill epithelium. *Journal of Protozoology.* **32**, 305-310.

Ewing, M.S. and Kocan, K.M. (1986). *Ichthyophthirius multifiliis*, (ciliophora). Development in the gill epithelium. *Journal of Protozoology*. **33**, 369-374.

Ewing, M.S., Lynn, M.E. and Ewing, S.A. (1986). Critical periods in the development of *I. multifiliis* (ciliophora) populations. *Journal of Protozoology*. **33**, 388-391.

Ewing, M.S. and Kocan, K.M. (1987). *Ichthyophthirius multifiliis*, (ciliophora). Exit from the gill epithelium. *Journal of Protozoology*. **34**, 309-312.

Ewing, M.S., Ewing, S.A. and Kocan, K.M. (1988). *Ichthyophthirius* (Ciliophora): Population studies suggest reproduction within host epithelium. *Journal of Protozoology*. **35**, 549-552.

Ewing, M.S., Hefler, C., Kocan, K.M. and Stebler, E. (1991). Extracellular calcium concentration affects infectivity of *Ichthyophthirius multifiliis* (Ciliophora) theronts. *Journal of Protozoology*. **3**, 178-181.

Ewing, M.S. and Kocan, K.M. (1992). Invasion and development strategies of *Ichthyophthirius multifiliis*, a parasitic ciliate of fish. *Parasitology Today* . **8**, 205-208.

Farley, D.G. and Heckmann R. (1980). Attempts to control *Icthyophthirius multifiliis* Fouquet (ciliophora) by chemotherapy and electrotherapy. *Journal of Fish Disease*. **3**, 203-212.

Fernandez R.D., Yoshimizu M., Kimura, T., and Ezura, Y., (1993). Establishment and characterisation of seven continuous cell lines from freshwater fishes. *Journal of Aquatic Animal Health*. **5**, 137-147

Fok, A.K., Lee, Y. and Allen, R.D. (1982) The correlation of digestive vacuole pH and size with the digestive cycle in *Paramecium caudatum*. *Journal of Protozoology*. **29**, 409-414.

Freshney, R.I. (1987), *Culture of Animal Cells, A Manual of Basic Techniques.* Second Edition. Alan R. Liss, Inc., New York. 397pp.

Fryer, J.L., Yusha, A. and Pilcher, S. (1965). The *in vitro* cultivation of tissues and cells of Pacific salmon and Steelhead trout. *Annals of New York Academy of Sciences*. **126**, 566-586.

Geisslinger, M. (1987). Observations on the caudal cilium of the tomite of *Ichthyophthirius multifiliis* Fouquet, 1876. *Journal of Protozoology*: **341**,180-182.

Glaser, R.W. and Coria, N.A. (1930). Methods for the pure culture of certain Protozoa. *Journal of Experimental Medicine*. **51**, 787.

Gospodarowicz, D., Delago, D. and Vlodavsky, I. (1980). Permissive effects of the extracellular matrix on cell proliferation *in vitro*. *Proceedings of the National Academy of Scientists of USA*. **77**, 4094-4098.

Goven, B.A., Dawe, D.L. and Gratzek, J.B. (1980). Protection of channel catfish *Itcalurus punctatus* against *Ichthyophthirius multifiliis* by immunisation. *Journal of Fish Biology.* **17**, 311-316

Grasse, P.P. (1977). Premiers stades du developpement experimental en cultures, de spores de la Myxosporidie *Myxobolus exiguus* Thelohan. *Academy Des Seience.***285**, 681-683

Gravell, M. and Malsberger, R.G. (1965). A permanent cell line from the fathead minnow (*Pimephales promelas*). *Annals of the New York Academy of Scientists.* **126**, 555-565.

Graztzek, J.B., Gilbert, J.P., Lohr, A.L., Shotts, E.B. and Brown, J. (1983). Ultraviolet light control of *Ichthyophthirius multifiliis* Fouquet in a closed fish culture recirculation system. *Journal of Fish Diseases.* **6**, 145-153.

Grutzner, L. (1958). *In vitro*-Zuchtung des Leber und Nierengewebes von Tinca vulgaris Cuv. in trypsinierten Einschichtgewebekulturen. *Zentre Bakteriol Parasitenk, Abt. I. Orig.* **173**, 81-96.

Hauser M. (1972). The intranuclear mitosis of the ciliates Paracineta limbata and Ichthyophthirius multifiliis. Chromosoma. **36,** 158-175.

Hetherington, A. (1934). The sterilisation of Protozoa. *Biological Bulletin.* ***67**, 315-321.*

Hines, R.S. and Spira, D.T. (1973a). *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp, *Cyprinus carpio* (L.). I. Course of infection. *Journal of Fish Biology.* **5**, 385-392.

Hines, R.S. and Spira, D. (1973b). *Ichthyophthirius multifiliis* (Fouquet) in the mirror carp, *Cyprinis carpio* (L.). II. Leukocyte response. *Journal of Fish Biology.* **5**, 527-534.

Hines, R.S. and Spira, D.T. (1974a). *Ichthyophthirius multifiliis* in the mirror carp, *Cyprinus carpio* (L.). III. Pathology. *Journal of Fish Biology.* **6**, 189-196.

Hines R.S. and Spira, D.T. (1974b). *Ichthyophthirius multifiliis* in the mirror carp, *Cyprinus carpio* (L.). IV. Physiological dysfunction. *Journal of Fish Biology.* **6**, 365-371.

Hines, R.S. and Spira, D.T. (1974c). *Ichthyophthirius multifiliis* in the mirror carp, *Cyprinus carpio* (L.). V. Aquired immunity. *Journal of Fish Biology.* **6**, 373-378.

Hlond, B. (1966). Experiments in *in vitro* culture of *Ichthyophthirius multifiliis*. *FAO Fisheries report.* **44**, 365-368.

Hoffman, G. (1978). Ciliates of freshwater fishes. In: *Parasitic Protozoa II: Intestinal flagellates, Histomonads, Trichomonads, Amoeba, Opalinids and Ciliates.* (ed. by Kreier, P.J.) Academic Press, pp583-632.

Holz, G.G.Jr. (1973). The nutrition of *Tetrahymena*: Essential nutrients, feeding and digestion. In *Biology of Tetrahymena*. (Ed. Elliot, A.M. Dowden, Hutchinson and Ross). pp.89-98.

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

Houghton, G. (1987). The immune response in carp, *Cyprinus carpio* to *Ichthyophthirius multifiliis* (Fouquet, 1876). Ph.D Thesis, Plymouth Polytechnic.

Houghton, G. and Matthews, R.A. (1990). Immunosuppression in juvenile carp, *Cyprinus carpio*: the effects of the corticosteroids triamcinolone acetonide and hydrocortisone 21-hemisuccinate (cortisol) on acquired immunity and the humoral antibody response to *Ichthyophthirius multifiliis* Fouquet. *Journal of Fish Diseases.* **13**, 269-280.

Houghton, G., Healey, L.J. and Matthews, R.A. (1992). The cellular proliferative responses, humoral antibody response, and cross reactivity studies of *Tetrahymena pyriformis* with *Ichthyophthirius multifiliis* in juvenile carp (*Cyprinus carpio* L.). *Developmental and Comparative Immunology.* **3**, 157-166.

Kabata, Z. (1985). *Parasites and Diseases of Fish Cultured in the Tropics.* Taylor and Francis (Eds). Philadelphia, Pensylvania.

Kamei, Y., Yoshimizu, Y., Ezura and Kimura, T. (1992). Isolation and characterisation of antiviral substances against salmonid viruses, 46NW-04A produced by an aquatic bacterium, *Pseudomonas fluorescens* 46NW-04. In Salmonid Diseases (ed. Kimura, T.). Hokkaido University Press, Sapporo, Japan. pp 293-300.

Kidder G.W. and Dewey, V.C. (1951). The biochemistry of ciliates in pure culture. In *Biochemistry and Physiology of Protozoa*. Vol 1, pp232-400

Kleinman, H.K., McGoodwin, E.B., Rennard, S.I. and Martin, G.R. (1979). Preparation of collagen substrates for cell attachment: Effect of collagen concentration and phosphate buffer. *Analytical Biochemistry*. **94**, 308-312.

Kozel, T.R. (1976). The occurrence of *Ichthyophthirius multifiliis*: Ciliata; Hymenostomadidae in Kentucky waters. *Transactions of the Kentucky Academy of Science*. **37**, 41-44.

Kozel, T.R. (1979). *In vitro* maintenance of *Ichthyophthirius multifiliis* with cytology and electron microscopy of the infective stage. Ph.D Thesis, University of Louisville, Louisville, Kentucky.

Kozel, T.R. (1980). Contact and penetration of *Ictalurus punctatus* by *Ichthyophthirius multifiliis*. *Proceedings of the American Society of Parasitologists*. **55**, 201.

Kozel, T.R. and Whittaker, (1982). Ectoparasites of the Rainbow Darter, *Etheostoma caeruleum* Storer from Harrods Creek, Oldham County, Kentucky. *Proceedings of the Helminthological Society Washington*. **49**, 138-139.

Kozel, T.R. (1986). Scanning electron microscopy of theronts of *Ichthyophthirius multifiliis*: their penetration into host tissue. *Transactions of the American Microscopical Society*. **105**, 357-364.

Leick, V. and Hellung-Larson, P. (1985). Chemosensory responses in *Tetrahymena:* the involvement of peptides and other signal substances. *Journal of Protozoology*. **32**, 550-553.

Levandowsky, M., Cheng, T., Kehr, A., Kim, J., Gardner, L., Silvern, L., Tsang, G., Chung, C. and Prakasyh, E. (1984) Chemosensory responses to amino acids and certain amines cy the ciliate *Tetrahymena*: a flat capillary assay. *Biological Bulletin*. **167**, 322-330.

Lillie, J.H., MacCallum, D.K. and Jepsen, A. (1980). Fine structure of subcultivated stratified squamous epithelium grown on collagen rafts. *Experimental Cell Research*. **125**, 153-165.

Lin, T.L. and Dickerson, H.W. (1992). Purification and partial characterisation of immobilisation antigens from *Ichthyophthirius multifiliis*. *Journal of Protozoology*. **39**, 457-463.

Lobo-Da-Cuhna, A. and Azevedo, C. (1988a). Ultrastructural and cytochemical study of food vacuoles and primary lysosomes in *Ichthyophthirius multifiliis* (ciliophora). *European Journal of Protistologica*. **24**, 60-63.

Lobo-Da-Cuhna, A. and Azevedo, C. (1988b). Association between xenosomes and glycogen in the cytoplasm of the ciliate *Ichthyophthirius multifiliis*. *Endocytobiosis and Cell Research*. **5**, 225-231.

Lobo-Da-Cuhna, A. and Azevedo, C. (1990). Enzyme cytochemistry of the alveolar sacs and golgian like cisternae in the ciliate *Ichthyophthirius multifiliis*. *Journal of Protozoology*. **37**, 206-211.

Lobo-Da-Cuhna, A. and Azevedo, C. (1993). Processing of food vacuoles in the parasitic ciliate *Ichthyophthirius multifiliis* after exit from the host. *Parasitology Research*. **79**, 272-278.

Loefer J.B. (1949). Penicillin and streptomycin sensitivity of some protozoa. *Texas Journal of Science*. **1**, 92-94.

Lom, J., and Cerkasovova, A. (1974). Host finding in invasive stages of *Ichthyophthirius multifiliis*. *Journal of Protozoology*. **21**, 457.

Lom, J., and Suchankova E., (1974). Comments on the life cycle of trypanosoma danilewskyi. *Proceedings of the III. International Conference on Parasitology*. Vol. 1, pp66-67.

Lom J. (1979). Biology of the Typanosomes and Trypanoplasms of fish. In: *Biology of Kinetoplastida*. Vol II Academic Press. pp229-337.

Lom, J. and Dykova, I. (1992). Protozoan parasites of Fishes. *Developments in Aquaculture and Fisheries Sciences*. **26**. London, Elsevier

Luck J.M. and Sheets, G. (1931). The sterilisation of protozoa. *Archiv fur Protistenkunde*. **75**, 255-269.

Lyman Allen, S. and Nerard, T.A. (1978) Methods for the simultaneous establishment of many axenic cultures of *Paramecium*. *Journal of Protozoology*. **25**,134-139.

Lynn, D.H. and Corliss, J.O. (1992). Ciliophora. In: *Microscopic Anatomy of Inveretbrates*. Vol. 1 Protozoa. New York: Wiley-Liss. pp.333-469

MacLennan, R.F. (1935) Observations on the life cycle of *Ichthyophthirius* a ciliate parasitic on fish. *Northwest Science*. **9**, 12-14.

MacLennan, R.F. (1936). Dedifferentiation and redifferentiation in *Ichthyophthirius*. II The origin and function of cytoplasmic granules. *Archives of Protozoology*. **86**, 404-426.

MacLennan, R.F. (1937). Growth in the ciliate *Ichthyophthirius multifiliis*. I. Maturity and encystment. *Journal of Experimental Zoology*. **76**, 423-440.

MacLennan, R.F. (1942). Growth in the ciliate *Ichthyophthirius multifiliis*. II. Volume *Journal of Experimental Zoology*. **91**, 1-13.

Matthews, R.A., (1994). in *Parasitic Diseases Of Fish*, eds. Pike, A.W. and Lewis, J.W. (Samara, Dyfed, U.K.), pp. 17-42.

Matthews, R.A., Matthews, B.F. and Ekless, L.M. (1996). *Ichthyophthirius multifiliis*: observations on the life-cycle and indications of a possible sexual phase. *Folia Parasitologica*. **43**, 203-208.

Maupas, E. (1889). La rajeunissement karyogamique chez les cilies. *Arch. Zool. Exp. Gen.* **7**, 149-517.

McCallum, H.I. (1982). Infection dynamics of *Ichthyophthirius multifiliis*. *Parasitology*. **85**, 475-488.

McCallum, H.I. (1985). Population effects of parasite survival on host death: experimental studies of the interaction of *Ichthyophthirius multifiliis* and its fish host. *Parasitology*. **90**, 529-547.

McCallum, H.I. (1986). Acquired resistance of black mollies *Poecilia latipinna* to infection by *Ichthyophthirius multifiliis*. *Parasitology*. **93**, 251-261.

McCardle, E.W., Berquist, B.L. and Ehret, C.F. (1980). Structural changes in *Tetrahymena rostrata* during induced encystment. *Journal of Protozoology*. **27**, 388-397.

Milsan, and Smith-Somerville, (1986). Food vacuole morphology and membrane retrieval in the microstomal form of *Tetrahymena vorax*. *Journal of Protozoology*. **33**, 172-179.

Miyake, A. (1981). Physiology and biochemistry of conjugation in ciliates. In *Biochemistry and Physiology of Protozoa*, Edition 2, Volume 4. Academic Press, NY. pp 125-198.

Moewus-Kobb, L. (1965). Studies with IPN virus in marine hosts. *Annals of the New York Academy of Sciences*. **126**, 328-342.

Mosevitch, T.N. (1965). Electron microscopic study of the structure of the contractile vacuole in the ciliate *Ichthyophthirius multifiliis* (Fouquet). *Acta Protozoologica*. **3**, 61-67.

Muller M.and Rohlich P., (1961). Studies on feeding and digestion in protozoa. II. Food vacuole formation in *Tetrahymena corlissi*. *Acta Morphologica Academy Sciences Hungary*. **10**, 297-305.

Muller, M. and Toro I. (1962). Studies on feeding and digestion in protozoa III. Acid phosphatase activity in food vacuoles of *Paramecium micronucleatum. Journal of Protozoology.* **9**, 98-1126.

Nanney, D.L. (1974). Ageing and long-term temporal regulation in ciliated protozoa. A critical review. *Mechanisms of Ageing Development.* **3**, 81-105

Nanney, D.L. (1980). *Experimental Ciliatology: an introduction to Genetic and Developmental Analysis in Ciliates.* John Wiley and Sons, New York, pp 304.

Nicholson, B.L. (1985). Techniques in fish cell culture. In: *Techniques in the Life Sciences, Setting up and Maintenance of Tissue and Cell Cultures.* 105, 1-16.

Nicholson, B.L. and Byrne C. (1973). An established cell line from the Atlantic salmon (*Salmo salar*). *Journal of Fisheries Research Board Canada.* 30, 913-916.

Nickell T.A. and Ewing M.S. (1989). Dispersal of *Ichthyophthirius multifiliis* (Ciliophora). *Proceedings of the Oklahoma Academy of Science.* **69**, 23-25.

Nie-Dashu, D. and Lie-Siang, L. (1960). Studies on the morphology and life cycle of *Ichthyophthirius multifiliis* and its control with a description of a new species. *Acta Hydrobiology Sinica.* **2**, 197-255.

Nigrelli, R.F., Pokorny, K.S. and Ruggieri, G.D. (1976). Notes on *Ichthyophthirius multifiliis* a ciliate parasitic on freshwater fishes, with some remarks on possible

physiological races and species. *Transactions of the American Microscopical Society*. **95**, 607-613.

Nilsson, J.R. (1972). Further studies on vacuole formation in *Tetrahymena pyriformis* GL. *Comptes Rendus des Travaux du Laboratoire Carlsberg*. **39**,119-141.

Nilsson, J.R. (1976). Physiology and structural studies on *Tetrahymena pyriformis* GL. *Comptes Rendus des Travaux du Laboratoire Carlsberg*. **40**, 215-355.

Noe, J.G. and Dickerson H.W. (1995) Sustained growth of *Ichthyophthirius multifiliis* at low temperature in the laboratory. *Journal of Parasitology* . **81**, 1022-1024.

Noga, E.J. and Hartmann, J.X. (1981). Establishment of walking catfish (*Clarias batrachus)* cell lines and development of a channel catfish virus vaccine. *Canadian Journal of Fisheries and Aquatic Science*. **38**, 925-930.

Noga, E.J. (1987). Propagation in cell culture of the dinoflagellate *Amyloodinium ocellatum* an ectoparasite of marine fishes. *Science*. **236**, 1302-1304.

Noga, E.J. and Bower, C.E. (1987). Propagation of the marine dinoflagellate *Amyloodinium ocellatum* under germ-free conditions. *Journal of Parasitology*. **73**, 924-928.

Noga E.J. (1989). Culture conditions affecting the *in vitro* culture of *Amyloodinium ocellatum*. *Diseases of Aquatic Organisms*. **6**, 137-143.

Oestmann, D.J. and Lewis, D.H. (1996). Improved cell culture propagation of *Amyloodinium ocellatum*. *Diseases of Aquatic Organisms*. **24**,173-178.

Orias, E. and Rasmussen, L. (1976). Dual capacity for nutrient uptake in *Tetrahymena*. IV. Growth without food vacuoles and it's implications. *Experimental Cell Research*. **59**, 457-463.

Pantin C.F.A.(1962). *Notes on Microscopical Techniques for Zoologists*. Cambridge University Press, 77pp.

Parpart, A.K. (1928). The bacterial sterilisation of *Paramecium*. *Biological Bulletin*. **35**, 113-120.

Pitelka, D.R. (1961). Fine structure of the silverline and fibrillar sysyems of three tetrahymenid ciliates. *Journal of Protozoology*. **8**, 75-81.

Pyle, S.W. and Dawe, D.L. (1985). Stage-dependent protein composition in the life cycle of synchronous *Ichthyophthirius multifiliis*, a ciliate fish parasite. *Journal of Protozoology*. **32**, 355-359.

Putz R.E. (1972). Biological studies on the hemoflagellates *Crytobia cataractae* and *Crytobia salmositica*. *Bureau of Sport Fisheries and Wildlife (U.S.) Technical Papers*. **63**, 3-25.

Qadri S.S. (1962). The development in culture of *Trypanosoma striati* from an indian fish. *Parasitology*. **52**, 229-235.

Rasmussen, L. (1974). Food vacuole membrane in nutrient uptake by *Tetrahymenia*. *Nature*. **250**,157-158.

Rasmussen, L. and Orias, E. (1975). *Tetrahymenia*: growth without phagocytosis. *Science*. **190**, 464-465.

Reichenbach-Kinke, H. and Landolt, M. (1965). *The Principal Diseases of Lower Invertebrates. Book 1. Diseases of Fishes.* TFH, Neptune City, NJ.

Roque M., Puytorac P. and Lom, J.L. (1967). L' architecture buccale et la stomatogenese d' *Ichthyophthirius multiflis* Fouquet, 1876. *Protistologica*. **3**, 77-90

Ross, L.G. and Geddes, J.A. (1979). Sedation of warm-water fish in aquaculture research. *Aquaculture*. **16**, 183-186.

Seaman G.R. (1947). Penicillin as an agent for sterilisation of protozoan culture. *Science*. **106**, 327.

Seaman G.R. (1961). Some aspects of phagotrophy in *Tetrahymena*. *Journal of Protozoology*. **8**, 204-212.

Schmahl, G., Taraschewski, H. and Melhorn, H. (1989a). Chemotherapy of fish parasites. *Parasitology Research*. **75**, 503-511.

Schmahl, G., Taraschewski, H. and Melhorn, H. (1989b). Treatment of fish parasites. 5. The effects of sym. triazinone (toltrazuril) on fish parasitic Ciliophora (*Ichthyophthirius multifiliis* Fouquet, 1876, *Apisoma amoeba* Grenfell, 1884, *Trichodina* sp. Ehrenberg, 1831). *European Journal of Protistology*. **24**, 152-161.

Schmahl, G., Ruider, S., Melhorn, H., Schmidt, H. and Ritter, G. (1992). Effects of a medicated food containing malachite green on *Ichthyophthirius multifiliis* Fouquet, 1876 (Hymenostomatida, Ciliophora) in ornamental fish. *Parasitology Research*. **78**, 183-192.

Selosse, P.M. and Rowland, S.J. (1990). Use of common salt to treat Ichthyophthiriasis in Australian warmwater fishes. *The Progressive Fish Culturist*. **52**, 124-127.

Smith, S.A., Noga, E.J., Levy, M.G. and Gerig, T.M. (1993). Effect of serum from tilapia *Oreochromis aureus* immunised with the dinospore *Amyloodinium ocellatum* on the motility, infectivity and growth of the parasite in cell culture. *Diseases of Aquatic Organisms*. **15**, 73-80.

Smolikova, V., Lom, J. and Suchankova, E. (1977). Growth of the carp trypanosome *T. danilowskyi* in fish tissue culture. *Journal of Protozoology*. **24**, 54.

Stiles, C.W. (1894). Reports on a parasitic protozoan observed on fish in the aquarium. *Bulletin of the United States Fisheries Society.* **100**, 173-190.

Straus, D.L. (1993). Prevention *Ichthyophthirius multifiliis* infestation in channel catfish fingerlings by copper sulphate treatment. *Journal of Aquatic Animal Health.* **5**, 152-154.

Subasinghe R.P. and Sommerville C. (1989). An experimental study into the possible protection in fry from *Ichthyophthirius multifiliis* (Fouquet) infections during mouthbrooding in *Oreochromis mossambicus* (Peters). *Journal of Fish Diseases.* **12**, 143-149.

Svensson, R., (1955). On the resistance to heating and cooling of Balantidium coli in culture and some observations regarding conjugation. *Experimental Parasitology.* **4**, 502-525.

Suzuki, J. (1935). On the reproduction of *Ichthyophthirius multifiliis* Fouquet in relation to water temperature. *Bulletin of the Japanese Society of Scientific Fisheries.* **3**, 265-272.

Taylor, A.E.R. and Baker, J.R. (1987). In Vitro *Methods for Parasite Cultivation.* Academic Press, London.

Uspenska, A.V. (1963). Hyaluronidase in the various stages of the life history

of*Ichthyophthirius multifiliis*. *Doklady Academia Nauk SSSR, Novaia serviia*. **151**, 1031-1033.

Uzmann, J. and Hayduk, S. (1963). *In vitro* culture of the flagellate protozoan *Hexamita salmonis*. *Science*. **140**, 290-292.

Valtonen, E.T. and Keranen, A. (1981). Ichthyophthiriasis of Atlantic salmon, *Salmo salar* L., Montta hatchery in Northern Finland in 1978-1979. *Journal of Fish Diseases*. **4**, 405-411.

van Houton, J., Houser, D.C.R. and Levandowsky, M. (1981). Chemosensory behaviour in protozoa. In *Biochemistry and Physiology of Protozoa* (eds. Levandowsky, M. and Hutner, S.M.), Academic Press, NY. pp.67-124.

van Wagtendonk W.J. and Hackett P.L. (1949). The culture of *Paramecium aurelia* in the absence of other living organisms. *Proceedings of the National Academy of Sciences of the U.S.A.* **35**, 155-159.

van Wagtendonk, W.J. and Soldo, A.T. (1970). Methods used in the axenic cultivation of *Paramecium aurelia*. In *Methods of Cell Physiology*. **4**, 117-130.

Ventura, M.T. and Paperna, I. (1985). Histopathology of *Ichthyophthirius multifiliis* infections in fishes. *Journal of Fish Biology*. **27**, 185-203.

Wagner, G. (1960). Der Entwicklungszyklus von *Ichthyophthirius multifiliis*, Fouquet)

und der Einfluss physikalischer und chemischer aussenfaktoren. *Zeitschrift Fisherei.* **9**, 425-443.

Wahli T., Meier W. and Pfister, K. (1986). Ascorbic acid induced immune-mediated decrease in mortality in *Ichthyophthirius multifiliis* infected rainbow trout (*Salmo gairdneri). Acta Tropica.* **43**. 287-289

Wahli T., Meier W. and Scmitt M. (1991). Affinity of *Ichtyophthirius multifiliis* theronts to light and or the fish. *Journal of Applied Ichthyology.* **7**, 244-248.

Willetts, N. and Cobon, G. (1993). Antiparasite vaccines. In *Vaccines for Veterinary Applications* (ed. Peters, A.R.) pp 259-294.

Wolf, K. (1973). Freshwater fishes. In: *Tissue Culture, Methods and Applications.* pp138-143

Wolf, K. and Quimby, M.C., Pyle, E.A. and Dexter, R.P., (1960). Preparation of monolayer cell cultures from tissues of some lower vertebrates. *Science.* **132**, 1890-1891

Wolf, K. and Quimby, M.C. (1962). Established eurythermic line of fish cells *in vitro. Science.* **135**,1065-1066.

Wolf, K. and Quimby, M.C. (1969). Fish cell and tissue culture. In: *Fish Physiology. Volume III.* (Ed. by Hoar, W.S. and Randall, D.J.). Academic Press, London, pp253-301.

Wolf, K. and Mann, J.A. (1980) Poikilotherm vertebrate cell lines and viruses: a current listing for fishes. *In Vitro.* **16**, 168-179.

Wolf, K. and Markiw, A.A. (1982). Ichthyophthiriasis: Immersion immunisation of rainbow trout (*Salmo gairdneri*) using *Tetrahymena thermophila* as a protective immunogen. *Canadian Journal of Aquatic Sciences.* **39**, 1722-1725.

Woo P.K. and Sen Li (1990). *In vitro* attenuation of *Cryptobia salmositica* and its use as a live vaccine against crytobiosis in *Onchorynchus mykiss. Journal of Parasitology.* **76**, 752-755.

Wurtsbaugh, W.A. and Tapia, R.A. (1988). Mass mortality in Lake Titicaca (Peru-Bolivia) associated with the protozoan parasite *Ichthyophthiris multifiliis. Transactions of the American Fisheries Society.* **117**, 213-217.

Young, J.Z. (1933). The preparation of isostonic solutions for use in experiments with fish. *Pubblicazioni della Stazioni Zoologica di Napoli.* **12**, 425-431.

Zaman,V. (1978). *Balantidium coli.* In: *Parasitic Protozoa II: Intestinal flagellates, Histomonads, Trichomonads, Amoeba, Opalinids and Ciliates.* (ed. by Kreier, P.J.) Academic Press, pp633-658.

Media for the cryopreservation of AS cell lines

30ml EMEM (FLOW)

10ml FCS (IMPERIAL)

10ml glycerol

0.5ml L-glutamine (200mM) (FLOW)

0.5ml penicillin:streptomycin (FLOW)