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CRYPTOCARYON IRRITANS BROWN, 1951 (CILIOPHORA): TRANSMISSION AND IMMUNE RESPONSE IN THE MULLET CHELON LABROSUS (RISSO, 1826)

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CRYPTOCARYON IRRITANS BROWN, 1951 (CILIOPHORA): TRANSMISSION AND IMMUNE RESPONSE IN THE MULLET *CHELON LABROSUS* (RISSO, 1826)

by

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DECLARATION

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

Signed.....P. J. Burgess.....
Candidate

Date.....22/9/92.....

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

Signed.....P. J. Burgess.....
Candidate

Date.....22/9/92.....

Signed.....
Supervisor

Date.....22/9/92.....

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ABSTRACT

P J Burgess. *Cryptocaryon irritans* Brown, 1951 (Ciliophora): transmission and immune response in the mullet *Chelon labrosus* (Risso, 1826).

A standardised procedure was established for the laboratory maintenance of *C.irritans* in thick-lipped mullet. Nine isolates of *C.irritans* were obtained of which eight were successfully established for up to 48 weeks. Studies on individual life cycle stages with regards to longevity, viability, and susceptibility to a chemotherapeutic agent, revealed the difficulties in eradicating the cysts. Transmission of the parasite both to and from the host correlated with darkness. High infection levels resulted in the death of host fish within five days following exposure to theronts. An acquired protective immune response developed in host mullet within 14 days after exposure to sub-lethal infection. The degree of immunity appeared to relate to infection dose, and was not fully protective in all fish. Protection persisted for six months after infection and appeared specific to *C.irritans*. Specific antibodies to trophont antigen were identified in mullet serum but not epithelial mucus following either natural exposure to theronts or intraperitoneal immunisation with trophont antigens. Serum from intraperitoneally immunised fish caused theront immobilisation and agglutination *in vitro*; however no evidence was found for a protective role for specific antibody. Major polypeptides were identified and characterised by molecular weight for both trophont and theront stages using SDS-PAGE. Significant homology in major polypeptide profiles was found between *C.irritans* and *I.multifiliis*, in respect to trophonts and particularly theronts. Murine monoclonal antibodies raised to trophonts identified two polypeptide components of molecular weights 20-21kDa and 68-69kDa, the latter being homologous with host immunoglobulin heavy chain. These results are discussed in relation to future management and control strategies for cryptocaryosis in warmwater mariculture systems and aquaria.

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CHAPTER ONE

INTRODUCTION

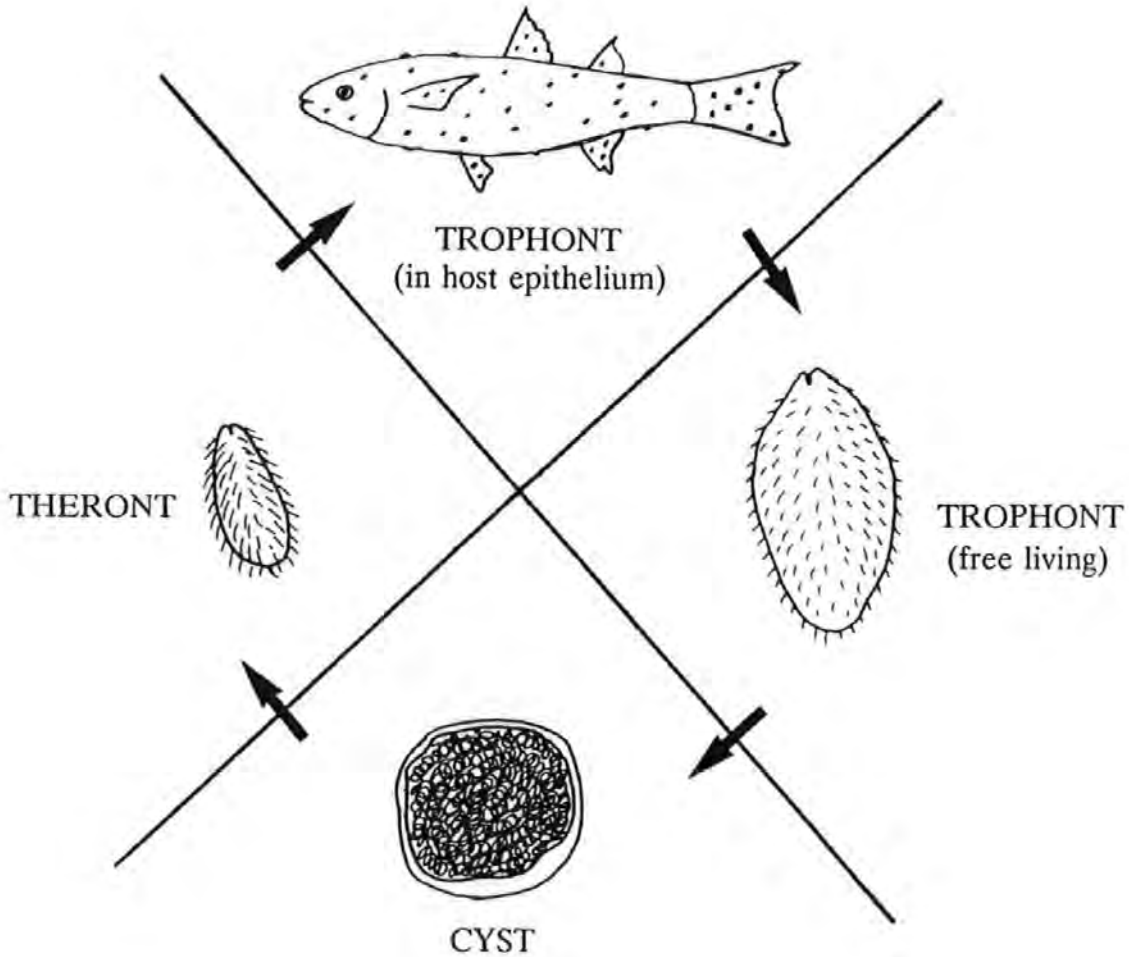
Cryptocaryosis is a major disease of warmwater marine teleosts, accounting for epizootics and acute mortalities in captive fish populations (Nigrelli and Ruggieri, 1966; Blasiola, 1976). Until recently, the causative agent, *Cryptocaryon irritans* Brown 1951, was chiefly associated with ornamental coral reef fishes (De Graaf, 1973). Within the last decade, however, this pathogen has been increasingly reported as a serious problem in the foodfish industry, causing high mortalities in commercially important species as red snapper, sea bream, sea bass, grouper and flounder (Huff and Burns, 1981; Colorni, 1985; Kaige and Miyazaki, 1985; Rasheed, 1989; Tookwinas, 1990a,b). The economic importance of cryptocaryosis seems likely to increase further, in view of the substantial growth in both the ornamental fish and warmwater mariculture industries (MINTEL, 1988; Andrews, 1990).

C.irritans is an obligate parasite with a direct transmission. Its life cycle, as described by Sikama (1937, 1938) is conveniently divided into four phases: i) trophont, the growth phase within the host epidermis; ii) free-living trophont; iii) reproductive cyst; and iv) theront, the stage infective to the fish (Fig. 1).

Currently there is no satisfactory method for control of *C.irritans*, present procedures being largely reliant upon chemical immersion treatments which are toxic to both fish and the environment; these are ineffective against the parasitic trophont and reproductive cyst (Kingsford, 1975; Herwig, 1978; Andrews *et al.*, 1988). New approaches for the prevention and control of *C.irritans* will require a greater understanding of the parasite's biology and its interaction with host, however few

Figure 1

Life cycle stages of *Cryptocaryon irritans*.


TROPHONT
(in host epithelium)
THERONT
TROPHONT
(free living)
CYST

studies have been undertaken since the parasite's initial description by Sikama in 1937. Of the limited work on *C.irritans*, most has focused on aspects of the life cycle and morphological investigations, with little information regarding its geographical range, habitat, and environmental factors influencing its transmission. There have been no investigations on the host response to infection with *C.irritans*; such information is vital for considering an immunological approach to control by vaccination, as has been investigated for *Ichthyophthirius multifiliis*, a closely related ciliate (Dickerson *et al.*, 1984; Clark *et al.*, 1988).

The major objectives of this study are to establish a standardised system for the laboratory maintenance of *C.irritans* as a basis to the investigation of parasite transmission, molecular composition, and interaction with host. This study will hopefully contribute information leading towards safe and effective control and management strategies.

CHAPTER TWO

REVIEW

No previous major review of *C.irritans* has been undertaken. This chapter attempts to cover literature concerning the parasite's biology, economic importance, morphology, host range, pathology, geographical distribution, and control and treatment. In view of the parasite's location in the host epithelium it is appropriate here to also review teleost skin in relation to its defense mechanisms against infection.

1. CRYPTOCARYON IRRITANS

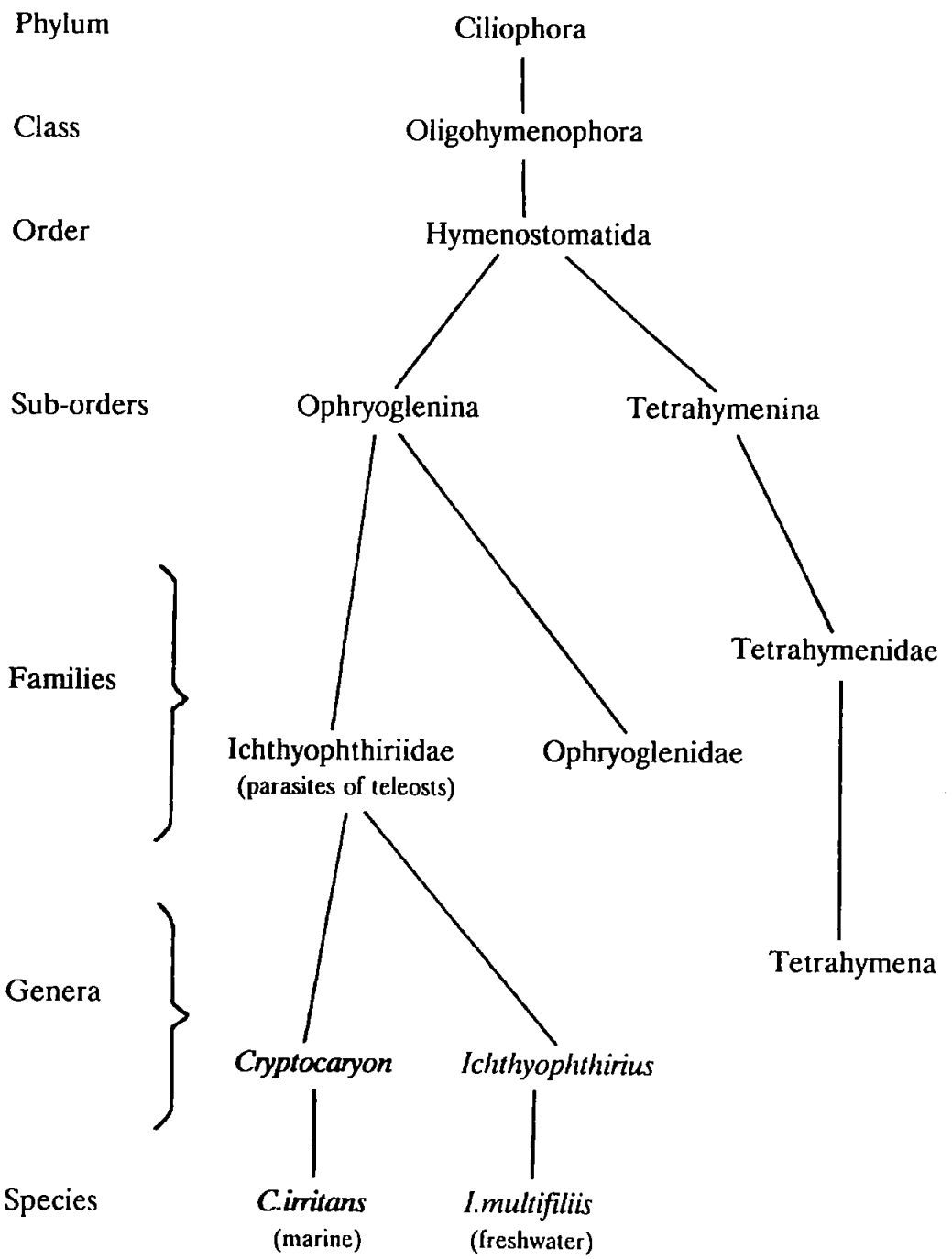
Biology

Cryptocaryon irritans Brown, 1951 is a holotrichous ciliated protozoan, belonging to the family Ichthyophthiriidae (Corliss, 1979) (Fig. 2). The trophic stage is parasitic in marine teleost fishes, occurring within the skin and gill epithelium and eye cornea. *C.irritans* causes the disease known under various names such as "marine whitespot", "marine ich", "cryptocaryoniasis" and "cryptocaryasis", but will here be termed "cryptocaryosis" in accordance with recent proposals (Baker, 1989).

C.irritans was first described in 1937 by Sikama in Japan as a marine form of *Ichthyophthirius multifiliis* (Fouquet), the causative agent of whitespot disease in freshwater fishes. Sikama (1961) later named the parasite *Ichthyophthirius marinus*, unaware that Brown (1951) had previously described the protozoan as *Cryptocaryon irritans*. Brown (1951), in her brief description, erected the new genus *Cryptocaryon*

Figure 2

Taxonomic position of *C.irritans*, after Corliss (1979).


Phylum
Ciliophora
Class
Oligohymenophora
Order
Hymenostomatida
Sub-orders
Ophryoglenina
Tetrahymenina
Families
Ichthyophthiriidae
(parasites of teleosts)
Ophryoglenidae
Tetrahymenidae
Genera
Cryptocaryon
Ichthyophthirius
Tetrahymena
Species
C.irritans
(marine)
I.multifiliis
(freshwater)

which is currently placed together with *Ichthyophthirius* in the family Ichthyophthiriidae. As yet, no other species have been assigned to *Cryptocaryon.*

Economic importance

At present, *C.irritans* appears primarily of economic importance to the ornamental fish industry, causing high mortalities in coral reef fishes (De Graff, 1973; Herwig, 1978; Moe, 1982; Andrews *et al.*, 1988). As a disease pathogen of importance in tropical marine aquaria it ranks only second to *Amyloodinium* (Moe, 1982), however the economic impact of *C.irritans* to the ornamental fish industry has not been assessed. With regards to the economic effect of *C.irritans* on the warmwater mariculture of food fish, only a few references have been made to outbreaks of cryptocaryosis prior to 1980, although it has been increasingly reported over the past ten years. Recent outbreaks have occurred in Japanese flounder, *Paralichthys olivaceous* (Kaige and Miyazaki, 1985) and gilt-head bream, *Sparus aurata* (Colorni, 1985). In the Southern United States, epizootics of *C.irritans* have impaired the culture of red snapper, *Lutjanus campechanus*, causing acute, total mortalities (Huff and Burns, 1981). The parasite is apparently a major problem in the development of red snapper farming in Australia and New Zealand (Hine, 1982, and pers. comm.). In Kuwait, the parasite has infected sparids and sea-adapted *Tilapia aurea* (Tareen, 1980) and caused mass mortalities of tank-reared grouper, *Epinephelus tauvina*, which succumbed within one month of diagnosis (Rasheed, 1989). In the French West Indies, *C.irritans* has been considered the most dangerous of parasites encountered in the tank rearing of lutjanids, carangids, and seabass (Gallet de Saint Aurin *et al.*, 1990). *C.irritans* is also known to infect salmonids (Roberts and Shepherd, 1986).

Morphology

Morphological studies of *C.irritans* have been undertaken using light microscopy (Sikama, 1937, 1938; Brown, 1951, 1963; Nigrelli and Ruggieri, 1966; Colorni, 1988) and scanning electron microscopy (Cheung *et al.*, 1981). Structural features of each life cycle stage are considered below.

Trophont

Brown (1951) described the trophont as an opaque white ciliate, oval in its early stages, becoming spherical later. Its length ranged from around 70μm to 450μm (Brown, 1951; Sikama, 1961). The ciliature were studied by Brown (1951) who reported their uniform covering, arranged in rows which terminate at the edge of an oral pit. Lengths of individual cilia were recorded as 7μm to 9μm (Brown, 1951), however according to Cheung *et al.* (1981) these were slightly shorter, measuring only 3μm to 4μm. Studies of the nucleus by Brown (1951) revealed a well defined membrane enclosing granular chromatin with non-chromatin inclusions. She observed several micronuclei, and a meganucleus of four or more parts which fuse in older trophonts. Nigrelli and Ruggieri (1966) noted that the meganucleus became obscured by densely packed granules, and observed numerous densely staining basophilic spherical-shaped bodies in vacuole-like areas. The trophont did not possess an organella of Lieberkuhn (Brown, 1951, 1963) nor contractile vacuoles (Nigrelli and Ruggieri, 1966). The oral pit has been studied by several workers, including Brown (1951) who described two regions, a broad depression (peristome or cytostome) and a narrower region (pharynx or vestibule). Brown (1951) observed a well defined protrusile membrane on one wall of the oral pit, with a smaller membrane situated opposite. Nigrelli and Ruggieri (1966) suggested the protrusile apparatus might

function to draw in host material, however, Cheung *et al.* (1981) concluded that it serves to adhere the trophont to the substratum prior to encystment. The buccal apparatus was studied in detail by Cheung *et al.* (1981) which they described as a simple ring comprising 65 to 75 cirri-like structures surrounding the oral opening. The simplicity of this structure and the absence of accessory oral membranes or membranelles which are characteristic of the Hymenostomatida Delage and Herouard, 1896, prompted Cheung *et al.* (1981) to question the placing of *Cryptocaryon* within this taxonomic order.

Cyst

Brown (1951) described the encysted parasite, noting the coiled ribbon-like macronucleus, numerous micronuclei and at division, the production of theronts by budding. Studies made by Sikama (1961) suggested the presence of a laminated outer region of the cyst with ten layers, at least six of which formed the outer cyst wall. Later investigations by Nigrelli and Ruggieri (1966) differentiated at least four membranes within the outer cyst region. These workers also recorded cyst size, giving ranges of 94.5μm X 170μm to 225μm X 441μm. Recent research on the cyst by Cheung *et al.* (1981) has revealed the gradual loss of body ciliature (by absorption ?) following encystment and subsequent division.

Theront

Following theront differentiation, it has been shown by Nigrelli and Ruggieri (1966) that these active free swimming stages escape from small openings on one side of the cyst wall. Brown (1951) described the theronts as pear-shaped, varying in length from 40μm to 56μm, with a uniform covering of cilia, each 8μm to 9μm in

length and arranged in about 40 rows. Brown (1951, 1963) recorded 4 to 7 micronuclei and an oval meganucleus in four parts, often arranged in a crescent; non-chromatin inclusions were not observed. No organella of Lieberkuhn was observed (Brown, 1951). Brown (1951) described a dorsal perforatorium which projects beyond the mouth region, which, according to Colorni (1988), may play an important role in the theront's ability to attach to its host.

Host specificity

C.irritans appears to be parasitic only to teleost fishes, but within this group the parasite has a broad host range. For example, amongst aquarium fishes, Nigrelli and Ruggieri (1966) recorded 27 species infected with *C.irritans*, and a further 92 species (spanning 33 families within 7 orders) were listed by Wilkie and Gordin (1969). *C.irritans* has been observed to infect both adult and juvenile fish, the latter as young as 3 weeks for *Sparus aurata* (Colorni, 1985). Aquarium observations by Wilkie and Gordin (1969) suggest that some species are refractory to infection, these belonged to the elasmobranch order Squaliformes (9 species) and the teleost orders Anguilliformes (3 spp), Pleuronectiformes (5 spp), and two species of gobiids (*Lythrypnus* spp.) of the order Perciformes. These authors noted that these "resistant" species comprised primarily those which lived in contact with the substrate, prompting speculation that the mechanisms which protect the fish from abrasion injuries may also provide resistance to *C.irritans*.

Cryptocaryosis: clinical signs and pathology

C.irritans manifests as opaque to greyish papules in the skin, gills and eyes of its host, which in moderate to severe infections may form clusters (Blasiola, 1976).

Infected fish may show loss of appetite, nervous or uncoordinated activity and exhibit scraping or "flashing" of the flanks against hard objects (Violetta, 1980). Heavy infections invariably result in host death (Nigrelli and Ruggieri, 1966). Disease manifestations, when based on visual examination alone, have often been confused by aquarists with the dinoflagellate *Amyloodinium ocellatum* which initiates a similar type of disease and is also characterised by white spots, however these are smaller (Violetta, 1980). Subdermal haemorrhaging has been associated with *C.irritans* infection, particularly on the fins (Wilkie and Gordin, 1969). Invasion of the epithelial gill lamellae results in tissue erosion and excessive effusion (Nigrelli and Ruggieri, 1966). Hyperplasia of gill epithelial cells and mucus cells has also been observed (Kaige and Miyazaki, 1985). Normal respiratory function is disrupted leading to respiratory stress and increased respiratory activity (Wilkie and Gordin, 1969; Violetta, 1980). Parasites invading the corneal epithelium may cause opacity and sometimes blindness (Wilkie and Gordin, 1969; Blasiola, 1976; Sindermann, 1977). The possibility that tissue invasion by *C.irritans* may give rise to bacterial infection has been suggested (Nigrelli and Ruggieri, 1966; Violetta, 1980); inconclusive evidence for this has come from the presumptive diagnosis of *Cryptocaryon* in cultured turbot (*Scophthalmus maximus*) presenting with skin ulcerations. The parasite was considered a possible primary agent in the ulcerative disease by enabling invasion of secondary opportunistic pathogens (Devesa *et al.*, 1989).

Geographical distribution

The majority of reports of *C.irritans* infections in fish relate to occurrences in marine aquaria and enclosed mariculture systems. Outbreaks of cryptocaryosis in aquaria have been reported in several countries, including England (Brown, 1951),

Germany (Dr Hofte, Tetra Ltd., pers. comm.), the Netherlands (De Graaf, 1962), United States (Nigrelli and Ruggieri, 1966; Wilkie and Gordin, 1969), Canada (Shapiro, Marine Aquarium Society of Toronto, pers. comm.), Israel (Colorni, 1985) and Japan (Sikama, 1938). Nigrelli and Ruggieri (1966) consider that the parasite has become established in North American aquaria through marine fishes imported from Hawaii and the Indo-Pacific region; a similar explanation may account for the observed aquarium outbreaks in other countries, particularly in those situated outside the parasite's known temperature range. With regards mariculture systems, *C.irritans* has been recorded in sea bass (*Lates calcarifer*) in Thailand (Tookwinas, 1990a,b, and pers. comm.) and other parts of South East Asia (Cheong, 1990), in sea bream (*Sparus aurata*) in Israel (Colorni, 1985), in grouper (*Epinephelus tauvina*) in Kuwait (Rasheed, 1989), and in red snapper (*Lutjanus campechanus*) in Florida, U.S.A. (Huff and Burns, 1981). However, these reports did not address the possibility of an extraneous source of infection.

Observations on *C.irritans* in wild fishes are very few, however such studies are necessary to determine the parasite's possible origin and natural distribution. In Fiji, examination of 36 fish species collected from a coral reef revealed only one species, *Epinephelus merra*, harbouring *C.irritans*, infection in this host being very light (Laird, 1956). Sikama (1961) reported the parasite in fishes collected from the coastal waters of Japan. A presumptive identification of *C.irritans* in wild adult and larval butterflyfishes (Chaetodontidae) on a coral reef was made by Burgess (1978), but the locality or species infected were not given. Wilkie and Gordin (1969) recorded *C.irritans* on a single specimen of opaleye (*Girella nigricans*), collected from a tide pool near the Scripp's Institute, California, which they considered may have originated via effluent water from the Institute's aquaria which had a history of *C.irritans* outbreaks.

The distribution of *C.irritans* in the wild appears to be limited by temperature. Under aquarium conditions, *C.irritans* has not been shown to develop or transmit below 19°C to 20°C (Wilkie and Gordin, 1969; Cheung *et al.*, 1979) or above 30°C (Cheung *et al.*, 1979). Based on this information, it is considered that *C.irritans* is restricted to warmwater marine environments, although recent observations by Diamant *et al.* (1991) suggest that *C.irritans* may have a counterpart existing in the cooler waters of the eastern Mediterranean. This assumption was based on reports of disease outbreaks caused by a *Cryptocaryon*- like ciliate which was believed to have originated from cultured fish stocks from Cyprus and northern Israel.

Control and treatment

Several methods for the control and treatment of cryptocaryosis have been described. Of these, chemical treatments and salinity manipulations appear to have been most widely used.

A wide range of chemical compounds and mixtures, virtually all applied to the water, have been reported for use against *C.irritans*. The efficacy of chemical treatments, when used at levels which are non- or low-toxic to fish, are thought to be restricted to destroying the free-living stages (Andrews *et al.*, 1988), the cysts and parasitic trophonts being resistant (Herwig, 1978). Direct comparisons of different chemical treatments under conditions of similar water chemistry, host species and infection levels appear not to have been undertaken. The following review can provide, therefore, only a rough guide to the relative efficacies and contra-indications of the chemical treatments.

One group of chemical compounds, originally developed as dyes and biological stains, have been widely used over many years to control parasitic protozoal diseases

of fishes, including *I.multifiliis* (Van Duijn, 1973; Herwig, 1979). Amongst this group, acriflavine, malachite green and methylene blue have been employed either alone or in conjunction with other chemicals (e.g. formalin) to control *C.irritans* (Herwig, 1978; Tookwinas, 1990b). These compounds, which are generally administered as a prolonged bath, are reported to be toxic to fish, causing damage to the epithelium with resultant susceptibility to bacterial invasion and, in the case of malachite green, may cause sterility (Kingsford, 1975; Herwig, 1978). Copper treatments have been widely used. Herwig (1978) reported that copper sulphate, when used at 0.15 to 0.25 ppm as a long-term bath, is inhibitory to *C.irritans*. Wilkie and Gordin (1969) found that continual exposure to copper sulphate alone (at levels up to 0.4 ppm Cu) had no deleterious effect on the parasitic trophonts but was effective when used in conjunction with formalin as a short-term bath. Copper sulphate, however, is highly toxic to most fish species and its efficacy is impaired by carbonate compounds present in seawater (Wilkie and Gordin, 1969; Blasiola, 1976; Keith, 1981; Colorni, 1987). Furthermore, copper compounds in general are highly toxic to certain marine invertebrates (De Graaf, 1973; Andrews *et al.*, 1988), which largely precludes their use in aquaria housing mixed fish and invertebrate species. Formalin has been used by several workers to control *C.irritans*, with varying degrees of success (Herwig, 1978; Moe, 1982; Rasheed, 1989). Herwig (1978) reported that formalin at a level of 0.2 ml/gallon destroyed theronts, whereas Rasheed (1989) observed that twice weekly treatments of 30 to 50mg/litre formalin failed to prevent reinfections in cultured grouper, *Epinephelus tauvina*. Formalin has been used in conjunction with copper compounds to control *C.irritans*. For example, Nigrelli and Ruggieri (1966) used a mixture of formalin, cupric acetate and tris buffer as an effective single dose treatment, but warned of its potential high toxicity to fish. Moe (1982) reported the

successful treatment of *C.irritans* in aquaria using a series of one hourly formalin baths for the infected fish, in combination with a systemic copper treatment for the aquarium.

Amongst the chemicals found to be effective in controlling *C.irritans* are the group of quinine derivatives, which have proven efficacy in destroying certain medically important protozoa, by inhibiting nucleic acid synthesis (Gutteridge and Coombs, 1977). Those employed against *C.irritans* include chloroquine phosphate, primaquine phosphate, quinine hydrochloride and quinine sulphate; these compounds are reported to vary in their toxicity to fish (De Graaf, 1973; Kingsford, 1975; Herwig, 1978; Huff and Burns, 1981). Quinacrine (= atebrine) hydrochloride has been considered by some workers as the treatment of choice for controlling *C.irritans* in marine aquaria (Kingsford, 1975; Herwig, 1978). Other groups of anti-protozoal compounds, developed for medical and veterinary use, have also been applied to control *C.irritans*. These include metronidazole, which is reported to be non-toxic to fish at a therapeutic level of 25mg/ gallon (Herwig, 1978) and pyrimethamine which, although effective in controlling *C.irritans* when used at 4 to 8mg/ gallon, is toxic to fish, sometimes causing death (Kingsford, 1975).

A variety of other chemicals have been applied against *C.irritans* although their efficacy and toxicity are less well documented. These include potassium permanganate (reported to damage fish gills), sodium chlorite, suphathiazole, nitrofurazone, and penicillin (Wilkie and Gordin, 1969; Herwig, 1978, 1979). In the aquarium literature there has been reference to different strains of *C.irritans* (Herwig, 1978), some of which are considered to be "drug-resistant" (aquatic traders, pers. comms.) although there appears to be no scientific evidence to support these statements.

Manipulation of salinity (normally around 32 ‰), to create hypo- or hyper-saline conditions, has been employed by several workers for controlling *C.irritans* (Cheung *et al.*, 1979; Huff and Burns, 1981; Colorni, 1985). Freshwater dips in conjunction with chemical treatments have been reported by Herwig (1978) to destroy, by osmotic pressure, the parasitic trophont. However, Colorni (1985) found that freshwater immersions alone for periods up to 18 hours did not prevent development of trophonts within the fish epidermis. Effects of hyposalinity on the cyst stage was investigated by Cheung *et al.* (1979) who found that cyst development was prevented when exposed to a salinity of 16 ‰ (= 50% seawater: 50% freshwater) and lower. Colorni (1985), in a detailed study of salinity effects on the free-living stages of *C.irritans*, found that viability was retained over the following salinity ranges: trophonts, 15 to 60 ‰; cysts, 15 to 70 ‰; theronts, 25 to 50 ‰. Survival at higher salinities was not investigated. On the basis of this work, Colorni (1985) devised a successful strategy to control *C.irritans* in sea bream, by hyposaline destruction of the cyst before excystment. The regime involved four treatments at three day intervals, each comprising three hours immersion in 10 ‰ salinity. However, because of osmotic effects on host fish, the use of hyposaline treatment may need to be restricted to euryhaline species (Huff and Burns, 1981; Colorni, 1985). Hypersaline conditions have also been applied for the control of *C.irritans*. Huff and Burns (1981) found that immersion of infected red snapper in 60 ‰ salinity for five minutes caused the fish to shed mucus containing embedded trophonts which underwent cellular disruption. This treatment proved to be unsatisfactory, causing severe stress to the fish which died within seven days post-immersion. By using less extreme hypersaline conditions (45‰) in conjunction with quinine- based treatments, these authors succeeded in eradicating *C.irritans* without causing high fish mortalities. Two other methods for the control of

C.irritans have been reported in the literature: ultraviolet (U.V.) irradiation and ozone (O_3). Both U.V. irradiation units and ozone generators are commonly installed in marine aquaria, partly to kill microbial pathogens (Andrews *et al.*, 1988; Thiel, 1989). Neither method has been carefully evaluated in terms of its efficacy in destroying *C.irritans*. U.V. irradiation, around 2537 A°, has been recognised as effective in destroying certain fish pathogens, including bacteria (Bullock and Stuckey, 1977) and protozoa (Hoffman, 1974; Gratzek *et al.*, 1983). Spotte (1979) estimated from the size of the theront stage that the minimal lethal dose of U.V. needed to destroy *C.irritans* would be 800,000 $\mu W\ sec^{-2}$. As stated for *I.multifiliis* by Gratzek *et al.* (1983), U.V. can prevent the spread of infection between multi-aquaria closed systems but is not effective in controlling infection within an individual aquarium; the same probably applies to *C.irritans*. Regarding the use of ozone, Wilkie and Gordin (1969) reported that administration of 8 mg O_3/hour/15 gallons prevented infection in the opaleye fish, *Girella nigricans* which were held for 21 days in an infection aquarium. No other studies on ozone for controlling *C.irritans* appear to have been undertaken. One control method which does not require chemical intervention was described by Colorni (1987) in which the cyst stage was eliminated by repeated removal and replacement of the substrate at three day intervals.

In summary, chemical immersion treatments remain the control method of choice for *C.irritans*, being relatively cheap and simple to administer. Despite their toxicity to fish, copper compounds and formalin have been widely used in mariculture systems, whereas less harmful chemicals, such as quinines, have been favoured for the treatment of ornamental coral reef fishes. At present, there is no simple, effective and safe method for controlling *C.irritans*.

2. IMMUNE DEFENSE MECHANISMS IN SKIN AND GILL EPITHELIA OF TELEOST FISHES

The parasitic stage of *C.irritans* appears confined to the epithelium of the skin and gills of its teleost host (Colorni, 1985), with no reports of invasion via the basal lamina to subdermal tissues.

Both the skin and gills of teleosts have been exploited by many other protozoa including *Ichthyophthirius*, *Amyloodinium*, *Ichthyobodo*, *Chilodonella* and *Trichodina* (Joyon and Lom, 1969; Hines and Spira, 1973a; Lom and Lawler, 1973; Roberts and Shepherd, 1986). The general structure of teleost skin has been well documented (Whitear, 1970; Roberts and Bullock, 1980) and contrasts with that of higher vertebrates by containing an epidermis which is comprised of non-keratinised living cells (Bullock *et al.*, 1978). Several defense mechanisms exist to prevent epidermal invasion by pathogens, and these have received attention in recent years, both from a comparative immunological viewpoint and in relation to studies on vaccination delivery by external routes.

The non-specific ("innate") and acquired immune mechanisms in fish have been the subject of several reviews (Corbel, 1975; Ellis, 1982, 1989; MacArthur and Fletcher, 1985). The underlying mechanisms, comprising non-specific and specific elements, will be discussed separately here, with respect to skin and gill epithelia.

Non-specific defense mechanisms

The defense role of epidermal mucus secretions in entrapping microorganisms and inhibiting microbial colonisation has been reported (Pickering, 1974; Ourth, 1980; Ellis, 1989), with evidence of increased mucus production in response to certain

infections (Ellis, 1989). Rapid healing and tissue hyperplasia responses have also been reported to limit epithelial disruption by pathogens (Bullock *et al.*, 1978), and gill hyperplasia has been frequently observed in parasitic diseases, such as costiasis (Ellis, 1989) and myxosporidiosis (caused by *Henneguya* spp.) (Dykova and Lom, 1978). Whether these responses are effective against protozoal invasions is not clear.

A range of non-specific defense substances have been found in teleost epidermal mucus, including microbial growth inhibitors such as transferrin which has bacteriostatic and fungistatic action (Winter *et al.*, 1980), bacteriolytic enzymes, including protease (Hjelmeland *et al.*, 1983) and trypsin (Braun *et al.*, 1990) and bacterial enzyme inhibitors (Ellis and Grisley, 1985). Lysozyme, which causes lysis of bacterial cell walls, has also been detected in the mucus of several teleost species (Fletcher and White, 1973; Ourth, 1980; Hjelmeland *et al.*, 1983). Acute phase substances have been found, including the antiviral agent, interferon (De Kinkelin *et al.*, 1982) and C-reactive protein, the latter recorded in low levels in the skin mucus of tilapia (Ramos and Smith, 1978). Natural haemagglutinins have been found in the skin mucus of rainbow trout (Hjelmeland *et al.*, 1983) and catfish, *Tachysaurus australis* (Di Conza, 1970), and may possess functional anti-bacterial activity. Whether such haemagglutinins are truly non-specific or immunoglobulins directed to uncharacterised antigens, is not clear. Complement components, homologous to those found in mammals, are recorded from fish serum (Nonaka *et al.*, 1981) and may also be present in mucus (Harrell *et al.*, 1976), although there appears to be no evidence for complement-mediated cytotoxicity occurring in fish mucus. Certain fish species possess skin toxins which may have anti-pathogen as well as anti-predator functions (Randall *et al.*, 1971; Hori *et al.*, 1979). However, there appear to be few reports of non-specific non-cellular defense mechanisms in fish epithelial mucus directed against

protozoa. Non-specific cellular immunity in fishes is associated with either phagocytes or cytotoxic cells. In mammals, the cellular components important in non-specific immunity are the phagocytes (Roitt *et al.*, 1985) of which several types, including macrophages, have been identified in teleosts in the skin (Roberts *et al.*, 1971; Phromsuthirak, 1977; Peleteiro and Richards, 1990) and in gills (Chilmonczyk and Monge, 1980). Non-specific cytotoxic cells (NCC) have been recognised in the immune response of teleosts (Etlinger *et al.*, 1977); these cells show certain properties which are similar to mammalian natural killer (NK) cells, a heterogeneous group comprising mainly large granular lymphocytes (Roitt *et al.*, 1985). Anti-protozoal action of these cells has been reported by Graves *et al.* (1985) who showed that channel catfish NCC were able to kill *Tetrahymena pyriformis* trophozoites following immobilisation with specific antibodies. However, these authors believed that the parasite killing was not attributable to antibody-dependent cellular cytotoxicity (ADCC)-like mechanisms. Graves *et al.* (1985) suggested that immobilisation followed by NCC activity may operate in fish mucus to kill invading *I.multifiliis* theronts, although the possible defense role *in vivo* of NCC is still to be proven.

Specific defense mechanisms

Specific defense mechanisms in fish and higher vertebrates comprise both cellular and humoral factors, the latter characterised by antigen-specific immunoglobulins: antibodies. In teleost fish, the predominant immunoglobulin (Ig) type is tetrameric, termed IgM, indicating its similarity to the pentameric IgM of mammals (Litman, 1976). The presence of immunoglobulins and specific antibodies in fish serum has been well documented. Immunoglobulins have also been shown to exist in the skin mucus from a wide taxonomic range of teleosts (Fletcher and Grant,

1969; Di Conza and Halliday, 1971; Fletcher and White, 1973; Lobb and Clem, 1981a; Rombout *et al.*, 1989b) and in the holostean *Lepisosteus platyrhinchus* (Bradshaw *et al.*, 1971). Specific antibodies in skin mucus have been induced by immunisation with erythrocytes or bacterial antigens, the latter delivered by either intra-peritoneal (i.p.) or bath immersion routes (Fletcher and Grant, 1969; Fletcher and White, 1973; Harrell *et al.*, 1976; Ourth, 1980; St. Louis-Cormier *et al.*, 1984; Lobb, 1987). The origin of mucosal immunoglobulin has been addressed by several workers. For example, Ourth (1980) detected antibodies in skin mucus directed to *Salmonella paratyphi* in channel catfish immunised with these bacteria by the i.p. route and suggested it probable that they may originate from the serum by transudation, although did not rule out the possibility of their local synthesis. Lobb (1987), also working with channel catfish, demonstrated that skin mucus antibodies can be elicited to antigens applied externally (bath immersion) and in the absence of a serum antibody response. Lobb's results suggests that mucus antibodies may have originated from local synthesis within the skin epithelium or from a common mucosal immune system similar to that described for mammals (Mestecky, 1987). Conversely, Tatner and Horne (1986) were unable to detect a mucosal antibody response in rainbow trout to a *Vibrio* bath vaccine despite significant serum antibody titres. Comparative studies on the molecular structures of immunoglobulins from skin mucus and serum have revealed differences which might suggest distinct origins and/or functions. Lobb and Clem (1981b), studying immunoglobulins in the sheepshead *Archosargus probatocephalus*, found both tetrameric and dimeric Ig in the skin mucus, but only tetrameric Ig in the serum. The dimeric Ig from the mucus was resolved by Lobb and Clem (1981a) into two different molecular weight populations, the higher of which contained a 90kDa component similar in size to the secretory piece of mammalian

secretory IgA which is also dimeric (Roitt *et al.*, 1985). These structural differences between serum and mucosal Ig molecules possibly reflect functional heterogeneity, as found amongst different Ig classes in mammals (Roitt *et al.*, 1985). Further evidence for local immunoglobulin synthesis comes from kinetic experiments using radiolabelled Ig (Lobb and Clem, 1981b).

Studies on the cells present in teleost skin mucus have also indicated the possibility of local Ig synthesis. Lymphocytes were detected in the skin epidermis and mucus of channel catfish (Lobb, 1987; Ourth, 1980) and diffuse collections of lymphoid cells were observed in the skin mucus of catfish, *T.australis* (Di Conza and Halliday, 1971). An increase in epidermal lymphocytes was recorded in brown trout, *Salmo trutta*, infected with the fungus *Saprolegnia* sp. (Pickering and Richards, 1980). Further evidence for localised Ig synthesis has come from immunolabelling techniques which have enabled the identification of immunoglobulin- containing cells in the epidermis of rainbow trout (St. Loius-Cormier *et al.*, 1984; Peleteiro and Richards, 1988). However, the specific location of Ig associated with these cells was not determined. Specific antibodies were detected in rainbow trout epithelial cells by Peleteiro and Richards (1985) which were directed to a *Vibrio anguillarum* vaccine delivered by direct immersion.

A possible gut origin for cutaneous mucus antibodies has been proposed in the case of carp by Rombout *et al.* (1989b) who detected antigen- specific antibodies in the skin mucus following oral or anal administration of *V.anguillarum*. Evidence for antibody production in the gut of carp comes from immunocytochemical studies revealing an internal epithelium rich in lymphoid cells with surface bound immunoglobulin, macrophages and granulocytes (Rombout *et al.*, 1989a). Local production of specific antibodies in the gut has also been shown for rainbow trout

(Davidson, 1991).

Whether the presence of antibodies in fish epidermis has any direct action on parasites inhabiting this organ is not clear.

Antigen presentation

Studies referred to above indicate that key components of the specific defense mechanisms are present in fish epithelium, together with evidence for local production of antibody. Mechanisms may therefore function locally to allow the immune system to be presented with and recognise antigens associated with an epithelial-dwelling pathogen. In the mammalian epidermis, antigen "trapping" and presentation is known to be undertaken by Langerhans cells (Shelley and Juhlin, 1976; Braathen and Thorsby, 1980), however similar cells have rarely been reported from fish epidermis (Mittal *et al.*, 1980) and antigen uptake within teleost skin is considered to be poor (Hockney, 1985). Peleteiro and Richards (1990), being unable to demonstrate Langerhans cells in rainbow trout epidermis, considered that epidermal macrophages might instead play the role of antigen trapping. It is evident that further studies on the epidermal cell types and functions are necessary in order to understand their role in local acquired immunity. Consideration must also be given to a possible role by the gills in specific immune mechanisms, given that *C.irritans* is known to parasitize gill epithelia. The gills are considered to be an important organ of antigen uptake, especially particulate antigens (Smith, 1982) and are known to contain phagocytic cells, including macrophages (Chilomonczyk and Monge, 1980; Ellis, 1989). Both gill epithelial cells and mononuclear phagocytes have shown to be involved in uptake of bacterin antigens in salmonids (Zapata *et al.*, 1987).

Immune responses to C.irritans

No studies have been reported on the immune response in fish to *C.irritans*, although aquarium observations have led some authors to conclude that a degree of host protection develops following infection with this parasite. Nigrelli and Ruggieri (1966) suggested that fish maintain immunity to *C.irritans* by premunition but provided no supporting evidence for this. Colorni (1985) observed that fish which survived several exposures to *C.irritans* theronts sustained little or no infection with the parasite for several months, and presumed this reflected acquired immunity.

CHAPTER THREE

BIOLOGY OF CRYPTOCARYON IRRITANS

INTRODUCTION

Previous studies concerning the biology of *C.irritans*, with regards to its life cycle and transmission, have been limited to work by Nigrelli and Ruggieri (1966) and Colorni (1985). The present study sets out to establish the laboratory procedures for the maintenance of *C.irritans* as a basis for biological investigations. Experimental conditions were maintained within the temperature range 23-27°C, chosen to lie within the optimum range of both the parasite (Cheung *et al.*, 1979; Colorni, 1987) and the coral reef fish (De Graaf, 1973; Nybakken, 1988) which provided the sources of *C.irritans* isolates. Photoperiod was also standardised following preliminary investigations, for maintenance of free-living stages. This entailed a controlled photoperiod of 12 hours light and 12 hours dark (12L:12D) using artificial fluorescent lighting in order to simulate the day-length conditions in equatorial regions where the coral reef habitats are found (Nybakken, 1988).

Studies here on the biology of *C.irritans* have necessitated selection of a suitable laboratory host fish for routine parasite passage and experimental infections. The thick-lipped mullet, *Chelon labrosus* (Risso, 1826), a species shown to be susceptible to infection with *C.irritans* during preliminary experiments, was chosen on the following grounds. Wild mullet were locally available throughout most of the year, small specimens (< 10cm TL) being caught by seine net (see methods). Mature mullet, suitable for immunisation, were available from the University aquarium.

Mullet taken from British waters are outside the known geographical range of *C.irritans* and therefore naive to the parasite. They are relatively hardy and non-aggressive, and adapt well to laboratory conditions. Studies with this host would also build upon the extensive knowledge concerning the biology of grey mullet and its interactions with eucaryotic parasites, already undertaken at Plymouth (Pulsford and Matthews, 1982; Ralphs and Matthews, 1986; Mughal and Manning, 1986; Wood and Matthews, 1987; Wood, 1990).

The aims of the present study are to gain a further understanding of the life cycle and general biology of *C.irritans* under different controlled conditions. In particular, to provide information on the behaviour of *C.irritans* which might indicate strategies for its transmission in the wild state, and to investigate for morphometric or behavioral differences between isolates, possibly indicative of strains. Fundamental to these studies is the need to develop optimal laboratory conditions for the maintenance of *C.irritans* for routine passage and the supply of parasite material for experimental applications.

MATERIALS AND METHODS

1. FISH

Fourteen species of fish were used in the present investigation, including 8 tropical marine species which served as donors of *C.irritans* (listed in results). Mullet and other selected species were used for the laboratory maintenance of the parasite and for experimental procedures.

1.1 Sources and collection

Marine tropicals infected with *C.irritans* were bought from aquatic traders nationwide. Thick lipped grey mullet, *Chelon labrosus* (Risso, 1826), were seine netted at low tide from St. John's lake, a creek of the River Tamar estuary, Cornwall. Collections were mostly made between April and October when groups 0+ and 1+ fish were available (Table 1). Mullet, which are euryhaline, were acclimated from native saline conditions (S.G. 1.004) to full seawater (SW, S.G. 1.024) over a period of two weeks, or if intended for stock, were immediately transferred to 25% seawater. Large *C.labrosus* (> 15cm TL), used for immunisation procedures, were selected from stock which had been held upwards of 12 months in the University aquarium. The host of choice for maintenance of *C.irritans* was *C.labrosus* of 0+ and 1+ age groups. Other hosts comprised: thin lipped mullet, *Liza ramada* (Risso), collected in Cornwall at Landulph and the River Fowey at Lostwithiel; common gobies, *Pomatoschistus microps* (Kroyer), from St. John's lake; bream, *Sparus aurata* (L.); tilapia, *Oreochromis mossambicus* (Peters); molly fish, *Poecilia latipinna* (Lesuer, 1821), an ovoviviparous species, purchased from the aquarium retail outlets. The latter were acclimated to full strength seawater and a breeding population established.

Table 1

Pooled monthly collections of 0+ and 1+ group *Chelon labrosus* from St. John's lake, Cornwall.

Collection batch	Date of collection		Number collected
	Month	Year	
1	May	1989	60
2	June		86
3	August		60
4	September		150
5	October		140
6	February	1990	20
7	April		60
8	May		750
9	July		100
10	May	1991	80
			Σ = 1506

1.2 Maintenance

All fish used for parasite maintenance and experimentation were held in a small temperature controlled room maintained at 25±2°C. Reserve stocks of mullet, held at ambient RT, were maintained in 25% seawater as a precaution against accidental infection with *C.irritans* (Colorni, 1985). Further stocks were held at 12°C in full seawater within the main University aquarium.

Both natural and synthetic seawater were used at some stage of the work, the latter only in the case of emergency and for parasite culture and the maintenance of coral reef fish. Natural seawater is supplied to the University on a regular basis, being transported from the Plymouth Marine Laboratory by tanker and stored in subterranean tanks. This water originated from the Plymouth Sound deep channel, and is pumped to shore only when the salinity lies within a SG of 1.023 to 1.024 (at 25°C) and a pH of around 7.9. For some parasite handling procedures, filter sterilised seawater (FS-SW) was prepared by passing natural seawater through a 0.2μm membrane (Sartorius Ltd.) fitted to a 10ml syringe. Synthetic SW, prepared from commercially formulated dry salt mixes (Tropic Marin, Germany or Waterlife Research, U.K.), was maintained at S.G. 1.023 to 1.025, nitrite < 0.1mg N/litre.

Water quality was maintained with the aid of one of the following filtration systems. External canister filters (Eheim Ltd., Germany) containing polymer wool (Interpet Ltd., U.K.) were used for 30-50 litre stock aquaria. Internal powerhead sponge filters (Eheim, Ltd., Germany) were employed in small aquaria where high water quality was required (e.g. for coral fishes). Water quality in the 4-10 litre experimental aquaria, was maintained with the aid of undergravel filters or with air powered internal sponge filters. Filtration was avoided for infection and challenge experiments; water being replaced every 1 or 2 days. All aquaria were aerated.

Water quality was regularly monitored. A falling pH, attributed to organic build up, was readjusted by stepwise buffering with 6:1 (v/v) sodium bicarbonate and sodium carbonate (De Graaf, 1973). Partial buffering was achieved by the use of a commercially prepared calcium and magnesium based substrate ("Calcium Plus", Underworld products, England) which usually maintained the pH above 8.0. High nitrite levels, occasionally exceeding 1.0 mg N/litre, were a particular problem in recently established synthetic SW systems and precluded the introduction of fish until levels had fallen to < 0.1mg N/litre, which usually required a delay of 2-4 weeks. Synthetic SW was used for the maintenance of coral reef fishes which are known to be intolerant of organic and inorganic pollutants, unfortunately present in natural seawater collected within British coastal regions.

Wild caught mullet and gobies were given a prophylactic 14 day bath of broad spectrum bactericide (Technical Aquatic Products Ltd., U.K.) immediately following capture and fed pelleted food ("Promin", U.K.) pre-coated with oxolinic acid antibiotic (3mg/Kg feed; Sigma, product 0-0877).

Feeds, usually given twice daily, comprised pelleted food ("Promin") occasionally supplemented with mixed flake (Aquarian, U.K.), spirulina pellets (Hikari, Japan) and macerated coley fillets enriched with a vitamin mix. Certain species of coral reef fishes required specialised diets including brine shrimp nauplii, and chopped mussels (*Mytilus*).

1.3 Anaesthesia

Fish were anaesthetised using either benzocaine (ethyl-p-amino benzoate) or MS222 (methane-tricaine sulphonate), both supplied by Sigma Ltd. Benzocaine was generally preferred to MS222, being less excitant to fish and with fewer long term

cardiac effects (Ross and Geddies, 1979). Benzocaine, prepared as a stock solution, 100mg/ml in absolute methanol, and stored at 10°C, was added drop wise to the water to give the required level of anaesthesia. For small mullet (≤ 10g weight), 25 drops/litre was effective. Fish were recovered by transferring to clean, well-aerated seawater. Recovery from deep anaesthesia was sometimes assisted by passing a stream of seawater under the gill arches. Recovery in the molly fish (*P.latipinna*) was monitored until resumption of swimming activity, as this species had a tendency to relapse even after recommencement of normal opercular movement.

2. CRYPTOCARYON IRRITANS

2.1 Source

Isolates of *C.irritans* were obtained from infected marine tropical fishes purchased from various aquatic traders throughout the country. Infected fish known to have received recent chemotherapy were avoided. One isolate, originally collected from the sparid, *Diplodus noct* (Cuvier and Valenciennes), was received from Israel, viable cysts being sent air mail at ambient temperatures. Host fish species which provided the *C.irritans* isolates (listed in the results) were identified from reference sources (Carcasson, 1977; Smith, 1977; Burgess *et al.*, 1988).

2.2 Laboratory maintenance

2.2.1 Isolation and establishment

Newly purchased infected marine fishes were held in 10 litre aquaria for 5 days during which period trophonts would have exited from the fish epidermis to encyst. Donor fish were then removed and the aquarium restocked with 2 or more mullet,

naive to *C.irritans*. The number of mullet added was dependent upon the estimated level of infection in the source fish, being increased in cases of heavy infections. Mullet were held in the infection aquaria until the white spots of *C.irritans* were visible and then transferred to other aquaria for further passage. Replacement mullet were added every 2 to 3 days until infection levels subsided.

2.2.2 Passage

Passage of *C.irritans* between mullet was performed by either co-habitation of infected and uninfected fish or by exposure to controlled numbers of theronts. In the former method, recipient mullet, naive to *C.irritans*, were introduced into the aquarium at various intervals, the numbers of fish being gauged to the levels of theront release, with the aim of establishing sublethal infections. The controlled method provided a better means of quantifying standardised infection procedures using a known number of theronts. Infected fish were removed as required for trophont harvesting, as described below.

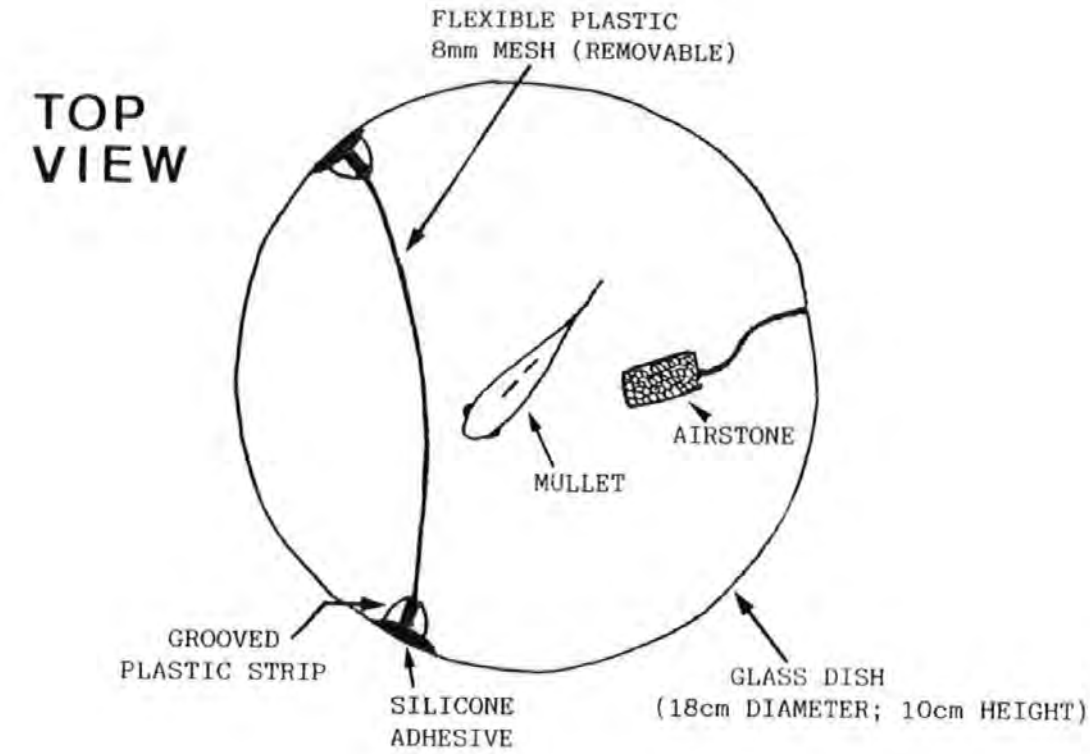
2.3 Collection of trophonts

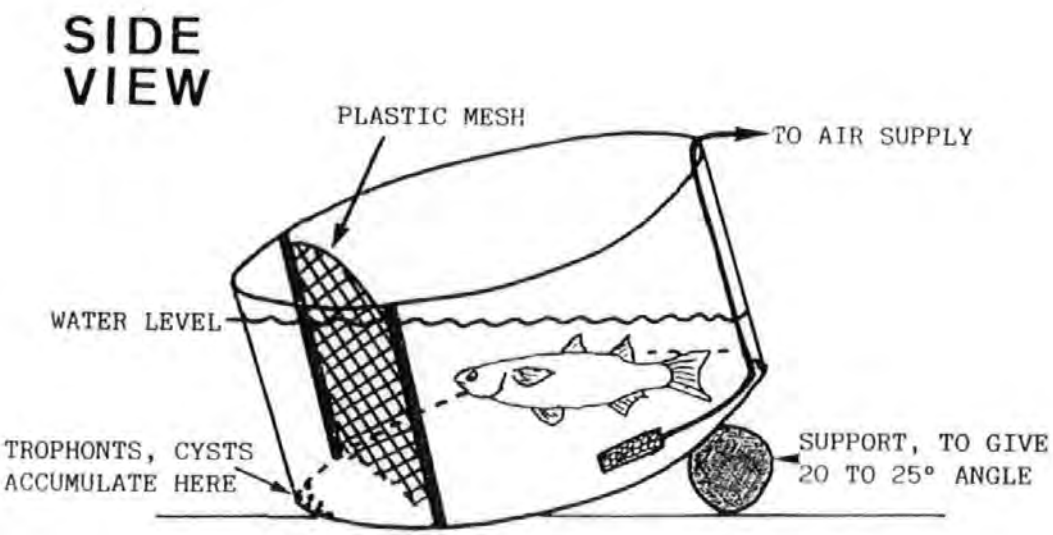
As mullet were observed to feed on trophonts and cysts, it was necessary to design a container which would separate the free-living parasites from the host fish upon trophont release. The "trophont harvester", based on a 17-19cm diameter crystallising bowl, is shown diagrammatically in Fig. 3. The bowl is separated into 2 chambers, one large and one small, by a removable plastic mesh which allows parasites but not fish to pass through. The dish is raised to a 20-25° angle, so that the larger chamber is uppermost. One to 2 infected mullet (< 80mm TL) were held in the upper chamber until trophont release. Upon exit from the host, the trophonts

Figure 3

Trophont harvester.

For the collection of trophonts and cysts of *C.irritans*.
The harvester holds 0.9 to 1.2 litres of seawater.


TOP VIEW
FLEXIBLE PLASTIC
8mm MESH (REMOVABLE)
AIRSTONE
MULLET
GROOVED
PLASTIC STRIP
SILICONE
ADHESIVE
GLASS DISH
(18cm DIAMETER; 10cm HEIGHT)


SIDE VIEW
PLASTIC MESH
TO AIR SUPPLY
WATER LEVEL
TROPHONTS, CYSTS
ACCUMULATE HERE
SUPPORT, TO GIVE
20 TO 25° ANGLE

sediment to the base of the dish and move through or under the mesh to the lower chamber where they accumulate along the lower rim. Depending on their subsequent use, trophonts were either collected by pipette before encystment, or allowed to encyst before being scraped off with the edge of a glass coverslip.

2.4 Trophont encystment in culture plates

Trophonts collected from the trophont harvester were pipetted into solid 4ml watchglasses and observed under a stereomicroscope. Host epidermal tissue, scales, and other particulate matter were removed by pipette and the trophonts washed in several changes of FS-SW at 25°C. Washed trophonts were allowed to encyst in wells of a 24 well polystyrene tissue culture plate (Corning Ltd., U.S.A.) up to a density of about 400/well. Wells were filled with 2ml FS-SW.

2.5 Enumeration of parasite stages

Free-living trophonts were enumerated as they were collected by pipette, with the aid of a stereomicroscope. Enumeration of cysts was less laborious due to their immobility. Cysts were counted in the trophont harvesters aided by a transparent squared template affixed under the dish. For theronts, estimates of total numbers were made as follows. Theronts released from cysts held in culture dishes were transferred by pipette into a small glass container and the volume adjusted to a known value. Following gentle agitation to evenly disperse the theronts, two 50μl samples were taken and each spotted onto a Sedgewick-Rafter counting chamber. Theronts were immobilised by killing with a drop of formalin. Total counts were made for each 50μl sample, and the combined counts used to estimate the number of theronts in the original sample. Repeat samples were estimated in cases where theront counts in the

two 50μl samples differed by twofold or greater.

2.6 Morphometric analyses

Theronts and cysts were measured in a Sedgewick-Rafter chamber; theronts were killed-immobilised with 1-2 drops of formalin. Morphometric studies were made using a compound microscope equipped with an ocular micrometer.

2.7 Incubation of reproductive cysts

Parasites, at either the trophont or cyst stage, were transferred to culture plates, covered with a lid and placed in a humidification chamber at 23-27°C. FS-SW was generally replaced 1 to 2 times/day. Culture plates containing the adhered cysts were conveniently emptied by inversion; this facilitated thorough rinsing thereby minimising microbial contamination. Later in the study, cysts were held under a photoperiod of 12L:12D.

2.8 Control of contaminating organisms during parasite incubation

In situations where trophonts or cysts could not easily be separated from host material, or where the water had been fouled by dead fish, bacterial contaminants were controlled by a commercial preparation of penicillin (10,000 IU/ml) and streptomycin (10,000μl/ml) (= "pen-strep", Gibco Ltd.) diluted to 1% in FS-SW. Free-living ciliates (mostly hypotrichs), flatworms, and less commonly, nematodes, were occasionally observed in the *C.irritans* cultures. These were removed by pipette; flatworms and some ciliates were selectively destroyed by brief (< 1 min) immersion in tap water without harmful effects to *C.irritans* cyst development.

2.9 Quantification of theront activity

Swimming activity of emerged theronts was quantified visually on a five point scale (Table 2). This subjective scoring, based on numerous observations, was used as a possible indicator of theront infectivity, and also as a basis for assessing the effects of chemotherapeutants. Somatic activity was recorded with the aid of a microscope using transmitted light. Cilia movement was observed on an inverted stereomicroscope at 200 X magnification.

2.10 Measurement of photoresponses of theronts

A Sedgewick-Rafter chamber, 50mm X 20mm, was positioned on the stage of a stereomicroscope placed within light proof conditions, allowing access to the eyepieces. A system of lighting was arranged using a cool fibre optic source which illuminated a restricted area of approximately one quarter at one end of the chamber. A seawater suspension of theronts were pipetted onto the centre of the chamber and exposed to the localised light source. After a known period of exposure, 4-5 drops of formalin were pipetted along the centre line of the chamber to prevent the theronts from crossing from one side to the other. A further 2-3 drops of formalin were gently added to each side of the chamber in order to kill all theronts without altering their spatial positions. Theront counts were made for each side of the chamber with the aid of normal substage illumination.

2.11 Cloning from an isolate

A mullet, naive to *C.irritans*, was exposed to progeny obtained from a single cyst of isolate CI-CR. Trophonts released from this fish were harvested for further passages. The clone ("CI-CR clone") was maintained over 8 passages after which

Table 2

A five point scale for theront activity.

Scale	Activity	Criteria
+ + +	Normal	Based on observations of recently (< 1 hour) excysted theronts. Fast speed, in mid-water.
+ +	Moderate	Motile in mid-water, but speed is markedly less than "+ + +".
+	Low	Motile, but mostly on the substrate, with infrequent mid-water movement.
O	None	Remain on substrate. No significant somatic movement, but cilial movement observed.
OD	Dead	As "O" but no cilial movement (= NCM). Presumed dead.

viability was assumed to be lost.

2.12 Control of photoperiod

An artificial light/dark cycle was imposed on the free living stages of *C.irritans* and during host infection with the parasite during certain maintenance procedures. These conditions were established by construction of a light proof chamber fitted with a timer controlled 30 watt fluorescent light (Thorn, "warmlight") emitting ≈1,700 lux, set horizontally 40cm above the parasite container. This facilitated manipulation of the number of hours of light and dark (L:D) per 24 hour period.

3. PHOTOMICROSCOPY

Motile stages of *C.irritans* were first immobilised by killing with formalin. Photographs were taken using a VANOX-T microscope (Olympus, Japan). For colour work, the resolution of internal structures was enhanced using a differential interference contrast attachment which gave a false colour image. Films used were Fujichrome 100 ASA colour (reciprocity number =2) and TMAX 100 ASA black and white (Kodak).

EXPERIMENTS AND RESULTS

1. CRYPTOCARYON IRRITANS

1.1 Isolates

During the course of the study, isolates from 9 different sources were acquired, 8 of these being successfully passaged through experimental fish (Table 3). Each isolate was designated a 1 to 3 letter code, based on the latin name of the donor species.

1.1.1 Host specificity of isolates

Infections between different species of fish were recorded throughout the study to provide information concerning the host specificity of *C.irritans*. Nine isolates and a clone of CI-CR were exposed to a total of 6 different fish species, from 5 taxonomic families. Initial passages from the donor fishes were performed using thick lipped mullet as recipient hosts. The routes of transmission, recorded here between combinations of the 9 donor fishes (7 coral reef species and one sparid) and 6 experimental fish species, are shown in Fig. 4. The results show that for all combinations of passage attempted between host species, all but one resulted in successful transmission. The exception, an isolate from the chaetodontid, *Chelmon rostratus*, (designated CI-ROS), did not passage to mullet from the donor fish. The CI-CR clone was successfully passaged from thick lipped mullet to thin lipped mullet, mollies, and gobies, thereby transcending three taxonomic families of hosts. This investigation provided no evidence for host specificity by *C.irritans*.

Table 3

Marine species of fish from which *C.irritans* was isolated.

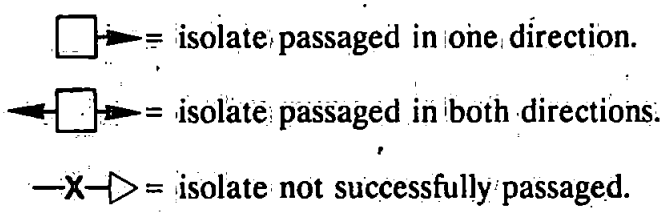
Isolate code	Host			Passage into mullet?
	Species	Family	Common name	
CI-CE	*Centropyge eibli* [1]	Pomacanthidae	Pygmy angelfish	yes
CI-FL	*Forcipiger longirostris* (Broussonet) 1782	Chaetodontidae	Long-nosed butterflyfish	yes
CI-CR	*Chelmon rostratus* (L.) 1758	Chaetodontidae	Copperband butterflyfish	yes
CI-HT	*Holacanthus tricolor* [1]	Pomacanthidae	Rock beauty	yes
CI-ROS	*Chelmon rostratus* (L.) 1758	Chaetodontidae	Copperband butterflyfish	no
CI-DN	*Diplodus noct* (Cuvier and Valenciennes)	Sparidae	-	yes
CI-AE	*Amphiprion ephippium* (Bloch) 1790	Amphiprionidae	Black-faced clownfish	yes
CI-GS	*Grammistes sexlineatus* Thunberg, 1792	Grammistidae	Six striped grouper	yes
CI-O	*Omegophora* sp.[2]	Tetrodontidae	Dog-faced pufferfish	yes

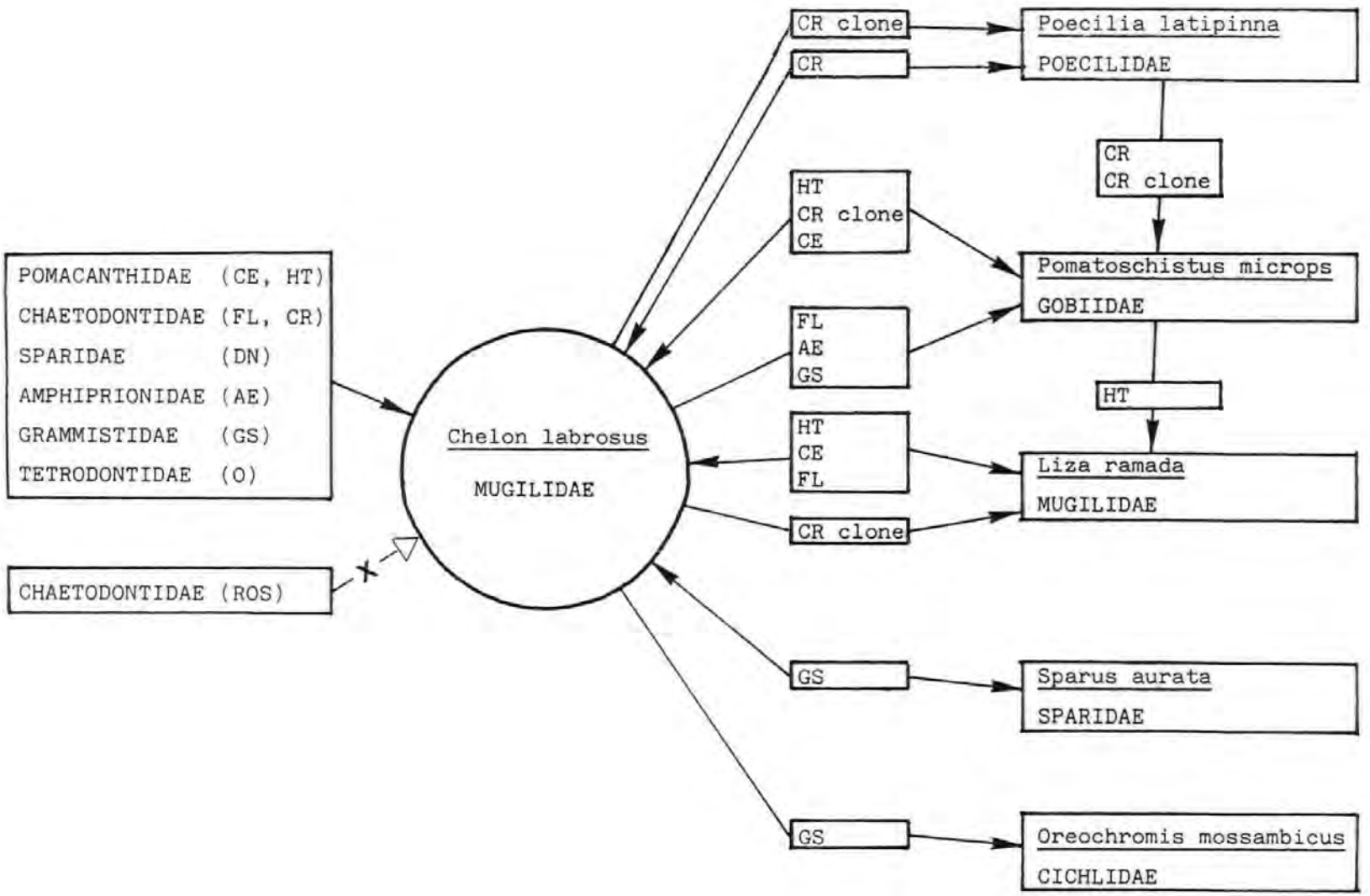
[1] Authority not known.

[2] Presumptive identification, based on genus description by Burgess *et al.* (1988).

Figure 4

Passage of *C.irritans* isolates between different fish species.




POMACANTHIDAE (CE, HT)
CHAETODONTIDAE (FL, CR)
SPARIDAE (DN)
AMPHIPRIONIDAE (AE)
GRAMMISTIDAE (GS)
TETRODONTIDAE (O)
CHAETODONTIDAE (ROS)
Chelon labrosus
MUGILIDAE
CR clone
CR
HT
CR clone
CE
FL
AE
GS
HT
CE
FL
CR clone
GS
GS
Poecilia latipinna
POECILIDAE
CR
CR clone
Pomatoschistus microps
GOBIIDAE
HT
Liza ramada
MUGILIDAE
Sparus aurata
SPARIDAE
Oreochromis mossambicus
CICHLIDAE

1.1.2 Distribution and effect of trophonts within host mullet epithelium

The parasitic trophonts were observed to infect the epithelium of the skin and gills, but rarely the eye (Fig. 5). Although no pathological studies were undertaken, it was noted that high levels of infection resulted in severe skin haemorrhaging, host death usually occurring within 4 days after exposure.

1.1.3 Survival of laboratory isolates

Isolates were maintained through successive transmissions for a maximum of 48 weeks. Due to the varied duration of the cyst stage, development being asynchronous even for the clone, the true number of passages for each isolate could not be determined. Nevertheless, an estimate was made, based according to the average time for each life cycle. Maintenance times and approximate number of passages are shown for all isolates in Table 4. The results show a wide range in maintenance times for the various isolates, between 0 and 48 weeks ($\approx$ 0 - 34 passages). Loss of an isolate was not here associated with any particular period or season of the year.

1.1.4 Senescence

A gradual loss of viability and infectivity over a few passages of isolates CI-CE and CI-GS was found to precede the loss of the isolate (termed isolate "crash"). The sequence of developmental failures, between the stages of free-living trophont and theront, was recorded for CI-CE. Observations were based on a sample of 230 trophonts, harvested from a primary infection of mullet, and subsequently monitored in tissue culture plates (Fig. 6). Of these, only 25.7% underwent normal encystment, of which 32 cysts (= 14% of trophonts studied) were monitored for theront

Figure 5

Distribution and effect of *C.irritans* within mullet epithelium.

A: Heavy infection at 3 days.
Note distribution of trophonts within skin epithelium of body and fins.

B: Infection at 3.5 days.
Some trophonts have exited from the fish. Note slight body surface haemorrhaging.

C: Infection at 4 days.
All trophonts have exited from the fish. Note extensive body surface haemorrhaging and white fletches on fins.

Scale bar = 1cm.


A


B


C

Table 4

Duration of *C.irritans* isolates.

Isolate designation[1]	Duration maintained (weeks)	Approx. number of passages[2]
CI-CE	31	21
CI-FL	11	7
CI-CR	25	18
CI-HT	31	21
CI-ROS[3]	<1	0
CI-DN	5	3
CI-AE	3	1
CI-GS	48	34
CI-O	12	8

[1] Details of isolates given in Table 3.

[2] Estimate for average time of life cycle being 10 days, comprising 3 days as the parasitic trophont and 7 days average time between encystment and 50% excystment.

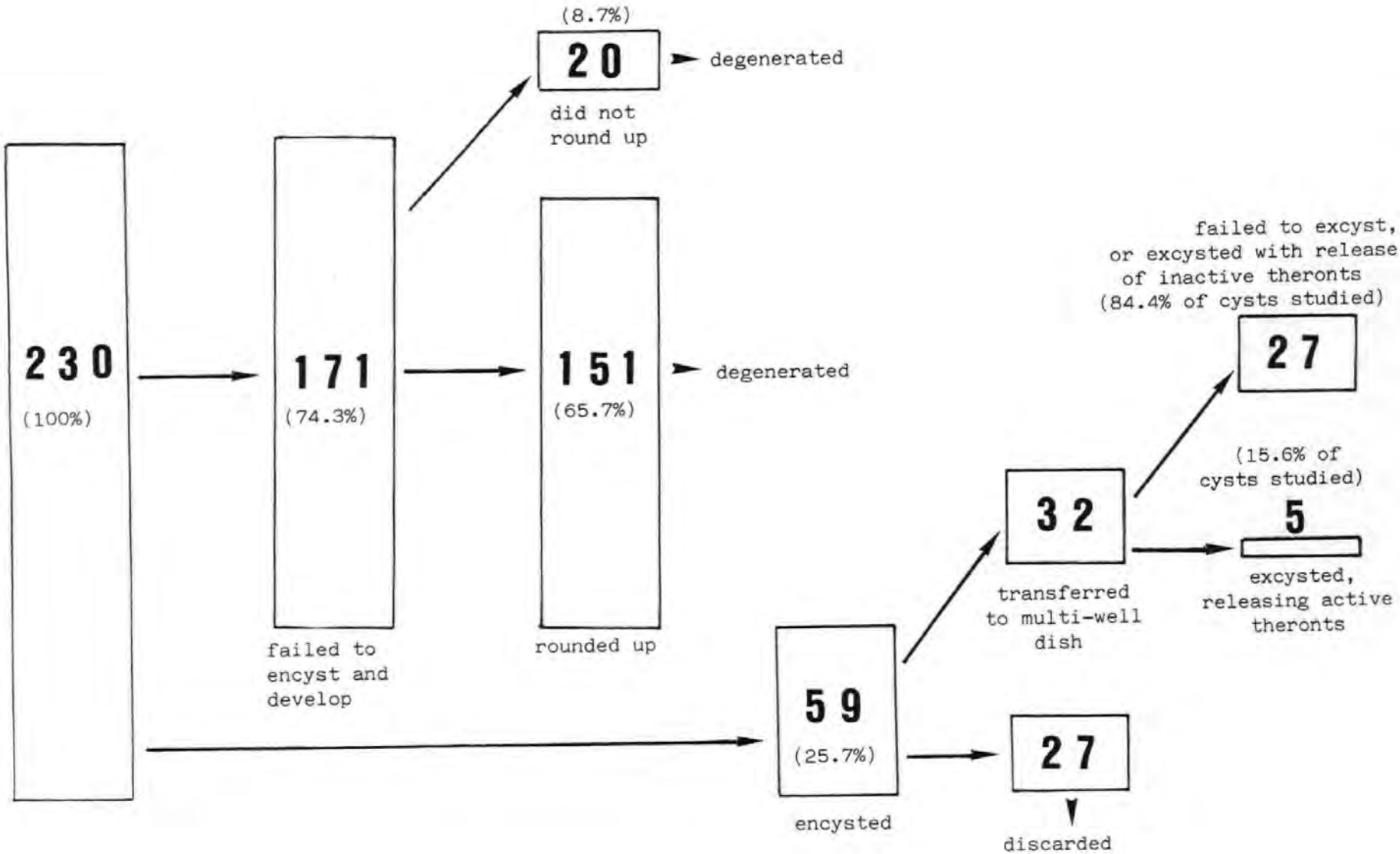
[3] Live, motile *C.irritans* trophonts confirmed by light microscopy, but unable to passage into mullet.

Figure 6

Senescence of *C.irritans* following primary infection of mullet.

Based on 230 trophonts (CI-CE).

Height of blocks proportional to numbers of parasites.


230
(100%)
171
(74.3%)
failed to encyst and develop
(8.7%)
20
did not round up
degenerated
151
(65.7%)
rounded up
degenerated
59
(25.7%)
encysted
32
transferred to multi-well dish
failed to excyst, or excysted with release of inactive theronts (84.4% of cysts studied)
27
(15.6% of cysts studied)
5
excysted, releasing active theronts
27
discarded

production. The percentage encystment is low as compared with 70% to 93% encystment in "healthy" isolates (data from Table 9). Failure to encyst was associated with an inability of trophonts to round up or to adhere to the substratum. Of those cysts monitored for development, only 15.6% (5/32) released active theronts, equivalent to 4% of the sampled 230 trophonts (after adjustment for those cysts not studied). In the 5 instances of excystment, the duration of the cyst stage was between 13 and 15 days; this was longer than the peak time for excystment under normal conditions (5-7 days). Theront infectivity was not investigated.

2. QUANTITATIVE STUDIES ON THE LIFE CYCLE STAGES

2.1 Morphometric

Morphometric analyses (summarised in Table 5), made on the following stages: theront; cyst; and parasitic trophont, *in situ* development, are considered separately below.

2.1.1 Theront

Preliminary observations on size differences between theronts from a single cyst prompted an investigation for distinct populations. Theronts were collected on emergence from a random sample of 14 cysts (isolate CI-HT) between days 7 and 16 post-encystment. Measurements of length and width were recorded from random samples of 10 theronts from each cyst. Size distribution of the 140 theronts is shown in Fig. 7. The distribution appears unimodal, as based on frequency per 5μm length interval, with no evidence for more than one size population. Theronts varied from 40-69μm in length (mean = 55.9μm) and 22-44μm in width (mean = 32.3μm).

Table 5

Summary of morphometric and other measurement data for *C.irritans*, and comparison with data from other sources.

Parameters	Authority				
	Present study	Brown (1951)	Sikama (1961)	Nigrelli & Ruggieri (1966)	Colorni (1985)
Duration of trophont on host (days)	2.9 - 4.7 ($\bar{x}$ = 3.5) (24-26 °C)	-	-	-	3 - 7 (peak = 4-5) (24-27 °C)
Duration of cyst (days)	3.5 - 35 (peak = 5-7) (24-26 °C)	-	-	6 - 9 (peak = 8) (22-25 °C)	3 - 28 (peak = 4-8) (24-27 °C)
Number of theronts per cyst	119 - 292 ($\bar{x}$ = 198)	-	up to 100 or more	up to 200 or more	not greater than 200
Theront longevity (hours)	< 24 (24-26°C)	-	-	< 24 (approx.)	generally ≥ 30-36; up to 48
Theront infectivity (hours)	< 18 (24-26°C)	-	-	-	-
Theront size ($\bar{x}$: length X width, µm)	55.9 X 32.3 (r: 40-69 X 22-44)	40-56 (length)	35 X 65	35 X 56.5	25 x 57 (r: 50-70 X 20-30)
Cyst size ($\bar{x}$: µm)	326 X 306 (r: 154 X 160 to 369 X 406)	-	200-300 diam; (r: 90-400 diam)	r: 95 X 170 to 252 X 441	214 X 241 (r: 160 X 150 to 310 X 370)
Trophont size ($\bar{x}$: µm)	[1] 353 X 205 (r: 129-332 X 203-452)	(r:70-400 length)	(r: 66 X 34 to 360 X 452)	(r: 48 X 27 to 450 X 350)	-

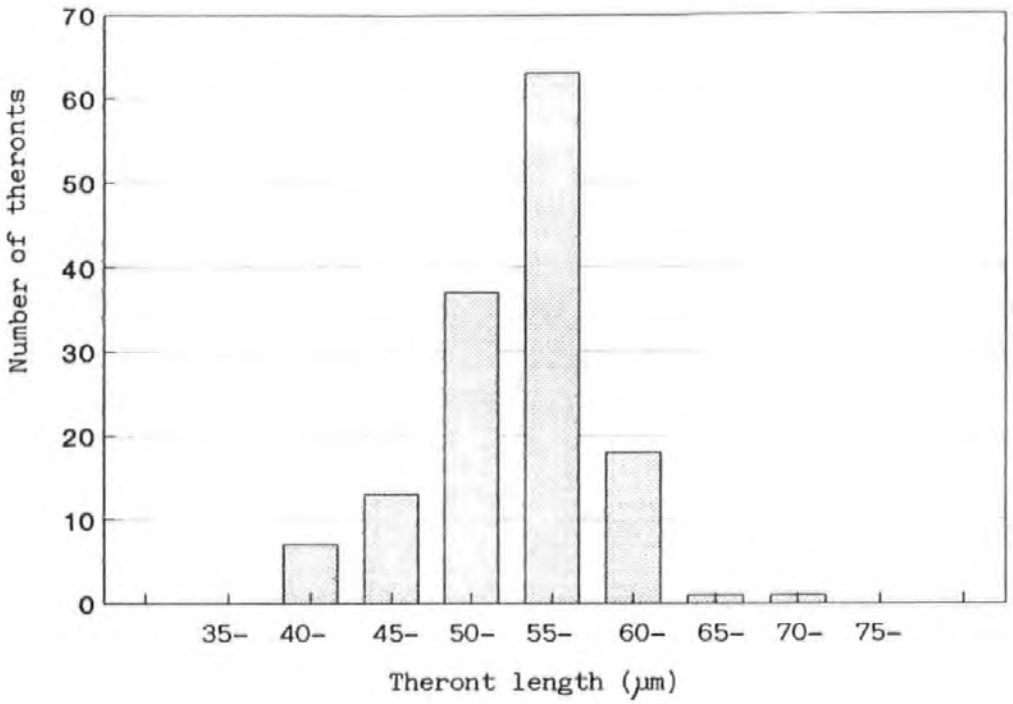
r = range

[1] size at 72 hours post-infection, 24-26°C.

Figure 7

Frequency distribution of theront length, released from a population of cysts (CI-HT).

Mean theront length = 55.9μm (n=140).


70
60
50
40
30
20
10
0
Number of theronts
35-
40-
45-
50-
55-
60-
65-
70-
75-
Theront length (μm)

2.1.2 Trophont growth within host epidermis

Eight mullet (3-6g) were each exposed to a primary infection of 2,000 theronts (CI-GS) and killed at either 2, 24, 48, or 72 h post-exposure. The ventral, anal, and caudal fins were excised for measurement of the live trophonts *in situ* (Fig. 8). Trophont body length and mid-region width, excluding the cilia, was recorded from 14 to 39 randomly sampled trophonts for each of the 4 time periods post-exposure (Fig. 9). A total of 118 trophonts were measured. Over the observation period, which terminated 6 to 17 h before anticipated time of trophont release, both the mean length and mean width of the trophonts showed approximate doubling at each 24 hour period. The mean length:width (L:W) ratio remained fairly constant over the four sample times, ranging between 1.00(L):0.63(W) at 72 h and 1.00(L):0.70(W) at 48 h.

2.1.3 Cyst

A total of 215 cysts were measured from three isolates, CI-HT, CI-AE, and CI-GS. Cysts were derived from trophonts following normal development in a primary infection of mullet. Random samples of cysts, collected for each isolate, were measured across the long axis (= maximum diameter between the outer cyst walls) and cross axis (= diameter across the mid-line bisecting the long axis). Cyst size ranges were recorded within individual isolates, between isolates, and for the same isolate following development in different mullet hosts. Measurements of 125 cysts from isolate CI-GS was recorded first as a basis for comparative studies with the other isolates. Results for CI-GS gave a size range of 160μm to 406μm (long axis) with mean cyst size of 325μm (long axis) by 305μm (cross axis), equivalent to a long:cross axis ratio of 1.00:0.94.

A comparison of cyst sizes from different isolates and from different host

Figure 8

Growth of *C.irritans* trophont within fin epithelium of host mullet at four different times post-infection.

Times, post-infection: A: 2 h (arrowed); B: 24 h; C: 48 h; D: 72 h.

Scale bar = 50μm.

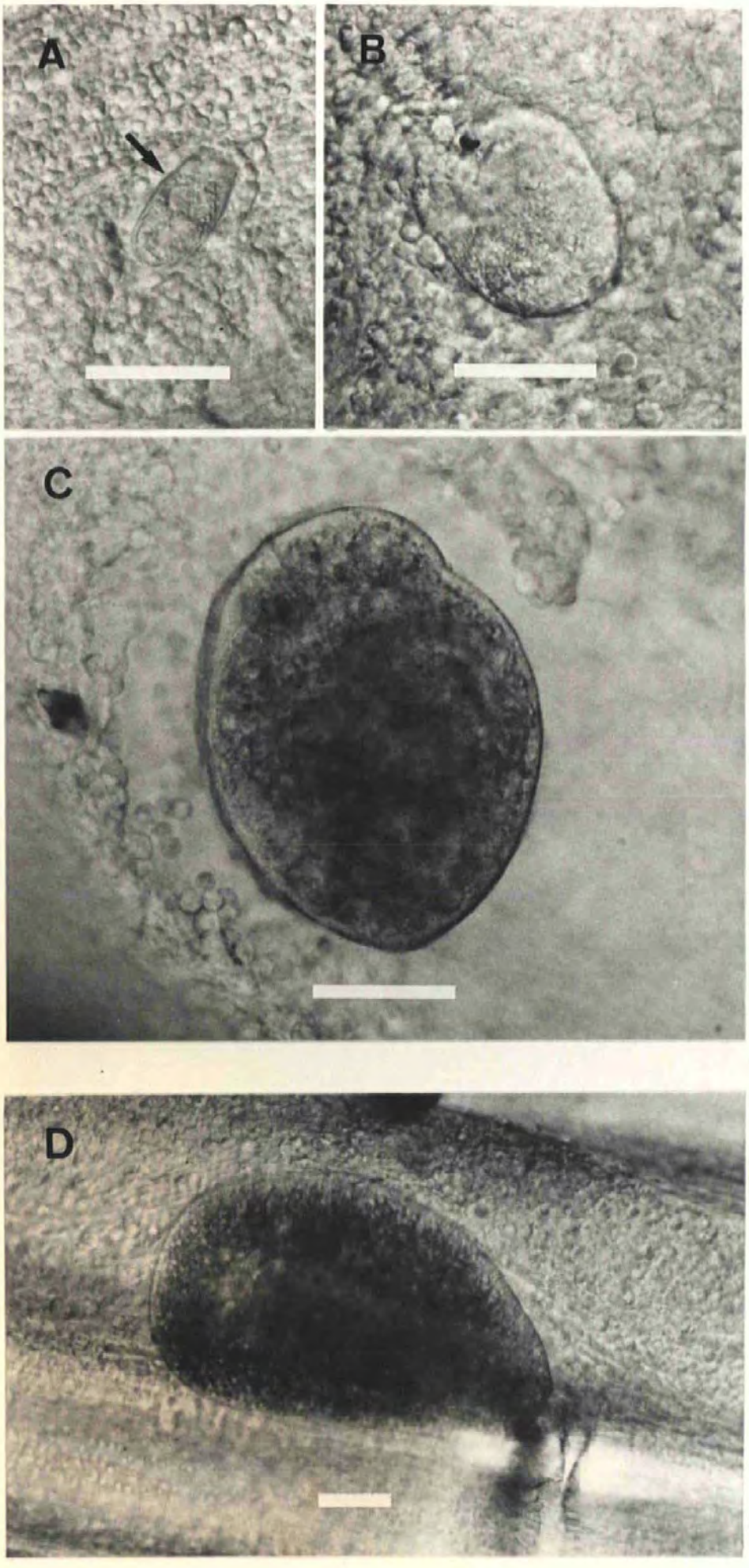

A
B
C
D

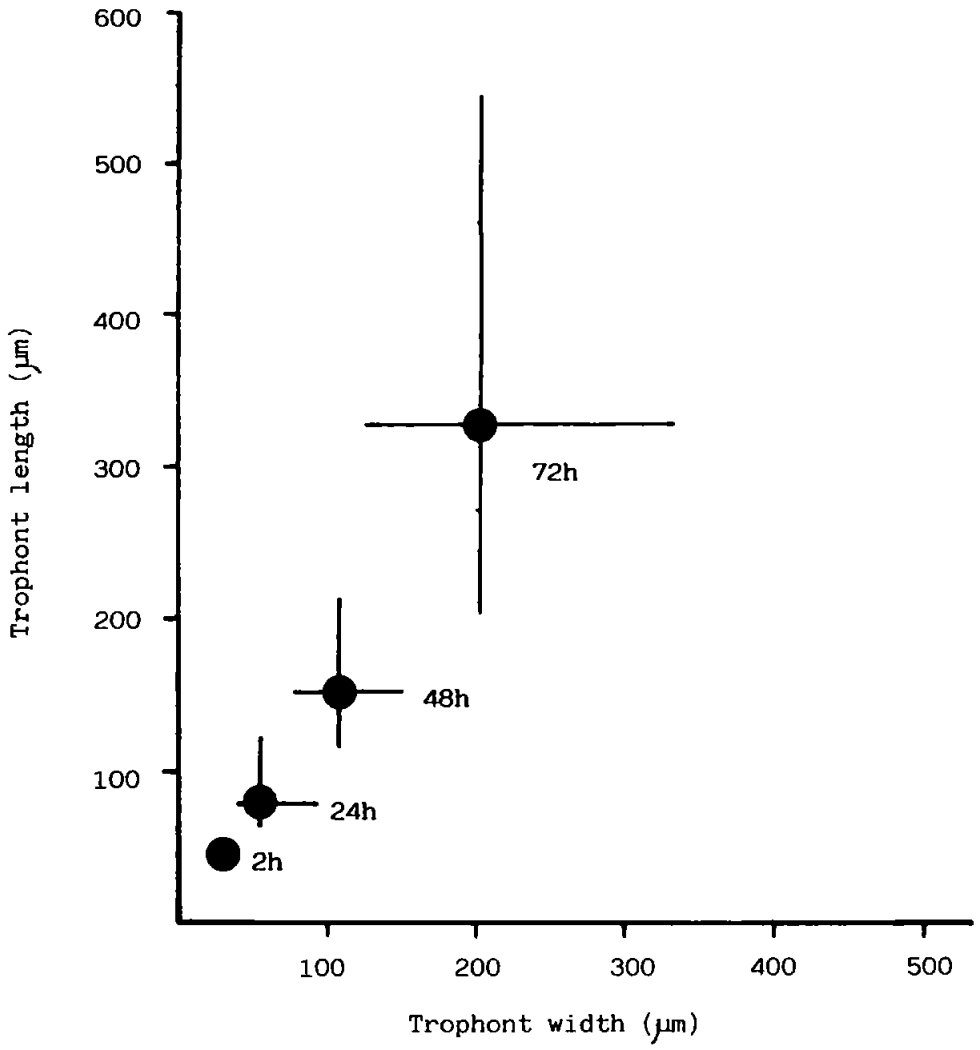
Figure 9

Growth of *C.irritans* trophont within fin epithelium of host mullet.

h = hours post-infection.

● = mean size.

┼ = size range.


600
500
400
300
200
100
Trophont length (µm)
72h
48h
24h
2h
100
200
300
400
500
Trophont width (µm)

specimens, based on long axis only, is shown in Table 6.

Mean cyst size was significantly different between CI-GS isolate (from fish #3) and CI-HT (fish #1) ($p = 0.001$) and between CI-GS (fish #3) and CI-AE (fish #2) ($p = 0.001$). Mean cyst size, however, also differed significantly ($p = 0.001$) between sample populations of cysts derived from different fish infected with the same isolate (CI-GS); for example, fish #3 versus fish #5, and fish #4 versus fish #5. Cyst morphometric data from fish #1,2,3,4 were compared to determine any association between infection intensity and cyst size, which might be suggestive of a "crowding effect" inhibiting trophont growth. The results show no significant correlation between mean cyst size and the number of cysts harvested (correlation coefficient, $r = -0.26$; $n=4$), or, by standardising for fish weight, with the number of cysts harvested per gram of donor fish ($r = -0.27$; $n=4$). Maximum cyst wall thickness, recorded from a CI-AE cyst, measured 32μm across the widest point of the translucent zone.

3. PHOTORESPONSES

Photoperiod responses were recorded for both trophont release from the host and for excystment; observations were made following manipulations of the light cycle. Any effects of light and dark on encystment and on the theront stage were also investigated.

3.1 Periodicity of trophont release from the host

Preliminary observations that the release of trophonts occurred predominantly at night prompted further studies. The influence of photoperiod on trophont release from mullet was investigated as follows. Eight mullet, naive to *C.irritans*, were acclimated for ≈7 days to 12L:12D, the light phase commencing at 9pm. Each fish

Table 6

Comparison of cyst sizes from three different isolates of *C.irritans* determined from primary infection in mullet.

Isolate	Fish		Cysts				
	Identity number	Weight (g)	Total released	Number measured	Mean size (μm)	Size range (μm)	Size SD_{n-1}
CI-HT	1	3.98	276	20	263.4	209-302	24.62
CI-AE	2	3.84	nd[1]	20	282.8	228-345	31.38
CI-GS	3	3.42	514	125	325.9	160-406	50.02
	4	3.21	684	25	238.1	201-315	28.92
	5	3.38	172	25	289.2	239-353	32.14

[1] Not done

was then exposed to 5,000 theronts (CI-GS) for 2 h during the dark phase after which the fish were transferred to separate trophont harvesters. Three of the 8 fish were maintained under 12L:12D; the remaining 5 fish were held under identical conditions except for an extended (36 hours) light period between 66-101 hours post-exposure to encompass the usual period for trophont release. At 12 h intervals, representing the end of each light or dark phase, the numbers of released parasites were recorded. At each inspection, the fish were transferred to a clean harvester under similar conditions. All fish were monitored until 137 h post-exposure.

Patterns of trophont release for the 3 fish held under the repeating 12L:12D photoperiod and for the 5 fish under the extended light period are compared in Figs. 10 and 11. In the "normal" photoperiod (Fig. 10) all trophonts were released during darkness, and most (98%) between 78 to 89 h post-exposure. The remaining 2% of trophonts were released over the following dark phase at 102 to 113 h post-exposure, but no releases occurred during the intervening light period. Thus, release appeared to be determined by both the duration of infection and by the photoperiodicity. When the photoperiod was adjusted to give continual light (from 66 to 101 h post-exposure) during the critical period of trophont release (Fig. 11) the majority of trophonts (99%) released at the same period post-exposure (78 to 89 h) but in this case in the light. No trophonts were released over the following 12 h of light (90 to 101 h post-exposure), however 15 trophonts, all from the same fish, were released after resumption of darkness between 102 and 113 h post-exposure, suggesting some influence of photoperiodicity. Observations on trophont release following routine passage by co-habitation occasionally revealed a small proportion of trophonts ($<10\%$) exiting the host fish during the light phase of a normal photoperiod (12L:12D).

Figures 10 and 11

Periodicity of *C.irritans* trophont release from mullet epithelium under different photoperiods.

Figure 10

Normal photoperiod (12L:12D).

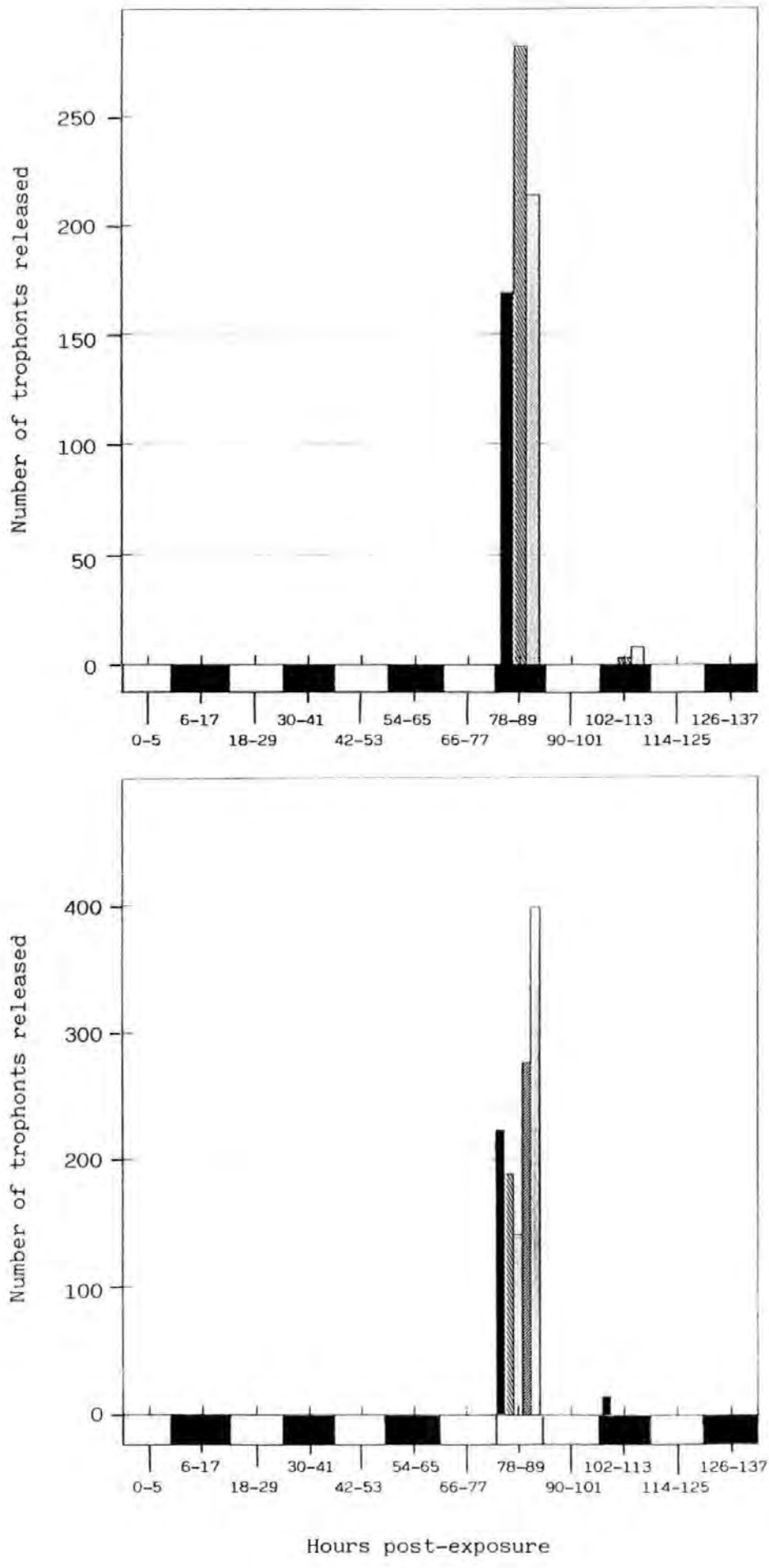
n= 3 fish.

Figure 11

Normal photoperiod with extended light phase between 66-101 hours post-infection.

n= 5 fish.

For both figures: individual fish represented by different bar shadings.


Number of trophonts released
250
200
150
100
50
0
0-5
6-17
18-29
30-41
42-53
54-65
66-77
78-89
90-101
102-113
114-125
126-137
Number of trophonts released
400
300
200
100
0
0-5
6-17
18-29
30-41
42-53
54-65
66-77
78-89
90-101
102-113
114-125
126-137
Hours post-exposure

3.2 Effects of light or dark on encystment

In view of the positive influence of darkness on trophont release, investigations were undertaken to determine whether photoperiod also influenced the timing of encystment. A total of 179 trophonts (CI-HT) were harvested within 2 h of release from 2 infected mullet and distributed between two tissue culture plates, with 10-20 trophonts/ well. Both plates were placed in a 25°C light incubator ("Fi-Totron 600", Fisons Ltd.), one held within a light-proof container, the other exposed to a light intensity of 4400 lux. After 7 h, the number of encystments were counted. The results indicated 100% encystment, under conditions of both continual light (n = 101) and continual dark (n = 78). Cyst viability in both groups was investigated by exposing the cysts to 12L:12D and monitored until excystment. Theront release was observed from both dark and light encysted parasites, each commencing at 5 days post-encystment and occurring in the dark.

3.3 Periodicity of theront emergence

Emergence times of theronts were difficult to determine accurately in view of their nocturnal release. Approximately 160 CI-GS cysts, which had encysted the previous day, were distributed amongst wells of a cell culture plate, ≈ 20 cysts/ well, and held for 9 days under a 12L:12D photoperiod. The seawater was changed regularly during monitoring. Water temperature was monitored in control wells containing seawater only. Cysts, day 10 post-encystment, were monitored at various intervals over a 66 h period, and emerged theronts at each interval were removed, pooled, and their numbers estimated. The pattern of theront emergence over 66 h (Fig. 12) indicates a strong association with periods of darkness. Emergence was observed as early as 3 h after onset of darkness, continuing on to the end of the dark

Figure 12

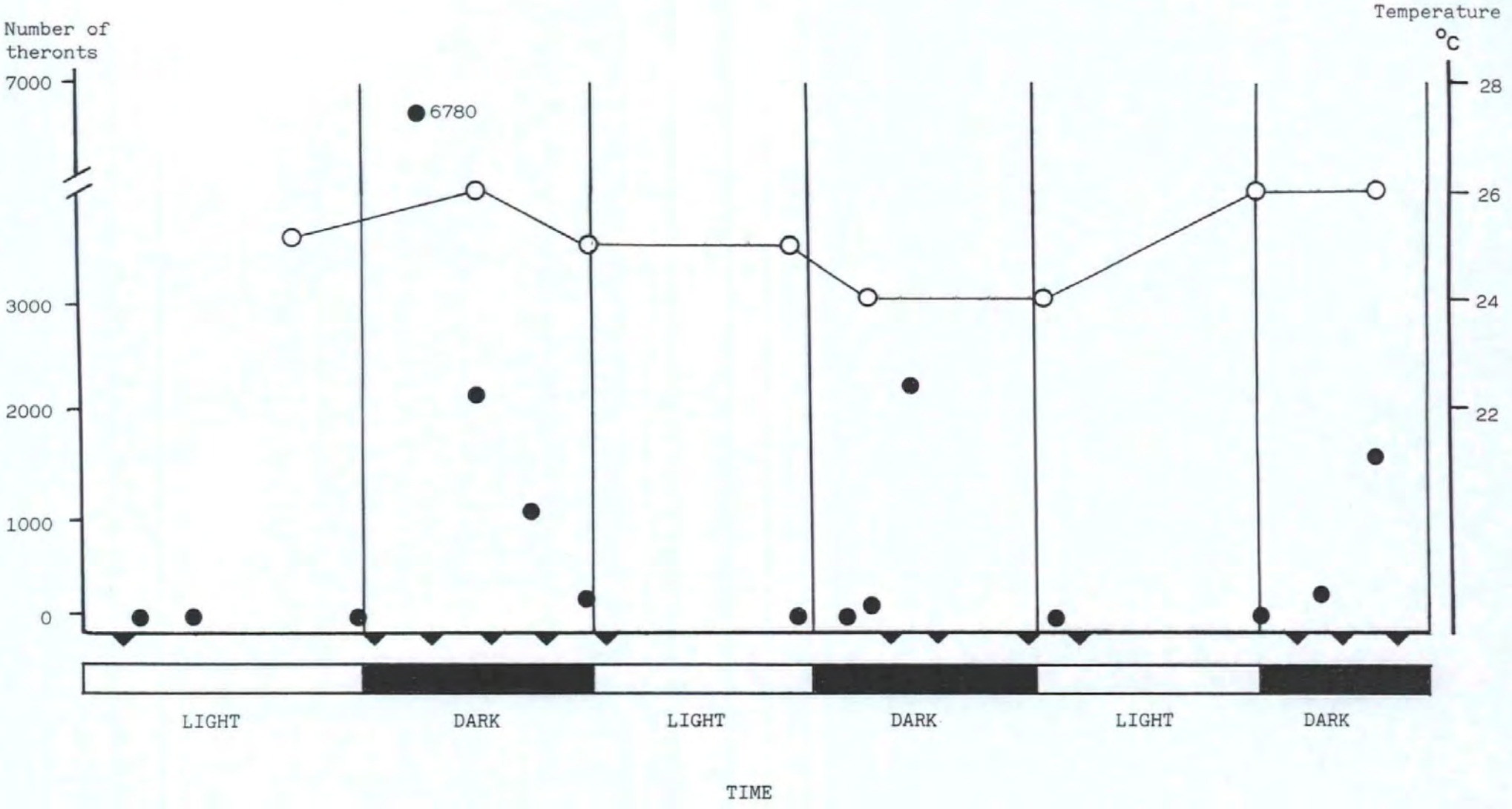
Influence of photoperiod on *C.irritans* excystation and theront emergence.

Photoperiod = 12L:12D.

O–O = temperature, °C.

● = number of theronts.

▼ = water change.


Number of theronts
7000
3000
2000
1000
0
6780
Temperature °C
28
26
24
22
LIGHT
DARK
LIGHT
DARK
LIGHT
DARK
TIME

period. In total, over 14,000 theronts emerged during periods of darkness whereas none emerged during periods of light. Water temperature ranged between 24°C to 26°C over the experimental period, with no fluctuations in response to light or dark. Emergence appeared to be unaffected by seawater changes or water temperature.

3.4 Photoresponses of theronts

Evidence for the responses of theronts to light was investigated. Seawater suspensions of between 112-343 theronts (CI-GS), < 1.5 h post-emergence, were added to a Sedgewick-Rafter chamber and exposed to a localised light source for a given time (see methods, section 2.10) after which any spatial congregations were noted. Initially, observations were made after 2 to 30 min, with the aid of brief periods of substage lighting. Subsequent investigations were standardised to a single 15 min uninterrupted exposure to the localised light.

Initial trial runs provided some evidence of a positive response to light. This result was further supported in the 4 standardised trials (Table 7) in which the light source was alternated between left and right. On all four occasions, the majority of theronts were recorded on the illuminated side of the chamber; overall, the positive phototactic response to light was statistically significant (Chi^2: $p<0.001$). Although a response to light was not observed in all theronts, the percentage responders was similar for each run, ranging between 57.1% to 68.9% on the light side (mean =65.2%).

3.5 Effects of photoperiod on duration of theront activity

Activity of theronts with time was investigated using two different photoperiod regimes. Theronts (CI-GS), < 1 h post-excystment, were pooled and distributed

Table 7

Photoresponses of *C.irritans* theronts provided with light and dark options.

Trial	Theronts				
	Numbers			Percentage in light	Chi^{2} [a]
	Total	Light	Dark		
1	343	236	107	68.9	48.50[b]
2	114	78	36	68.4	15.47[b]
3	112	64	48	57.1	2.29[c]
4	213	132	81	62.0	12.21[b]
Σ	782	510	272	65.2	72.43[b]

[a] At 1 degree freedom.

[b] Significant at $P < 0.001$.

[c] Not significant.

amongst wells of two tissue culture plates, 8-17 theronts/ well in 2 ml FS-SW, and held at 25±1°C. One culture plate, containing 107 theronts, was held under a normal 12L:12D regime, the other containing 119 theronts was held under continual light. Theront activity in both groups was monitored at various intervals up to 18 h.

Fig. 13 compares activity in both groups. Under normal photoperiod (encompassing 10 h dark followed by 8 h light), the percentage of active theronts is initially 100%, falling only slightly to 90% at 6 h post-excystment. A significant reduction in activity is observed by 10 h with only 56% active, and by 18 h the majority of theronts were inactive. Immobility was observed as early as 3 h with some deaths at 10 h. Comparison of the two activity profiles suggests that loss of activity is more rapid under continuous light than under a normal photoperiod, these differences being particularly notable at 6 h and 10 h post-excystment.

4. LONGEVITY AND VIABILITY OF FREE-LIVING STAGES

The duration of the free-living trophont and cyst stages, and the effect of temperature on stage longevity were recorded. For the theront stage, viability with time, and the effect of antibiotics on activity with time, were investigated.

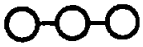
4.1 Trophont

The duration of the trophont stage was recorded as the time between host exit and encystment. Forty-eight trophonts (CI-CE), from a primary infection of mullet, were collected immediately after release and transferred to individual wells of a tissue culture plate. The numbers of trophonts encysting were recorded at timed intervals with the aid of an inverted microscope. The percentage encystment with time is shown in Fig. 14. Extrapolation from the graph indicates that the time for 50% of

Figure 13

Effect of light and dark on theront activity.

 = theronts exposed to darkness (0-10 h), then light (10-18 h) (n=107).

 = theronts exposed to continuous light (n=119).

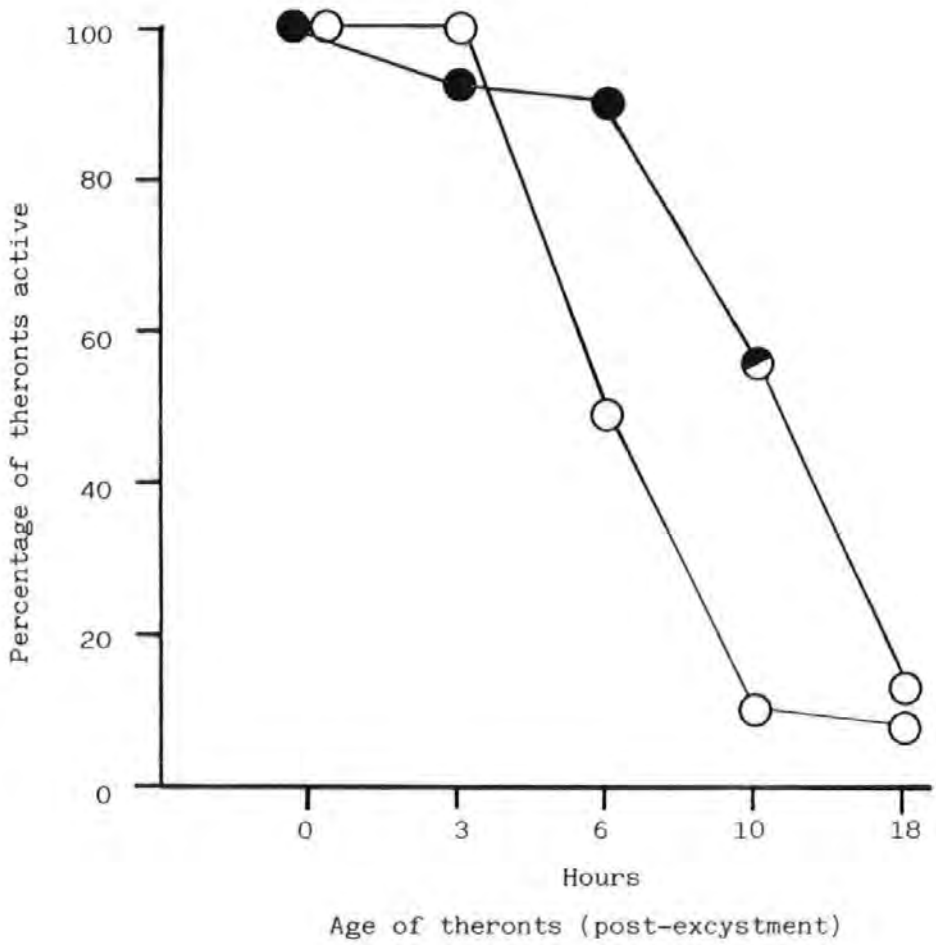
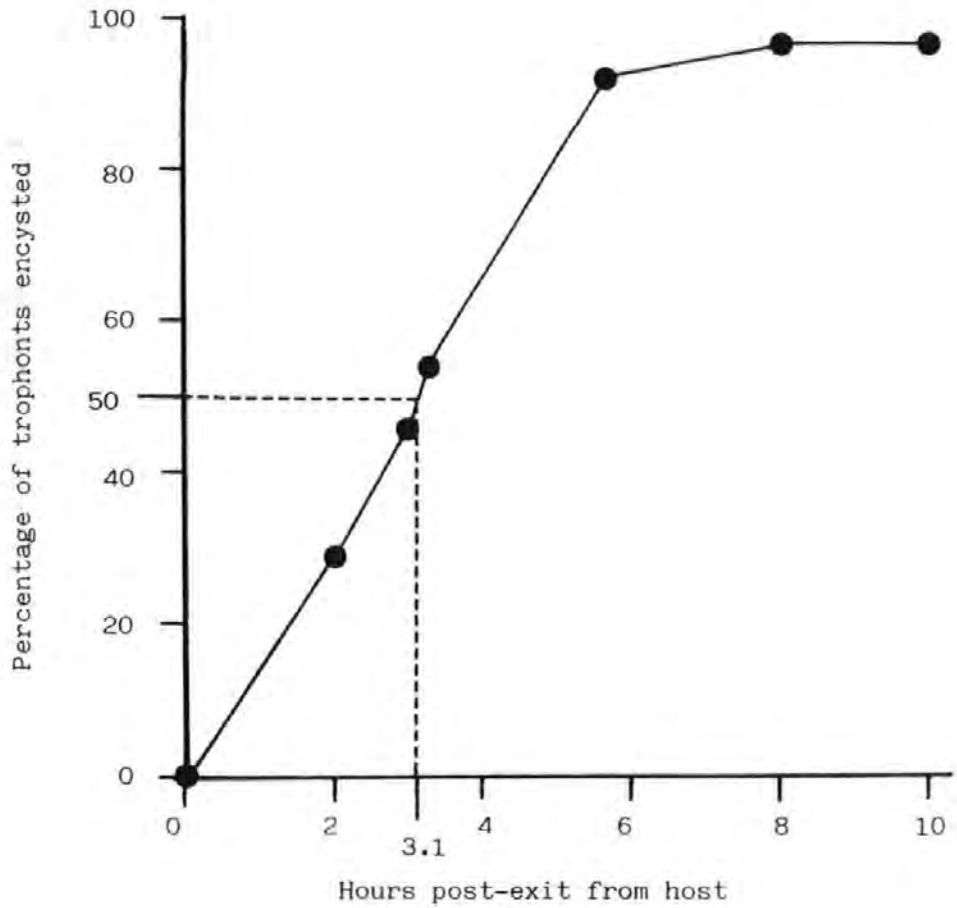

100
80
60
40
20
0
Percentage of theronts active
0
3
6
10
18
Hours
Age of theronts (post-excystment)

Figure 14

Duration of free-living trophont stage of *C.irritans*.

Based on time, h, between exit from host mullet and encystment.

Hatched line shows extrapolated time of 3.1 h for 50% of trophonts to encyst.


100
80
60
50
40
20
0
Percentage of trophonts encysted
0
2
3.1
4
6
8
10
Hours post-exit from host

trophonts to encyst was 3.1 h after release from host. Forty six of the 48 trophonts (=96%) eventually encysted, the process being completed within 8 h post-release. The two remaining trophonts failed to encyst although both had rounded up by 23 h post-release.

4.1.1 Effects of low temperature on trophont release and development

The experiment was designed to determine whether trophont exit from host and subsequent development could occur at temperatures below the parasite's lower tolerance range of 19-20°C. Four mullet (3- 4.5g), naive to *C.irritans*, were each exposed 72 h previously to 2,000 theronts (CI-HT) at 25°C. After 72 h the infected fish were isolated in trophont harvesters, 2 being held at 25°C, the other 2 acclimated over 3-4 h to ambient laboratory temperatures (12-15°C). Trophonts were released from all four fish at between 76 and 88 h after exposure. Random samples of ca. 75 motile trophonts collected from each of the two fish held at 25°C were divided into three groups of ca. 50: A,B, and C, comprising ca. 25 trophonts from each fish. Trophonts from the 2 fish held at 12-15°C were similarly separated into groups D,E, and F. The effects of temperature on further trophont development was investigated as follows. Each of the six groups of trophonts (A to F) were distributed amongst wells of a tissue culture plate (10-20 trophonts/well) and held at either 4°C, 12-17°C, or 25°C (Table 8). The seawater was replaced every 1-2 days and the parasites observed at various intervals up to 12 days post-release. Any trophonts which had encysted were monitored for theront release up to day 21. The results (Table 8) show that all trophonts exhibited normal rounding up and loss of motility within 6 h of harvesting. Under optimal temperature conditions (group A) all trophonts encysted, of which 94% eventually produced theronts. Trophonts released at low temperature

Table 8

Development of *C.irritans* trophonts at different temperatures.

Temp. for parasite exit from mullet	Parasites						
	Group	Maintenance temp.	No. trophonts sampled	Number (%)			
				Rounded up	Attached	Encysted	Excysted
25°C	A	25°C	50	50 (100%)	50 (100%)	50 (100%)	47 (94%)
	B	12-17°C	50	50 (100%)	11 (22%)	11 (22%)	0 (0%)
	C	4°C	47	47 (100%)	0 (0%)	0 (0%)	0 (0%)
12-15°C	D	25°C	50	50 (100%)	50 (100%)	50 (100%)	31 (62%)
	E	12-17°C	49	49 (100%)	4 (8%)	6 (12%)	0 (0%)
	F	4°C	50	50 (100%)	0 (0%)	0 (0%)	0 (0%)

(12-15°C) and subsequently maintained optimally at 25°C (group D) also achieved 100% encystment, however, the percentage excystment was lower than that for group A. These results indicate that the temperature at which the trophonts were released had no effect on the ability to encyst, but release at low temperature may have adversely affected subsequent cyst development. Overall, cyst formation did appear to be influenced by the temperature at which the parasites were maintained following exit from host; percentage encystment falling with temperature. Trophonts maintained at the lowest temperature, 4°C (groups C,F) did not encyst, with some degenerating by days 6 to 7, and all by day 12. Encystment was usually associated with adherence to the plastic well, although some trophonts (all from group E) encysted without adherence. Comparison of groups D,E, and F, show that trophonts released from the host at low temperature were able to complete normal development and excystment only if subsequently returned to an optimal 25°C (group D) and not when maintained at sub-optimal temperatures (groups E and F). Cyst formation occurred in only a few parasites held at low temperature (12-17°C = groups B and E) but no excystations were observed up to day 12. Further incubation of these cysts at 25°C until day 21 did not lead to theront production. Infectivity of the excysted theronts, from groups A and D, was not investigated.

4.2 Cyst

4.2.1 Duration of cyst stage and theront release

The time between trophont encystment and theront release was investigated as well as the peak time for theront production in a cyst population following synchronised encystment. Trophonts which had recently exited following a primary infection of mullet were pipetted into separate wells of 96 well polystyrene flat

bottomed plates and allowed to encyst. Trophonts which had not encysted by 8 h post-emergence were excluded from the study. Cysts, held under a 12L:12D photoperiod, were observed every 24 h during the initial 1-2 h of the light phase and the number of excystments recorded. The duration of the cyst stage was investigated for each of 4 isolates: CI-GS, CI-HT, CI-CR, and CI-CR clone. A fifth group of cysts (CI-HT) were obtained from a partially immune mullet following a second exposure to 5,000 theronts. Each group of cysts was monitored for at least 15 days and thereafter until a minimum of 4 consecutive days had passed without theront release. Results are presented in Figs. 15 to 19 and Table 9. The pattern of excystment does not closely follow a normal distribution with time, for all 5 groups. A peak time for excystment (i.e. maximum number of excystments in 24 h) at days 5 to 7 was recorded for all groups except B (CI-HT) which was days 9 to 10 (Table 9). The time taken for 50% of cysts to release was 6 to 7 days for all groups, again except B which was 9 to 10 days. No apparent difference was found in the pattern of excystment of CI-HT between those cysts derived from a primary infection (group B, Fig. 16) and those from a fish with partial protective immunity to *C.irritans* (group C, Fig. 17), suggesting no influence of host immunity on cyst development time. The clone of CI-CR (group E, Fig. 19) showed a similar distribution of excystment as that of the parent isolate (group D, Fig. 18). When combining the results from all groups, it was observed that the minimum time for excystment was 4 days and the maximum 24 days. However, observations here on other cyst populations, have shown a minimum period between encystment and excystment of 3.5 days (≈ 84 h) recorded for isolates CI-HT and CI-GS, and a maximum of 35 days recorded for CI-GS.

It was noted from two observations of excystment that theront exit was synchronous, taking between 1 to 2 minutes for all to leave the cyst.

Figures 15 to 17

Duration of *C.irritans* cysts in different populations.

Figure 15

Isolate CI-GS.

n= 50.

Figure 16

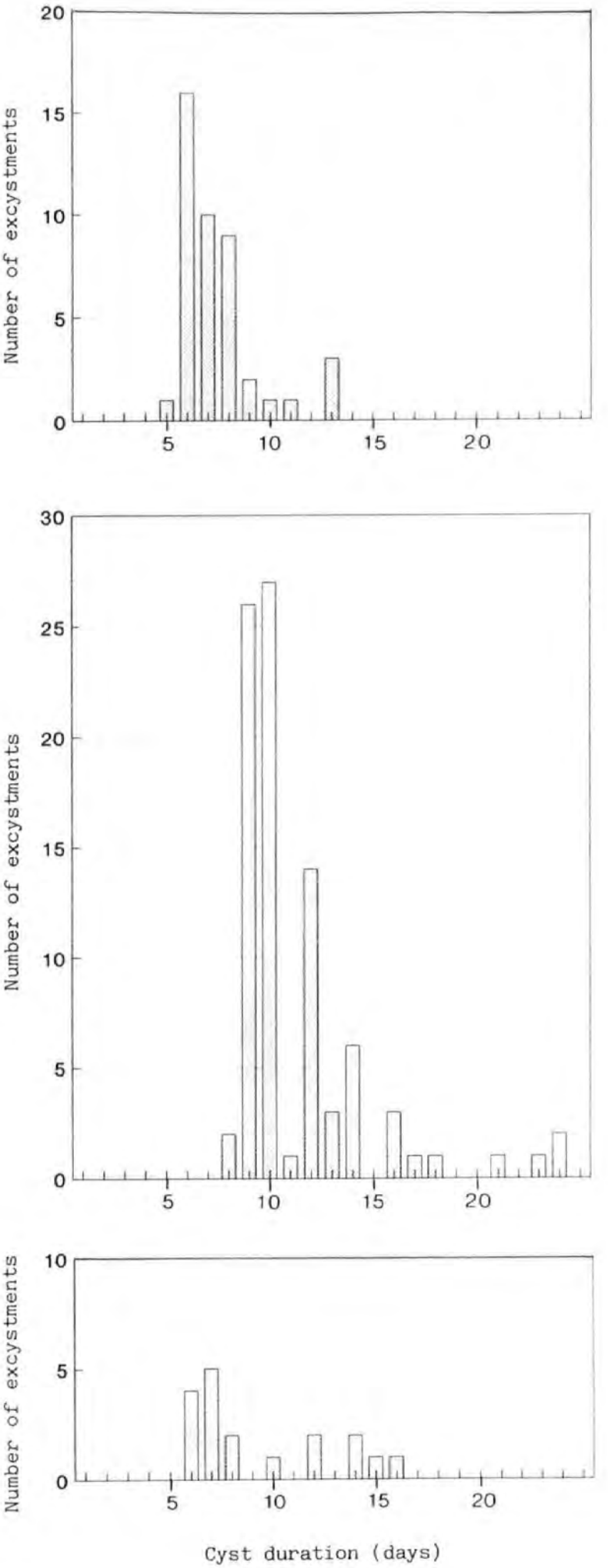
Isolate CI-HT from a primary infection of mullet.

n= 95.

Figure 17

Isolate CI-HT from a partially immune mullet.

n= 20.


Number of excystments
20
15
10
5
0
5
10
15
20
Number of excystments
30
25
20
15
10
5
0
5
10
15
20
Number of excystments
10
5
0
5
10
15
20
Cyst duration (days)

Figures 18 and 19

Duration of *C.irritans* cysts in different populations.

Figure 18

Isolate CI-CR parent line.

n= 132.

Figure 19

Isolate CI-CR clone.

n= 127.

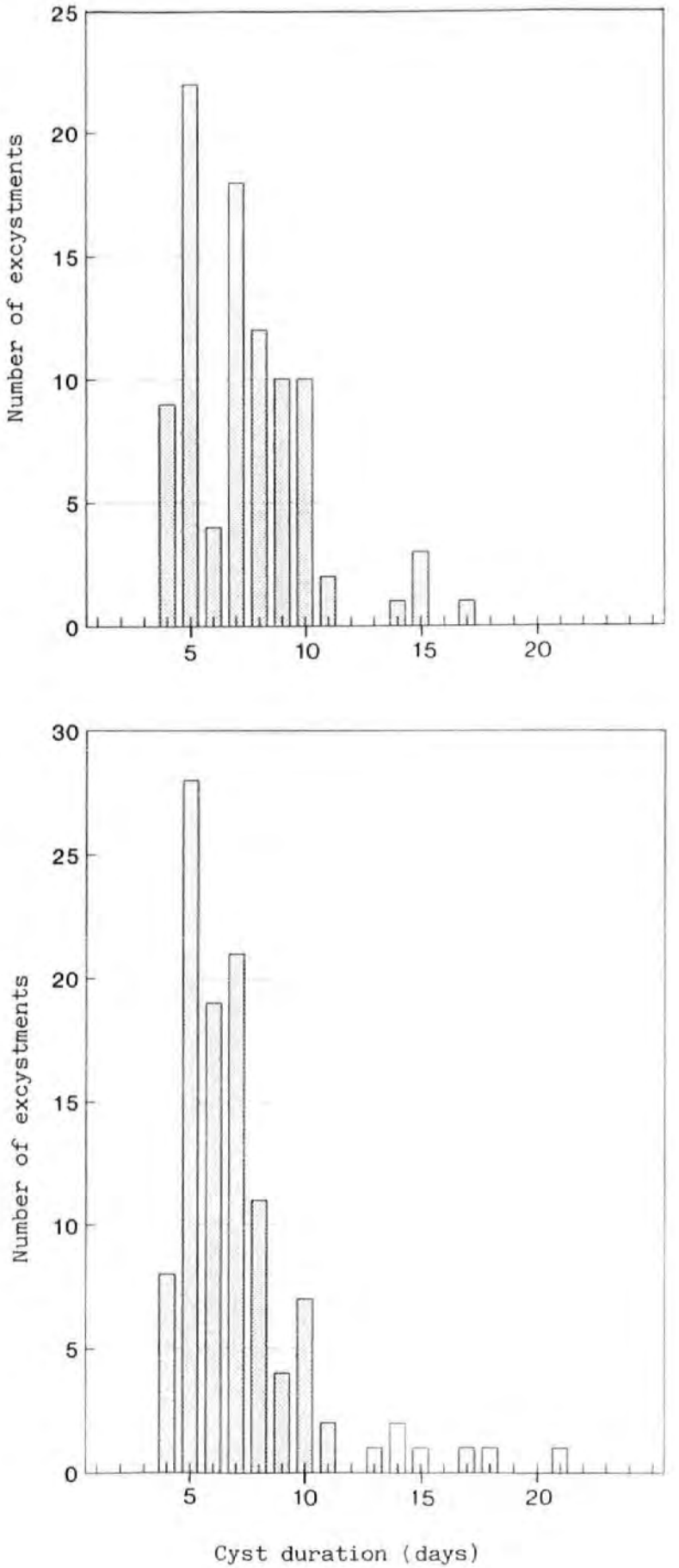

Number of excystments
25
20
15
10
5
0
5
10
15
20
Number of excystments
30
25
20
15
10
5
0
5
10
15
20
Cyst duration (days)

Table 9

Summary of *C.irritans* excystment times for cysts from four different isolates.

Cyst group	Isolate	No. cysts	Time (days) for peak excystment	Time (days) for 50% excystment	Percentage (proportion) excysted[1]
A	CI-GS	50	6	7	86 (43/50)
B	CI-HT (naive[2])	95	9-10	9-10	93 (88/95)
C	CI-HT (immune[3])	20	7	7	90 (18/20)
D	CI-CR (parent)	132	5	7	70 (92/132)
E	CI-CR (clone)	127	5	6	84 (107/127)

[1] Recorded for duration of experiment; true % excysted values may be slightly higher.

[2] Cysts harvested from a primary infection of mullet.

[3] Harvested from mullet with partial protective immunity.

4.2.2 Theront productivity with age of cyst

Possible associations between the duration of cyst stage and the numbers and sizes of theronts produced were investigated following a synchronised primary infection of mullet. Twenty cysts (CI-HT) were individually maintained in wells of a microtitre plate. Subsequent investigations were undertaken on 16 of these which excysted between days 7 and 23. For each excystment, the numbers of theronts were counted and a random sample of ten measured by length. The results (Fig. 20) show no significant correlation between the number of theronts released per cyst and age of cyst ($r = 0.25$; $n = 16$). There was no significant correlation between theront length and the number of theronts produced per cyst ($r = 0.19$; $n = 14$). It was noted that the minimum number of theronts from any one cyst was 117, and the maximum, 377 (mean = 198; median = 183).

4.3 Theront

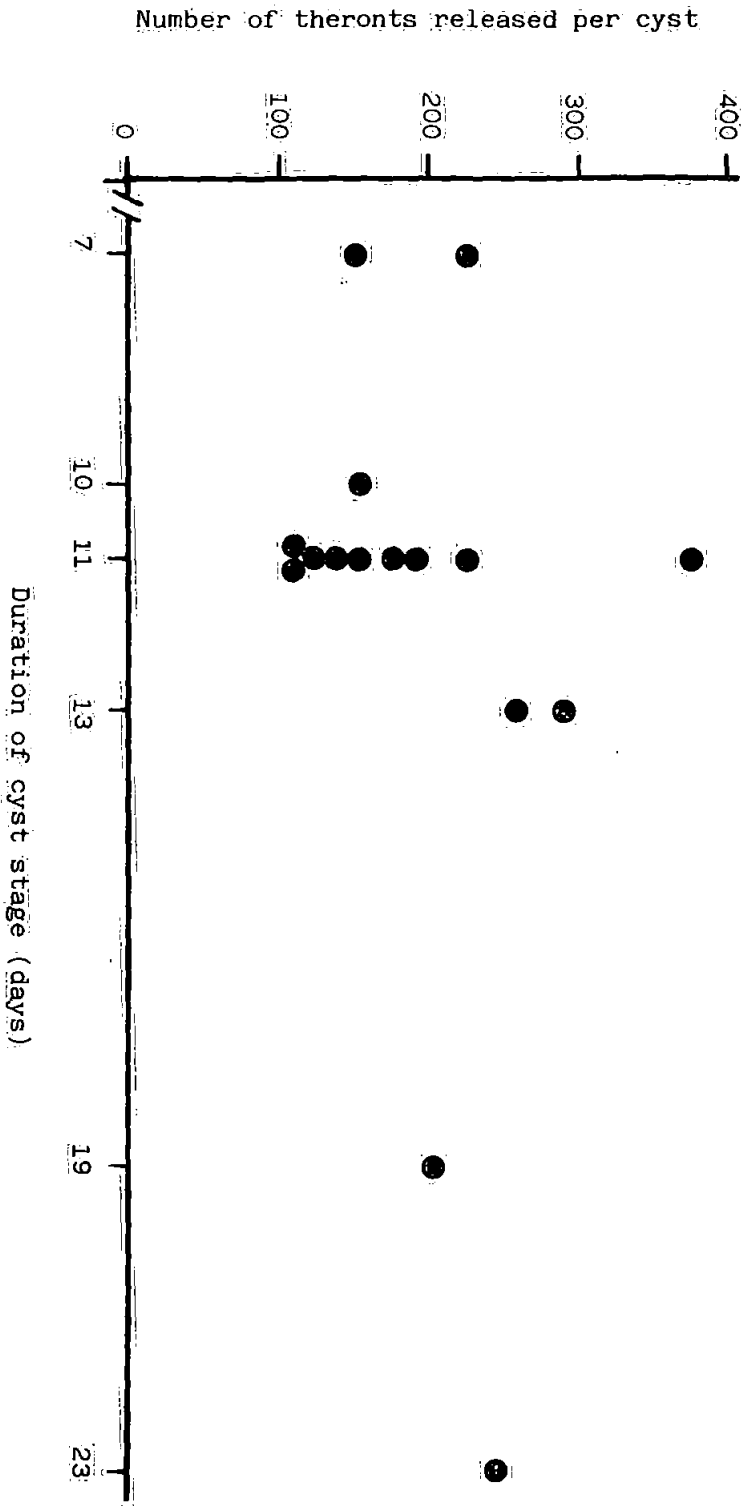
4.3.1 Theront activity and behaviour at excystment

Theronts (CI-HT), harvested within 1 h of excystment, were distributed amongst wells of a culture plate, each well containing 1cm depth of static seawater at 25°C. Theronts were observed using a low power stereoscope with substage illumination and their activity confirmed as normal (activity score +++, Table 2) under these conditions. Theront speed was measured in a Sedgewick-Rafter chamber. The theronts, which are denser than seawater, displayed negative geotaxis, tending to accumulate near the surface. Swimming behaviour, which involved helical gyration, included repeated migrations between the water surface and the base of the well. Upon contact with the base the theronts would pause for a fraction of a second before beginning their upward journey. Occasional bursts of relatively high speed swimming,

Figure 20

Relationship between duration of cyst stage and number of theronts produced per cyst.

Correlation coefficient, r = 0.25 (n=16).


Number of theronts released per cyst
0
100
200
300
400
Duration of cyst stage (days)
7
10
11
13
19
23

lasting for a few seconds, were observed. Velocities up to 1,500 μm /second (≈5.4 metres /h) were recorded in a horizontal plane, being three times faster than *Tetrahymena* trophozoites of similar body length (70μm) and cilia length (7μm) (Roberts, 1981). Motile theronts were observed within cysts up to 12 h before emergence.

4.3.2 Effects of penicillin-streptomycin on theront activity with time

The effect on theront activity of antibiotics was investigated. A total of 193 theronts were collected within 1 h emergence from cysts (CI-GS) held under a 12L:12D photoperiod. Theronts were divided into 2 groups, each distributed amongst 2ml wells of a culture plate and held in the dark. One group was maintained in FS-SW (n= 107), the other in FS-SW containing 1% penicillin /streptomycin ("pen-strep", Gibco Ltd.) (n=86). Theront activity was scored according to the five point scale (Table 2) at various intervals over 18 h, with the aid of a stereomicroscope with substage lighting. The results (Figs. 21 and 22) show normal (+ + +) activity in all theronts at the beginning of the experiment. At 6 h, 72% of theronts held in seawater showed normal to low activity (Fig. 21), as compared with only 20% of those exposed to antibiotics (Fig. 22). At 10 h, 44% of theronts held in seawater had become inactive with 10% dead, as compared with values of 88% and 66%, respectively, for theronts in pen-strep. At 18 h, only low activity was observed, occurring in a small proportion of theronts held in seawater alone. Exposure for 18 h to pen-strep did not harm free-living hypotrich ciliates which were used as controls.

4.3.3 Theront viability with time

The ability of theronts of different ages post-excystment to establish infection

Figures 21 and 22

Activity of *C.irritans* theronts with time.

Figure 21

Activity in seawater.

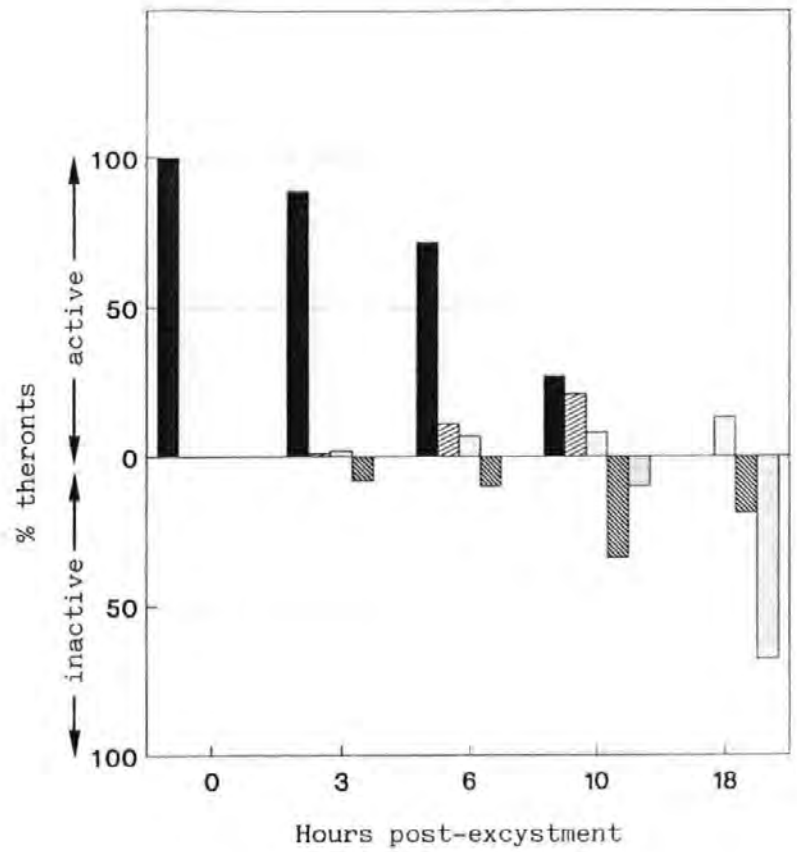
n = 107 theronts.

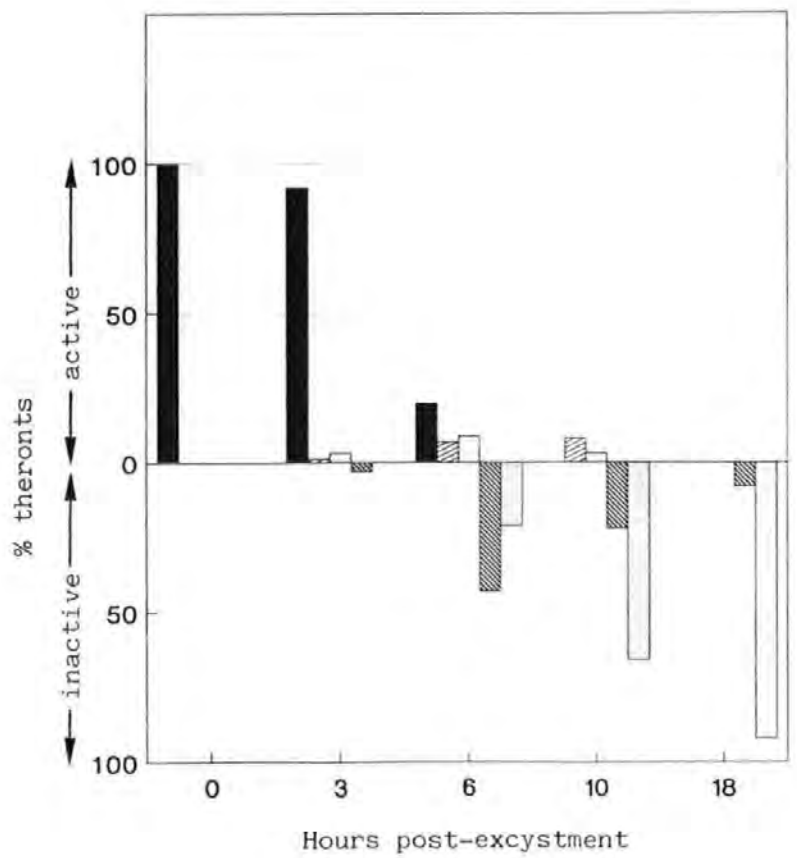
Figure 22

Activity in seawater containing pen-strep.

n = 86 theronts.

For both figures: activity scores =


% theronts
active
inactive
100
50
0
50
100
0
3
6
10
18
Hours post-excystment


% theronts
active
inactive
100
50
0
50
100
0
3
6
10
18
Hours post-excystment

through to normal trophont release was assessed. Theronts (CI-GS), <1 h post-excystment, were pooled, enumerated, and divided into 5 approximately equal groups, A-E. These were held for different periods of time in 20ml FS-SW as follows:- A, 0-1 h; B, 3-4 h; C, 6-7 h; D, 10-12 h; E, 18 h. Each group was tested for viability at the appropriate time by exposure to mullet (4-6g) naive to *C.irritans*, at levels of 5,000 theronts /fish. 10 mullet were used for group A, 5 for groups B-E. Exposed fish were held in aquaria and moved to trophont harvesters, the numbers of trophonts being counted as an indicator of theront viability. The relationship between theront age and viability is shown in Fig. 23. Analysis by T test suggests that viability remained relatively high during the first 4 h post-excystment, however there was a significant reduction by 6-7 h. No theronts were viable by 18 h. An assumption was made that all theronts establishing infection survive to trophont release and that no divisional stages occur on the fish. Small white fletches observed on the fins of 2 fish exposed to theronts at 10-12 h and 1 fish exposed to theronts at 18 h were thought to indicate transitory invasion without establishment.

5. SUBSTRATE SUITABILITY FOR ENCYSTMENT

A variety of materials used for furnishing or constructing marine aquaria or sea cages were investigated as substrates for attachment and encystment of trophonts. These included synthetic materials (e.g. glass, plastic, steel) and natural materials either dead (e.g. wood, shells, coral) or living (e.g. fish epithelial scrapings, invertebrates).

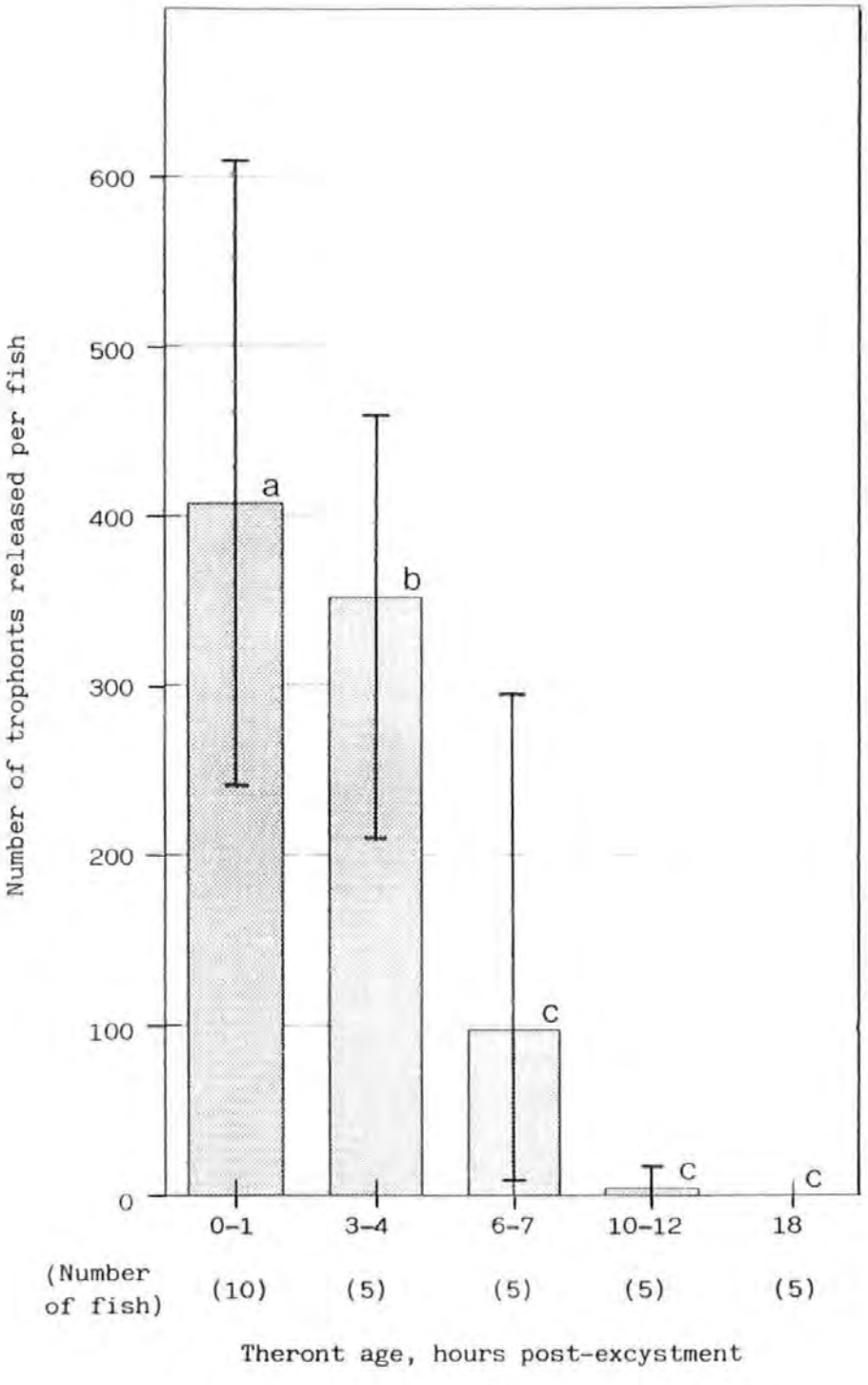
Motile trophonts (CI-CR), collected within 2 h of leaving the fish host, were washed rapidly by repeated resuspension in FS-SW and pipetted onto the appropriate test substrates immersed in FS-SW. Those materials which were not themselves

Figure 23

Theront viability with time.

Bars and vertical lines show mean and range, respectively.

Letters above bars represent T test statistics for viability, where:
a versus b = not significant ($0.5 > P > 0.1$)
a versus c = significant ($P < 0.001$); both at 13 degrees of freedom.


Number of trophonts released per fish
600
500
400
300
200
100
0
a
b
c
c
c
0-1
3-4
6-7
10-12
18
(Number of fish)
(10)
(5)
(5)
(5)
(5)
Theront age, hours post-excystment

receptacles were held in plastic culture plates or glass dishes. A minimum of 25 trophonts were allowed to sediment onto each test material and these examined at various times for evidence of encystment. Trophont adherence was designated as "normal" if the trophonts or cysts could not be dislodged by a direct stream of water expelled from a pasteur pipette, or "moderate" or "weak" if a low or high proportion, respectively, of parasites were dislodged by this action. Those trophonts which encysted were monitored for several days for evidence of theront emergence. The range of substrates to which trophonts can adhere is shown in Table 10. These included most hard, non-living surfaces although only a proportion of trophonts adhered to polished glass, steel, wood, and hemp. The hemp sample appeared toxic to the parasite, causing trophont degeneration within 2 days. Comparison of adherence on the comparatively inert glass and plastic surfaces suggested that the strength of adherence might be positively related to the degree of roughness of the material. In support of this, it was observed that trophonts released into glass dishes containing fish scales would accumulate at a higher density per unit area on the scales than on the surrounding glass. Trophonts appeared unable to attach to the living surfaces of the echinoderms, *Asteria* sp. (starfish) and *Echinus* sp. (sea urchin). With those cysts monitored until excystment, swimming activity of the theronts appeared normal. However, theront infectivity was not investigated, except from parasites routinely allowed to encyst on polystyrene and glass receptacles.

6. EVALUATION OF A CHEMICAL TREATMENT

The need for an effective chemotherapeutic against *C.irritans* prompted an assessment of "Oomed™" (Tetra Ltd., Germany), a recently introduced treatment for protozoal infections of coral reef fishes, which claimed to be effective against the cyst

Table 10

C.irritans trophont attachment and cyst development on a variety of natural and synthetic substrates.

Material		Trophont attachment	Encystment	Theront release	Observations
Type	Details				
Glass	aquaria, dishes	Yes (most)	Yes	Yes	Attachment less than 100% on polished glass surface. Otherwise normal adherence
Plastic	polystyrene	Yes	Yes	Yes	Normal adherence
	polyvinyl chloride	Yes	Yes	Yes	Normal adherence
Gravel	aquarium gravel (0.7mm diameter)	Yes	Yes	Yes	Normal adherence
Metal	steel plate	Yes (some)	Yes (some)	n.t.	15% (4/27) trophonts adhered
Fibre	hemp	Yes (some)	No	No	28% (7/25) trophonts adhered. All degenerated within 48 hours
Wood	-	Yes (some)	Yes (some)	n.t.	42% (11/26) trophonts adhered
Shell materials	shell gravel, commercial prepn.	Yes	Yes	Yes	Normal adherence
	cleaned *Mytilus edulis* shell	Yes	Yes	n.t.	Normal adherence to inner and outer surfaces of shell
	exoskeleton, caridean shrimp	Yes	Yes	n.t.	Moderate adherence
	staghorn coral[1]	Yes	Yes	Yes	Normal adherence
Fish tissues	mullet epidermal mucus	Yes	Yes	n.t.	Weak adherence - mucus degenerated
	mullet scales	Yes	Yes	Yes	Normal adherence
Live organisms	*Asteria* starfish	No	-	-	Trophonts delivered to the dorsal surfaces of these organisms, but none attached
	Echinus sea-urchin	No	-	-	

[1] Staghorn coral (*Acropora*) - dead, bleached and washed.

n.t. = not tested.

stage of *C.irritans*. Oomed has the following composition:

Hexamethyl para-rosaniline	75.7mg
Quinine hydrochloride dihydrate	1261.7mg
9-aminoacridine hydrochloride monohydrate	252.3mg
Benzyl dodecyl-bis-(2-hydroxyethyl) ammonium chloride	504.7mg
Aqueous solvent	100 ml.

Oomed, diluted in seawater, was used at one or more of the three manufacturer's recommended doses:

1:25,000 = low dose (aquaria containing living corals);

1:12,500 = medium dose (aquaria containing any species of invertebrate);

1:6,250 = high dose (aquaria containing fishes only).

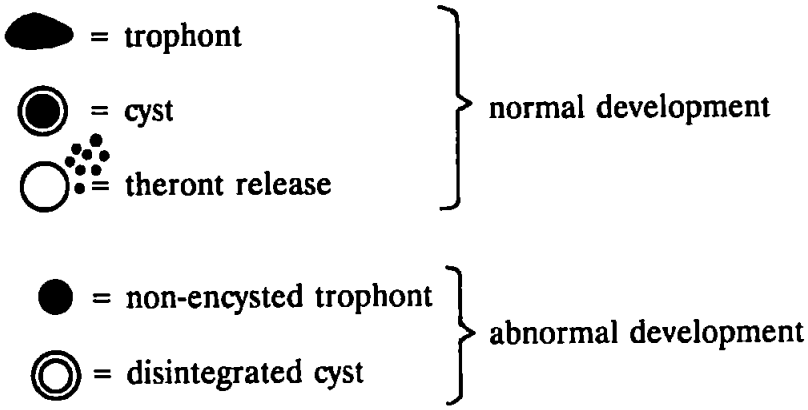
The efficacy of Oomed in destroying trophont, cyst, and theront stages of *C.irritans* (CI-HT) was assessed in addition to its effect on theront infectivity. All tests were carried out on parasite stages transmitted through mullet naive to *C.irritans*, and held under a 12L:12D photoperiod.

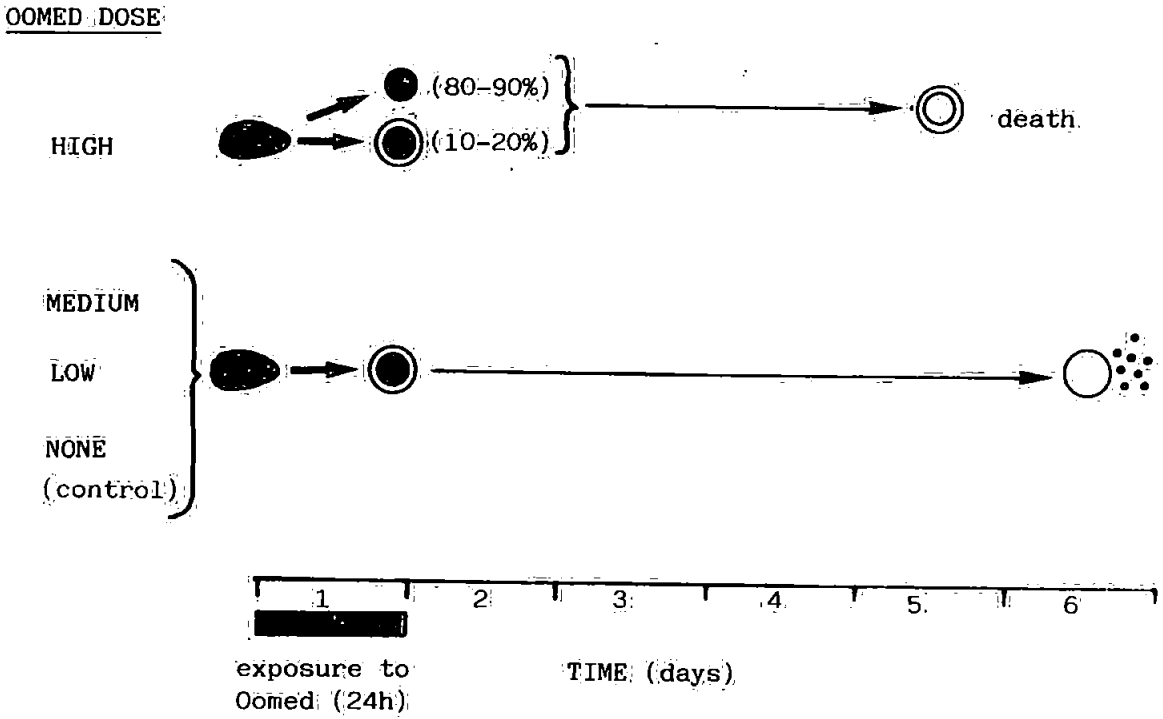
6.1 Trophont

Recently emerged trophonts were collected into 4 wells of a tissue culture plate, 10 trophonts/ well, containing Oomed at either "low", "medium" or "high" doses in seawater, plus controls containing no reagent. The trophonts were exposed to the reagent for 24 h after which they were washed free of Oomed in FS-SW. Parasite development was monitored for 6 days, with FS-SW being replaced daily. The results (Fig. 24), based on two sets of experiments, showed that Oomed had no effect on reducing trophont mobility over the first hour of exposure, at any of the three dose levels. Cyst formation and development with subsequent release of active theronts

Figure 24

Effect of Oomed™ on development of free-living trophonts of *C.irritans*.




OOMED DOSE
HIGH
(80-90%)
(10-20%)
death
MEDIUM
LOW
NONE
(control)
1
2
3
4
5
6
exposure to
Oomed (24h)
TIME (days)

occurred in all trophonts exposed to medium and low doses of Oomed as well as the unexposed controls. With those trophonts exposed to high dose Oomed, only 10 to 20% encysted and all had degenerated by day 5 following exposure, with no release of theronts.

6.2 Cyst

Fifty cysts, collected 24 h post-encystment, were dispensed into wells of a tissue culture plate, 10 cysts/ well, and exposed to Oomed at high dose for 48 h. Cysts were then washed free of Oomed in FS-SW, transferred to new wells containing FS-SW which was replaced daily, and monitored for theront release. It was found that 48/50 cysts (96%) produced theronts, with peak excystments at 6 -8 days, these values being comparable to that of unexposed control cysts (results not shown). The reagent appeared to be ineffective in destroying the cyst stage of *C.irritans*.

6.3 Theront

Theronts, <2 h post-excystment, were divided into 3 approximately equal groups of ca. 25, and each group exposed to one of the three doses of Oomed within a 2ml culture plate well. A further group of 25 theronts, held in FS-SW only, served as controls. Theront activity was assessed using the five score system described earlier (Table 2). The results (Table 11), based on two separate trials, show that the reagent, at all three dose levels, significantly reduced theront activity within 2 h exposure, the extent of reduction being dose- dependent. At high dose, the reagent effected a rapid reduction in theront activity, with theront death as early as 10 min, and with no theronts surviving longer than 30-60 min. Medium and low doses of Oomed failed to destroy all theronts during the 2 h exposure period.

Table 11

Effect of Oomed™ on activity of *C.irritans* theronts.

Exposure time (min)	Oomed dose			
	High	Medium	Low	None
<1	+++ / +++	+++ / +++	+++ / +++	+++ / +++
5	++ / ++	+++ / +++	+++ / +++	+++ / +++
10	+ / o (75%)	+++ / ++	+++ / +++	+++ / +++
30	o / o (88%)(92%)	++ / +	+++ / ++	+++ / +++
60	o / o (100%)(100%)	++ / o (100%)	++ / ++	+++ / +++
120	o / o (100%)(100%)	+ / o (100%)	++ / o (25%)	+++ / +++

Results based on 2 sets of observations.

Theront activity scored: o; +; ++; +++.

Values in parentheses are percentage theronts with no cilial activity (= presumed dead).

117

6.3.1 Theront viability

Three groups of 4 mullet (6-8g), naive to *C.irritans*, were used. Each fish was transferred to a 1 litre aquarium containing either low or high dose Oomed or no Oomed (=controls) to which was then added 5,000 theronts, <2h post-excystment. Following 4 h exposure the fish were separately transferred to 5 litre aquaria containing seawater alone until the time for trophont release when they were finally moved to individual trophont harvesters. The number of cysts released per fish was recorded as an indication of theront viability. The results (Fig. 25) show that Oomed was only partially effective at both dilutions tested in reducing theront viability, the extent of which was dose-dependent. It was noted that, even when exposed to high dose Oomed, total prevention against infection was not achieved.

6.4 Oomed efficacy- summary

Overall, the results show that Oomed, when used at high dose, was partially effective in destroying free-living trophonts and theronts, but not the cysts of *C.irritans*. When used at medium or low dose, Oomed was effective only against the theront stage.

7. SUMMARY CHARACTERISTICS OF C.IRRITANS

A summary of the life cycle stages of *C.irritans* maintained by passage through mullet, together with morphometric data and other information gained in this chapter is shown in Fig. 26. Individual stages are illustrated in Fig. 27.

Figure 25

Effect of Ooomed ™ on viability of *C.irritans* theronts.

Bars and vertical lines show mean and range, respectively (n= 4 fish/ dose level).

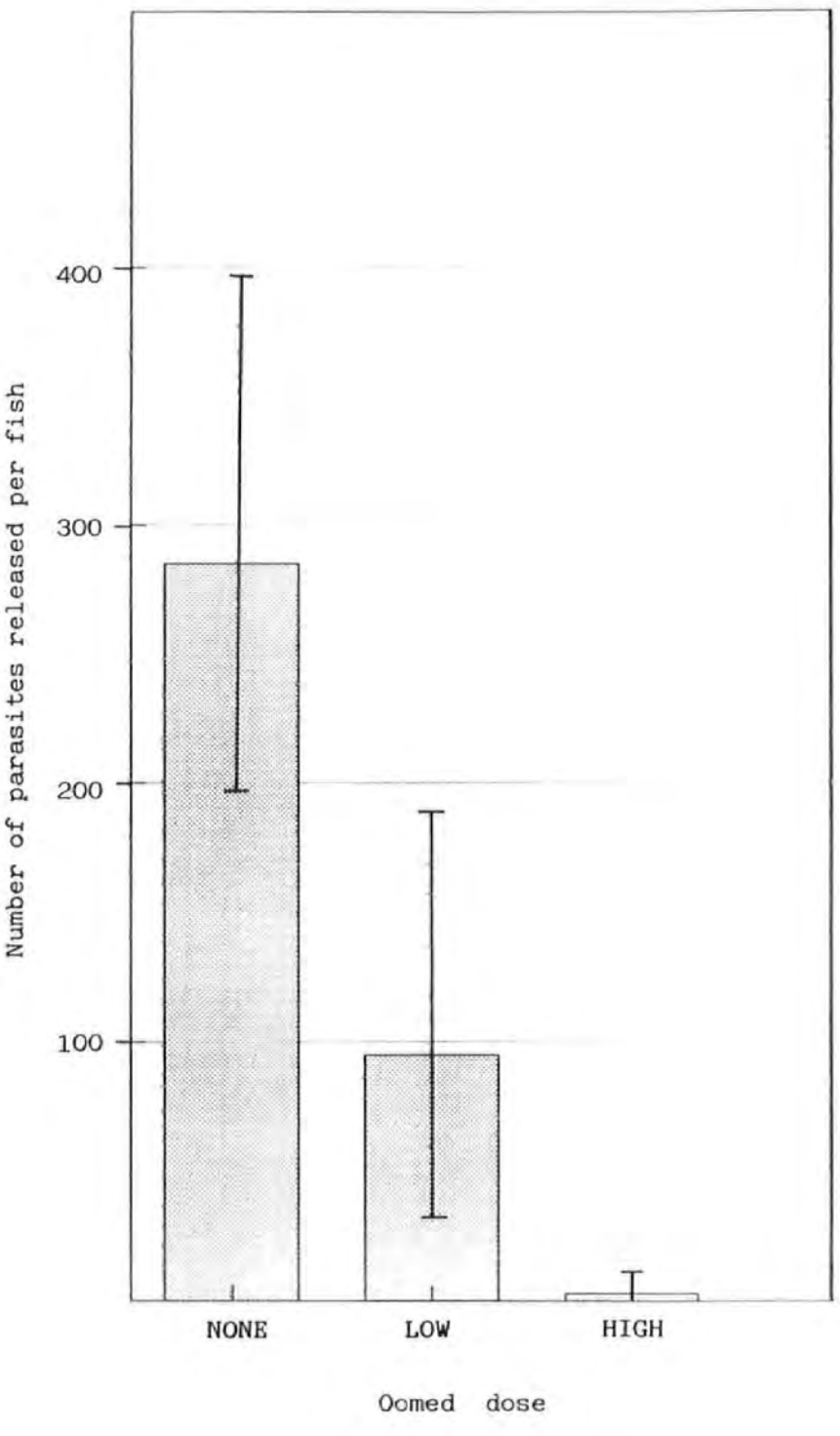

400
300
200
100
Number of parasites released per fish
NONE
LOW
HIGH
Oomed dose

Figure 26

Summary characteristics of *C.irritans* life cycle using mullet as hosts.

Numbers in outer circle represent duration in hours (h) or days (d) for each stage, expressed as mean (and range).

Scale bars show long axis measurements.

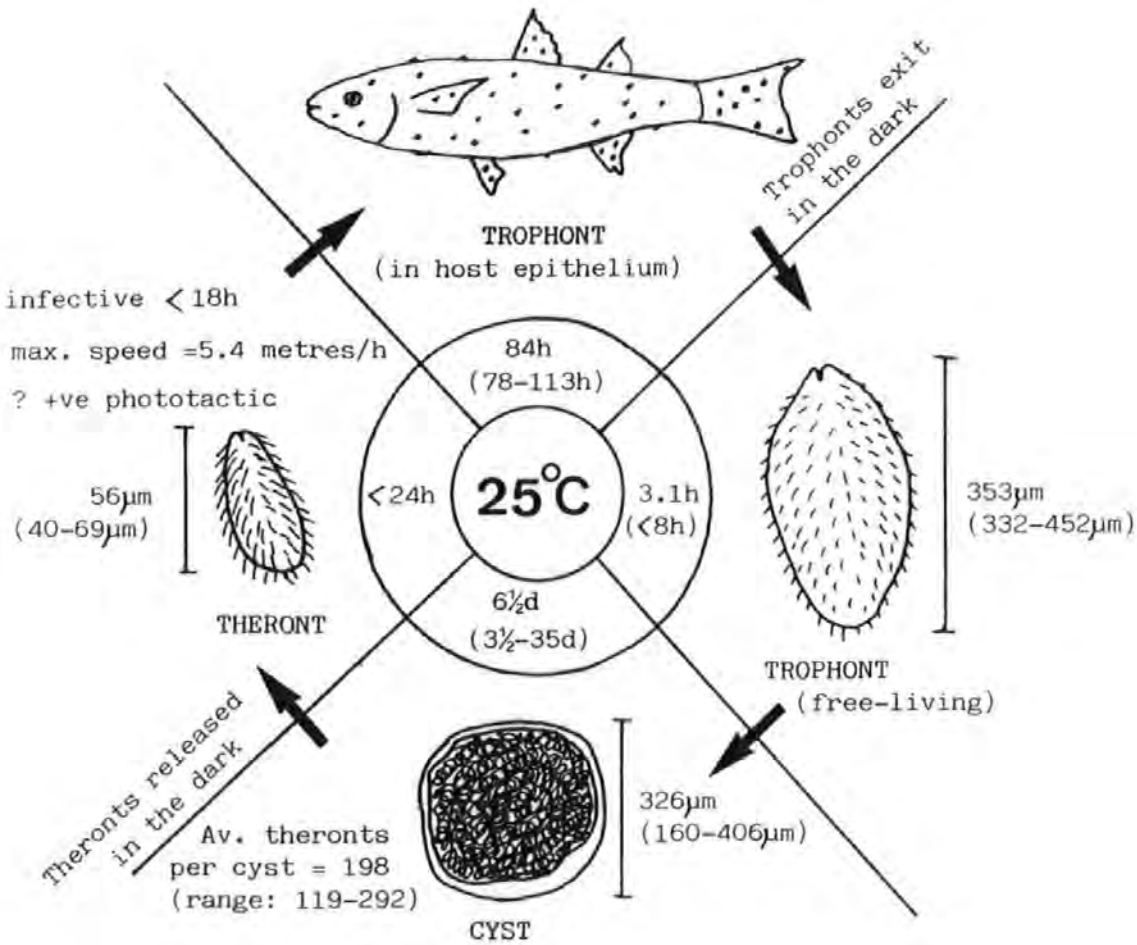

TROPHONT
(in host epithelium)
Trophonts exit
in the dark
infective < 18h
max. speed =5.4 metres/h
? +ve phototactic
84h
(78-113h)
<24h
25°C
3.1h
(<8h)
6½d
(3½-35d)
56µm
(40-69µm)
THERONT
353µm
(332-452µm)
TROPHONT
(free-living)
Theronts released
in the dark
Av. theronts
per cyst = 198
(range: 119-292)
326µm
(160-406µm)
CYST

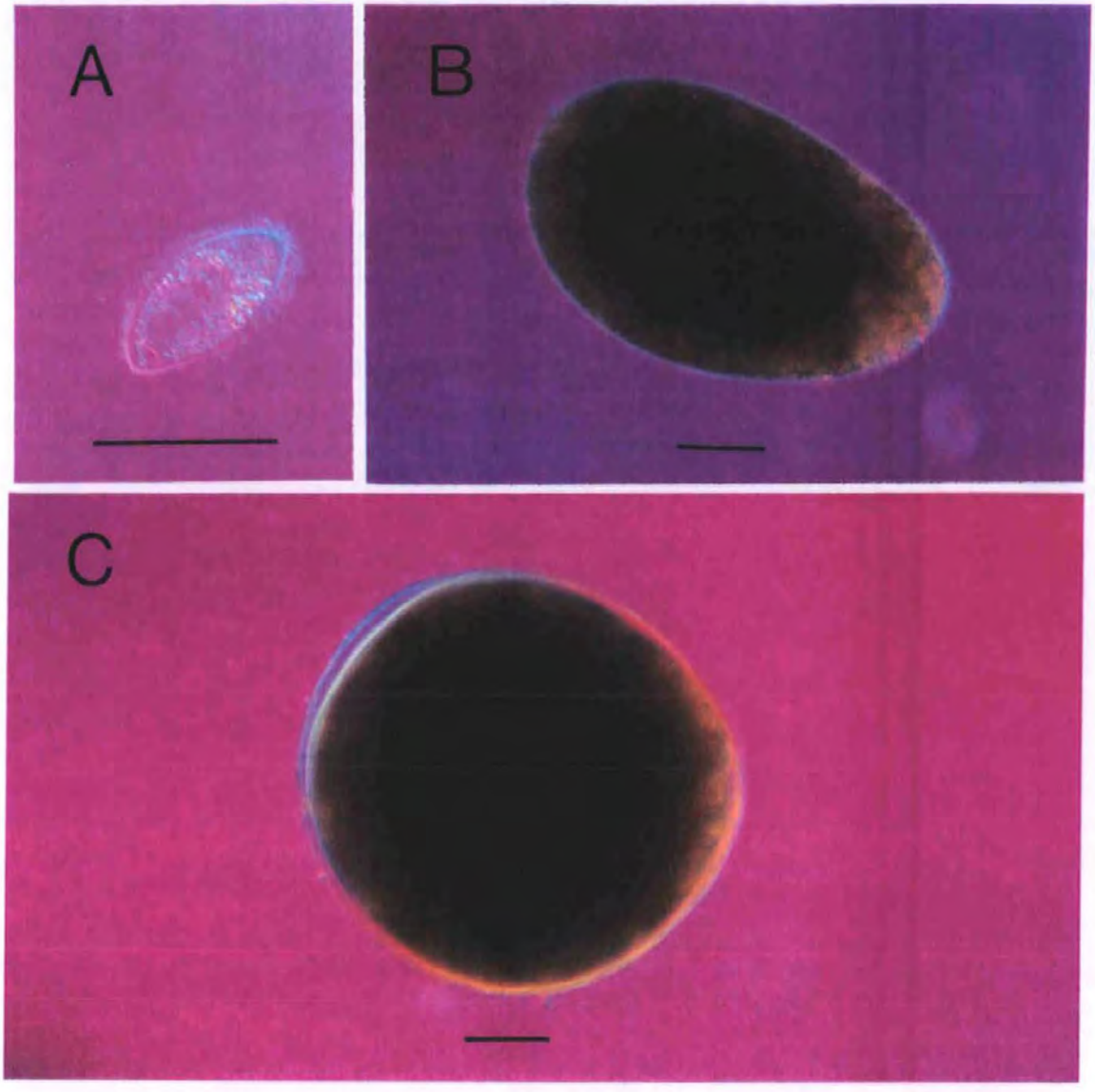
Figure 27

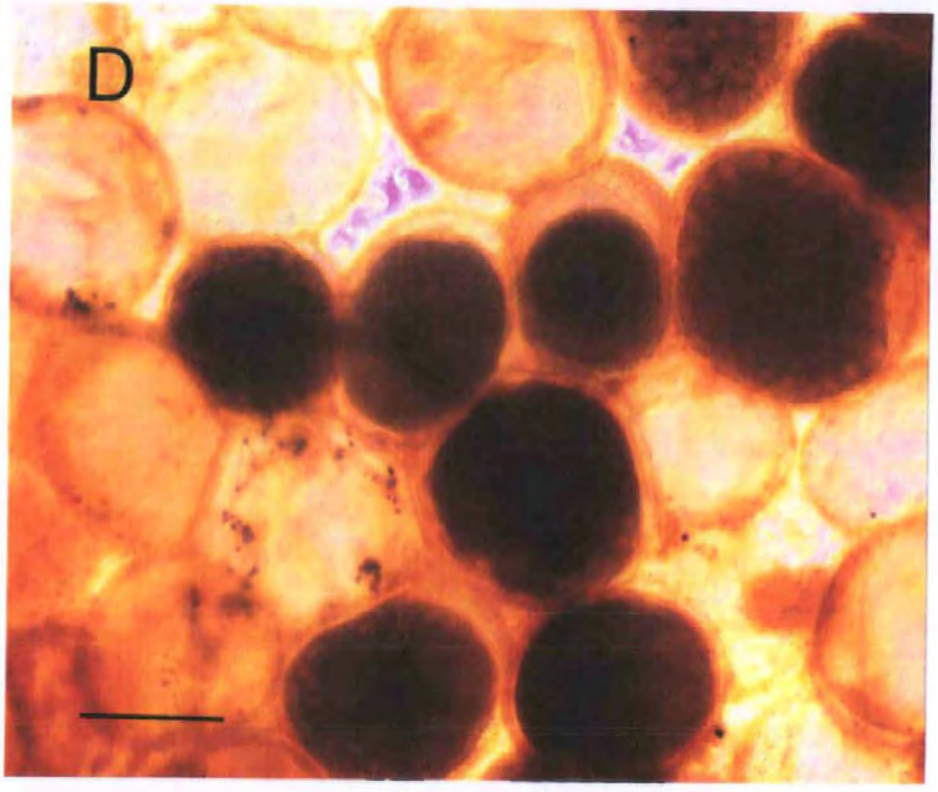
Free-living stages of *C.irritans*.

A: theront.
B: trophont.
C: cyst.
D: cysts, monolayer aggregation, showing excysted and unexcysted forms.

Scale bar: A,B,C = 50μm
D = 200μm.

label excysted with excysted forms.


A
B
C


D

DISCUSSION

The establishment of *C.irritans* within a controlled laboratory system has enabled detailed investigations concerning host parasite interactions and transmission.

Studies of *C.irritans* using mullet, *Chelon labrosus*, as an experimental host confirm the general features of the life history as described by others. The parasite's growth and development within the fish epidermis, entering the marine environment to encyst, multiply, and produce new infective stages are events well documented by previous workers (Sikama, 1937, 1938; Nigrelli and Ruggieri, 1966; Colorni, 1987). In addition, however, this study has contributed new information concerning transmission, including theront viability, timing of development, influence of environmental factors, and isolate "senescence", and these aspects are discussed below.

Although a total of eight isolates of *C.irritans* were established and successfully passaged in mullet none survived more than 34 cycles under laboratory conditions. Similar problems in the maintenance of this parasite were encountered by Colorni (pers. comm.) whereas Houghton and Matthews (1986) and Burkart *et al.* (1990) record loss of the related ciliate *I.multifiliis* following up to 10 months of serial transmission. Loss of *C.irritans* recorded here was associated with a decline in viability, suggestive of senescence. Ageing within cell lines is well recognised in Ciliophora in the absence of genetic exchange, however, sexual reproduction has not been recorded in either *C.irritans* or *I.multifiliis*. Nevertheless, a lack of complimentary partners ("mating types") within the populations of these parasites could account for isolate loss under conditions of laboratory maintenance. The effect of mixing complimentary mating types on initiating conjugation has been demonstrated for other holotrich ciliates, namely, *Tetrahymena* and *Balantidium coli* (Svennson, 1955; Adair

et al., 1978) as well as other ciliate species (reviewed by Miyake, 1981). That senescence is recorded here in *C.irritans* following extended *in vivo* maintenance gives some support to the view that genetic exchange is a requirement within the life cycle of this parasite. Matthews (pers. comm.) has recorded four micronuclei in theronts of *I.multifiliis*, suggestive of autogamy. It is possible, therefore, that sexual reproduction occurs in these parasitic ciliates but that the process is sufficiently brief as to go unnoticed. During conjugation of *B.coli*, for example, the two partners are only temporarily attached to each other by their anterior ends (Zaman, 1978). Possible opportunities for sexual reproduction in *C.irritans* were noted here when parasites closely associate within the host and at encystment. Trophonts have been observed in physical contact within a gallery of host epithelium (Fig. 28); such formations have also been described for *I.multifiliis* (Ewing *et al.*, 1988; Matthews, pers. comm.). Aggregations of the cysts of *C.irritans* have been observed comprising up to several hundred individuals forming a monolayer (Fig. 27, plate D); also reported by Diamant *et al.* (1991). Aggregation of trophonts on the substrate could theoretically enable genetic exchange to take place between adjacent cells prior to cyst wall formation. The theront stage, on emergence into the marine environment, provides a further opportunity for physical contact.

The encysted reproductive stage of *C. irritans* was shown here to be of variable duration, from 3.5 to 35 days, even for individuals resulting from a synchronised infection. This contrasts with the short cyst stage in the life cycle of *I.multifiliis*, encystment and theront production being completed within approximately 24 hours at 20°C (Dickerson *et al.*, 1985; Cross, 1990). Asynchronous release of theronts over an extended time period in *C.irritans* could provide a more effective strategy for transmission within the marine environment, compensating for adverse conditions

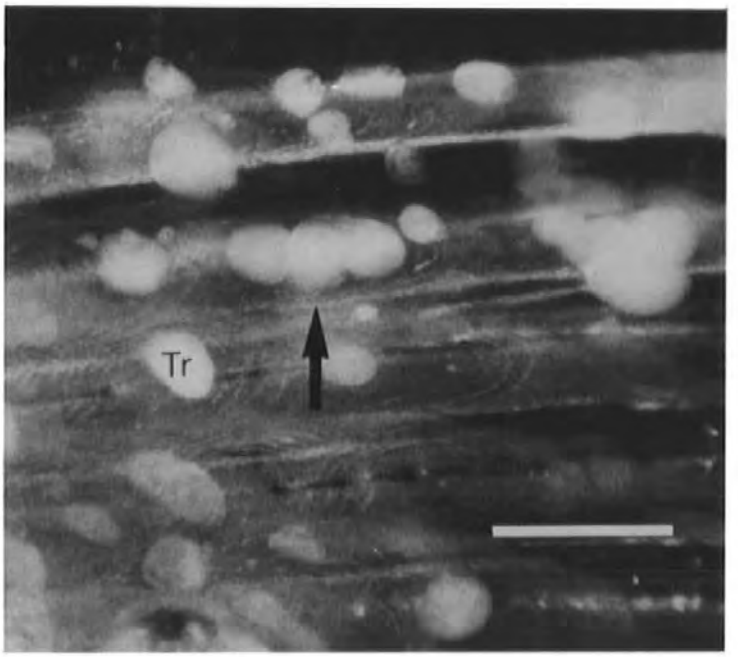
Figure 28

Aggregation of *C.irritans* trophonts within mullet fin epithelium.

Tr = trophont.

Arrow shows cell contact between three trophonts.

Scale bar = 1mm.


Tr

imposed by tide, weather and changes in fish host behaviour. The gradual dissemination of the theront population with time might also favour transmission by minimising the loss of potential hosts through the development of protective acquired immunity and acute disease.

C.irritans has a low degree of host specificity to teleosts, all of fourteen species, from diverse taxonomic groups, being shown here to be susceptible to infection. The parasite's wide host range within teleosts is further supported by observations on aquarium fish by Nigrelli and Ruggieri (1966) and Wilkie and Gordin (1969). That specificity is not limited to fish within either the seawater habitat of this parasite or within its assumed geographical range is indicated in the present study; infections being established in tropical fish which normally inhabit freshwater or slightly brackish environments, such as *Poecilia latipinna* (Jacobs, 1971) and *Oreochromis mossambicus* (Trewavas, 1983), and also representatives from temperate zones including the goby (*P.microps*) and mullet (*C.labrosus*, *L.ramada*). In this respect, *C.irritans* shows similarities with *I.multifiliis* which has been recorded from most freshwater teleosts world wide (reviewed by Houghton, 1987) and whose capability to infect marine species under experimental conditions was demonstrated here. The success of both species must reflect the effectiveness of direct transmission and infection of the epidermis, permitting unrestricted access between host and aquatic environment, together with the opportunity to exploit a wide range of teleost hosts.

Although *C.irritans* has been isolated here from several host species originating from different geographical regions, it was not possible to confirm whether these isolates represented different strains of the parasite. In general, the course of infection and longevity of free living stages of all isolates fell within expected parameters. That four species of fish representing three taxonomic families were

equally susceptible to a cloned isolate of *C. irritans* indicated that a wide host range did not necessarily reflect the presence of different host-specific strains within the captive parasite population. Nevertheless, further investigations of virulence and temperature tolerance are identified here as highly relevant in assessing the importance of this parasite within a rapidly developing mariculture industry. Recent work by Diamant *et al.* (1991) has already revealed a presumptive strain of *C.irritans* in the Mediterranean which differs from the Red Sea isolate described by Colorni (1985) in its distribution pattern on the host and its ability to establish infections in more northerly regions than previously recorded.

That the behaviour of *C.irritans* is influenced by photoperiod has been shown here for the first time, both trophont exit from the host and excystment strongly coinciding with darkness. The ability of *C.irritans* to respond to light and dark is not unique amongst the Hymenostomatida, having been shown for other parasitic as well as free- living species, namely *I.multifiliis* (Lom and Cerkasovova, 1974; Nickell and Ewing, 1989) and *Tetrahymena* (Wille and Ehret, 1968), respectively. Circadian rhythms based on photoperiod have also been reported for other taxonomic groups of protozoa, as reviewed by Wille (1979). The mechanisms of response to darkness are not known, however it might be significant that certain ciliates, including *Stentor,* possess photoreceptor pigments (reviewed by Pill-Soon and Walker, 1981). These pigments could theoretically function in the parasitic trophonts and the encysted theronts, as both these stages are exposed to light and dark cycles, in the latter stage being facilitated by a translucent cyst wall. Darkness, however, is clearly not the only stimulus for trophont exit which occurs only after a certain critical time period on the host, shown here to be between 78 and 89 hours post-infection. Observations that trophont exit during this time period can also occur under an artificially extended

photoperiod of light, suggests an overriding force of trophont maturation in determining release from the host. However, exit in the light arising from an altered photoperiod could be mediated by an endogenous circadian rhythm. In support of this, it is well documented that certain protozoa and other organisms possess photoresponses which are controlled by a temperature-independent biological clock which although synchronised ("entrained") by the external photoperiod, can exhibit an endogenous rhythm (Lofts, 1970; Wille, 1979). It is significant that the few trophonts which remained in the host after 89 hours did not release until the next dark phase (at 102-113 hours), thereby further indicating the influence of the dark cycle in trophont exit. It is possible that the trigger for trophont exit is host-mediated, possibly by physiological changes which have been shown to relate to circadian rhythms in fish (Schwassmann, 1971). Studies here were undertaken using diurnally active host fishes; it is not known whether the diel pattern of trophont release from nocturnally active hosts is different. It has been observed in the present study that host death also effects trophont exit, irrespective of photoperiod; similar observations having also been recorded for *I.multifiliis* (Ewing and Kocan, 1987). The underlying cause for this is unknown but might relate to falling oxygen supplies to the parasite.

Circadian rhythms have been recorded for several species of parasites and have been shown to improve the chances of survival and/or transmission (Kennedy, 1975). The possible benefits to transmission of photoperiod responses by *C.irritans* are considered below within the context of a reef environment. There are several reasons to consider the coral reef as the major habitat of *C.irritans*. First, the few recordings of *C.irritans* in wild fish have involved reef fishes (Laird, 1956; Burgess, 1978; Colorni, 1985). Furthermore, all but one of the *C.irritans* isolates acquired for this study were obtained from infected wild-caught reef fishes which had been imported into the U.K.

for the ornamental aquarium hobby. Further evidence in support of an association between *C.irritans* and the reef habitat is that a lower temperature threshold of 18°C - 20°C is common to both parasite (Wilkie and Gordin, 1969; Cheung *et al.*, 1979) and the hermatypic (reef-building) antozoan corals (Guilcher, 1988; Nybakken, 1988). The coral reef, characterised by its wide diversity of fish species (Nybakken, 1988) would favour a parasite with a broad host range. Assuming, therefore, that *C.irritans* is naturally associated with the coral reef, the observed nocturnal transmission of parasite between host and environment might be geared to diel behaviour patterns of the reef fishes. It is well recorded that the majority of fish species which are closely associated with the reef are diurnally active, hiding at night in crevices and caves (Stark and Davis, 1966; Thresher, 1980; Lowe-McConnell, 1987), with some, such as chaetodontids, resting in a state of torpor (Randall, 1968). The nocturnal inactivity of these fishes and their close physical contact with the substrate might therefore offer transmission benefits to *C.irritans* by minimising the distance travelled between exit from the host and encystment on the substrate, and subsequently, offer a nearby stationary target for the excysted theronts. In contrast, diurnal transmission of the parasite, at a period when many fish species are actively feeding, would require the theronts being capable of contacting a moving fish under relatively turbulent waters which occur around the reef (Nybakken, 1988); the effects of strong water currents on reducing the chances of theront transmission has been considered in the case of *Ichthyophthirius* (MacLennan, 1935). Photoperiod responses by *C.irritans* might also serve to reduce the chances of predation. In the present study it was observed that trophonts of *C.irritans* sediment towards the substrate immediately following host exit, as compared with those of *I.multifiliis* which are capable of some swimming activity (Nickell and Ewing, 1989; pers. obs.). The rate of trophont sedimentation by

C.irritans, measured here to be 14 metres per hour, together with the close proximity of the reef fish to the substrate (Lowe-McConnell, 1987), suggests that trophont settlement also occurs in the dark. Such a behaviour pattern could serve to avoid predation by planktonivorous fishes which are mostly diurnal, *C.irritans* being within the micro- to meso-plankton size ranges (Nybakken, 1988; Dr N. Polunin, Newcastle University, pers. comm.). Furthermore, the rapid formation of a hard cyst, shown here to occur within an average of 3 hours of host exit, may further deter predation.

Excystment of *C.irritans* was also shown to be strongly associated with darkness, with theront release commencing within three hours after onset of the dark phase. In view of the limited time for theronts to successfully infect a fish, viability being significantly reduced within 6 -7 hours after emergence, it is evident that a large proportion of theronts must locate and invade a host in the absence of light, if they are to survive. The ability of theronts to infect in the dark could arise if host contact occurs by chance. Alternatively, host location may involve a chemotaxic response to substances released from fish, although Lom and Cerkasovova (1974) were unable to demonstrate this for *I.multifiliis*, or possibly by a positive rheotactic response by the theront to fish movement; protozoan cilia are known to be receptors for such responses (Grell, 1973).

The important role of photoperiod responses in the behaviour of *C.irritans* in the aquatic environment has been discussed above. Work here has provided some evidence that theronts, upon emergence, respond to light, with positive phototactic responses being observed in a slight majority of theronts. Positive photoresponses by theronts have also been reported for *C.irritans* by Nigrelli and Ruggieri (1966) and for *I.multifiliis* by Lom and Cerkasovova (1974) and Wahli *et al.* (1991). It is difficult to perceive the significance of positive phototactism in a parasite which appears to be

nocturnally released, however this phenomenon is not unique, being also reported for the aquatic cercariae of the rodent helminth parasite, *Schistosomatium douthitti* (Olivier, 1951).

Although the coral reef is considered here to be the natural habitat of *C.irritans*, it is possible that the parasite occurs in other marine environments, such as coastal regions and open sea. Findings from this study provide some evidence that *C.irritans* has the potential to exploit these habitats, as indicated by its wide host range and ability to settle and encyst on a variety of substrates. That cryptocaryosis is recorded within intensive fish rearing systems (Huff and Burns, 1981; Rasheed, 1989) suggests some degree of tolerance to water conditions which are less favourable than those found around coral reefs. The recent discovery of a *Cryptocaryon*- like organism infecting fish cultured in the Eastern Mediterranean (Diamant *et al.*, 1991) may suggest a strain not associated with coral reefs, however conclusive evidence for this will depend upon the demonstration of infected wild fish in these waters. Further searches for the presence of *C.irritans* in wild fish populations are required in order to reveal the parasite's true environmental distribution.

A detailed evaluation of a proprietary chemotherapeutant against individual stages of *C.irritans* has provided conclusive evidence that the cysts are resistant to its chemical action, supporting aquarium observations following other drug therapies (Herwig, 1978). It is probable that the cyst wall, formed of several layers (Sikama, 1961; Nigrelli and Ruggieri, 1966), provides an impervious barrier to chemical entry. These findings underline the urgent need for a safe, effective chemical treatment for the control of *C.irritans*, the future prospects for which are discussed in Chapter 8.

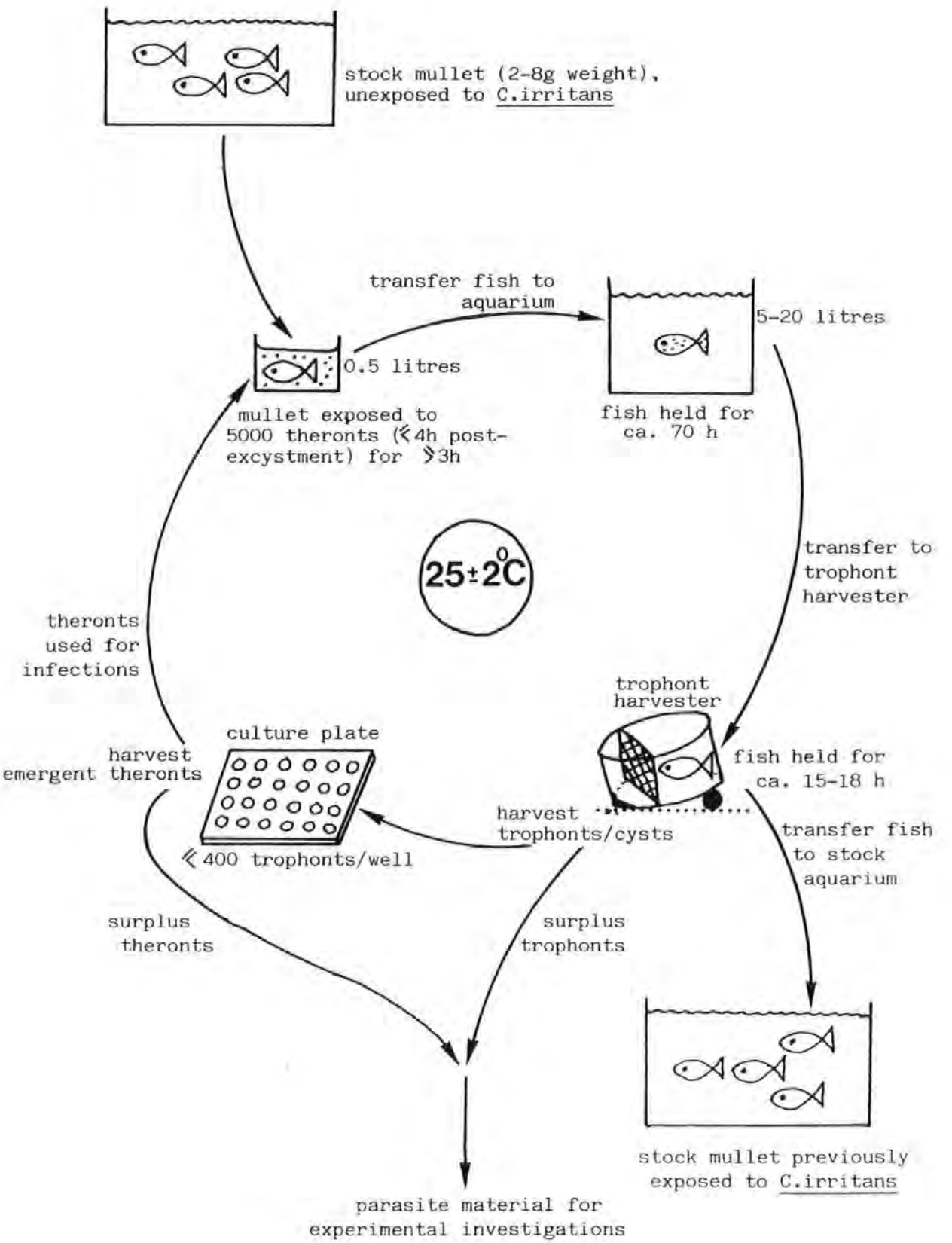
The laboratory maintenance of *C.irritans* has entailed standardised procedures for infection which provides the first opportunity for detailed investigations of the

immune response of fish to *C.irritans*. In addition it is now possible to collect specific stages in parasite development, in sufficient quantities, for antigen characterisation. Both these areas of research will be addressed in subsequent chapters.

A summary procedure for the laboratory maintenance of *C.irritans* is given in Fig. 29.

Figure 29

Summary procedure for the laboratory maintenance of *C.irritans* by controlled infections in mullet, *Chelon labrosus*.


stock mullet (2-8g weight), unexposed to C.irritans
transfer fish to aquarium
5-20 litres
0.5 litres
mullet exposed to 5000 theronts (≤4h post-excystment) for ≥3h
fish held for ca. 70 h
25±2°C
transfer to trophont harvester
theronts used for infections
trophont harvester
culture plate
harvest emergent theronts
fish held for ca. 15-18 h
harvest trophonts/cysts
transfer fish to stock aquarium
≤400 trophonts/well
surplus theronts
surplus trophonts
stock mullet previously exposed to C.irritans
parasite material for experimental investigations

CHAPTER FOUR

INFECTION AND IMMUNITY

INTRODUCTION

The occurrence of acquired protective immunity in fish has been demonstrated for only a few protozoal parasites, including *I.multifiliis* (Hines and Spira, 1974b), haemoflagellates (Woo, 1979; Woo *et al.*, 1987), *Ichthyobodo necator* (Robertson, 1979), and the PKX agent of proliferative kidney diseases (Klontz *et al.*, 1986). No infection and challenge experiments have been undertaken to investigate for acquired host immunity to *C.irritans*.

With regards *I.multifiliis*, Hines and Spira (1974b), using carp, were the first to demonstrate that fish experimentally infected with this parasite were refractory to potentially lethal challenge. Acquired immunity to *I.multifiliis* has subsequently been demonstrated in other teleosts, including trout (Wahli and Meier, 1985), mollies (Poeciliidae) (McCallum, 1986), and channel catfish (Clark *et al.*, 1988). Experimental methods for infection of fish with *I.multifiliis* have included exposure to infected fish (Goven *et al.*, 1980) or by exposure to a controlled number of trophonts (Hines and Spira, 1974b) or cysts (Wahli and Meier, 1985). However, these procedures are prone to inaccuracy with regards the exposure dose as they do not control for the significant variations observed between the numbers of theronts derived from each cyst (Ewing *et al.*, 1986) and assume that all trophonts or cysts will successfully complete development and produce viable progeny. The requirement for an accurate quantification of the infection dose in order to understand the infection dynamics and

to assess for any correlation with the degree of subsequent host protection, has been shown for *I.multifiliis* by McCallum (1982). Improved experimental infection procedures were established by Dickerson *et al.* (1981) who exposed fish to known numbers of theronts under standardised conditions.

For studies with *C.irritans*, infection and challenge procedures are complicated by the extended cyst stage and the asynchrony of theront production within a population of cysts, which present a major problem in procuring sufficient numbers of theronts at any one time for immunisation or challenge experiments. Furthermore, studies on *C.irritans* (Chapter 3) revealed a significant decline in theront viability within 6 hours post-encystment, with 4 hours being considered the upper time limit for infection procedures.

Fundamental to the current study will be the establishment of standardised procedures for immunising fish by controlled infections. Following optimisation of these procedures, the aims will be to investigate the capability of mullet to develop protective immunity to *C.irritans* and to correlate protection with exposure level. Further, in the event of acquired immunity being established, to investigate evidence for a memory component and specificity to *C.irritans*.

MATERIALS AND METHODS

1. FISH

Mullet, *Chelon labrosus* (Table 1, collection batch 8), selected for similar size, were used for experimental infections. Where necessary, mullet were individually identified by subcutaneous marking with Alcian blue (10mg/ml in ethanol) administered by a Panjet needleless injector (Wright Health Group Ltd., U.K.). When marking small mullet (< 10cm length) the Panjet force was reduced by placing paper sheets over the target area on the fish's body.

Fish hosts for *I.multifiliis* included cyprinids (*Cyprinus carpio* L., *Carassius auratus* L. and *Carassius carassius* L.), stickleback (*Gasterosteus aculeatus* L.) and tropical freshwater species. These were purchased from commercial fish farms or aquarium retail outlets, the stickleback being caught locally in the River Erme.

2. C. IRRITANS

Isolate CI-GS was used for all infection and challenge studies presented in this chapter.

2.1 Infection procedure

Infection procedures were standardised as follows. Theronts were harvested within 4 h post-excystment and enumerated. Mullet were individually exposed to known numbers of theronts in 500ml seawater at 25°C, in the dark, for 3-4h. Gentle aeration (≈ 50-100 cm^3 air/ min) was maintained throughout. Following exposure, the fish were held in small aquaria under a 12L:12D photoperiod until ca. 70 h post-exposure when they were transferred to trophont harvesters for trophont release.

2.2 Control for theront viability

In order to control for any day to day variations in theront viability, studies on re-exposure and challenge of mullet were undertaken using pairs of fish, matched by weight, comprising one previously exposed test fish and a control not exposed to *C.irritans*. Each pair were separately exposed to theronts obtained from a common pool. Exposure methods were as described above (section 2.1).

The percentage of theronts successfully establishing infection (%PEI) in individual mullet was based on the following calculation:

$$\frac{\text{number of trophonts released from fish}}{\text{number of theronts used for exposure}} \times 100\% .$$

2.3 Assessment of immune protection to C.irritans in mullet

Mullet were considered to have significant immune protection to a controlled exposure if the %PEI was ≤ 1% and the number of trophonts released was < 5% that from a paired control mullet, matched for weight and with no previous exposure to *C.irritans*.

2.4 Prevention of accidental infection

For certain long-term experiments, accidental infection of mullet with *C.irritans* was prevented by adapting the fish to hyposaline conditions of ≈ 8‰ salinity (prepared from 1 part seawater to 3 parts freshwater), which has been shown by Colorni (1985) to prevent transmission of the parasite.

3. I. MULTIFILIIS

I.multifiliis was selected to evaluate the specificity of the mullet immune

response to *C.irritans*.

3.1 Sources of isolates

Isolates were acquired either from ornamental aquarium fishes, mostly cyprinids, or from wild caught sticklebacks.

3.2 Routine maintenance

I.multifiliis was passaged through captive reared cyprinids (*C.carpio*, *C.carassius*, and *C. auratus*). An infected population of these fish was maintained in 20 litre aquaria at ambient laboratory temperature. Water quality was maintained by external power filters (Eheim Ltd., Germany) and aeration.

3.3 Controlled infections

Carp (<30 g), naive to *I.multifiliis*, were each exposed to 1,000 theronts for 3 h in a darkened 2 litre aquarium containing dechlorinated tap water at RT. Aeration was reduced throughout exposure so as not to interfere with theront behaviour. Exposed fish were transferred to 10 - 20 litre aquaria until day 5 when they were returned to 2 litre aquaria for trophont release. Free-swimming trophonts were pipetted into 2 ml wells of a tissue culture dish and held overnight at RT in the dark. The following morning, emergent theronts were collected, enumerated in a Sedgewick-Rafter (see Chapter 3, methods section 2.5), and the numbers adjusted for challenge or exposure to further fish hosts.

3.4 Infection of mullet

Mullet, temporarily acclimated to freshwater, were each exposed to 5,000

theronts of *I.multifiliis* using the procedure outlined above (section 3.3). Infections were not allowed to proceed to trophont release as mullet are intolerant to long periods (> 2 days) in freshwater, manifested by inappetence, sluggish activity and susceptibility to *Saprolegnia* infections. At 48h post-exposure, infected mullet were killed by anaesthetic overdose and the number of trophonts counted within a known area of caudal fin. Infection levels were expressed as the number of trophonts/ mm^2 fin.

EXPERIMENTS AND RESULTS

1. THERONT VIABILITY

Percentage PEI values were determined for a large group (n=67) of mullet of average weight 4g (range= 1.8- 8.0g) following primary exposures to 5,000 theronts per fish under standardised conditions (described earlier). The frequency distribution for %PEI is given in Fig. 30, using 2% infection intervals. The histogram, which exhibits a slight positive skewness, shows a wide range of %PEI values from 2.0% to 18.1% (mean = 6.97%; median = 6%).

To control for differences in fish size, %PEI was expressed as a frequency distribution giving the number of trophonts released per gram of host fish. The results (Fig. 31) show a wide range of infection levels, from 19.0 to 266.8 trophonts per gram of fish. There was no significant correlation between the number of trophonts released and total weight of the fish host (r= -0.13; n=67). These results suggest that, at the 5,000 exposure level, the %PEI is not influenced by host weight (and therefore host surface area), under the conditions tested here.

1.1 Relationship between exposure level and percentage PEI

Percentage PEI results from the 67 mullet exposed to 5,000 theronts, described above, were used as a reference for comparison with smaller groups of fish exposed to one of the following numbers of theronts: 500 (10 fish); 1,000 (4 fish); 2,000 (13 fish); 10,000 (11 fish). The results (Fig. 32), expressed as median, mean and range of %PEI values, show no relationship between %PEI and theront exposure level. The slight rising trend of upper %PEI limits observed over the 1,000 to 10,000 theront exposure levels represents high parasite levels which occurred in a small proportion

Figure 30

Primary infection of mullet with *C.irritans*: frequency distribution of percentage number of parasites establishing infection (%PEI).

Exposure level = 5,000 theronts per fish.

n = 67 fish.

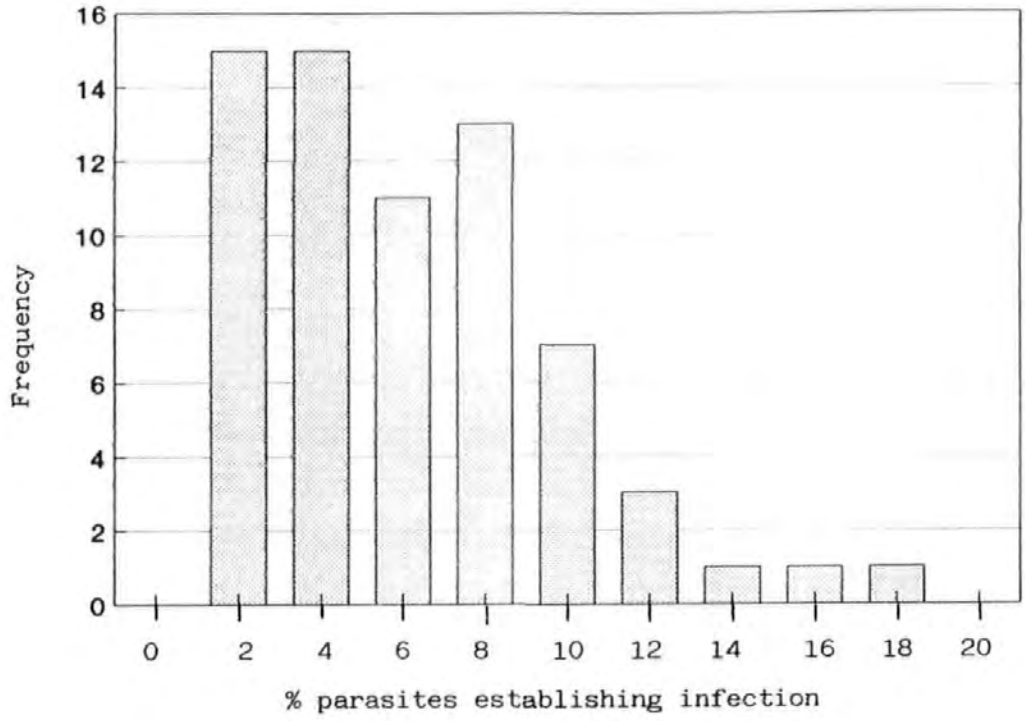

16
14
12
10
8
6
4
2
0
Frequency
0
2
4
6
8
10
12
14
16
18
20
% parasites establishing infection

Figure 31

Primary infection of mullet with *C.irritans*: frequency distribution of numbers of parasites released per gram weight of host.

Exposure level = 5,000 theronts per fish.

n = 67 fish.

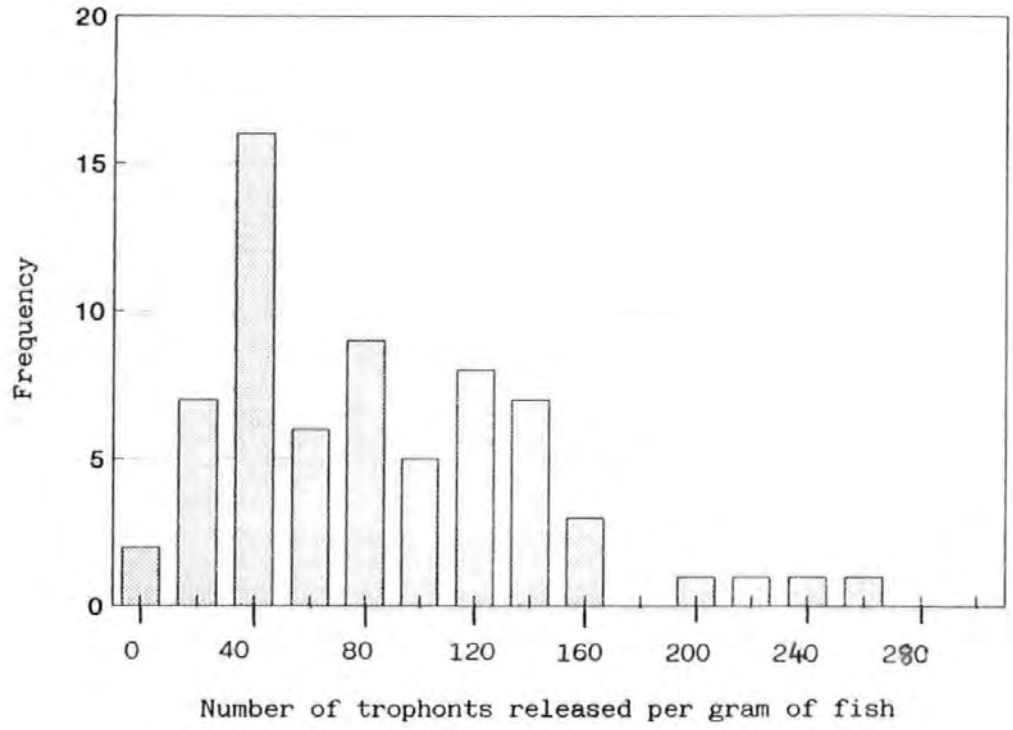
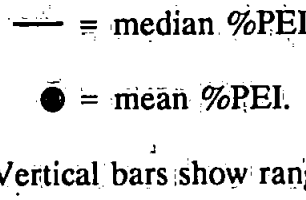
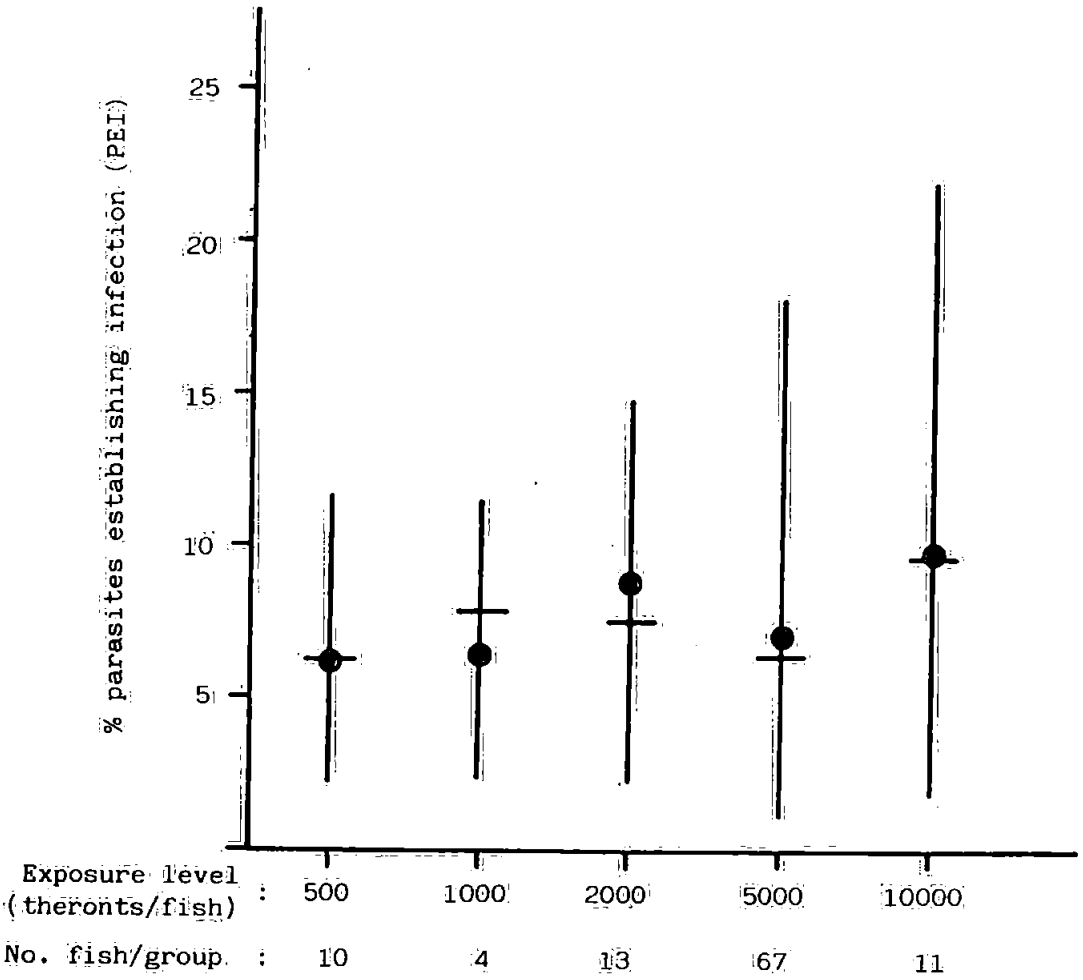

20
15
10
5
0
Frequency
0
40
80
120
160
200
240
280
Number of trophonts released per gram of fish

Figure 32

Primary infection of mullet with *C.irritans*: relationship between %PEI and the number of theronts used for exposure.




% parasites establishing infection (PEI)
25
20
15
10
5
Exposure level (theronts/fish) : 500 1000 2000 5000 10000
No. fish/group : 10 4 13 67 11

of fish. This could be indicative of a possible attraction to entry sites on the host, however the trend was not substantiated by the mean and median %PEI values and so may be insignificant. Fig. 33 shows a strong linear relationship (r = 0.99) between the number of parasites established in a host and the number of theronts to which it was exposed, with no evidence of a threshold effect.

2. LETHAL THRESHOLD

Lethal levels of theronts were evaluated by retrospective analysis of data on 105 mullet following primary exposure to between 500 and 10,000 per fish. Mortality was attributed to *C.irritans* if death occurred within 10 days after exposure. The relationship between mortality and the level of primary exposure is presented in Table 12. No mortalities occurred in mullet exposed to 2,000 or less theronts, whereas 13.4% and 81.8% mortalities occurred at theront exposure levels of 5,000 and 10,000, respectively. There was a strong temporal association between trophont release and mortality. Of the combined mortalities, 72% (13/18) coincided with trophont exit from the fish; the remaining deaths occurring either within 24 h following trophont release (2/18 fish) or between 24 h and 7 days after release (3/18 fish). This may reflect mortality caused by host epidermal disruption during trophont exit, as shown in Fig. 5. The number of trophonts released per gram of host fish, expressed as a frequency distribution, along with cumulative percentage mortalities, is shown in Fig. 34. With the exception of two fish, a threshold effect was observed, with mortalities occurring when parasite levels exceeded 100 trophonts per gram of host fish. No clear threshold effect was evident when the mortality data were not controlled for fish weight (results not shown). The results indicated a 90% probability of death following parasite levels of >200 trophonts/g host weight. Deaths in the two fish supporting

Figure 33

Primary infection of mullet with *C.irritans*: relationship between the exposure level and the number of trophonts released per fish.

n = 105 fish.

● = mean.

Vertical bar = range.

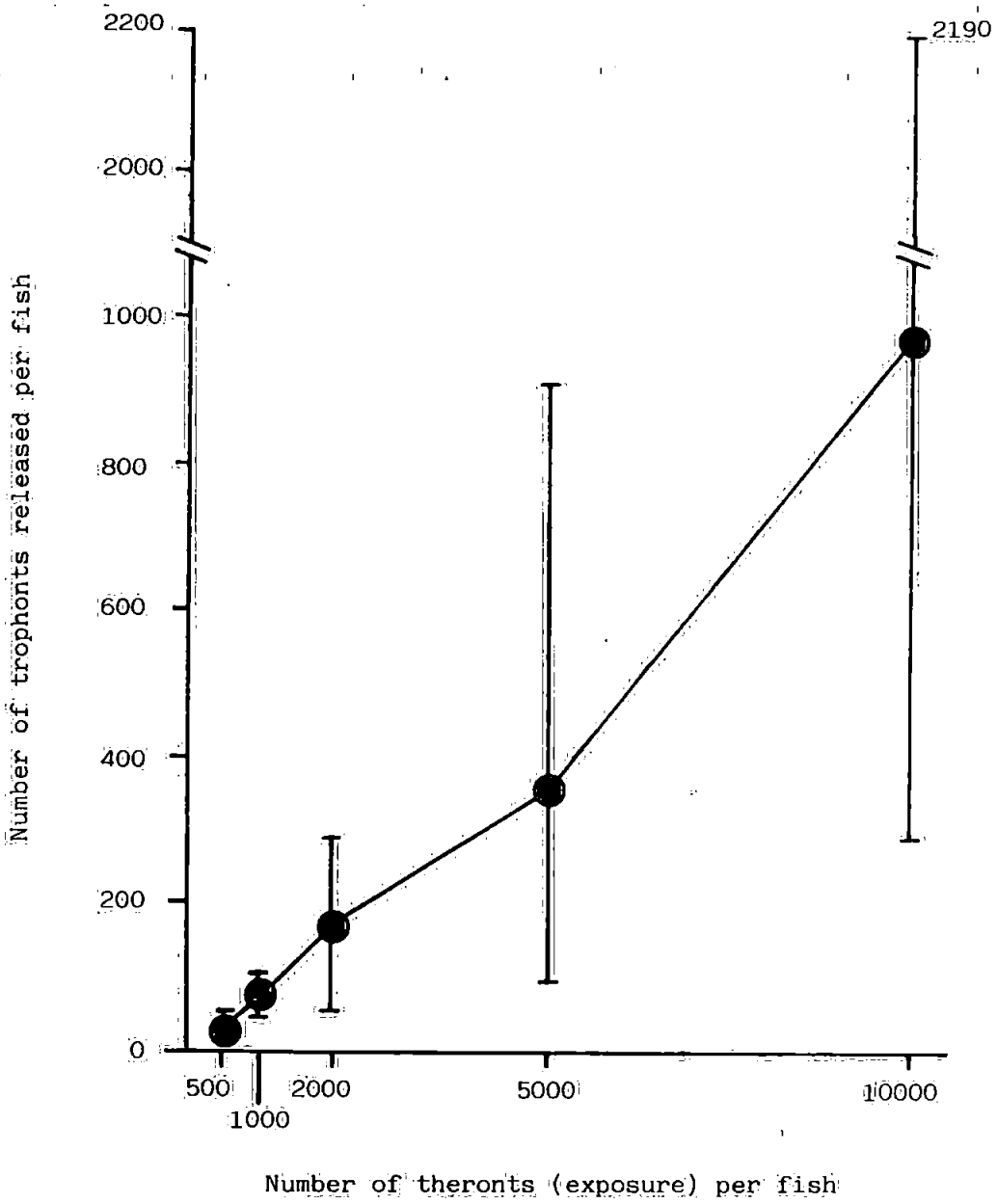

2200
2000
1000
800
600
400
200
0
2190
500
1000
2000
5000
10000
Number of trophonts released per fish
Number of theronts (exposure) per fish

Table 12

Mortalities in mullet related to exposure level of primary infection with *C.irritans*.

Number of theronts per fish	Fish		
	Number per group	Number (%) deaths	Number (%) deaths concurrent with trophont release
500	10	0 (0)	-
1,000	4	0 (0)	-
2,000	13	0 (0)	-
5,000	67	9 (13.4)	6 (67)
10,000	11	9 (81.8)	7 (78)

Figure 34

Frequency distribution of mortalities in mullet following primary exposure with *C.irritans*.

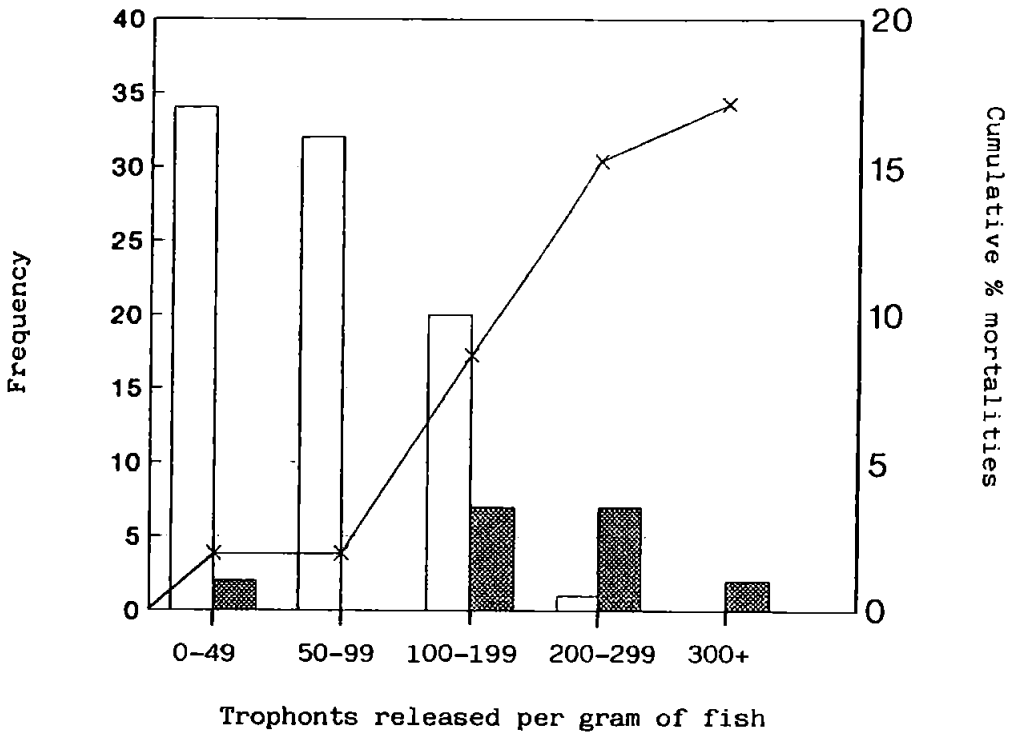
n = 105 fish, exposed to between 500 and 10,000 theronts /fish.

Bars show frequency.

Line shows cumulative percentage mortalities.

□ = alive > 10 days post-exposure.

▩ = dead ≤ 10 days post-exposure.


Frequency
40
35
30
25
20
15
10
5
0
20
15
10
5
0
Cumulative % mortalities
0-49
50-99
100-199
200-299
300+
Trophonts released per gram of fish

relatively low levels of parasites, of 19 and 44 trophonts/g, were discordant with the remaining mortality data; however, as with all mortalities, the possibility of death arising from causes other than cryptocaryosis could not be ruled out. Analysis of trophont counts from individual fish showed that the highest parasite intensity was 243.5/g, recorded from a 3.15g fish which released 767 trophonts.

3. INFECTION AND CHALLENGE

The ability of mullet to develop protection against *C.irritans* following exposure to a known number of theronts was assessed. The asynchronous development of cysts and release of theronts over an extended time scale necessitated staggering the infections over 14 weeks.

The infection procedure, shown in Fig. 35, was as follows: 87 mullet, naive to *C.irritans*, were divided into three experimental groups, A-C (n=36), plus two control groups, #1 (n=15) and #2 (n=36). Groups A, B, and C were exposed to 500, 2,000, or 5,000 theronts per fish, respectively, followed by a second exposure of the same level given 14 days later. After a further 14 days, all fish were individually challenged with 5,000 theronts, and a mullet from control group #2 was also exposed to 5,000 theronts, to confirm theront viability (methods, section 2.2). The degree of protection following each exposure was assessed in 15 mullet from experimental groups A,B, and C. For each of these fish, at the second exposure, a weight matched control fish from group #1 was separately given an equivalent exposure of theronts from the same source, to confirm theront viability. The results (Table 13) show that the numbers of trophonts sustained by groups A-C fish following a challenge exposure are significantly lower than those of control group #2. Further, the results suggest that the degree of host immunity is dependent upon the numbers of theronts used for 1°

Figure 35

Experimental procedure for the immunisation of mullet by exposure to theronts of *C.irritans*.

Numbers (500; 2,000; 5,000) represent levels of theronts per exposure.

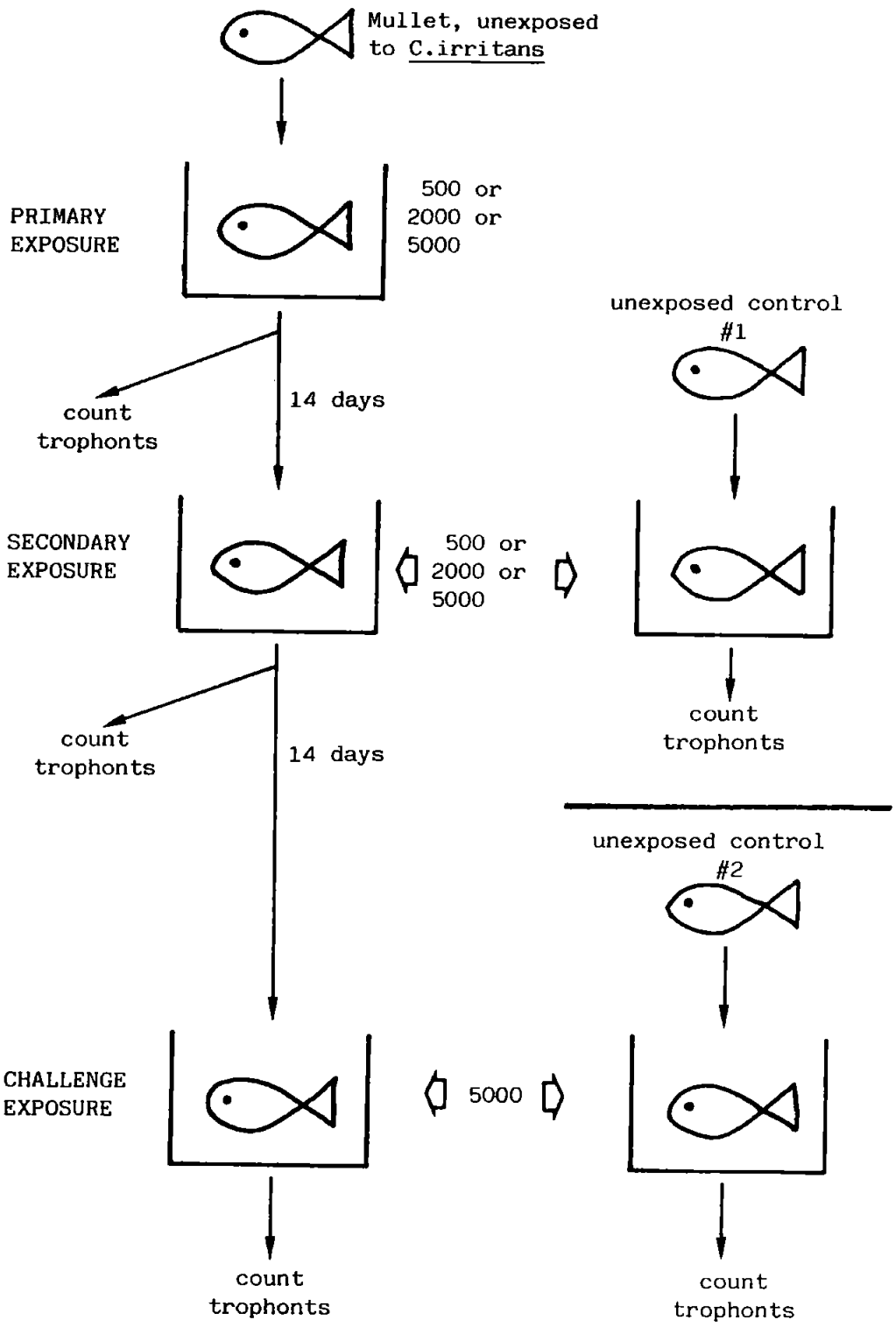

Mullet, unexposed
to C.irritans
PRIMARY
EXPOSURE
500 or
2000 or
5000
unexposed control
#1
count
trophonts
14 days
SECONDARY
EXPOSURE
500 or
2000 or
5000
count
trophonts
count
trophonts
14 days
unexposed control
#2
CHALLENGE
EXPOSURE
5000
count
trophonts
count
trophonts

Table 13

Post-challenge levels of *C.irritans* in mullet in relation to different primary and secondary exposure levels.

Fish			Number of theronts/exposure		Number of trophonts released per fish following challenge: median (mean) range	Median %PEI	Number (%) of fish with no parasites
Group		n	1° & 2°	challenge			
Exposed	A	13	500	5,000	3.0 (13.2) 0-81	0.06	4 (31)
	B	12	2,000	5,000	2.5 (7.4) 0-42	0.05	5 (42)
	C	11	5,000	5,000	0.0 (0.5) 0-3	0.00	9 (82)
Control	#2	36	0	5,000	307.5 (330.3) 102-767	6.14	0 (0)

and 2° exposures, being particularly notable when the relationship is assessed from the mean and range numbers of trophonts released. Fish which sustained no parasites following challenge are considered to have acquired full protection, the proportion of which shows a positive relationship with the numbers of theronts used for 1° and 2° exposures, increasing from 31% for 500 theronts/fish to 82% for 5,000 theronts/fish. However, none of the three 1° and 2° exposure levels used here resulted in full protection to challenge in all fish.

Results for the 15 experimental fish monitored for parasite levels following each exposure are shown in Fig. 36. Of the 10 fish subjected to a primary exposure of 2,000 or 5,000 theronts all but one sustained no or few (≤ 4) parasites following 2° and challenge exposures (median %PEI = 0). In contrast, the group of 5 fish subjected to a 1° exposure of 500 theronts sustained relatively higher numbers of parasites upon 2° and challenge exposures, with median %PEI values of 0.4 and 0.3, respectively; two of these fish sustained a higher %PEI upon challenge as compared with 2° exposure.

4. EFFECTS OF PARTIAL HOST IMMUNITY ON PARASITE GROWTH

Possible effects of host immune response on parasite growth was assessed on the basis of cyst size, taking a single measurement across the long axis. Four mullet, naive to *C.irritans*, were selected to provide 2 pairs of matched weight. One fish from each pair was subjected to a single exposure of 500 theronts and maintained without re-exposure for 14 days; the remaining 2 fish being kept as unexposed controls. All 4 fish were then individually exposed to 5,000 theronts from a common pool. Fish were held individually in trophont harvesters until parasite exit and encystment. A random sample of 25 cysts were collected from each mullet for measurement. Comparisons of mean cyst sizes from the two pairs (Table 14) showed no significant

Figure 36

Percentage of parasites establishing infection (%PEI) in mullet following primary, secondary and challenge exposures to theronts of *C.irritans*.

Number of fish (per 1°, 2° exposure levels): 5 (500); 4 (2,000); 6 (5,000).

Arrows show primary (1), secondary (2), and challenge (Ch) exposures.

Numbers of theronts /exposure shown in parentheses.

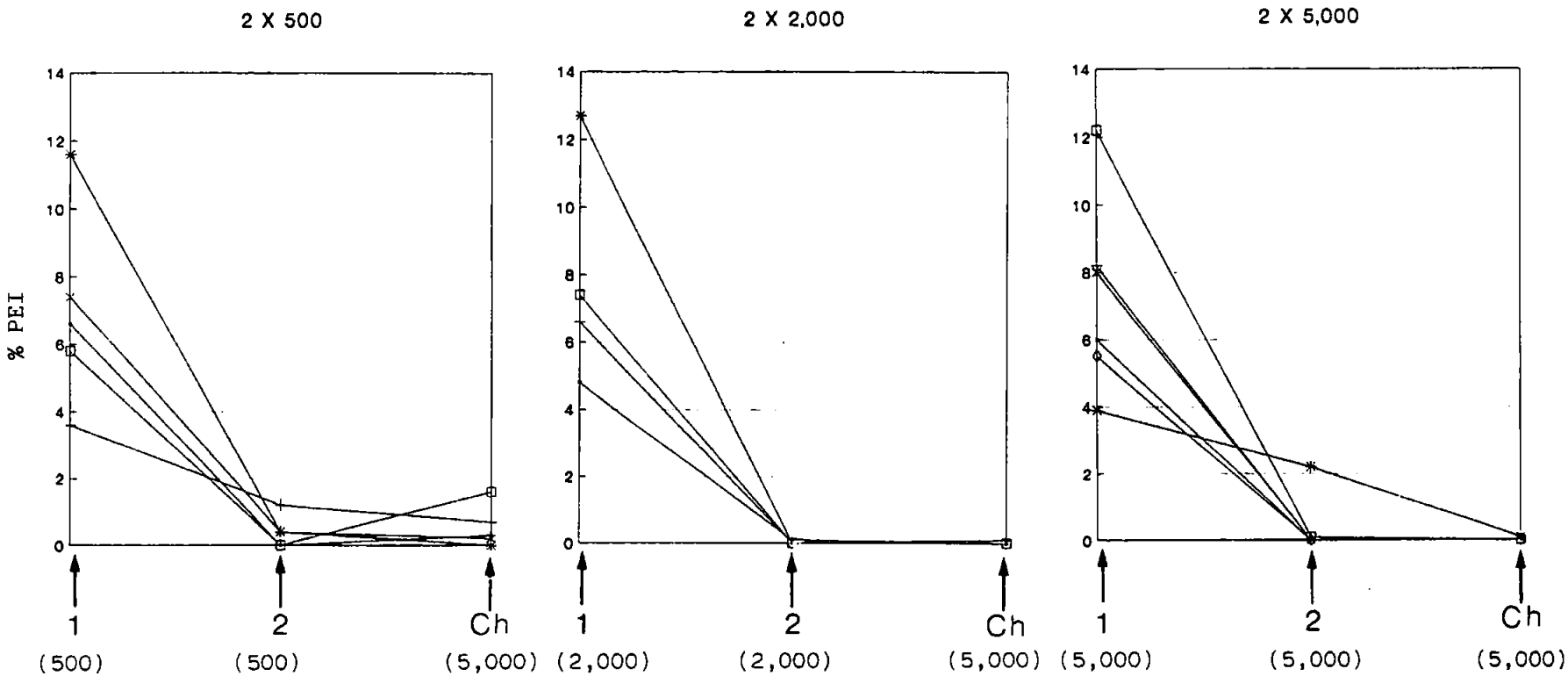

2 X 500
2 X 2,000
2 X 5,000
% PEI
14
12
10
8
6
4
2
0
1
2
Ch
(500)
(500)
(5,000)
(2,000)
(2,000)
(5,000)
(5,000)
(5,000)
(5,000)

Table 14

Long axis measurements of cysts of *C.irritans* from primary and secondary infections of mullet.

FISH			Number of theronts		No. trophonts released	CYSTS			
Pr.	Ident	Wt. (g)	previous exposure	challenge exposure		Number sampled	Mean Size (μ)	Size range (μ)	Size SD_{n-1}
A	1	3.20	500	5,000	35	25	233.0	181-287	24.21
	2	3.21	None	5,000	684	25	238.1	201-315	28.92
B	3	3.21	500	5,000	31	25	283.8	229-344	34.16
	4	3.38	None	5,000	172	25	289.2	239-353	32.14

difference between cysts from primary and secondary infections (p= 0.05) and hence no evidence of any association between host immune status and parasite growth. Partial immunity of the previously exposed fish of each group was confirmed by low %PEI values following challenge as compared with those from the unexposed fish (0.7% vs. 13.6% - pair A; 0.6% vs. 3.4% - pair B). It was noted that the duration of trophont development within the fish appeared unaffected by host immune status, with trophont exit occurring between 76-88 h for all 4 fish.

5. DURATION OF IMMUNE PROTECTION

Twelve mullet were immunised against *C.irritans* following three exposures each to 5,000 theronts, administered 14 days apart. At the third exposure, for each experimental fish, a weight matched control mullet, naive to *C.irritans*, was separately exposed to an equivalent number of theronts obtained from the same source in order to control for theront viability (methods, section 2.2); significant immune protection was confirmed in all experimental fish, according to criteria given earlier (methods 2.3). The 12 fish were divided into three groups and held under non-stressful hyposaline conditions without further exposure to *C.irritans* for either 1.5 months (5 fish), 3 months (5 fish), or. 6 months (2 fish). At the appropriate time, fish were individually challenged with 5,000 theronts, along with weight matched control mullet previously unexposed to *C.irritans*. Numbers of trophonts released were recorded for each fish (Table 15), the degree of immune protection being indicated by the numbers sustained following challenge as compared with that from the paired control. All 12 experimental fish exhibited significant immune protection to challenge. Very low parasite levels (%PEI $\leq$ 0.2%), indicating a high degree of sustained immune protection, were observed in fish held for up to 3 months, with full protection being

Table 15

Duration of immune protection in mullet against *C.irritans*.

Duration (months)[1]	Fish group	Number per group	Number of trophonts released per fish: median (mean) range	%PEI median (range)
1.5	Exposed[2]	5	0 (2.6) 0-9	0 (0.0-0.2)
	Control[3]	5	217 (262.0) 146-402	4.3 (2.9-8.0)
3.0	Exposed	5	0 (0) 0	0 (0)
	Control	5	214 (206.0) 141-283	4.3 (2.8-5.7)
6.0	Exposed	2	11 (10.5) 4-17	0.2 (0.1-0.3)
	Control	2	272 (271.5) 216-327	5.4 (4.3-6.5)

[1] Time between third exposure to *C.irritans* and challenge.

[2] Mullet which had received three exposures to *C.irritans* prior to challenge.

[3] Weight matched control mullet not exposed to *C.irritans* prior to challenge.

recorded in 60% at 1.5 months and 100% at 3 months. For the 2 fish held for 6 months, a relatively lower level of immune protection was apparent, with higher numbers of parasites being sustained following challenge as compared with fish held up to 3 months, and with neither fish showing full protection.

6. SPECIFICITY OF ACQUIRED IMMUNE PROTECTION

The specificity of the immune response of mullet to *C.irritans* was assessed by cross-challenge with the closely related ciliate, *I.multifiliis* which also occupies the same preferred site within its fish host. The infection and cross-challenge procedure devised here is shown in Fig. 37. Five mullet were each immunised against *C.irritans* by two exposures to 5,000 theronts, administered 14 days apart. At the second exposure, the 5 fish were considered to have acquired significant immune protection as based on comparison with paired naive controls (methods, section 2.3). The 5 mullet were rested for 7 days after which they were acclimated to freshwater, via 2-7 days in 25% SW, for challenge with *I.multifiliis*. Each of the 5 fish was challenged with 5,000 theronts of *I.multifiliis*, as previously described, along with a weight matched control mullet naive to both *C.irritans* and *I.multifiliis*. Levels of infection with *I.multifiliis* sustained by the 5 test fish and their paired controls, at 48h post-exposure, are shown in Table 16. The mean numbers of trophonts of *I.multifiliis* per mm^2 caudal fin were 0.54 for the 5 mullet immune to *C.irritans* and 0.49 for the 5 previously unexposed control fish; this difference being insignificant according to T test analysis by the paired comparisons method ($P > 0.5$; $n = 5$ pairs) (Swinscow, 1980). These results provide no evidence for cross-protection against *I.multifiliis* in mullet possessing immunity to *C.irritans*.

Figure 37

Procedure for challenge of mullet with *I.multifiliis* following primary and secondary exposures to *C.irritans*.

CI = *C.irritans*.

IM = *I.multifiliis*.

5,000 = number of theronts per exposure.

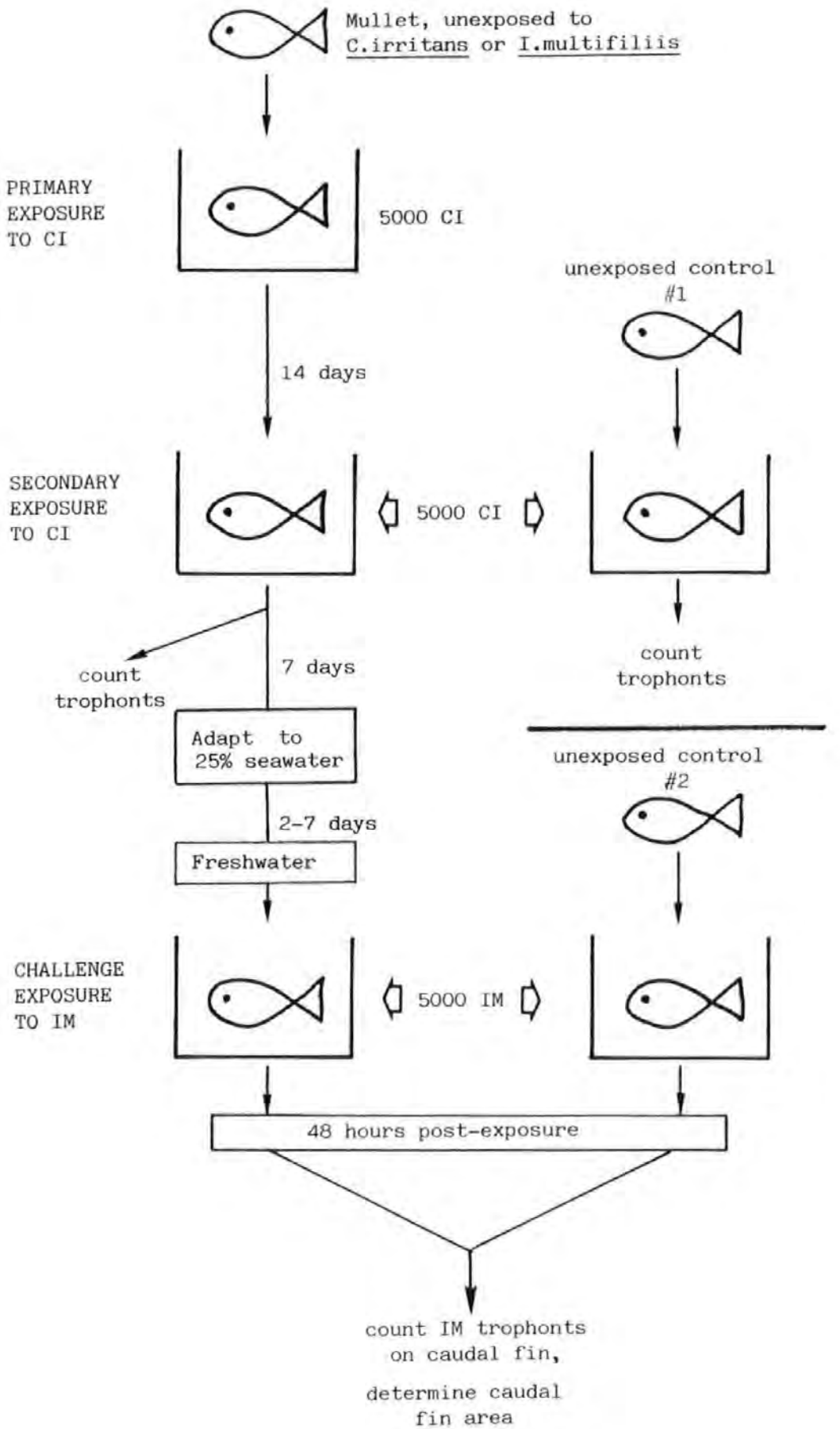

Mullet, unexposed to C.irritans or I.multifiliis
PRIMARY EXPOSURE TO CI
5000 CI
unexposed control #1
14 days
SECONDARY EXPOSURE TO CI
5000 CI
count trophonts
7 days
count trophonts
Adapt to 25% seawater
unexposed control #2
2-7 days
Freshwater
CHALLENGE EXPOSURE TO IM
5000 IM
48 hours post-exposure
count IM trophonts on caudal fin,
determine caudal fin area

Table 16

Effect of immune status of mullet to *C.irritans* on protection against infection with *I.multifiliis*.

Fish pair	Fish #	Immunity to *C.irritans*	Parasite levels in caudal fin following challenge with *I.multifiliis*		
			No. trophonts in fin	Area of fin examined (mm^2)	No.trophonts /mm^2 fin
A	1	yes	18	36	0.50
	2	no	11	28	0.39
B	3	yes	28	32	0.88
	4	no	6	27	0.22
C	5	yes	9	34	0.26
	6	no	16	32	0.50
D	7	yes	15	35	0.43
	8	no	33	34	0.97
E	9	yes	19	30	0.63
	10	no	15	41	0.37

DISCUSSION

Mullet are shown to be susceptible to *C.irritans*, sustaining infection levels of up to 267 parasites per gram of fish. The linear relationship between the numbers of parasites establishing infection in naive mullet and the numbers of theronts to which the fish was exposed, provide no evidence for a density-dependent or intra-specific competition in parasite establishment, even at infection levels resulting in significant host mortalities. A similar finding was made from studies on the infection dynamics of *I.multifiliis* in mollies, *Poecilia latipinna*, by McCallum (1982). Although mullet were clearly susceptible to *C.irritans*, only a small percentage of theronts, not more than 18%, were able to establish infection in naive hosts. Houghton (1987) noted that only 25% of theronts of *I.multifiliis* established infection in carp under experimental conditions. McCallum (1982) similarly recorded unexpectedly low infection levels of *I.multifiliis* in mollies and suggested that this might be attributed to loss of viability, resulting from reduced nutritional or energy status in the majority of theronts. Theront viability would be affected by failure to fully differentiate within the reproductive cyst and with age following excystment, as there appears to be no evidence of energy resources being replenished from the external environment. In the present study, however, mullet were exposed to theronts of *C.irritans* within four hours of excystment, within the period of maximum viability, as predetermined earlier. It is probable that host factors also account for the low percentage of theronts establishing infection. McCallum (1982), in considering differences in host susceptibility of mollies to *I.multifiliis*, speculated that a previous unknown exposure to the parasite, resulting in partial immunity, might have accounted for such variation. This was not the case, however, in the present study, as mullet for experimental

infections were collected from British coastal waters outside the geographical range of *C.irritans*. Clayton and Price (1992) have shown that variability in guppies associated with genetically inherited traits influences their susceptibility to *I.multifiliis*. Such factors could explain results here in which mullet exposed to the same number of theronts of *C.irritans* under identical conditions developed significantly different levels of primary infection, varying from 2-18% of the initial exposure. Little is known of the role of innate immunity in controlling the invasion of fish by protozoa although epithelial mucus can inhibit the attachment of certain other microbial pathogens (Ourth, 1980; Ellis, 1989). Contact, recognition and infection of fish hosts might be expected to pose many difficulties for an infective agent such as the theront of *C.irritans*, particularly as there is no evidence in support of a host finding mechanism. The degree of activity of mullet during exposure may be significant in this respect in effecting the chances of random host location and attachment by the theronts of *C.irritans*. Host recognition factors associated with ectoparasites and endoparasites invading the skin of fish have been detected within the mucus layer (Kearn, 1988; Lom and Cerkasovova, 1974). The possibility cannot be ruled out, therefore, that many theronts become entrapped in excess mucus shed from mullet under conditions of stress within experimental infection aquaria. The low percentage of theronts of *C.irritans* which established primary infections in mullet here may, therefore, reflect their probability to contact the host surface rather than be an expression of viability.

Cryptocaryosis is a potentially acute disease, mullet deaths occurring within five days in primary infections following exposure to high numbers of theronts. It is not surprising, therefore, that sudden deaths and mass mortalities of fish in mariculture have been attributed to this pathogen (Huff and Burns, 1981; Rasheed, 1989). The cause of death from cryptocaryosis was not investigated here, although it is probable

that gross epidermal disruption associated with trophont release would result in osmotic imbalance and respiratory stress. Hines and Spira (1974a) considered these factors to be the major cause of death in carp infected with *I.multifiliis*. It was important in the present study, therefore, to establish a sub-lethal exposure level for immunisation and challenge infections of mullet with *C.irritans*. Five thousand theronts per 4g mullet, provided a level sufficient to immunise with a single exposure, with minimal host deaths.

Infection and challenge experiments using mullet have provided the first conclusive evidence that fish have a capability to acquire protective immunity with memory to *C.irritans*. This is as might be expected as many species of freshwater teleosts develop protection against the closely related ciliate *I.multifiliis* which has a similar course of infection invading the epithelium (Lahav and Sarig, 1973; Hines and Spira, 1974b; Valtonen and Keranen, 1981; McCallum, 1986; Houghton, 1987). The demonstration of protection in mullet against reinfection with *C.irritans* within 14 days following a single exposure also compares favourably with that recorded in carp to *I.multifiliis* (Houghton, 1987). Although temperature has a positive influence on the kinetics of fish immune mechanisms (Hildermann, 1957; Rijkers, 1982) it was not possible to determine whether tropical species respond more rapidly to *C.irritans*. The immune response of mullet collected here from temperate waters and maintained at temperatures in excess of 23°C could not be considered representative of normal host species from parasite endemic regions.

The degree of acquired immunity to *C.irritans* was positively related here to the number and size of exposures. Establishment of partial protection in mullet following a single exposure level of only 500 theronts suggests the existence of a critical lower threshold to elicit a protective response for this pathogen. The possible implications

of an immune threshold in the development of vaccination programmes is considered in the final discussion.

That immunological memory is established in mullet against *C.irritans* has clearly been demonstrated here with sustained protection of at least six months in the absence of re-exposure to the parasite. Memory is well recognised in fish for both humoral and cell mediated immunity (Rijkers *et al.*, 1980, Rijkers, 1982), including mullet, *C.labrosus* (Mughal, 1984), the duration being influenced by temperature and the dose and nature of antigen administered at immunisation (Ellis, 1988). To what extent the duration and amplitude of the anamnestic response to *C.irritans* can be enhanced in marine species of fish maintained under tropical conditions warrants further investigation. Such studies will be vital in assessing the feasibility of affording protection to this parasite by vaccination.

Immunisation of mullet against *C.irritans* provided no cross- protection against the related ciliate *I.multifiliis*. Nevertheless, some evidence exists for cross-protection between *I.multifiliis* and *T.pyriformis* in freshwater fish hosts (Wolf and Markiw, 1982; Dickerson *et al.*, 1984) although more recent studies have questioned the involvement of a specific immune response (Graves *et al.*, 1985). That *T.pyriformis* and other adjuvants could provide some protection against *I.multifiliis* by enhancing non-specific mechanisms is suggested from *in vitro* studies of killer cells by Graves *et al.* (1985). The application of such an approach to the control of cryptocaryosis may well provide some alleviation from acute disease whilst acquired immunity becomes established.

The establishment of low numbers of *C.irritans* on some fish following two or more exposures to the parasite indicates a significant, but incomplete immune protection. Hines & Spira (1974a), McCallum (1986) and Houghton (1987) have made similar observations in fish immunised against *I.multifiliis*, small numbers of

parasites establishing on challenge completing normal development. In these instances (Hines & Spira, 1974a; Houghton, 1987), the trophonts were confined to the periphery of the fins, possibly indicative of poor immune response in sites of reduced blood flow. No such distribution of *C.irritans* on immune mullet was observed here. It is possible that host protective mechanisms operate during or shortly following parasite invasion of the epidermis, subsequent immune responses directed at the parasite becoming ameliorated by normal feeding activity with lysis and break down of host tissue. Occasional observations of white fletches on the fins of immune mullet following exposure to theronts but in the absence of established trophonts, suggests some brief interaction between host and parasite. It is possible that a proportion of theronts which invade immune fish are subsequently destroyed *in situ* or prematurely exit from the host. Evidence for a brief invasion of immune fish by *I.multifiliis* theronts comes from studies by Ewing *et al.* (1986) and Cross (1990). Further evidence that immune protection against *C.irritans* functions early in the infection process comes from observations on immune mullet, which although sustaining few parasites, as compared with controls, showed no inhibitory effects on those trophonts which established infection, both in terms of trophont growth rate and duration on host. The underlying effector mechanisms for immune protection could not be ascertained from these studies; examination for specific antibody production to *C.irritans* will be investigated in Chapter 6.

CHAPTER FIVE

CHARACTERISATION OF MAJOR POLYPEPTIDES

INTRODUCTION

There has been no previous research into the molecular biology of *C.irritans*, however, the characterisation of proteins and polypeptides for other aquatic ciliates, including parasites of fish, is briefly reviewed.

Studies of the major proteins of ciliates which are closely related to *C.irritans* have been investigated by several workers. Pyle and Dawe (1985), working on *I.multifiliis*, used polyacrylamide gel electrophoresis (PAGE) to reveal quantitative and possibly qualitative differences in the protein patterns of different life-cycle stages of the parasite. Unfortunately, these authors did not characterise the proteins by molecular weight, thereby preventing comparison with other species. Further compositional analysis of *I.multifiliis* has identified ciliary antigens (Clark *et al.*, 1988) and possible membrane-associated immobilisation antigens (Dickerson *et al.*, 1989) which may be important in eliciting the host response. Protein analyses on *Tetrahymena*, a ciliate which can be easily propagated *in vitro*, have been extensively undertaken (Buhse and Williams, 1982; Williams *et al.*, 1984; Doerder and Berkowitz, 1986); these studies may provide data for comparison with *C.irritans*. Little is known of the molecular composition of other ciliate parasites of fish, the majority of which have not been successfully maintained *in vivo* or *in vitro*, or have not attracted

attention as economically important pathogens. With regards to ciliates which are obligate parasites, a major limiting factor has been the difficulty in obtaining sufficient parasite material, in the absence of *in vitro* propagation methods.

The objectives of the present study were first to identify the major polypeptides of *C.irritans* by PAGE analysis. Secondly, to compare polypeptide profiles between different isolates with a view to strain detection, and between the theront and trophont stages in order to detect any stage-specific molecules. Further, polypeptide analysis of the theronts and trophonts of *I.multifiliis* and trophozoites of *T.pyriformis* would be undertaken to allow comparative studies between these closely related ciliates. Elucidation of the polypeptide composition of *C.irritans* should provide a basis for the future characterisation, identification, and isolation of parasite molecules which may be relevant in eliciting host protection, and therefore offer potential as candidate vaccines.

MATERIALS AND METHODS

1. CILIATES

In addition to *C.irritans*, the related ciliates *I.multifiliis* and *Tetrahymena pyriformis* were also used for antigen analysis. Trophonts and theronts of *C.irritans* and *I.multifiliis* were available through *in vivo* maintenance, as described earlier (Chapters 3 and 4, methods).

1.1 Maintenance of T.pyriformis

T.pyriformis strain 1630/W, obtained from the Freshwater Biological Association, Windermere, U.K., was cultured axenically at RT in 1% proteose peptone (Lab M) containing 0.25% yeast extract (Oxoid), and sub-cultured weekly. Cells were harvested during their growth phase. Medium-free cells were obtained by repeated centrifugation at 2,000*g* and resuspension in distilled water or PBS.

1.2 Ultrasonic disruption of ciliate cells

Ciliate cells for sonication were maintained alive or stockpiled by cryopreservation until required. Material was sonicated at 14μm amplitude in an MSE ultrasonic disruptor fitted with a 3mm diameter microprobe. In order to prevent overheating, the cells were kept on ice and pulse sonicated at 15 sec intervals for a total sonication period of 2 to 5 min. Sample solubilisation was monitored microscopically at 30 to 60 sec intervals, samples being centrifuged at 8,000*g* until a constant size pellet was obtained.

1.3 Protein and lipid assays for C.irritans

1.3.1 Protein

The protein estimation method of Lowry *et al.* (1951) was modified to a micro-assay in view of the small sample sizes available. Trophonts of isolate CI-HT, obtained from a primary infection, were enumerated, the numbers adjusted to samples of 100 or 200, and washed in FS-SW. Trophonts were resuspended in PBS and sonicated for 2 min according to the method given above. Sonicated trophonts were solubilised in 2% Na_2CO_3 in 0.1M NaOH, prior to reaction. Protein standards comprised bovine albumin (Sigma, product A-7906) diluted over the range 25μg/ml to 1mg/ml in PBS. Colour reactions were read against PBS at 625nm and protein concentrations of test samples extrapolated from the standard curve.

1.3.2 Total lipids

The sulphophosphovanilin colorimetric method of Zollner and Kirsch (1962) was followed, using a proprietary kit (Boehringer, Germany). Trophonts numbering 1,400 of CI-HT isolate, were washed and sonicated using the methods given above for protein estimation. The manufacturer's total lipids standards were used. Colour reactions were read at 530nm.

2. MULLET IMMUNOGLOBULIN

Immunoglobulin from mullet, obtained from Ultrogel fractionation of whole serum (Chapter 6), was analysed by SDS-PAGE on a 11% gel.

3. POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

One-dimensional PAGE was performed either under reducing conditions, using

2-mercaptoethanol to disrupt disulphide bonds, or under non-reducing conditions, as follows. Vertical slab polyacrylamide gels, 75 X 100mm, 0.75mm thick, were cast on a stand (Mini-protean II, Biorad, USA). Between 4μl and 10μl samples were loaded per track (10 well combs) and electrophoresed in a water cooled apparatus (LKB, "Midget 2050") at a constant 100V through the stacking gel, increased to 200V through the separating gel. Protein migration was monitored using a bromo-phenol blue dye marker incorporated with the sample buffer. Buffers were prepared according to the Sigma technical bulletin (MWS-877L).

3.1 Sodium dodecyl sulphate (SDS)-PAGE

Seven or 11% separating gels were overlaid with a 3% stacking gel, using the discontinuous buffer system of Laemmli (1970). Gels were run for 30 to 60 min. Molecular weight standards comprised, for 11% gels: bovine milk alpha-lactalbumin (14.2kDa), soybean trypsin inhibitor (20.1kDa), bovine pancreas trypsinogen (24kDa), bovine erythrocytes carbonic anhydrase (29kDa), rabbit muscle glyceraldehyde-3-phosphate dehydrogenase subunit (36kDa), egg albumin (45kDa), bovine albumin (66kDa) (Sigma, MW marker kit, MW-SDS-70L), and for 7% gels: carbonic anhydrase (29kDa), egg albumin (45kDa), bovine albumin (66kDa), rabbit muscle phosphorylase B subunit (97.4kDa), *Escherichia coli* beta-galactosidase subunit (116kDa), rabbit muscle myosin subunit (205kDa) (Sigma, MW marker kit, MW-SDS-200).

3.2 Gel staining for protein and carbohydrate

3.2.1 Protein

Gels were stained with 0.125% Coomassie brilliant blue in 40% (v/v) methanol, 7% (v/v) glacial acetic acid fixative (Johnstone and Thorpe, 1982).

Enhanced sensitivity was achieved by monochromatic silver staining (Wray *et al.*, 1981). Polypeptide sizes were estimated from molecular weight standard curves constructed for each gel.

3.2.2 Carbohydrate

Gels were stained for the presence of carbohydrate by the periodic acid - Schiff's method, red-staining bands indicating glycoproteins (Johnstone and Thorpe, 1982).

EXPERIMENTS AND RESULTS

The polypeptide composition of *C.irritans* and related ciliates was investigated using biochemical and electrophoretic techniques.

1. BIOCHEMICAL ANALYSIS OF C.IRRITANS TROPHONTS

Biochemical analysis of trophonts indicates a protein value of 1.9 to 2.2μg per trophont and total lipids 0.35μg per trophont.

2. SDS-PAGE ANALYSIS

2.1 C.irritans trophonts

Slight quantitative and qualitative differences in polypeptides were found between cell preparations obtained from the same *C.irritans* isolate which were possibly attributable to the varying extent of solubilisation between samples. Sonication prior to solubilisation and electrophoresis reduced variation, but was necessarily limited to larger samples. Comparative studies were therefore based on routinely observed polypeptide profiles, gained from repeated runs.

2.1.1 Comparison between trophont isolates

Eleven percent SDS-PAGE analysis of trophont sonicate preparations from three isolates of *C.irritans*, namely, CI-FL, CI-HT, and CI-GS, is shown in Fig. 38. At least 25 bands were resolved for each isolate, most occurring between 20kDa and 100kDa. A broad diffusely stained region was detected over the low MW range (< 20kDa). Seven percent SDS-PAGE analysis of *C.irritans* trophonts resolved a further 7 bands above 100kDa, of which 2 were predominant, occurring at 107 and 148kDa

Figure 38

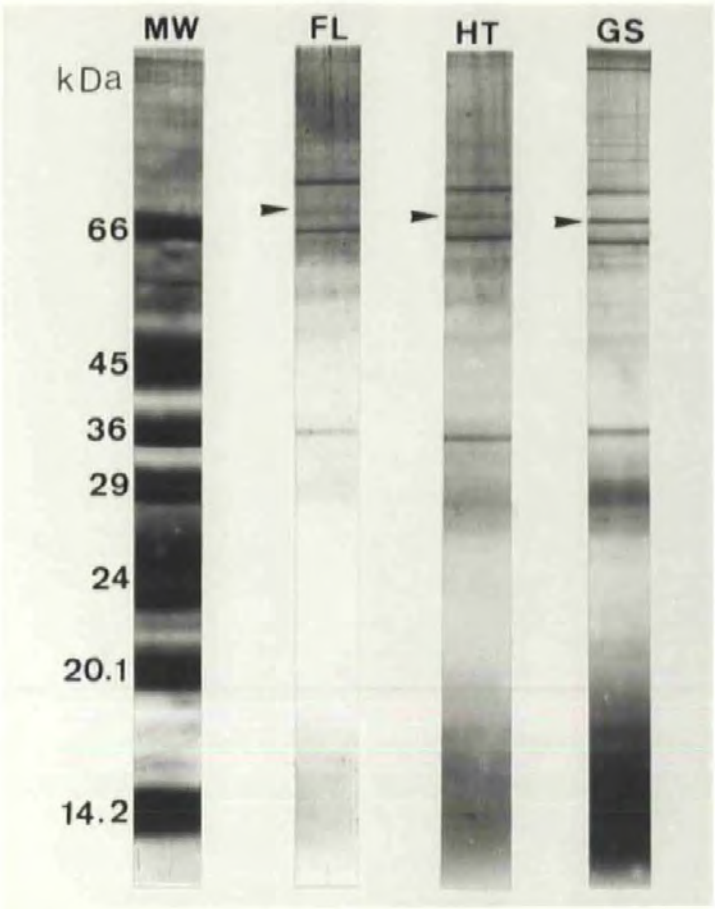
Comparison of polypeptide profiles of trophonts from three isolates of *C.irritans*, by SDS-PAGE.

Electrophoresis performed in 11% gel under reducing conditions, and stained with Coomassie blue.

FL, HT, and GS = isolates of *C.irritans*.

MW = molecular weight marker set.

Arrows show 67-68 kDa positions.


MW
FL
HT
GS
kDa
66
45
36
29
24
20.1
14.2

positions (not shown). No significant qualitative polypeptide differences between the three isolates were observed. A major quantitative difference was found with the 67-68kDa component of trophonts which stained heavily in the CI-GS preparation, moderately in CI-HT and weakly in CI-FL.

2.2 Comparison between C.irritans trophonts and other ciliates

Comparative polypeptide profiles, following 11% SDS-PAGE, were made for the trophont and theront stages of both *C.irritans* (CI-GS) and *I.multifiliis* (isolate IM-CC) and the trophozoites of *T.pyriformis* strain 1630/W. Coomassie blue staining of trophont and trophozoite preparations of all ciliate species tested revealed at least 30 bands each, but only 8 to 10 bands in theront preparations. Silver staining of theronts increased resolution to >20 bands, however when applied to trophonts and *T.pyriformis*, this caused overstaining, restricting interpretation. Major polypeptide bands are shown diagrammatically in Fig. 39, compiled from consistent results obtained using Coomassie and silver staining. Comparison between *C.irritans* trophont and theront stages of the same isolate suggests both common and possibly stage-specific polypeptides; a similar pattern is observed for *I.multifiliis*. A degree of homology with regards to five major trophont polypeptides is seen between *C.irritans* and *I.multifiliis*. Less homology is observed between the trophont of either species and *T.pyriformis*.

A major band was detected in all five preparations at the 55- 56kDa position, being least strongly staining in *C.irritans* trophonts. A major 44- 45kDa band was present in the theronts of *C.irritans* and in both theronts and trophonts of *I.multifiliis* but appeared as very faint in the trophonts of *C.irritans* and absent in *T.pyriformis*. A 68kDa polypeptide, which varied quantitatively between *C.irritans* trophont isolates,

Figure 39

Comparison of major polypeptides of trophonts and theronts of *C.irritans*, trophonts and theronts of *I.multifiliis*, and trophozoites of *T.pyriformis*, by SDS-PAGE.

Electrophoresis performed in 11% gel under reducing conditions.

CI = *C.irritans*

IM = *I.multifiliis*

TP = *T.pyriformis*

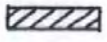
MW = molecular weight marker set

Arrows show molecular weight (kDa)

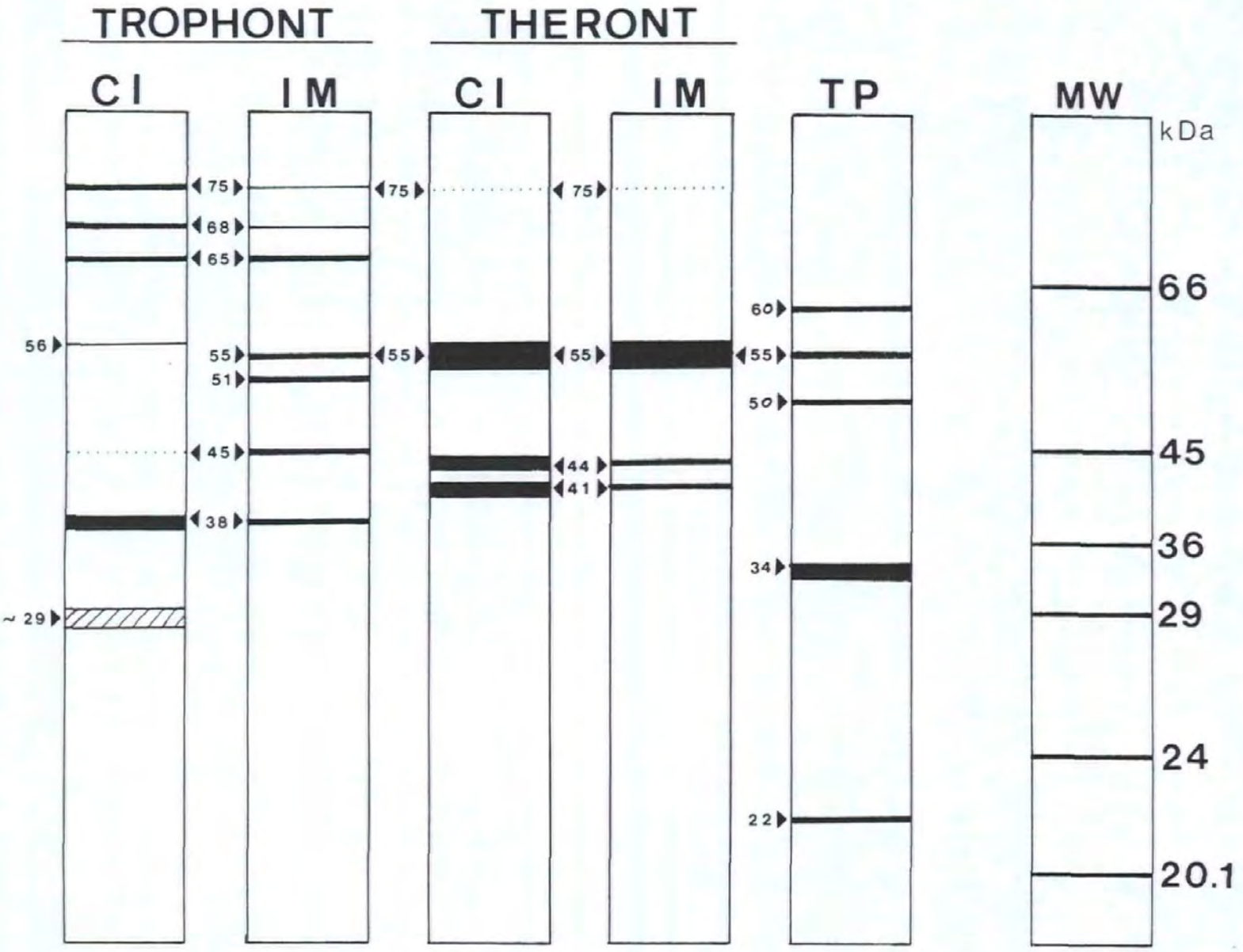
Intensity of staining:

..... weak (included for comparative purposes)

increasing intensity



diffusely stained region


TROPHONT
THERONT
CI
IM
CI
IM
TP
MW
kDa
75
68
65
56
55
51
45
38
~ 29
75
55
75
55
44
41
60
55
50
34
22
66
45
36
29
24
20.1

was also present in *I.multifiliis* trophonts, but absent in the theront stage of both species, as well as in *T.pyriformis*. Staining of all ciliate preparations for carbohydrate resulted in reactivity only with *C.irritans* trophonts, with a diffuse stained area extending across the low molecular weight region, between ca. 2- 17kDa.

Greater polypeptide homology is observed between the theront stages of *C.irritans* and *I.multifiliis* than compared with trophont polypeptides, as revealed by silver staining, (Fig. 40). Twenty-one distinct bands were resolved for the *C.irritans* theronts and 35 for the same stage of *I.multifiliis*; additional diffusely stained bands of low molecular weight were recorded for both species. The three major polypeptide bands at 41, 44, and 55kDa appeared common to both species; a further 11 minor bands also showed molecular weight homology.

2.3 Mullet immunoglobulin

SDS-PAGE separation of mullet immunoglobulin (MIg) revealed two major bands (Fig. 41). These comprised a discrete, intensely-staining polypeptide at 67-68kDa and a moderately stained diffuse region between 27.5- 29kDa, roughly corresponding in molecular weight to immunoglobulin heavy (H) and light (L) chains, respectively. The 67- 68kDa H chain of mullet serum corresponds with an equivalent sized dominant polypeptide in trophonts of both *C.irritans* and *I.multifiliis*, but absent in theronts of both these species and also absent in trophozoites of *T.pyriformis*. The 27.5- 29kDa L chain of mullet immunoglobulin, which contains 2 minor bands, showed no molecular weight counterpart in any of the ciliate preparations examined. Estimated molecular weight of the intact mullet Ig molecule is 766kDa, based on the formula: [2H + 2L] X4; this assumes mullet serum Ig is tetrameric.

Figure 40

Comparison of polypeptides of theronts between *C.irritans* and *I.multifiliis*, by SDS-PAGE.

Electrophoresis performed in 11% gel under reducing conditions, followed by monochromatic silver staining.

CI = *C.irritans*

IM = *I.multifiliis*

MW = molecular weight marker set.

Arrows show molecular weights (kDa) of major bands common to both species.

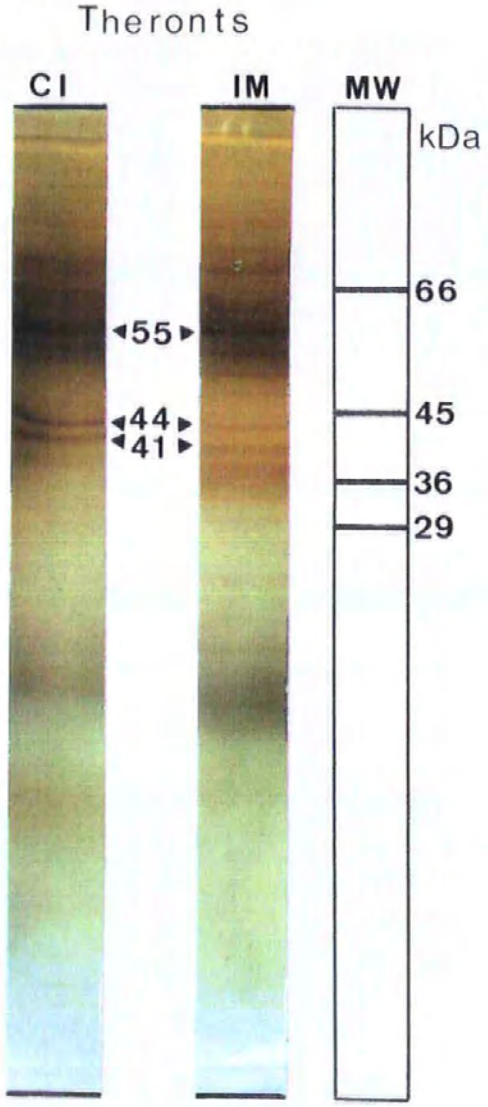

Theronts
CI
IM
MW
kDa
66
55
45
44
41
36
29

Figure 41

SDS-PAGE analysis of mullet serum immunoglobulin (MIg), Ultrogel fraction.

Electrophoresis performed in 11% gel under reducing conditions, followed by monochromatic silver staining.

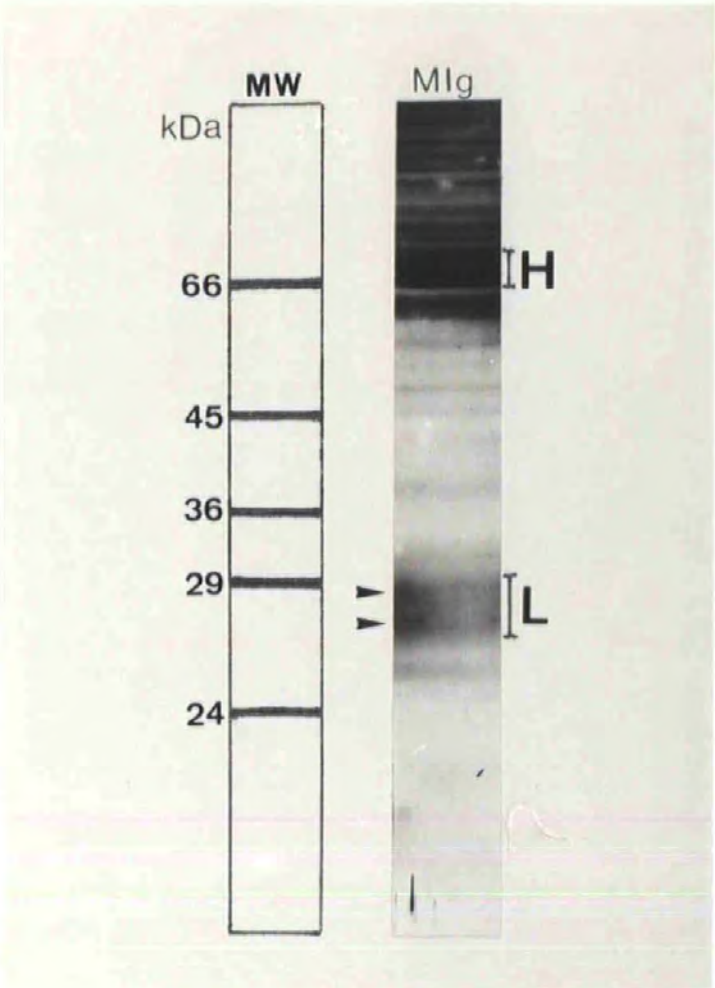
H = heavy chain region, 67-68 kDa.

L = light chain region, 27.5-29 kDa.

MIg = mullet immunoglobulin.

MW = molecular weight marker set.

Arrows show molecular weight sub-populations of L chain.


MW
kDa
66
45
36
29
24
MIg
H
L

DISCUSSION

SDS-PAGE analysis of *C.irritans* routinely resolved at least 20 polypeptide components in both the trophont and theront stages. Five predominant polypeptides, occurring between 38kDa and 75kDa, were recorded for trophonts and three, between 41kDa and 55kDa, for theronts, using 11% gels.

The absence of qualitative differences between polypeptide profiles of trophonts obtained from three isolates of *C.irritans* provided no evidence for the existence of strains. The 67-68kDa polypeptide, which differed quantitatively between the three isolates, is discussed later. Given the impossibility of tracing the history of the *C.irritans* isolates studied here, it is not possible to exclude a common origin accounting for their similar polypeptide profiles. Strain differences have been well documented for *Tetrahymena* and protein analysis has formed the basis for describing new species within this genus (Williams *et al.*, 1984). Further investigation for possible strains of *C.irritans* would therefore seem worth pursuing, particularly as an isolate has recently been described from the cooler waters of the Eastern Mediterranean by Diamant *et al.* (1991).

Qualitative differences in the polypeptide profiles between theronts and trophonts of *C.irritans* suggested the presence of stage-specific proteins. The occurrence of such proteins has been well documented for medically important parasitic protozoa (Arujo *et al.*, 1982; McMahon-Pratt and David, 1982; Martinez, 1987). In contrast, few such studies appear to have been reported for fish protozoan parasites, with the exception of *I.multifiliis* where stage-specific components were demonstrated by SDS-PAGE analysis following studies of the parasite from its free-living trophont stage through to the theront (Pyle and Dawe, 1985). The existence of

stage-specific proteins of *C.irritans* seems probable, given the differing functional requirements of the life cycle stages. It is likely that some stage-specific proteins are enzymes. For example, hyaluronidase, which causes breakdown of host intercellular material, has been detected in the theronts but not the trophonts of *C.irritans* (Colorni, 1988).

Major polypeptides, showing quantitative, rather than qualitative, differences between theronts and trophonts probably include structural molecules. The 55-56kDa polypeptide, found in all ciliate preparations, appeared more predominant in the theronts of *C.irritans* as compared with trophonts, and may be the 55kDa tubulin polypeptide identified from the cilia of *I.multifiliis* by Clark *et al.* (1988). Similarly, the 44-45kDa band was quantitatively greater in theronts as compared with trophonts of *C.irritans*. The greater degree of ciliation in theronts as compared to trophonts, observed here, could be significant in accounting for quantitative differences in both the 44-45kDa and 55-56kDa bands. The 44-45kDa polypeptide, which was also found in theronts and trophonts of *I.multifiliis*, closely correlates with a major *I.multifiliis* component reported by Pyle and Dawe (1985), and may represent the 43kDa cilial membrane polypeptide which has been identified in the theronts of *I.multifiliis* by Clark *et al.* (1988) and Dickerson *et al.* (1989). The 43kDa polypeptide has been identified as a predominant surface antigen (Dickerson *et al.*, 1989), possibly analogous to the 44-52kDa immobilisation ("i")-antigens of *Tetrahymena* (Williams *et al.*, 1985; Doerder and Berkowitz, 1986) and the low molecular weight population i-antigens of the ciliate *Paramecium* (Eisenbach *et al.*, 1983; Capdeville *et al.*, 1985). Given the molecular weight heterogeneity of i-antigens amongst the ciliates, the absence of a predominant ≈43kDa component in the trophonts of *C.irritans* does not preclude the possibility of other molecular weight components functioning as i-

antigens. Studies here have shown a high degree of apparent homology in major polypeptides between *C.irritans* and *I.multifiliis* with respect to trophonts, and in particular, theronts. Such inter-species homology appears greater than that between the two life-cycle stages for either parasite. However, the major polypeptides of *C.irritans* and *I.multifiliis* showed less homology with *T.pyriformis* trophozoites. These polypeptide patterns lend some support to the current classification of *C.irritans* and *I.multifiliis* within the same family (Fig. 2), as based on morphological characteristics (Corliss, 1979).

The demonstration of polypeptides shared between *C.irritans* and non-parasitic ciliate species could be beneficial to vaccination studies, by identifying possible antigens of easily cultured free-living species which may elicit cross-protection. This approach has been attempted using *T.pyriformis* as a vaccine against ichthyophthiriasis (Goven *et al.*, 1980; Wolf and Markiw, 1982; Dickerson *et al.*, 1984). However, structural polypeptides may be of less importance in terms of antigenicity. For example, the presumptive 55kDa tubulin molecule, shown here to be present in all three ciliate species tested, is a highly conserved polypeptide and therefore unlikely to be highly antigenic (Benjamin *et al.*, 1984). In view of observed differences in cross-protective efficacy between strains of *Tetrahymena* (Dickerson *et al.*, 1984), with some strains giving no cross-protection (Houghton, 1987), it would seem valuable to use polypeptide analysis to identify strains of *T.pyriformis* possessing possible shared antigens, for selection of candidate vaccines.

Care must be taken in the interpretation of polypeptide profiles, for the following reasons. Limitations in the level of resolution achieved here together with slight compositional variations in the ciliate preparations has restricted comparative studies to major polypeptides; such major components appear to include structural

polypeptides which may not be important antigenically, as discussed above. It is likely that numerous non-structural minor polypeptides, some possibly important antigens, were present in the ciliate preparations but were either undetected or insufficiently resolved to be included in the comparative analyses. Furthermore, the protein composition of ciliates may be altered in response to changing environmental conditions, as shown for *Tetrahymena vorax* trophozoites by Buhse and Williams (1982). The influence of culture conditions may therefore confuse comparative polypeptide profile analyses between different laboratories. Finally, the possibility that certain polypeptide components identified in the *C.irritans* and *I.multifiliis* stages are derived from either bacteria - "xenosomes" (Corliss, 1985) or the host fish, cannot be excluded. Gram negative bacteria, existing as endocytobionts, have been found in all stages of *I.multifiliis* (Roque *et al.*, 1967; Lobo-da-Cunha and Azevedo, 1988) and in the marine ciliate, *Parauronema acutum* (Soldo *et al.*, 1974). With regards to host polypeptides, of particular interest is the 68kDa component, present in the trophonts but not theronts of both *C.irritans* and *I.multifiliis*. This polypeptide shows molecular weight homology with the presumptive H chain of mullet serum immunoglobulin. The presence of host immunoglobulin within the trophonts of *C.irritans* could be a result of the parasite's feeding in the epidermis, given that immunoglobulin has been identified in the skin epidermal tissue of teleosts (reviewed in Chapter 2). Further evidence for host immunoglobulin within *C.irritans* trophonts is presented in Chapters 6 and 7.

It is worth mentioning that studies here have permitted molecular weight characterisation of mullet immunoglobulin H and L chains, calculated to be 67-68 kDa and 27.5-29 kDa, respectively. These values are similar to those reported for other teleosts, including salmonids: H ≈72-75kDa; L ≈23-26kDa (Cisar and Fryer, 1974;

Kobayashi *et al.*, 1982), carp: H ≈71-76kDa; L ≈24-26kDa (Marchalonis *et al.*, 1971; Vilain *et al.*, 1984), cod: H ≈81kDa; L ≈27.5kDa (Pilstrom and Petersson, 1991), channel catfish: H ≈70kDa; L ≈22-26kDa (Lobb and Olson, 1988), and flounder: H ≈72 kDa; L ≈22-28 kDa (Glynn and Pulsford, 1990). The presence in mullet immunoglobulin of a diffuse L chain region containing two bands suggests distinct molecular weight L chain populations, as identified in channel catfish L chain by Lobb and Olson (1988). The observed molecular weight heterogeneity may reflect differing functional roles of the immunoglobulin molecules.

CHAPTER SIX

SEROLOGY

INTRODUCTION

No studies have been reported on the immune responses in fish to *C.irritans*. Immunological investigations are necessary in order to understand the underlying mechanisms responsible for acquired protection, observed here for mullet, following infection with *C.irritans*. Methods to investigate specific immunity in fish have mostly comprised antibody detection assays which have provided useful information on host immune recognition and response to pathogens (Trump and Hildemann, 1970; Desvaux and Charlemagne, 1981; Wood and Matthews, 1987), including protozoa (Rijkers *et al.*, 1980; Sypek and Burreson, 1983; Burreson and Frizzle, 1986; Laudan *et al.*, 1986; Clark *et al.*, 1988; Gravil, 1991). Serological tests are now well established for medical and veterinary use (Voller *et al.*, 1976; Rose and Bigazzi, 1980; Stites, 1980; Wilson and Simpson, 1980), however, their application to fish has been restricted by the lack of commercially available immunological reagents, particularly antisera for the detection of fish antibody. Such reagents are a necessary pre-requisite for the development of the enzyme-linked immunosorbent assay (ELISA) (Thuvander *et al.*, 1987), a highly sensitive test which has gained increasing use (Edwards, 1985). The ELISA is ideally suited for studies on microparasites which cannot be grown *in vitro* (Burgess, 1990), as the test requires little antigen. ELISA has been applied to measure antibody responses in fish to a variety of pathogens including viruses and bacteria (Dixon and Hill, 1983; Cossarini-Dunier, 1985; Klesius *et al.*,

1991; Lorenzen and Dixon, 1991), as well as eucaryotic parasites (McArthur and Sengupta, 1982; Bortz *et al.*, 1984; Whyte *et al.*, 1987; Grayson *et al.*, 1991) including ciliated protozoa (Cross, 1990; Gravil, 1991).

In the absence of any previous studies on the immune response to *C.irritans*, it was considered appropriate to investigate for a specific antibody response to the parasite. This requires the production of specific antiserum reagents and parasite antigens for the development of serological assays to measure antibody. The serological tests would be applied to determine whether mullet produce an antibody response following exposure to *C.irritans* by natural infection or immunisation. Specific antibodies, if detected, would be investigated for any correlation between their levels and the degree of protection to *C.irritans* infection, and the duration of the antibody response investigated in the absence of further exposure to parasite. The specific mammalian antisera would also be applied in serological assays as probes for antigenic cross-reactivity between *C.irritans* and related ciliates.

MATERIALS AND METHODS

1. PROTEIN ASSAYS

Total protein was estimated by either the Folin phenol colorimetric method (Lowry *et al.*, 1951) or by Ultra-violet (U.V.) absorption at 280nm, the latter method being less sensitive but non-destructive to the test material. Bovine serum albumin (BSA, fraction V, Sigma), was used to prepare standards for each method.

2. FISH SERUM COLLECTION AND STORAGE

Blood samples were collected from anaesthetised (benzocaine) mullet by caudal venepuncture, using either a 21G or 23G needle, depending on the size of fish. Volumes of blood less than 50μl were successfully taken from small mullet. In these instances, blood was recovered from the 23G needle barrel by folding the latter into a polypropylene tube and centrifuging at 3,000 *g*, 5-10 sec. All blood samples were transferred to glass tubes or LP3 tubes (Luckham Ltd.), allowed to stand for 1 h at RT, and refrigerated overnight at 10°C for clot retraction. Samples were then centrifuged at 3,000*g*, 10 min, and the serum aliquoted amongst LP2 tubes (Luckham Ltd.) and stored at -20°C or in liquid nitrogen.

Plasma fractions from whole blood, using an anticoagulant (e.g. heparin), were rarely taken due to problems with samples clotting following cryostorage.

3. FISH MUCUS COLLECTION AND STORAGE

Epithelial body mucus was collected from live mullet by one of the following methods, depending on fish size. Fish over 30cm length were briefly held in a dry polythene bag and the body gently massaged against the polythene. Following

removal of the fish, detached mucus within the bag was collected with a plastic spatula. For smaller fish (≈10-30cm) body mucus was removed using a cotton wool swabstick (Boots the Chemist Ltd., England) which was repeatedly applied in a head to tail direction whilst rotating the swabstick on its axis, until saturated. Swabstick absorbing capacity was determined as ≈ 0.2ml fluid (mean capacity = 220 ± 50μl, based on 20 swabsticks). Swabsticks were eluted in LP3 tubes containing PBS by repeatedly squeezing the cotton bud against the tube wall. Mucus samples, collected by either method, were diluted fivefold (v/v) with PBS and stored at -20°C. For ELISA testing, mucus samples were further diluted to a final 1 in 10 in ELISA incubation buffer containing double strength Tween 20 (buffers given in appendix).

It is noted here that mullet yield relatively small quantities of epithelial mucus as compared with carp or trout of a similar size.

4. MAMMALIAN SERUM COLLECTION AND STORAGE

Blood samples were taken from rabbits by marginal ear venepuncture. Rats were test bled from the caudal vein, larger quantities being collected by cardiac puncture immediately following death. All serum fractions were prepared and stored as for fish serum, described earlier.

5. PURIFICATION OF MULLET IMMUNOGLOBULIN

5.1 Gel fractionation

A highly enriched immunoglobulin (Ig) fraction of mullet serum was prepared, based on the method described by Glynn and Pulsford (1990) for flounder. Gel filtration was performed using Ultrogel AcA22 polyacrylamide/agarose gel (LKB, Sweden), selected for its high resolution over the fractionation range 10^5 to 1.2 X 10^6

kDa (manufacturer's specifications). Three millilitres of serum was pooled from samples collected the previous day from three healthy mullet, each ≈ 20cm length, and naive to *C.irritans*. Pooled serum was loaded onto a 1.6 X 90cm gel bed which had been equilibrated for 48 h with carrier buffer (PBS pH 7.6). Fractionation was performed at RT with a flow rate of 4ml/h. The eluent was directed through a 280nm U.V. absorbance detector and into a fraction collector, set at 30 min/fraction (= 2ml/ fraction). Fractions were stored at 10°C until analysis.

5.2 Protein estimation of fractions

Fractions containing Ig, previously identified by SDS-PAGE analysis, were measured for absorbance at 280nm. Protein estimations were obtained from the extinction coefficient (EC_{280}) value for human IgM (EC_{280} = 11.8) (Johnstone and Thorpe, 1982).

5.3 Concentration of immunoglobulin fractions

Fractions containing Ig were concentrated by polysulphone membrane centrifugation using Milipore "ultrafree" membranes of 10,000 MW limit (Milipore, USA). Fractions were spun at 1,000 to 2,500 *g* for various times, depending on the degree of concentration required.

5.4 Electrophoretic analysis

Concentrated Ig fractions were analysed under reducing conditions for the presence of Ig heavy and light chains, using the Phast-gel apparatus (Pharmacia Ltd.). Spot samples of previously boiled fractions (diluted 1:1 in sample buffer) were loaded onto a commercially prepared 12.5% homogeneous gel ("Phastgel", Pharmacia.).

Reference samples comprised a molecular weight marker set (MW SDS-6H, Sigma, USA) and purified fractions of bovine Ig and flounder Ig, applied separately (the latter prepared by Dr P. Glynn, University of Plymouth). The gel was stained for protein with Coomassie blue, followed by the monochromatic silver staining method of Wray *et al.* (1981).

6. PREPARATION OF CILIATE ANTIGENS

The following antigens were prepared and stored at -20°C and in liquid nitrogen.

6.1 T.pyriformis trophozoite

T.pyriformis strain 1630/W was used for raising antiserum in rats and sonicated trophozoites used as antigen for ELISA and for electrophoretic analysis. *T.pyriformis* cells, washed free of medium and resuspended in distilled water, were enumerated using a Sedgewick-Rafter counting chamber, and adjusted to 200,000 cells/ml with distilled water. Aliquots, 0.5ml, of cells were frozen at -20°C for subsequent immunisations of rats. Antigen for ELISA and immunoelectrophoresis was prepared from 3 ml cell suspension which was sonicated on ice in an ultrasonic disruptor (MSE Ltd.) using a 3mm tip microprobe. Sonication was performed at 18μm amplitude for a total of 2 min on a 50% cycle (30 sec on; 30 sec off). The sonicate was spun at 11,000*g*, 4 min, to sediment any particulate debris and the supernate retained, leaving a small pellet (< 10μl) which was discarded. Total protein of the soluble sonicate was estimated using the Folin phenol method. The sonicate was aliquoted in 50μl and 100μl volumes and stored frozen (-20°C or liquid N_2) until required.

6.2 C.irritans trophont

C.irritans trophonts were prepared for rabbit immunisation, ELISA antigen, and electrophoretic analysis. Trophonts which had recently exited from host mullet were washed by suspension in several changes of either filter sterilised synthetic seawater or saline (0.85g NaCl/litre). Information regarding isolate type, trophont density, and preparation number, were recorded for each antigen batch.

6.2.1 C.irritans soluble antigen

Soluble trophont antigen was prepared from two separate isolates, CI-CR (preparation 13) and CI-GS (pooled preparations 44/46), both of which had previously been shown to possess similar protein profiles by SDS-PAGE analysis. Antigen CI-GS(44/46) was prepared from 1,400 trophonts diluted in 2 ml ELISA carbonate coating buffer (buffer details in appendix). Trophonts were then sonicated on ice at 18μm amplitude for a total of 6 min, using the MSE ultrasonic disruptor set at a 50% cycle time, as described earlier for *T.pyriformis*. The sonicate was centrifuged at 11,000*g*, 5 min, and the supernate retained, adjusted to 3 ml with ELISA coating buffer, and distributed in 50μl and 100μl aliquots at -20°C and in liquid N_2. Antigen CI-CR(13) was similarly prepared from 2,000 trophonts.

7. PRODUCTION OF MAMMALIAN ANTISERA

The following antisera were raised for use in ELISA and other serological tests:

- Rabbit antiserum to mullet immunoglobulins;
- Rabbit antiserum to *C.irritans* trophonts;
- Rat antiserum to *Tetrahymena pyriformis*.

Laboratory mammals were immunised according to established procedures (Johnstone and Thorpe, 1982; Dresser, 1986), small quantities of antigen being used in accordance with current recommendations (Goding, 1983).

7.1 Rabbit antiserum to mullet immunoglobulins (RABantiMIg)

The mullet Ig "Ultrogel" fraction, adjusted to 500 μg/ml protein in PBS pH 7.6, was emulsified with an equal volume of Freund's complete adjuvant (FCA)(Sigma, USA) and 1.0ml (= 250μg Ig) of the mixture administered subcutaneously at two sites on the hindquarters of a male Dutch rabbit. Booster immunisations, using equivalent quantities of Ig in Freund's incomplete adjuvant (FIA), were similarly administered at 10 days, 25 days, and 30 weeks later. Blood samples were collected pre- and post-immunisation by marginal ear venepuncture, and the serum fraction collected in order to monitor specific antibody levels to mullet Ig by ELISA and immunoelectrophoresis.

7.2 Rabbit antiserum to C.irritans (RABantiCI)

Trophonts of *C.irritans* (CI-CR), released from a primary infection of mullet, were repeatedly washed in sterile saline to remove host material. Five hundred trophonts (≈ 1mg protein) in 0.5 ml sterile saline, were emulsified with an equal volume of FCA and administered to a male Dutch rabbit, according to the method for production of RABantiMIg production, given above. Booster immunisations, each with 500 trophonts in FIA, were similarly administered 14 days, 30 days, and 29 weeks later. Pre- and post-immunisation blood samples were collected for specific antibody monitoring by ELISA.

7.3 Rat antiserum to T.pyriformis (RATantiTP)

Antiserum to *T.pyriformis* was raised in two 300g male CBRI WISTAR rats. Each rat was immunised on day 1 with 25,000 *T.pyriformis* (strain 1630/W) diluted to 1ml with either 1:1 (v/v) FCA (rat 1) or 1:1 (v/v) distilled water (rat 2), administered subcutaneously in the both axillary and inguinal sites. The number of *T.pyriformis* cells was selected to give 50μg protein per immunisation, as recommended for rats by Dresser (1986). A booster immunisation of equivalent numbers of cells in FIA (rat 1) or distilled water (rat 2) was administered by the same route at day 11. Test samples of blood were taken from the tail of pre- and post-immunised rats. Both rats were bled out by cardiac puncture at day 20, immediately following death.

8. PEROXIDASE CONJUGATION OF RABantiMIg

Two millilitres of rabbit antiserum to mullet Ig (RABantiMIg) was linked to horseradish peroxidase using the two step procedure of Avrameas and Ternynck (1971), modified by Dr. A. Voller (Nuffield Laboratories, London, pers. comm.) Briefly, RABantiMIg, purified by ammonium sulphate precipitation and dialysed against sodium chloride, was assayed for protein at 280nm and conjugated with twice its protein weight of horseradish peroxidase (E.C. 1.11.1.7, type V, Sigma Co., product P-8375) in the presence of glutaraldehyde. The immunoglobulin-peroxidase conjugate, diluted in ELISA carbonate buffer (see appendix), was dialysed against PBS and purified by ammonium sulphate precipitation. Dialysed conjugate was mixed with an equal volume of glycerol to facilitate storage at -20°C without freezing. The working dilution for ELISA was determined by titration using the method described in the results, section 3.1.4.

9. SEROLOGICAL ASSAYS

Serological investigations were undertaken using immunoelectrophoresis, ELISA, and immunofluorescence.

9.1 Immunoelectrophoresis of rabbit antisera

Sera from rabbits immunised with mullet Ig or trophonts of *C.irritans* (CI-CR) were immunoelectrophoresed against these antigens, according to standard procedures (Hudson and Hay, 1980). A 1% agarose gel was enriched with 3% PEG 6000 to improve precipitation ("The Binding Site" information leaflet, Birmingham, U.K.). Mullet Ig (≈1 mg/ml protein) and trophont antigen, were spotted onto separate agar wells in 80 x 80 mm glass plates, and electrophoresed at 30mA under constant voltage. Electrophoresis was monitored by the inclusion of a 0.0005% bromophenol blue tracker dye. Central troughs were filled with 180μl of the relevant rabbit antiserum, diluted 1 in 10, and the samples allowed to diffuse overnight. Gels were then cleared in saline, blot dried, and stained with Coomassie blue (Hudson and Hay, 1980; Johnstone and Thorpe, 1982).

9.2 Enzyme-linked immunosorbent assay (ELISA)

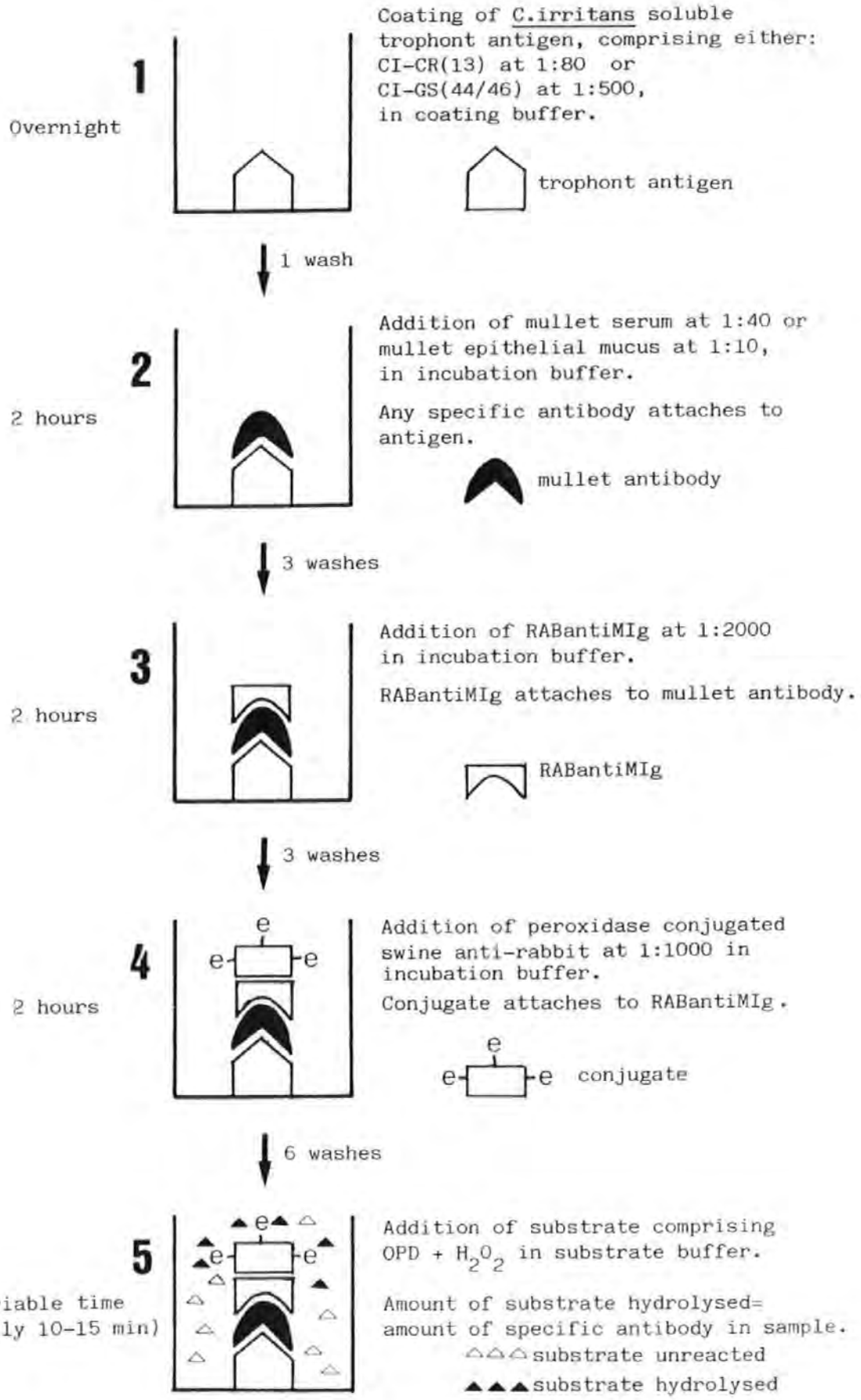
The generalised indirect ELISA of Voller *et al.* (1979) was employed for the measurement of antibodies to antigens adsorbed to a solid phase. Fig. 42 shows the five- stage ELISA procedure for the measurement of fish antibodies, giving the optimised conditions developed here. A four- stage ELISA, which omits the second antibody step (Fig 42, step 3), was used for development of the mammalian (rabbit, rat, mouse) antibody assays. All ELISAs were performed at RT in 96-well flat bottom polystyrene plates (Linbro, U.K.), selected on the basis of their low non-specific

Figure 42

Procedure for the indirect ELISA for the measurement of antibodies in mullet serum and epithelial mucus to trophont antigen of *C.irritans*.

The assay was performed at RT in polystyrene 96 well plates (Linbro, Flow Labs).

Buffer formulations are given in the appendix.


1
Overnight
Coating of C.irritans soluble trophont antigen, comprising either:
CI-CR(13) at 1:80 or
CI-GS(44/46) at 1:500,
in coating buffer.
trophont antigen
1 wash
2
2 hours
Addition of mullet serum at 1:40 or mullet epithelial mucus at 1:10, in incubation buffer.
Any specific antibody attaches to antigen.
mullet antibody
3 washes
3
2 hours
Addition of RABantiMIg at 1:2000 in incubation buffer.
RABantiMIg attaches to mullet antibody.
RABantiMIg
3 washes
4
2 hours
e
Addition of peroxidase conjugated swine anti-rabbit at 1:1000 in incubation buffer.
Conjugate attaches to RABantiMIg.
conjugate
6 washes
5
Variable time
(usually 10-15 min)
Addition of substrate comprising OPD + H_2O_2 in substrate buffer.
Amount of substrate hydrolysed= amount of specific antibody in sample.
substrate unreacted
substrate hydrolysed

binding properties (Burgess, 1988) and high antigen adsorption (Denmark and Chessum, 1978). Horseradish peroxidase and ortho-phenylenediamine (OPD) were selected as enzyme and chromogen, respectively (Voller *et al.*, 1979). ELISA buffers, advocated by Voller *et al.* (1979), are detailed in the appendix. ELISA results were read through-the-well in a photometric plate scanner (Titertek, Flow Labs), linked to a microcomputer loaded with ELISA analysis software.

9.3 Indirect fluorescent antibody test (IFAT)

The IFAT was used for the detection of specific antibodies in mullet and rabbit to theronts of *C.irritans*. Initial difficulties experienced with poor adhesion of theronts to glass microscope slides were overcome by pre-coating with 0.2μm membrane filtered 10% poly-L-lysine (p-L-lys, Sigma Diagnostics) in water (Mazia *et al.*, 1975). Satisfactory yields (10-40 theronts/5mm spot) were achieved using formalin (10%) fixed theronts delivered onto p-L-lys coated slides by Cytospin (Shandon Ltd.), optimised at 300-400 rpm, 10 min. Theront coated slides were washed free of salt in distilled water and dried overnight at RT. Prior to use, the slides were briefly rinsed with PBS followed by 30 min immersion in fresh buffer.

The IFAT procedure was performed at RT as follows. Test sera from rabbit and mullet, each optimally diluted in PBS pH 7.6, were dispensed onto the theront coated slides and incubated for 1-2 h. For fish antibody assays, the secondary antibody comprised a rabbit anti-mullet Ig (RABantiMIg) serum at 1:50 dilution. For both fish and rabbit assays, a commercial fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit Ig (Sigma) was used at 1:80 (according to the manufacturer's recommendations). The conjugate was diluted in PBS containing either 0.1% or 0.04% (w/v) Evans blue (Gurr, England) as counterstain to reduce

non-specific fluorescence. Following each incubation, slides were rinsed in PBS and immersed in fresh buffer for 30 min, in order to remove unbound reagent. Control slides included theronts incubated with PBS only during the serum stage. Preparations were mounted with glycerol mountant ("Citifluor") and observed using epifluorescence from a mercury vapour lamp. Fluorescence in test preparations was visually compared with controls and scored on the increasing fluorescence intensity scale: -; +/-; +; ++; +++.

10. IMMUNOBLOTTING

Polypeptide bands, resolved by SDS-PAGE under reducing conditions, were electroeluted onto nitrocellulose membranes (NC, 0.2μm, Sigma) using a water cooled Midget multiblot apparatus (Pharmacia/LBK Ltd.). The procedure was carried out at a constant 30mA, 18h, in a transfer buffer containing 25mM Tris, 192mM glycine, pH 7.8, and 25% methanol (Johnstone and Thorpe, 1982). Electroeluted gels were stained with Coomassie blue to confirm polypeptide elution. Membranes were prepared for antibody testing using PBS pH 7.6 as diluent. Membranes were first rinsed and blocked in 3% skimmed milk (Tesco Stores Ltd.), 30 min, and divided into tracks. Replicate tracks and molecular weight marker tracks were stained with Amido black (Towbin *et al.*, 1979) to confirm the presence and relative mobilities of the eluted polypeptides. The membranes were washed, 30 min, and immersed into LP2 tubes containing the appropriate antibody stage. Each stage was incubated for 2 h, RT, followed by a 30 min wash. A three stage system was used for antigen probing with mullet antibodies: primary incubation with mullet serum at 1:10 to 1:50, followed by a RABantiMIg at 1:250, and then a swine anti-rabbit peroxidase conjugate (Dako Ltd.) at 1:500. For probing with rabbit antibodies, a two-stage system involved

primary incubation with rabbit serum at 1:250, followed by a swine anti-rabbit peroxidase conjugate, as previously described. For both systems, the final substrate colour reaction comprised incubation of the NC membranes with 0.005% (w/v) diaminobenzidine, 0.003% (v/v) hydrogen peroxide in ELISA citrate phosphate buffer. Colour formation was monitored visually and terminated by washing the membranes under tap water. The presence and molecular weights of stained bands were recorded, with the aid of Amido black controls. Immunoblots were stored dry in the dark.

EXPERIMENTS AND RESULTS

1. ISOLATION AND CHARACTERISATION OF MULLET IMMUNOGLOBULIN

The Ultrogel elution profile of mullet whole serum is shown in Fig. 43. Electrophoretic analysis under reducing conditions of selected Ultrogel fractions indicated Ig within the second major protein peak eluted (= fractions 37 to 40, inclusive). Major polypeptide bands were obtained at ≈67-68kDa and ≈27.5-29kDa positions, indicating heavy and light chain components, respectively. Ig fractions 37 to 40 were pooled (= 8 ml total) and concentrated to 2.2 ml by polysulphone membrane centrifugation. Protein estimation of the pooled Ig concentrate was 0.99mg/ml, as measured by 280nm absorption; a value of 1.0 mg/ml was assumed. Aliquots of the Ig were stored at -20°C or in liquid N_2.

2. CHARACTERISATION OF RABBIT ANTISERA

2.1 Immunoelectrophoresis

The RABantiMIg and RABantiCI antisera were each reacted by immunoelectrophoresis with the soluble sonicate trophont antigen of *C.irritans* (CI-CR(13)) and the mullet Ig Ultrogel preparation. The results (Fig. 44) reveal a single precipitin arc between RABantiMIg and the immunising mullet Ig antigen, the low mobility of the arc closely resembled that for human IgM (Roitt, 1980). No precipitation was observed between the RABantiMIg and the trophont antigen. No reaction was observed between RABantiCI and either the immunising trophont antigen or mullet Ig.

Figure 43

Elution profile of mullet whole serum on Ultrogel AcA22.

Pooled serum (3ml) was eluted in PBS, pH 7.6. Fractions of 2ml were collected at a flow rate of 4ml/h, at RT.

Stippled area shows the immunoglobulin fraction (fraction numbers 37-40).

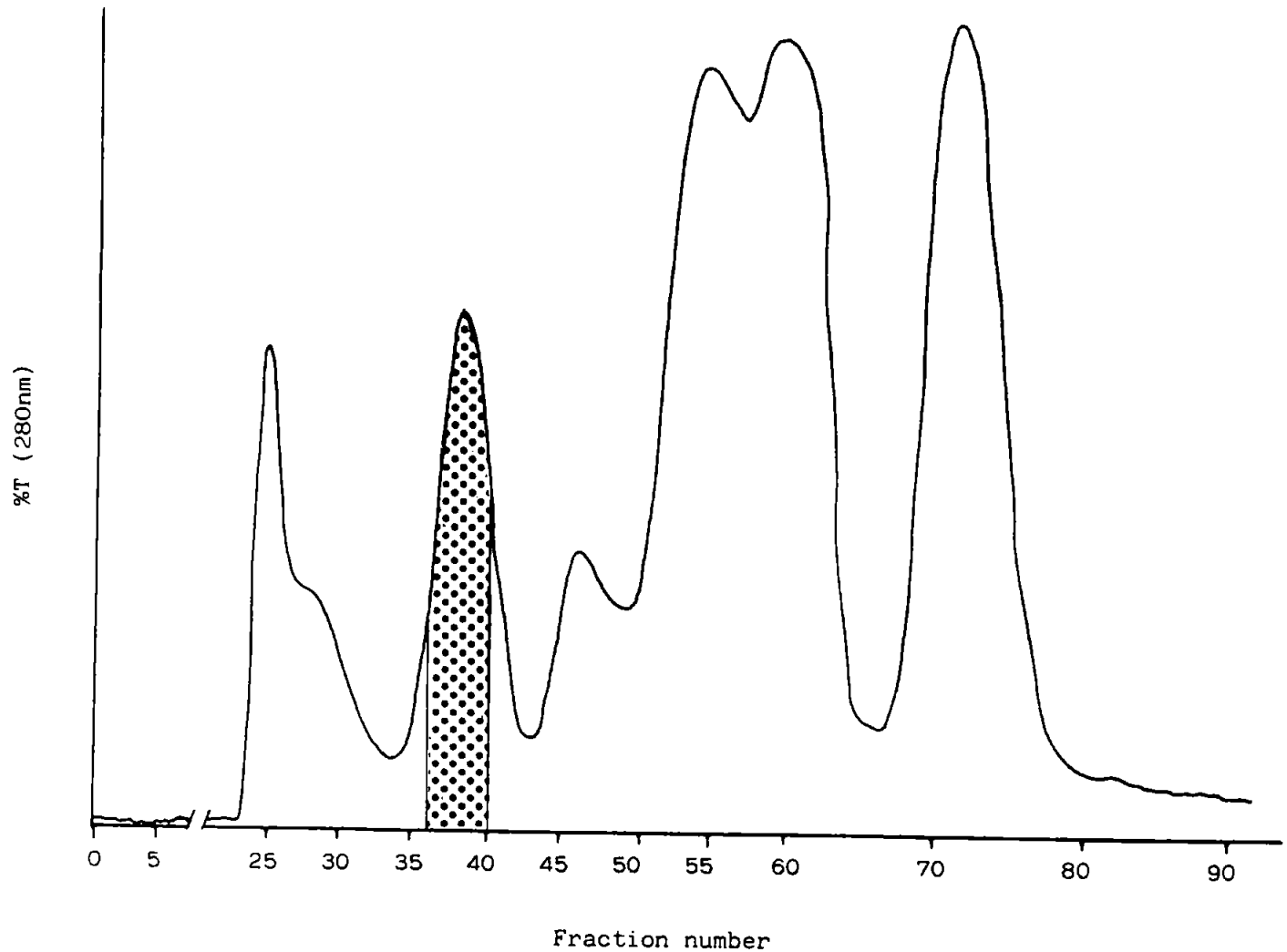
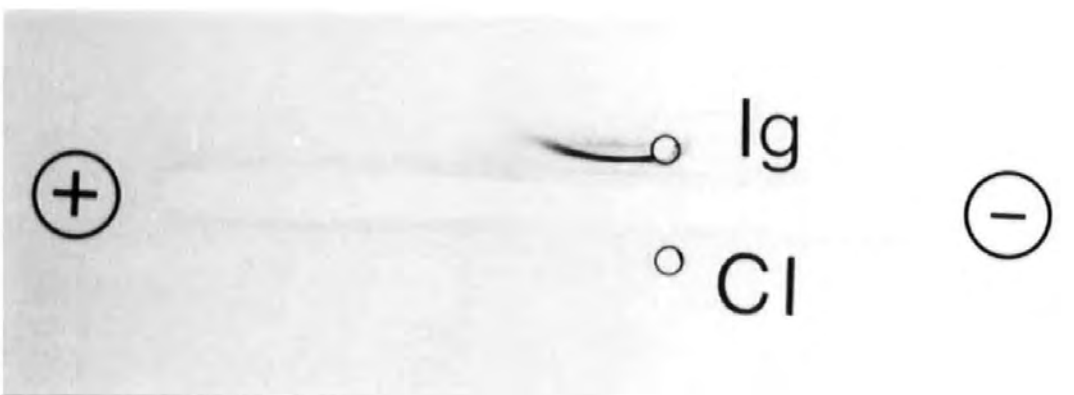

%T (280nm)
0
5
25
30
35
40
45
50
55
60
70
80
90
Fraction number

Figure 44

Analysis of rabbit antiserum to mullet immunoglobulin (RABantiMIg) by immunoprecipitation.

Precipitation occurs with mullet immunoglobulin (Ig) but not *C.irritans* trophonts (CI).


Ig
Cl

2.2 Immunoblotting

Immunoblotting of mullet Ig followed by incubation with RABantiMIg showed recognition of the H chain (around 67-68kDa) and weak recognition of the L chain (28-29kDa). Immunoblots revealed recognition by RABantiMIg of a faint 67-68kDa band in the CI-CR(13) trophont antigen.

3. ELISA

Indirect ELISAs were developed for the detection and quantification of the following:

i) Mullet and mammalian antibodies to *C.irritans*;

ii) Mullet and mammalian antibodies to *T.pyriformis*;

iii) Mammalian antibodies to mullet immunoglobulins.

3.1 Optimisation of assays

3.1.1 C.irritans antigen density

Soluble sonicate preparations of two trophont isolates, CI-CR(13) and CI-GS(44/46), were used separately in succession as antigens in ELISA. Optimal antigen density for each trophont preparation was determined by two-fold titration against rabbit sera at 1:100 dilutions, comprising a negative ("N") serum from a pre-immunised rabbit and a presumptive specific antibody positive ("P"= RABantiCI) serum from the same animal at 5 days following a third immunisation with *C.irritans* trophonts. The conjugate stage comprised a peroxidase labelled swine anti-rabbit immunoglobulin (Dakopatts, Denmark, product P217), diluted to 1:1000. The enzyme-substrate (E-S) reaction was allowed to proceed until moderate colour had developed in the positive serum wells (ca. 12 min). The results (Fig. 45) confirm the

Figure 45

ELISA titration of trophont antigens of *C.irritans*, CI-CR(13) and CI-GS(44/46).

□–□ CI-CR(13)

■–■ CI-GS(44/46)

● no serum blank

P = seropositive rabbit serum

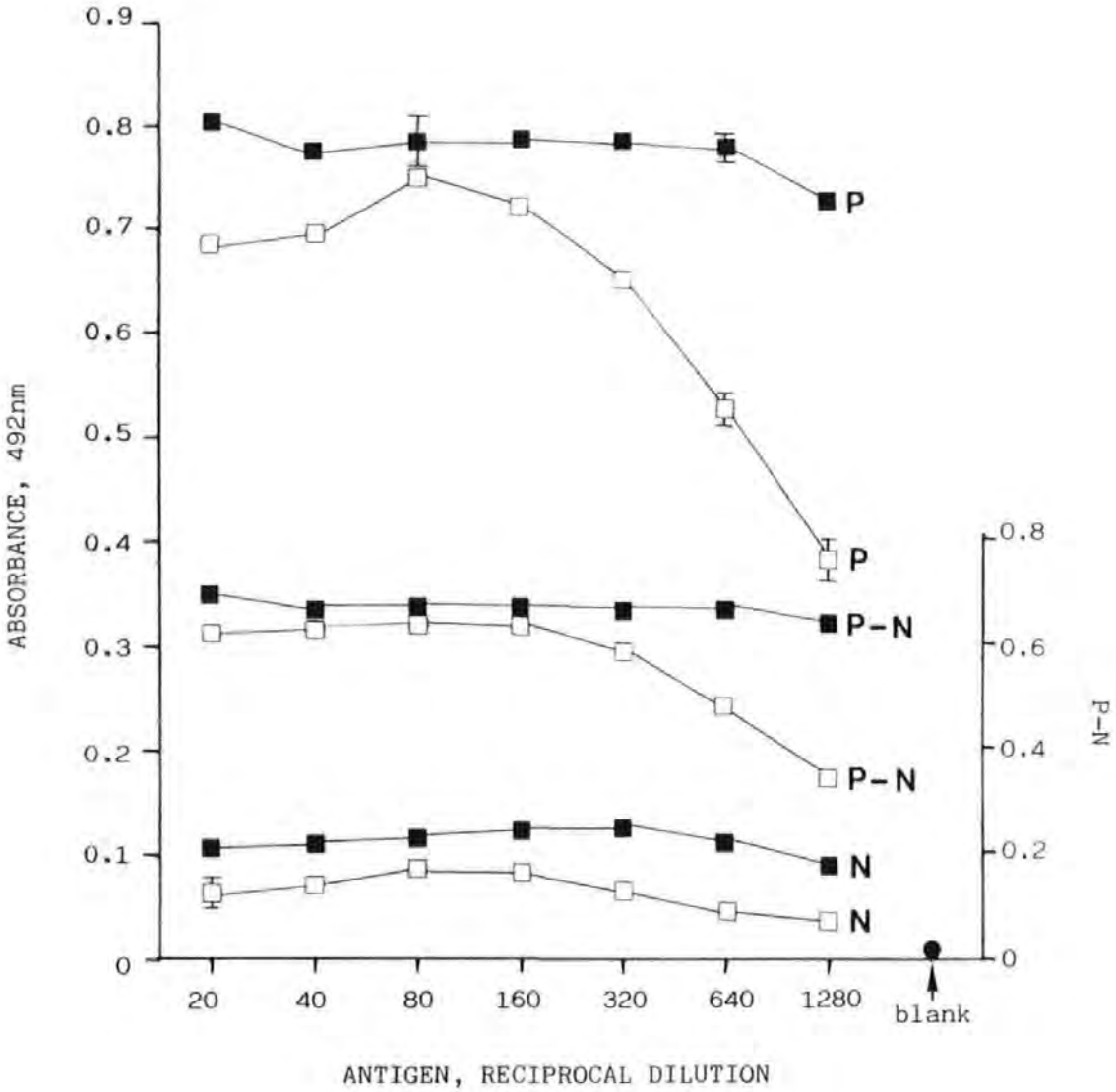
N = seronegative rabbit serum

P-N = $absorbance_{positive}$ minus $absorbance_{negative}$

(P-N = specific antibody activity)

} values on right hand axis

Vertical bars show absorbance range.


0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE, 492nm
P
P
P-N
P-N
N
N
0.8
0.6
0.4
0.2
0
P-N
20
40
80
160
320
640
1280
blank
ANTIGEN, RECIPROCAL DILUTION

seropositivity of the RABantiCI serum ("P") which gave absorbance values considerably higher than those of the negative serum ("N") collected before immunisation. At dilutions of 1:320 and greater, the CI-CR(13) antigen appeared to be limiting, causing a reduction in the specific antibody activity of the RABantiCI serum. The slight fall in absorbance values at 1:20 and 1:40 dilutions of CI-CR(13) antigen may indicate a prozone ("hook") effect (Edwards, 1985) reflecting antigen over-coating causing subsequent detachment along with bound antibody (McLaren *et al*, 1981). An intermediate CI-CR(13) dilution of 1:80 was selected. Antigen CI-GS(44/46) was more reactive than CI-CR(13) in ELISA, with antigen limiting at above 1:640 dilution; an optimal dilution of 1:500 was therefore chosen. Positive minus negative (P-N) values, used as an indicator of test performance, were very similar for both *C.irritans* preparations over their non-limiting dilution ranges. A control blank, in which the serum stage was substituted for buffer only, showed no non-specific binding by the conjugate to either antigen. The optimised antigen densities were used for all subsequent ELISAs measuring specific antibody to *C.irritans* in mullet and mammals, including MABs.

3.1.2 RABantiCI dilution

The RABantiCI serum (P) and negative control rabbit serum (N) were optimised at 1:500, being the dilution giving maximal P-N values using the CI-CR(13) antigen. These two rabbit sera were used as reference controls in ELISAs for measurement of mullet antibodies to *C.irritans*.

3.1.3 Mullet immunoglobulin antigen density

Optimisation of antigen coating density was assessed by two-fold titration

against the RABantiMIg and pre-immunised rabbit sera, both diluted to 1:100, following the above method for trophont antigens. The results indicated limiting Ig density at 1:1280 (≈ 0.75μg protein/ml); therefore an optimum of 1:640 (≈ 1.5μg protein/ml) was chosen for all subsequent ELISAs. At optimal antigen dilution, a P-N value of >0.8 absorbance units was obtained using the rabbit sera, with negligible (<0.02 units) non-specific binding to antigen by the negative rabbit serum.

3.1.4 RABantiMIg dilution

The RABantiMIg was optimised at 1:2000 dilution for use as an antiserum reagent for the detection of mullet antibodies to *C.irritans* (Fig. 42, step 3). This gave moderate colour formation (ELISA absorbance >0.3 units) in trophont coated wells containing a seropositive mullet serum after an appropriate E-S time of ca. 15 min.

3.1.5 Rat anti-T.pyriformis ELISA

The *T.pyriformis* sonicate antigen, TP(9), was optimised at 10μg protein/ml and the rat sera at 1:200 dilution, based on optimisation methods described earlier for the *C.irritans* ELISA. A commercially prepared peroxidase labelled goat anti-rat immunoglobulin antiserum (Dakopatts, Denmark, product P162) was used at 1:1000, according to manufacturer's specifications. Higher levels of antibody activity were present in the serum from the rat immunised with TP(9) plus adjuvant, as compared with that from the rat immunised with TP(9) alone, the former serum being used for further serological studies. Non-specific antibody binding to TP(9) antigen was negligible (<0.1 absorbance units), as measured using sera from two non-immunised rats.

4. SEROLOGICAL CROSS-REACTIVITY BETWEEN CILIATES

Evidence for the presence of shared or structurally similar antigens between the three ciliates, *C.irritans*, *I.multifiliis*, and *T.pyriformis*, was investigated by cross-reactivity tests using RABantiCI and RATantiTP antisera.

The following soluble sonicate antigens were used: *C.irritans* trophont CI-CR(13), *T.pyriformis* trophozoite TP(9), and *I.multifiliis* trophont (optimised at 10μg protein/ml). ELISAs were performed under their respective optimised conditions, described earlier. Table 17 gives the serological results of the antigen-antiserum combinations tested. Specific antibody activity values were obtained by subtracting the mean absorbance value of the negative serum from that of the positive (P-N). The results show high specific antibody activity between the RABantiCI, RATantiTP and their respective immunising antigens. No serological cross-reactivity was found between *T.pyriformis* and either *C.irritans* or *I.multifiliis*. However, slight activity was observed between RABantiCI and the *I.multifiliis* trophont antigen, indicating antigens(s) common to *C.irritans* and *I.multifiliis*. Homologous reactivity to the *I.multifiliis* antigen was not evaluated, due to lack of antiserum.

5. INVESTIGATION INTO THE CROSS-REACTIVITY BETWEEN RABantiCI AND MULLET IMMUNOGLOBULIN

Preliminary ELISA studies revealed cross-reactivity between the RABantiCI and mullet Ig. The extent and underlying causes of such cross-reaction were investigated by ELISA. Antibody activity was measured in the RABantiCI and a negative control rabbit serum, each titrated against the following optimally coated antigen preparations: CI-CR(13), mullet Ig, and whole serum from a healthy mullet, the latter optimised at 1:500 dilution. The results (Fig. 46) show specific (P-N)

Table 17

Serological reactivity by ELISA between antigens prepared from trophonts of *C.irritans* and *I.multifiliis* and trophozoites of *T.pyriformis*, using rabbit and rat antisera.

Serum		Antigen		
		C.irritans CI-CR(13) (trophont)	*I.multifiliis* (trophont)	*T.pyriformis* TP9 (trophozoite)
RABBIT antiCI	P-N	**0.61**	0.16	0.03
	P	**0.63 (0.59-0.66)**	0.19 (0.18-0.20)	0.10 (0.08-0.12)
	N	**0.02 (0.01-0.04)**	0.03 (0.02-0.03)	0.07 (0.06-0.08)
RAT antiTP	P-N	0.00	0.02	**0.77**
	P	0.05 (0.04-0.07)	0.05 (0.03-0.06)	**0.85 (0.82-0.91)**
	N	0.06 (0.05-0.09)	0.03 (0.01-0.04)	**0.08 (0.08-0.11)**

Values are ELISA absorbance at 492nm

P= positive antiserum.
N= negative control serum from unimmunised animal.
P-N = positive minus negative absorbance (= specific antibody activity).

P and N values represent mean and (range), n=3.

Bold cells show homologous activity between antiserum and respective antigen.

Figure 46

ELISA titration of RABantiCI against trophont antigen of *C.irritans*, mullet Ig, and mullet whole serum.

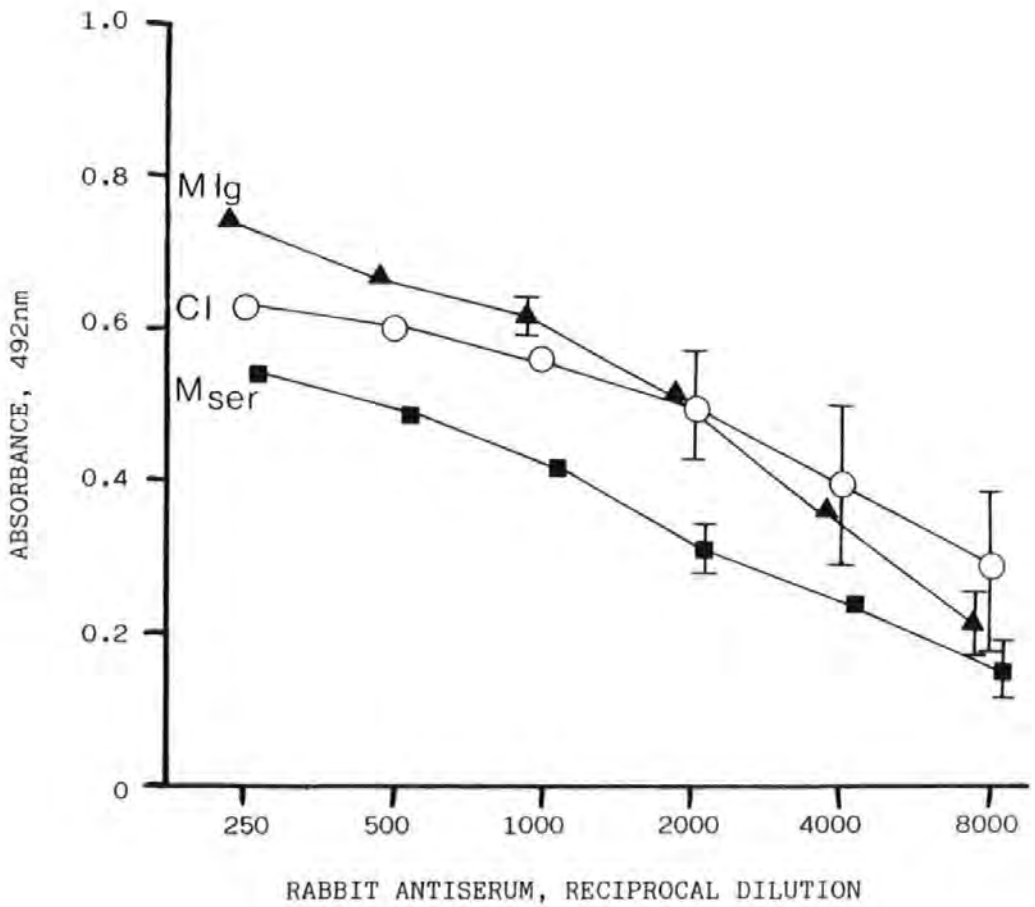
Results show specific (P-N) antibody activity

○ *C.irritans* trophont antigen (CI).

▲ mullet Ig (AcA22 preparation) (MIg).

■ mullet whole serum (M_{ser}).

Vertical bars show absorbance range (n=2).


1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE, 492nm
M Ig
Cl
M ser
250
500
1000
2000
4000
8000
RABBIT ANTISERUM, RECIPROCAL DILUTION

antibody activity in the RABantiCI to the immunising *C.irritans* CI-CR(13). Significant cross-reactivity was observed between the RABantiCI and both mullet Ig and whole serum. There was no significant reactivity between the negative rabbit serum and any of the three antigens (absorbance values ≤ 0.03 units; not shown).

5.1 Selective absorption of RABantiCI

Further investigations of the cross-reactivity between RABantiCI and mullet Ig were undertaken using selective absorption (quenching) procedures. Three 100μl aliquots of RABantiCI were each diluted 1:1 (v/v) with either: 50μl normal mullet whole serum + 50μl saline; 50μl mullet Ig Ultrogel fraction (1 mg protein/ml) + 50μl saline; or 100μl saline (= unabsorbed control). Serum from the pre-immunised rabbit, diluted 1:1 (v/v) with saline, was also tested as a negative control. The serum preparations were incubated with gentle agitation for 1 h at RT before testing by ELISA. Titration profiles of the four preparations reacted with CI-CR(13) antigen and mullet Ig are given in Figs. 47 and 48, respectively. Comparison of the titration profiles between absorbed and saline control antisera showed no significant differences in antibody activity to *C.irritans* (Fig. 47), indicating that a significant proportion of antibody activity was directed to the parasite. However, absorption had a marked effect in reducing antiserum activity to mullet Ig (Fig. 48) with antibody levels ranked as: non-absorbed (highest) > mullet Ig absorbed > mullet serum absorbed. Greater absorption using mullet whole serum as compared with the Ig fraction may have reflected quantitative differences with higher levels of Ig in the serum as compared with the purified fraction. A qualitative difference may be partly the cause, with antigenic components present in serum, but not the Ig fraction, to which a proportion of the antibody repertoire is directed. Absorption with mullet

Figures 47 and 48

Effects of absorption of RABantiCI with mullet serum or mullet Ig on antibody activity against trophont antigen of *C.irritans* or mullet Ig, by ELISA.

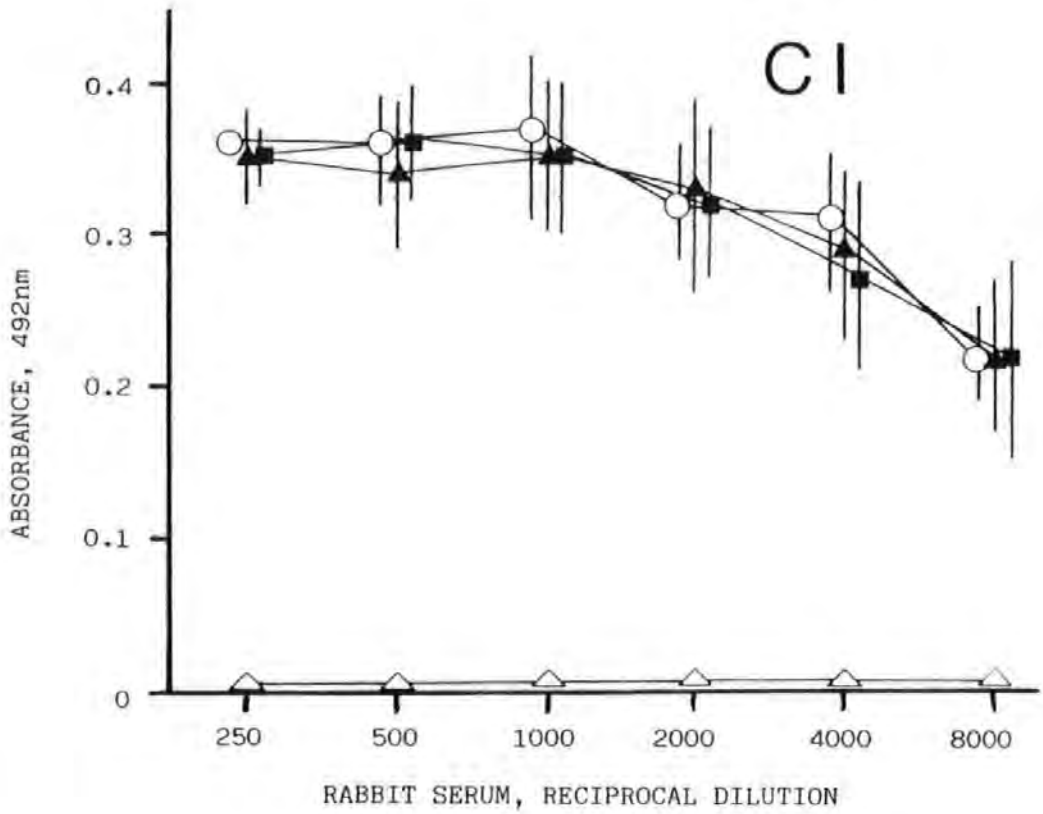
Figure 47 (upper)
C.irritans antigen.

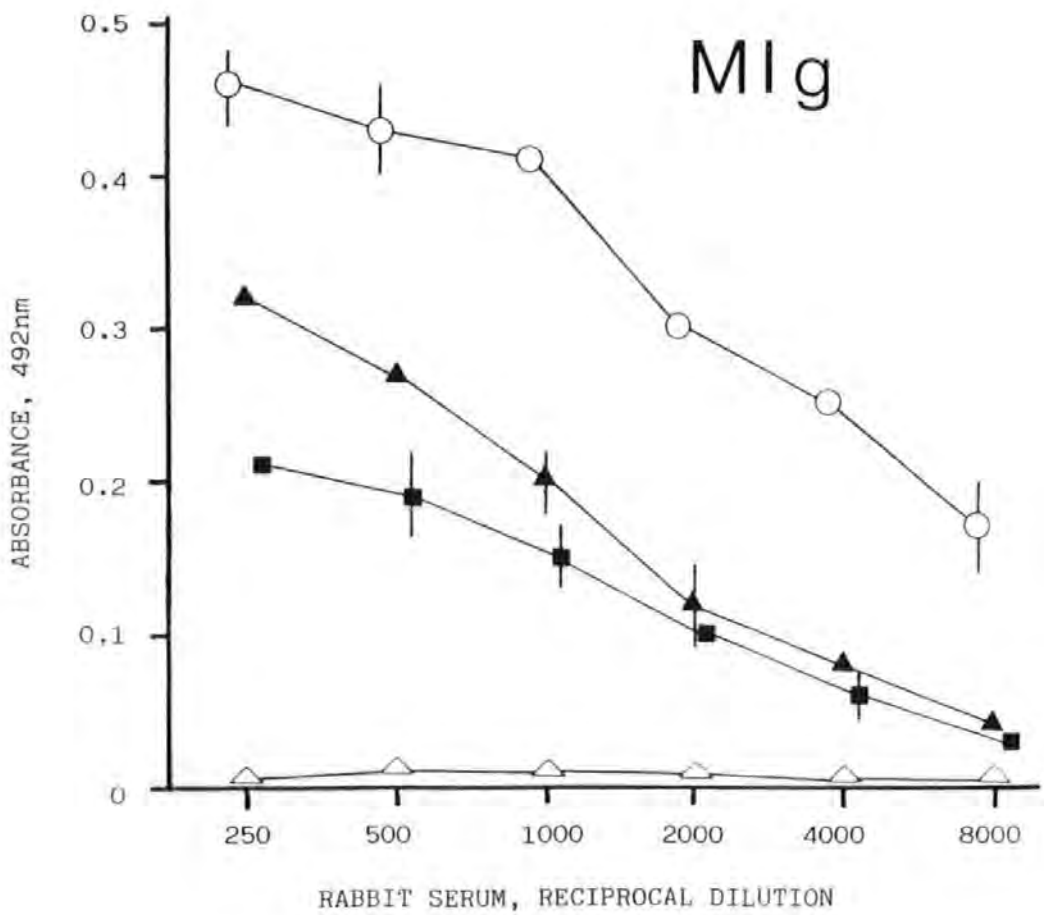
Figure 48 (lower)
Mullet Ig antigen.

Both figures:

○ RABantiCI, non-absorbed.
▲ " , absorbed with mullet Ig.
■ " , absorbed with mullet serum.
△ negative control rabbit serum, non-absorbed.

Vertical bars show mean and range (n=2).


CI
0.4
0.3
0.2
0.1
0
ABSORBANCE, 492nm
250
500
1000
2000
4000
8000
RABBIT SERUM, RECIPROCAL DILUTION


MIg
0.5
0.4
0.3
0.2
0,1
0
ABSORBANCE, 492nm
250
500
1000
2000
4000
8000
RABBIT SERUM, RECIPROCAL DILUTION

serum reduced antibody activity to the Ig antigen by 0·15 to 0·25 absorbance units over the titration range; complete absorption of rabbit antibody activity to mullet Ig was not achieved. The use of higher proportions of absorbent in subsequent studies did not further reduce anti-Ig activity (results not shown).

Based on these results, absorption of the RABantiCI with mullet Ig was routinely performed in subsequent serological studies in order to reduce cross-reactivity with host contaminants.

6. ANTIBODY RESPONSES IN THE MULLET TO C.IRRITANS

Serological responses to C.irritans in naturally infected or immunised mullet were investigated with the aid of ELISA, IFAT, and theront agglutination assays.

6.1 ELISA

The optimised assay for the measurement of specific antibodies to *C.irritans* in serum and epithelial mucus, was used (Fig. 42).

6.1.1. Antibody response in serum following immunisations with C.irritans trophonts plus adjuvant

A single adult mullet (≈30cm length), maintained at RT, was immunised against *C.irritans* by intraperitoneal injection with trophonts, administered on 5 separate occasions over a period of 141 days. The fish received 130-250 trophonts for the first 3 immunisations, this being increased to 3,000 trophonts for the final two immunisations. Freund's complete adjuvant was used for the first immunisation, and Freund's incomplete adjuvant for the remainder. Serum samples, collected before and during the immunisation programme, were each tested in duplicate by ELISA against

the CI-GS(44/46) trophont antigen. A panel of negative control sera, collected from 5 mullet not exposed to *C.irritans*, were simultaneously tested to determine the ELISA absorbance value for seropositivity, based on mean plus 3 standard deviations (Balfour and Harford, 1990). Longitudinal serological response in the mullet to i.p. administered trophonts is shown in Fig. 49, together with the times for immunisations (numbered arrows); with the °1 immunisation at day 1. Seropositivity was set at ≥0.105 absorbance units, based on the negative control results (mean absorbance = 0.048; 3SD = 0.057; n = 5). The experimental fish remained seronegative for at least 20 days following primary immunisation. Conversion to seropositive occurred sometime between days 20 and 56, an interval encompassing the secondary immunisation. Antibody levels elevated to around 0.4 absorbance units. Increased doses of parasite antigen, administered at the °4 and °5 immunisations, failed to further elevate specific antibody levels. The serum collected on day 400 was negative (≈260 days after the final immunisation).

6.1.2 Antibody response in serum and epithelial mucus following exposure to C.irritans theronts

Five mullet (47-77g), A-E, were immunised against *C.irritans* (CI-GS), each fish being exposed to 15,000 theronts on two separate occasions, 21-22 days apart. At the second exposure, 3 smaller mullet (4.2-5.1g), naive to *C.irritans*, were simultaneously exposed to 5,000 theronts from the same pool, to check for parasite viability. The percentage number of parasites establishing infection (%PEI) was determined for each of the 5 fish following primary and secondary exposures, although fish B died during the second infection. Sera and epithelial mucus samples were collected from fish A-E before and after exposure to theronts, and tested by ELISA at 1:40 (sera)

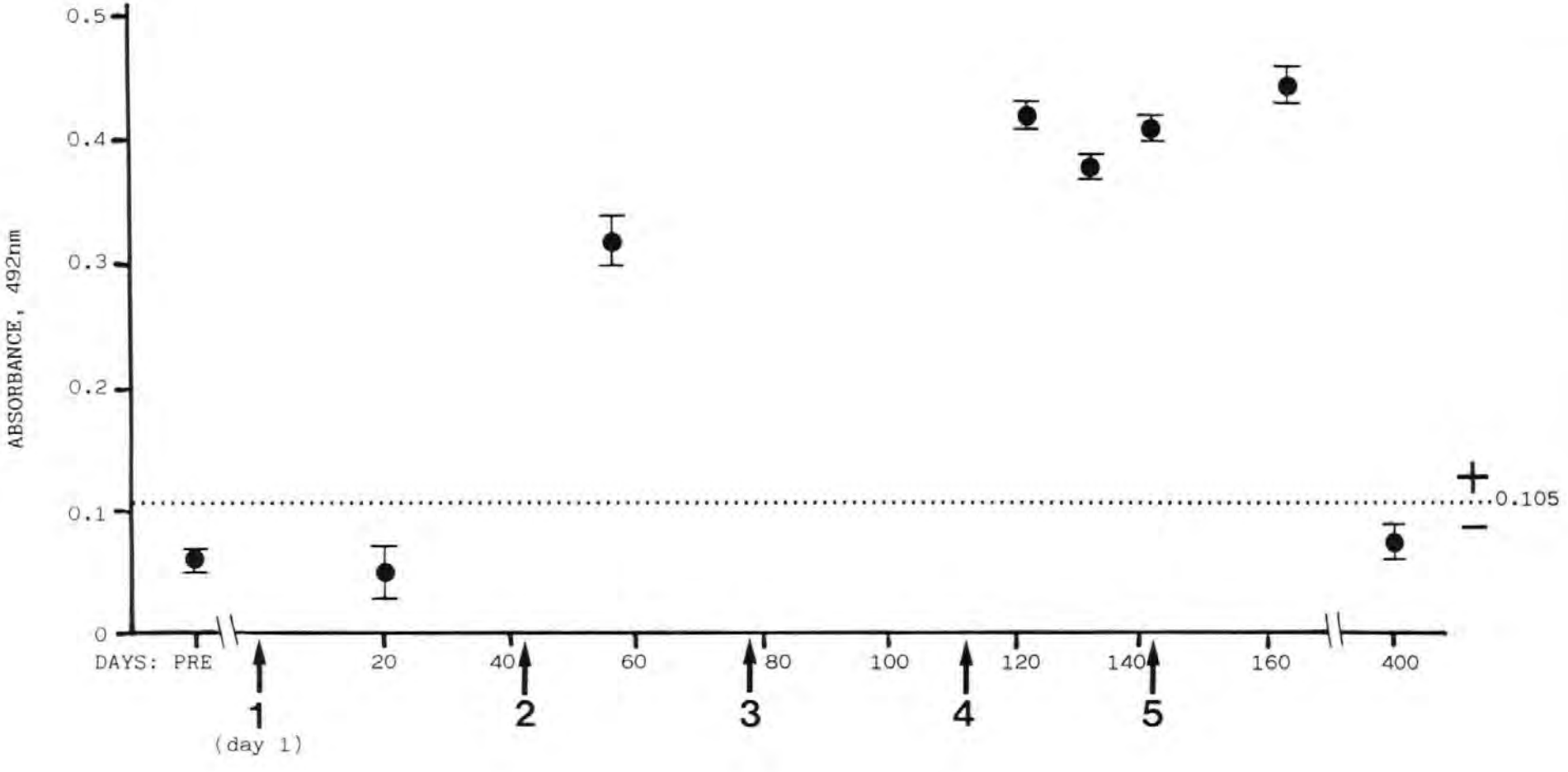
Figure 49

Serum antibody response of mullet to trophonts of *C.irritans* administered by i.p. immunisations, measured by ELISA.

Vertical bars show mean and range (n=2).

Arrowed numbers show immunisations.

Seropositive set at ≥ 0.105 absorbance units, based on mean plus 3SD of negative controls (n=5).


ABSORBANCE, 492nm
0.5
0.4
0.3
0.2
0.1
0
0.105
+
−
DAYS: PRE
20
40
60
80
100
120
140
160
400
1
(day 1)
2
3
4
5

and 1:10 (mucus) dilutions against trophont antigen CI-GS(44/46). Samples were also collected from control mullet, #1 and #2 (59g and 73g), naive to *C.irritans*. A panel of 8 normal mullet sera were also tested by ELISA in order to establish a level for seropositivity, according to the method given earlier.

The results (Fig. 50) indicate that fish A,C,D, and E sustained a reduced parasite burden following the second exposure, with a lower percentage of parasites establishing infection as compared with naive controls. No fish, however, was fully protected against a second exposure. Viability of the theronts used for the second exposures was confirmed by infecting 3 naive control mullet, which sustained a median %PEI of 7.24 (range= 5.94 to 9.70). Fig. 51 gives the serum antibody response to *C.irritans* antigen in the five exposed (A to E) and 2 control (1 and 2) mullet. Seropositivity was set at ≥0.095 absorbance units (mean absorbance of controls= 0.050; 3SD = 0.045; n=8). The time scale (X axis) is approximately the same for both figures, allowing temporal comparison between %PEI values and antibody levels. Rising antibody levels were observed in 2 mullet (C,E). Mullet C seroconverted to positive by day 14 following the primary exposure to theronts and before the second exposure. Mullet E seroconverted to positive by day 27, which was 5 days after the second exposure. Mullet A and D remained seronegative throughout the experiment, despite these fish sustaining reduced parasite burdens following secondary exposure. No specific antibodies to *C.irritans* were detected in the epithelial mucus samples of fish following either primary or secondary exposures at days 5 and 27-28, respectively (absorbance values ≤ 0.01).

Figure 50

Percentage parasites establishing infection (%PEI) in mullet following primary and secondary exposures to theronts of *C.irritans*.

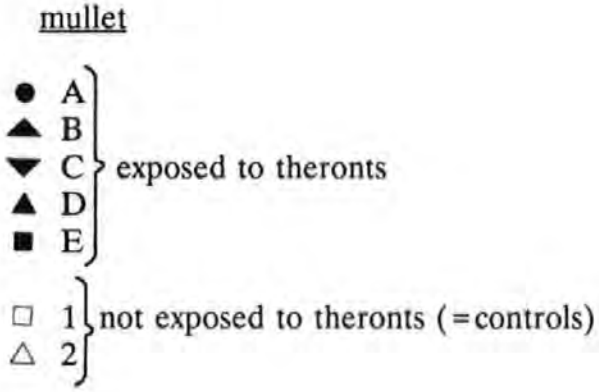
● Controls: median %PEI value for 3 mullet naive to *C.irritans* following primary exposure.

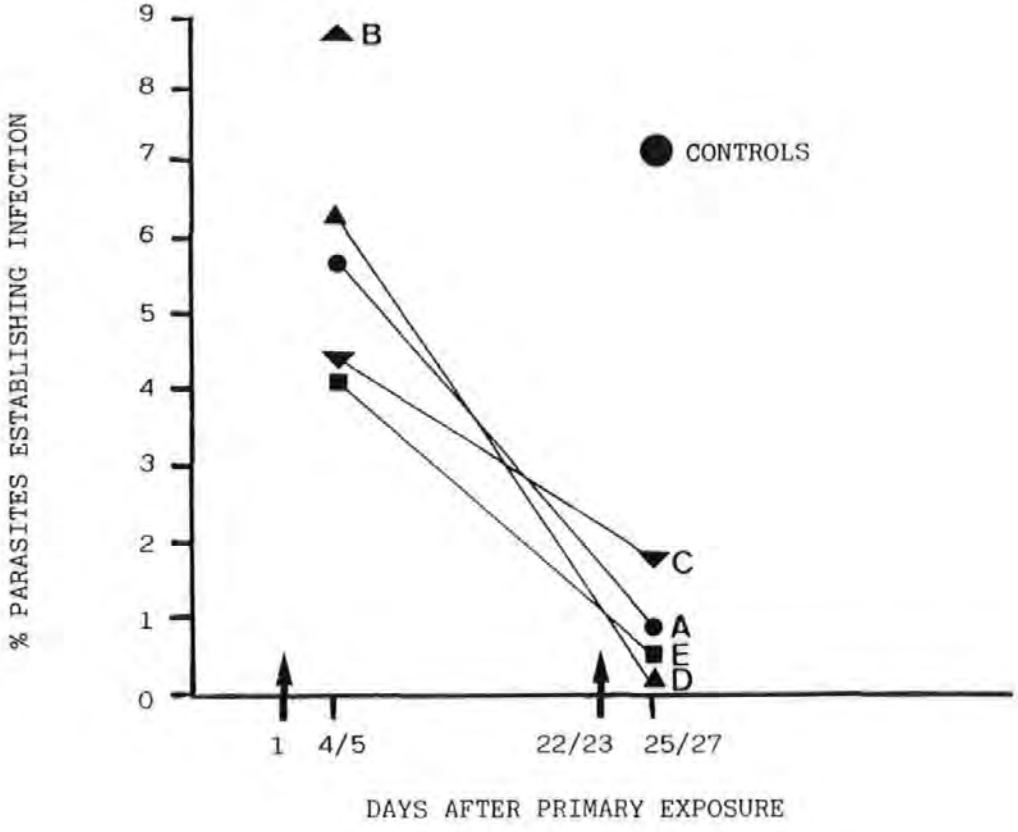
Figure 51

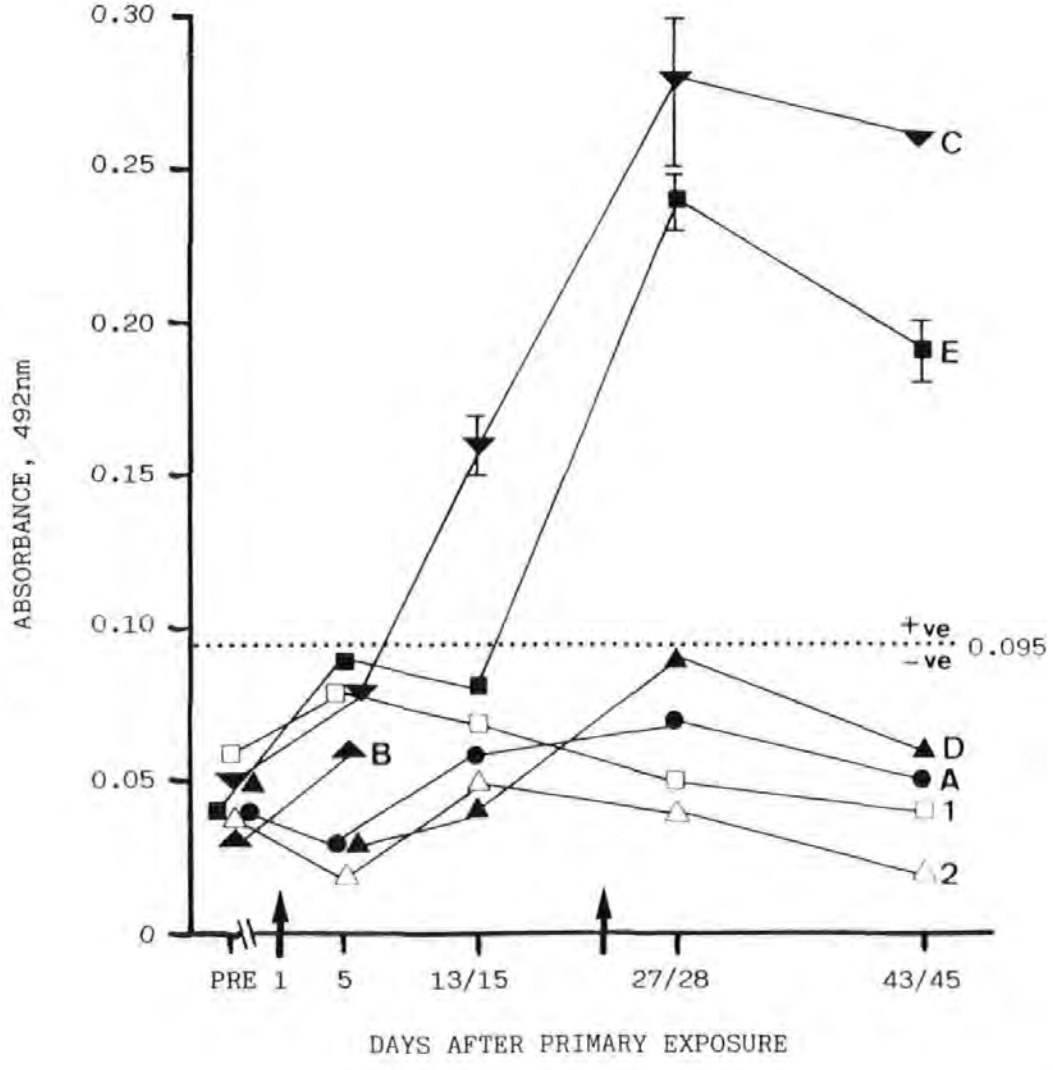
Serum antibody response to trophont antigen of *C.irritans* in mullet following primary and secondary exposures to theronts, measured by ELISA.

Both figures:

Arrows show times of exposures to theronts.




% PARASITES ESTABLISHING INFECTION
9
8
7
6
5
4
3
2
1
0
B
CONTROLS
C
A
E
D
1
4/5
22/23
25/27
DAYS AFTER PRIMARY EXPOSURE


ABSORBANCE, 492nm
0.30
0.25
0.20
0.15
0.10
0.05
0
C
E
+ve
-ve
0.095
B
D
A
1
2
PRE
1
5
13/15
27/28
43/45
DAYS AFTER PRIMARY EXPOSURE

6.1.3 Investigation for possible inhibitory effects of epithelial mucus on specific antibodies to C.irritans

The absence of specific antibodies in the epithelial mucus of mullet immunised against *C.irritans* prompted further investigations to eliminate possible inhibitory factors such as antibody degradation during storage or masking in ELISA.

Epithelial mucus was obtained from a mullet, 5 days following a secondary exposure to *C.irritans* and from a control mullet naive to the parasite. Each mucus sample was divided into two aliquots and each diluted to 1:10 in ELISA incubation buffer. Aliquots were then seeded with one of the following test antibodies to *C.irritans*: a murine MAB ("MABantiCI", see Chapter 7) diluted 1:10, and a seropositive serum from a mullet i.p. immunised with *C.irritans*, diluted 1:40. Negative antibody controls comprised monoclonal antibody medium and serum from a mullet naive to *C.irritans*, each appropriately diluted in incubation buffer. Reference antibody samples not seeded in mucus comprised the MABantiCI and mullet immune serum diluted in incubation buffer only. All samples were stored for 7 days at -20°C before testing by ELISA. The results (Fig. 52) shows no inhibitory effects of epithelial mucus on specific antibody activity to *C.irritans* in either the MAB or mullet serum samples, thereby providing no evidence of an inhibitory effect of mucus on specific antibodies to *C.irritans*.

6.2 IFAT

The IFAT was used to determine specific antibody activity to whole theronts of *C.irritans* (CI-HT) in mullet following natural infection and in mullet and a rabbit following i.p. injections with whole trophonts.

Figure 52

Effect of antibody activity to *C.irritans* in mullet serum and murine monoclonal antibody following cryostorage and ELISA testing in the presence of mullet epithelial mucus.

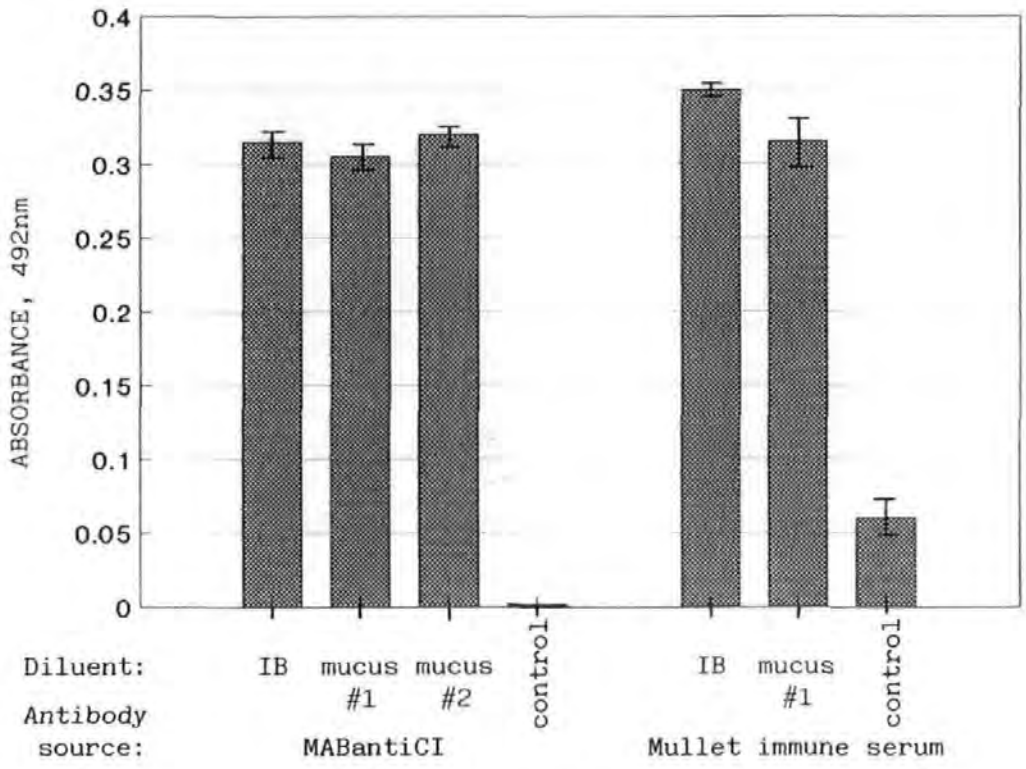
Vertical bars show absorbance range (n=2).

MABantiCI = murine monoclonal antibody to *C.irritans* trophont antigen.

Mucus #1 from mullet naive to *C.irritans*.

Mucus #2 from mullet exhibiting partial protection following secondary exposure to *C.irritans*.

Negative controls comprised monoclonal culture medium and serum from a non-exposed mullet, each diluted in incubation buffer.


0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
ABSORBANCE, 492nm
Diluent:
IB
mucus #1
mucus #2
control
IB
mucus #1
control
Antibody source:
MABantiCI
Mullet immune serum

6.2.1 Measurement of specific antibodies to C.irritans in the rabbit

Preliminary evaluation of the IFAT was made using RABantiCI and the pre-immunised rabbit sera as control, tested against formalin fixed theronts. Theronts fluoresced strongly with the RABantiCI antiserum at all dilutions from 1:10 to 1:40 (Table 18). The results indicated common antigens between the test theronts used as antigen and the trophonts to which the antiserum had been raised. Fluorescence appeared restricted to the surface of the theront including the buccal region. For any given test and serum dilution, the intensity of fluorescence was similar amongst all theronts. Non-specific fluorescence levels were acceptable, being absent or weak in the pre-immune rabbit serum and PBS controls.

6.2.2 Investigation for specific antibodies to C.irritans in mullet

Serum samples from mullet immunised against *C.irritans* by natural infection and by i.p. injection of trophonts were tested against formalin fixed theronts, sera being optimised at 1:20 for IFAT; lower dilutions giving high levels of non-specific fluorescence. Sera from mullet naive to *C.irritans* were used as controls. Serum samples from all but one exposed mullet were seropositive to *C.irritans* by ELISA. The results (Table 19) show no clear resolution in intensity of theront fluorescence between sera from exposed and non-exposed fish. Furthermore, there was no overall agreement between ELISA results and fluorescence intensity. Results of controls indicated that the high levels of non-specific fluorescence obtained with the negative sera was not caused by antigen binding with either the second antibody (RABantiMIg) or the FITC conjugate. The IFAT was unable to demonstrate a specific antibody response to the theront stage of *C.irritans* in any mullet serum tested.

Table 18

Serum antibody activity to *C.irritans* theronts in immunised and control rabbits, by IFAT.

Rabbit serum	Serum dilution		
	1:10	1:20	1:40
RABantiCI	+++ ; +++	+++ ; ++	+++ ; ++
Control[1]	+/- ; +/-	+/- ; -	+/- ; -
None[2]	- ; -		

Results based on 2 replicate tests.

[1] From a rabbit not exposed to *C.irritans*.

[2] PBS only.

Table 19

Serum antibody activity to theronts of *C.irritans* in mullet.

Fish identity #	Exposure status to *C.irritans*	ELISA result (absorbance value)	Fluorescence (replicate tests)
1	non-exposed	- (0.07)	+ ; +
	immunised[3]	+ (0.32)	+ ; +
	immunised[4]	+ (0.42)	++ ; ++
2[1]	infected[5]	- (0.07)	+ ; +
3[2]	infected[5]	+ (0.28)	+ ; +/-
	infected[6]	+ (0.26)	+ ; +
4	non-exposed	- (0.04)	+ ; ++
5	non-exposed	- (0.07)	+/- ; +/-
6	non-exposed	not done	+ ; +
7	non-exposed	not done	+ ; +

Controls	
No serum	- ; -
No RABantiMIg	- ; -
No conjugate	- ; -

[1] Fish "A" in Figs. 50 and 51.

[2] Fish "C" in Figs. 50 and 51.

[3] 14 days following 2° i.p. immunisation with trophonts.

[4] 9 days following 4° i.p. immunisation with trophonts.

[5] 5 days following 2° exposure to theronts.

[6] 22 days following 2° exposure to theronts.

6.3 Immunoblotting

Immonoblotting of SDS-PAGE separated trophonts of *C.irritans* (CI-GS(44/46)) with serum from mullet exposed to *C.irritans* by natural infection with the same isolate or by i.p. immunisation with trophonts failed to show parasite antigens. Immunoblotting of CI-GS(44/46) with RABantiCI serum, however, resulted in recognition of at least 15 antigen components (molecular weights not calculated).

7. AGGLUTINATION AND IMMOBILISATION TESTS

The *in vitro* effect on *C.irritans* theronts and other ciliates of sera from mullet or rabbit immunised with *C.irritans* was investigated.

7.1. Effects of RABantiCI on theronts of C.irritans and I.multifiliis and trophozoites of T.pyriformis

Theronts of *C.irritans* (CI-AE) and *I.multifiliis*, both harvested within 4h post-emergence, were transferred to FS-SW or mineral water ("Volvic"), respectively. *T.pyriformis* trophozoites were washed free of culture medium and resuspended in mineral water. Ciliate suspensions were adjusted to ca. 500 cells/ml and 100μl volumes of each were added to separate "U" well polystyrene microtitre plates (Sterilin), giving ca. 50 cells/well. Equal volumes of either RABantiCI or pre-immune rabbit control serum were titrated over the two-fold range 1 in 10 to 1:160 in the appropriate medium (final volume = 200μl/well). Additional controls for each ciliate species were incubated in FS-SW or mineral water only. Plates were held at 25°C for theronts and at RT (≈20°C) for *I.multifiliis* and *T.pyriformis*. Cells were observed at various intervals with the aid of a stereomicroscope. Ciliate activity was scored according to the five point scale used for *C.irritans* (Chapter 3, Table 2), + + +

representing normal activity.

The results (Table 20) are presented separately below for each ciliate species.

7.1.1 C.irritans theronts

An overall reduced theront activity from + + + (=normal) to + + / + occurred within 5 min exposure to 1:10 and 1:20 dilutions of the RABantiCI serum, however a small proportion (≈ 25%) of theronts remained unaffected and continued normal behaviour. Theronts in higher dilutions of RABantiCI and those in pre-immune control serum retained + + + activity. At 10 min exposure, theront agglutination was observed in wells containing RABantiCI at dilutions up to 1:40. Theronts exposed for 30 min to RABantiCI serum up to 1:80 dilution agglutinated in clumps of up to 14 and showed reduced activity. No dead theronts were observed at 30 min exposure. Incubation with the pre-immune rabbit serum had no significant effect on the theronts.

7.1.2 I.multifiliis theronts

No agglutination was observed in any wells at 30 min exposure to rabbit sera. A slight reduction in theront activity was observed in the presence of 1:10 dilutions of both RABantiCI and normal rabbit serum, suggesting a non-specific effect.

7.1.3 T.pyriformis trophozoites

No agglutination of trophozoites was observed in any wells at 30 min exposure to rabbit sera. Rounding up of some cells (< 10%) was observed in the presence of low dilutions (1:10- 1:20) of both RABantiCI and pre-immune control rabbit sera, possibly indicating an osmotic effect.

Table 20

In vitro immobilisation and agglutination test using rabbit antiserum to trophonts of *C.irritans* incubated with theronts of *C.irritans* and *I.multifiliis* and trophozoites of *T.pyriformis*.

Cells	Rabbit serum	Serum dilution					
		1:10	1:20	1:40	1:80	1:160	None
CI	POS[1]	+a	+a	+a/++	++a/+++	+++	+++
	NEG[2]	++/+++	++/+++	++/+++	+++	+++	
IM	POS	++	+++	+++	+++	+++	+++
	NEG	++	+++	+++	+++	+++	
TP	POS	++r	+++/+++r	+++	+++	+++	+++
	NEG	++r/+++r	+++	+++	+++	+++	

Results, based on 2 or 3 replicate tests, show activity of the majority of protozoal cells in each test well after 30 min exposure to serum.

CI = *C.irritans*.

IM = *I.multifiliis*.

TP = *T.pyriformis*.

[1] Serum from rabbit immunised i.p. with *C.irritans* trophonts (=RABantiCI).

[2] Serum from unimmunised control rabbit.

a = agglutination between 3 or more cells.

r = rounding up of cells.

7.2 Effect of mullet sera on C.irritans theronts

Serum samples were collected from 4 mullet immunised against *C.irritans*, 2 by natural exposure to theronts, and 2 by i.p. administration of trophonts. In addition, samples were collected from 4 control mullet not exposed to *C.irritans*. Each serum was titrated two-fold in seawater and 100μl aliquots added to separate wells, each containing 50 theronts (CI-AE) per well. The results (Table 21), based on observations at 30 min, show agglutinating titres of 1:40 and 1:80 in the sera from i.p. immunised mullet but no agglutination with sera from either naturally infected mullet or naive controls. Theront inactivity was observed in the lowest serum dilution tested (1:10) from some fish, irrespective of immune status, suggesting a non-specific effect.

Fig. 53 shows agglutination between 23 theronts, following 60 min incubation in 1:20 serum from a mullet previously immunised with *C.irritans* trophonts by i.p. injection.

Table 21

In vitro immobilisation and agglutination of theronts of *C.irritans* in sera from naive and exposed mullet.

Mullet		Mullet serum dilution				
Exposure status to *C.irritans*	#	1:10	1:20	1:40	1:80	1:160
non-exposed	1	0	+/+ +	+ + +	+ + +	+ + +
	2	0/+	+ + +	+ + +	+ + +	+ + +
	3	+ +/+ + +	+ + +	+ + +	+ + +	+ + +
	4	0/+ +	+ +/+ + +	+ + +	+ + +	nd
i.p. immunised (trophonts)	5[1]	0/0a	0a/0a	0a/+ +	0a/+ + +	+ + +
	6[2]	0a	0a	0a/+ +	+ + +	+ + +
naturally infected (theronts)	7[3]	0	+/+ +	+ + +	+ + +	+ + +
	8[4]	0/+	+ + +	+ + +	+ + +	+ + +
				No serum controls		+ + +

Results, based on two replicate tests, show activity of the majority of theronts in each test well, after 30 min exposure to serum.

a = agglutination between 3 or more theronts.

nd = not done.

[1] 9 days after 4° immunisation; ELISA positive.

[2] 22 days after 5° immunisation; ELISA positive.

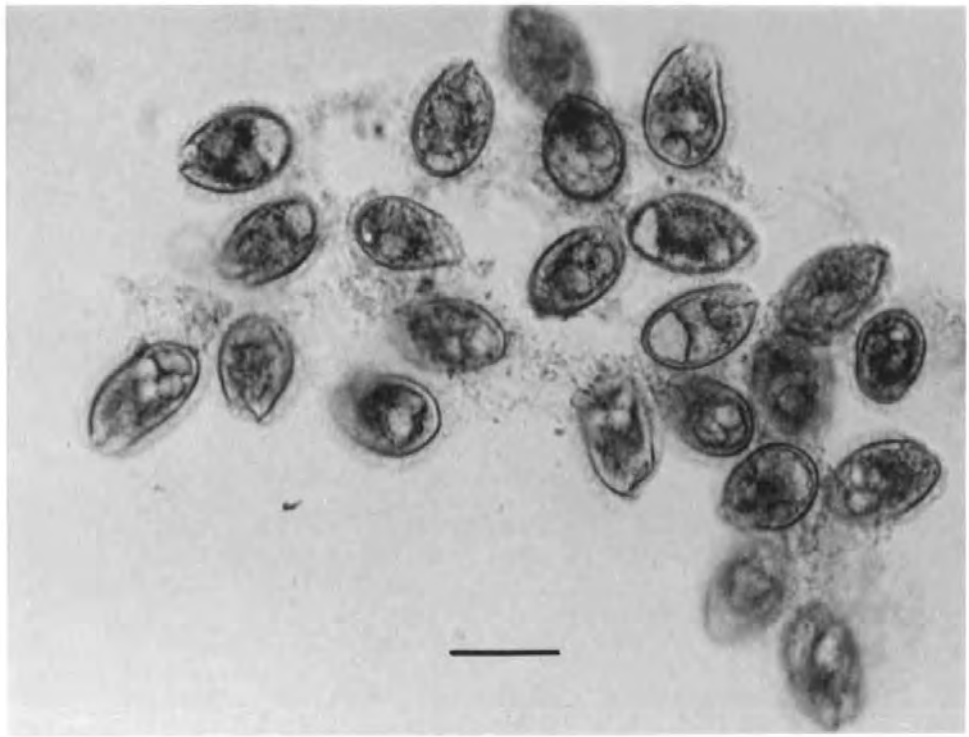
[3] 7 days after 3rd exposure to theronts; %PEI <0.1.

[4] 26 days after 3rd exposure to theronts; %PEI <0.1.

Figure 53

Agglutination of theronts of *C.irritans* in the presence of mullet immune serum.

Scale bar = 50μm.



DISCUSSION

The detection of specific antibodies to *C.irritans* in fish following natural infection has been reported here for the first time, using mullet as an experimental host. Nevertheless, the application of ELISA as the major investigative technique for monitoring antibody responses does introduce limitations in the interpretation of results. The sensitivity of ELISA, although greater than most other antibody assays (Edwards, 1985), may have been insufficient to detect low levels of specific antibody occurring, for example, during the early stages of an immune response and in epithelial mucus (St. Louis-Cormier *et al.*, 1984; Ellis, 1989). Previous studies (Cross, 1990) have attempted to amplify the detection of mucus antibodies by concentration. Unfortunately, mullet could not be induced to release copious amounts of epithelial mucus, in contrast to certain other species of teleosts, and therefore this was not a practical approach to pursue. In terms of qualitative limitations, it must be recognised that certain antigens, notably carbohydrates and glycolipids, do not bind to the solid phase under normal ELISA conditions (Kelsoe and Weller, 1978; Reggiardo *et al.*, 1980; Kurstak, 1985; Burgess, 1989; Wood and Wreghitt, 1990) and therefore antibodies directed to these molecules will not be detected. The importance of these compounds as antigens is, however, apparent from studies on human protozoan diseases (Crane *et al.*, 1982; Zenian and Kierszenbaum, 1982; Handman *et al.*, 1987); their role in fish immune responses to protozoa therefore also seems likely. With regards the kinetics of the antibody response, production of specific antibodies in mullet within 14 days following a primary exposure to *C.irritans* is in close agreement with the period of onset of primary antibody response in this host to the digenean,

Cryptocotyle lingua, as recorded by Wood and Matthews (1987). The timing also agrees with that recorded to a variety of antigens in other teleosts, including trout, *Oncorhynchus mykiss* (Chiller *et al.*, 1969) and sunfish, *Lepomis macrochirus* (Smith *et al.*, 1967). The kinetics of the antibody response to *C.irritans* is likely to be influenced by temperature, within the physiological limits of the host, as is well recognised for teleosts antibody responses in general (Rijkers *et al.*, 1981; Rijkers, 1982; Bly and Clem, 1992). The timing may also vary according to the host species, and there is some evidence to support this (Rijkers, 1982). Sailendri and Muthukkaruppan (1975), using *Tilapia mossambica*, have shown that under tropical conditions (30°C) a primary antibody response can be elicited within as short a period as five days after exposure to antigen. Although the species of mullet used here has a southerly distribution, extending to the Mediterranean (Lythgoe and Lythgoe, 1971), the speed of its immune response might not be representative of tropical marine fish species normally encountered by *C.irritans*. The delay in antibody response, recorded here, following intraperitoneal injection could also be attributed to temperature, as mullet immunised by this route were maintained at 5-10°C lower than those exposed to *C.irritans* by natural infection. Alternatively, this might reflect a faster recognition and response to antigens presented within the skin epithelium as opposed to the peritoneal cavity. The latter explanation is supported by evidence for a secretory immune system functioning within teleost skin epithelium (Lobb and Clem, 1981b; Peleteiro and Richards, 1988), although it is not clear whether locally synthesised antibodies enter the circulatory system. Despite the slow antibody response in mullet to *C.irritans* administered intraperitoneally, this immunisation route resulted in higher levels of specific antibodies in the serum, as compared with immunisation by natural infection. This might be attributable to quantitative and/or qualitative differences in the parasite

antigen to which the fish was exposed, the site of antigen presentation, or the immunopotentiating effects of the Freund's adjuvant used in conjunction with i.p. immunisations, all of these factors being known to affect the immune response of fish (Ellis, 1988). The occurrence of elevated antibody levels to *C.irritans* in some mullet following secondary as compared with primary exposure to theronts might be indicative of an anamnestic response although this was not observed with the intraperitoneally immunised fish. This observation may therefore have simply reflected an increasing response to the primary infection. Evidence that a memory response in teleosts does occur comes from studies on other species immunised to various antigens and pathogens (Sailendri and Muthukkaruppan, 1975; Anderson and Dixon, 1980; Rijkers *et al.*, 1980), although the height of the secondary response in fish appears far less pronounced than that in mammals (Roitt *et al.*, 1985). With the present study, firm evidence for an anamnestic response to *C.irritans* in mullet will necessitate longitudinal serological monitoring of these fish. Further studies are warranted, as the ability of fish to develop a heightened secondary response to *C.irritans* is an important feature when considering vaccination against this parasite. Studies here suggest that antibody screening, for example by ELISA, could prove a useful indicator of an anamnestic response, however further evaluation of the assay is required.

Although mullet exhibited protective immunity to *C.irritans* this was not always associated with the presence of specific antibody. A similar lack of correlation between serum antibody levels in fish and immune protection has also been shown for certain bacterial diseases (Michel and Faivre, 1982; Cipriano, 1983). It should be mentioned, however, that the trophont stage only was utilised as antigen for the ELISA developed for measuring humoral responses. It is possible, therefore, that

host protection is afforded by antibodies directed to antigens specific to the invasive theront, as supportive evidence for stage-specific proteins was provided from polypeptide analyses, described in Chapter 5. Although antibody may play no direct role in protection against *C.irritans*, it was of interest that mullet antisera induced theront immobilisation and agglutination under *in vitro* conditions in the present study. Similar studies have been undertaken using *I.multifiliis* in which fish immune sera were shown to immobilise the parasite at the theront stage (Houghton, 1987; Clark *et al*, 1987; Cross, 1990) and at the trophont stage (Hines and Spira, 1984b; Wahli and Meier, 1985), the latter workers also demonstrating immobilisation with epithelial mucus from immune fish. To what extent these observations reflect *in vivo* responses by the host remains unresolved. Nevertheless, Hines and Spira (1984b) and Clark *et al.* (1988) have suggested that protection against *I.multifiliis* might be provided by the immobilising activity of antibody, which according to Clark *et al.* (1987) is possibly directed to cilial antigens. The lack of correlation between specific antibody production and protective immunity would clearly present an important limitation to the assessment of vaccine efficacy by serological methods. Evidence for a protective role for antibody in response to parasite infections is largely conjectural, whereas the ability of specific antibody to protect fish against bacterial pathogens has come from studies by Horne and Baxendale (1983). The absence of specific antibodies in the serum of immune protected mullet here, does not preclude the possibility of antibody functioning locally at the infection site, as shown by Lobb (1987) using channel catfish administered with externally applied antigen. In the present study, however, the inability to detect specific antibodies to *C.irritans* in the epithelial mucus of mullet following natural infection, even in fish with specific antibodies in the serum, provides no evidence for acquired humoral immune responses operating within host skin

epithelium.

The demonstration of an antibody response by mullet to *C.irritans* raises the important question as to which parasite components are targeted by host antibodies, such knowledge being vital for the selection and development of sub-unit vaccines against cryptocaryosis. Unfortunately, the present study failed to demonstrate recognition of individual trophont antigens of *C.irritans* by immune mullet serum, with the aid of immunoblotting techniques. This failure might be associated with the level of specific antibodies, as a positive immunostaining was obtained with immune rabbit serum of high antibody titre. Low avidity of the mullet antibodies directed to target components of *C.irritans* could also account for negative immunostaining, as low avidity antibodies are thought to dissociate from antigen during the immunoblotting process (Ramlau, 1988). Whether teleosts produce low avidity antibodies, however, is not clear, although this appears to be the case for elasmobranchs (Shankey and Clem, 1980). Results obtained here do provide some insight into the species- and stage- specificity of these target antigens. Evidence for antigens common to the theront and trophont stages of *C.irritans* is provided from *in vitro* agglutination and immunofluorescence studies, theronts reacting *in vitro* with sera from rabbit and mullet immunised with trophonts. Given the distinct profiles of predominant polypeptides between theronts and trophonts, demonstrated here, it seems likely that the common antigens are quantitatively relatively minor components. Interestingly, these studies have also demonstrated inter- specific antigens common to *C.irritans* and *I.multifiliis* although there was no evidence of any cross-protection between these two ciliates. That these inter- specific antigens were detected using mammalian antisera probes raised to disrupted parasite preparations might not be indicative that teleosts could cross-recognise these antigens following natural infection. The absence of

serological cross-reactivity between *T.pyriformis* and *C.irritans* (as well as *I.multifiliis)* provides no evidence for the suitability of *T.pyriformis* as a candidate vaccine against cryptocaryosis. These results are in contrast to those of Goven *et al.* (1981b) who demonstrated cross-reactions between *T.pyriformis* and *I.multifiliis*, also using rabbit antisera. The use of different strains of *T.pyriformis* between the study by Goven *et al.* (1981b) and that here may account for the observed disparity in cross-reactivity, given that strains of *T.pyriformis* have been shown to differ serologically (Loefer *et al.*, 1958). Similarly, strain differences amongst *I.multifiliis* may also occur, as considered by Nigrelli *et al.* (1976); strain differences having also been shown for other ciliates, including *Balantidium coli* (Zaman, 1964). Studies here were based on results using antisera raised to *C.irritans* trophonts; the possibility of antigens shared between the theront stage of *C.irritans* and *T.pyriformis* was not investigated. The present study clearly underlines the possible pitfalls in selecting antigens from heterologous species as candidate vaccines based on serological cross-reactivity alone. Although these results show little promise for development of novel vaccines, the serological findings concur with polypeptide analyses (Chapter 5) in supporting the taxonomic positioning of *C.irritans* closer to *I.multifiliis* than to *T.pyriformis*, as according to the scheme by Corliss (1979).

These studies have demonstrated a humoral immune response to *C.irritans*, however, to what extent humoral factors are involved in protective immunity requires further investigation. That protection can be provided by specific antibody alone now appears a too simplistic explanation. Future investigations for a role of cellular components and their interaction with specific antibody in conferring host protection to *C.irritans* seem warranted. This view is supported by evidence for the involvement of leucocytes, macrophages and non-cytotoxic cells in immune response and protection

in fish to ciliates (Hines and Spira, 1973b; Graves *et al.*, 1985; Cross, 1990) as well as evidence of antibody-dependent cellular cytotoxic mechanisms, as reported for rainbow trout infected with the digenean *Diplostomum spathaceum* (Whyte *et al.*, 1990).

CHAPTER SEVEN

MONOCLONAL ANTIBODY STUDIES

INTRODUCTION

The application of monoclonal antibody technology to the investigation of fish diseases has been chiefly directed towards viral and bacterial pathogens (Plumb and Klesius, 1988; Lorenzen *et al.*, 1988; Hui-Min *et al.*, 1991; Ristow *et al.*, 1991; Rockey *et al.*, 1991). Few studies have involved eucaryotes, however, it is of some relevance that Dickerson *et al.* (1986) have produced MABs to the theront stage of the ciliate, *I.multifiliis*. These workers obtained three different MABs recognising either protein, glycoprotein, and carbohydrate antigens. Only one was characterised, a protein of 200kDa.

Hybridoma technology has also facilitated production of MABs to immunoglobulins of teleost fish, including channel catfish (Lobb and Clem, 1982), carp (Secombes *et al.*, 1983), rainbow trout (DeLuca *et al.*, 1983; Thuvander *et al.*, 1990), and cod (Pilstrom and Petersson, 1991). Such MABs have demonstrated immunoglobulin heterogeneity within individual fish species (Lobb *et al.*, 1984; Elcombe *et al.*, 1985), as well as lymphocyte heterogeneity with regards surface Ig expression (Lobb and Clem, 1982; DeLuca *et al.*, 1983; Secombes *et al.*, 1983; Thuvander *et al.*, 1990), the latter suggesting a possible division of T- and B- type cells, analogous to that of higher vertebrates.

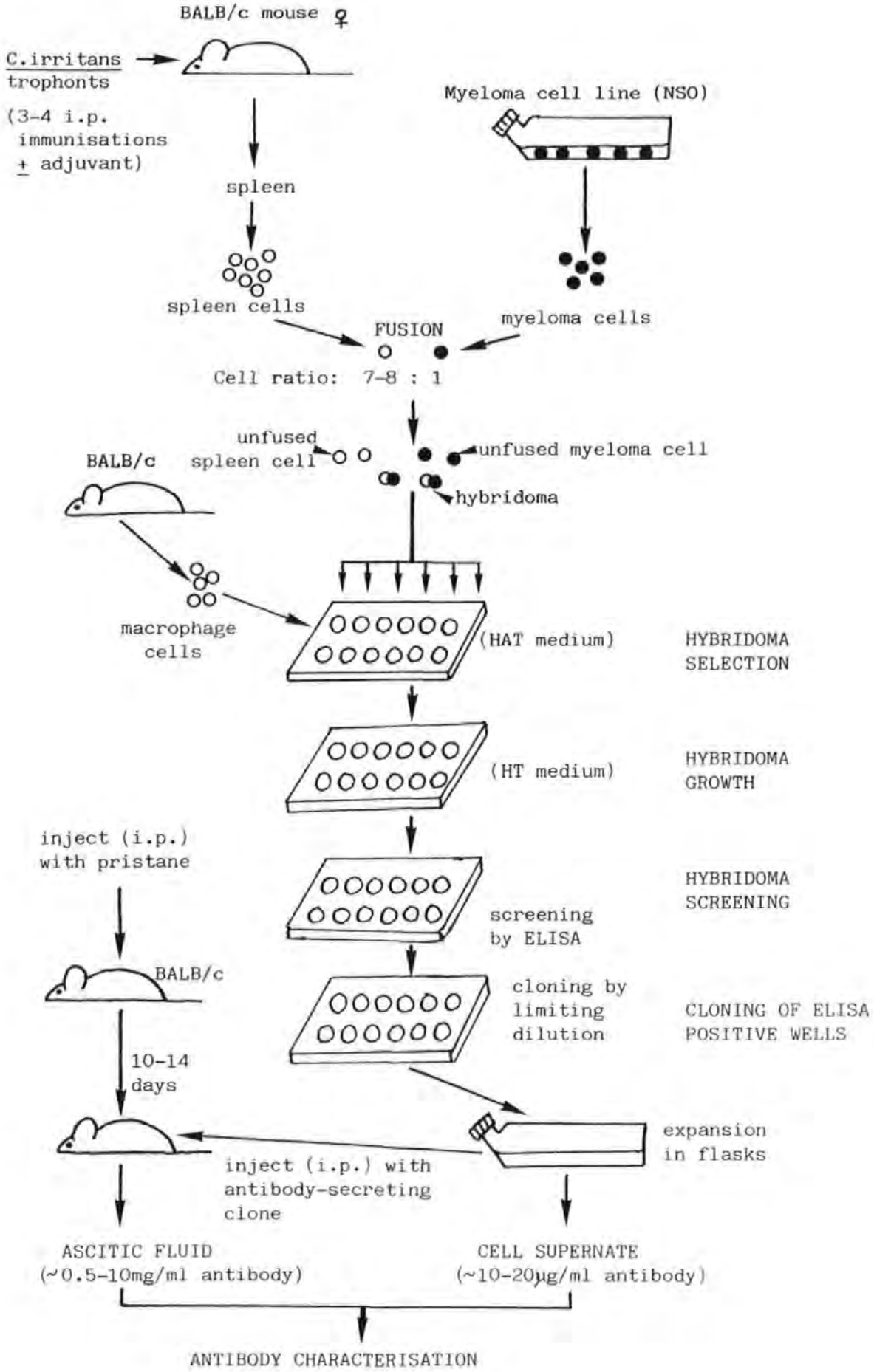
The present study aimed to produce MABs to *C.irritans* using the "shotgun" fusion technique as is frequently performed for mammalian parasite studies (Pearson

and Clarke, 1986). Any MABs produced would be used to probe for antigenic differences between isolates of *C.irritans* (parasite typing), between trophonts and theronts (stage-specific antigens), and between *C.irritans* and closely related ciliates (species-specific antigens).

A summary of the procedure for MAB production used in this study is given in Fig. 54.

Figure 54

Procedure for the production of murine monoclonal antibodies to *C.irritans* trophonts.


BALB/c mouse ♀
C.irritans trophonts
(3-4 i.p. immunisations ± adjuvant)
spleen
spleen cells
Myeloma cell line (NSO)
myeloma cells
FUSION
Cell ratio: 7-8 : 1
unfused spleen cell
unfused myeloma cell
hybridoma
BALB/c
macrophage cells
(HAT medium)
HYBRIDOMA SELECTION
(HT medium)
HYBRIDOMA GROWTH
inject (i.p.) with pristane
screening by ELISA
HYBRIDOMA SCREENING
BALB/c
cloning by limiting dilution
CLONING OF ELISA POSITIVE WELLS
10-14 days
expansion in flasks
inject (i.p.) with antibody-secreting clone
ASCITIC FLUID
(~0.5-10mg/ml antibody)
CELL SUPERNATE
(~10-20µg/ml antibody)
ANTIBODY CHARACTERISATION

MATERIALS AND METHODS

Three separate immunisation-fusion programmes were undertaken over a period of two years.

1. IMMUNISATION PROCEDURES

Female BALB/c mice were selected as spleen cell donors (Newell *et al.*, 1988). Each mouse received 3 or 4 i.p. immunisations of trophonts of *C.irritans* (CI-PE; CI-CR; CI-HT; CI-GS) presented alive or following cryostorage in liquid N_2, ca. 14 days apart. Trophonts were delivered in 0.5ml saline only (immunisation programme #1) or in saline containing either 50% FCA (1° immunisation), 50% FIA (2°), or saline only (3°, 4°)(programmes #2, #3), in accordance with recommended procedures (Dresser, 1986). Antigen-adjuvant emulsions were stored unfrozen for up to 1 month at 10°C. Immunisation regimes, together with numbers of trophonts administered, are given in Table 22.

2. COLLECTION OF MOUSE BLOOD

The caudal vein was exposed by tail snip and blood drops collected into a microcentrifuge tube. Samples of 100μl were taken at 1 to 2 days prior to planned fusion and specific antibody production to *C.irritans* detected by ELISA (see below). Serum fractions were prepared as described previously (Chapter 6, section 2).

3. MYELOMA CELL LINE (NSO)

NS0, a non-Ig producer (Galfre and Milstein, 1981) derived from the P3-X63-Ag8 parent cell line (Newell *et al.*, 1988) was selected as the myeloma fusion partner

Table 22

Immunisation regimes for eight BALB/c mice administered *C.irritans* trophonts.

Immunisation programme #	Mouse #	Immunisations				
		Number of trophonts per immunisation				Adjuvant
		1°	2°	3°	4°	
1	A	150[a]	300[a]	500[a]	200[c]	no
	B	500[c]	200[a]	200[a]	200[a]	
	C	200[a]	200[a]	200[a]	-	
2	D	650[a]	650[a]	550[a]	-	yes[1]
	E					
	F					
3	G	500[c]	500[c]	125[a]	-	yes[1]
	H					

[1]Adjuvant:
FCA administered with 1° immunisation.
FIA " " 2° "
None " " 3°,4° "

a = trophonts administered alive.

c = trophonts administered following cryopreservation.

for BALB/c spleen cells (NSO supplied courtesy of ECAAC, Porton Down, U.K.). NS0 stocks were cryopreserved in liquid N_2 at a density of 1-5X10^6 cells/ml in 1:9 v/v dimethylsulphoxide (DMSO): foetal calf serum (FCS) (Gibco Ltd.). Cell cultures were set up 10 to 14 days before planned fusion. Cryopreserved cells were rapidly thawed in a 37°C water bath, and immediately diluted in monoclonal culture medium (MCM) comprising RPMI-1640 (Gibco Ltd.) containing 10% FCS (see appendix) to minimise the known toxic effects of DMSO at > 4°C. Cells were distributed in MCM in 25ml culture flasks (Flow laboratories, U.K.) and incubated at 37°C in a humidified 5% CO_2 atmosphere (LEEC CO_2 incubator). Cultures were split every 2 to 3 days, to maintain a cell density of 2-5X10^5 cells/ml.

3.1 HAT sensitivity testing

The absence of hypoxanthine phosphoribosyl transferase (HPRT) in the myeloma cell line was confirmed prior to fusion by inability to grow in a mixture of hypoxanthine, aminopterin and thymidine (HAT) according to the method of Newell *et al.* (1988). Cell death was confirmed by the trypan blue exclusion test (Newell *et al.*, 1988).

4. HYBRIDOMA PRODUCTION AND SCREENING

4.1 Myeloma - spleen cell fusion

The general procedure recommended by the Royal Postgraduate Medical School (RPMS, Hammersmith, London, monoclonal course guidebook, undated) was employed. Briefly, fusions were performed 3 to 4 days following the final mouse immunisation. Spleen cells of the immunised mouse were teased from the aseptically

removed organ into PBS-J (see appendix) and counted in a Neubauer haemocytometer. NS0 cells, harvested from cultures during the exponential growth phase, were enumerated as above and the numbers adjusted to give a spleen:plasmacytoma cell ratio of about 7:1 to 8:1. Fusion was facilitated by the addition of polyethylene glycol (PEG 1500, BDH Ltd.) to the pelleted cell mixture.

4.2 Macrophage feeder cells

Low density hybridoma cultures were supplemented with macrophage feeder cells to provide growth factors (Newell *et al.*, 1988). These were obtained from a freshly killed unimmunised BALB/c mouse by i.p. injection of 5ml cold RPMI medium containing 10% FCS followed by gentle palpation of the abdomen. The medium plus peritoneal cells was aspirated and a sample enumerated in a haemocytometer and viability checked using trypan blue.

4.3 Hybridoma culture

Unfused myeloma cells were selectively destroyed by the HAT medium as follows. Fused hybrids were resuspended in MCM containing HAT to ca. 2 - $5X10^5$ cells/ml and peritoneal macrophages added to ca. 2 - $5X10^4$ cells/ml. The hybridoma/ macrophage cell mixture was plated out to ca. $5.2X10^6$ cells/ml in a 24 well tissue culture plate (Corning, U.S.A) and incubated at 37°C in a humid 5% CO_2 atmosphere. The MCM/HAT was replaced after 7 days. Following HAT selection, hybridoma cultures were split every 2 to 3 days in MCM containing hypoxanthine and thymidine (HT).

4.4 Screening hybridomas for specific antibody

Tissue culture wells with high cell growth, usually between 10 - 14 days post-fusion, were initially screened by ELISA for specific antibodies to trophont antigen of *C.irritans* (CI-GS(44/46)). Selected antibody positive cultures were further tested by ELISA against *T.pyriformis* and mullet Ig (Ultrogel fraction) antigens. Antigens were coated at previously optimised dilutions (Chapter 6). Mouse sera were tested over the range 1:50 to 1:1600. Hybridoma cell supernates were tested at 1 in 2, diluted in ELISA incubation buffer containing double strength Tween 20. Mouse ascites were tested at 1:40 to 1:1000. Appropriate serum or hybridoma controls were included with each test. A commercially prepared rabbit anti-mouse Ig peroxidase conjugate (Dako, Denmark, product P260) was used at 1:1000.

4.5 Hybridoma cloning

Hybridomas with specific antibody activity to an appropriate antigen were selected for cloning on the basis of those with highest ELISA values. Usually 2 - 10% of the highest antibody reactors were selected, the actual percentage being determined by the handling capacity and the availability of antigen. Selected hybridomas were cloned by limiting dilution in 96 well tissue culture plates (Sterilin, U.K.), initially to 6 cells/well; antibody positives from this were plated out to a theoretical density of 0.3 cells/well (*i.e.* average of 1 cell per 3 wells).

4.6 Cryopreservation of hybridomas and NS0 cells

Reserve stocks of NS0 cells and selected antibody- secreting hybridoma clones were cryopreserved in liquid N_2. Antibody secreting hybridoma cultures awaiting cloning were also cryopreserved. Cultures for cryostorage were centrifuged at 500g,

10 min, and the cells resuspended to 1-9X10^6/ml in 90% FCS, 10% DMSO. Cell suspensions were transferred to cryotubes (NUNC Ltd.), held in ice for 2 h and then placed in an insulated polystyrene box to facilitate slow cooling to -70°C in a freezer, after which they were stored in liquid N_2.

5. MONOCLONAL ANTIBODIES (MABS)

5.1 Bulk production

Antibody positive clones were scaled up for bulk MAB production by either expansion in 25ml and 75ml flasks or by ascites production, the latter providing 25 to 1000 times the concentration of MABs yielded by cell culture (Newell *et al.*, 1988). The method for ascites production, as described by the RPMS monoclonal course guidebook, was followed. Briefly, BALB/c mice were administered by i.p. 0.5ml pristane (2,6,10,14-tetramethyl-pentadecane, Aldrich Ltd.) followed 10 to 14 days later by i.p. injection with 1-4X10^6 cloned hybridoma cells in ca. 0.5ml PBS. Mice showing ascites were killed and the fluid aspirated using a fine pasteur pipette inserted into the peritoneal cavity. Ascitic fluid was centrifuged at 500*g*, 10 min, and the supernate aliquoted for storage at -20°C or in liquid N_2.

5.2 Isotype analysis

Culture supernates from specific antibody producing clones were tested by ELISA in replicate at 1:2 dilution, against their target antigen. MAB isotype was identified by the use of a panel of peroxidase conjugated sheep antisera to mouse Ig H and L chains (Mouse immunoglobulin set, Serotec Ltd.).

6. CHARACTERISATION OF TARGET ANTIGENS

The target antigens, against which the MABs were directed, were further characterised by immunoblot analysis of gel fractionated ciliate and fish immunoglobulin preparations.

The SDS-PAGE procedure was as described in Chapter 5, methods section 3.

6.1 Native PAGE

The PAGE procedure, described in Chapter 5, was modified for native gels as follows. A 7% separating gel was overlaid with a 3% stacking gel. Gels were run for approximately 2 h. Molecular weight markers comprised: bovine milk alpha-lactalbumin (14.2kDa), bovine erythrocytes carbonic anhydrase (29kDa), chicken egg albumin (45kDa), bovine albumin (monomer, 66kDa; dimer 132kDa), jack bean urease (trimer, 272kDa; hexamer, 545kDa) (Sigma MW marker kit MW-ND-500). Buffers and reagents were as described in the Sigma technical bulletin, MKR-137.

6.2 Immunoblotting

Immunoblots of selected MABs against PAGE separated ciliate and immunoglobulin preparations were performed using the basic methods described earlier (Chapter 6, methods section 10). Antigen preparations for MAB screening were loaded onto a wide comb for PAGE. They were then electroeluted onto 0.2μm nitrocellulose (NC) paper which was subsequently cut into 0.5cm strips in order to reduce the quantities of MAB required. NC strips were blocked in 3% skimmed milk powder (Tesco Stores Ltd.) in PBS, pH 7.6. for 40 min at RT. NC strips were incubated for 1 h in individual disposable plastic tubes containing 6 ml undiluted MAB cell culture supernate, previously pH stabilised with 20mM HEPES buffer

(Gibco Ltd.) as recommended by Newell *et al.* (1988). Ascitic fluids were tested at 1:50 in PBS. After washing, the NC strips were similarly incubated with a rabbit anti-mouse peroxidase conjugate (DAKO, Denmark, product P260) at 1:500, followed by further washing prior to addition of diaminobenzidine (DAB) substrate. Stained bands were characterised by MW by comparison with non-blotted Coomassie blue and amido black stained controls and with MW markers which had been run simultaneously.

7. THERONT IMMOBILISATION

The theront immobilisation/ agglutination test, described in Chapter 6, was performed using CI-GS theronts, 50/well, incubated in hybridoma cell culture supernates or ascites, both titrated over the range 1:10 to 1:160 in filter sterilised seawater (FS-SW). A rabbit antiserum to *C.irritans* (RABantiCI) and a negative rabbit serum, each tested at 1:20 dilution, were included as controls. Observations were made at 30 min.

EXPERIMENTS AND RESULTS

1. SELECTION OF MICE FOR HYBRIDOMA PRODUCTION

Serological results and spleen cell harvests of 8 mice, A-H, prepared for fusion are shown in Table 23. Mice were bled on the day prior to harvest and their sera tested at 1:200 for antibody activity to *C.irritans* by ELISA. Of the 5 mice tested all were seropositive. Spleen cell harvests from 7 mice were sufficient for fusion, ranging between $7.4X10^7$ to $1.1X10^8$ cells/mouse. A total of 7 mice, including 2 not screened by ELISA, were used for fusions.

2. SELECTION OF HYBRIDOMAS

The selection of hybridomas for cloning was based on ranking them according to the level of specific antibody activity to *C.irritans* by ELISA, those with highest antibody levels being chosen. An example of the frequency distribution of antibody activity to *C.irritans* is shown in Fig. 55 for 311 hybridomas derived from mouse D. The distribution shows a bimodal effect, broadly distinguishing two major populations: high antibody reactors ($\approx > 0.4$ absorbance units) and low/non-reactors ($\approx < 0.1$ units). From these results, the highest 5% of antibody reactors were selected for further cloning by limiting dilution methods.

Hybridomas derived from spleen cell donor mice D and G were screened by ELISA. From ca. 2100 ELISA tests, a total of 20 hybridomas were identified with antibody reactivity against one or more of the three ELISA antigens.

Mouse D yielded 4 hybridoma cultures (termed 1A1, 1D2, 2B5, and 2C4), each producing a unique reactivity pattern with the ELISA antigens (Fig. 56). Cross-reactivity with *T.pyriformis* trophozoites and mullet Ig was observed, with one

Table 23

Antibody production and cell fusion data for eight BALB/c mice immunised with *C.irritans* trophonts.

Immunisation programme	Mouse			Fusion	Hybridoma
	#	Serum antibody[1] (ELISA)	Spleen cell harvest X 10^7	S:P ratio[2]	Antibody production
1	A	nd	9.6	8.0:1	no[FC]
	B	nd	8.8	8.0:1	no
	C	nd	7.6	8.0:1	no
2	D	0.73	7.4	8.0:1	yes
	E	0.72	8.0	8.0:1	no[FC]
	F	0.49	11.0	6.9:1	no[FC]
3	G	1.04	8.8	8.1:1	yes
	H	0.74	nd	-	-

[1] Mean ELISA result (n=2) of tail bleed sample, collected 1 day prior to fusion, and tested at 1:200 against CI-GS(44/46). Sera from non-immunised mice: ≤ 0.07 absorbance units (n=2).

[2] S:P = spleen cell : plasmacytoma cell ratio.

nd = not done.

[FC] = fungal contamination, hybridoma culture destroyed. Identified as *Aspergillus* sp. for mouse F hybridomas.

Figure 55

Frequency distribution of antibody activity to *C.irritans* trophonts by ELISA in 311 murine hybridoma lines.

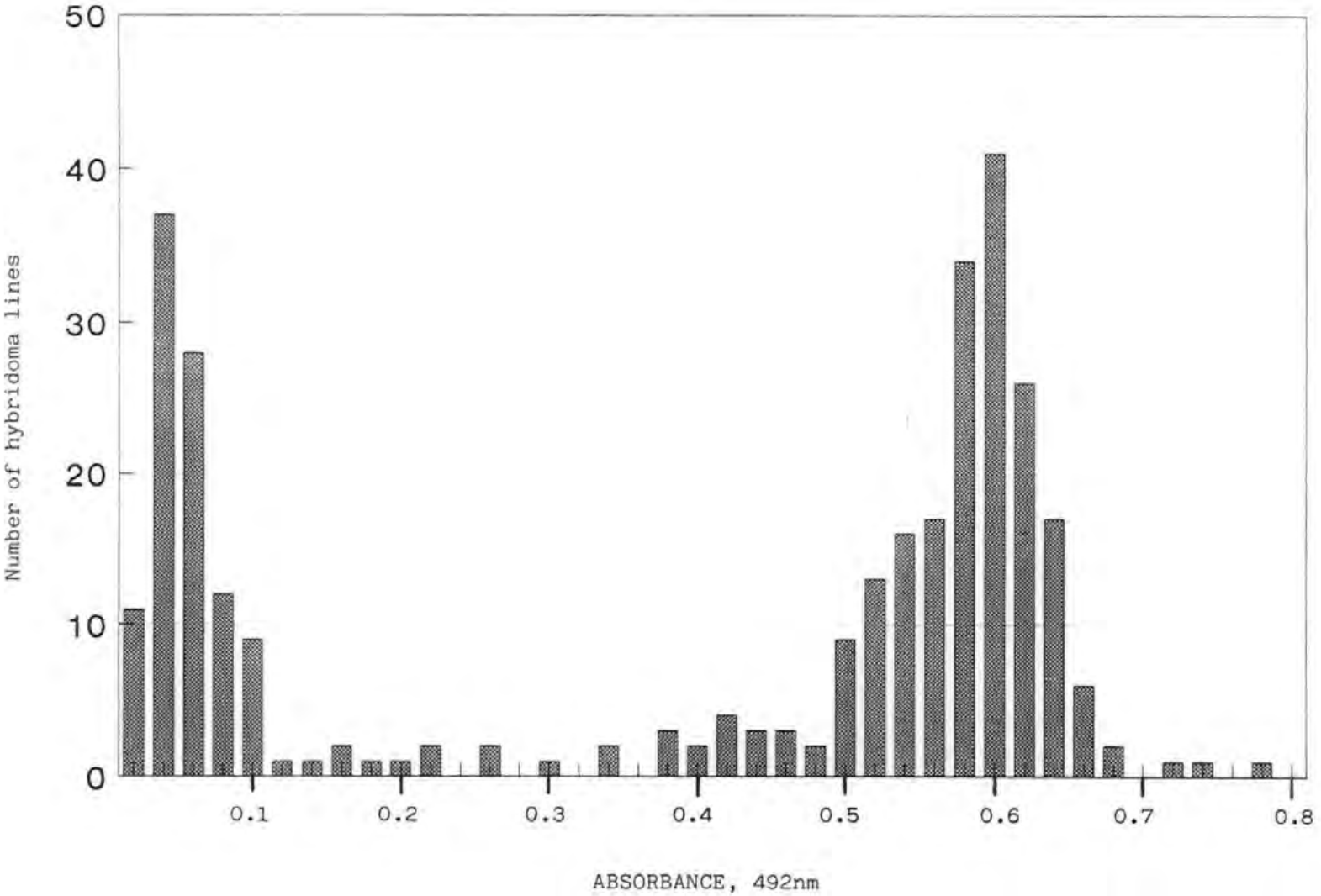

Number of hybridoma lines
50
40
30
20
10
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
ABSORBANCE, 492nm

Figure 56

Monoclonal antibody activity to trophonts of *C.irritans*, trophozoites of *T.pyriformis*, and mullet immunoglobulin, in hybridomas from two mice, measured by ELISA.

Mouse D:
Hybridomas = 1A1, 1D2, 2B5, and 2C4 (n= 4).

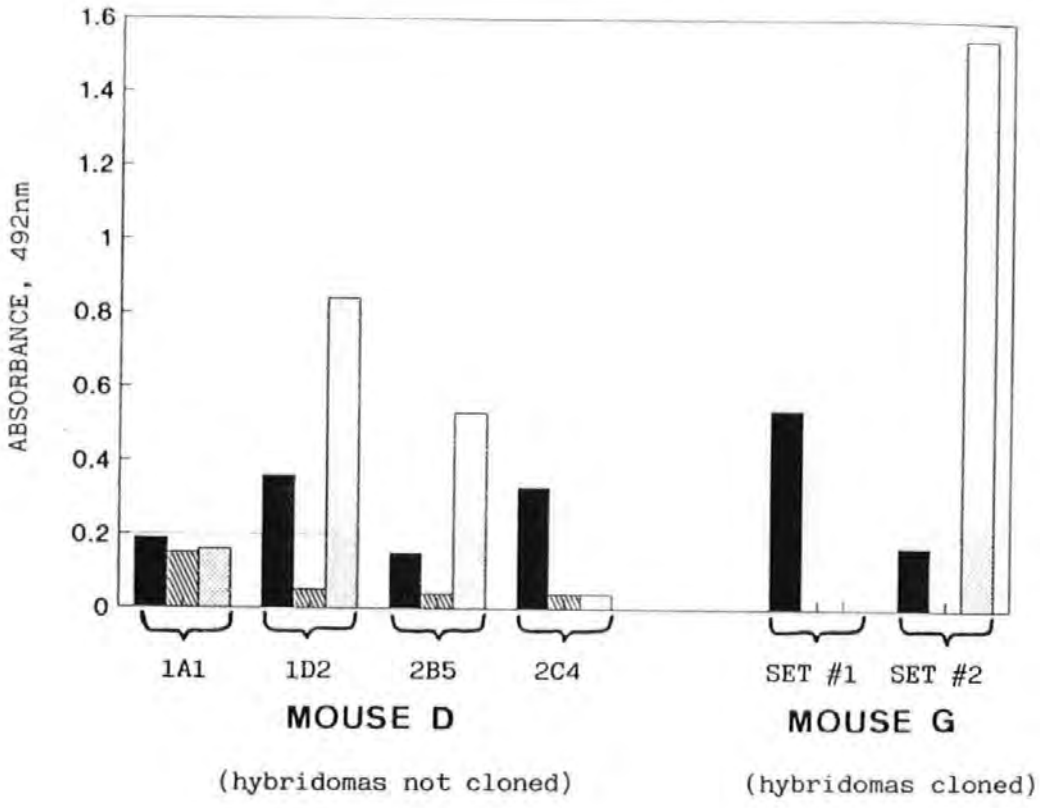
Mouse G:
Set #1 hybridomas comprise clones 5,6,7,11,19,20,29,31, and 32 (n= 9).
Set #2 hybridomas comprise clones 34,35,36,37,38,39, and 40 (n= 7).

Antigens:


C.irritans


T.pyriformis


Mullet Ig


ABSORBANCE, 492nm
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1A1
1D2
2B5
2C4
SET #1
SET #2
MOUSE D
(hybridomas not cloned)
MOUSE G
(hybridomas cloned)

hybridoma culture giving high antibody levels to mullet Ig but weak levels to *C.irritans* antigen. However, cloning of these cultures resulted in diminishing antibody levels eventually becoming negative by ELISA, at which point all clones were discarded.

Mouse G yielded 16 hybridoma cultures all of which were successfully cloned. Of these, 9 clones (=set #1) showed similar ELISA reactivities with *C.irritans* only; the other 7 clones (=set #2) reacted weakly with *C.irritans* but strongly with mullet Ig (Fig. 56), indicating the presence of two distinct types of MABs. None of the 16 hybridomas reacted with *T.pyriformis*.

3. MONOCLONAL ANTIBODIES (MABS)

3.1 Production

MABs were routinely harvested from hybridoma cell culture supernates. However, higher concentrations of MABs were obtained from ascites. Table 24 shows antibody activity in ascitic fluids obtained from pristane treated mice, administered with various numbers of antibody- secreting hybridoma cells. Of 16 mice administered hybridoma cells, 12 produced ascites, with yields up to 20ml per mouse. Serum antibody levels were much higher in ascites fluids than in culture supernates, permitting the former to be screened in ELISA at 1:1000 dilution, compared with 1:2 for supernates.

3.2 Isotype analysis

Isotype analysis by ELISA revealed IgG1 and IgM class MABs. The 9 hybridomas reacting specifically with *C.irritans* trophonts were all IgG1; the 7 reacting strongly with mullet Ig were all IgM.

Table 24

Murine ascites production and antibody activity to trophonts of *C.irritans* and mullet immunoglobulin, measured by ELISA.

Target antigen, MW	Hybridoma, designatory number	Mouse #	No. hybridoma cells x10^6 per mouse	Ascites production	Ascites antibody activity (ELISA absorbance)[1]	
					CI	MIg
20-21kDa	5	5-1	2.3	yes	0.19	0.01
		5-2			0.16	0.01
	6	6-1	2.3		0.18	0.01
		6-2			0.18	0.02
	7	7-1	0.4		nt	nt
		7-2			nt	nt
	11	11-1	1.3		0.18	0.01
	29	29-1	1.7		nt	nt
	31	31-1	1.5	no	-	-
	32	32-1	1.1		-	-
68-69kDa	34	34-1	3.9	yes	0.09	0.51
		34-2			nt	nt
		34-3			nt	nt
	37	37-1	5.0		0.08	0.50
		37-2		no	-	-
		37-3			-	-

[1] Mean antibody activity (n=2) in ascites at 1:1000 dilution. Control hybridoma supernate (non-antibody secretor) = 0.03 absorbance units for *C.irritans* antigen, and 0.02 for mullet Ig antigen.

CI = *C.irritans* trophont antigen.

MIg = mullet immunoglobulin.

nt = not tested.

3.3 Target antigen characterisation by immunoblotting

MABs from cloned hybridoma supernates were characterised for antigen recognition by immunoblotting against electroeluted reduced SDS-PAGE preparations of the ciliates, namely, *C.irritans*, *I.multifiliis*, and *T.pyriformis*, and against immunoglobulin preparations from mullet, rainbow trout and flounder. MABs recognised proteins in trophonts of both *C.irritans* and *I.multifiliis* and in mullet Ig. These results are detailed below.

3.3.1 Reactivity with C.irritans trophonts

The 9 MABs which were specific to *C.irritans* by ELISA gave similar immunoblot reactions, identifying a single protein component of approximately 20-21kDa, corresponding with a moderately intensive amido stained band on the electrophoresed *C.irritans* preparation. The 20-21kDa component was recognised in trophonts of all isolates tested, namely, CI-CE, CI-FL, CI-CR, CI-HT and CI-GS (Fig. 57). Electrophoresis of a *C.irritans* trophont preparation under non-reducing conditions on a 7% gel, followed by immunoblotting, also resulted in MAB recognition of a single band of undetermined molecular weight. Very faint bands were observed at the 45-46kDa and 56kDa positions on some heavily loaded trophont preparations of *C.irritans*.

3.3.2 C.irritans stage and species specificity

A cocktail of 3 MABS (#7, #29, #32) directed to the 20-21kDa protein were reacted with NC strips containing reduced SDS-PAGE preparations from whole trophonts and theronts of both *C.irritans* and *I.multifiliis*, trophozoites of *T.pyriformis*, and mullet immunoglobulin, each separately loaded at concentrations detectable by

Figures 57 and 58

Immunoblot assays using the MAB directed to the 20-21kDa polypeptide.

Figure 57 (=A)
Assay of SDS-PAGE preparations of trophonts from five isolates of *C.irritans*.

Isolates: CE, FL, CR, HT, GS.

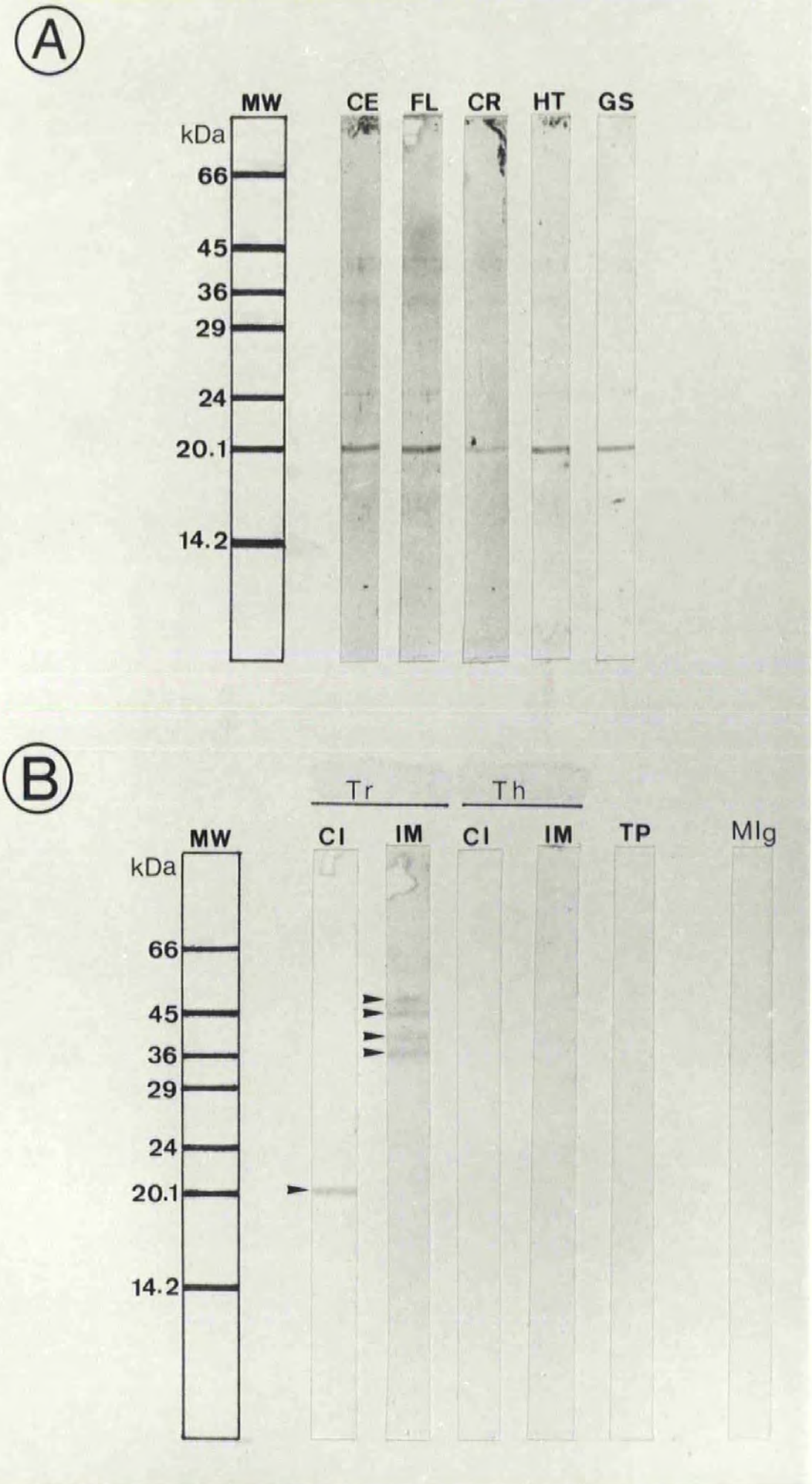
MAB reactivity is observed at ca. 20-21kDa position for all five isolates.

Figure 58 (=B)
Assay of SDS-PAGE preparations of theronts and trophonts of *C.irritans* and *I.multifiliis*, trophozoites of *T.pyriformis*, and mullet immunoglobulin.

Tr = trophont.
Th = theront.
CI = *C.irritans*.
IM = *I.multifiliis*.
TP = *T.pyriformis*.
MIg = mullet immunoglobulin.

MAB reactivity is observed with trophonts only, at ca. 20-21kDa position for *C.irritans* (arrow), and ca. 41, 46, 50, and 56kDa positions for *I.multifiliis* (arrows).

For both figures: molecular weight marker positions shown in left hand lane.


A
MW
kDa
66
45
36
29
24
20.1
14.2
CE
FL
CR
HT
GS
B
Tr
Th
MW
kDa
66
45
36
29
24
20.1
14.2
CI
IM
CI
IM
TP
Mlg

Coomassie and amido staining. The results (Fig. 58) show MAB recognition of a 20-21kDa protein in the *C.irritans* trophont preparation only. However, the MABs reacted with 4 components of the *I.multifiliis* trophont preparation at 41, 46, 50 and 56kDa positions.

3.3.3 MAB reactivity with mullet immunoglobulin

Recognition of mullet immunoglobulin by MABs was investigated. Mullet Ig (Ultrogel fraction), electrophoresed by SDS-PAGE under reducing conditions, was separately probed with 3 MAB clones reactive by ELISA specifically with *C.irritans* (#7, #11, #29) and with 7 reactive by ELISA with both *C.irritans* and mullet Ig (#34 to #40, inclusive). A rabbit antiserum to mullet Ig (RABantiMIg) was also reacted, for comparison. The results (Fig. 59) show strong recognition of a single protein component at 68- 69kDa corresponding in MW to the presumptive H chain of mullet Ig. Only those MABs which were positive to mullet Ig antigen by ELISA gave immunoblot reactivity. None of the MABs recognised the L chain. The rabbit antiserum to mullet Ig (RABantiMIg) also reacted strongly with mullet Ig H chain but gave a weak reaction with the L chain (results not shown).

3.3.4 Teleost species specificity

A cocktail of MABs (# 35, 36, 39), all reactive to mullet Ig by ELISA, were immunoblotted against electroeluted reduced SDS-PAGE preparations of purified mullet Ig, rainbow trout Ig, purified flounder Ig, and mullet whole serum, all from fish in a good state of health. The results (Fig. 60) show strong MAB recognition of the H chain in mullet Ig and weak recognition in mullet whole serum preparations but no recognition of the Ig from either rainbow trout or flounder.

Figures 59 and 60

Immunoblot assays using the MAB directed to the 68-69kDa polypeptide.

Figure 59 (=A)
Assay of SDS-PAGE preparations of mullet immunoglobulin by a panel of ten MAB clones.

MAB clone numbers shown at the top of each lane.

Mullet immunoglobulin (MIg) heavy (H) and light (L) chain positions are shown for reference, in the right hand lane.

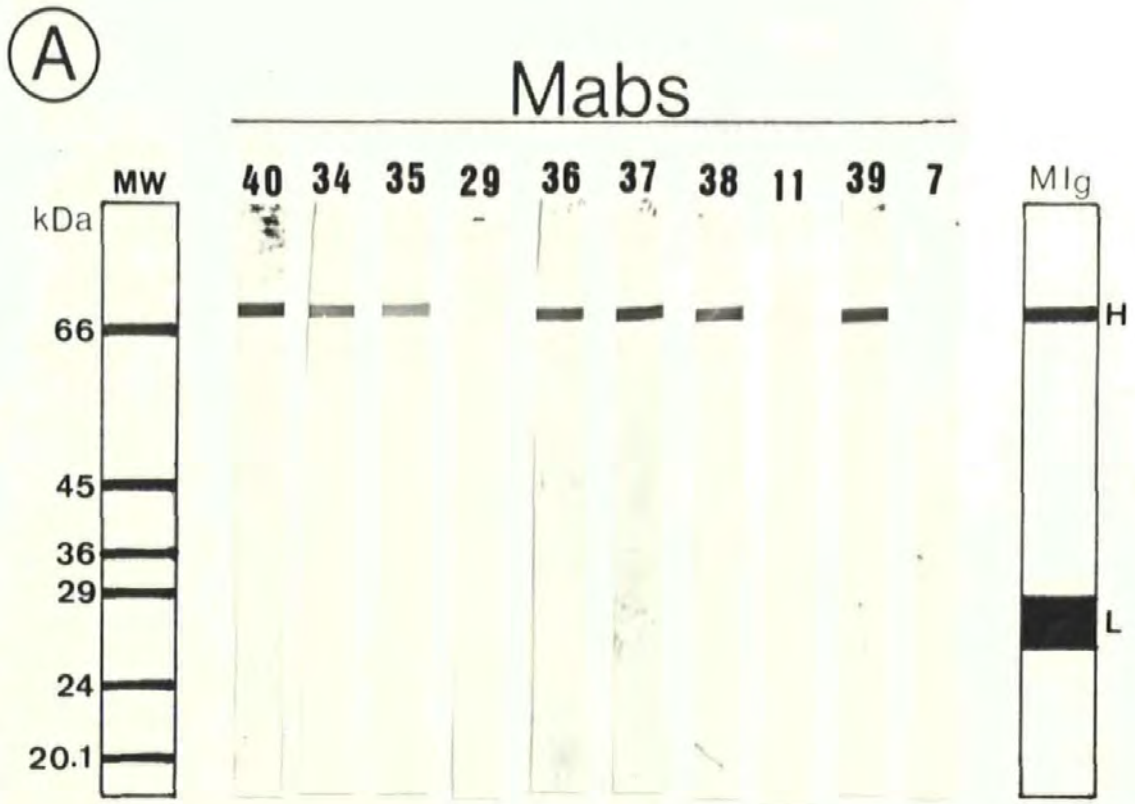
Reactivity is observed for seven of the ten MAB clones.

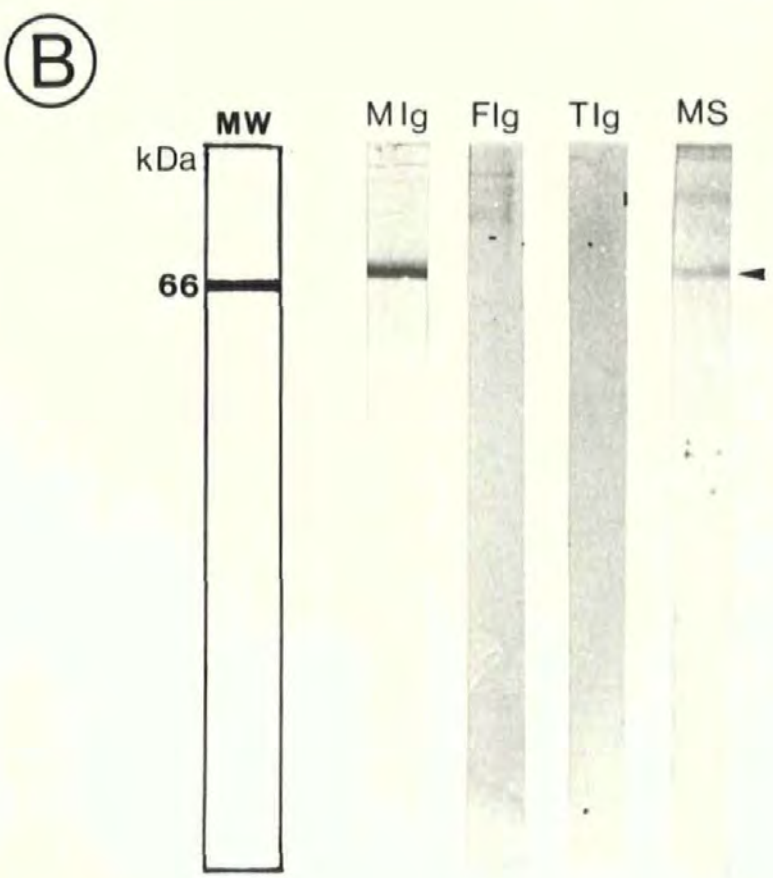
Figure 60 (=B)
Assay of SDS-PAGE preparations of immunoglobulin and serum from three teleost species.

MIg, FIg, TIg, = immunoglobulins of mullet, flounder and trout, respectively.
MS = mullet serum.

MAB reactivity is observed for immunoglobulin and serum (arrow) of mullet only.

For both figures:
molecular weight marker positions shown in left hand lane.


A
Mabs
MW
kDa
66
45
36
29
24
20.1
40
34
35
29
36
37
38
11
39
7
MIg
H
L


B
MW
kDa
66
MIg
FIg
TIg
MS

3.3.5 Recognition of mullet immunoglobulin in C.irritans trophonts

Mouse ascites (#34-2), previously shown to recognise mullet Ig H chain, was immunoblotted separately against reduced SDS-PAGE preparations of trophonts and theronts of both *C.irritans* (CI-GS) and *I.multifiliis*, and against *T.pyriformis* trophozoites. The results (not shown) revealed very weak banding at ≈ 68-69kDa on the *C.irritans* trophont preparation only.

The mouse ascites #34-2 revealed the *in situ* localisation of mullet Ig within the food vacuoles of trophonts of *C.irritans* (CI-GS), as shown by immunogold labelling (Fig. 61).

3.4 Agglutination and immobilisation tests with C.irritans theronts

Neither species of MAB caused agglutination of *C.irritans* theronts over the dilution range tested. Theront deaths, comprising between 52% and 96% of parasites, occurred within 30 min of incubation in all dilutions of ascitic fluids from both MAB species, suggesting this was a non-specific effect. No deaths occurred in the presence of cell culture supernates. Theront agglutination occurred in the presence of the positive control RABantiCI, but not in the serum from a non-immunised control rabbit.

3.5 Summary characteristics

A summary of MAB characteristics and target antigens is shown in Table 25.

Figure 61

In situ localisation of mullet immunoglobulin within food vacuoles of trophonts of *C.irritans*.

Intense immunostaining is observed in areas which are less electron dense (asterisks).

Scale bar = 500nm.

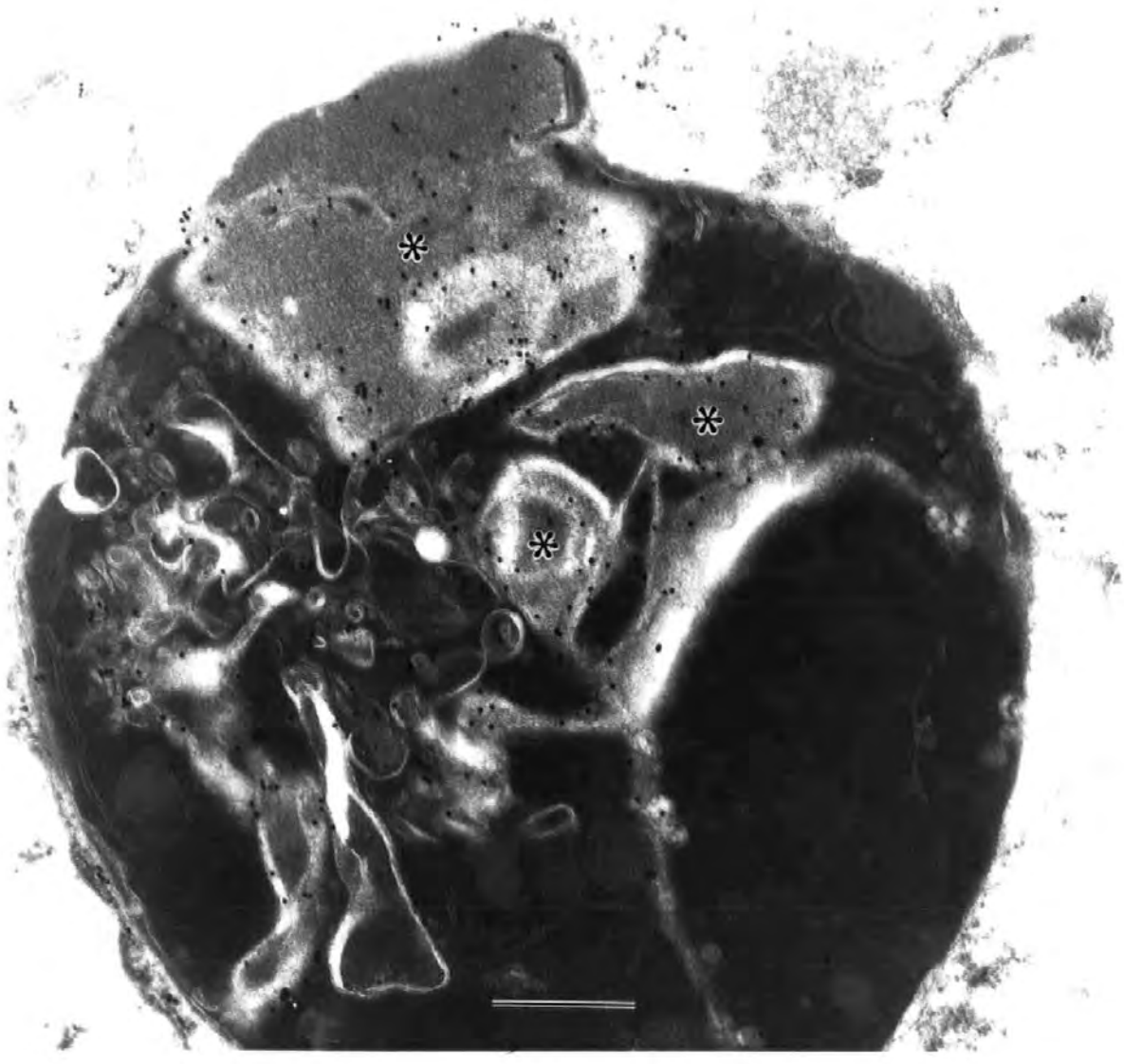


Table 25

Summary characteristics of two murine monoclonal antibodies raised to trophonts of *C.irritans*.

Characteristic		MAB target antigen MW	
		20-21kDa	68-69kDa
Target antigen characterisation		*C.irritans* polypeptide	mullet immunoglobulin heavy chain
In situ localisation by immunogold labelling		no	yes (within food vacuoles of trophonts)
Isotype		IgG1	IgM
Serological activity by ELISA with:	CI trophont	yes	yes (weak)
	Mullet Ig	no	yes
Immunoblot cross- reactivity with:	*C.irritans* theronts	no	no
	I.multifiliis trophonts	yes (weak) · (at 4 high MW positions)	no
	I.multifiliis theronts	no	no
	T.pyriformis trophozoites	no	no
	Mullet Ig	no	yes (H chain only)
	Flounder Ig	nt	no
	Trout Ig	nt	no
In vitro agglutination of *C.irritans* theronts		no	no

nt = not tested.

DISCUSSION

The generation of hybridomas with antibody activity to *C.irritans* trophonts has yielded two distinct monoclonal antibody (MAB) species, reacting with 20-21kDa and 68-69kDa components, respectively. This represents the first record of MABs directed to *C.irritans*.

The MAB directed to the 20-21kDa trophont polypeptide of *C.irritans* revealed this component to be stage specific, its absence from the theronts of both *C.irritans* and *I.multifiliis* suggesting a possible association with growth within the fish host. The MAB did not detect a similar sized trophont polypeptide from *I.multifiliis*, however, recognition of four higher molecular weight components of this parasite was unexpected. This might reflect complexing of the 20-21kDa polypeptide with other molecules, possibly resulting from inadequate reducing conditions during PAGE analysis. Alternatively, it might be relevant that Ghosh and Campbell (1986) reported incidences of MAB binding to unrelated antigens possessing partial epitope identity as well as spurious irrelevant MAB binding to heterologous antigens with high epitope density. Weak MAB reactivity with two major structural proteins of *C.irritans*, namely, 45-46kDa cilia membrane polypeptide and 56kDa tubulin, was not entirely unexpected as MABs frequently cause spurious immunoblot reactions with structural proteins or other major protein components (Ghosh and Campbell, 1986). Although the 20-21kDa component is associated with trophonts of *C.irritans*, it has not been possible so far to locate the molecule *in situ* using immunogold labelling techniques, despite most major parasite organellae being investigated. It is possible that the immunogold procedure may have caused detachment of the 20-21kDa component from the trophont preparation, or altered its structure so as to prevent MAB binding.

It is remotely possible that the MAB was directed to an endosymbiotic bacterium not present in those trophonts prepared for electron microscopy. Such bacteria, termed "xenosomes" (Corliss, 1985), have been reported in *Ichthyophthirius* (Roque *et al.*, 1967; Lobo-da-Cunha and Azevedo, 1988) and in large numbers in the marine ciliate *Parauronema acutum* (Soldo *et al.*, 1974).

The MAB directed to a 68-69kDa component reacted weakly with the trophont stage of *C.irritans* with no recognition of the theront stage of *C.irritans* or either stages of *I.multifiliis*. Of considerable interest was the strong recognition of host mullet immunoglobulin by this MAB. That the MAB was, in fact, directed to mullet immunoglobulin heavy (H) chain, known to be 67-68kDa, was shown from ELISA and immunoblotting. Conclusive evidence comes from the successful application of the MAB as second antibody in ELISA for the detection of mullet antibodies to *C.irritans* and to a digenean parasite, *Cryptocotyle lingua*, the latter assay also developed by the author. The possibility that the 68-69kDa polypeptide also represents a parasite component serving to mimic host immunoglobulin as a protective role, was considered highly unlikely given the cosmopolitan host range of *C.irritans* and the apparent specificity of the MAB which did not recognise immunoglobulins from two other teleost species. Further, the detection of immunoglobulin H chain polypeptide within the food vacuoles of trophonts, with the aid of immunogold labelling, provides an acceptable explanation of its identification in trophont homogenates. That host immunoglobulin is ingested by parasitic trophonts has also been demonstrated for *I.multifiliis* by Cross (1990). It is notable that all hybridoma clones secreting antibodies to mullet immunoglobulin were reactive only with the H chain component. No obvious explanation for this can be found, except that MABs directed to the L chain were missed during the ELISA screening procedure, discussed below. It would

seem likely that mullet L chain is immunogenic to mice given the successful production of murine MABs to L chains of other teleosts, including cod (Pilstrom and Petersson, 1991) and channel catfish (Lobb *et al.*, 1984).

At least thirty two polypeptide components of *C.irritans* were introduced into mice in the trophont preparations. It was surprising, therefore, that only two MAB species were identified and that only one of these was directed to a parasite antigen. Nevertheless, several factors could account for the low yield of MAB species. It is possible that the two MAB-directed polypeptides were the major antigen targets of the mouse's immune response, reflecting either predominant immunogenicity, and/ or greater stability within the mouse peritoneal cavity, favouring immune recognition. The specific properties which make for a strong immunogen are poorly understood, although it seems that quantity is not a crucial factor, given that minor impurities within an antigen preparation have evoked strong antibody responses (Goding, 1983). Low yield of MAB species may also be attributable to the initial selection and screening procedures employed in the study. The cloning procedures may also have selected against MAB-secreting hybridomas, particularly as cloning delays could result in overgrowth by more vigorous non-secreting cells (Galfre and Milstein, 1981). Failure to detect all hybridomas producing MABs to *C.irritans* might be attributed to the ELISA screening procedure, as it is known that some cell components, such as lipids and polysaccharides, do not remain bound throughout this assay, as discussed in Chapter 6. Further, different MABs have been shown to vary in their avidity to target antigen, and are susceptible to minor changes in pH and salt concentration (Goding, 1983; Craig *et al.*, 1989). The buffer conditions used in the screening ELISA may, therefore, have favoured only a proportion of the total MAB repertoire to bind with adequate avidity to target antigens. The hybridoma selection procedure, based

on ELISA reactivity, may therefore have contributed to the limited MAB species as a result of stringent elimination of weakly reacting clones. Such weak antibody reactors would also comprise MABs directed to quantitatively minor antigens of *C.irritans*. Screening of further hybridoma cell populations, currently cryopreserved, might hopefully yield MABs reactive with other components of *C.irritans*.

In the present study, it was not feasible to use the theront stage of *C.irritans* for raising MABs, due to insufficient quantities of parasite material. The production of MABs to the infective stage could, however, prove valuable in the light of evidence for acquired host protection directed against this stage, as suggested earlier (Chapter 4). MABs directed to host-protective antigens would provide a useful immunological tool for the characterisation and isolation of the relevant molecules, as successfully applied for protozoa of medical importance (Yoshida *et al.*, 1980; Kasper *et al.*, 1983; Wright *et al.*, 1983; Musoke *et al.*, 1984).

Although not a direct aim of the present study, mention should be given to the potential value of the MAB directed to mullet immunoglobulin as an immunological reagent for serological studies as well as a candidate probe for T and B cell-like lymphocyte sub-populations in the mullet, as already investigated in other teleosts using MABs (DeLuca *et al.*, 1983; Secombes *et al.*, 1983).

CHAPTER EIGHT

DISCUSSION

This study has contributed new information on the biology of *C.irritans* and its interaction with host which should find application in the design of control and management strategies for cryptocaryosis in both foodfish and ornamental fish culture.

In mariculture, the increasing use of sea cages and other "open" systems presents a serious risk of disease transmission from the local wild fish populations (Shepherd, 1978). The broad host range for *C.irritans* and direct transmission are two factors which underline the vulnerability of cage cultured fish to cryptocaryosis. A first consideration for the control of *C.irritans* in open systems would be the identification of high risk localities and their avoidance at the stage of site selection. The wide salinity tolerance of *C.irritans* would theoretically enable it to exist in all oceans, however, this study has highlighted two environmental factors which are considered to restrict the parasite's global range, namely temperature and light. The inability of *C.irritans* to complete development below 20°C, as shown here and by other workers (Wilkie and Gordin, 1969; Cheung *et al.*, 1979) would limit the parasite to the area encompassed by the 20°C surface isotherm (Fig. 62). It is within this area that temperature would also restrict the parasite's vertical distribution to relatively shallow waters, within the epipelagic zone which extends to around 200 metres in depth; at greater depths the temperature falling below 20°C (Ingmanson and Wallace, 1973). Further evidence that the parasite inhabits only shallow waters comes from observations on the strong influence of photoperiod on two developmental phases within its life cycle: trophont exit from host and excystation. The influence of

Figure 62

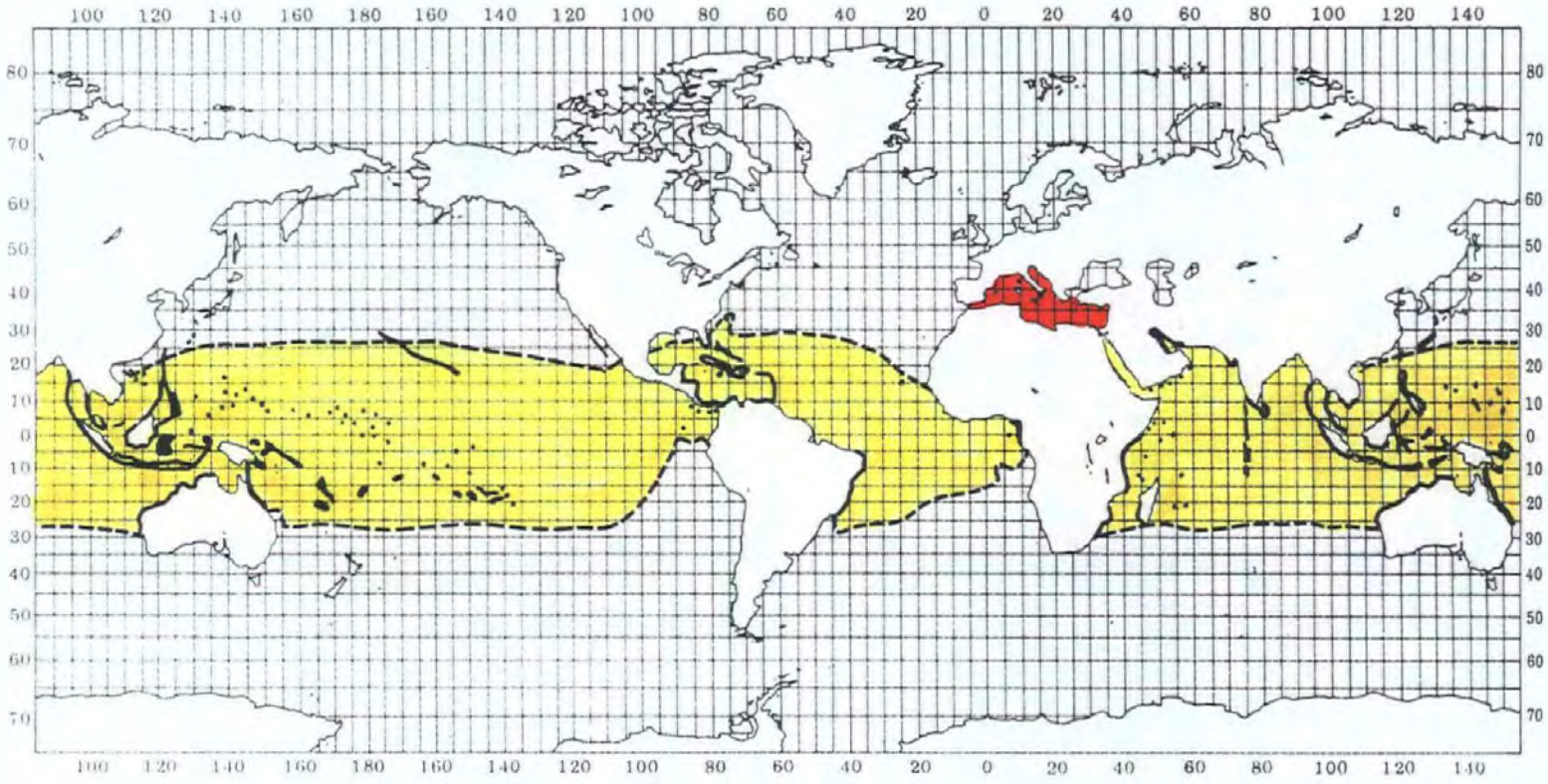
The global 20°C surface isotherm.

Adapted from Nybakken (1988).

Stippled line = 20°C surface isotherm.

Yellow = area with surface isotherm of 20°C or greater.

Red = possible region for the low temperature Mediterranean isolate.


100 120 140 160 180 160 140 120 100 80 60 40 20 0 20 40 60 80 100 120 140
80 70 60 50 40 30 20 10 0 10 20 30 40 50 60 70

photoperiod on the latter phase, and the requirement for a substrate for cyst settlement suggests that *C.irritans* is limited to regions where water clarity is high and the sea floor is exposed to light. The maximum depth of the lighted region ("photic" zone) of the ocean is generally considered to be 100-200 metres (Nybakken, 1988), which again points to *C.irritans* inhabiting the epipelagic zone. The environment for *C.irritans*, here considered to be clear, shallow, sunlit waters above 20°C, is typical of that colonised by hard (hermatypic) corals (Lowe-McConnel, 1987; Nybakken, 1988), and further supports earlier views that *C.irritans* is primarily a parasite of reef dwelling fishes (Chapter 3). The dense population of fishes on the coral reef (Lowe-McConnell, 1987; pers. obs.) would also favour transmission of *C.irritans* given the limited period over which the infective theronts are viable. The present study has confirmed the broad host range of *C.irritans*, which would clearly be beneficial to a parasite associated with a coral reef habitat, typified by a diversity of fish species (Lowe-McConnel, 1987; Nybakken, 1988). This finding provides little prospect for the discovery of food fish species which are innately resistant to *C.irritans*. Furthermore, the potential host range for *C.irritans* extends to fresh- or brackish-water teleosts, such as mollies and tilapia, which can be acclimated to marine environments, as experimentally shown here.' This poses a serious constraint with regards tilapia as there is a growing interest in the use of these salt-tolerant cichlids for cage mariculture, with trials off coastal Southern United States (Watanabe *et al.*, 1990) and Malta (Dr Agius, Ministry of Agriculture and Fisheries, Malta, pers. comm.). It would therefore seem advisable to assume that all habitats which satisfy the environmental conditions outlined above, present a potential risk of cryptocaryosis to all species of farmed fish.

Confirmatory evidence for *C.irritans* in natural waters can only realistically be

achieved by the detection of parasitic trophonts in the wild fish population, however, this stage represents a relatively brief period within the parasite's life cycle, being shown here to average 84 hours. Effective detection of *C.irritans* in wild fish would be further exacerbated as the development of protective immunity against the parasite, demonstrated here in mullet, would result in low or zero infections. This could possibly explain the low prevalence of *C.irritans* in the few recorded cases in wild fish (Laird, 1956; Wilkie and Gordin, 1969). The inability to demonstrate *C.irritans* in wild fish could therefore result in an area being erroneously designated risk -free. Confirmation of presumptive risk areas might be achieved with the aid of sentinel fish which are exposed to the local waters. Such an approach has been applied to the detection of other parasites, notably the use of mice for the detection of aquatic cercariae of schistosomes (Jordan, 1985). The brief parasitic stage of *C.irritans* would necessitate inspection of fish sentinels at least every three days; low infection levels being detected by transferring exposed fish to trophont harvesters and enumerating cysts.

In assessing potential risk areas for *C.irritans*, consideration must be given to the introduction of the parasite into waters within the 20°C isotherm but at sites naturally unsuitable for its establishment. This could result from aquaculture practices, as shown for other fish diseases (Kabata, 1983). For example, sea cages, which house a high density of fish, may provide ideal conditions for the transmission of *C.irritans* in deep waters where the natural substratum occurs below the photic zone. Transmission within the sea cage would be aided by the parasite's ability to encyst on unnatural substrates, as shown here for wood and steel, both these materials being used for cage construction (Crook, 1985; Moffatt, 1991). This situation could lead to infection of the wild fish population which are often attracted to sea cages for

food and shelter (Carss, 1990; pers. obs.), with the risk of these fish establishing a reservoir of *C.irritans*. Evidence that the parasite's geographical range may already extend to sub-tropical marine environments comes from the recent report of a *Cryptocaryon*-like organism from the Eastern Mediterranean (Diamant *et al.*, 1991). Whether this isolate represents a new species or an adaptation of *C.irritans* to more temperate conditions is not known. Studies presented here on the behaviour, morphology, molecular composition and antigenicity of tropical isolates of *C.irritans* now provides a basis for assessment of the Mediterranean form.

Prior to the present study, there has been no method available for the assessment of chemotherapeutic agents against cryptocaryosis, although the use of chemicals has provided the major approach to disease control (Herwig, 1978; Andrews *et al.*, 1988). Chemical treatments have involved the application of a wide range of products including those intentionally used against fish parasites in general, such as copper, malachite green, and formalin (Nigrelli and Ruggieri, 1966; Wilkie and Gordin, 1969; Kingsford, 1975; Herwig, 1978; Moe, 1982; Rasheed, 1989) and drugs originally developed for medical and veterinary use, including the quinines and antibiotics (De Graaf, 1973; Kingsford, 1975; Herwig, 1978, 1979; Huff and Burns, 1981). The selection and evaluation of these compounds against *C.irritans* has been based largely on empirical grounds, with efficacy assessed from aquarium or mariculture observations under non-standardised conditions. The establishment of a system for the laboratory maintenance of *C.irritans* now provides an effective method for the screening of drugs against the parasite; the controlled conditions permitting direct comparisons between different drugs and the determination of therapeutic doses. The pilot screen developed here for Oomed™ represents the first detailed evaluation of a commercial chemical treatment against *C.irritans*, and has already

revealed the limitation of this product, a newly formulated drug which was claimed to destroy the cyst stage (Dr. Pool, Tetra Ltd., pers. comm.). The significant differences observed between the cysts and other free-living stages of *C.irritans* in their susceptibility to Oomed™ leads to the recommendation that future candidate drugs are screened against all life cycle stages. It is clear from studies here that a search for more effective drugs against cryptocaryosis is urgently required, although in view of the high costs of drug development it seems inevitable that novel formulations will be adapted from those designed for medical and veterinary applications. Several promising compounds have been produced, including toltrazuril, an anti-coccidian (Mehlhorn *et al.*, 1984), shown to destroy trophozoites of *I.multifiliis* (Schmahl *et al.*, 1989). Other candidate drugs might include those effective against the mammalian ciliate pathogen, *Balantidium coli*, such as ampicillin and paromomycin (Manson-Bahr and Apted, 1982; Farthing and Rolston, 1990). Reports of success using the anti-malarial quinines against *C.irritans* (Kingsford, 1975; Herwig, 1978) warrants the evaluation of novel quinine derivatives which inhibit protozoal nucleic acid synthesis (Gutteridge and Coombs, 1977) as well as heterologous compounds such as the phenanthrene methanols (Horton, 1988). Pilot studies undertaken here on the polypeptide composition of *C.irritans* provides a basis for the future analysis of their biological functions. Identification of key molecules, for example enzymes, could obviate the need for empirical drug screening by enabling the selection of existing drugs with known target action. This approach would benefit from our increased understanding of the molecular action of anti-protozoal drugs in addition to advances in rational drug design (Hart *et al.*, 1989). The incorporation of drugs with feeds, as recently assessed using malachite green against ichthyophthiriosis (Schmahl *et al.*, 1992) could enable the systemic application of novel chemicals for controlling

C.irritans. However, as drugs with more selective action are employed against *C.irritans*, consideration must be given to the emergence of drug-resistance, particularly against antibiotics and quinines, in the light of extensive problems with quinine -resistant forms of malaria (Kean, 1979). It is therefore anticipated that drug-screening may usefully be applied for the surveillance for resistant forms of *C.irritans*.

The demonstration of an acquired protective immunity in mullet to *C.irritans* following a single exposure, possibly with immunological memory, provides some optimism for the development of a vaccine against cryptocaryosis. An effective vaccine could obviate many of the disadvantages of chemical control methods, particularly ichthyotoxicity (Herwig, 1979; Williams and Wootten, 1981) and environmental damage (Alderman, 1982). This study has contributed fundamental information which would assist in vaccine development programmes. The characterisation of several major polypeptides of *C.irritans* could eventually be linked with identification of their antigenic properties, enabling selection of parasite components which stimulate a protective host response; this information being crucial to the rational development of sub-unit vaccines (Horne and Ellis, 1988). The successful application of monoclonal antibody technology to *C.irritans*, achieved here, provides a means by which protective antigens could be isolated, for example using MAB- linked affinity chromatographic techniques (Sikora and Smedley, 1984), enabling their individual evaluation in conferring host protection. The possibility that host protective antigens of *C.irritans* are stage-specific has been suggested in the present study, as based on SDS-PAGE analyses, and underlines the future need to compare both trophont- and theront- derived antigens as candidate vaccines. In support of this, differences in levels of host protection have been demonstrated between these two stages in *I.multifiliis* (Burkart *et al.*, 1990). Clearly, however, an

effective vaccine against the theront stage would be most beneficial by preventing the establishment of infection, this concept being similar to that envisaged for the sporozoite vaccine against human malaria (Cochrane *et al.*, 1980; McGregor, 1985). The demonstration here of a humoral immune recognition of *C.irritans* antigens presented by intraperitoneal injection offers hope for vaccine delivery via this route, which is widely considered the most effective method of artificial immunisation (Ellis, 1988). The ability of intraperitoneal administration of antigen to confer some protection against an epithelial dwelling ciliate is supported by work on *I.multifiliis* (Goven *et al.*, 1980). Despite these encouraging findings, two major drawbacks are foreseen which are likely to restrict the development and efficacy of vaccines against cryptocaryosis. First, observations here that some fish develop incomplete protection to *C.irritans* even after repeated exposure to immunizing doses of theronts, suggests that *C.irritans* could persist in a population of vaccinated fish. Incomplete protection is not unusual, being also reported in fish vaccinated against bacterial diseases (Hastings, 1988; Smith, 1988). The potential for an asymptomatic carrier status in vaccinated fish could lead to renewed outbreaks of cryptocaryosis in situations where vaccine protection declines (=loss of immunological memory), or as a result of other conditions known to compromise immunocompetence, such as stress, vitamin deficiencies, pollutants, or antibiotic therapy (reviewed by Ellis, 1981, 1988). The possibility that outbreaks could recur following the addition of new stock which are immunologically unprotected against the parasite necessitates the need to vaccinate all fish before introduction to the system. The second limitation is the difficulty in procuring significant quantities of parasite material for vaccine production. Laboratory passage of *C.irritans* through mullet has enabled small scale immunisation studies, presented here, but this work is time-consuming and requires a continual

supply of susceptible fish. Furthermore, it was not possible to produce sufficient theront material for immunisation studies with this stage. With regards trophonts, contamination with host material, including ingested components such as immunoglobulin, presents difficulties in obtaining pure preparations. Furthermore, maintenance of an isolate was achieved over a limited number of passages, with eventual loss, attributable to senescence; the regular need for replacement isolates introduced the possibility of antigenic variations. *In vivo* maintenance of the parasite through fish hosts is evidently not a practical approach to obtaining material for vaccines. Some of these problems could be overcome by the ability to culture *C.irritans in vitro* as well as the capability to cryopreserve viable stocks of strains, however, it may be significant that neither of these technologies have been successfully applied to *I.multifiliis*, despite claims to the contrary (Beeler, 1980). The problem could be resolved by the use of heterologous vaccines against cryptocaryosis, similar to the use of *T.pyriformis* to immunise fish against ichthyophthiriosis (Goven *et al.*, 1980, 1981a; Wolf and Markiw, 1982; Dickerson *et al.*, 1984). However, the absence of cross-protection by natural infection of mullet between *C.irritans* and its closest relative, *I.multifiliis* provides little optimism for this approach. The development of synthetic vaccines against *C.irritans*, with the aid of recombinant technology, is not considered to be realistic at present, being expensive and unreliable; it is significant that no commercial synthetic vaccine exists for any eucaryotic pathogen.

New information presented here concerning the biology of *C.irritans* should provide a better understanding of the requirements of an integrated control and management programme for cryptocaryosis. One important finding of the present study is the acute nature of cryptocaryosis, with host deaths occurring within five days of a primary infection. The rapidity of death is more similar to that resulting from

bacterial infections (Munro, 1982) rather than eucaryotic diseases in general, and contrasts with ichthyophthiriosis in which host survival may extend beyond 20 days following high level infections (Hines and Spira, 1973a). Clearly, measures used to combat chronic parasitic infections are not appropriate for cryptocaryosis, and the need exists for management based on prevention rather than cure. Several recommendations are proposed for the routine management of fish stocks, which will reduce the risks of exposure to *C.irritans*, namely identification of sources of infection, movement restrictions, quarantine procedures, and surveillance. With regards sources of infection, this study reveals the importance of the cyst stage which is capable of settlement on a variety of substrates. Fish managers must therefore consider materials such as filter media, water pipes and tank flooring as potential sites for cyst adherence which will require disinfection following known or suspected exposure to *C.irritans*. Demonstration here of the long duration of the cyst stage of *C.irritans*, for up to 35 days, combined with its resistance to chemical treatments, presents the major problem in terms of control measures in that single chemical applications will not eradicate the parasite. Two procedures are, however, recommended to eliminate viable cysts: either continual treatment using chemicals effective against the theront stage, or isolation of contaminated materials away from fish, both these measures requiring 35 days, this being the minimum period to ensure cysts are no longer viable. With regards aquaria, the present study has shown the possibility of *C.irritans* being transferred between tanks via decorative items including non-living hard corals and shells. A serious risk comes from popular decorative rock fragments, purchased for their attached invertebrate colonies ("living rock"), which therefore cannot be sterilised prior to introduction into aquaria; these too requiring 35 days isolation.

Dissemination of *C.irritans* via infected fish is considered a potential hazard and

for this reason it is recommended that regulations are imposed on the movements of fish which have been exposed to the parasite. Carrier status in fish stocks may be difficult to assess, as discussed above. However, the serological detection of specific antibodies to *C.irritans* could prove a useful indicator of recent infection in suspected stock. Quarantine methods have been widely applied as preventative measures against fish diseases (Kabata, 1985; Roberts and Shepherd, 1986; Andrews *et al.*, 1988). Knowledge of the time base for the life cycle of *C.irritans* allows effective quarantine procedures. In view of the brief parasitic phase, lasting less than five days, and with no evidence of a reproductive stage in the fish, it is suggested that fish are quarantined for six days. However, observations here that theront release can occur within 3.5 days after encystment, reveals the risk of reinfection during quarantine. It is therefore essential that fish are regularly moved to clean holding facilities, at least every two days. This system has also proved useful for eliminating *C.irritans* from certain host species, particularly coral reef fishes, which are sensitive to chemical treatments. Where serial movement is impractical, effective levels of chemotherapeutic agents must be maintained in an attempt to destroy any excysted theronts; although judging from the drug trial undertaken in the present study, destruction of all theronts cannot be assured.

The existence of *C.irritans* within the aquatic medium, as the free-living trophont and theront, although shown here to be brief, not exceeding eight and 24 hours, respectively, provides a possible route of infection via the water. This would be an important source of transmission in open systems as well as in intensive systems which rely on seawater pumped from local waters known to be endemic for the parasite. With the latter system, certain procedures can be undertaken to render the water safe. The use of mechanical (sand) filtration has been reported effective in

removing spores of *Myxosoma cerebralis* (Herman, 1970); for *C.irritans*, morphometric analyses of theronts, the smallest life cycle stage, indicates the need for a filter pore diameter not exceeding 22μm. It would be beneficial, where possible, to store freshly pumped seawater for 24 hours before exposure to fish, in order to kill the theronts.

Given the rapidity with which lethal populations of *C.irritans* can build up, as observed here from aquarium infections, the regular surveillance for *C.irritans* (and other diseases) is recommended for any population considered at risk, and should in any case form part of the general husbandry programme (Brown and Gratzek, 1980). The onset of inappetence, abnormal swimming, or body "flashing", as observed here with experimentally infected mullet, should alert the fish manager to consider cryptocaryosis amongst the possible underlying causes.

In summary, it is considered that the control of cryptocaryosis, for the immediate future, will rely heavily on preventative measures, based on recommendations given here, supported by chemical treatment regimes. It is envisaged that improvements in control will be influenced by the economic impact of *C.irritans* upon the ornamental and foodfish industries, as well as advances in chemical and vaccine control methods for protozoa of medical and veterinary importance. Despite the clear advantages of vaccination over chemotherapy, the absence of a commercial vaccine for eucaryotic diseases of fish (Houghton *et al.*, 1988) and for human protozoal diseases (Liew, 1989) offers little optimism for the production of an effective vaccine against *C.irritans* in the foreseeable future.

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APPENDIX

1. BUFFERS AND REAGENTS

PBS pH 7.6

Na_2HPO_4	1.28g
$NaH_2PO_4.2H_2O$	0.156g
NaCl	8.5g
Dist. H_2O	1 litre

PBS-J

KH_2PO_4	2g
Na_2HPO_4	8.56g
NaCl	4.5g
Dist. H_2O	1 litre

Adjust pH to 7.4 and filter sterilise.

ELISA coating buffer pH 9.6

Na_2CO_3	1.59g
$NaHCO_3$	2.93g
Dist. H_2O	1 litre

ELISA incubation buffer

PBS, pH 7.6	1 litre
Tween 20	0.5ml

ELISA wash solution

NaCl	45g
Tween 20	2.5ml
Dist. H_2O	5 litres

ELISA substrate buffer

Na_2HPO_4	7.19g
Citric acid	5.19g
Dist. H_2O	1 litre

ELISA substrate

OPD*	100mg
Methanol	10ml

*ortho-phenylenediamine.
Store in the dark, stable for < 1 week.

ELISA substrate (working solution)

OPD/methanol	1ml
H_2O_2 (6%)	0.05ml

Prepare within 15 min of use.

2. CULTURE MEDIA

Monoclonal culture medium (MCM)

RPMI	100ml
FCS*	10ml
Pen/Strep, (10,000 i.u./ml)	1ml
L-glutamine, (11mg/ml)	1ml
Na pyruvate (200mM)	1.25ml

* heat inactivated, 56°C, 30 min.

ABBREVIATIONS

DAB	diamino benzidine
DMSO	dimethyl sulphoxide
ELISA	enzyme linked immunosorbent assay
E-S	enzyme-substrate
FCA	Freund's complete adjuvant
FCS	foetal calf serum
FIA	Freund's incomplete adjuvant
FITC	flourescein isothiocyanate
FS-SW	filter sterilised seawater
HAT	hypoxanthine, aminopterin and thymidine
HT	hypoxanthine and thymidine
IFAT	indirect fluorescent antibody test
Ig	immunoglobulin
i.p.	intraperitoneal
kDa	kilodalton
MAB	monoclonal antibody
MABantiMIg	monoclonal antibody to mullet immunoglobulin
MABantiCI	monoclonal antibody to *C.irritans* (trophonts)
MCM	monoclonal culture medium
MIg	mullet immunoglobulin
MW	molecular weight
NC	nitrocellulose
OPD	ortho-phenylene diamine
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
%PEI	percentage of parasites establishing infection
p-L-lys	poly-L-lysine
ppm	parts per million
RABantiCI	rabbit antiserum to *C.irritans* (trophonts)
RABantiMIg	rabbit antiserum to mullet immunoglobulin
RATantiTP	rat antiserum to *T.pyriformis*
RT	room temperature
SDS	sodium dodecyl sulphate
SG	specific gravity
SW	seawater
TL	total length
UV	ultraviolet