THE IMMUNE RESPONSE OF PLAICE WITH PARTICULAR EMPHASIS ON ANTIGENIC STIMULATION BY TISSUE PARASITES.

BY

Barrie J. Cottrell, B.Sc. (London)

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The University of London, (External).

SUMMARY

Antibody responses to soluble and cellular antigens have been demonstrated in juvenile and adult plaice, Pleuronectes platessa L. A relatively strong precipitating antibody was produced to calf serum, but not to bovine serum albumin. The onset, and magnitude of antibody production was temperature dependant and physico-chemical characterisation of plaice precipitins indicated they were of the IgM class. Plaice also produced haemagglutinins, following injection of rabbit erythrocytes, with a suggestion of a heightened response upon secondary stimulation with the antigen.

An evaluation was made of the humoral immune response of the fish to three tissue parasites; Rhipidocotyle johnstonei and Cryptocotyle lingua, the metacercariae of which develop in the musculature and connective tissue of plaice; and Trypanosoma platessae, a haemoflagellate. Elevated levels of β-globulin were detected in the sera of T. platessae infected plaice, and it is suggested that this may be associated with antibody secretion to the parasite. Further studies were made on the biology of T. platessae, including efforts to maintain the parasite, by passage, in the laboratory.

Natural and experimental infections with the metacercariae of C. lingua and R. johnstonei induced a temperature dependant, precipitin response in plaice. Application of the indirect fluorescent antibody technique suggested that the functional antigens of both parasites included somatic elements, however the antigens of R. johnstonei were also clearly associated with secretory tissues.

A 'natural' antibody, present in the sera of the majority of test plaice, was found to precipitate in agar gel with an antigenic extract of the nematode, Proleptus obtusus. This was considered of particular

interest, as P. obtusus is a parasite specific to the dogfish and is unknown in plaice. The precipitin, first suspected of being non-specific C-reactive protein, was later characterised as 19S, IgM.

The nature of serological and immunological changes in fish subjected to immunisation and parasitic infection was reviewed and discussed in relation to the findings of the present project.

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INTRODUCTION

The problems of disease in fish farming have been discussed in several recent symposia, the proceedings of which have been edited by Snieszko (1970, a) Taylor and Muller (1970) and Mawdesley-Thomas (1972). It is indicative that the main topics of these meetings related to diseases in freshwater systems, as the farming of these fish is long established. In contrast, the study of parasitic disease in the marine environment has been neglected, but, with the recent advent of marine fish farming in the U.K., research in this field is of some urgency. Further, Swift (1970), in discussing the future of marine aquaculture listed disease as one of the significant areas for research in the future.

At the start of the present work the plaice, Pleuronectes platessa L., was being used by the White Fish Authority in feasibility trials and for this reason was selected as a suitable host for study. Matthews (1968), and Mackenzie and Gibson (1970) had previously drawn attention to the importance of certain parasitic diseases in young plaice. The object of the present study was to make a critical investigation of the plaice's immune response to selected parasites. Although there is now much information available on the fundamental principles of fish immunology, reviews on the subject being presented by Clem and Leslie (1969) and Cushing (1971), there has been little emphasis on the role of immunity in parasitic disease. Of particular note are the reports of Nigrelli and Breder (1934) and Hines and Spira (1974), who demonstrated acquired protective immunity in fish to parasitic agents, and that of Harris (1972) who characterised both the antigen and antibody involved in a fish host/parasite system.

In the present instance, three tissue parasites of plaice were selected for study, as it was considered these would provoke a more pronounced host immunity than external or intestinal parasites. These parasites were the Digenea Cryptocotyle lingua and Rhipidocotyle johnstonei, and the haemoflagellate Trypanosoma platessae. The biology and life cycles of C. lingua and R. johnstonei have been described by Stunkard (1930) and Matthews (1968), respectively. The metacercariae of both species occur in the muscles and connective tissue of the host, but they differ significantly in that R. johnstonei does not secrete a cyst and is progenetic in the plaice.

Prior to investigating these selected models, however, it became necessary to study some of the basic features of the plaice's serology and immunology. An evaluation of the fish's humoral response to specific antigen, under controlled laboratory conditions, assisted in the interpretation of the more complex host/parasite systems. The latter demanded different experimental approaches which were largely dictated by the availability of the parasite species. Of the two digeneans, experimental infections were only possible with C. lingua, a source of cercariae being available from Littorina littorea. Studies on R. johnstonei were restricted to naturally infected, wild '0' group plaice. It was demonstrated that during the six months after initial infection with R. johnstonei, increasing numbers of the plaice population produced specific antibody to the parasite. In studying both metacercarial infections, particular attention was given to the identification of the antibody-antigen complexes, and to the use of the fluorescent antibody technique in labelling parasitic antigen.

The low levels of T. platessae infection in wild fish and its apparent inability to multiply in the plaice, made routine collection and laboratory maintenance of this parasite impracticable. Consequently a full experimental analysis of immunity to T. platessae was not possible, but certain aspects of the serology of infected plaice suggested a seasonal secretion of antibody to this parasite, probably mediated by temperature.

Investigations on the potential cross-reactivity of plaice sera to a number of helminth antigens, revealed a serum precipitin to a nematode, Proleptus obtusus. This was considered to be of particular interest as P. obtusus is an intestinal parasite of the dogfish and is not known to infect plaice. Further, physico-chemical studies revealed this precipitin to be an antibody and not a non-specific factor such as C-reactive protein, and the significance of this is discussed.

To summarise, the aims of the present research were to provide fundamental information on the immune response of plaice, especially in the disease situation, where relevant data may prove vital to the future prospects of commercial marine cultivation.

Chapter 1

REVIEW

Past work is discussed under two headings, namely fish immunology and marine trypanosomes. The latter is considered relevant in view of investigations presented here on Trypanosoma platessae in the plaice, and further, as far as is known, there has been no recent review of the literature concerning trypanosomes of seawater fish.

FISH IMMUNOLOGY

Particular emphasis has been placed in this section on the phylogenetic development of fish immunoglobulins, and on the role of the immune system in disease.

The ability of fish to produce antibody to a wide variety of antigens has been known for some time and initial studies in this field have been reviewed by Good and Papermaster (1964) and Smith et al (1966). It is evident from these that up to the mid 1960's little was known of the physico-chemical nature of fish immunoglobulins. Clem and Leslie (1969) have discussed the progress in this aspect of fish immunology. The more accurate analysis of piscine antibodies has allowed comparisons with the well defined classes of mammalian immunoglobulins, with the general concept emerging of only one immunoglobulin class being present in fish. This immunoglobulin has heavy chains of the μ- type and in this and other criteria represents an IgM class.

The subclass Cyclostomata, comprised of hagfish and lampreys, represents a group of vertebrates phylogenetically more primitive than the cartilaginous and boney fishes (figure 1). Good and Papermaster (1961) and Papermaster et al (1964) failed to demonstrate an immune response in the hagfish, Epatretus stoutii, held at 10°C, to Brucella

Evolution of Fish (after Romer)

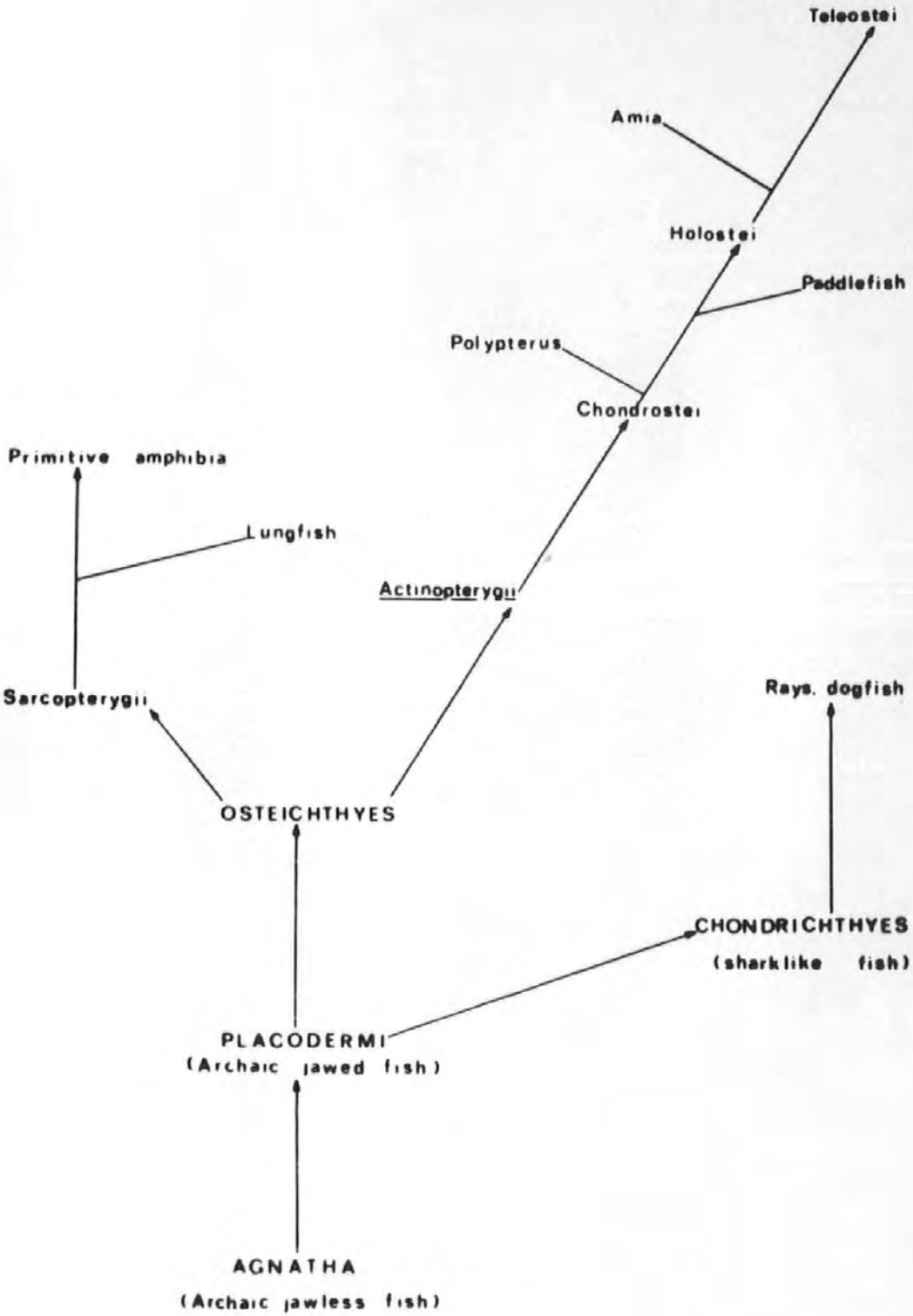


Figure I. To show the phylogenetic relationships and general evolution of the Teleostei, (based on Romer 1967).

abortus, BSA, haemocyanin and viral antigens. Further, they were unable to detect an antibody response to T_2 phage antigen in the more highly evolved lamprey. Finstad et al (1964) and Good and Finstad (1964) established the presence in hagfish, of lymphocytes, suggesting the existence of a mechanism for antibody production. Although they could not induce a humoral response to BSA, and bovine gamma-globulin, they did demonstrate agglutinating antibodies to the Brucella antigens.

Thoenes and Hildemann (1969) showed that hagfish produced antibodies to keyhole limpet haemocyanin (KLH) at 20°C. The antibody was in the macroglobulin fractions of the serum and had a sedimentation coefficient of approximately 28S. Clem and Leslie (1969) have suggested that the hagfish immunoglobulin may be a dimer of the 19S IgM molecule found in the higher fishes. Neutralising antibodies to the bacteriophage f2 were found in the 6.6S and 14S fractions of immunised lamprey serum by Marchalonis and Edelman (1968). Thoenes and Hildemann (1969) reported typical graft rejection in hagfish at 19°C. Allografts, which were rejected after approximately 72 days followed the same rejection mechanism as higher vertebrates. Moreover, the decreased rejection time for second and third set grafts pointed to an anamnestic response. Finstad and Good (1964) induced systemic anaphylactic shock when they repeatedly injected an adjuvant-antigen emulsion into hagfish. They also reported delayed hypersensitivity to tuberculin antigen, but did not substantiate this with further histological and cell transfer studies.

Preliminary reports of antibody production in elasmobranchs by Clem and Sigel (1963, 1965) and Papermaster (1965) indicated the presence of high molecular weight antibodies, the activity of which could be destroyed by 2-mercaptoethanol (2-ME). Marchalonis and Edelman (1965) were able to induce a 17S antibody after immunisation

with Limulus haemocyanin in Mustelus canis. They also noted a 7S protein in the serum, which, although not possessing antibody activity, cross-reacted with the 17S protein in immunodiffusion tests. Both the 17S and 7S proteins were antigenically identical and appeared to possess light and heavy polypeptide chains comparable to those of higher vertebrates. They suggested that M. canis had only one class of immunoglobulin resembling the IgM of mammals.

Clem and Small (1967) and Clem et al (1967) induced 7S immunoglobulins in lemon and nurse sharks after prolonged periods of immunisation. 17S and 7S immunoglobulins, antigenically identical, were demonstrated in the leopard shark Triakis semifasciata by Suran et al (1967) after intense stimulation with a viral antigen and KLH.

The most primitive elasmobranch studied to date Heterodontus francisci was shown by Frommel et al (1971) to possess 18.5S and 7S immunoglobulins after immunisation with Brucella abortus antigens. Although H. francisci possessed two antibodies of differing molecular weights, like other elasmobranchs, these were confined to one class, by virtue of the heavy chain, which in both cases resembled the μ-type chain of mammals. Upon reduction with cysteine the 18.5S immunoglobulin gave 7S fragments that still retained agglutinating activity to B. abortus.

In contrast to this Thomas et al (1972) were able to demonstrate antibody to T2H phage antigen, in Platyrhinoides triseriata, in the macroglobulin serum fraction only. No antibody activity could be detected in the smaller protein molecules in the immune serum, but this may have resulted from the short immunisation period used.

More recent studies of elasmobranch immunoglobulins have centred upon a more detailed analysis of physical structure and function. Voss et al (1971) investigated the functional binding properties of a

'natural' shark antibody (IgM) against a 2, 4-dinitrophenol antigenic determinant, and found that the shark IgM had low affinity for the tested ligands. Klapper and Clem (1972) produced 7S IgM from pentameric shark 19S IgM, following reduction and alkylation. They considered that 5 disulphide bonds were reduced in the cleavage of the 19S molecule, and the bonds were labile, confined to the H-chains and involved only one cysteine molecule per H-chain.

Sledge et al (1974) determined in nurse shark the terminal 28 amino-acids of the L- and H-chains from specifically induced antibody. Stanton et al (1974) noted a relatively constant pentapeptide sequence in the variable (V) regions of shark, bird and mammalian light chains. They considered that this constant region must serve some important function to have conserved its sequence of amino-acid residues over 400 million years of phylogenetic development.

Limited studies of the immune competence of the primitive boney fish, namely the holosteans and chondrosteans, have been made (figure 1). The holostean, Lepisosteus platyrhincus, was investigated by Bradshaw et al (1969). Litman et al (1971, a) isolated from Amia calva serum, 13.6S and 6.3S antibodies using B. abortus as antigen. Comparisons of the two revealed their basic similarity and close relationship to human IgM, both the low and high molecular weight proteins having slow beta electrophoretic mobility. Litman et al (1971, b) made a more detailed study of the subunit nature of the A. calva, immunoglobulins, from which they postulated the 13.6S antibody was a tetrameric form of IgM. This, in turn, could be reduced to monomeric units, the H- and L-chains of which resembled human μ and κ chains respectively. The H- chains of the tetramer and monomer were compared antigenically and found to be identical, thus confirming a single class of immunoglobulin, IgM, in this species.

The chondrosteans appear to manifest similar features to the holosteans. Polyodon spathula produced macroglobulin antibody with beta mobility after Fish et al (1966) injected them with Brucella and Salmonella antigens. They noted, however, that even after prolonged immunisation no shift to low molecular antibody occurred.

The ability of teleost fish to produce agglutinating antibody to bacterial infections has been known for some time. Smith (1940) and Bisset (1947, 1948 a) utilised the ability of teleosts to produce agglutinins to bacteria, to study basic features of the immune response of fish. Clem and Sigel (1963) produced relatively low agglutin titres following immunisation of Haemulon albium with Salmonella paratyphi B. organisms.

Production of agglutinins to Salmonella paratyphi H. antigen A was noted by Harris (1973). After 75 days, at 18°C, fish had an optimum antibody titre of 1:512, as opposed to an optimum of 1:128 at 5°C. A similar effect of temperature on production of bacterial agglutinins was noted by Nybelin (1968) in Tinca tinca injected with Vibrio anguillarum. From a temperature range of 10 - 25°C the response was closely related to the ambient temperature. At 5°C, however, dace did not secrete any detectable antibody. Upon transference to water at 20°C these fish exhibited high agglutinin titres after 24 hours.

Lewis and Savage (1972), using the indirect fluorescent antibody technique, detected anti-Aeromonas liquefaciens immunoglobulin in three teleost species. Schachte and Mora (1973) indicated that the warm water species Ictalurus punctatus, produced high antibody titres after injection with Chondrococcus columnaris. There were no significant differences in antibody titre using the sub-cutaneous and intra-muscular routes of injection. Fujihara et al (1964) demonstrated a response in the trout to C. columnaris similar to that of the channel catfish.

Evidence for an anamnestic response in sockeye salmon to a bacterium, causing salmonid kidney disease, has been given by Evelyn (1971). He found a concomitant increase in serum proteins of gamma and beta electrophoretic mobility, with onset of antibody production to the bacterium.

Agglutinins have also been induced in teleosts to particulate antigens other than bacteria. Isohaemagglutinins have been demonstrated in the goldfish by Hildemann (1956, 1958) and in rainbow trout, and sockeye salmon by Ridgway (1962), and Ridgway and Klontz (1960).

There are relatively few reports in the literature of neutralising antibody to viruses. Uhr et al (1962), Papermaster (1964), Clem and Sigel (1963) and Wolf and Quimby (1969) have shown that freshwater and marine teleosts produced antibody in response to infection with viral particles.

Teleosts have shown the ability to respond to soluble antigens by the production of precipitating antibodies. Ridgway et al (1966) reviewed early reports of precipitin production in teleosts. Post (1963) found precipitins in the sera of trout immunised with a protein extract from a bacterium. Clem and Sigel (1966) induced weak precipitins in margates and grey snappers after repeated injection with BSA. They also reported an anamnestic response to BSA in the marine teleosts, but not in the 'more primitive' gar. Margates and snappers, whilst lacking gamma globulin, seemed to be efficient synthesizers of antibody. These antibodies were 2-ME sensitive macroglobulins, with electrophoretic mobilities comparable to the IgM of man as defined by Miller and Metzger (1965).

Hodgins et al (1967) administered BSA and KLH to rainbow trout. Anti-haemocyanin precipitins were detected in the macroglobulin fraction of the serum. Anti-BSA precipitins were associated with two distinct

proteins demonstrable by immunoelectrophoresis, having sedimentation coefficients of 13S and 10.5S. Trump and Hildemann (1970) discovered two populations of antibody in goldfish immunised with BSA. Further confirmation of this was given by Trump (1970) who separated two proteins with anti-BSA activity by immunoelectrophoresis, the sedimentation coefficients of which were 16.4S and 15.3S respectively.

Marchalonis (1971) reported that Carassius auratus and Cyprinus carpio responded to soluble protein antigens by the production of IgM-type antibody. The IgM molecules of C. carpio were viewed under an electron microscope by Shelton and Smith (1970) who found them to have a tetrameric form. Everhart and Shefner (1966) compared the specificity and avidity of rabbit and C. auratus anti-BSA antibody. They found that the fish serum had greater levels of antibody, but this had lower avidity than the rabbit anti-BSA.

Leslie and Clem (1969) reported the ability of several poikilothermic species to produce specific antibody to a DNP determinant. Clem (1971) utilised this to study the physico-chemical nature of anti-DNP immunoglobulin in the giant grouper, Epinephelus itaira. The serum contained 16S and 6.4S immunoglobulins, the former having an approximate molecular weight of 700,000, being composed of approximately equimolar amounts of heavy and light polypeptide chains. It was suggested that the 16S protein was a tetrameric form of IgM, instead of the more 'typical' pentameric molecule. The valencies and association constants of this anti-DNP immunoglobulin were further studied by Clem and Small (1970). A similar tetrameric antibody from rainbow trout was reported by Dorson (1972).

Fletcher and Grant (1969) detected antibodies to KLH, and two particulate antigens in the serum and mucus of immunised plaice. The immunoglobulin was of large molecular weight, resembling a 'fast'

immunoglobulin of the higher vertebrates. Upon reduction and alkylation the 12.4S immunoglobulins yielded light and heavy polypeptide chains. These results were largely repeated by Fletcher and White (1973) using a soluble antigen extract from Vibrio anguillarum. They found, however, that oral immunisation with Vibrio antigen induced higher antibody titres in the intestinal mucus than in the serum.

Klontz (1970) using agar gel diffusion partially elucidated the nature of precipitins to protein extracts of Aeromonas salmonicida in salmonids. Antibody activity was confined to protein with beta-2 electrophoretic mobility, there being no antibody activity in the gamma-globulin fraction.

The accumulated knowledge of fish immunology has found practical application in the treatment and understanding of fish disease. As indicated above, teleost fish may produce a variety of antibodies with different functional properties. There are, however, very few records of antibody production to parasites.

Knowledge of immunity to metazoan parasites was reviewed by Snieszko (1969) who reiterated the observations of Bauer (1958) on the sparse and fragmentary evidence of immunity in fish disease. Finn (1970) has also reviewed fish diseases with special emphasis on cellular and inflammatory responses of fish to their pathogenic organisms.

Molnar and Berczi (1965), using precipitation in agar gel, reported the presence of specific precipitins in the serum of Abramis brama infected in the cestode Ligula intestinalis. Harris (1972) demonstrated antibody in the sera of chub, Leuciscus cephalus infected with Pomphorhynchus laevis, that specifically precipitated with a saline extract of the parasite. Lethal factors in the serum of Raja radiata to the cestode Acanthobothrium quadripartitum were described by McVicar and Fletcher

(1970). They postulated that an antibody-complement system, already well documented in fish by Legler et al (1967), was responsible for the death of the cestode in the serum of R. radiata.

The presence of immunoglobulins in fish mucus is of obvious survival value to the host. Indeed, some of the earliest observations of immunity in fish were made by Nigrelli and Breder (1934) studying the protection afforded by fish mucus to monogenetic trematodes. Nigrelli (1937) studied the immunity of marine aquarium fishes to Benedenia melleni. Fish that had overcome previous infestations of this external parasite were able to resist further attacks. Live B. melleni specimens placed in mucous preparations from immune dogfish, grouper and ray were moribund within 3 - 4 hours. In the mucus of fish susceptible to infestation, the parasites survived for 24 hours, and for three days in seawater. A similar situation was reported by Reuling (1919) who demonstrated that gar, Lepisosteus sp., after external infection with the mussel glochidia from Lampsilis luteola, could successfully reject further glochidia. Ring precipitation tests indicated low levels of antibody in the serum, and glochidia were inactivated by mucous preparations from immune fish. Hines and Spira (1974) demonstrated immobilising antibody to Ichthyophthirius multifiliis in the mucus of Cyprinus carpio that had overcome sub-lethal doses of this protozoan parasite.

More recent work has elucidated the nature of the protective immunoglobulins in fish mucus. Barry and O'Rourke (1959) showed by chromatography that each species of fish has its own characteristic mucus. O'Rourke (1961) reported that rabbit anti-bass serum cross-reacted, in immunodiffusion tests, with bass mucus indicating the presence of serum proteins in the bass mucus.

Dobson (1972) has reviewed the literature concerning the immune response of mammals to gastrointestinal helminths, pointing to the importance of specific, secretory antibody in the mucus of the IgM class. At present, no specialised secretory immunoglobulin class has been demonstrated in fish mucus. Harris (1972) identified weak precipitins in gut mucus extracts of Leuciscus cephalus, infected with the acanthocephalan, Pomphorhynchus laevis. The mucous antibody appeared identical to the serum anti- P. laevis immunoglobulins. The latter, on the basis of molecular weight and sensitivity to 2-ME, was designated IgM.

A 12.4S, IgM was demonstrated in the cutaneous mucus of plaice by Fletcher and Grant (1969). Bradshaw et al (1969) found IgM in the serum and mucus of the holostean, Lepisosteus platyrhincus. The immunoglobulin in the mucus was totally susceptible to 2-ME reduction, and rabbit anti-gar IgM neutralised the antibody activity of this immunoglobulin.

It has recently become apparent that fish antibody, with associated complement, may not be the only biochemical means of defence against pathogenic organisms. Lysozyme, the bacteriolytic enzyme, was shown to be present in the sera of several species of flatfish by Fletcher and White (1973). Lysozyme was also found in body tissues and cutaneous mucus of plaice.

Baldo and Fletcher (1973) pointed to the existence of C-reactive protein in the serum of plaice. CRP precipitated in agar gels with saline extracts of nematodes, bacteria and fungi. This CRP seemed to be present in healthy plaice, as opposed to mammals where it only occurs in the acute phases of specified diseases. Ellis (1973) inferred the potential value of CRP in poikilothermic animals with temperature dependant immune responses.

Attempts at the systematic control of disease, using our knowledge of fish immunology, have been largely confined to bacterial pathogens. Klontz and Anderson (1970) and Snieszko (1970) have reviewed the immunisation of fishes against bacteria with particular emphasis on oral immunisation. This method has proved more convenient than parental immunisation and, as such, has greater potential in the vaccination of large numbers of fish. Two pathogenic bacteria have been studied in detail, namely Aeromonas hydrophila causing "redmouth disease" in trout, and Aeromonas salmonicida the causative agent of furunculosis. As the problems and methods of approach have been much the same for both diseases, attention in this review will be confined to A. salmonicida. For further details on A. hydrophila the reader is referred to the work of Ross and Klontz (1965) and Post (1963, 1966 a, 1966 b).

Oral immunisation of Salmo clarkii against A. salmonicida was successfully carried out by Duff (1942). Snieszko and Friddle (1942) carried out further investigations with this bacterium in Salvelinus fontinalis. Following oral immunisation, however, the trout were not fully protected against parental challenge with the bacteria. These results do not negate Duff's earlier report of success, as he challenged his immunised trout with external exposure of virulent bacteria.

Klontz (1966) extracted a toxin from A. salmonicida and added this to the normal diet of brook trout over 30 days. Using immunofluorescence he was able to demonstrate the presence of homologous antibody in the lymphoid cells of the fish. Further experiements by Klontz (1967, 1968) on coho salmon, Oncorhynchus kisutch, using an oral immunisation procedure with A. salmonicida antigen, indicated increased protection from furunculosis in this fish.

Since these early reports of success, Klontz (1969) has reported equivocal results on the effectiveness of oral immunisation against furunculosis. The suggestion has been made by Krantz (1964) that oral vaccination with living or killed cells of A. salmonicida is not as effective as parental administration of the bacterial antigens.

The first record of a trypanosome from a fish host was that of Valentin (1841), who observed a protozoan blood parasite from the sea trout, Salmo furio. Trypanosomes were subsequently reported from a variety of freshwater fish including tench, perch, gudgeon and pike by Remak (1841), Gros (1845), Berg (1843), Wedl (1850) and Chausat (1850).

Danilewsky (1885) described trypanosomes in Cyprinus carpio, Tinca tinca, Cobitis fossilis, Cobitis barbatula, Esox lucius, Perca fluviatilis and Carassius vulgaris. He distinguished two morphological types, both of which possessed an undulating membrane and free flagellum. Trypanosomes from freshwater fish were reported by Lingard (1899) which, in form, resembled those described by Mitrophanow (1883).

Laveran and Mesnil (1901) noted for the first time, a trypanosome from a marine host. 25% of Solea solea they investigated were infected with a species which they designated Trypanosoma solea. Brumpt and Lebailly (1904) and Lebailly (1904) described 9 new species of Trypanosoma from marine fish, five of which were flatfish (table 1). Lebailly (1904) noted that 1 of 6 plaice was infected with T. platessae. The latter coexisted in plaice blood with Haemogregarina platessae. Similarly, T. flesi occurred in Pleuronectes flesus along with H. flesi.

Lebailly (1905) in a review of marine haematozoa described all of the marine trypanosomes recorded up to 1905, and also indicated the criteria for the naming of new species. These were based chiefly on morphological features, and to a lesser extent on the specificity of the fish host. Lebailly (1905) was unsuccessful in passaging trypanosomes from one host species to another. For instance, he attempted, without success, to transmit T. limandae from Limanda limanda, the natural host, to Anguilla anguilla. T. platessae, T. callionymi and

TABLE 1. Records of All Species of Marine Trypanosoma with Morphological Descriptions.

TRYPANOSOMA SPECIES	HOST SPECIES AND LOCATION	ORIGINAL DESCRIPTION	MORPHOMETRIC RECORD						NOTES ON OTHER DISTINCTIVE FEATURES
			Total Length	Flagellum Length	Body Length	Width	Distance of Kinetoplast - Post. Tip	Widt' Undulating Membrane	
T. soleae	Solea vulgaris sole (France)	Laveran and Mesnil (1901)	40μ	8	32				
T. granulosum	Anguilla vulgaris eel (France)	Laveran and Mesnil (1901)	80μ	25	55	2.5-3.0			Nucleus occupies full body width
T. scylliumi	Scyllium stellare dogfish (France)	Laveran and Mesnil (1902)	70-75μ	14	61	5-6			
T. rajae	Raja punctata, Raja mosaica, skates (France)	Laveran and Mesnil (1902)	75-80μ	20	60				Kudo (1923) reports nucleus occupies full body width.
T. gobii	Gobius niger, goby (France)	Brumpt and Lebailly (1904)	66μ	10	56	5-5.5	7		
T. callionymi	Callionymus dracunculus (France)	Brumpt and Lebailly (1904)	70μ	5	65	5	11		
T. cotti	Cottus bubalis (France)	Brumpt and Lebailly (1904)	53μ	8	45	5	7		
T. delagei	Blennius pholis blenny (France)	Brumpt and Lebailly (1904)	33μ	12	21	2.5	7	6-7	Undulating membrane as wide as body. Nucleus 3 x as long as wide.

Table 1. (Continued)

TRYPANOSOMA SPECIES	HOST SPECIES AND LOCATION	ORIGINAL DESCRIPTION	MORPHOMETRIC RECORD						NOTES ON OTHER DISTINCTIVE FEATURES
			Total Length	Flagellum Length	Body Length	Width	Distance of Kinetoplast - Post. Tip	Width Undulating Membrane	
T. limandae	Limanda platessoides dab (France)	Brumpt and Lebailly (1904)	45μ	20	25	2-2.5	2		Nucleus is 4 x as long as wide.
T. bothi	Bothus rhombus, (France)	Lebailly (1904)	42μ	13	29	3	4		Nucleus oval 2.5μ long by 2μ wide
T. platessae	Platessa vulgaris plaice (France)	Lebailly (1904)	52μ	12	40	3-3.5	5		
T. flesi	Flesus vulgaris flounder (France)	Lebailly (1904)	55μ	10	45	5	3.5		
T. laternae	Platophrys laternae (France)	Lebailly (1904)	65μ	8	57	5-6	5.5		
T. carchariasi	Cacharias sp. Shark (France)	Laveran (1908)	60-70μ	25-30	35-40				
T. variable	Raja punctata (Germany)	Neumann (1909)	90-100μ	25-30	65-70				Polymorphic species smaller form 31μ, 8μ free flagellum
T. giganteum	Raja oxyrhincus ray (Germany)	Neumann (1909)	125-130μ	25-30	100				
T. triglae	(Trigla corax) Tubgunard Trigla lucerna (Germany)	Neumann (1909)	45μ	15	30	8	4		Nucleus oval 5μ long x 4μ wide.

Table 1. (Continued)

TRYPANOSOMA SPECIES	HOST SPECIES AND LOCATION	ORIGINAL DESCRIPTION	MORPHOMETRIC RECORD						NOTES ON OTHER DISTINCTIVE FEATURES
			Total Length	Flagellum Length	Body Length	Width	Distance of Kinetoplast - Post. Tip	Width Undulating Membrane	
T. scorpaenae	Scorpaena ustula (Germany)	Neumann (1909)	65-70μ	5-7	60-63				Very broad undulatin membrane.
T. zeugopteri	Zeugopterus punctata (U.K.)	Henry (1913)							No description given
T. cataphracti	Agonus cataphractus (U.K.)	Henry (1913)							No description given
T. aeglefini	(Gadus aeglefinus) Melanogrammus aeglefisius (U.K.)	Henry (1913)							No description given
T. capigobii	Gobius nudiceps (S. Africa)	Fantham (1919)							Hardly any free flagellum
T. nudigobii	Gobius nudiceps (S. Africa)	Fantham (1919)				6			Nucleus extends across body width.
T.sp.	Box salpa (S. Africa)	Fantham (1919)			29	2			
T. sp.	Dentex argyrozona (S. Africa)	Fantham (1919)	22.06μ	2.6	20.6	1.3			
T. pulchra	Gilbertia semicineta and Parma microlepis (Australia)	Mackerras and Mackerras (1925)	48.3μ	7.3	41	3.5	1.7	1-1.5	Aflagellar and beak shaped.

Table 1. (Continued)

TRYPANOSOMA SPECIES	HOST SPECIES AND LOCATION	ORIGINAL DESCRIPTION	MORPHOMETRIC RECORD						NOTES ON OTHER DISTINCTIVE FEATURES
			Total Length	Flagellum Length	Body Length	Width	Distance of Kinetoplast - Post. Tip	Width Undulating Membrane	
T. aulopi	Aulopus purpurissatus Australia	Mackerras and Mackerras (1925)	Range 57.1 - 29.1μ						Separated from T. pulchra, because of marked pleomorphism.
T. murmanensis	Gadus morhua U.S.S.R.	Nikitin (1927)	83.5μ	6.9	76.6	3.8	9.1		Nuclear Index = 1.2. Undulating membrane not easily seen. (Data from Khan, 197
T. blenniclini	Blennius cornutus and Clinus anguillaris. S. Africa	Fantham (1930)	50-77μ			3-7	6-9		No free flagellum
T. percae var canadensis	Perca flavescens Canada	Fantham et al (1942)	34.3 - 39.3μ	5.3-6.9	29-32.5				
T. myxocephali	Myxocephalus octodecimspinosus Canada	Fantham et al (1942)	44-53μ	3	41.5-49	3			Nucleus 6.7 - 7.7 μx 2.2 - 3.0 μ
T. marplatensis	Psammotics microps ray S. America	Bacigalupo and de la Plaza (1948)	60-65μ	15	45	1.2		3-3.4	Polymorphic species. Dimensions of large form given.
T. heptatreti	Hepatretus cirrhatus New Zealand	Laird (1948)	82.5μ	22	60.5	3.4		2.8	

Table 1. (Continued)

TRYPANOSOMA SPECIES	HOST SPECIES AND LOCATION	ORIGINAL DESCRIPTION	MORPHOMETRIC RECORD					NOTES ON OTHER DISTINCTIVE FEATURES
			Total Length	Flagellum Length	Body Length	Width	Distance of Kinetoplast - Post. Tip	Width Undulating Membrane	
T. gargantua	Raja nasuta New Zealand	Laird (1951)	114.7μ			14.3		2.9	Considerable degree of polymorphism. Dimensions of large forms only.
T. coelorhynchi	Coelorhyncus australis Physiculus bachus New Zealand	Laird (1951)	66.8μ	11	55.8	3.2		1.1	Polymorphic
T. caulopsettae	Caulopsetta scapha Rhombosolea plebia New Zealand	Laird (1951)	61.5-70.1μ						Polymorphic. Intermediate forms dimensions. Undulating membrane same width as body.
T. tripterygium	Tripterygium varium New Zealand	Laird (1951)	93.1μ	14	79.1	5.3		0.8	
T. congiopodi	Congiopodus leucopaecilus New Zealand	Laird (1951)	43.8μ			4.5			No free flagellum.
T. parapercis	Parapercis colias New Zealand	Laird (1951)							Nucleus extends full width of body; no free flagellum.

Table 1. (Continued)

TRYPANOSOMA SPECIES	HOST SPECIES AND LOCATION	ORIGINAL DESCRIPTION	MORPHOMETRIC RECORD						NOTES ON OTHER DISTINCTIVE FEATURES
			Total Length	Flagellum Length	Body Length	Width	Distance of Kinetoplast - Post. Tip	Width Undulating Membrane	
T. balistes	Balistes capriscus Florida, U.S.A.	Saunders (1959)	55.5μ	12	43.5	3	12	1	Length of nucleus 1.0, width 0.6
T. sp.	Glyptocephalus cynoglossus Canada	So (1972)	65.7μ	6.5	59.2	3.2	3.0	1.5	
T. pacifica	Parophrys vetulus Oregon, U.S.A.	Burreson and Pratt (1972)	36μ	15.4	20.5	1.5	3.3		Nuclear index 1.1; undulating membrane relatively broad.

T. granulosum all failed to develop after they had been inoculated into species of fish other than their natural host.

Four new species of trypanosomes were named by Neumann (1909) (table 1). Henry (1910, 1911, 1913) made the first observations of marine trypanosomes from British waters. Henry (1913) named three new species, but unfortunately gave no description of these. The new species were only justified as such because they were recorded from new host fish. During the period 1909-1913 he sampled fish from the Irish Channel, North Sea and English Channel and noted a further seven species already recorded by the French workers. Henry (1913) found that 25% of the plaice at Port Erin were infected with T. platessae. By contrast, he was unable to detect the parasite in plaice caught at Plymouth.

From this period until 1951, 18 new species were recorded from marine fish, by various workers notably Fantham (1919) and Laird (1951) (table 1). As far as is known, only two new species have been named since 1951. Saunders (1959) recorded T. balistes from Balistes capriscus in the U.S.A. T. pacifica was noted by Burreson and Pratt (1972) from the English sole Parophrys vetulus. This seemed to be morphologically distinct from T. solea and other species from flatfish hosts. So (1972) found a single trypanosome in a new host fish Glyptocephalus cynoglossus, and in the absence of further specimens, designated it Trypanosoma sp. until he investigated further material.

The vectors of fish trypanosomes as far as is known are aquatic leeches of the family the Hirudinea. Leydig (1859) recorded trypanosomes in the guts of two leeches, Pontobdella and Piscicola that had just taken a feed from an infected fish. The suggestion that leeches were implicated in the transmission of fish flagellates was first made by Danilewsky (1885). Brumpt (1904, 1906 a, b,) analysed and described

trypanosome infections of freshwater and marine leeches. Leger (1904) saw trypanosomes in the gut of Piscicola sp. after they had fed on Cobitis barbatula infected with T. barbatulae. He also noted trypanosomes dividing and in different states of differentiation in the guts of these leeches. Using the leech, Piscicola geometra, Keyselitz (1904, 1906) experimentally transmitted trypanosomes of carp, tench and pike.

Robertson (1912) in a series of experiments, evaluated the role of leeches in trypanosome transmission. He successfully infected goldfish with trypanosomes from bream, perch and other goldfish using Hemiclepsis as vector. In contrast to this, he was unable to directly inoculate trypanosomes into recipients, using infected blood from other donor species.

There is no evidence to suggest that fish trypanosomes are transmitted by agents other than leeches. Minchin (1909) and Robertson (1912) using a variety of other ectoparasites unsuccessfully tried to transmit trypanosomes from fish to fish.

Barrow (1953) showed that the development of metacyclic forms of T. diemyctyli in the leech Batrochobdella pista took from 12-16 days, depending upon the ambient temperature and nutritional state of the host. Development culminated in metacyclic forms which were regurgitated into the vertebrate host. Qadri (1962) demonstrated that three hours after Hemiclepsis marginata fed on fish infected with T. danilewskyi, the latter appeared in the crop. Metacyclic forms developed within seven days and on the tenth day migrated to the proboscis sheath ready for transmission to the vertebrate host.

That marine trypanosomes are relatively rare with low parasitaemias, is evident from the literature. Bullock (1958), for example, recorded that only 14 fish, representing 6 species, out of a total

of 209, representing 43 species, had protozoan blood parasites. Only one fish, Raja erinacea, had an extremely light infection of trypanosomes. Saunders (1960) studied 1,451 fish, comprising some 104 species, from the Red Sea and found no trypanosome infections in any of the blood preparations.

There is little or no evidence to suggest marine trypanosomes actively divide in the blood of their fish host. Fantham et al (1942) noted dividing form of T. myxocephali in the kidney of Myxocephalus octodecimspinosus. Others, such as Laird (1951) and Robertson (1909) who have recorded divisional trypanosomes in the blood, have attributed these to physiological changes associated either with in vitro preparations, or with the death of the host fish.

Polymorphism has been described in many species of Trypanosoma from marine fish. T. variable, recorded by Neumann (1909), displays marked polymorphism with large forms 90-100 μ long and smaller forms 30 μ in length. Laird (1951) recorded polymorphism in T. gargantua, T. coelorhynci and T. caulopsettae. Qadri (1962) suggested that the polymorphism of T. danilewskyi in the blood of Cyprinus carpio, may have resulted from differences of size of metacyclic forms in the infected leech.

Immunity of poikilotherms to protozoan diseases, as Lom (1969) has indicated, is a much neglected field, and there are very few records in the literature of immunity in fish to trypanosome infections. Nine European fish species, evidently infected with the same trypanosome, were studied by Barrow (1954). The fish suffered their highest parasitaemias just after the winter season, and this was probably associated with a decrease of lytic antibody secretion at low temperature. Fish maintained at 5°C were unable to produce antibody, whereas those at

20°C produced appreciable amounts of anti-trypanosome immunoglobulin. This work repeated Barrow's earlier observations (1948) on the effects of temperature on T. diemyctyli in its poikilothermic host. At 10°C the immune response is lowered, but the lower temperatures inhibit the rapid division of the trypanosomes. Barrow (1955) studied the effects of ambient temperature and nutritional status on the ability of tench and perch to overcome trypanosome infections. Fish that were feeding were able to eliminate the parasite at 20°C, but were unable to do so at 15, 10 and 5°C. The antibody remained effective for 2 - 3 weeks after the flagellates had been destroyed.

There is some evidence of antibody production to T. rotarium in frogs. Doflein (1913) suggested an 'ablastin' was instrumental in the acquired immunity of frogs against T. rotarium. He was unable to infect immune frogs with infective leeches or culture crithidial forms. Doflein's work was largely confirmed by Ogawa (1913) and Mendeleeff-Goldberg (1913).

Khan (1972) studied the effects of splenectomy upon the susceptibility of certain marine fish to inoculations of T. murmanensis from the cod, Gadus morhua. T. murmanensis failed to develop in all of the following: 3 splenectomised and 3 normal Pseudopleuronectes americanus; 2 splenectomised and 2 normal Myxocephalus octodecemspinosus. T. murmanensis did survive, however, when inoculated into G. morhua, the natural host. Khan deduced from this that the failure of trypanosomes to develop in other species was due to innate factors and not to specific acquired immunity of the host to the parasite.

Chapter 2

METHODS AND MATERIALS

FISH

Collection. Plaice, Pleuronectes platessa L. was the species most predominantly studied. Other heterosomatous fish were selected for study namely: dab, Limanda limanda (L.); turbot, Scophthalamus maximus (L.); sole, Solea solea (L.); flounder, Pleuronectes flesus (L.); and brill, Scophthalamus rhombus (L.).

'O' group flatfish were caught using the Riley net (1973). Collections were made along the shore within one hour of low water, preferably on spring tides for the best catches.

Older fish were captured offshore aboard commercial trawlers and research vessels, as noted in Table 2.

Fish were transferred immediately upon capture, to tanks of aerated seawater and transported without delay to the aquaria. Delays of up to 10 hours, however, were often unavoidable when commercial trawlers were used for collection. Consequently fish from this source were often in poor condition upon arrival at the laboratory.

Maintenance The fish were maintained in temperature controlled seawater aquaria, as described by Iddon (1973). Wild fish introduced into the aquaria were often slow to feed, especially older fish from '2' group onwards. These, therefore, were force fed using a method successfully exploited by Edwards (1970) for the experimental feeding of plaice. Food was carefully introduced into the gut by means of a small length of plastic tubing attached to a syringe. The tubing was carefully introduced through the buccal cavity into the stomach, and the contents of the syringe expelled.

Table 2. Listing species of adult flatfish caught, together with location, vessels used, and dates of collections.

DATE	VESSEL	LOCATION	DESCRIPTION OF FISH	
			Species	Age
Oct., 1971 - March, 1972	Lady Cinderella	Looe	Plaice Dab Sole	All adult fish
Oct., 1971 - Sept., 1973	R.V. Sarsia (M.B.A.)	Looe	Plaice Dab Sole	All adult fish
Sept., 1973	R.V. Sepia (M.B.A.)	Tamar estuary	Plaice Flounder	'1' - gp.
Oct., 1973	R.V. Prince Madoc	Anglessey	Plaice	'1' - and '2' gp.
Feb., 1973 - June, 1974	R.V. Portunus	Brixham	Plaice	Adult

Table 3. Indicating the source of helminth antigens used in tests for C-reactive protein in plaice serum.

TAXONOMIC GROUP	SPECIES	HOST	LOCATION IN HOST
Trematoda	*Fasciola hepatica*	Cow	Bile duct
Cestoda	*Hymenolepis diminuta*	Mouse	Small Intestine
Nematoda	*Ascaris suum*	Pig	Small Intestine
	Proleptus obtusus	Dog fish	Stomach and Intestine

All fish were fed on a diet, recommended by the White Fish Authority, as follows:-

Minced beef or fish (100 parts),

A synthetic binder - Methofas (ICI) (3-4 parts),

Vitamin premix ("Beta" Animal feed (1-2 parts) supplements),

Red shrimp meal (10 parts).

For feeding '0' group fish, the meat mix was dispensed into aquaria, through a syringe, simulating a worm shape of approximately the right size.

'0' group plaice, which were less than three months old, were fed initially with nauplii of Artemia salina, before being introduced to the diet above.

Stock fish were maintained at a temperature of 10 - 15°C and were gradually acclimatised to the appropriate temperature, prior to experimentation. Sea water was obtained from the M.B.A. where it had been carefully monitored to ensure high quality and salinity. Seawater in all aquaria was changed at regular intervals of 21 days.

COLLECTION AND MAINTENANCE OF PARASITES

Trypanosoma platessae

Only low level infections of T. platessae occurred in Pleuronectes platessa and detection of the flagellates, by means of stained thin blood smears, was, therefore, unreliable. Two other methods were used, therefore, based on the concentrating of the trypanosomes in the blood. The three methods are outlined below.

Thin blood smears. These were prepared, air-dried and fixed in absolute methanol for two minutes. The smear was then stained with Giemsa

(Hopkins and Williams) diluted 1:15 with phosphate buffered water at pH 7.2, for 15 - 20 minutes. The advantage of this method was that many smears could be prepared at sea, and later stained and examined in the laboratory at convenient times.

Examination of fresh clotted blood. This method has successfully been used by Strout (1962) for the detection of low level infections of trypanosomes. A small drop of blood was placed on a slide and allowed to clot under a coverslip. Any trypanosomes could be seen, under the microscope, to be actively swimming from the contracting clot into the clear serum.

Centrifugation of blood in micro-haematocrit tubes. This method is based on that described by Bennet (1962) for the detection of avian trypanosomes, and Khan (1972) has diagnosed the presence of fish trypanosomes using this technique. Blood was taken from fish and immediately transferred to heparinised 5 ml. bijou bottles. These could be stored at $4^{o}C$ for periods of 5 hours at this stage. The blood was drawn into Benjamin haematocrit tubes (Harshaw Chemicals, Ltd.), the ends of the tubes sealed and spun down in a micro-haematocrit centrifuge for 5 minutes. By breaking the tube between the erythrocyte-leucocyte interface the buffy coloured layer of white blood cells could be removed and examined, under the microscope, for the presence of live trypanosomes. Immediately after centrifugation, the haematocrit value (i.e. percentage volume of packed blood cells to serum) was read and recorded.

Cryptocotyle lingua

Littorina littorea were collected at the Yealm estuary, South Devon. As the infection rate is low (less than 5% infection) large numbers of L. littorea had to be examined to obtain a satisfactory number of infected winkles, and the following procedure was used to

screen them. L. Littorea were placed in groups of three into 100 ml. beakers filled with filtered seawater, and subjected to bright, overhead illumination. The presence of actively swimming cercariae, clearly visible to the naked eye, indicated infected specimens. Infected winkles were successfully maintained in well aerated, seawater at 15°C, and fed on Ulva lactua. When large numbers of Cryptocotyle lingua cercariae were required, the following method was used to collect and concentrate them. 50 L. littorea were placed in 500 ml. flask of seawater and maintained in an environmental chamber under conditions of constant light and temperature. The positive phototactic behaviour of these cercariae enabled them to be concentrated by the use of a spot light source. Estimation of cercariae per unit volume was effected by counting the numbers present in three 0.1 ml. samples and taking an average value.

Rhipidocotyle johnstonei

Plaice infected with the metacercariae were collected from Broadsands, Torbay. The metacercaria were clearly visible to the naked eye, through the body wall of the '0' group fish. Parasites were carefully dissected out from the caudal region and washed three times in 0.75% saline. These survived in this simple saline, at 10°C, for periods up to 7 days.

ANTIGENS

Antigens were prepared in phosphate buffered saline at pH 7.2. When combined with adjuvant the following method was employed. Equal volumes of Freund's Complete Adjuvant (F.C.A.) (Difco) and antigen solution were measured and the antigen forcefully injected into the F.C.A. by means of a syringe. The mixture was shaken vigorously and recycled through the syring until a satisfactory water in oil emulsion had been achieved. The following antigens were used in this study.

Fish parasite antigens

Antigens were prepared from two fish parasites, namely Cryptocotyle lingua and Rhipidocotyle johnstonei. Live material was washed twice in P.B.S. and then centrifuged at 1,000 r.p.m. to produce a thick suspension of parasites. The supernatant was discarded and the parasites resuspended in 1-2 mls. of P.B.S. The material was then chilled on ice and homogenised in an ultra-sonic disintegrator. The homogenate was spun down in a refrigerated centrifuge at 6,000 r.p.m. for 30 minutes, the supernate decanted and labelled the soluble Cryptocotyle or Rhipidocotyle antigen, and finally delipidated using diethyl ether. This soluble antigen was then concentrated to 5-10 mg. protein per ml. by means of Lyphogel granules (Gelman Hawksley). These polyacrylamide granules are of particular use in concentrating solutions of macromolecules as they will absorb five times their weight of water, but will exclude the larger molecules.

The centrifuged pellet of cellular debris was resuspended in P.B.S. and centrifuged once again at 6,000 r.p.m. This procedure was repeated twice so that the insoluble parasite material was thoroughly washed. This material was labelled insoluble Cryptocotyle or Rhipidocotyle and stored at -20°C. This antigen was solubilised by the addition of 6 mg. of the detergent Digitonin, to 1 ml. of the antigen suspension. This was then dialysed against several changes of P.B.S. to remove excess detergent from the solution. Both the insoluble and soluble parasite antigens were used, when possible, freshly prepared. If, however, they were stored for any length of time, Merthiolate was added and they were kept at -20°C.

Antigens administered parentally to fish

Bovine serum albumin (B.S.A.). This was prepared in P.B.S., pH 7.2, using Fraction V Cohn, from whole bovine serum (B.D.H. Chemicals). It was administered at a concentration of 10 mg. per ml.

Rabbit erythrocytes. Fresh blood from a rabbit was collected in Alsever's solution, centrifuged at 2,500 r.p.m., and the cells given two washes in P.B.S. The washed cells were resuspended in P.B.S. at a concentration of 10%

Whole calf serum. This was obtained commercially (Burroughs Welcome) and administered to the fish undiluted.

Antigens to test for the presence of C-reactive protein

All antigens used were homogenates of the test organisms in P.B.S. (Table 3). After treatment in the ultra-sonic disintegrator, each antigen was dialysed for 48 hours against several changes of distilled water to remove salts. They were then freeze dried and stored until they were required for tests, when they were reconstituted in saline at a concentration of 10 mg. of freeze dried material per ml.

PRODUCTION OF ANTISERA

Rabbits

Antibodies were raised by parental injection of the appropriate antigen into white New Zealand rabbits of approximate average weight of 1.5 Kgm. Prior to injection the back of the rabbit was shaved in two small areas and the exposed skin treated with 70% ethanol. The first dose of antigen, injected sub-cutaneously, was invariably given with Freund's Complete Adjuvant, and thereafter the antigen was presented in P.B.S. alone. Many schedules for the immunisation of

rabbits have been given including those of Holborrow and Johnston (1968), and Campbell et al (1970). In the present instance, the animals were injected every 7 days for the first 28 days, and thereafter every 14 days until a sufficiently high titre of antibody had been produced. Assay for the presence of antibody was performed every 14 days after immunisation had commenced. 0.2 mls. volumes of blood were collected from the external marginal vein of the pinna. This provided sufficient serum to test for antibody activity using standard tests. The animals were terminally bled by cardiac puncture, this providing 30 - 50 mls. of immune serum.

Serum was collected from the whole blood by standard haematological procedures. Blood was allowed to clot at room temperature for one hour and left overnight at 4°C so that the clot contracted. The clot was removed and the serum centrifuged at 2,500 r.p.m. for 10 minutes to spin down remaining blood cells. The antiserum was then decanted, Merthiolate added, and stored at -70°C.

Plaice

Several experiments involved the production of antisera by plaice to parentally administered antigens. The antigen was almost invariably presented by the intraperitoneal route, although in some cases it was administered intra-muscularly into the flanks of the fish. The volume of antigen introduced was regulated to the size of the fish. '0' group fish received not more than 0.25 mls. and older fish 0.5 - 1 ml. volumes of antigen. The total amount of antigen per fish, however, was always adjusted to 10 mg. of protein, unless otherwise stated .

Blood, when required, was taken from the caudal vein of the plaice. Viewing the fish from the topographical dorsal aspect, the vein was located to the right of the lateral line. Up to 0.2 ml.

of blood could be drawn from 'O' group plaice and 1 - 2 mls. from adult fish. Serum was prepared as described for the rabbit. Fish blood characteristically clots more rapidly than that of mammals and, consequently, many previous workers have advocated the use of special techniques for its collection, including the use of pre-chilled needles and syringes and heparin. In the present study, however, these methods were not found necessary. If plasma was required, blood was collected in syringes that had been previously rinsed with a solution of heparin. The blood was then centrifuged at 3,000 r.p.m. and the plasma decanted and stored.

METHODS OF DETECTING ANTIBODY-ANTIGEN INTERACTION

Immunodiffusion

The immunodiffusion precipitation test is a method whereby antigen-antibody complexes may be viewed when they precipitate out, in the presence of ions, in clear agar or cellulose acetate membrane. Antigen and antibody solutions diffuse towards each other and, at the point of optimal proportions, a visible white precipitate forms. The methods of immunodiffusion are useful in resolving complex precipitating systems. The now commonly used technique of double diffusion in gels was introduced by Ouchterlony (1948). The following modified method of double diffusion was used in this study. A 1% (w/v) solution of Ionagar (Difco) was prepared in P.B.S. at pH 7.2, and Merthiolate added at a concentration of 1:10,000 to suppress bacterial growth. A thin sealing layer of the gel was poured onto the bottom of a sterile, plastic petri dish. This was followed after solidification by a further 20 ml. of molten agar to give a final depth of 4 mms. These gel plates could be stored for 14 days at $4^{o}C$. Three basic well patterns were utilised (figure 2) 4-well and 18-well Feinberg well cutters (Shandon) were used to make the patterns. The

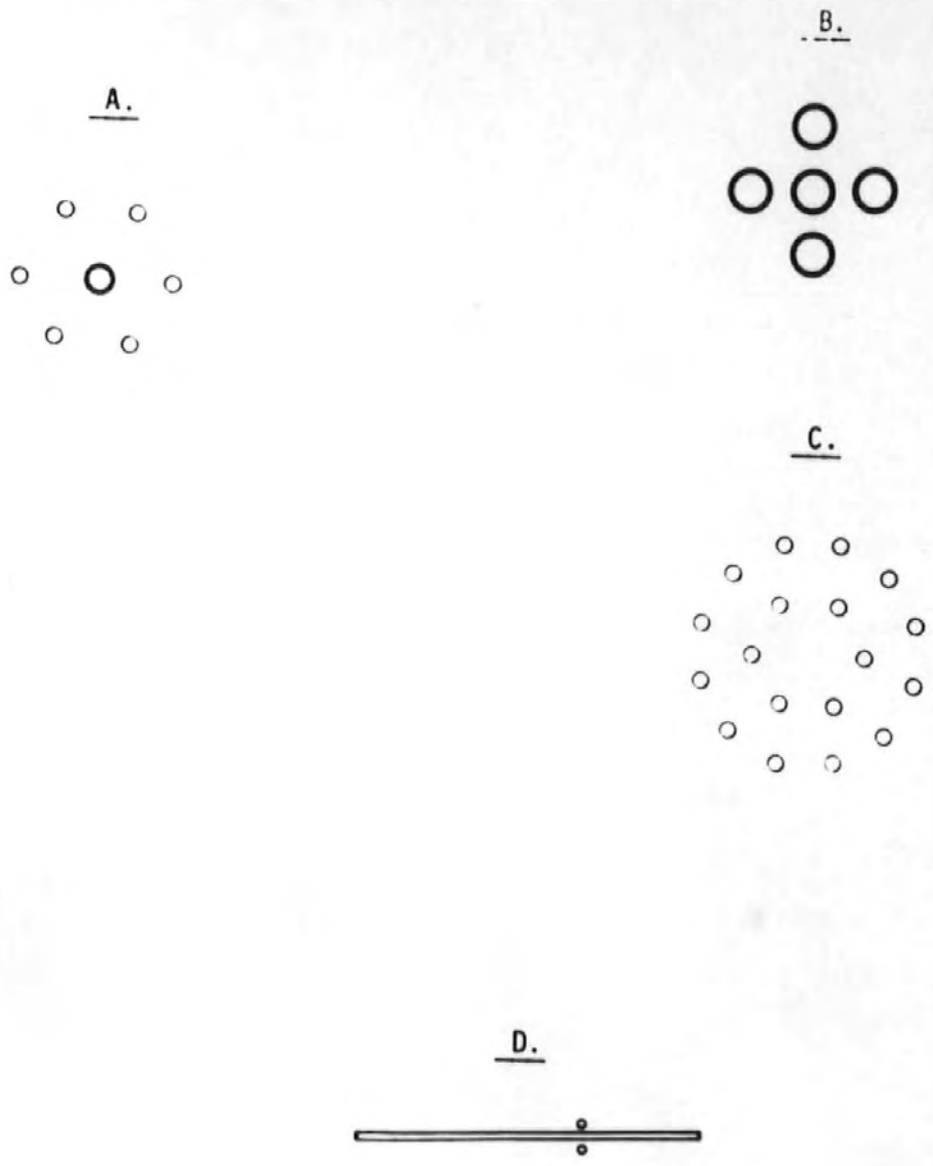


Figure 2. To illustrate the actual size and patterns of the templates used in immunodiffusion studies. A, B and C are Ouchterlony well borer patterns, and D shows the basic immuno-electrophoresis template.

7-well pattern, including one central well and 6 peripheral wells, was constructed using a perspex template and cork borers. Following application of the well borers, the plugs of gel were removed with a fine needle, however, the sealing layer of gel remained to prevent seepage of solutions from the bottom of the well. Antigen solutions and antisera were carefully layered into these wells according to the dictates of the experiment.

Immunoelectrophoresis

This technique combines a preliminary electrophoretic separation of antigen or antibody followed by double diffusion of the antigen-antibody system. First described by Grabar and Williams (1953) a great variety of immunoelectrophoretic methods are now available for qualitative and quantitative analysis of immunoprecipitating materials. The procedure adopted in the present study utilised the micro-immunoelectrophoretic apparatus developed by Shandon. The apparatus consisted of the electrophoresis tank (after Kohn) model U.77; the Vokam constant voltage-current power supply and associated accessories.

A 1% Ionagar (Difco) solution was prepared in 0.05 M. Barbitol buffer, pH 8.6, and Merthiolate added (1:10,000). The molten agar was gently poured onto eight microscope slides, fitted into the Shandon slide tray to a depth of 2 mm. Wells and troughs were cut on the agar coated slides using the Shandon cutters, in the pattern indicated in figure 2. The agar plugs were removed using a fine needle and the appropriate antigen or antibody added to the wells. Electrophoresis was carried out in 0.05 M. Barbitol buffer, contact with the agar slides and buffer being made by means of filter paper wicks. A voltage of 7 - 8 volts, per cm. of agar slide, was applied for 90 - 120 minutes. The gel plates were then taken from the tank and the

agar strips removed from the trough patterns with a fine needle. Antigen or antiserum was then introduced into these troughs by means of a pipette.

Precipitation patterns were developed in a moist atmosphere at 20°C for 7 days, during which time they were checked regularly for the development of arcs. The agar plates were then washed in saline for 48 hours to remove excess unreacted protein.

Photographic records were made of both the immunoelectrophoretic patterns and the Ouchterlony plates. A scattered light source was found most effective in differentiating precipitates which, in these conditions, appeared as white arcs against the dark background of the plate. A suitable darkground illumination was improvised by using a circular fluorescent light bulb of 40 cm. diameter. This was placed in the bottom of a box (40 cm. square), the interior of which had been painted black. Oblique illumination was achieved by placing an agar plate directly over a central opening in the top of the box. Some plates, after photography, were preserved in 40% ethanol or stained with Ponceau S. dye fixative using the following procedure. Whatman filter paper was placed on the agar surface of the plate and the agar dried to a thin film at 37°C. The filter paper helped to absorb minerals from the agar and also prevented excessive cracking of the agar as it dried. The plates were then stained with Ponceau S. in 3% w/v. trichloracetic acid for 30 minutes. These were then differentiated by removing excess stain in several changes of 5% acetic acid and dried and stored.

The capillary tube agglutination test

The test described by Hudson and Mudd (1935), as modified by Luoto (1956), was used in this study specifically to test antibody activity to a particulate antigen, namely rabbit erythrocytes. Capillary tubes, 7 cms. long with and internal bore of 0.2 mm., filled approximately with $\frac{1}{3}$ antigen and $\frac{2}{3}$ test antisera. The tubes were inverted, erythrocytes uppermost, and maintained in a vertical position in blocks of clay. Following incubation for 1 hour at 20°C., the tubes were viewed under bright illumination for the presence of floccules, indicating a positive reaction. The highest dilution of serum showing flocculation of the rabbit erythrocytes was taken as the end point, and the reciprocal of this dilution recorded as the titre of the test serum.

Passive haemagglutination

This is a highly sensitive method for the assay of antibody and Borduas and Grabar (1953) have indicated that the test is sensitive enough to detect 0.003 to 0.006 µg of antibody nitrogen. Red blood cells treated with tannic acid and protein antigen will specifically agglutinate in homologous antiserum. The method used in this study was based on that given by Stavitsky (1954).

1 ml. of sheep erythrocytes (Burroughs Welcome) in Alsever's solution were centrifuged at 2,500 r.p.m., the Alsevers decanted, and the cells resuspended in 0.85% saline. This procedure was repeated twice and, after washing, the pellet of erythrocytes was mixed thoroughly with an equal volume of 0.005% (w/v) tannic acid, and left at room temperature for 10 minutes. The tanned cells were then centrifuged and given two washes with P.B.S., to remove any unadsorbed tannic acid, and the final volume adjusted to 2 ml.

At this stage, the tanned erythrocytes could be stored at 4°C. for periods of twenty-four hours, whilst preparing the serial dilutions of the test sera. The passive haemagglutination test was used in the present study to detect antibody to two antigens namely, bovine serum albumin (B.S.A.) and the cercarial antigen of Cryptocotyle lingua. 0.5 ml. of S.R.B.C. was added to each of two test tubes. To one tube 0.5 mls. of antigen solution were added, to the other 0.5 ml. of saline, as a control. B.S.A. was used at a concentration of 0.025% (w/v) in saline, and C. lingua antigen at 0.1% (w/v) of freeze-dried material dissolved in 0.85% saline. Both the experimental and control tubes were left for 10 minutes then, after centrifugation, were given two washes with serum saline (0.85% saline with 1% normal, inactivated rabbit serum). After the final wash, the volume of the cells was adjusted to 5 ml. with the serum saline. These cells were then ready for the titration.

Prior to haemagglutination all test sera were inactivated at 56°C for 30 minutes, and adsorbed once with an equal volume of S.R.B.C. for 10 minutes, at room temperature. Haemagglutination was performed in specially constructed lucite plates (Flow Laboratories, Ltd.) using micro-pipettes to prepare the double dilutions of sera, and dispense the coated cells. Each test serum was taken through a series of doubling dilutions in serum saline from 1:1 to 1:256. To each serum dilution an equal volume (0.025 ml.) of antigen coated erythrocytes were added. For each serum a control consisting of tanned, uncoated S.R.B.C. was used. Coated S.R.B.C. were tested against serum saline alone, and against known negative serum as further controls.

The haemagglutination plate was covered and left at room temperature for three hours, after which time the plates were read for any observable reaction. The presence of antibody produced positive agglutination that was indicated by the S.R.B.C. forming a thin layer

of cells across the surface of the U-shaped well. The absence of antibody, however, was indicated by the S.R.B.C. precipitating into a distinct button of cells at the bottom of the well. The reciprocal of that dilution of serum giving positive agglutination was taken to be the titre of antibody present in the test antiserum.

Immunofluorescence

The indirect immunofluorescence technique of Weller and Coons (1954) was used for the detection of antigen and antibody. The method may be summarised thus:

Fluorescent anti-gamma globulin
+
Specific Ab
+
Antigen

Using this technique it is possible not only to detect the production of antibody, but also resolve the precise site of its secretion, and its eventual location on an antigenic organism or tissue. High titre antisera, namely anti-plaice globulin (raised in rabbits), and anti-rabbit globulin (raised in sheep), were chemically bound to fluorescein isothiocyanate (F.I.T.C.). The anti-rabbit globulin was obtained already bound to F.I.T.C. from Burroughs Welcome, Ltd. Anti-plaice globulin, however, was labelled in the laboratory using the following procedure.

Production of anti-plaice globulin in rabbits. Antiserum to plaice globulin was raised in rabbits using the immunisation schedule outlined above. As a high titre of antibody was necessary, the double diffusion in agar test was selected, as a relatively insensitive method of antibody assay, that would only yield positive results when antibody levels were high. When a suitable antiserum had been raised it was necessary to 'salt out' the gamma globulins from the serum albumin. The latter combine preferentially with the fluorochromes to form highly negatively

charged molecules that may become a source of non-specific fluorescence. The method depended upon the relative insolubility of gamma globulin in 30 - 40% ammonium sulphate. Accordingly, to 2 volumes of antiserum, 1 volume of saturated ammonium sulphate was added dropwise. The precipitated globulin was centrifuged at 6,000 r.p.m. and given 2 washes with 30% saturated ammonium sulphate. The precipitate was then dissolved in a minimum volume of saline and dialysed against the saline to remove excess ammonium sulphate from the globulin solution.

Conjugation of the globulin and fluorescein isothiocyanate. By measuring the absorbance of the globulin solution at a wavelength of 280 μ, the concentration of protein in mg. per ml. was approximately calculated. For every mg. of globulin 12.5 μg of F.I.T.C. was used for conjugation. The F.I.T.C. was dissolved in 2 ml. of 0.1 M. Na_2HPO_4. This solution (pH 9.0) was unstable and was used within two hours. To the 4 ml. globulin sample, 1 ml. of 0.2 M. Na_2HPO_4 was added dropwise, over a three minute period. Following this the F.I.T.C. was added in the same manner, and the pH of the solution adjusted to pH 9.5 with drops of 0.1 M. Na_3PO_4. This solution was poured into a measuring cylinder and sufficient 0.145 M. saline added to make the volume 8 ml. The reaction was allowed to proceed at room temperature for 30 minutes and at 4°C for a further 30 mintues.

The conjugate was restored to a pH 7.2 and excess unreacted fluorochrome molecules removed, by passing it through a column of Sephadex G25 (expanded in P.B.S., pH 7.2; 0.01 M. for phosphate, and 0.15 M. for saline). Sephadex G25 retained molecules up to 5,000 M. wt. (including the unreacted fluorescein), while conjugated globulin, M. wt. 150,000, passes straight through the column and was collected in 3 ml. fractions. Those fractions containing conjugate

were pooled and concentrated back to the original 8 ml. volume using Lyphogel particles.

Incubation of the conjugate against plaice tissue homogenates removed any remaining conjugated molecules that may have adsorbed non-specifically onto the tissues. Plaice muscle and liver was homogenised in cold saline, frozen to -76°C and then thawed. The deposit was washed twice in P.B.S. and finally the suspension centrifuged at 8,500 r.p.m. in a refrigerated centrifuge, in amounts designed to give 1 ml. of packed tissue in each centrifuge tube. Absorption was carried out by stirring 2 mls. of conjugate and 1 ml. of packed homogenate. This was then incubated at 37°C for 45 minutes, the mixture centrifuged, the supernate decanted and stored in 0.5 ml. amounts at -20°C.

Preparation of the tissue for staining. Tissue blocks approximately 2 - 5 mm. square were prepared and stored in small polythene bags, and the ends of the bags sealed. These were then 'snap frozen' by immersing them in liquid nitrogen for 5 seconds. Using forceps, the frozen tissue was transferred for sectioning or storage at -70°C.

Frozen section cutting. Sections of 5 μ thickness were cut on a Slee rotary cryostat microtome. Sections were removed on coverslips, air-dried, and immersed briefly in cold, absolute acetone (-20°C) for 1 minute.

Fluorescent antibody staining technique. Frozen mounted sections were irrigated with P.B.S. for 30 seconds. Excess P.B.S. was removed, and the test serum was added dropwise to the section and incubated for 30 minutes at 20°C. Following incubation, sections were washed for 10 minutes in P.B.S. and then treated with the F.I.T.C. anti-globulin conjugate for 30 minutes. The sections were washed once again with

P.B.S. for 10 minutes and mounted in phosphate buffered glycerol on a clean glass slide.

The sections were viewed under a Vickers fluorescent microscope employing a mercury vapour lamp as a u.v. light source. Bright apple-green fluorescence in a section indicated a positive result, i.e. F.I.T.C.-anti-globulin conjugate had attached to any globulin on the section. Photographic records were made using the Vickers camera attachment. Photographs were taken on High Speed Ektachrome Film (A.S.A. 160) using four separate exposures of 30 seconds, 1 minute, 3 minutes and 5 minutes.

Three controls were employed for the indirect fluorescent antibody test. These were:-

(a) application of F.I.T.C.-anti-globulin conjugate directly to a section;

(b) application of negative serum to the section followed by F.I.T.C.-anti-globulin conjugate;

(c) application of positive serum, incubation with unlabelled anti-globulin, followed by labelled anti-globulin.

PHYSICAL METHODS FOR THE SEPARATION OF ANTIBODIES AND ANTIGENS

Several techniques were used in this study to fractionate and analyse the complex macromolecular mixtures investigated. The physical methods, outlined below, exploited either differences in molecular weight or net electrical charge of the molecules, as a basis of their separation.

Zone electrophoresis

In the present study, electrophoresis was performed on two inert media, cellulose acetate, and polyacrylamide gel. After completion of the electrophoretic separation, the zones could be characterised by the usual physico-chemical or biochemical means.

Cellulose acetate electrophoresis. In the present study, the complete Millipore cellulose acetate electrophoresis system was used (figure 3). Such equipment provided a consistency and quality that aided a comparative quantitative analysis of fish serum proteins. A comprehensive account of this system is given in the Millipore technical manual (product bulletin PS, 1969), but for the sake of completeness, an outline of the method used here is given.

The cellulose acetate slides (Millipore phoroslides) pre-buffered in 0.075 M. veronal buffer, pH 8.6, were inserted into the Millipore electrophoresis cell, using the phoroslide forceps. Using the Millipore cell, a total of eight separations could be achieved simultaneously on one run. 0.005 ml. volumes of serum were accurately applied to the strips by means of the sample applicators which were gently lowered onto the phoroslides and allowed to deliver their contents for 2 minutes. Following serum application, electrophoresis was performed, with 0.075 M. veronal buffer in the cell, at 100 volts for exactly 20 minutes. The slides were then immersed in Ponceau S dye fixative for 10 minutes, destained in 5% (v/v) acetic acid, and air-dried. The opaque phoroslides were cleared in a solution of the following composition, ethyl acetate (30 volumes) and glacial acetic acid (70 volumes) and dried in an oven at 40^{o}C for 2 - 3 hours. The cleared strips, with the stained bands of protein, were then ready for quantification on the Phoroscope densitometer. This apparatus converted the pattern of stained strips into graphical form, which

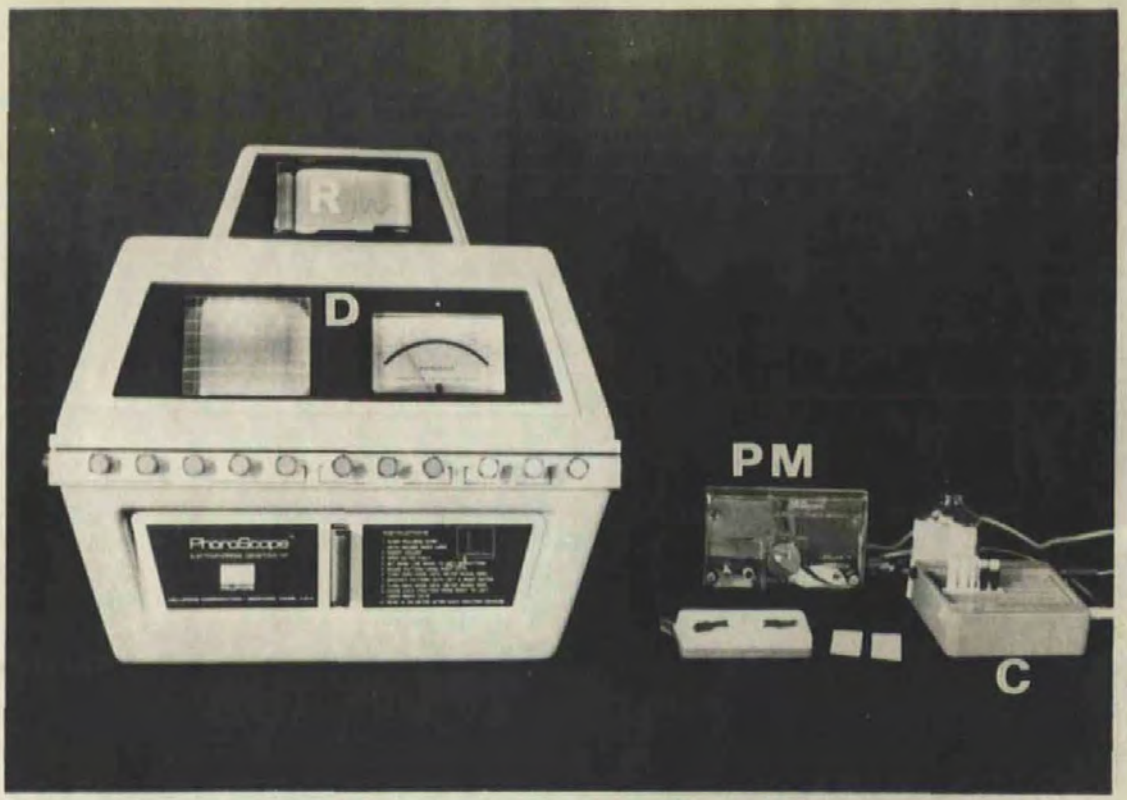


Figure 3. The Millipore cellulose acetate, electrophoresis equipment. Separation of the sera was performed in the cell (C.), using 100 volts D.C. from the power module (P.M.). Stained and cleared cellulose acetate slides were then quantified on the Phoroscope densitometer (D.). A permanent record of the separation was traced on the recorder (R.).

appeared on a fluorescent screen (figure 3). Moreover, using the apparatus, it was possible to integrate the peaks and obtain a numerical value for each zone of stained protein, and thus calculate a percentage value of the total serum protein. For each separation a permanent graphical record was made and stored along with observed values of each of the resolved protein bands in that separation.

Polyacrylamide electrophoresis. Complex mixtures of charged molecules were separated on this gel medium using the Shandon equipment and following the disc electrophoresis method of Davies and Ornstein (1961). Only a brief outline of this method is given as full details of it may be found in Sargent's (1969) book. Three separate gels were poured into glass tubes (2.5" long and 5 mm. inside diameter). These were the separating gel, the spacer gel and the sampler gel. During the run the protein ran from the sample gel, through the large pore spacer gel and finally concentrated at the interface of the spacer gel and the small pore separating gel. The proteins then entered the separating gel as a sharp discrete band, this giving much clearer resolution of the protein bands. Before each separation a few drops of bromophenol blue were added to the buffer in the upper cathode well, to indicate the rate of electrophoresis through the polyacrylamide tubes. Electrophoresis was performed at 100 volts for 20 - 40 minutes, after which time the gels were removed from the tubes and stained with naphthalene black 12 B (1% w/v in 7% acetic acid) for one hour. The stained gels were then washed for several days in 7% acetic acid until all excess stain had been removed. The gels were either photographed against a white background or quantified by a light absorption method using the Chromoscan equipment, which provides, in a graphical form, a permanent record of the separation.

Column Chromatography

A technique of gel filtration employing Sephadex (Pharmacia), a modified dextran, was used to separate macromolecules on the basis of their molecular weight. The dextran macromolecules are cross-linked to give a three-dimensional network of polysaccharide chains. Because of the large number of hydroxyl groups, Sephadex beads swell considerably in electrolyte solutions, and gels are available that expand to variable degrees. This fact is used in the separation of molecules with a wide range of molecular weights. For instance, gels in which the matrix is the minor component are used for the fractionation of high molecular weight substances, whereas compact gels are used for the separation of low molecular weight compounds. Sephadex is supplied in the form of minute beads, which has several advantages in that it imparts good flow and separating properties to chromatographic materials. Three grades of Sephadex were used in this study, the properties of which are listed (table 4).

Sephadex gels have the property, as chromatographic material, to separate substances according to their molecular weight, and over a considerable range, the elution volume of a given substance is approximately a linear function of the logarithm of the molecular weight.

Sephadex G200 chromatography. A column, vertically mounted, 100 cms. long with a diameter of 1.5 cm. was packed with Sephadex that had been previously swollen in buffer for 48 hours at room temperature. The buffer used both for the swelling of the gels and the chromatographic separation was 0.1 M Tris-HCl pH 8.0 with 0.2 M Na Cl and 0.02% sodium azide to prevent microbial growth in the column. After packing, buffer was eluted through the column for 48 hours. Prior to the separation, the buffer was allowed to drain to the level of the gel and buffer flow stopped. The material to be separated, usually in 1 ml. volumes,

Table 4. Listing properties of the grades of Sephadex.

SEPHADEX TYPE	PARTICLE DIAMETER (μ)	BED VOLUME ml./g DRY SEPHADEX	FRACTIONATION RANGE	
			Peptides and globular protein	Dextran
Sephadex G25 Medium	5 - 150	4 - 6	1,000 - 5,000	1,000 - 5,000
Sephadex G100 Medium	40 - 120	15 - 20	4,000 - 150,000	1,000 - 100,000
Sephadex G200	40 - 120	30 - 40	5,000 - 800,000	1,000 - 200,000

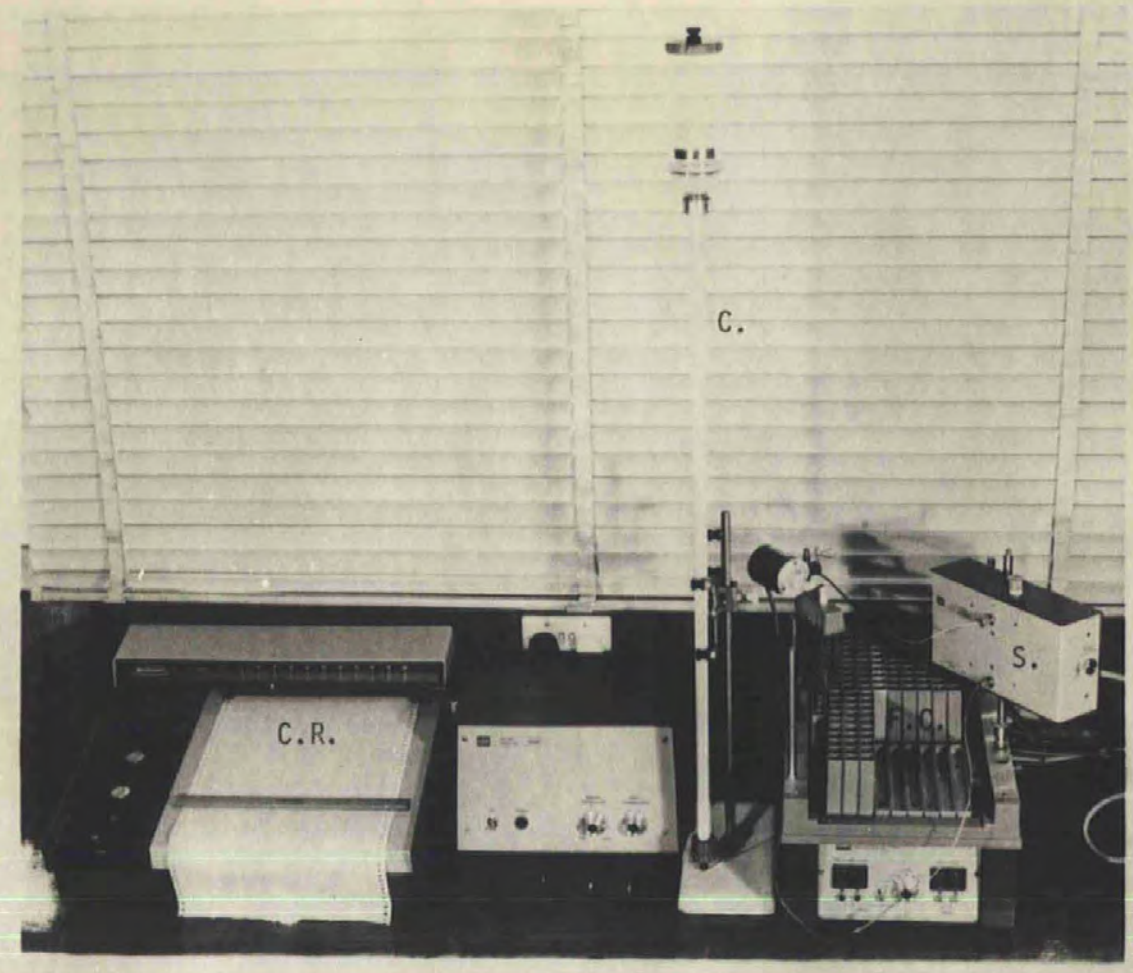


Figure 4. To show the equipment used in column chromatography studies. Proteins eluted from the column (C.), packed with Sephadex G.200, were scanned in the L.K.B. Uvichord apparatus (S.) at 280 nm. and the optical density the fractions charted on a Beckman recorder (C.R.). 2.5 ml. volumes of the protein eluate were automatically taken in the L.K.B. fraction collector (F.C.).

was carefully layered onto the column and allowed to run into the gel. Following this, the reservoir at the head of the column was filled with 200 ml. of buffer and the run commenced. Proteins eluted from the column were measured by their absorbance of a u.v. light source (280 μ wavelength), using L.K.B. (Sweden) scanning equipment fitted to a chart recorder. The fractions were collected in an L.K.B. fraction collector, preset to change tubes every 15 minutes. The flow rate of buffer through the column was adjusted to 15 ml. per hour so that each fraction contained approximately 3.8 ml. volumes (figure 4). By reference to the peaks of a chart recorder graph, fractionated proteins from the original sample could be obtained of known elution volume. To obtain values of the approximate molecular weights of eluted proteins, the following procedure was followed. Firstly, the void volume of the column was estimated using blue dextran. The elution volumes of four protein standards was noted. These proteins were: bovine serum albumin, human immunoglobulin G, salmon immunoglobulin M, and human haemoglobin. A calibration graph was then plotted using the value $\frac{Ve}{Vo}$ (Ve = elution volume of protein; Vo = void volume of column) of each standard protein against the log. of the molecular weight. From the calibrated graph the unknown molecular weights of proteins from any separation could be calculated. Assay for antibody activity was carried out on pooled fractions of protein corresponding to the relevant peak on the graph. Pooled fractions were first dialysed against distilled water, lyophilised, and stored until required for testing.

D.E.A.E. column chromatography. Columns (50 x 1 cm.) were packed with D.E.A.E. (diethylamino-ethyl cellulose) previously swollen in 0.01 M phosphate buffer, pH 7.2. Serum samples were fractionated as for G200 Sephadex gel. However, as this is an ion-exchange gel, at the slightly alkaline pH used, the albumins were bound to the

column and the globulins passed through. This method was used, therefore, to extract gamma-globulin from serum samples.

Analytical Ultracentrifugation

Sedimentation velocity measurements were performed in an M.S.E. Analytical Ultracentrifuge. Sedimentation coefficients were determined at 55,000 r.p.m., at 20°C. The sedimentation of the protein peak was viewed with Schlieren optics, and the rate of sedimentation (S_{20} w) was calculated using the following formula:

$$w^2 = \left(\frac{2\pi, \text{ rpm}}{60}\right)^2$$

The protein was centrifuged at one concentration only, 10 mg/ml, and the sedimentation coefficient was not corrected for concentration.

Chapter 3

SEROLOGY AND IMMUNOLOGY OF PLAICE AND SELECTED HETEROSOMATA. EXPERIMENTS AND RESULTS

Before undertaking a study of the potential immunity of plaice to their parasites, it was considered necessary to investigate their basic serology and immunology. By studying the immune response of plaice to specific antigens, under defined conditions, it was hoped that the information gained would aid in the evaluation of the role of immunity in the more complex host-parasite relationship. Furthermore, as '0'-group plaice were used extensively in this project, it was necessary to determine their immune competence, as there is little or no information on the immunology of these young fish.

Experiment 1. Serum protein patterns of '0'-group and adult plaice. Whilst investigating the basic electrophoretic patterns of plaice sera, a qualitative difference was noted between the '0'-group and adult fish, taken as two years old or more. Serum samples of all age groups captured in February, 1973 were compared electrophoretically and the results recorded in table 5. Five fractions designated I, II, III, IV and V were separated on the cellulose acetate strips (figure 5). The largest fraction, designated II, corresponds to the albumin on comparable mammalian serum separations. A small percentage of the serum protein migrates diffusely ahead of fraction II and is called fraction I. Fraction III is not always completely obvious and occasionally is indistinguishable from fraction II. Fraction IV corresponds to the point of serum application and is usually well defined in all plaice sera. A small amount of protein migrates cathodically and, where this is clearly separated from IV, is called fraction V.

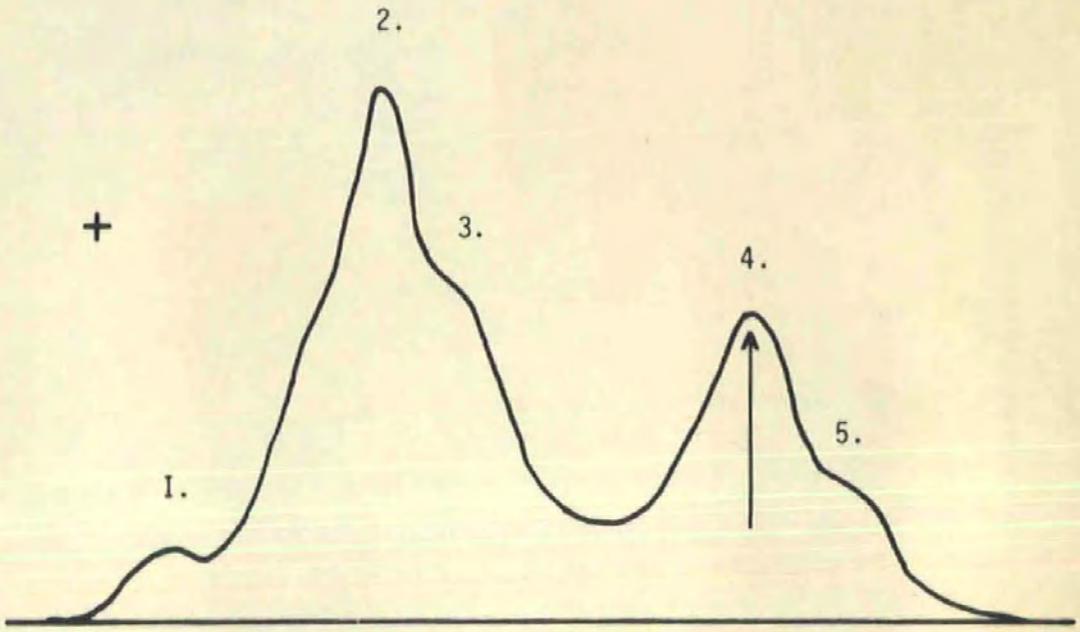


Figure 5. To show a typical serum protein pattern of plaice, after electrophoresis on cellulose acetate strips, indicating the designated fractions I-5. Arrow indicates the point of serum application.

Table 5. Comparing the serum protein patterns of '0'-group and adult plaice following electrophoresis on cellulose acetate strips. All fish were captured in February, 1972. Fish nos. 1 - 5 are '0'-group and nos. 6 - 12 are adult plaice.

FISH No.	PERCENTAGE COMPOSITION OF PLAICE SERUM FRACTIONS				
	I	II	III	IV	V
1	5	46	20	28	1
2	2	73	10	15	—
3	4	53	23	20	—
4	5	56	24	15	—
5	16	52	16	16	0
6	11	48	26	15	—
7	3, 8	66	11	11	1
8	9	74	—	15	2
9	2, 10	55	15	13	5
10	4, 12	51	19	14	—
11	10, 14	46	9	21	—
12	4, 13	56	13	13	1

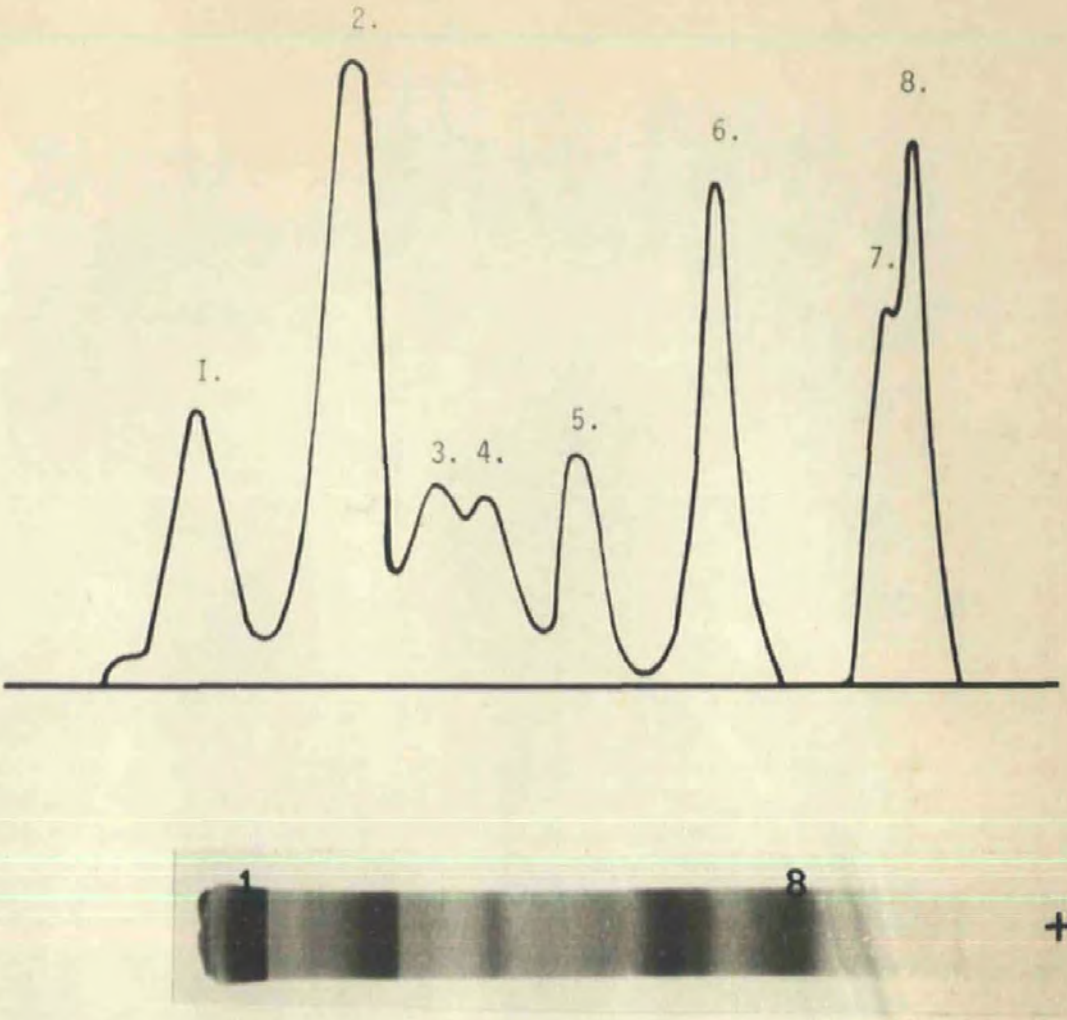


Figure 6. To show a typical serum protein pattern of a plaice following electrophoretic separation on polyacrylamide gel rods. Upper figure is a Chromascan trace of the stained gel shown in the lower photograph.

Generally, the adult fish differed from the juvenile '0'-groups in three respects (table 5). First, the adult plaice display a more complex pattern with smaller fractions appearing in the gamma-migrating proteins. Secondly, fraction III is quite clear in adults whereas it is not always discernible in '0'-group plaice. Thirdly, in the sera of adult fish, the pre-albumins or fraction I, often give rise to two distinct protein bands. Apart from the stated differences, however, both '0'-group and adult plaice have very similar electrophoretic patterns. The results presented here suggest that the protein patterns of plaice serum elaborate with maturity.

In general, electrophoresis of plaice serum on cellulose acetate revealed five major fractions. Greater resolution of the sera, however, was gained by using polyacrylamide gel as the inert base for electrophoresis. Electrophoresis on a comparative basis was performed on sera numbers 9, 10, 11 and 12 using both methods. The results (figure 6) illustrate the improved resolution using the polyacrylamide gel. Eight major fractions of serum number 10 are obvious, compared with the five fractions obtainable on cellulose acetate.

Experiment 2. The serum proteins of '0'-group plaice compared with three other species of Heterosomata. Electrophoretic patterns on cellulose acetate strips were obtained from two specimens of each of the following fish: plaice, sole, flounder and turbot. These were quantified on the Millipore densitometer, following staining and clearing of the strips. The fast, anode migrating fraction, discernible as the most intensely staining band, has in all cases been designated as fraction II. Apart from this, no homology of the protein fractions is implied in the numbering of the fractions from the four species (table 6 and figure 7).

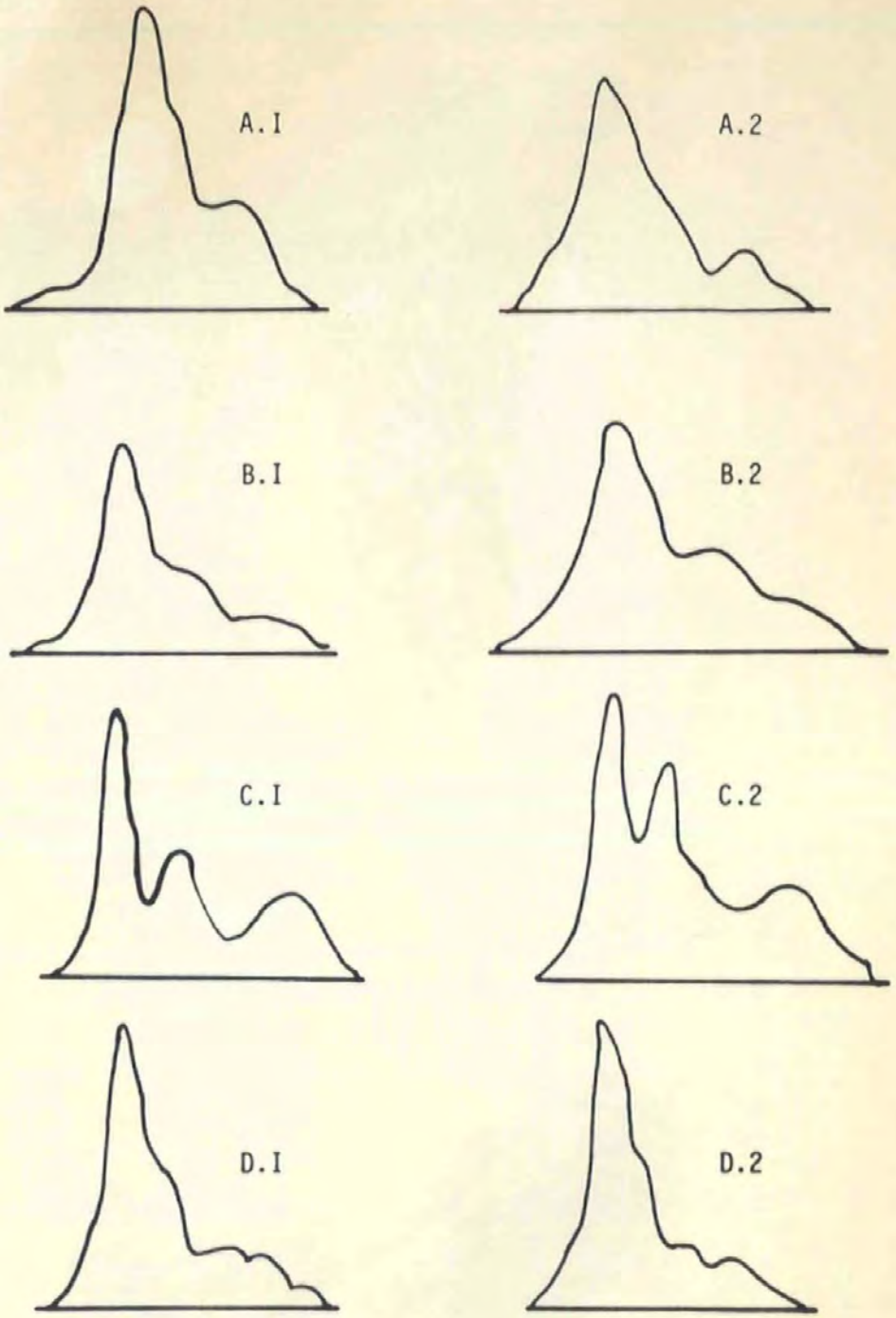


Figure 7. The electrophoretic patterns of serum proteins from four species of '0' group, Heterosomata.

A.I and A.2 are plaice.

B.I and B.2 are flounder.

C.I and C.2 are sole.

D.I and D.2 are turbot.

Table 6. Comparing the serum protein patterns obtained from four Heterosomata, following electrophoresis on cellulose acetate.

SPECIES		RELATIVE PERCENTAGE OF FRACTIONS				
		I	II	III	IV	V
Plaice,	A	5	51	24	15	——
	B	12	55	22	11	——
Flounder,	A	16	62	13	9	0
	B	12	60	20	8	0
Sole,	A	6	38	31	25	0
	B	3	43	34	20	0
Turbot,	A	4	51	26	10	9
	B	6	57	23	9	5

Table 7. To illustrate cross reactions of anti-plaice serum (rabbit) with the sera of other fish species. (+) denotes positive reaction of identity.

FISH SPECIES	No.	PRECIPITATION WITH ANTI-PLAICE SERUM
Dab	1	+
	2	+
Turbot	1	-
	2	-
Flounder	1	+
	2	+
Sole	1	+
	2	+
Dogfish	1	-
	2	-
Plaice	1	+
	2	+

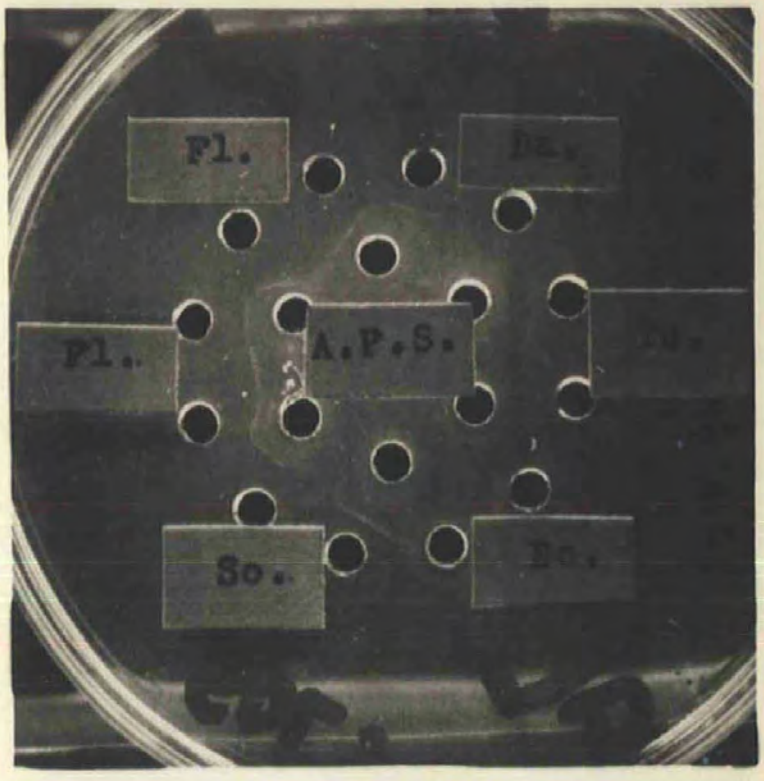


Figure 8. To show the agar gel diffusion of rabbit anti-plaice serum A.P.S., in the central well, against the sera of the following fish:

Flounder (Fl.)

Dab (Da.)

Turbot (Tu.)

Dogfish (Do.)

Sole (So.)

Plaice (Pl.)

Of the four species, plaice and flounder are the most closely related in their electrophoretic patterns. In both cases, fraction II is predominant comprising at least 50% of the total serum protein. A difference nevertheless is indicated by the slightly elevated levels in the fraction III of plaice serum. In both cases there is a small percentage of cathode migrating protein which forms a diffuse band, not as readily distinct as the other fractions.

The electrophoretic pattern of turbot serum protein patterns is quite distinct from plaice in the greater elaboration of the slow, and cathode migrating protein. The sole possesses the most distinctive pattern of these four heterosomatous species. A notable feature of their electrophoretic separations is the relatively lower proportion of fraction II.

Although the species investigated above illustrated broadly similar electrophoretic patterns, a further experiment was necessary to determine whether the serum proteins of these flatfish shared common antigens. First, a high titre antiserum to plaice serum was produced in rabbits. The anti-plaice serum was placed in the central well of an agar diffusion plate and allowed to diffuse against the sera of selected species in the peripheral wells (figure 8). The plaice antiserum reacted with all the heterosomatous species except turbot, and all positive sera showed reactions of identity with the plaice serum (table 7). A negative result was obtained for the dogfish serum.

Experiment 3. Immunoelectrophoresis of plaice serum. Pooled serum from three adult plaice was subjected to electrophoresis for 200 minutes at 100 volts, 10 mamps. Following the initial separation rabbit anti-plaice serum was layered into the troughs, and the gel plate left in a moist atmosphere for 48 hours. From the results (figure 9) it is possible to discern at least 7 major components. 6 of these are anodic

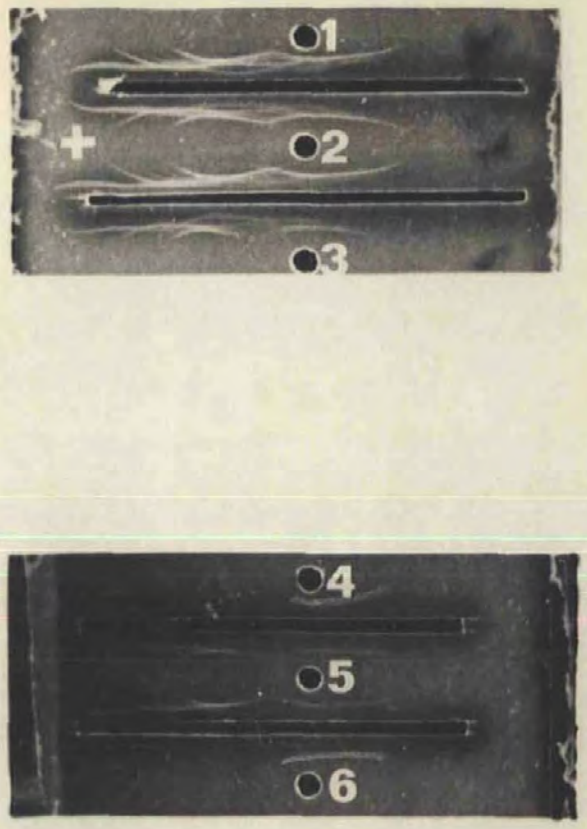


Figure 9. Immunoelectrophoresis of plaice serum against homologous rabbit antiserum. Wells I,2,3 and 5 contained whole serum, and wells 4 and 6 contained the precipitated protein from plaice serum treated with 33% saturated ammonium sulphate. All the troughs were filled with the anti-plaice serum.

migrating, whilst there is clearly one component migrating slightly towards the cathode in the position of a slow β- or γ-globulin.

This latter protein was extracted from whole plaice serum by the following procedure. Saturated ammonium sulphate (SAS) was added to pooled plaice serum to a concentration of 33%. The precipitated protein was washed and subjected to immunoelectrophoresis. When developed against rabbit anti-plaice serum, only one arc developed, in the same position as the cathode migrating protein from the whole plaice serum (figure 9).

Experiment 4. The antibody response of plaice to the parental administration of bovine serum albumin (BSA). As a preliminary experiment, plaice were subjected to multiple injections of BSA and tested at regular intervals for antibody production using the passive haemagglutination (PHA), and Ouchterlony techniques. Fish were divided into experimental groups as listed below:-

(a) 10, '0'-groups and 5 adults at 10°C.

(b) 20, '0'-groups and 5 adult fish at 20°C.

Over the 40 day experimental period all fish received four inoculations of antigens on days 1, 10, 20 and 30. The first injection of BSA was incorporated into F.C.A. and thereafter was administered in saline alone. Adult fish were injected intraperitoneally with 10 mg BSA on each occasion, whilst the '0'-group fish were given 1 mg of BSA per injection. Tests for antibody were performed on days 10, 20, 30 and 40 respectively. Because of their small size '0'-group fish were terminally bled when tested for antibody, whereas individual adults could be continuously screened over the 40 days.

Table 8. The antibody response of plaice to intraperitoneal injections of BSA, at 20°C.

(A) '0'-group plaice

FISH	DAY	ANTIBODY TEST. TITRES	
		OUCHTERLONY	PHA.
1	1	0	N.D.
2	1	0	N.D.
3	10	0	N.D.
4	10	0	N.D.
5	20	0	0
6	20	0	0
7	30	0	4
8	30	0	16
9	40	0	4
10	40	*	*

(B) Adult plaice

FISH	DAY	ANTIBODY TEST. TITRES	
		OUCHTERLONY	PHA.
A	1	0	N.D.
B	1	0	N.D.
C	1	0	N.D.
D	1	0	N.D.
E	1	0	N.D.
A	10	0	N.D.
B	10	0	N.D.
C	10	0	N.D.
D	10	0	N.D.
E	10	0	N.D.
A	20	0	4
B	20	*	*
C	20	0	2
D	20	0	0
E	20	0	0
A	30	0	8
C	30	0	2
D	30	0	16
E	30	0	4
A	40	0	N.D.
C	40	0	N.D.
D	40	0	N.D.
E	40	0	N.D.

(*) indicates fish dead.
(N.D.) indicates test not performed.

None of the fish held at 10°C produced antibody to BSA over the 40 day period. At 20°C, it was possible to detect antibody activity in moderately high titres in adult and '0'-group plaice (table 8).

In the adult fish the onset of antibody production occurred by day 20, in 2 of 5 plaice. By day 30, all surviving fish had detectable antibody to BSA with one fish having a titre of 16. Unfortunately, it was not possible to screen the adult plaice sera on day 40 using the PHA technique. Antibodies, however, were not detected during the 40 days using the precipitation in agar technique of Ouchterlony.

'0'-group plaice did produce antibody by day 30 as detected by PHA, but no precipitins to BSA were noted using the Ouchterlony technique (table 8).

Experiment 5. The antibody response of plaice to parental administration whole calf serum (W.C.S.). Following the limited success of the initial experiment it was hoped to demonstrate the production of precipitating antibody by the administration of W.C.S. Plaice were divided into the following experimental groups:-

(a) 10, '0'-groups at 10°C.

(b) 25, '0'-groups at 20°C.

(c) 5, adults at 20°C.

'0'-groups and adults received 0.1 ml. and 0.5 ml. volumes of undiluted W.C.S. respectively on days 1, 10 and 20, the first dose being incorporated into F.C.A. Detection of antibody was by means of the Ouchterlony technique, tests being made on days 1, 10, 20, 30 and 40 using the homologous antigen. Titres of test sera were determined by plating them against serial dilutions of W.C.S. The reciprocal of the antigen dilution giving visible precipitation was taken as the titre for that serum.

Table 9. The production of precipitins in 'O'-group plaice to calf serum (W.C.S.).

(A) 20°C

DAY	Nos. OF FISH TESTED	ANTIBODY TITRES OF TEST FISH
1	2	O, O
7	2	O, O
10	2	O, O
20	4	N, 1, 1, 1
30	4	2, 4, 4, 8
40	5	4, 4, 8, 32, 32

(b) 10°C

DAY	Nos. OF FISH TESTED	ANTIBODY TITRES OF TEST FISH
1	2	O, O
10	2	O, O
20	2	O, O
30	2	N, O
40	2	N, O

(N) indicates a positive reaction to neat antigen

Table 10. The production of precipitins in adult plaice to whole calf serum (W.C.S.) at 20°C.

DAY	ANTIBODY TITRES OF TEST FISH Nos. 1-5				
	1	2	3	4	5
1	0	0	0	0	0
10	0	0	0	0	0
20	1	1	1	N	N
30	16	*	4	4	*
40	64	*	64	8	*

(*) indicates fish dead

(N) indicates a positive reaction with neat antigen

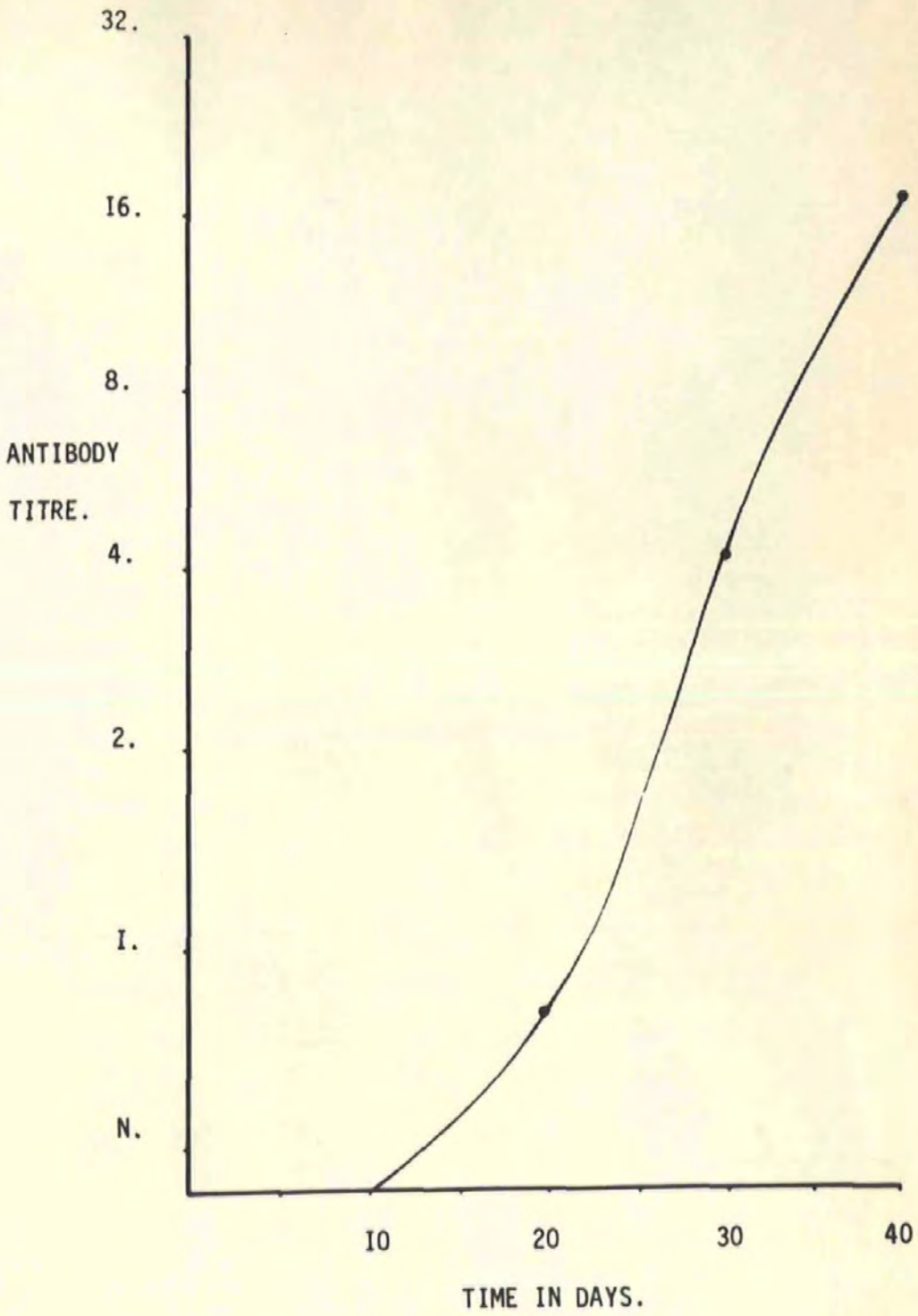


Figure 10. To show the antibody response of '0'group plaice to calf serum antigen, at 20'C. On the graph N. denotes a positive precipitin response to neat antigen. Each point on the graph represents the average antibody titre of several individual plaice.

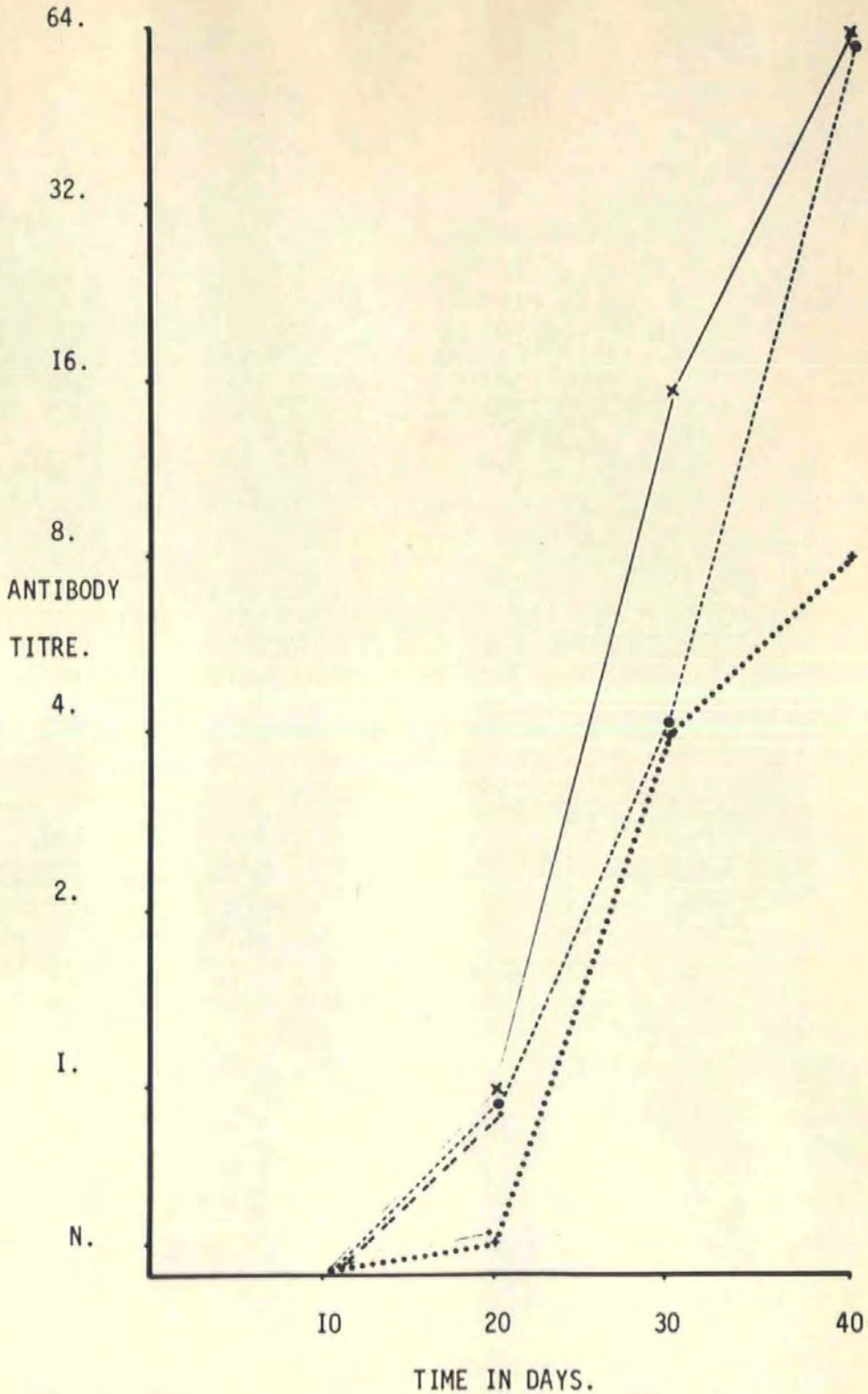


Figure II. To show the antibody response of five adult plaice to calf serum antigen, at 20'C.

Both '0'-group and adult plaice showed a good response to the calf serum antigen at 20°C. In both cases precipitins were detected on day 20 and titres increased until day 40 when maximum titres of 64 in adults and 32 in juveniles were recorded (tables 9, 10 and figures 10, 11). A correlation between the period of antibody induction and ambient temperature was evident in the '0'-group plaice. Precipitins were not seen in plaice held at 10°C until day 30, whereas higher precipitin titres were obvious on day 20 in plaice held at 20°C.

Experiment 6. The antibody response of '0'-group turbot to parental administration of W.C.S. at 20°C. To compare the precipitin production of another '0'-group species of Heterosomata, 15 juvenile turbot were given weekly injections of 0.1 ml. of W.C.S. Precipitin production was tested on days 1, 7, 14 and 21 using the Ouchterlony technique. The induction period of precipitin secretion was between days 14 - 21 at 20°C (table 11).

Experiment 7. Partial characterisation of plaice anti-W.C.S. antibody.

(a) 2 - Mercaptoethanol (2 - ME) sensitivity. Immune serum was first dialysed against 0.2 M, 2 - ME for 12 hours and then against physiological saline for 24 hours. Serum thus treated, and untreated immune serum, were then pipetted into the wells of an Ouchterlony plate and allowed to diffuse against the homologous antigen. A line of precipitate formed between the W.C.S. and normal immune plaice serum, but not between the antigen and 2 - ME treated plaice serum. This indicated that the 2 - ME had, at least partially, reduced the fish immunoglobulin, making it ineffective as a precipitin.

Table 11. The production of precipitins in '0'-group turbot to W.C.S., at 20°C. N indicates a positive reaction with neat antigen.

DAY	TITRES OF TEST FISH
1	0, 0
7	0, 0
14	0, 0, 0, 0
21	N, N, 1

Table 12. The haemagglutination response of '0'-group plaice to intraperitoneal injections of rabbit erythrocytes at 15°C. Figures in brackets denote fish given secondary dose of antigen.

DAY	HAEMAGGLUTINATION TITRES OF TEST FISH
10	2, 8, 0
20	2, 4, 16
40	4, 8, 0
40	(32), (16)

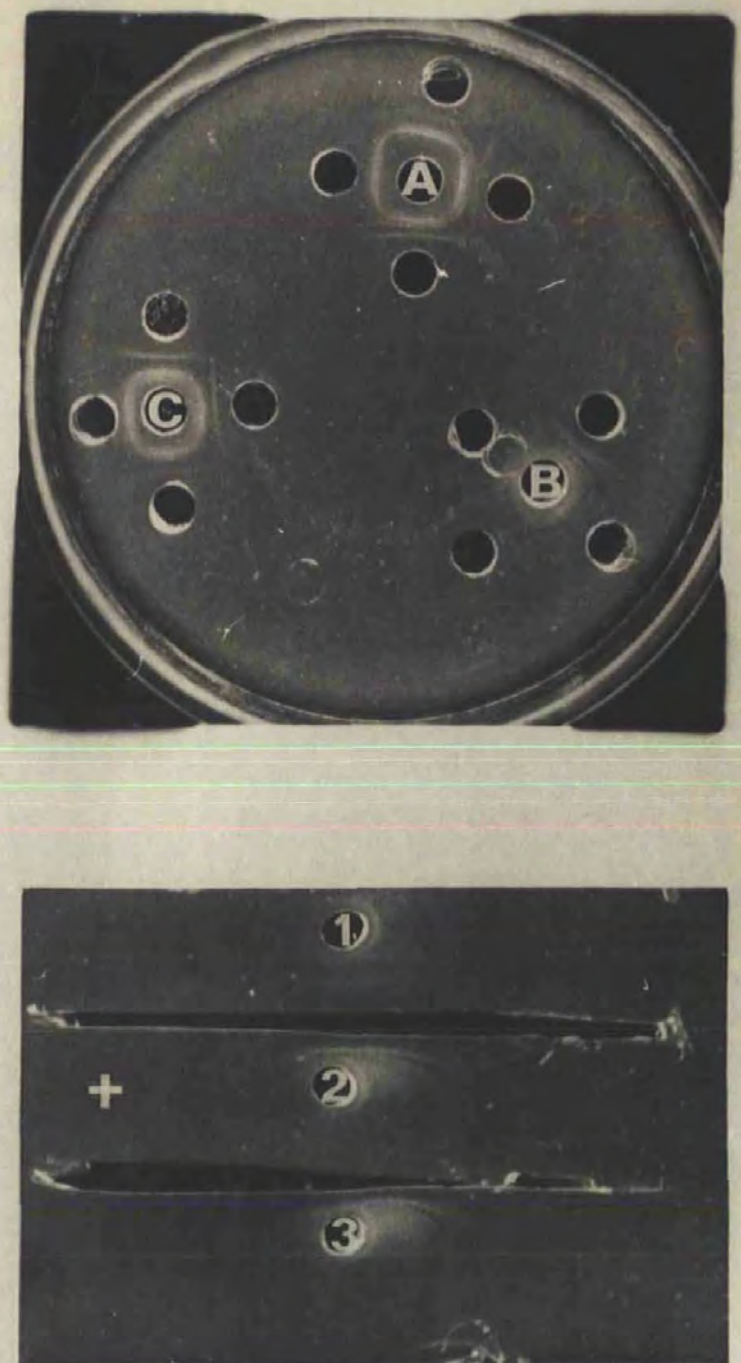


Figure I2. Illustrating agar gel diffusion studies on immune plaice serum to the calf serum antigen. Upper figure shows the multiple lines of precipitates that formed in Ouchterlony tests between the test sera , in central wells A,B and C, and outer wells that contained serial dilutions of the antigen. Lower figure shows the immunoelectrophoresis of plaice serum, in wells I,2 and 3, against calf serum in the troughs.

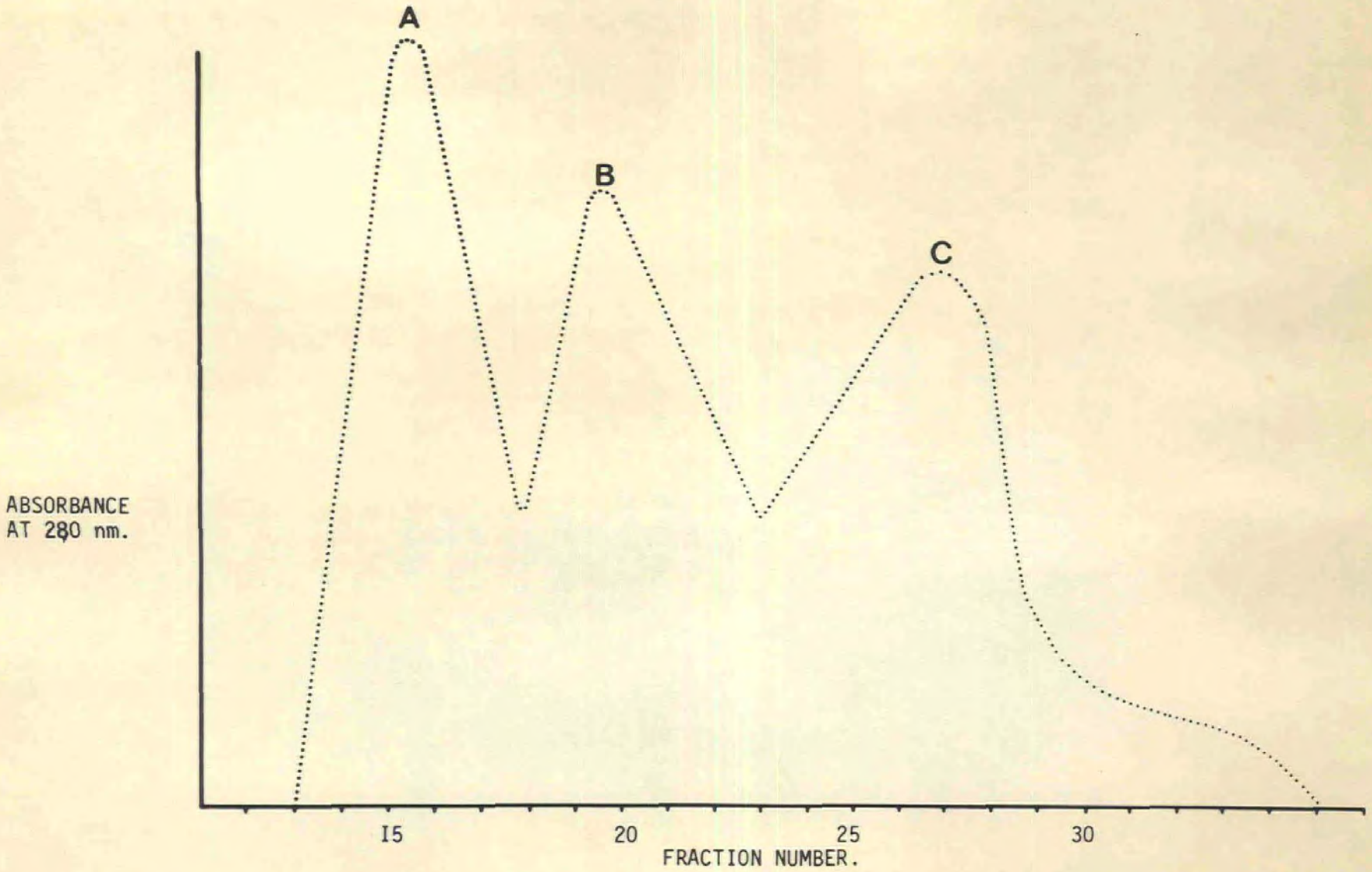


Figure I3. The fractionation of plaice anti-calf serum on a column of Sephadex G.200, dimensions 90xI.5 cm.

(b) Immunoelectrophoresis. First, immune plaice serum was separated electrophoretically and then allowed to diffuse against calf serum diluted 1:1 with saline. After 24 hours a single arc of precipitate was seen in the β migrating area (figure 12).

Secondly, calf serum was separated by electrophoresis followed by diffusion against plaice anti-W.C.S. serum. At least three antigen-antibody reactions were evident, indicating that the plaice had secreted specific antibody to more than one of the protein classes of the calf serum. No lines of precipitate formed in the regions of fast migrating protein, supporting the view that B.S.A. is a poor immunogen in plaice.

(c) Column chromatography. 1 ml. of plaice antiserum was fractionated on a 90 x 1.5 cm. column of Sephadex G200. The serum separated into three major peaks, designated A, B, and C (figure 13). Fractions corresponding to the highest protein concentrations of each peak were pooled, dialysed against water and freeze dried. After reconstitution in saline the proteins of peaks A, B, and C were then tested by agar gel diffusion against W.C.S. Only protein from fraction A precipitated with the test antigen. By reference to the elution volumes of standard proteins of known molecular weight, eluted on the same column, the molecular weight of the protein from fraction A was calculated to be approximately 850,000 - 950,000. The molecular weight of plaice anti-W.C.S. precipitin therefore falls within the range of that of human IgM.

Experiment 8. The immune response of '0'-group plaice to parental administration of rabbit erythrocytes at 15°C. This experiment was designed to investigate whether plaice could produce agglutinating antibody to a particulate antigen and also to test for an anamnestic, or secondary response to the antigen. Twelve '0'-group plaice, maintained at 15°C, were given 0.2 ml. intraperitoneal injections of a

25% suspension of rabbit erythrocytes in saline. Two further control fish were injected with 0.2 ml. of saline. Fish haemagglutinins were assayed using the capillary tube method, with the antibody titre being taken as the reciprocal of the highest serum dilution causing visible agglutination of the erythrocytes.

Three fish were tested for antibody on day 10, following the first injection, and a further three tested on day 20. Of the six remaining plaice, three received further 0.2 ml. injections of the erythrocyte suspension and three were given 0.2 ml. injections of saline only. 20 days later, on day 40, the remaining six plaice were tested for haemagglutination activity.

Controls tested on day 20 and 40 respectively were negative. The fish receiving erythrocyte suspensions, however, clearly responded with the production of haemagglutinins.

Antibodies were evident on day 10 in 2 of 3 plaice tested. There was no appreciable increase in the titres of those fish given a single injection of erythrocytes. In plaice that were given a second dose of antigen, however, there was a suggestion of a heightened antibody response on day 40 (table 12).

Chapter 4

IMMUNE RESPONSE OF PLAICE TO TISSUE PARASITES.

EXPERIMENTS AND RESULTS

Following the preliminary work on the immune response of plaice, attention was directed to the role of the immune system in disease. The three selected tissue parasties, namely Cryptocotyle lingua, Rhipidocotyle johnstonei and Trypanosoma platessae are considered. The life cycles of these species are diagrammatically represented in figure 14.

CRYPTOCOTYLE LINGUA

Cryptocotyle lingua infections in young plaice have already been used in the Plymouth laboratory by Iddon (1973) to study host reaction to parasitic infection. The parasties have been routinely maintained by storing infected L. littorea (figure 14). In the present study the humoral antibody response of plaice to C. lingua metacercariae was investigated by experimentally infecting them with known numbers of infective cercariae.

Initial tests on the sera of wild plaice indicated a restricted number had detectable precipitins to C. lingua antigen. In March 1973, a total of 23 adult plaice were tested using the Ouchterlony technique and of these, only 2 formed precipitates with the C. lingua antigen. The precipitating activity of these sera was abolished by 2 - ME treatment. Unfortunately, even after concention of the immune serum with Lyphogel, it was not possible to produce detectable arcs of precipitation using immunoelectrophoresis. It is possible, however, that the

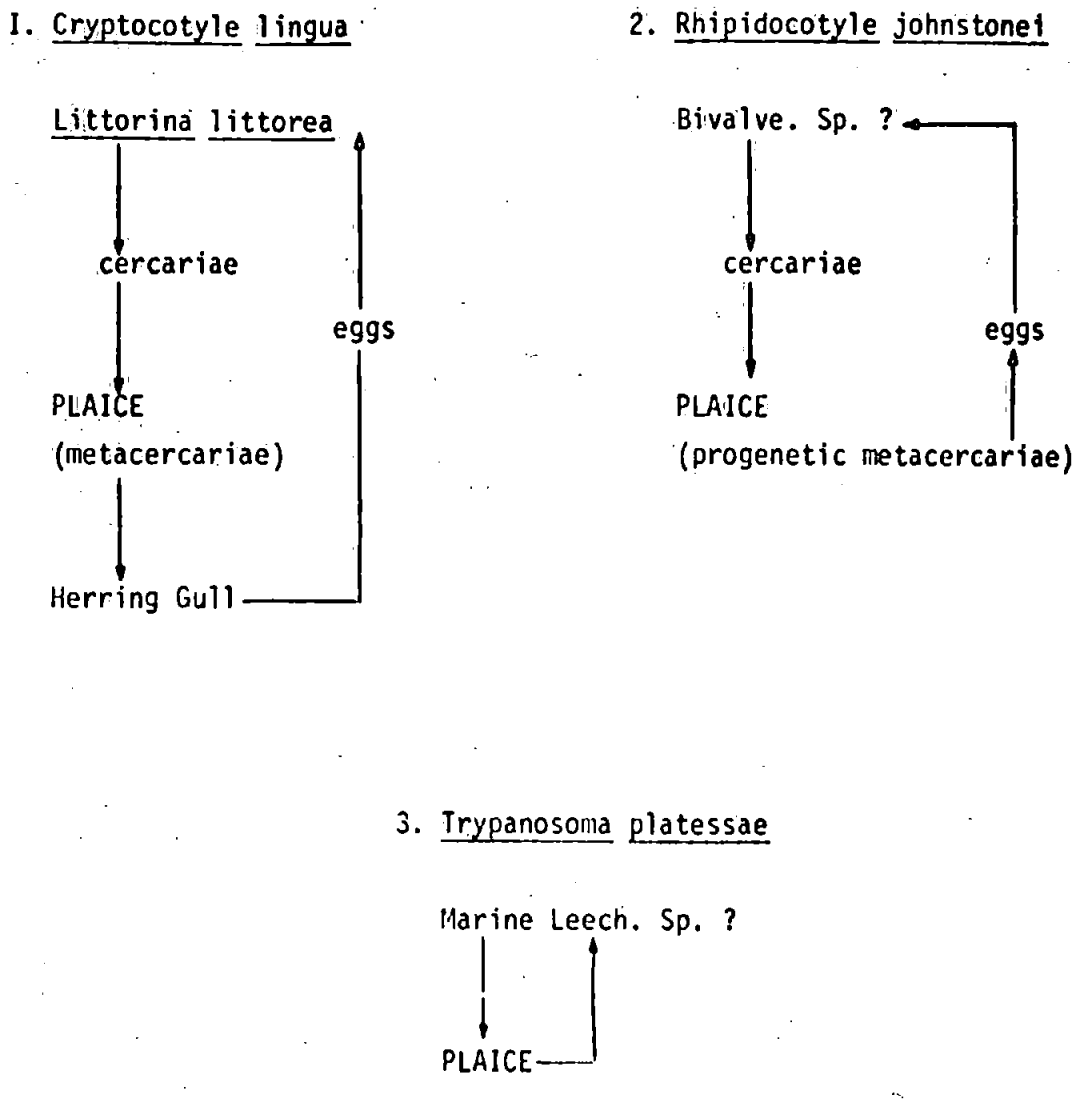


Figure 14. To show the life cycles of the selected parasites utilised in the present study.

precipitins detected were specifically secreted antibodies to C. lingua as the two fish giving positive reactions were lightly infected with C. lingua metacercariae.

In 1973, in this laboratory, Iddon conducted a series of experiments where previously uninfected plaice, approximately 11 months old, were subjected to weekly infections of 800 cercariae per fish. The plaice were maintained at 18°C and received a total of eight of these weekly exposures. Three weeks after the final exposure each fish was challenged with 2,500 cercariae.

On the termination of this experiment, a number of the fish were bled and the sera tested for antibody to C. lingua using the passive haemagglutination (PHA) and Ouchterlony techniques. Mucus samples were also tested for the presence of precipitins using the Ouchterlony technique. Unfortunately, due to the small size of the plaice, it was not possible to collect sufficient serum and mucus from each fish to employ the full range of antibody tests on each individual.

A total of 9 fish sera were tested with PHA, in which the erythrocytes were coated with the soluble C. lingua antigen. Of these one experimental and the uninfected control fish proved negative. Seven experimentally infected fish had antibody titres ranging from 1 to 64 (table 13). 8 of 12 infected plaice displayed detectable precipitins in the serum as indicated by the agar gel diffusion method of Ouchterlony (Table 13 and figure 15). Using the latter technique, no precipitins were detected in the mucus of 14 plaice, although the sera of 5 of these did have detectable antibody. The mucous samples collected, however, were not very concentrated and this, added to the relative insensitivity of the Ouchterlony method, possibly accounted for the negative results.

Following this initial work, further experiments were designed to investigate the effects of temperature and levels of parasite infection

Table 13. The results of immunological tests performed on '0' group plaice experimentally infected with a total of 9,000 cercariae at 18°C.

CODE NO. OF PLAICE	OUCHTERLONY TEST		PHA TITRES
	SERUM	MUCUS	
R 1	N.D.	-	N.D.
R 2	+	-	4
R 4	+	-	N.D.
R 5	+	-	N.D.
R 7	N.D.	-	N.D.
R 8	+	-	N.D.
R 9	N.D.	-	N.D.
R 10	+	N.D.	64
R 12	+	N.D.	N.D.
R 13	N.D.	-	N.D.
R 14	N.D.	-	18
R 15	-	N.D.	N.D.
R 18	N.D.	N.D.	2
B 2	N.D.	N.D.	4
B 10	-	-	-
B 13	-	-	N.D.
B 14	N.D.	N.D.	1
B 22	-	-	N.D.
G 6	+	-	8
C 11	-	-	-

(+) indicates response to antigen

(-) indicates no response

N.D. indicates test not done.

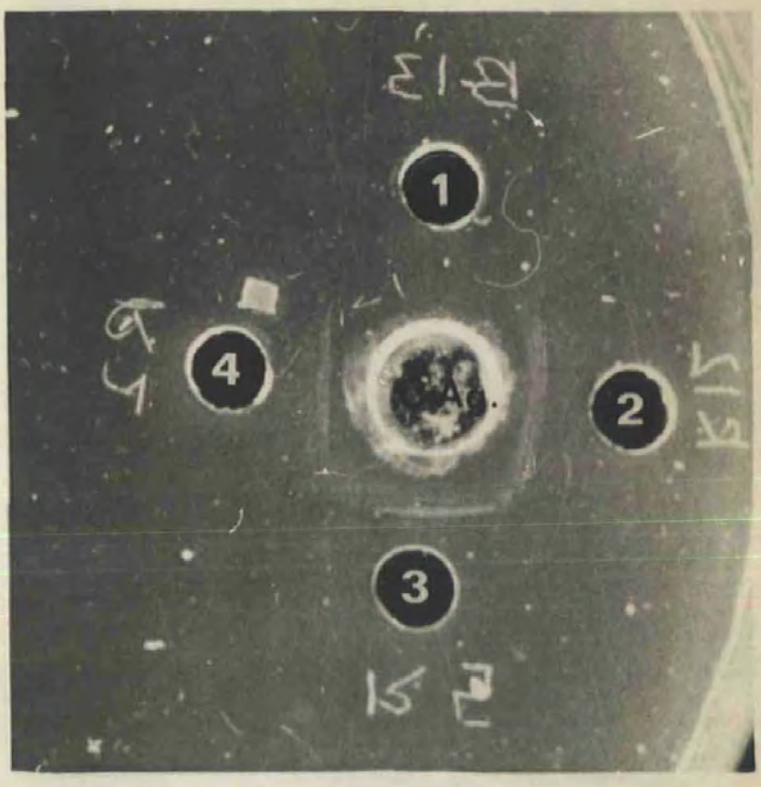


Figure I5. Agar gel diffusion plate showing the reaction of four plaice sera, in the outer wells (I-4), against Cryptocotyle lingua antigen (C. Ag.) in the central well. Note serum number I shows no apparent precipitin activity with the antigen.

on the humoral response of plaice. Adults were preferred for these experiments as it was possible to screen them continuously for the presence of serum antibodies. Also, adult fish provided sufficient amounts of immune serum for immunochemical analysis of anti- C. lingua antibodies.

Experiment 9. Effect of temperature on the immune response of plaice to C. lingua infection. 25 adult plaice, of mixed sexes and approximately the same length, were divided into three experimental groups. 6 plaice were held at 5°C, 13 plaice were held at 15°C and 6 plaice maintained at 25°C. Two fish in each of the three groups were kept as uninfected controls. The remainder received doses of 1,000 cercariae per fish on days 1, 10, 20, 30 and 40. The fish were tested for anti- C. lingua precipitins on days 20, 40 and 80 respectively, using the Ouchterlony technique. In addition, an electrophoretic analysis was carried out on the plaice held at 15°C. On day 1, and at the termination of the experiment on day 80, pooled serum of the control and experimental fish were compared following electrophoresis on cellulose acetate strips. Quantitative estimates of the plaice serum protein patterns were obtained by scanning the strips in the Millipore densitometer.

None of the plaice maintained at 5°C showed an antibody response to C. lingua. Fish held at 25°C, however, gave a positive precipitin response on day 20. Unfortunately, these fish died on day 34 of the experiment due to aerator failure. Fish held at 15°C showed a good response to C. lingua, 3 out of 11 plaice producing precipitins within 40 days (table 14). At day 80, 5 of the surviving 8 plaice had low levels of antibody to the parasite, 3 of the fish being negative. Comparisons of the serum albumin/globulin ratios, determined after electrophoresis of the pooled sera, indicated a change during the

Table 14. The antibody response of plaice exposed to repeated infections of C. lingua administered at 10 day intervals at 15°C.

DAY	NOS. POSITIVE	NOS. TESTED	ANTIBODY TITRES OF POSITIVE FISH
20	0	11	
40	3	11	N, N, N
*80	5	8	N, N, N, 1, 1

Table 15. The electrophoretic patterns and albumin/globulin (A/G) ratios of plaice infected with C. lingua at 15°C, and uninfected controls.

DAY	POOLED SERUM	SERUM FRACTION					A/G RATIO
		I	II	III	IV	V	
1	Experimental	20	58	8	24	—	2.12
1	Control	15	50	14	21	—	1.80
80	Experimental	3	62	11	24	—	1.85
80	Control	1	68	9	22	—	2.20

course of C. lingua infection over 80 days (table 15). In infected fish there was a discernible rise in the globulins with a parallel decrease in the albumin. In contrast, controls displayed a reversal of this trend with slight increases in albumin levels.

Experiment 10. The immune response of plaice to a single infection with 10,000 C. lingua cercariae. 15 plaice, between 15 - 20 cm. long, were divided into groups of 5 and 10 and held at 5^{o} and 20^{o} respectively. On day 1, all fish with the exception of two controls from each group, were placed into individual tanks and subjected to 10,000 cercariae each over 6 hours. Infected and uninfected control plaice were tested periodically for antibody on weeks 2, 4 and 8 following infection. 0.1 ml. samples of blood were removed from each fish, and the sera tested against C. lingua antigen using the Ouchterlony technique.

At 5^{o}C no precipitating antibody to C. lingua was detected in the experimental or control fish over the 8 weeks. Visual examination of the fish indicated that there had been limited development of the metacercariae at this temperature, as very few of the typical black cysts were evident.

At 20^{o}C, onset of antibody production occurred in one fish by the fourth week. By the eighth week all 6 surviving fish produced detectable antibody to C. lingua. In contrast to infected plaice at 5^{o}C, the 6 plaice at 20^{o}C displayed massive infections of metacercariae. Antibody was not detected in the control fish at 20^{o}C.

The 6 antibody producing fish were bled terminally, 9 weeks after initial infection, the sera from these pooled and concentrated from 6 to 2 ml. with Lyphogel, before proceeding with the following immunological tests. Positive plaice antiserum proved sensitive to 2 - ME treatment. Serum incubated with 2 - ME failed to precipitate with the

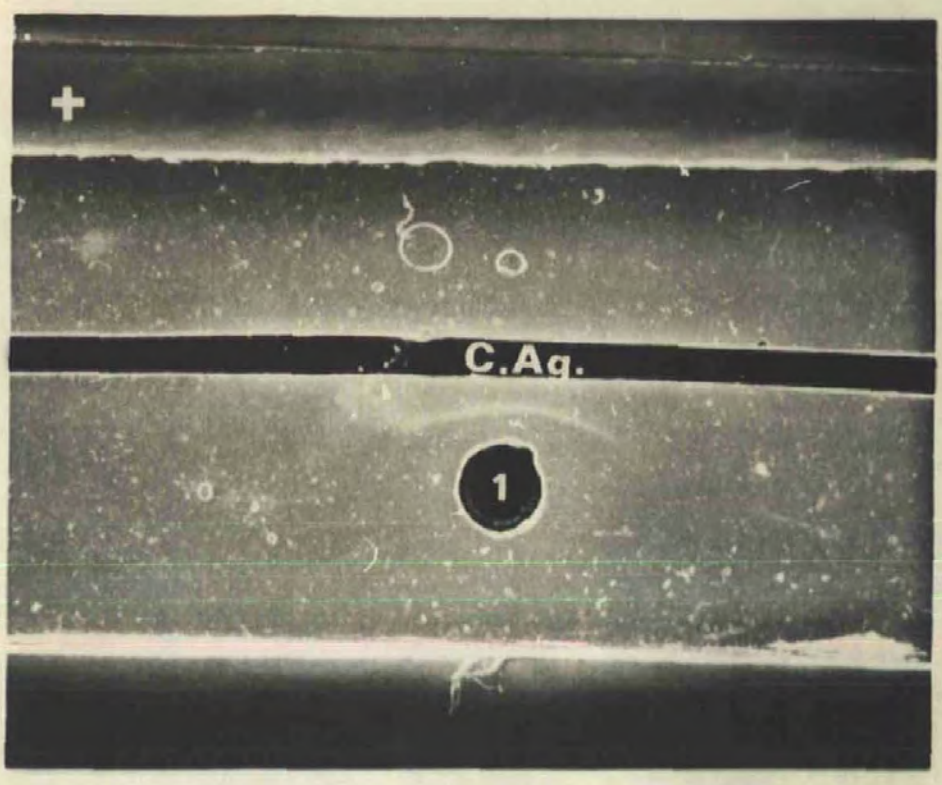


Figure I6. Immunoelectrophoresis of immune plaice serum, in well I, against Cryptocotyle lingua antigen (C.Ag.) in the trough.

C. lingua antigen, whereas untreated control serum gave positive reactions in Ouchterlony tests.

Immunoelectrophoresis. Serum was electrophoretically separated and concentrated C. lingua antigen containing 5 mg. protein per ml. allowed to diffuse against it on the agar slide. After 48 hours, a single faint arc of precipitation developed by the central well, indicating a molecule of β-mobility (figure 16).

Column chromatography. 0.75 ml. of the concentrated, pooled serum was passed down a column of Sephadex G200, dimensions 100 x 2 cms., using the methods outlined in Chapter 2. Three major peaks of protein were noted and antibody activity to the helminth antigen was only associated with the first eluted protein from the column.

Experiment 11. The biological activity of plaice anti- C. lingua immunoglobulin. Previous observations had indicated the potent toxicity of immune plaice serum for live C. lingua cercariae. The following experiment investigated this effect in more detail. To each of five microscope slides was added one drop of seawater containing 50 cercariae. One drop of the following was added to five slides:

1. Anti C. lingua serum from plaice.
2. Normal plaice serum.
3. Anti- C. lingua serum from rabbit.
4. Normal rabbit serum.
5. Seawater.

The slides were maintained in a moist atmosphere at 15°C, and viewed after 5, 30 and 60 minutes.

The cercariae survived over 60 minutes in both the immune and normal rabbit serum and the seawater. In serum from normal, uninfected plaice, precipitates formed around the cercariae after 30 minutes, but all parasites were moderately active over the 60 minutes. The immune plaice serum totally immobilised all cercariae within 5 minutes. Many of the cercariae had sloughed off their tails and were surrounded by visible precipitate.

Experiment 12. The immune response of plaice to parental administration of C. lingua antigens, at 20°C. 8 adult plaice between 15 - 25 cm. long and 20, '0' groups were given single intraperitoneal injections of C. lingua antigen incorporated in F.C.A. '0' group plaice received 0.1 ml. injections containing 1 mg. of protein and adults were given 0.5 ml., equivalent to 5 mg. of protein. '0' group fish were terminally bled on days 10, 20 30 and 40 and the sera tested using Ouchterlony technique. The individual adult fish were continuously screened for antibody production at 10 day intervals over the 40 days. The results (table 16) indicate that C. lingua extracts were successful in invoking a precipitin response in juvenile and adult plaice.

Precipitins were detected in 50% of the adult plaice within 10 days of the first injection, and on day 30 all fish proved positive. Only four fish survived until day 40, three of these being positive and one negative. Antibody titres were uniformly low during the 40 days, only two fish achieving titres of 4.

'0' groups secreted antibody to C. lingua antigen between days 10 and 20, and by day 40, three of five tested fish proved positive for precipitins. The serum antibody titres of these juveniles was not measured.

Table 16. (A) The antibody response of adult plaice to intraperitoneal injections of C. lingua antigen at 20°C.

DAY	NO. POSITIVE	NO. TESTED	ANTIBODY TITRES OF POSITIVE FISH
1	0	8	
10	4	8	N, N, N, 1
20	5	8	N, N, N, N, 1
30	5	5	N, N, 2, 4, 4
40	3	4	1, 4, 4.

(B) The antibody response of '0'-groups to intraperitoneal injections of C. lingua antigen at 20°C.

DAY	NO. POSITIVE	NO. TESTED	ANTIBODY TITRES OF POSITIVE FISH
10	0	5	Not done
20	2	5	" "
30	1	5	" "
40	3	5	" "

Table 17. The specific staining characteristics of soluble and insoluble C. lingua antigens, separated by polyacrylamide gel electrophoresis.

STAIN	STAIN SPECIFIC FOR:	CRYPTOCOTYLE ANTIGEN	
		SOLUBLE	INSOLUBLE
1. Amido Black	Protein	+	+
2. Toluidine Blue	Acid mucopolysaccharide Nuclear protein	+	+
3. P.A. Schiffs	Carbohydrate bound proteins	-	+
4. Sudan Black	Lipoproteins	-	+

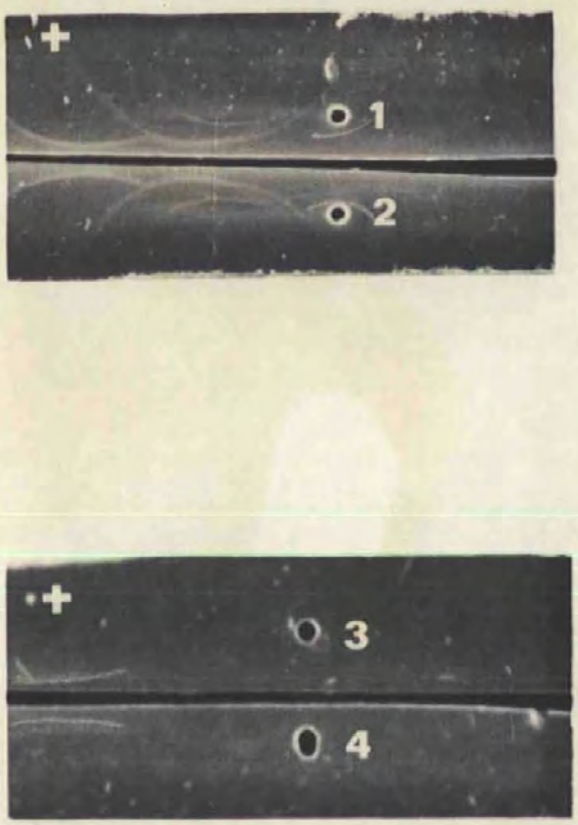


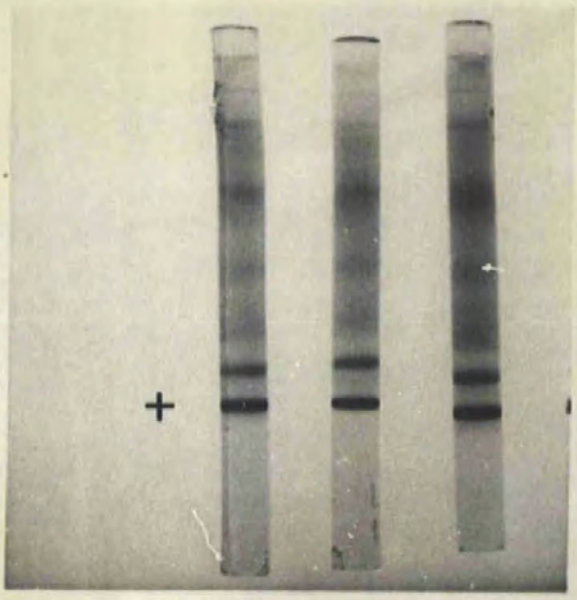
Figure I7. Immunoelectrophoresis of the SOLUBLE (wells I and 2); and INSOLUBLE (wells 3 and 4) Cryptocotyle lingua antigens against their homologous rabbit antisera.

Experiment 13. The partial analysis of C. lingua antigens. Following the demonstration of antibody production in the plaice to C. lingua, it became necessary to evaluate the antigenic nature of the parasite. The following techniques were used to ascertain the numbers and biochemical nature of the functional antigens involved.

Immunoelectrophoresis. C. lingua cercariae were disintegrated in PBS, pH 7.2 and the soluble and insoluble antigens prepared as previously described. Antisera to these were produced in rabbits by the administration of multiple injections of the soluble and insoluble antigens incorporated into Freund's Incomplete Adjuvant. When sufficiently high titres of antiserum had been produced to both antigens, the rabbits were terminally bled.

Both soluble and insoluble antigens were separated by electrophoresis, and allowed to diffuse, on the agar slides, against their homologous antisera. At least five antigen-antibody systems were evident in the soluble C. lingua preparation, one of these antigens clearly migrating towards the cathode (figure 17). The insoluble antigen contained only one antigenic constituent, which appeared to be fast migrating towards the anode (figure 17). To check that there was no cross-reactions between soluble and insoluble antigens, they were subjected to electrophoresis and diffused against their heterologous antisera, i.e. immunoelectrophoresis of soluble antigen against antiserum to insoluble antigen, and vice versa. No lines of precipitate were evident on either of the immunoelectrophoresis plates, indicating that the insoluble and soluble preparations did not share common antigens.

Specific staining of C. lingua antigens. Insoluble and soluble antigens were prepared at a concentration of 2.5 mg per ml. and separated electrophoretically using polyacrylamide gels. Gel rods of separated soluble and insoluble antigen were stained with the following



(A.)

(B.)

Figure I8. Polyacrylamide gel electrophoresis of (A.) soluble and (B.) insoluble Cryptocotyle lingua antigens. All the gel rods were stained for protein with Amido Black.

specific stains: amido black, toluidine blue, periodic acid Schiffs (PAS) and Sudan black. All gels were destained and the resulting patterns photographed (figure 18).

Although both antigens stained with general protein stain, Amido black, they differed qualitatively. The insoluble fraction displayed three bands, whereas the soluble antigen had a minimum of 10 such bands. The insoluble antigens, however, possessed a greater number of protein types with positive reactions for acid mucopolysaccharides, nuclear proteins, carbohydrate bound proteins and lipoproteins (table 17).

Fluorescent antibody staining. As the previous experiments had indicated two separate antigen groups in C. lingua, it was hoped to demonstrate, by fluorescent antibody methods, the location of soluble and insoluble antigens within the cercariae and metacercariae. Frozen sections of the metacercariae and cercariae were treated with rabbit antisera to the insoluble and soluble antigens, and following this with anti-rabbit globulin linked to FITC.

Results indicated that all preparations tested in this way fluoresced under U.V. light. The specific fluorescence in the cercariae and metacercariae tended to be confined to the tegument with duller generalised fluorescence within the parasites. The body wall of metacercariae treated with anti-insoluble antibody was observed to fluoresce more strongly than the anti-soluble preparations, but, unfortunately, no photographic records illustrating this qualitative difference could be obtained.

To evaluate where antibody from immune plaice serum may localise on the cercariae and metacercariae the following experiment was performed. Encysted metacercariae were sectioned in situ in tissues of infected

Figure I9. Showing the specific fluorescence of Cryptocotyle lingua cercaria, following the indirect fluorescent antibody test in which the parasite was incubated in plaice anti-C.lingua serum.

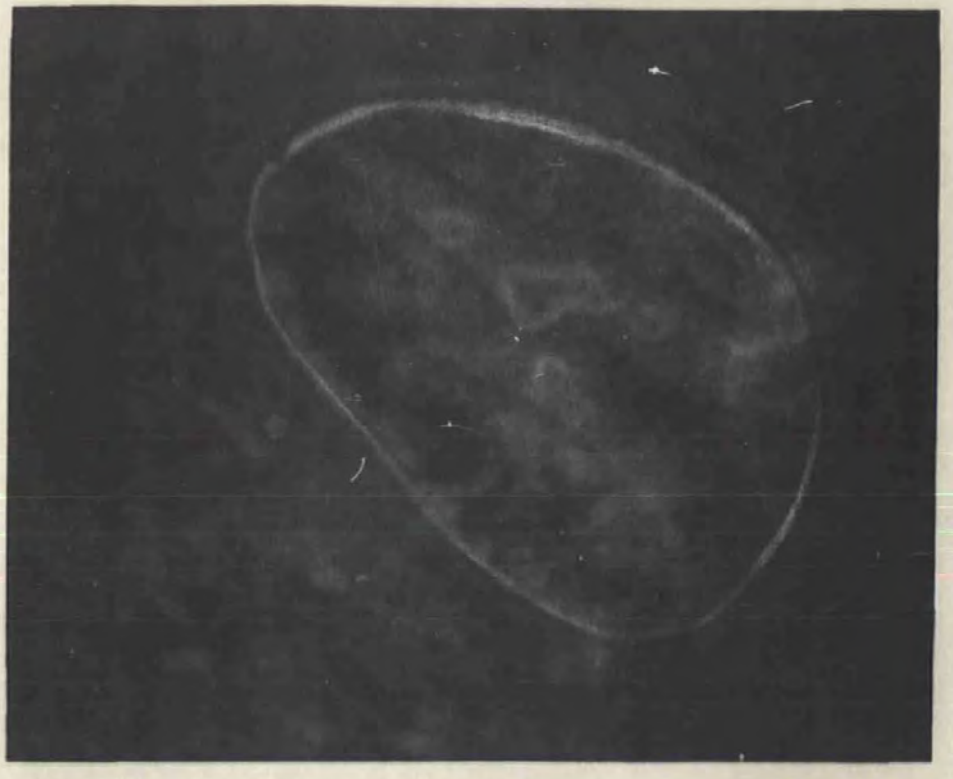


Figure 20. Showing specific fluorescence of sectioned Cryptocotyle lingua metacercaria, following the indirect fluorescent antibody test, in which the parasite was incubated with plaice anti-C.lingua serum. Note particularly the fluorescence of the inner cyst wall of parasitic origin.

'0' group plaice. These sections and heat killed cercariae were treated with anti- C. lingua serum from immune plaice. All preparations were then washed and treated with rabbit anti-plaice globulin conjugated to FITC and viewed for fluorescence.

Both the cercariae and metacercariae fluoresced with a bright apple green colour, indicating that the plaice antibody had attached to sites on the parasite (figures 19, 20). Fluorescence was observed on the cercariae. Sections of metacercariae in host tissue emitted dim fluorescence, with the parasite tegument fluorescing more intensely than the inner tissues (figure 20). Fluorescence was also recorded on the inner cyst wall of parasite origin.

RHIPIDOCOTYLE JOHNSTONEI

Between July and September, 1972, several catches of '0' group plaice were taken from Broadsands Bay, South Devon. A large majority of these fish were heavily infested with Rhipidocotyle johnstonei, the metacercariae of which live progenetically in the muscles and connective tissue of the host (figure 21). The lack of cyst wall around the parasite, the noticable host cellular reaction and the large numbers of R. johnstonei infesting young plaice, all suggested that the parasite might provoke a pronounced immunological response (figure 22).

In September, 1972, 10 '0' groups with heavy infections of R. johnstonei (on average 42 per fish) were terminally bled and their sera tested for antibody using PHA and precipitation in agar techniques. All fish gave weak precipitin bands against R. johnstonei antigen, on Ouchterlony plates. The antigen coated erythrocytes used in PHA, however, gave non-specific precipitation and results were, therefore, discarded. Such non-specific precipitation can be the result of coating the tanned blood cells with too great a concentration of antigen, or the use of impure antigen. The crude antigen extract of homogenised parasites used here, was therefore considered partly responsible for the inconsistency of the results.

Following these initial observations and the theoretically promising nature of this host-parasite system, it was decided to study, in more detail, the infection and immune response of plaice over a set period in 1973.

All fish used in this study were collected from Broadsands Bay, from June to December, 1973. From June to October it was possible to take a sample of at least 50 fish, but in the colder months of November

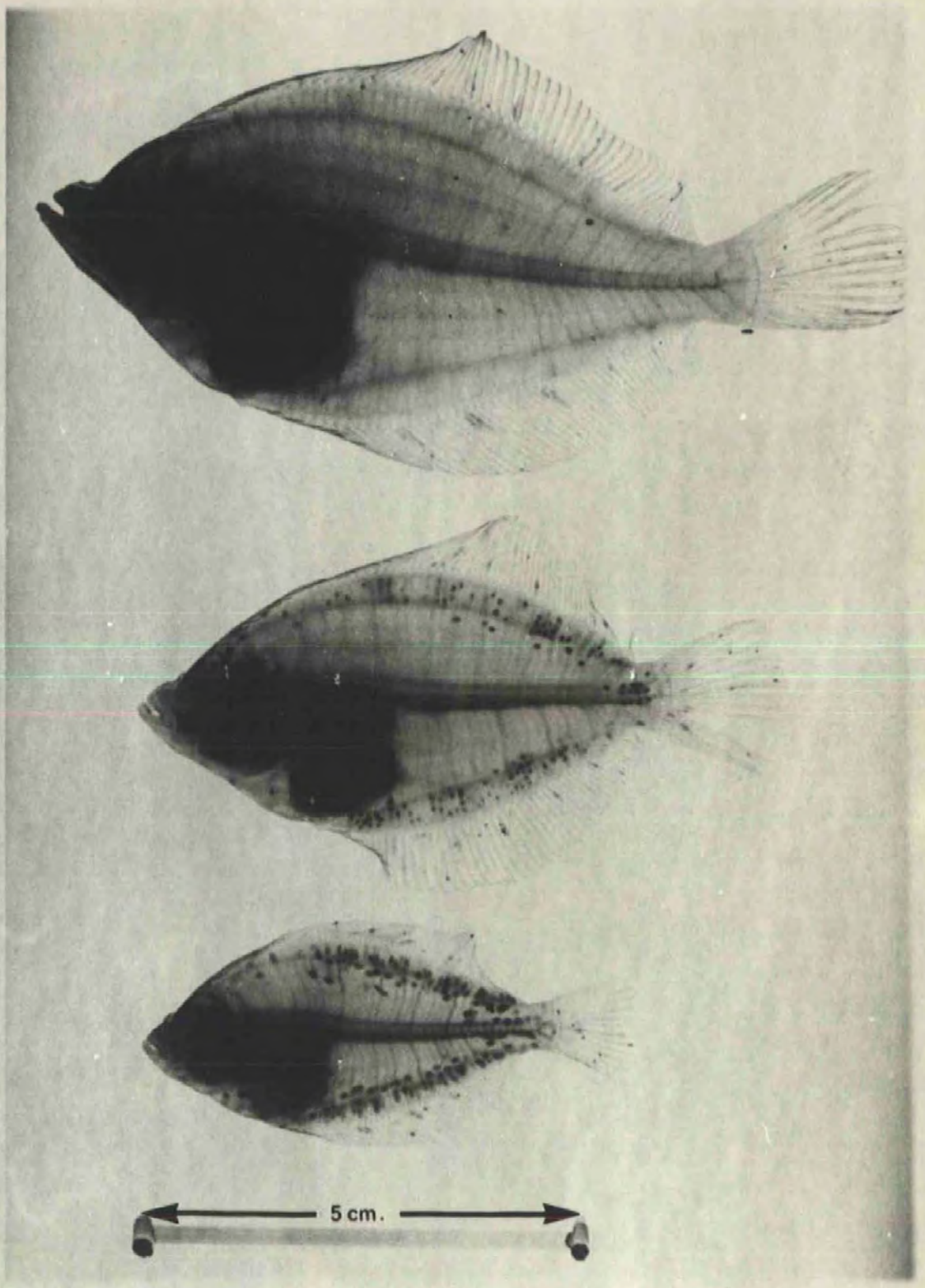


Figure 21. Rhipidocotyle johnstonei metacercariae in the muscles and connective tissues of '0' group plaice. The parasites are predominantly placed at the bases of the fins. The largest fish has only a very light infection.

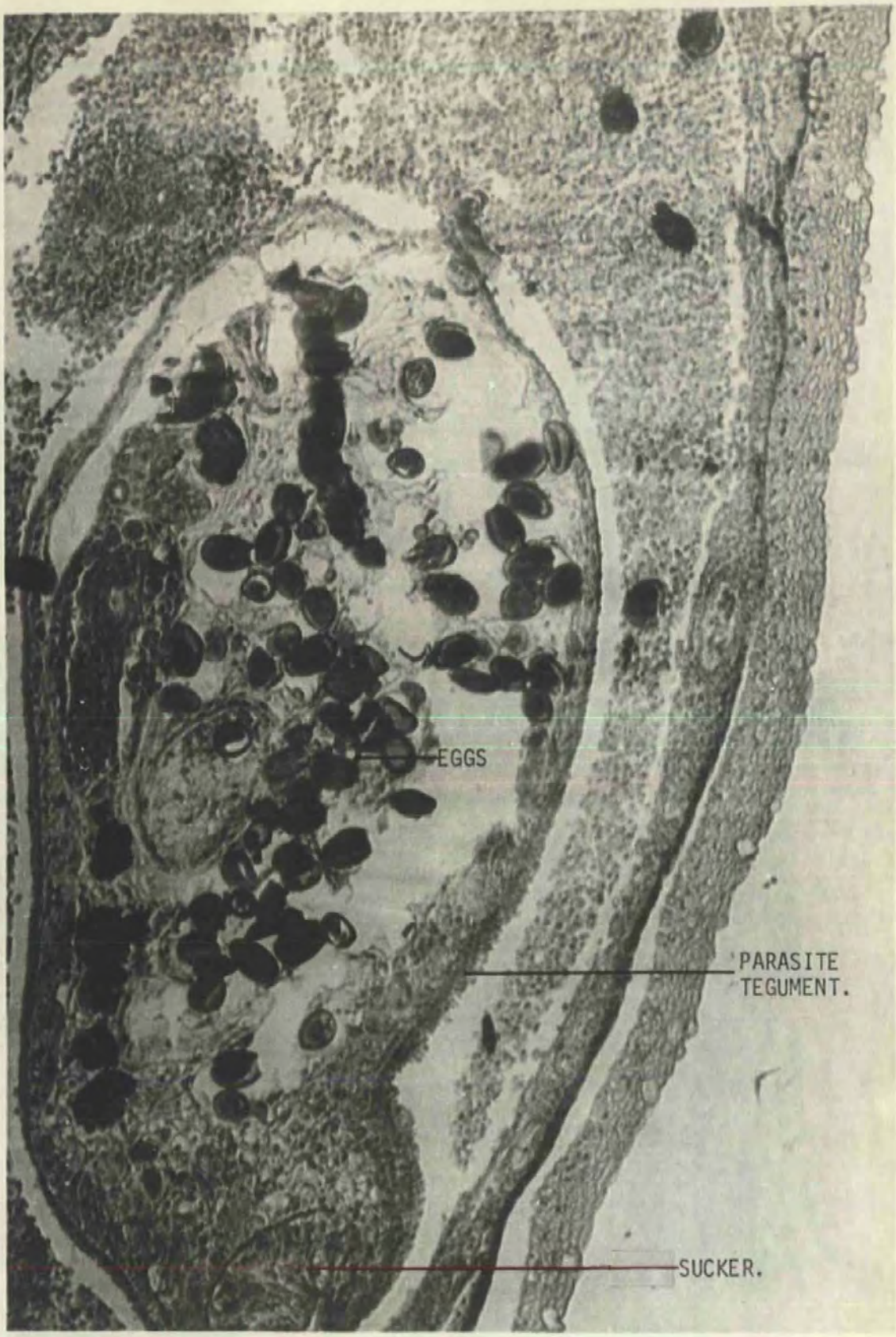


Figure 22. To show a section of a mature Rhipidocotyle johnstonei metacercaria in the caudal tissues of an '0' group plaice. Note the egg production within the parasite, and the intense host cellular reaction around the tegument.

and December only 8 and 23 fish, respectively, were caught. Measurements of the weight and length of all fish were recorded each month and their mean values calculated (table 18). The numbers of metacercariae per fish were determined, using the method of Matthews (1973), by placing the plaice on a lighted stand and counting directly the numbers of parasites. The average numbers and range of parasites each month were noted as were the sea temperatures.

Each month a random sample of 10 fish were tested for precipitating antibody to R. johnstonei, using the Ouchterlony method, the numbers giving a positive response being noted. All data collected is summarised in table 18 and figure 23. A regular, monthly histological examination of the plaice revealed that, although immature metacercariae were in evidence in July, the mature egg producing form was not noted until early August.

In May the plaice were too small to be bled for antibody tests, but thorough investigation of their musculature failed to reveal the presence of any parasites. R. johnstonei appeared in small numbers, on average three per plaice, in July, but no antibody to the parasite could be detected at this early time. By August, when '0' groups had grown to an average length of 6.2 cm., one fish had a maximum infection of 120 cercariae and on average fish were infested with 31 parasites each. Antibody was not evident, however, until September when 4 plaice gave precipitin reactions with the homologous antigen. In October, when plaice were of average length 9.6 cm. and infested with an average 37 metacercariae, all of the tested fish were positive (figure 24). A similar result was obtained in November, when 87% of the test fish were positive for antibody. Unfortunately, it was not possible to obtain blood samples from plaice captured in December as they died during transport to the laboratory. A record of their size and parasite infestation was taken, however, to complete the records for 1973.

Table 18. To show the growth of '0'-group plaice from May - December, 1973 and the development of antibody responses to infestations of Rhipidocotyle johnstonei.

Month	Plaice Av. weight (gms)	Plaice Av. length (cms.)	Nos. of Rhipido-cotyle per fish	Range of Rhipido-cotyle	OUCHTERLONY			Av. Month Sea Temps.
					Nos. Tested	Nos. +ve.	% +ve	
May	0.8	3.5	0	0	10	0	0	11.5°C
June	1.97	5.2	0	0	10	0	0	13.5
July	2.6	5.3	3	1-15	10	0	0	17.0
Aug.	2.7	6.2	31	8-120	10	0	0	17.0
Sept.	3.9	7.1	37	5-12	10	4	40	16.0
Oct.	8.8	9.6	37	2-120	10	10	100	15.0
Nov.	6.8	8.6	40	10-105	8	7	87	12.0
Dec.	7.2	8.1	35	1-103	0	0	0	10.5

Table 19. The effect of ambient temperature on the antibody response of '0'-group plaice to R. johnstonei infection.

Fish	OUCHTERLONY TEST		% Positive
	Nos. Positive	Nos. Tested	
Experimental plaice held at 5°C.	0	5	0
Experimental plaice held at 20°C.	5	5	100
Wild plaice in ambient sea temperature 16-17°C	4	10	40

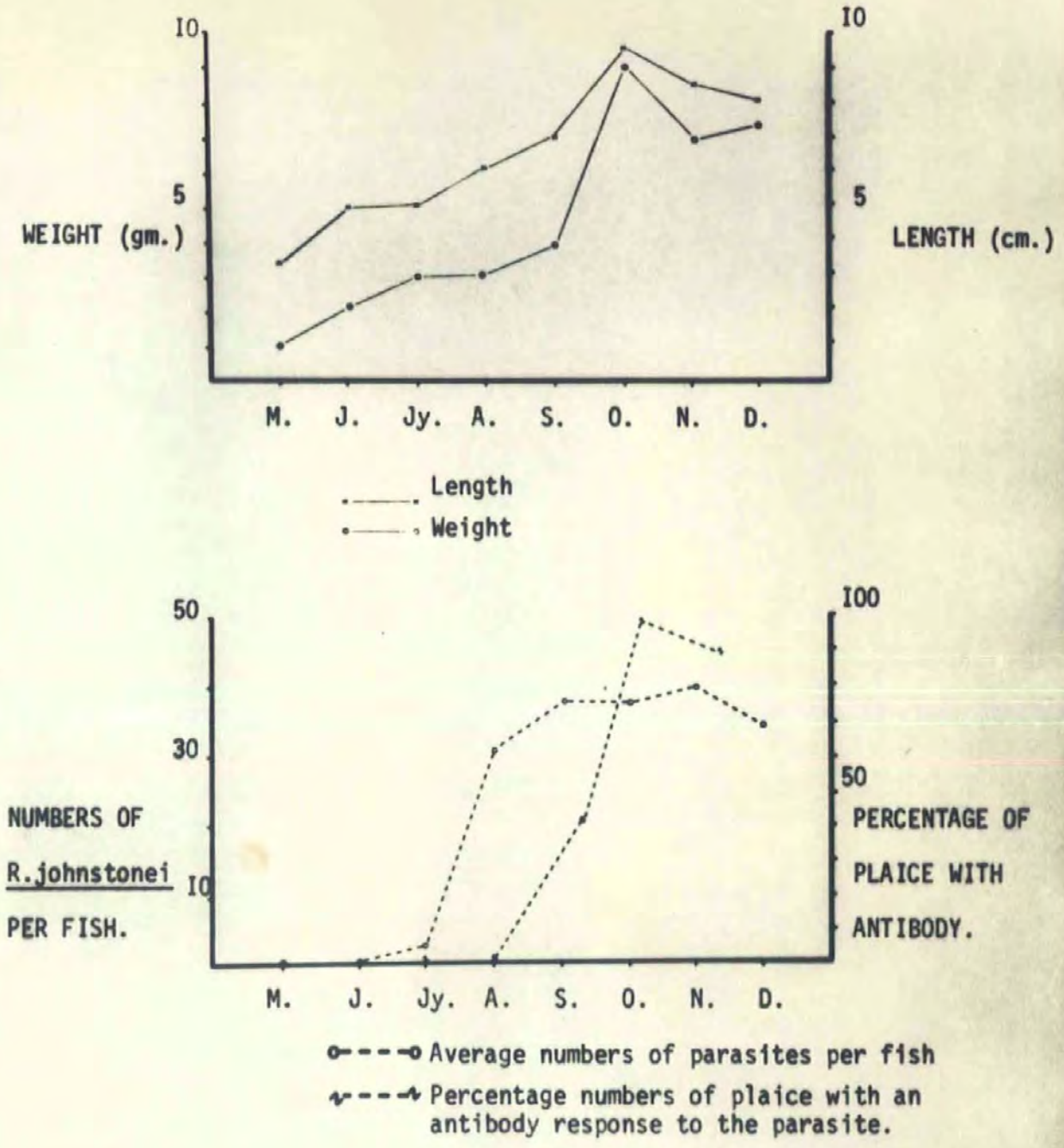


Figure 23. The growth of '0'group plaice from May to December 1973 (upper graph), and the associated average monthly levels of infection with Rhipidocotyle johnstonei (lower graph). Also indicated is the antibody response to this parasite, recorded as the percentage numbers of plaice with detectable precipitins to R. johnstonei antigen.

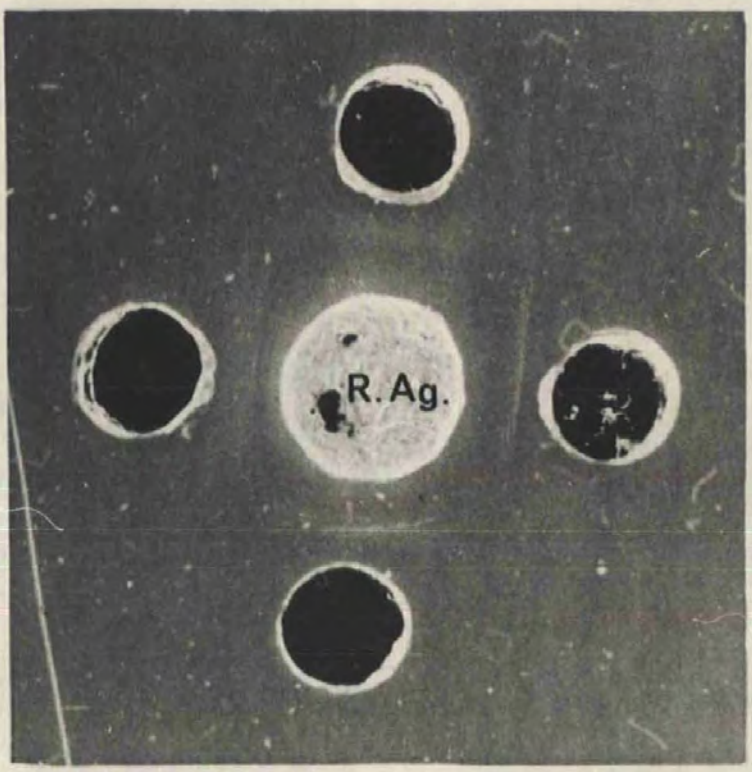


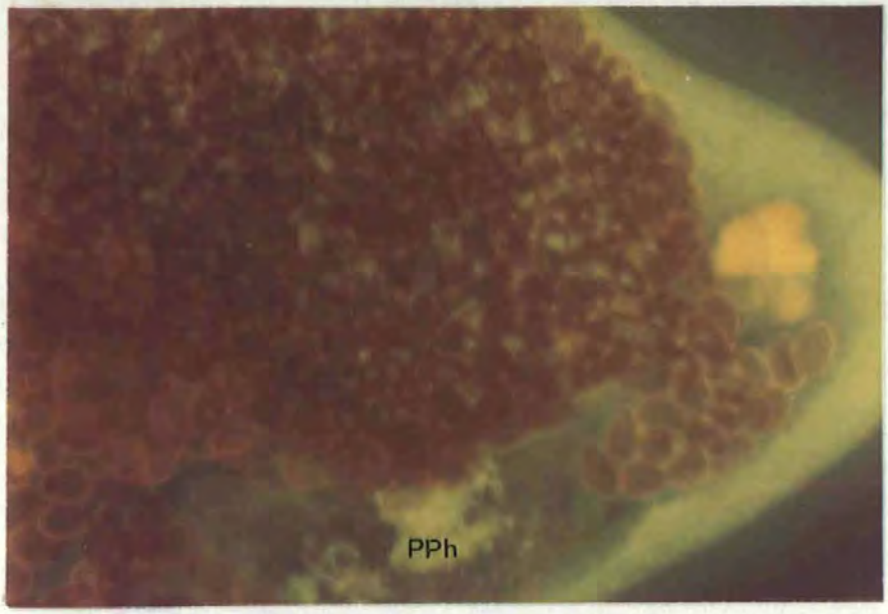
Figure 24. Agar gel diffusion plate showing the reaction of the sera of four infected "0" group plaice against Rhipidocotyle johnstonei antigen (R.Ag.) in the central well.

To summarise, after approximately eight weeks from the initial infection, 40% of the fish produced precipitating antibody to the parasites. Four weeks later, in mid-October, 100% of the plaice had anti- R. johnstonei precipitins.

Unfortunately, due to the small volumes of sera available from infected fish, it was not possible to fully characterise the immunoglobulins by standard immunochemical procedures. The sera were, however, sensitive to 2-ME treatment, untreated immune serum retaining its precipitin activity with R. johnstonei antigen.

Experiment 14. The effect of ambient temperature on the humoral antibody response of plaice to R. johnstonei. Ten fish collected in July when there was no detectable antibody to R. johnstonei, were selected for the following experiment. The fish were divided into two equally sized groups and maintained at 5°C and 20°C, respectively. These were maintained under constant conditions of diet and temperature until September, when they were compared immunologically with wild, infected plaice. Accordingly, in September, when 40% of the wild plaice were positive for anti- R. johnstonei antibody, the plaice at 5°C and 20°C were bled and their sera tested for antibody using the Ouchterlony technique. The results (table 19) suggest that an antibody response to the parasite was temperature dependant. All of those at 20°C produced a positive antibody response, whereas those maintained at 5°C were negative. The wild plaice, that had existed at temperatures approximately between the two experimental values, displayed only a 40% response.

Experiment 15. The determination of R. johnstonei antigens. In order to elucidate the nature of the antigen(s) of R. johnstonei, the indirect fluorescent antibody test was used to locate the areas of specific binding of plaice antibody onto the parasite. Whole



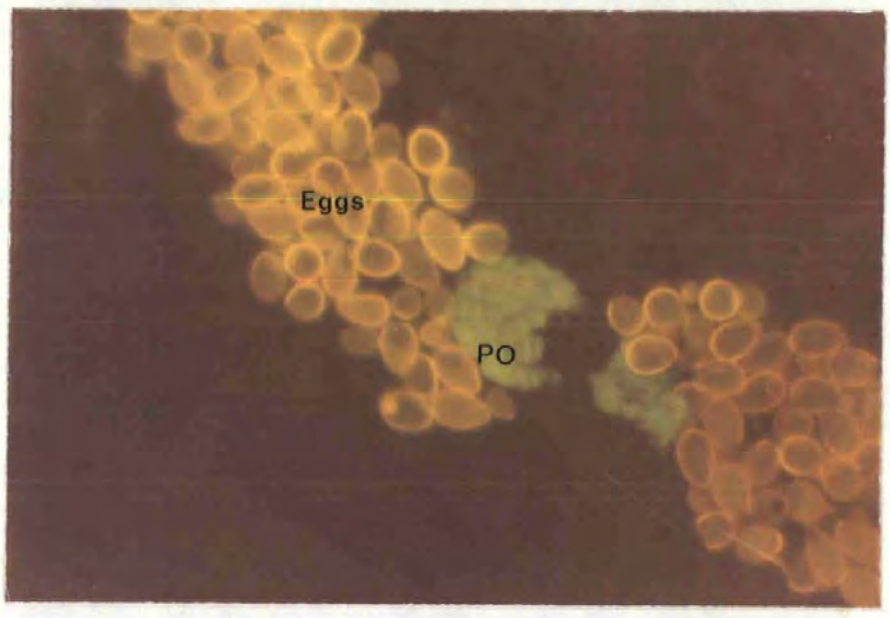


Figure 25. Showing the specific fluorescence of Rhipidocotyle johnstonei metacercariae, following the indirect fluorescent antibody test in which sections of the parasite were incubated in plaice antisera. Note particularly, the bright fluorescence associated with the glandular tissue located in the anterior of the parasite. In photographs, PPh. indicates the prepharyngeal gland and PO. the prostate gland.

metacercariae were dissected out from freshly killed hosts and given several washes in saline. These were treated with immune serum from infected plaice, washed and then finally immersed in rabbit anti-plaice globulin linked to FITC. The metacercariae were viewed under U.V. light using a fluorescence microscope. Where the fluorescence was sufficiently intense, a photograph was taken as a permanent record. Frozen sections of metacercariae, in situ in plaice tissue, were treated as above and viewed for fluorescence.

In the sections and whole metacercariae bright apple-green fluorescence was noted within specific areas of the parasite. In the whole metacercariae (figure 25) the fluorescing tissues were identified by Matthews as prepharyngeal and prostate glands, both of which tend to secrete materials into the surrounding host tissues. Slighter green fluorescence was also noted on the general external surface of the metacercariae. Unfortunately, lack of parasitic material, heavily infected plaice only being available three to four months a year, precluded a more thorough investigation of the biochemical nature of the parasite antigens.

The strong fluorescence of the glandular material confirmed that the functional antigens involved in provoking immunoglobulin production, were of a secretory nature (i.e. E.S. or excretory - secretory antigens). Therefore, live metacercariae were cultured in vitro in an attempt to collect E.S. antigens. Approximately 40, mature metacercariae were washed several times in 0.85% saline. These were then transferred to 5 ml. of sterile plaice ringer and maintained at 10°C for 72 hours. No attempts were made to keep the culture sterile, but gross bacterial contamination was not noted and all parasites survived over the three day period.

The plaice ringer medium was concentrated to 0.5 ml. volume and plated against a known positive homologous antiserum on an Ouchterlony plate. No line of precipitate appeared between the E.S. (?) antigen and plaice antiserum. Following this, the protein concentration of the E.S. antigen was assayed and found to be less than 1 mg. of protein per ml. It was, therefore, concluded that the negative result was caused by the use of a too dilute antigen.

PROLEPTUS OBTUSUS

During the course of the present study, results presented by Baldo and Fletcher (1973) demonstrated the presence of C-reactive protein (CRP) in the sera of normal, healthy plaice. They further showed that plaice CRP precipitated in agar gel with extracts of bacteria, plants and invertebrates including helminths. As indicated in the present study, plaice have been shown to produce precipitating antibody to the helminths C. lingua and R. johnstonei. It was considered relevant, therefore, to further investigate the importance of CRP with regard to helminth antigens.

Delipidated antigens, containing at least 10 mg. of protein per ml. were prepared from the following species: Proleptus obtusus, Ascaris suum (Nematoda), Hymenolepis diminuta (Cestoda) and Fasciola hepatica (Trematoda). Sera was obtained from 9 adult plaice between 25 - 35 cm. long, and allowed to diffuse in agar gel plates against each of the 4 antigens. In addition, similar tests were made on two dogfish with heavy infections of the spiuroid nematode P. obtusus. None of these test fish displayed any precipitin activity with the A. suum, H. diminuta and F. hepatica antigens. Sera from 7 of 9 plaice did, however, develop lines of precipitation with P. obtusus antigen, within as little as 12 hours. Neither of the two dogfish sera precipitated with the P. obtusus antigen.

Further investigations were carried out to determine whether the precipitin activity of plaice sera to P. obtusus antigen was due to CRP or a naturally occurring antibody. As reactivity to the antigen was evident in a large percentage of plaice, it was possible to collect the relatively large volumes of positive sera necessary for immunochemical investigation.

Figure 26. Immunoelectrophoresis of plaice serum against Proleptus obtusus antigen. In the upper figure plaice serum, in wells I,2 and 3, has been separated and reacted with the antigen in the troughs. In lower figure the P.obtusus antigen, in wells 4 and 5 has been reacted with plaice serum in the trough.

10 ml. of pooled serum was concentrated to 5 ml. with Lyphogel. This sample was found adequate for analysis by immunoelectrophoresis, column chromatography, ultracentrifugation and 2-ME sensitivity tests, given in order below.

Immunoelectrophoresis. Serum, separated electrophoretically on agar gel slides, was allowed to diffuse against the P. obtusus antigen, introduced into the troughs. Conversely, antigen was subjected to electrophoresis and allowed to react with the plaice serum.

Precipitates developed within 24 hours. the separated serum forming an arc of precipitation very near the central well, which corresponded to a protein of slow β mobility, similar to the IgM of mammals. The nematode antigen, following electrophoresis, formed only one arc of precipitation with the plaice serum and appeared to migrate as a fast β (figure 26).

Column Chromatography. 2 ml. of the concentrated plaice serum were passed through a 90 x 1.5 cm. column, packed with Sephadex G200. Three major protein fractions were eluted from the column (figure 27), but probably due to the relatively large volume of serum applied there was not a sharp resolution of the peaks.

The following fractions were pooled into three groups, corresponding to the peaks of the three eluted proteins:- A included fractions 14 and 15; B fractions 20 and 21; C fractions 26 and 27. These were then dialysed against distilled water, lyophilised, and reconstituted in saline to a concentration of 5 mg. of dried material per ml. The pooled proteins of peaks A, B and C were then tested for antibody activity against P. obtusus antigen using the Ouchterlony technique. Fractions B and C gave no visible precipitates with the nematode antigen, whereas the first eluted, macroglobulin, fraction A visibly precipitated with P. obtusus antigen.

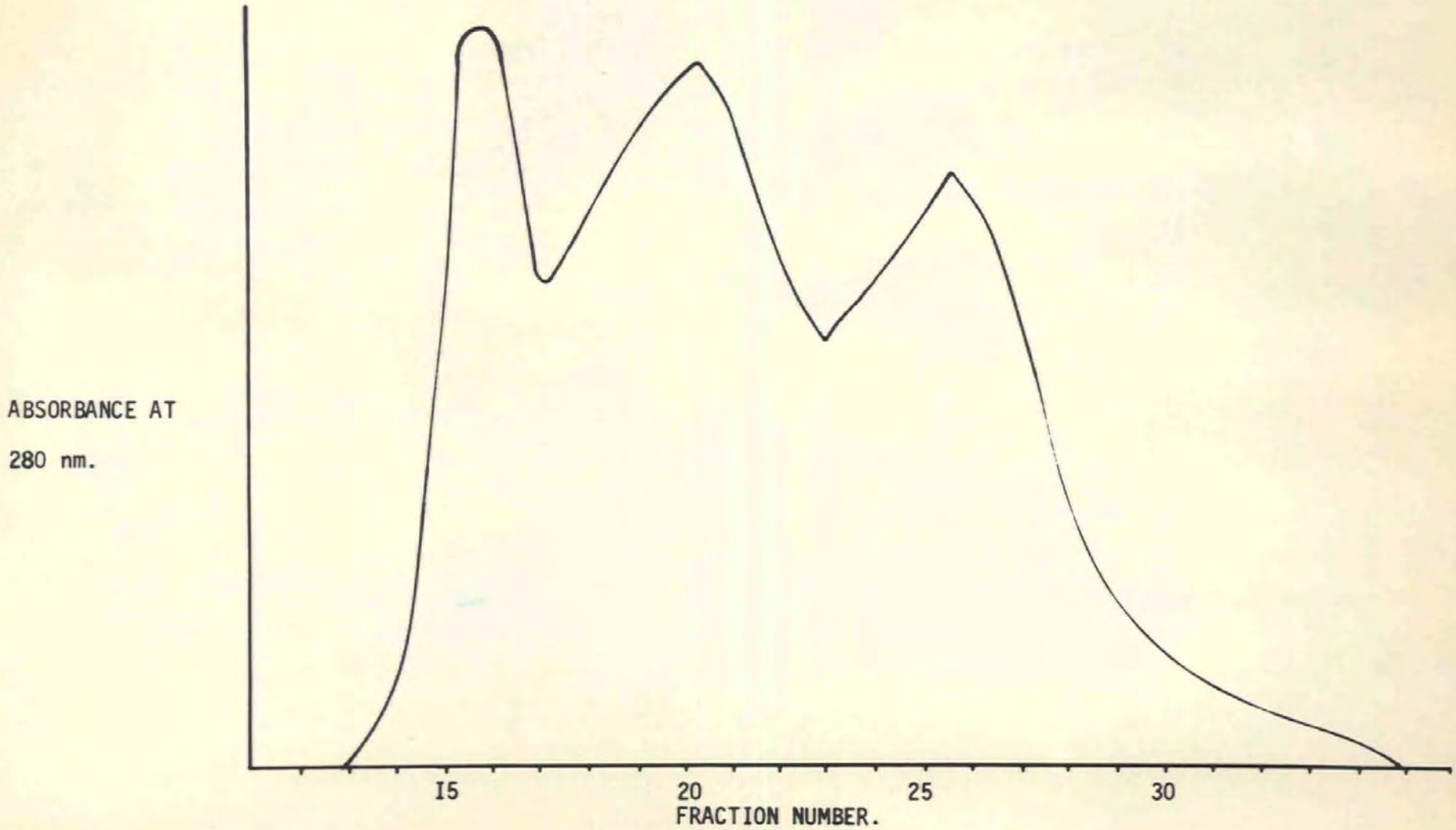


Figure 27. The fractionation of plaice anti-Proleptus obtusus serum on a column of Sephadex G.200. dimensions 90 x I.5 cm.

Table 20. To show the approximate calculation of the molecular weights of plaice serum proteins separated on Sephadex G200, by reference to the elution volumes of standard proteins.
Void volume of column (V_O) is 45 ml.

ELUTED PROTEIN	ELUTION VOLUME (ml.) (V)	V/V_O	LOG MOL. WT.	MOL. WT.
Human IgM	37.5	0.83	5.95	900,000
Human IgG	52.5	1.16	5.26	180,000
Bovine serum albumin	70.0	1.55	4.78	60,000
Plaice fraction A	37.5	0.83	5.95	900,000
Plaice fraction B	50.0	1.11	5.46	290,000
Plaice fraction C	67.5	1.50	4.82	66,000

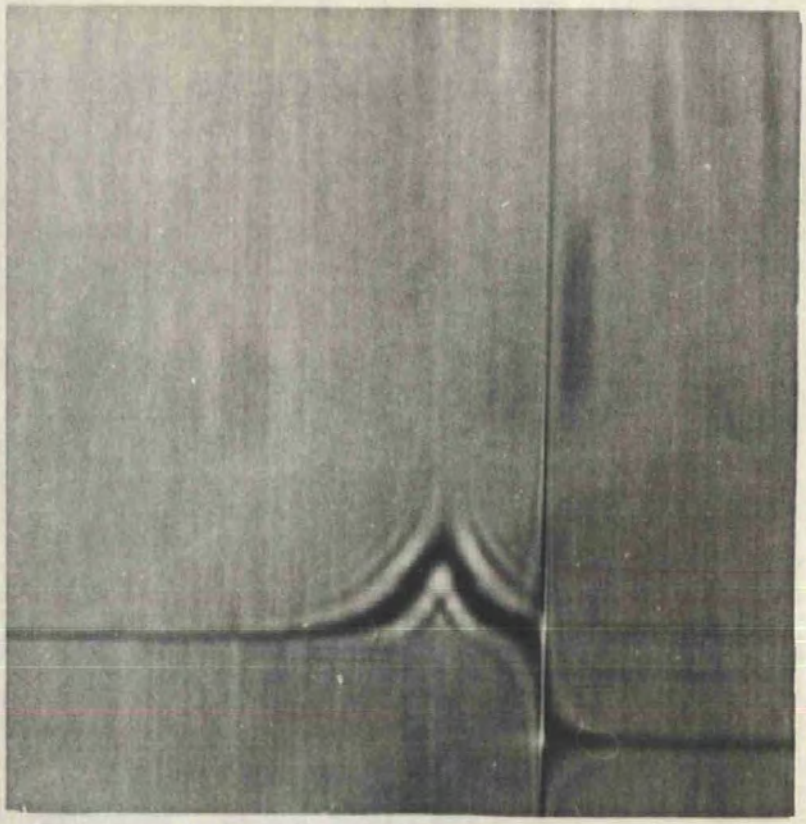


Figure 28. Showing the Schlieren peak of plaice purified precipitin to Proleptus obtusus antigen, following ultracentrifugation at 55,000 r.p.m. Sedimentation is from right to left.

An approximate value for the molecular weight of proteins in fractions A, B and C was calculated by running standard proteins through the column, namely, human IgM, human IgG, and bovine serum albumin (BSA). It can be seen that (table 20) human IgM and plaice serum fraction A had approximately the same elution volume, with the latter having a calculated molecular weight of approximately 900,000

Analytical Ultracentrifugation. To obtain a more accurate estimation of the molecular weight of fraction A, its sedimentation coefficient was calculated following high speed centrifugation. A single, pure protein from fraction A was obtained by first running plaice sera through DEAE Sephadex in PBS at pH 7.2, and then passing it down G200 columns. Protein from the first, eluted macroglobulin peak was dialysed against distilled water and freeze dried, until 15 mg. of material was obtained. The homogeneity of the protein was tested by polyacrylamide, gel, electrophoresis.

5 mg. of lyophilised material was dissolved in 1 ml. of 20 mM. phosphate buffer, pH 8.0 and centrifuged at 55,000 r.p.m. for 30 minutes. The sedimentation of the protein peak was viewed with Schlieren optics (figure 28). From the observed rate of sedimentation an uncorrected S_{w20} value of 19.628 was obtained.

2-Mercaptoethanol Sensitivity. The precipitin activity of plaice serum to P. obtusus, was abolished following incubation with 2-ME. Control sera dialysed against saline retained precipitin activity against the antigen.

Tests for CRP. Three Ouchterlony plates were prepared, where positive plaice serum was allowed to diffuse against P. obtusus antigen. Lines of precipitate developed in 24 hours. One plate was immersed in one of the following solutions: (1) 5% sodium citrate; (2) 0.1 M EDTA

in PBS, pH 7.2; (3) PBS, pH 7.2. These were left for 12 hours at room temperature and viewed for the presence of precipitates. None of the precipitates dissolved in the three solutions. Baldo and Fletcher (1973) have shown CRP precipitates redissolve in EDTA and sodium citrate. It was considered, therefore, that the anti-nematode precipitin recorded here was not CRP and that from the evidence presented, it closely resembled the IgM of mammals and other fish.

TRYPANOSOMA PLATESSAE

Morphology. As a basis for the experimental studies T. platessae was briefly redescribed to include details previously omitted by other workers. Scale drawings were prepared of 15 individual trypanosomes from two host fish. On average, T. platessae measured 50 μ in total length, including a free flagellum of 13.6 μ (table 21). Variations in size were slight and there was no evidence to suggest polymorphism in this species.

In Giemsa stained, blood smears, T. platessae generally assumed a coiled shape (figures 29, 30). The oval nucleus is situated considerably nearer the anterior tip than the posterior, with the prominent kinetoplast situated 1.6 μ from the posterior extremity.

Seasonal incidence of T. platessae. In order to maintain consistency in sampling, all plaice used in this study were 2 - 5 years old and obtained from one trawling ground, namely Looe Bay. Previous study of T. platessae had suggested that infection of younger fish, less than two years old, was extremely limited and that also distribution of the parasite may vary with geographical location of plaice. Monthly samples of at least 20 fish were examined for T. platessae, and the percentage numbers of infected plaice recorded from January - December, 1972. The results of these observations (figure 31) indicate a seasonal fluctuation in the incidence of T. platessae in the plaice stock at Looe.

Peak levels of infection were reached in March when 20% infections were recorded, following which there was a gradual decline until, in July, no infected plaice were noted in a sample of 28. As winter progressed the numbers of infected fish increased to 14% by October. In figure 31, the infection levels are plotted against the average sea temperatures for Plymouth waters as supplied by the Meteorological Office. This shows a possible correlation of infection with ambient

Table 21. To show the morphometric records of T. platessae.

	Mean (microns)	Range (microns)
Body length	37.3	35.8 - 38.4
Flagellum length	13.6	10.0 - 15.2
Total length	50.5	48.4 - 58.2
Width at nucleus	2.6	2.0 - 3.2
Width of undulating membrane	1.0	0.5 - 2.5
Distance of kinetoplast from posterior tip	1.6	1.0 - 2.0
Distance of nucleus from anterior tip	12.6	11.5 - 14.7
Distance of nucleus from posterior tip	24.2	23.6 - 26.7
Nuclear index N.P./N.A.	1.9	1.6 - 2.2
Nucleus length	2.6	2.0 - 3.2
Nucleus width	1.6	1.0 - 2.1

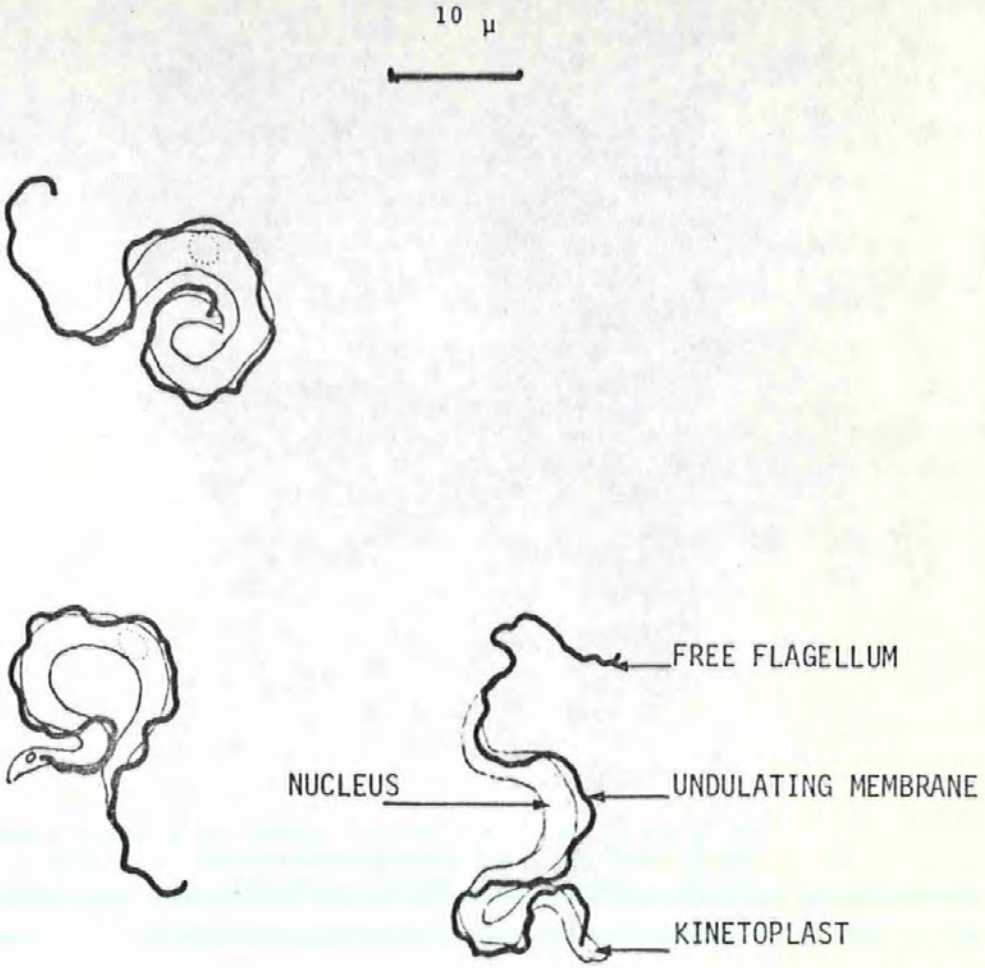


Figure 29. Scale diagrams of Trypanosoma platessae drawn from Giemsa stained thin blood films.

Figure 30. Photomicrograph of a Giemsa stained, Trypanosoma platessae. Key to the labelling:

N. nucleus. K. kinetoplast. U. undulating membrane.

F. free flagellum.

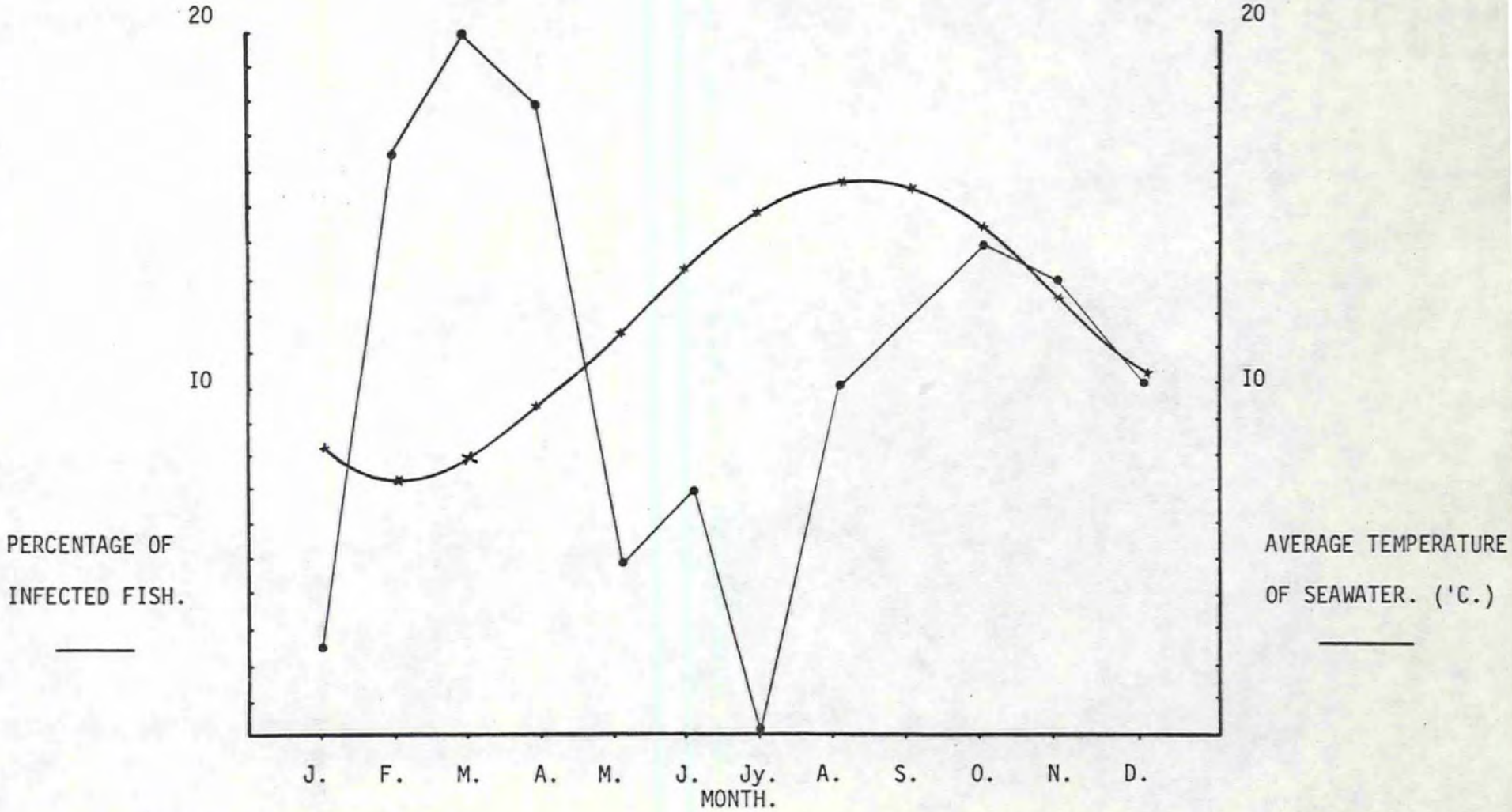


Figure 3I. To show percentage numbers of plaice infected with T. platessae in 1972, with average sea temperatures.

temperatures, highest infection levels of 20% coinciding with the lowest sea temperatures. Conversely, the lowest infection levels in July corresponded to some of the highest sea temperatures.

Distribution of T. platessae through the age classes of plaice. A total of 471 plaice were sexed, aged and screened for trypanosomes and of these only 25 were infected. In all cases, the numbers of trypanosomes occurring in the blood was very low and varied from approximately 100 - 5,600 organisms per ml. of blood. A notable feature of the results is the discontinuous distribution of infected fish through the age classes. In fish under 19 cms. in length, only 0.7% of the plaice were infected. The incidence increased dramatically, however, in older fish of approximately 2 - 4 years of age (table 22). The failure to record any infections in fish 40 - 49 cms. long may have the result of the low numbers sampled.

The χ^2 test was applied to the data of table 22, using the null hypothesis that trypanosome infected plaice were evenly distributed through the age groups. A value of 115 for χ^2 was obtained indicating that the null hypothesis had a probability of 0.001, or stated otherwise there was a 99.9% certainty of unequal distribution.

T. platessae seemed to be equally distributed among the sexes as is indicated by the fact that 8% of 123 males, and 6% of 202 females were infected with the flagellate.

Exp. 16. Passage of T. platessae in the plaice. In order to facilitate the maintenance of T. platessae in a laboratory system, experiments were initiated to determine whether the parasite could be passaged from one plaice to another. A source of infected fish was limited because of the low levels of infection encountered in the wild and, because of this, it

Table 22. Total figures for the distribution of Trypanosoma platessae through the age classes of Pleuronectes platessa.

Approximate age	Length (cms.)	Total nos. examined	Numbers infected	Percentage infected
0 - 1 years	0 - 9	146	1	0.7
1 - 2	10 - 19	26	0	0
2 - 3	20 - 29	148	14	9.4
3 - 4	30 - 39	121	10	8.5
4 plus	40 - 49	28	0	0
Totals		471	25	

Table 23. To show the establishment of experimental infections of T. platessae in plaice maintained at 10, 15 and 20°C.

DAYS AFTER INITIAL INFECTION	NUMBERS OF PLAICE INFECTED AT EACH TEMPERATURE					
	10°C		15°C		20°C	
	No. Exam.	No. Infected	No. Exam.	No. Infected	No.Exam.	No. Infected
0	2	0	4	0	4	0
5	2	0	4	0	4	1
10	2	0	4	1	4	1
20	2	1	4	2	4	1
30	2	1	4	2	3*	1

* 1 plaice dead

was not possible to obtain sufficient parasites for all the originally intended experiments.

Trypanosomes were obtained by terminally bleeding infected adult fish. Blood from these was stored in heparinised bottles at 4°C. Generally, blood from intected fish was injected intraperitoneally into uninfected recipient fish, previously screened for the presence of trypanosomes. Blood samples from recipient adult fish were taken at regular intervals to check for the presence of T. platessae. '0' groups were terminally bled, however, when screened for infection, because of their small size.

In a preliminary experiment, '0' groups received approximately 40 parasites each, by intraperitoneal injection of 0.1 ml. of infected blood. Two fish were examined every five days over a 20 day period. At no time were trypanosomes detected in any of the plaice.

In view of these results, further experiments were carried out to investigate the effects of ambient temperature and site of inoculcation on the establishment of T. platessae in the plaice.

Experiment 17. The effect of temperature upon the establishment of T. platessae. 13 fish were divided into 3 groups of 3, 5 and 5 and held at temperatures of 10°, 15° and 20°C, respectively. In each group one control fish was given 0.2 mls. of saline. All experimental plaice were injected with 0.2 ml. blood containing approximately 350 trypanosomes.

Samples of blood were taken from the fish at regular intervals over a 30 day experimental period. The results of these experiments (table 23) indicated a limited success in establishing this parasite at all three temperatures.

Experiment 18. Effect of introducing T. platessae directly into blood stream of plaice at 15°C. 2 routes of entry were chosen for injection of trypanosomes, namely intracardiac and intravenous routes. Five adult plaice were injected directly in the heart with 0.1 ml. of infected blood, and one control received 0.1 ml. of saline. A further 6 fish were treated in the same manner except that injections were given in the caudal vein.

All fish were examined every 5 days over the period of the experiment. Of the plaice injected via the cardiac route, trypanosomes were detected after 15 days when 1 of 4 recipient plaice was positive, and after 25 days when 2 of the 4 were found to harbour trypanosomes. The control was always negative. None of the 4 fish injected intravenously were found positive over the 25 days. The location of the caudal vein during injection was difficult and it is suggested that the nagative results noted here were due to the failure of trypanosomes to directly enter the blood vessel.

Experiment 19. Observations of T. platessae in 2 naturally infected plaice maintained at 15°C. There has been no previous investigation into the course of infection of T. platessae in plaice. The aim of this experiment, therefore, was to determine any fluctuations in the numbers of trypanosomes in the blood of naturally infected fish. Two plaice, with known infections, were examined at 5 day intervals for 25 days. Trypanosomes were detected in both fish on all occasions they were examined. There appeared to be no obvious increase in their numbers with consistently low parasitaemias being observed.

At the termination of the experiment one plaice was killed and the following tissues removed for histological investigation: liver, kidney, spleen and muscle. Giemsa stained, smear preparations were taken from each tissue and examined for the presence of trypanosomes. For more

detailed investigation fixed material was embedded in wax and sections of this examined. Trypanosomes were not detected in any of the above preparations.

Experiment 20. Passage of T. platessae into different species of Heterosomata. In view of the low parasitaemias and difficulty of passaging T. platessae from one plaice to another, it was considered that plaice may not be the natural host of the parasite. For this reason it was decided to attempt to transfer the trypanosome from plaice to other closely related species, namely: turbot (4 recipient fish), flounder (2), sole (4), dab (1), brill (3). All the recipient fish used in this experiment were '0'-group between 6 - 10 cms. long which had previously been found clear of trypanosomes. In addition to these 2 uninfected '0'-group plaice received injections of T. platessae to act as a positive control. All fish were injected intraperitoneally with 0.1 ml. of infected blood and tested for the presence of T. platessae at 5 and 10 day intervals. Of all the fish injected with the trypanosomes only the 2 '0'-group plaice proved positive on days 5 and 10, respectively.

Experiment 21. The establishment of Trypanosoma percae in the plaice. As indicated in the above experiments, the passage of T. platessae in plaice did not meet with uniform success. The restricted numbers of trypanosomes available from naturally infected fish restricted the variety of passage experiments, and it was felt that the initiation of a successful host-parasite system in the laboratory depended upon a more plentiful supply of trypanosomes. Attempts were made, therefore, to passage into plaice a trypanosome from the freshwater perch, Perca fluviatilis L. T. percae was found to occur in large numbers in the blood of perch and, furthermore, when available, a large percentage of perch were infected.

Table 24. The establishment of 1000 T. percae per fish in '0'-group plaice.

FISH No.	DAYS AFTER INFECTION	PRESENCE OF TRYPANOSOMES	
		Wet	Smears
1	1	+	0
2	1	+	0
3	2	++	0
4	2	+	0
5	6	++	0
6	6	++	0
7	14	+	0
8	14	+	0
9	21	+	0
10	21	+	0

Key to the above:- 0 Nil
+ Less than 1/10 sightings
++ Less than 1/1 sightings
+++ More than 1/1 sightings

(The positive signs indicate the number of T. percae seen in the microscope field as it scanned the blood clot.)

Table 25. The establishment of 12,000 T. percae per fish in '0'-group plaice.

FISH No.	DAYS AFTER INFECTION	PRESENCE OF TRYPANOSOMES	
		Wet	Smears
1	2	+++	0
2	2	++	0
3	5	++	0
4	5	++	0
5	10	++	0
6	10	++	0
7	15	+	0
8	15	++	0
9	21	++	0
10	21	+	0

Perch were captured at Slapton Ley Field Centre from August - October, 1972. The fish were terminally bled from the heart, and infected blood pooled in a sterile test tube and allowed to clot. Trypanosomes were extruded from the clot into the serum. Following this the numbers of T. percae extruded into the serum was calculated using a haemocytometer. This serum was then diluted with 0.85% saline according to the numbers of trypanosomes to be introduced into the '0'-group plaice. The latter, for the purposes of the experiment, were divided into two groups, each group receiving the following treatments:

Group 1. 12 experimental fish injected with 1,000 trypanosomes per fish, and 12 controls receiving 0.1 ml. injections of saline.

Group 2. 10 experimental fish injected with 12,000 trypanosomes per fish, and 10 controls as above.

Fish from each group were periodically examined for the presence of T. percae in the blood. Enough blood could be extracted (0.01 - 0.02 ml.) to make 2 thin blood smears, and one wet drop for examination of live trypanosomes. The latter was allowed to clot under a coverslip and the interface of the serum and blood clot systematically scanned under a microscope. A qualitative assessment was made of the numbers of trypanosomes present by noting the frequency they were observed in each field of the microscope (see tables 24, 25).

The results (tables 24, 25) show that T. percae was able to establish itself in plaice for up to 21 days. There was no indication of a discernible rise or fall in their numbers during this time. As far as is known this is the first record of a trypanosome from a fresh-water host being passaged into a marine fish. Unfortunately, no T. percae were evident in the blood smears of infected plaice so that morphological observations of the flagellate could not be made. In each experiment, all control fish, examined over the three week period, proved negative for trypanosomes.

Table 26. The haematocrit values of all plaice infected with T. platessae.

Month	Haematocrit Reading Mean value	Standard deviation	Haematocrit of infected plaice
January			
February	19.6	2.2	18%
March	23.9	1.5	25, 26
April	24.7	2.8	22, 23, 23
May	22.2	3.2	24
June	21.1	3.2	21
July	22.0	3.3	
August	23.2	2.8	22
September			
October	32.1	5.4	29
November			
December	19.8	3.6	18

Table 27. A comparison of the serum protein patterns of plaice infected with T. platessae and uninfected controls.

Ref. No. Fish	Infected T. platessae	% Composition of Serum I	II	III	IV	V	A/G Ratio
1	+	—	22	38	30	10	0.28
2	+	11	45	29	15	0	1.27
3	-	10	70	—	20	—	
4	-	9	74	—	15	2	
5	-	17	56	13	13	1	2.70
6	-	16	51	19	14	—	2.03
7	-	11	48	26	15	—	1.40
8	-	13	47	20	20	0	1.50

Experiment 22. The Correlation of Blood Parameters with Infections of T. platessae. The main emphasis of the present investigation was directed towards the host's response to parasitic invasion. During the 12 month survey, therefore, of T. platessae, detailed studies were made of the serology and packed cell volumes (P.C.V.) of both infected and uninfected fish. It was hoped that this would provide information on the pathology of the disease, and also, if extreme values in the 2 parameters were noted in fish with trypanosomes, these could aid diagnosis of the disease.

Packed cell volumes (P.C.V.). Each month the P.C.V. of all fish screened for T. platessae were taken and the mean and standard deviations for the haematocrit values recorded (table 26).

It can be seen that only 1 of 11 plaice infected with T. platessae had haematocrit values that fell outside the standard deviations for that month (figure 32). It was concluded, therefore, that there was no correlation between P.C.V. and infections with trypanosomes.

Serum protein patterns. In March 8 female fish between 30 - 35 cms. long were selected for study. These had been previously screened for trypanosomes and 2 of 8 were infected. The sera of all these fish was subjected to electrophoresis on cellulose acetate strips. The electrophoretic patterns thus obtained were scanned on the Millipore densitometer and quantified in the standard way. The protein levels are given in table 27, and all the traces reproduced in figure 33. The protein fractions were designated I - V as previously described.

The two plaice 1 and 2, had low parasitaemias of T. platessae, whereas the remaining fish, numbers 3 to 8 were not infected. There were notable increases in fractions III and IV of infected plaice with concomitant reductions in the albumin levels, namely fractions I and II. In addition, specimen I had a relatively high level of fraction V.

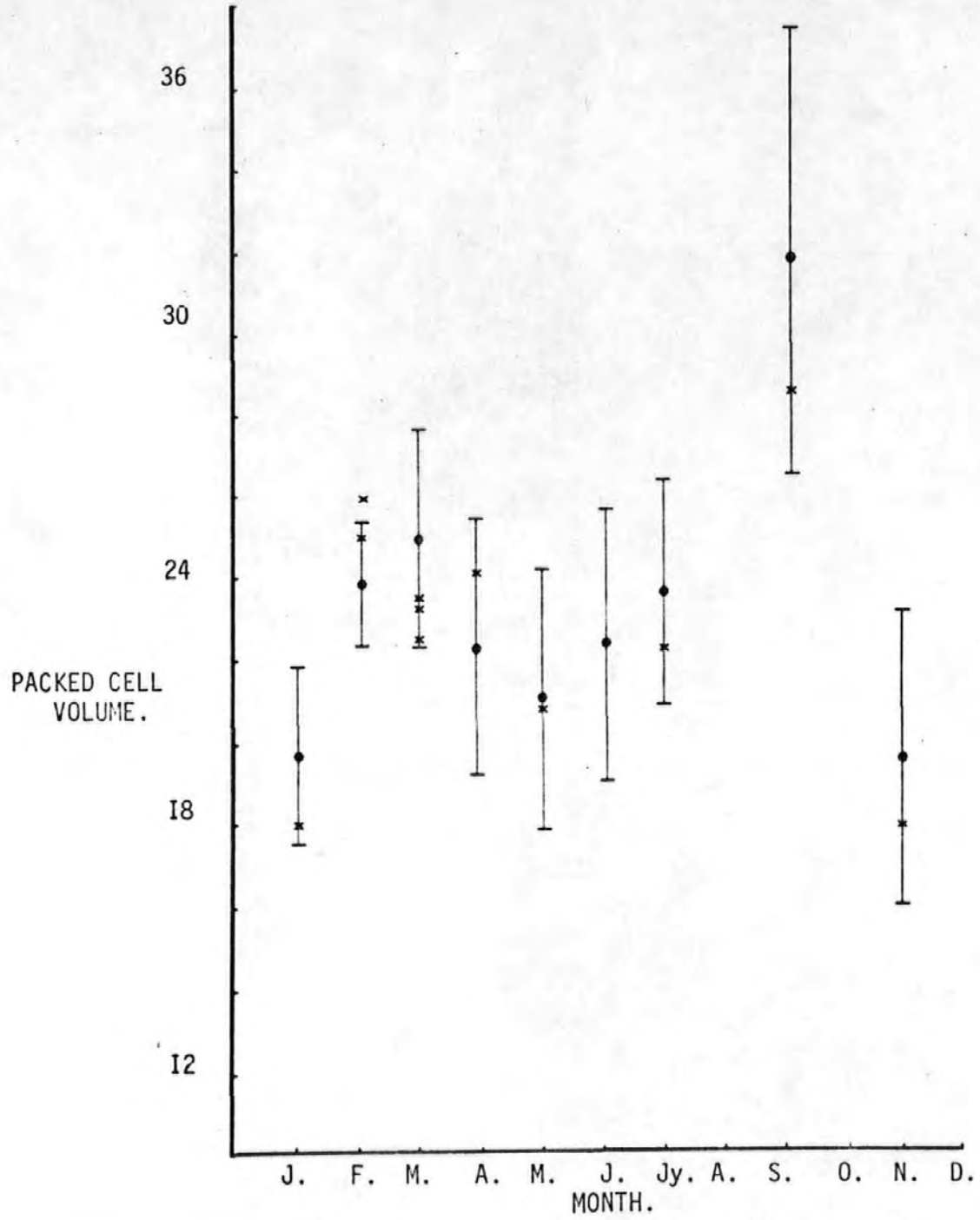


Figure 32. To show haematocrit values of individual plaice infected with Trypanosoma platessae, (indicated by x) together with the monthly mean values (indicated by ∘), and the standard deviation of all the uninfected plaice taken in I972.

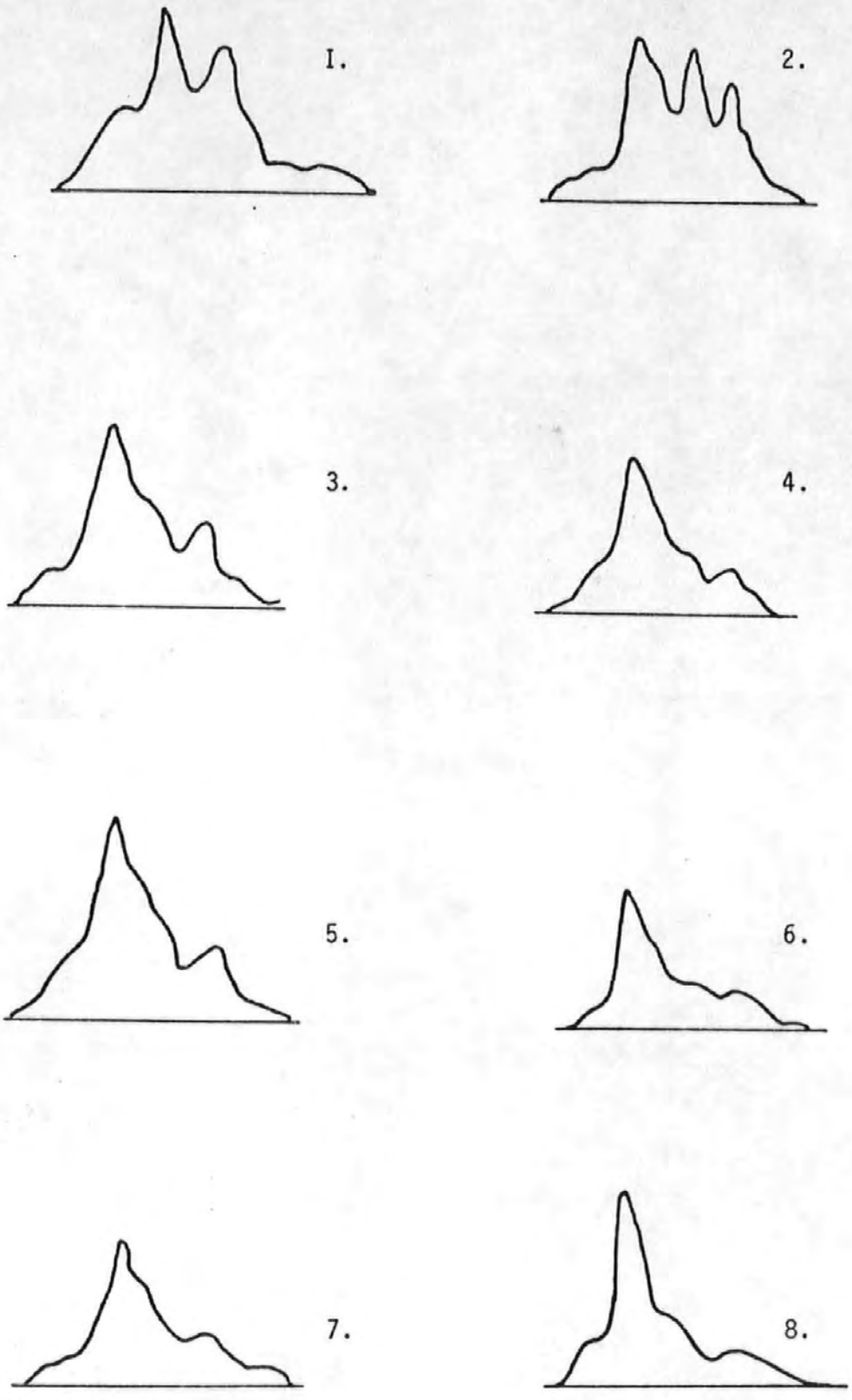


Figure 33. The electrophoretic patterns of serum proteins of two adult plaice infected with Trypanosoma platessae (I and 2) compared with the sera of six uninfected adults (3-8).

The general feature of increased globulin levels in the infected fish is illustrated in table 27. Fish number 1 had an extremely low A/G value of 0.28. Fish number 2 also had A/G ratio below that of any of the uninfected fish, the lowest value of which was 1.4.

Chapter 5

DISCUSSION

PLAICE IMMUNOLOGY AND SEROLOGY

The five major fractions separated from plaice serum by cellulose acetate electrophoresis were seen to correspond approximately with the albumin, and globulins of mammals, as described by Tiselius (1937). According to Summerfelt (1966, a), however, direct homology of fish and human proteins should not be made purely on the electrophoretic migration properties of the constituent proteins. For example, de Boutard (1966) has shown that a shark serum protein with immunoelectrophoretic mobility comparable to Ig G of humans, is transferrin. The fractions recorded from the plaice and other flatfish were, therefore designated I - V or VI. Other workers using cellulose acetate electrophoresis have recorded similar numbers of fractions, five being noted in the bass by O'Rourke (1960), five fractions in salmon by Carberry (1970) and five fractions in pike by Mulcahy (1971).

A small percentage of the plaice serum, fraction V, migrated towards the cathode in a position of a γ-globulin. There are variable reports in the literature concerning the presence of γ-globulin in fish, several marine teleosts being described as agammaglobulinaemic by Clem and Sigel (1963, 1966), Pollara et al (1966) and Engle and Woods (1960). The techniques used, however, may be important in the recognition of γ-globulins. Fletcher (1968) was unable to detect cathode migrating protein in plaice sera using starch gel electrophoresis. The same technique, however, failed to reveal appreciable amounts of γ-globulin in normal human serum. Using electrophoresis Becker et al (1958), Deutsch and McShan (1949), Drilhon (1960) and Engle et al (1958) have all shown low levels of γ-2 globulins in fish sera.

The more sensitive technique of immunoelectrophoresis has been used to display serum protein components with γ-mobility, not previously detected by electrophoretic techniques. Immunoelectrophoresis of plaice serum against homologous high titre, rabbit antiserum clearly revealed protein fractions which migrated towards the cathode. Similarly Krauel and Ridgway (1963), and Pollara et al (1966) have demonstrated γ-globulin in fish using the immunoelectrophoresis method.

Interspecific variations of serum proteins of Heterosomata, noted after cellulose acetate electrophoresis, supported views on the phylogenetic relationships of these fish. Thus plaice and flounder, included in the family Pleuronectidae, possessed very similar serum protein patterns, but were clearly distinct from the common sole, family Soleidae, and turbot, family Bothidae. Turbot proved to be the least similar to plaice, as an antiserum to the latter cross-reacted with flounder, dab and sole sera, but not with turbot sera. Such interspecific differences in the serum proteins of relatively closely related species have been noted in fish by Deutsch and Goodloe (1945), Sanders (1964), Nyman (1965) and Badawi (1971).

Intraspecific variations in the serum protein patterns were associated with the age classes of plaice. These differences were particularly noticable in the greater elaboration of the proteins in adults as compared with '0' group fish. Intraspecific serum protein polymorphism has been attributed to a number of parameters, including genetic, physiological and environmental factors. Stress, as mediated by water O_2 content, has also been shown by Bouck and Ball (1965, 1966) to affect the serum proteins of a species.

The intraspecific differences recorded here for plaice, may have been the direct result of age group variation in serum proteins, and there is evidence from other fish species that maturation does effect

the composition of serum. Saito (1957) demonstrated by electrophoresis that immature forms of elasmobranchs lacked some protein components of the serum that were present in adults. Magnin (1960), Rall et al (1961) and Vanstone and Ho (1961) all described differences in the serum, in the age groups of fish that were associated with the development of sexual maturity. Conversely, Harris (1974) could find no intraspecific differences in the sera of chub, attributable to degrees of maturity.

As stated, a whole range of external factors may alter fish serum proteins and these have been reviewed by Book (1964) and Thurston (1967). The variation of serum protein patterns noted here, may have resulted from the distinct diets of '0' group and adult fish. These differences in feeding habits have been shown by other workers to cause serum protein changes within a species. Lysak and Wojcik (1960) noted pattern variations in the sera of carp fed on varying amounts of protein, and similarly Thomas and McCrimmon (1964) recorded changes in the electrophoretic patterns of fasting and feeding lampreys.

Parental administration of soluble protein and cellular antigens induced antibody in the plaice. The strength and nature of the response, however, varied with the antigen. Bovine serum albumin (B.S.A.) proved to be ineffective in the induction of precipitating antibody, although limited antibody activity to B.S.A. was recorded using the more sensitive passive haemagglutination technique. No precipitins were noted in adult or juvenile plaice, some of which received a total of 40 mg of antigen during the immunisation schedule. The maximum antigen concentration of 10 mg B.S.A. per ml. employed in the Ouchterlony tests may have caused the failure to detect anti-B.S.A. precipitins. This concentration, although a relatively high one for an antigen, may have been insufficient as

Leslie and Clem (1970) have stated that high concentrations of antigens may be necessary for effective agar precipitation tests with fish sera.

Other workers have had variable success with B.S.A. in fish, and there certainly seems to be great variability of individual species to respond to a range of antigens. Clem and Sigel (1963, 1966) were able to provoke anti-B.S.A. precipitins in two marine teleosts, following repeated injection of the antigen. Hodgins et al (1967), Trump (1970) and Trump and Hildemann (1970) demonstrated in rainbow trout, that B.S.A. induced two populations of precipitins, belonging to the same immunoglobulin class. An anti-B.S.A. response was also demonstrated in a cyprinid by Everhart and Shefner (1966). In contrast to this, Harris (1973) and Trump (1970) were unable to demonstrate precipitins to B.S.A. in two cyprinid species, although the former did detect some antibody activity using the P.H.A. technique. Di Conza and Halliday (1971) had similar difficulty detecting anti-B.S.A. precipitins in the catfish, although they obtained high P.H.A. titres against the antigen after 90 days.

A much more pronounced antibody response was noted in plaice to the parental administration of whole calf serum (W.C.S.). Both juvenile and adult plaice responded to the antigen with relatively high precipitin titres, and in both cases, the onset of antibody secretion had occurred within 20 days at 20°C. At 10°C antibody was not detected in the juvenile fish until 30 days, and the antibody titres of the test fish were generally lower than the plaice maintained at 20°C. These results confirm, therefore, the importance of ambient temperature on the immune response of poikilotherms as reported by Allen and McDaniel (1937), Pliszka (1939), Cushing (1942), Bisset (1946, 1948, b) and Cone and Marchalonis (1971).

Earlier workers in this field, notably Bisset (1948, b) postulated that poikilotherms could not produce and release antibody into the circulation at lower temperatures. The results of Gee and Smith (1941) and Avtalion (1969), however, have shown that provided that antigenic stimulation occurs at higher temperatures, antibody secretion will continue at the lowered ones.

Ellis (1973), amongst others, has pointed out that the immunological responsiveness of fish has become adapted to the temperature range of their environment. Thus, Ridgway (1962) detected agglutinins in the coldwater sablefish, Anoploma fimbria at 5°C, whereas Fijan and Cvetnic (1964, 1966) and Avtalion (1968) were unable to demonstrate antibody production in the warmwater carp at temperatures below 14°C. In the present study, water temperatures used in experiments were related to the normal range of the English Channel plaice, although some of the higher temperatures were used to simulate conditions encountered on marine fish farms, where plaice are subjected to abnormally high temperatures to increase growth rate. The relatively strong response of plaice at 10°C noted here would seem to reflect their normal environmental temperatures which vary seasonally from 8 - 17°C. This concept may also account for the lack of immunological responsiveness of plaice at 5°C, when injected with soluble parasite antigens of Cryptocotyle lingua.

Several physico-chemical properties of plaice anti-W.C.S. immunoglobulin were investigated, to compare this molecule with the defined classes of antibody from other fish and mammals. Column chromatography of immune plaice serum revealed that antibody activity was confined to the macroglobulin fraction, there being no discernible reactivity in the other two smaller molecular weight fractions. This macroglobulin was calculated to have a molecular weight of 900,000.

Immunoelectrophoresis of immune plaice serum against the W.C.S. antigen revealed a single arc of precipitation, with the immunoglobulin migrating as a slow-β or fast γ-globulin. This data, allied to the 2-ME sensitivity of the precipitin indicated the presence of a single class of high molecular weight immunoglobulin, resembling the IgM of higher vertebrates. The findings of the present study agree with Fletcher and Grant's (1969) work on plaice immunoglobulin, however, their calculated sedimentation coefficient for plaice macroglobulin would indicate a molecular weight less than 900,000. Later reports, where Fletcher and White (1973) separated immune plaice serum on G200 columns, indicated antibody activity in the second, lower molecular fraction, but the authors considered that this may be a contamination product from the first eluted macroglobulin fraction.

Although, in the present study only one population of immunoglobulin was demonstrated, this may have been the result of the relatively short immunisation schedules, or due to the method of antibody assay. Thus, Clem and Sigel (1966) could only demonstrate a single 19S immunoglobulin in two marine teleosts following immunisation, but they considered, however, that the failure to demonstrate lower molecular weight antibody may have been artifactual.

In all of the elasmobranch species studied to date, two populations of molecules of differing molecular weights, but belonging to the same IgM class, have been identified. Clem and Small (1967) have shown in lemon sharks a 19S antibody which is followed in the latter stages of immunisation by 7S molecule, antigenically identical with the macroglobulin. Similar results have been presented, from other shark species by Clem et al (1967), Suran et al (1967) and Marchalonis and Edelman (1965).

The picture is not so consistent in the teleosts. Two populations of antibodies belonging to the same class have been described by Clem (1971), Uhr (1962), Hodgins et al (1967) and Trump and Hildemann (1970). Alternatively, only one population of antibody, of IgM type, has been attributed to a range of teleosts by Fish et al (1966) and Di Conza and Halliday (1971).

The serum immunoglobulin of plaice to W.C.S. possessed β-2 or γ-1 mobility. Immunoglobulins with similar electrophoretic mobility have been described from a wide range of fish species, following stimulation with soluble and cellular antigens. β-2 immunoglobulins have been demonstrated in the elasmobranchs by Suran et al (1967), Clem et al (1967), Thomas et al (1971), Frommel et al (1971) and Voss and Sigel (1972). Similarly, Boffa et al (1967) showed that cyclostomes have immunoglobulin with slow electrophoretic mobility. The following have demonstrated the ability of actinopterygians to produce β-2 or γ-1 immunoglobulin: Fine and Drilhon (1961), Clem and Sigel (1963), Fish et al (1966), Klontz et al (1966), Evelyn (1971), Bradshaw et al (1971), Dilonza and Halliday (1971), Marchalonis (1971), Watson et al (1968), Litman et al (1971), Acton et al (1971) and Fletcher and White (1973). Not all antibodies from fish species, however, posses γ- or β- mobility. Pollara et al (1970) reported an immunoglobulin from the lamprey with α mobility and Richter et al (1968) and Alexander et al (1973) have presented similar results from salmon and perch.

The response of juvenile plaice to rabbit erythrocytes, indicated that these young fish were capable of immunological stimulation by cellular or particulate antigen. Moreover, although there were only a limited number of specimens used in this experiment, there were indications of a heightened immunological response to secondary administration of antigen.

Isohaemoagglutinins have been demonstrated in goldfish by Hildemann (1956, 1958) with titres ranging from 1:4 to 1:2048 following 9 injections given over a period of three weeks. Haemagglutinins have also been reported from teleosts in the sera of salmon, trout, herring and catfish by Ridgway and Klontz (1960), Sanders and Wright (1962), Sinderman (1958) and Baldo (1972 a, b), and in elasmobranchs by Sigel and Clem (1966). Harris (1972) demonstrated a temperature dependant haemagglutinin response in dace following an intraperitoneal dose of human erythrocytes. Fletcher (1968) was able to obtain titres of 1:1,024 86 days after injection of erythrocytes into adult plaice. At around 40 days, however, the titres were relatively low (1:8 and 1:16) and are comparable to the plaice's response noted in the present study.

Secondary or anamnestic responses to antigenic stimulation have been observed in a wide range of fish. Finstad and Good (1966) reported the ability of lampreys to produce a heightened immunological response following the secondary administration of antigen. Similar anamnestic responses to soluble and particulate antigens have been reported from the elasmobranchs by Sigel and Clem (1966), Papermaster et al (1964), Sigel and Beasley (1967) and Clem et al (1967).

In contrast to the present study, Sigel and Clem (1966) were able to induce heightened secondary responses to PR8 virus in lemon sharks, but not with B.S.A. and erythrocytes. Such variability may be attributed to a number of factors including the period between primary and secondary stimulation with antigen. Clem et al (1967) and Sigel and Beasley (1967) have demonstrated that high levels of antibody in immunised fish may suppress the secondary response. Also, temperature has been implicated as a factor in the magnitude and latent period of the secondary reaction in fish.

Both '0' group and adult plaice, infected with metacercariae of Cryptocotyle lingua and Rhipidocotyle johnstonei, responded with the production of precipitating antibody to the antigens of these parasites. The low antibody titres to these parasites may be partly explained by the relative insensitivity of the Ouchterlony test. A possibility is, however, that a large proportion of the serum antibody may be bound to the parasite in vivo, and this could be significant where very large numbers of the metacercariae occur in the host. Thus, in one experiment, plaice were subjected to 10,000 cercariae, and it is possible that these excessive numbers of parasites were binding plaice antibody, therefore making very little available for the immunological assays.

As far as is known, there have been no previous reports of antibody production in marine teleosts to metacercarial infections. There are very few examples in the literature of a precipitin response, in fish, to parasitic infection. Molnar and Berczi (1965) and Harris (1972) demonstrated serum precipitins in the sera of freshwater fish to species of cestode and acanthocephala, respectively.

Although precipitins to parasitic antigens were detected in plaice sera, there was no evidence to suggest that antibody was present in the cutaneous mucus. This result was perhaps unexpected as Fletcher and Grant (1969) demonstrated antibody in plaice mucus to specific antigen. Further to this several workers have detected antibody or neutralising factors to parasites, in fish mucus. Harris (1972) found weak precipitin activity in the intestinal mucus of Leuciscus cephalus infected with the acanthocephalan, Pomphorhynchus laevis, and Nigrelli and Breder (1934), Reuling (1919), and Hines and Spira (1974) have all detected specific immobilising antibody

in the cutaneous mucus of fish infected with external protozoan and metazoan parasites.

The presence of specific secretory antibody in plaice cutaneous mucus to C. lingua and R. johnstonei would be of obvious benefit to the host in resisting penetration of cercariae, as both species enter fish by the cutaneous route. Parental administration of Vibrio antigen into the plaice was shown by Fletcher and White (1973) to induce high titres of serum antibody, lower titres being found in the intestinal and epithelial secretions. Thus, serum transudation into the mucus would seem to involve a dilution of antibody. It is suggested, therefore, that serum precipitin levels to C. lingua were too low to produce appreciable activity in the cutaneous mucus.

There are many examples in the literature of distinct changes in the serum protein patterns of fish resulting from either immunisation or infection with disease agents. In the present study, variations in plaice sera were recorded that could be associated with the presence of parasites. In adult plaice, infected with large numbers of Cryptocotyle lingua metacercariae, there were obvious rises in globulin levels in comparison with the uninfected controls. In this case, such changes were paralleled by the demonstable production of antibody to the parasite. Similarly, although there was no direct evidence for antibody production, elevated levels of fractions III, IV and V were seen in plaice infected with Trypanosoma platessae, which is discussed later.

The results obtained here agree, therefore, with the changes in protein levels recorded in other groups of animals. In mammals, for instance, infection with microbial or parasitic agents usually results in increases in the β- and γ- globulins with concommitant decreases in the albumins, and examples of this have been given by Dobson (1967).

Further Deutsch and McShan (1949) indicated that in fish and higher vertebrates there would be predictable changes in the serum proteins of infected individuals.

Phillips et al (1957) found that the A/G ratios of brown trout infected kidney disease were the reverse of values obtained from uninfected brown trut. Sindermann and Mairs (1958) discovered, using paper electrophoresis, drastic decreases in the albumin fraction of herring infected with a fungal pathogen. This was associated in some cases with the appearance of extra fractions in the globulin component of the serum. The changes noted by Mulcahy (1969) in the sera of U.D.N. infected salmon would seem to point to there being a specific serum pattern associated with the disease. Definite increases in the β-2 globulins with decreased albumin and α proteins were seen in infected trout by Klontz et al (1966).

As has already been discussed, the immunoglobulins in plaice and the majority of teleost species have β electrophoretic mobility. From this it is consistent that the sera of infected plaice should show increases in the globulins, especially in the β fraction. A similar situation occurs in teleosts immunised with antigens. Increases in the β-2 and ɣ-1 globulins with a fall in the albumin levels were noted in immunised golden shiners and goldfish by Summerfelt (1966, b) and Watson et al (1968). These results were largely repeated in the eel by Fine and Drilhon (1961), in carp by Sorvachev et al (1962), in salmon by Evelyn (1971) and in rainbow trout by Ingram (1974). In all these cases the increases in the β- and ɣ-globulins seemed to be associated with the production of homologous antibody to the antigen. Evelyn (1971), for example, noted elevated levels of β- and ɣ-globulins in bacteria-vaccinated sockeye salmon. Incubation of the immune serum with the antigen resulted in the adsorption of the β- and ɣ-globulins onto the bacterial cells.

Evidence for antibody secretion in the plaice to Trypanosoma platessae is here based on features of serology and aetiology of the disease, the former having already been discussed. It was not possible to demonstrate directly an anti-trypanosome immunoglobulin in the infected plaice due to the scarcity of the parasite. The lack of sufficient parasitic material made it impractible to prepare an adequate antigen for standard tests. To overcome this problem attention was given to the in vitro culture of T. platessae in diphasic media, in attempts to harvest sufficient organisms for antigen extracts. These attempts failed, however, but future work on plaice immunity to T. platessae would certainly benefit from such a supply of parasites.

Parasitaemias of T. platessae were invariably low, there being no significant increases of flagellates in plaice maintained in the laboratory for periods of several months. Resons for these low, chronic infections may be due to aspects of host immunity, both acquired or innate, or may be directly related to the biology of T. platessae. There is a possibility, for instance, that plaice is an accidental host for T. platessae. Partial innate immunity of the plaice would then account for the restricted levels of trypanosomes infecting the species. Historically, the only basis for naming several new species of marine Trypanosoma was the identification of trypanosomes in a new host, such an approach being employed by Henry (1913) for example. More definitive criteria for the naming of new Trypanosoma species were based on exact morphological description, and experiments to determine the specificity of trypanosomes for their fish host. By both these criteria T. platessae would seem to be a species unique to the plaice. The results presented here show that T. platessae displayed a degree of host specificity in that it failed to develop in several species of flatfish. Further, Lebailly (1905) was unable to experimentally transmit T. platessae to several species of marine

teleosts, other than the plaice. If, indeed, T. platessae were to be transmitted in the wild to other fish, the probability of these species inhabiting a similar ecological niche to the plaice is high and future work might experimentally investigate the cross-infection of T. platessae into such bottom living species of fish.

On the basis of morphology, T. platessae appears to be quite distinct from the trypanosomes recorded from other species of fish. Laird (1951) considered, however, that T. platessae and T. caulopsettae may be conspecific. If polymorphism occurred in T. platessae certain forms would indeed resemble T. caulopsettae, but the results of the present study indicate that this is not the case, as the size range of all measured parameters of the plaice trypanosome was very small.

From the above it would seem that T. platessae is a species, specifically parasitising the plaice and, as such, the low numbers of trypanosomes in the host cannot be attributed to innate immunity. There is some evidence, however, that the low levels of T. platessae may be due to the specific, acquired immunity of the host, and results from serology and aetiology of the disease indicated that plaice may secrete antibody to the parasite.

Doflein (1913) demonstrated acquired immunity in frogs to T. rotarium and suggested that the antibody involved was 'ablastin'. The latter, which inhibits division of trypomastigotes, could account for the failure to detect any divisional forms of T. platessae either in their blood films, or live preparations. The possibility that trypanosomes induce such an antibody in their fish hosts would also explain the general scarcity of marine Trypanosoma, and their low parasitaemias. Further, freshwater fishes, in overcoming trypanosome infections, have been shown by Barrow (1955) to produce trypanocidal antibody, so that the possibility that marine fish also employ similar antibodies cannot be overlooked.

A seasonal variation in the levels of T. platessae in wild plaice was noted over a period of one year, and it was considered that this may possibly be due to ecological factors. Plaice from Plymouth waters all known to spawn on common grounds in December and January, and in this time the vector, a marine leech, may have increased opportunity for transmitting T. platessae to other plaice. This would then explain the high incidence of infected fish noted in February and March. Such variations of T. platessae levels, however, may be consistent with a seasonal secretion of antibody to the trypanosomes. As has been discussed, temperature determines the time course and magnitude of the immune response in plaice and other fish, with increased antibody production at higher temperatures. The decrease, therefore, of the numbers of fish harbouring T. platessae in the summer may have resulted from the increased immunological competence of plaice with the elevated ambient temperatures. Conversely, the highest incidence of T. platessae occurred at the lowest environmental temperatures, when the immune response would be most depressed. Other workers, such as Sindermann and Honey (1964), have indicated that the levels of fish antibody may vary seasonally, being their lowest at times of minimum temperature. Vladimirov (1968) reported that the intensity of fish antibody production increased significantly in the warmer summer months.

Further to this reports of similar seasonal fluctuations of trypanosomes in poikilothermic hosts have been attributed to a temperature controlled antibody secretion. Barrow (1954) showed that nine European freshwater fish suffered their highest trypanosome parasitaemias just after the winter season, and this he associated with a decrease of lytic antibody secretion at low temperature. Anti-trypanosome antibody was evident at 20^{o}C, but not at 5^{o}C. These results were largely repeated by Barrow (1955) for tench and perch.

Similar seasonal variations to these, with decreases of trypanosome numbers in summer have been noted in the Anura by Bardsley (1969, 1972), Nigrelli (1929) and Peyrez-Reyes et al (1960).

A similar temperature mediated, humoral response was noted in plaice with infections of C. lingua and R. johnstonei. In both cases there was no antibody secretion to these parasites in fish experimentally maintained at 5°C for up to two months. At temperatures of 15°C and above, proportionately more plaice secreted antibody to their metacercarial infections. Elevated temperatures also shortened the induction period between initial infection and primary antibody response of the host.

As has already been discussed, this lack of responsiveness at 5°C may be due to experimental temperatures falling below the normal ambient range of the Plymouth plaice. Plaice living off the eastern Scottish coast, for instance, are certainly subjected to temperatures in winter of less than 5°C and it would be of interest to see if these fish produce anti-parasite immunoglobulins at 5°C or lower.

It should also be considered that temperature rises will cause elevations in the metabolic rate of a parasite with the consequence of increased growth rate, and production of excretory-secretory products. There is, therefore, a delicate balance between the host and parasite, and the effects of temperature on fish immunity to parasites cannot be considered to favour the host alone.

Partial physico-chemical analysis of the immunoglobulins to the three helminth antigens indicated a macroglobulin similar in all respects to the IgM of mammals (table 28), and the plaice anti- W.C.S. precipitin, which has already been discussed. The most complete characterisation was made of the plaice 'natural' antibody to the nematode, Proleptus

Table 28. Physico-chemical analysis of plaice precipitins to parasite antigens.

PARASITE ANTIGEN	2-ME SENSITIVITY	IMMUNOELECTROPHORETIC POSITION	COLUMN CHROMATOGRAPHY	SEDIMENTATION COEFFICIENT
C. lingua	+	β - 2	1st. eluted macroglobulin	Not done
R. johnstonei	+	β - 2	Not done	Not done
P. obtusus	+	β - 2	1st. eluted macroglobulin	19 S

obtusus. As the latter is not known to occur in the plaice, being host-specific to the dogfish, standard tests were employed to investigate whether precipitin was true antibody or a non-specific factor. C-reactive protein (C.R.P.) was demonstrated in the sera of healthy plaice by Baldo and Fletcher (1973), and this precipitated in diffusion in agar tests, with extracts of bacteria, fungi and nematodes, including Ascaris suum. The precipitin, in the present case, to P. obtusus was characterised as a 19S antibody of the IgM class. Furthermore, there was no observable reaction in the sera of plaice tested against Ascaris suum antigen. The apparent differences in the present results and those of Baldo and Fletcher (1973) may be explained by non-specific stress factors which are known to elevate C.R.P. levels. Baldo and Fletcher (1973) took their serum samples from aquarium maintained fish, which may be considered a long term stress situation. In the present study, stress was minimized by taking blood samples from plaice immediately upon capture. Although C.R.P. was not demonstrated here, it would be of obvious value to the plaice as a non-specific protective factor in the serum, especially at lower temperatures where the magnitude and speed of antibody production is depressed.

As the precipitin to P. obtusus was shown to be an immunoglobulin, the possibilities for its presence in plaice sera are discussed and two explanations given below. First, P. obtusus may share common antigenic specificities with a micro-organism normally resident in the plaice, as several reports have indicated that certain nematode antigens are serologically related to bacterial antigens. Oliver-Gonzalez and Kent (1961) found an antigen from Ascaris suum cuticle was serologically cross-reactive with Clostridium collagenase. Similarly, Diplococcus pneumoniae and Ascaris were shown to be antigenically related by Heidelberger et al (1954).

Secondly, serological cross-reactions can occur between species of nematodes and it may be significant that two species, Cucullanus minutus and C. heterochrous, closely related to P. obtusus in the Spirurida, are common parasites of the plaice. P. obtusus in the dogfish, and C. minutus in the plaice attach to the intestinal wall and secrete enzymes from their anterior glands which cause lysis and inflammation of the host tissue. Janiszewska (1939) and Schuurmans et al (1932) have shown that both these species caused lymphocyte infiltration with P. obtusus inducing local haemorrhaging at its point of attachment.

In view of the similarity of functions of the oesophageal glands of P. obtusus and C. minutus, and the close taxonomic relationship of these species in the Spirurida, it is suggested that a common source of antigen may be found in the enzymes of these secretions. In nematodes of mammals, the anterior gland secretions have been shown to provoke host antibody production. Thorson (1956) and Dusamic (1966) demonstrated specific antibody production to the proteolytic enzymes and lactic dehydrogenase of oesophageal secretions of Ancylostoma caninum and Trichinella spiralis. Rothwell and Merrit (1974) have further shown that acetylcholinesterase (A.Ch.E.) is antigenic and can stimulate specific host antibody. Workers, including Bremner et al (1973), Lee (1970) and McClaren et al (1974), have demonstrated high levels of A.Ch.E. in the oesophageal and/or excretory glands of many nematode species. From the above, therefore, it is possible that plaice are producing antibody to either a micro-organism or species of Cucullanus, and the antibody, in turn, is precipitating with the serologically related P. obtusus antigen.

That parasitic enzymes may be important in inducing specific anti-parasite immunoglobulins was first proposed, and later elaborated

by Chandler (1935, 1953). This anti-enzyme mechanism may partly explain why immunity induced by dead or disintegrated helminths is inferior to that of live infections, the absolute amounts of enzymes in non-living material being very small. From the evidence of the fluorescent antibody tests on Rhipidocotyle johnstonei it would seem that plaice antibody is produced to secretory products associated with the prostate and pharyngeal glands of the parasite. Fluorescence of these glands indicates that their products must find access to the plaice tissues and stimulate the immune mechanism of the host. It seems likely that the proteolytic secretions from the pharyngeal glands are enzymes with a digestive function. If this is so, then antibodies produced by '0' group plaice infected with R. johnstonei metacercariae, may neutralise the lytic effects of the pharyngeal secretions. Fluorescence was also noted on the tegument, indicating the attachment of plaice antibody either to somatic elements, or to the tegumental secretions. It is a commonly held view, however, as pointed out by Sinclair (1970), that somatic antigens are not so important in stimulating host immunity as the secretion and excretion products.

In C. lingua, there was no indication of plaice antibody being associated with the secretory or excretory glands of the parasite, and possibly the cyst formation around the metacercariae prevents access of C. lingua secretions to the plaice's immune system. Also the inner cyst wall, of parasitic origin, may serve to block antibody from the C. lingua, and act as an immunological barrier. Fluorescent antibody tests on sections of C. lingua metacercariae indicated that most of the plaice's immune serum attached to this inner cyst wall.

Certainly immune serum from plaice is highly toxic for C. lingua cercariae. Precipitates forming around cercariae incubated in immune serum, were shown, by fluorescent antibody tests, to contain plaice immunoglobulin. It is possible that antibody is produced to somatic

antigen on the tegumental surface of the cercariae, but more probable, however, that antibody attaches to the cystogenous secretions of the parasite. Iddon (1973) and the present report show that incubation of C. lingua cercariae in normal plaice sera induces secretion of substances from the cystogenous glands which rapidly covers the whole cercariae. Papirmeister and Bang (1948) demonstrated similar precipitates when incubating Schistosoma mansoni cercariae in immune, monkey serum. If the cercariae and metacercariae share common antigens, as they seem to, this should aid the infected plaice in combating further cercarial invasion. Antibody directed at established C. lingua metacercariae could then assist in the immobilisation of new infections of the parasite, migrating through the skin and musculature prior to encystment. In this context, antibody directed at enzymic secretions of the cercariae may inhibit the lysis of host tissues. There is some evidence that such anti-C. lingua antibody does assist in the resistance of plaice to invasion of the cercariae, as Iddon (1973) has shown that statistically fewer cercariae are able to penetrate those plaice with established infections of C. lingua. Soulsby (1962) has pointed to the possible importance of host inhibition of the skin penetrating enzymes of invasive helminths, and a serum factor responsible for the blocking of collagenase-like enzymes of S. mansoni and S. ratti has been described by Lee and Lewert (1957).

Double diffusion tests revealed only one line of precipitation between antigen preparations of R. johnstonei and C. lingua and their homologous plaice antisera. Although it was not possible to subject these antigens to immuno-electrophoresis, the Ouchterlony tests suggested there was not, in either case, a multiplicity of antigens reacting with plaice antibody.

Injections of C. lingua extracts into rabbits indicated, however, that there were at least six potential antigens, one of which was associated with the insoluble cellular debris. In addition, polyacrylamide gel electrophoresis revealed that there were significantly more protein fractions in the soluble C. lingua extract than in the insoluble one. It is probable that some of the soluble fractions included functional enzymes of the parasite, although no specific identifications were made, and future work to investigate the potential antigenicity of C. lingua enzymes would be of value.

Following electrophoresis of the insoluble extract, only 3 fast migrating protein bands were identified. From the results of immunoelectrophoresis one of these fractions must represent an antigen. Specific staining of these bands did little to reveal their biochemical nature as they both stained for a wide range of materials. There has been very little work of this nature on fish parasites and most of the immunological and biochemical analyses have been performed on antigens of mammalian parasites. In a review of the subject, Kagan (1967) pointed to the importance of such studies in the identification and purification of functional antigens, and, although only preliminary results on the biochemical nature of C. lingua were presented here, such an approach would be necessary in future research for the isolation of antigens used in immunisation schemes.

ASPECTS OF FISH IMMUNITY TO PARASITES

Sigel (1968) suggested that the immune responses of fish are not so fully evolved as higher vertebrates, and the single class of immunoglobulin in fish compared with number of classes in the mammals certainly supports this view. Whether this lack of a wide range of immunoglobulin classes makes the fish's response to parasites deficient is unclear. As

far as could be determined, all plaice antibody to digenean and nematode antigens, was of the IgM type, and there was no evidence to implicate any other class.

In the case of Cryptocotyle lingua, although antibody was produced by the plaice, this could not be correlated to absolute protection against recurrent infections of the parasite. Some examples of protective immunity have been given by Kuhn (1932) and Nigrelli and Breder (1934), who noted the ability of marine fish to develop protection against the external parasite Benedenia. Such examples of acquired immunity of fish to parasites are rare, however, and even where antibody production to the parasite has been demonstrated (see Harris 1972) protection against the disease has not been obvious.

As well as humoral factors, cell mediated immunity has to be considered. In the present study the role of cellular factors in resonse to parasites was not evaluated, however, the findings of Wardle (1971) and Ellis and de Sousa (1974) on the plaice lymphoid system indicate many functional and physiological similarities to the mammalian systems. Further to this, aspects of cellular mediated immunity, for example, graft rejection, have been demonstrated in fish by Hildemann and Cooper (1963), and there seems to be no reason why other important cellular functions such as phagocystosis and encapsulation may be important to fish in protection against parasites. The importance of humoral antibody in the protection of mammals against protozoan and helminth infections has been reviewed by Smithers (1967) and Sinclair (1970). Passive transfer of immune anti-parasite serum alone confers limited protection to recipients. On the other hand good immunity to Nippostrongylus brasiliensis was demonstrated by Kelly and Dineen (1972) following the passive transfer of mesenteric node lymphocytes from immune to non-immune rats. In any parasitic disease, therefore, both cellular and humoral factors have to be considered when evaluating the role of host immunity.

Several authors including Burnet (1962), Uhr (1964) and Papermaster et al (1964) have considered the possibility that the primitive vertebrate immune systems evolved primarily to deal with endogenous changes, for example, cell mutation. Once these were fully evolved they could fortuitously deal with exogenous factors such as parasites. As such, primitive immune mechanisms in the lower vertebrates would not be primarily adapted to combating parasite material, and this may partially explain the relative ineffectiveness of fish in combating disease.

As Sprent (1969) has considered, in any long evolved host/parasite relationship there should be a dynamic balance between the two where the damaging effects of the parasite are minimised. Dineen (1963 a, b) has suggested that the host's immune response acts as a form of selection pressure in reducing the antigenic stimulation of the parasite, as the relationship evolves. The end point of the process results in the "adaptation tolerance" of Sprent (1969) where the parasite can be considered as a successful graft in the host.

Such concepts as these may generally be applied to fish where the host/parasite relationship could be expected to have undergone significantly longer periods of evolution than the mammals for example. The evidence from the parasites studied in present case, suggests a minimal amount of antigenic imput, for example, the relatively small numbers of antigen/antibody complexes noted in immunodiffusion studies of immune plaice serum to metacercarial antigens.

In the case of R. johnstonei and T. platessae the parasites are in direct contact with host tissue, and it would be expected that there would be strong selective pressure on those parasites that reduced the 'antigenic disparity' between themselves and the host. Such a theory might explain the fact that relatively huge infections of R. johnstonei only provoke a minimal antibody response in their host.

Finally the potential importance of our knowledge of fish disease must be considered in relation to fish husbandry. In the case where flatfish, such as plaice and turbot, may be reared in mixed culture systems with shellfish, our knowledge of life cycles and biology of parasites may be crucial as pointed out by MacKenzie and Gibson (1970) and control of specific disease may be achieved by rearing fish in the absence of intermediate hosts or vectors, for example, leeches in the case of trypanosomes. The present author's work immunising plaice with C. lingua antigens and that of Fletcher and White (1973) who orally and parentally immunised plaice with Vibrio antigen, suggest the possibility of prophylaxis based on a sound knowledge of the immunology of plaice. It is to be hoped that the future husbandry of marine fish will absorb both these approaches in the prevention and control of disease.

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