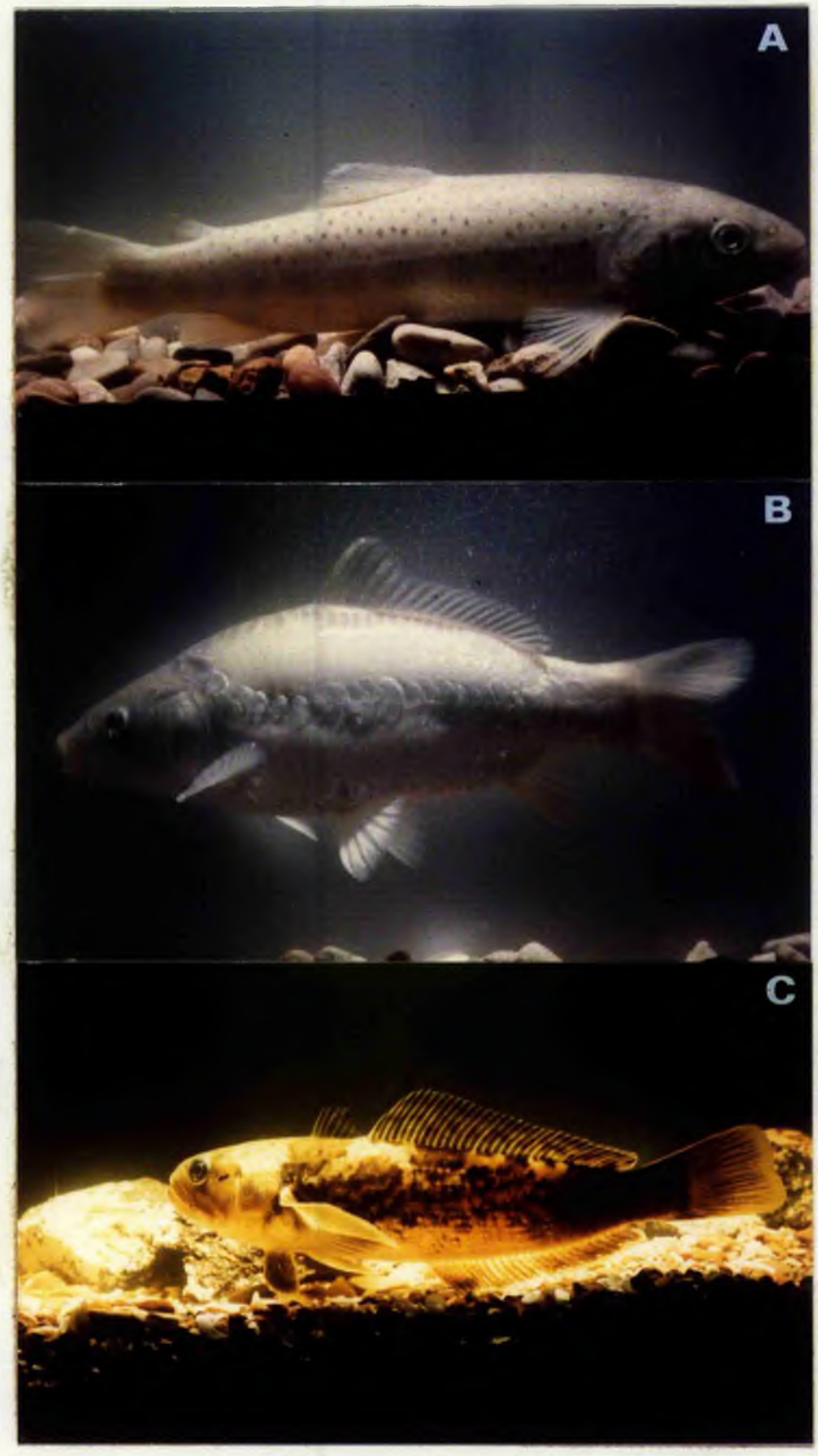
FRONTISPIECE

A. The Brown Trout: *Salmo trutta*

B. The Mirror Carp: *Cyprinus carpio*

C. The Antarctic Fish: *Notothenia rossii*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B
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THE IMMUNE RESPONSE IN TELEOSTS: THE EFFECTS OF TEMPERATURE AND HEAVY METALS

A Dissertation Submitted
to
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by

Julian G O'Neill B Sc (Leeds) M Sc (UCNW)

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Department of Life Sciences
Trent Polytechnic
Nottingham

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DECLARATION

This work has not been accepted in substance for any other degree, and is not concurrently being submitted in candidature for any other degree.

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

Signed Julian G. O'Neill .....
(Candidate)

Signed .....
(Director of Studies)

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ABSTRACT

The humoral neutralisation antibody titre response to single intraperitoneal inoculations of MS2 bacteriophage was followed in three species of teleost (Salmo trutta, Cyprinus carpio and Notothenia rossii) and was measured as a 50% bacteriophage neutralisation titre (SD_{50}). The neutralisation activity was confined to the heavy molecular weight fraction of the sera from both primary and secondary inoculated fish.

Primary and secondary antibody responses were observed, the latter showed enhancement and immune memory, and the quantitative antibody response was modified by different antigen concentrations and by the addition of Freund's adjuvants.

Water temperature was an important factor affecting the clearance of MS2 bacteriophage from the sera and modified the amount of humoral antibody formed. Inter-species adaptation of the humoral immune response to temperature was observed though evidence of acclimation at low temperatures was not demonstrated.

The actions of antigen concentration, adjuvants and temperature in the control of humoral immune response have been discussed.

A preliminary histological investigation was made of the uptake and distribution of intraperitoneal inoculations of MS2 bacteriophage and carbon in the tissues of S. trutta.

S. trutta and C. carpio were continuously exposed to sub-lethal levels of waterborne heavy metals (0.75 mg Ni dm^{-3}, 0.14 to 2.13 mg Zn dm^{-3}, 0.29 mg Cu dm^{-3} and 0.01 mg Cr dm^{-3}). The rate of MS2 bacteriophage clearance and the humoral antibody response were suppressed though antibody titres of the secondary response were found to be enhanced by exposure to nickel and 1.06 mg zinc dm^{-3}.

Intraperitoneally inoculated concentrations of lead (0.01 to 0.3 mg 100 g^{-1}) and cadmium (0.05 to 0.2 mg 100 g^{-1}) suppressed an already raised tertiary immune response to MS2 bacteriophage in S. trutta and the ability to respond to a further bacteriophage challenge was diminished.

The suppressive and adjuvant effects of heavy metals, their mode of action and stressor activity have been discussed in relation to humoral immunity and disease.

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TABLES

ABBREVIATIONS

ABS	Alkyl benzene sulphonate, a soft detergent
BAB	Blood agar base
BGG	Bovine gamma-globulin
BSA	Bovine serum albumin
^{o}C	Degree Centigrade (^{o}Celsius)
d	Day
D	Dalton, unit molecular weight
DDT	Dichloro-diphenyl-trichloroethane
DNP	Dinitrophenyl
EIFAC	European Inland Fisheries Advisory Commission
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
GPC	Guinea pig complement
H & E	Haematoxylin and eosin stains
h	Hours
HGG	Human gamma-globulin
HMW	Heavy molecular weight
Ig	Immunoglobulin, for example IgA, IgE, IgG, IgM and IgT
IHN	Infectious haematopoietic necrosis
IPN	Infectious pancreatic necrosis
k	Viral inactivation constant
KLH	Keyhole limpet haemocyanin
LC_{50} (LD_{50})	The concentration (dose) of a toxicant which kills 50% of the exposed population of fish
LMW	Low molecular weight
2-ME	2-Mercaptoethanol

$mg\ dm^{-3}$	Milligrammes per litre, parts per million (ppm)
min	Minutes
MS222	Tricaine methanesulphonate
NADH	Nicotinamide-adenine dinucleotide
OD	Optical density
17-OHCS	17-Hydroxycorticosteroid
OSD	Oregon sockeye disease
PCB	Polychlorinated biphenyls
PFU	Plaque forming units
RHS	Rabbit haemolytic serum
RE	Reticulo-endothelial
S	Svedberg units
SD_{50}	The dose of serum required to produce 50% inactivation of a virus
SE	Standard error
SRBC	Sheep red blood cells
SRCD	Sacramento River chinook disease
t	'Student's' t-statistic
VHS	Viral haemorrhagic septicaemia
VSV	Vesicular stomatitis virus
V/V	Volume for volume
w/v	Weight per volume
yr	Year

I INTRODUCTION

The investigation of the defence mechanisms of teleost fishes to disease has continued throughout this century, though it was not until the last decade that the immune responses of fishes became a focal point of attention. This has been in part due to the growth of salmonid culture both for food and sport, though considerable attention has also been placed on the examination of the phylogenetic relationships of the immune mechanisms in the vertebrates.

The study of the teleost's ability to mount an immune response against disease organisms (pathogens) has become the object of much research, especially since the successful expansion of intensive fish aquaculture has been found to be limited by the outbreak of diseases (Roberts, 1975a). Both natural and cultivated stocks of teleosts by necessity inhabit an environment which is prone to fluctuation in physico-chemical quality and which is intimately associated with the fishes. The aquatic environment also provides an ideal vehicle for the presentation of pathogens, many of which exist in close association with the host fish as commensals, some being obligate while others are free living, and are triggered from their latent pathogenic state by a complex of host, pathogen and environmental factors (Snieszko, 1974). Thus the diseases of teleosts and their defence mechanisms against the causative pathogens must be related to the complex effects of environmental factors such as temperature and water quality.

A. The immune response in teleosts

In the late nineteenth century Metchnikoff expanded the theory of cellular phagocytosis, and associated humoral components with this activity (reviewed by Metchnikoff, 1905). His work extended to the fishes and lower vertebrates, as did that of his contemporaries Mesnil (1898), Noguchi (1903) and Babes and Riegler (1903) who were the first to examine the natural

agglutinins of carp, perch, roach and pike to certain disease organisms of fishes.

It was not until the 1960's that interest was once more revived in the immunology of lower vertebrates, an interest created by studies into the phylogenetic origins of the immune response in vertebrates and advanced by the techniques now available to clinical immunologists (Hildemann, 1962; Good and Papermaster, 1964; Papermaster, Condie, Finstad and Good, 1964a; Finstad and Good, 1966).

Many authors have examined and reviewed the phylogenetic relationships of the immune system within the fishes. This work has shown the complexity of the response in fishes to be related to the developmental stage of the thymus, lymphoid cell series and other organs involved in the lymphoid system (Finstad, Papermaster and Good, 1964; Papermaster, Condie, Finstad and Good, 1964b; Finstad, Pollara and Gabrielsen, 1966; Good and Finstad, 1967; Burnet, 1968; Fänge, 1966, 1968; Fichtelius, Finstad and Good, 1968, 1969; Finstad, Fänge and Good, 1969).

The evolution and origins of the immunoglobulins formed by fishes have also been extensively reviewed (Marchalonis and Edelman, 1965; Hill, Delaney, Fellows and Lebovitz, 1966; Cushing, 1970; Kubo, Zimmerman and Grey, 1973; Nisonoff, Hopper and Spring, 1975).

Two types of immunological reaction can occur when antigen invades the body of mammals (Roitt, 1974). Firstly, there is synthesis and release of free humoral antibody specific to the antigen which neutralise or opsonise that antigen ready for enhanced phagocytosis. Secondly, "sensitised" lymphocytes are produced which have cell-bound antibody on their surface. The latter are the effectors of cell-mediated immunity involved in the rejection of grafts from xenogeneic and allogeneic sources and other cell-mediated hypersensitivity (Type IV, delayed-type) reactions.

i. Cellular response and immunocompetent tissues

Cells equivalent to the active-cells involved in the immune response of mammals have been found throughout the elasmobranchs and teleostomes (Good and Papermaster, 1964), though the follicular accumulation of these cells in lymphoid tissues was found to be less well organised in fishes.

The haematological responses to infection have been described for various teleosts: Salmo salar (Conroy, 1972, Salmo gairdneri (Klontz, 1972), Cyprinus carpio (Hines and Spira, 1973), and Tilapia zilli (Ezzatt, Shabana and Farghaly, 1974). The inflammatory response in teleosts was poorly documented until Finn and Nielsen (1971a) investigated the response in S. gairdneri. Further work has mainly looked at the effect of temperature on the inflammatory response in S. gairdneri (Finn and Nielsen, 1971b), Lepomis macrochirus (Hoffman and Putz, 1965), S. salar (Roberts, McQueen, Shearer and Young, 1973), Carassius auratus (Mawdesley-Thomas and Bucke, 1973) and Pleuronectes platessa (McQueen, MacKenzie, Roberts and Young, 1973).

The cells involved in the immune response of fishes have shown themselves difficult to differentiate, leading to different workers using nomenclature suited to their own techniques and indicating similarities with mammalian cell lines (Ellis, 1976, 1977). Ferguson (1975) using morphological studies at the electron microscope level, and Ellis (1976), using histochemical and physiological techniques, have attempted to characterise the lymphocytes and related cells in P. platessa. The cells identified were four types of thrombocyte, lymphocytes, plasma cells, monocytes, macrophages and a granulocyte histochemically resembling mammalian neutrophils. Earlier work by Ferguson (1975), and Ellis, Munro and Roberts (1976) had shown the phagocytic activity of plaice leucocytes and the role of reticulo-endothelial cells in the heart which on ingestion of inoculated carbon particles became free macrophages. This had also been observed by Mackmull and Michels (1932) in carbon inoculated

Tautogolabrus adspersus.

In mammals two major sub-populations of lymphocyte are found, the T-lymphocytes (T-cells) which, dependent on the thymus, are responsible for cell-mediated immunity, and B-lymphocytes (bursa of Fabricius dependent in birds) which are responsible for synthesis of humoral antibody. It is now known that the T-cell population can be further subdivided into groups dealing with one specific function: cell-mediated immunity cells, helper cells, amplifier cells, suppressor cells and killer cells. These populations of T-cells, and the B-cells, interact closely with macrophages, both physically and by use of mediating 'factors', to regulate both humoral and cellular based immunity (Feldmann, Beverley, Erb, Howie, Kontiainen, Maoz, Mathies, McKenzie and Woody, 1977).

The differentiation of lymphocytes in fishes is still not well understood, there being at this time no clear distinction of T- and B- populations. The use of fluorescent labelled immunoglobulin anti-sera has demonstrated surface-immunoglobulin on lymphocytes, a mammalian B-cell characteristic, in the thymus, spleen, anterior kidney and blood of teleosts. Emmrich, Richter and Ambrosius (1975) observed a high proportion of labelled lymphocytes (65 to 68%) in the thymus of C. carpio, as well as in the peripheral blood (30 to 58%), spleen (24 to 45%) and in the pronephric kidney. In P. platessa Ellis (1976) made the observation that all the lymphocytes had surface immunoglobulin, as did plasma cells in the kidney and 10% of the macrophages from the kidney and spleen. The surface immunoglobulin on the macrophages was found not to form 'caps' as had been observed to happen readily in the case of the lymphocytes, a mammalian B-cell characteristic. The plaice thymus had earlier been investigated by Ellis (cited by Ellis and Parkhouse, 1975) and he found no cells with internal immunoglobulin in this organ, though surface immunoglobulin was detected (Ellis, 1976).

Ortiz-Muniz and Sigel (1971) cultured tissues of Lutjanus griseus and Mycteroperca bonacia which had previously been

sensitised with BSA or BGG. They then used a fluorescent 'sandwich' technique to label the cells producing anti-BSA or anti-BGG antibodies. Cultures of thymus, spleen and anterior kidney lymphocytes were the only tissues to show positive labelling and many of the antibody producing cells had the appearance of plasma cells. Antibody forming cells were also found in the spleen and predominantly in the pronephros of SRBC sensitised L. macrochirus by Smith, Potter and Merchant (1967) using a modified Jerne-Nordin plaque assay for antibody producing cells. Whereas, Chiller, Hodgins, Chambers and Weiser (1969a), Chiller, Hodgins and Weiser (1968, 1969b) using SRBC sensitised S. gairdneri found various cells in the spleen and anterior kidney which would form rosettes with SRBC, indicating surface antibody. Lymphocytes, plasma cells, blast-like cells and eosinophil resembling cells were found to form rosettes. They also observed a small number of rosette forming macrophages, which may be linked to the findings of Ellis (1976) of surface antibody on plaice macrophages.

Those fish possessing a proportion of lymphocytes showing no surface immunoglobulin or synthesis, such as in the carp (Emmrich, Richter and Ambrosius, 1975) or plaice (Ellis and Parkhouse, 1975), may represent inactive cells or possibly a T-type cell. Ellis and Parkhouse (1975) point out, however, that many of the immunoglobulin negative cells described in the literature may have been thrombocytes. The latter cells were found to have a similar appearance to lymphocytes when blood smears were taken from stressed fish (Ellis, 1976, 1977).

The source and differentiation of lymphocytes are still not well understood in fishes. They cannot originate from the bone marrow as in mammals, though other tissues such as the mammalian liver are implicated in the production of lymphocyte precursors. In amphibia the larval thymus was shown to be the primary lymphoid organ for the production of both T- and B-lymphocyte analogues (Turpen, Volpe and Cohen, 1973; Volpe and Turpen, 1975). This may also be true of teleosts and other fishes, the formation of T- and B- like cells being a later

secondary stage in other lymphoid tissues.

Emmrich et al. (1975) suggest that the high proportion of surface immunoglobulin-carrying thymic lymphocytes in young adult carp was analagous to the situation found by Du Pasquier, Weiss and Loor (1972) in amphibian larvae. The population of surface immunoglobulin bearing thymic lymphocytes in the larvae (70%) was found to decrease to 9% in the adult stage. Lymphocytes are thus seeded throughout the body from the thymus and, as in the adult mammal, the seeded areas may have T and B activation properties not requiring contact with any one particular organ such as the thymus.

The influence of the lymphoid organs on the later function of formed or differentiating lymphocytes has been little examined. One account of splenectomy in L. griseus and Haemulon albium was unable to demonstrate any change in humoral antibody production against BSA (Ferren, 1967), which may have been due to the soluble nature of the antigen preventing efficient trapping in the spleen or other lymphoid organs in particular. Yu, Sarot, Filazzola and Perlmutter (1970) did, however, find Trichogaster trichopterus which had been splenectomised were less resistant to IPN virus. As yet no account exists on the effects of thymectomy or removal of the pronephric kidney in teleosts.

The existence in teleosts of most of the mammalian-like immunocompetent tissues and cells, and their involvement in the immune system has been recognised for some time (Good and Papermaster, 1964). The major organs that possess lymphoid activity are the thymus, spleen and pronephric kidney, though the latter two are not primary lymphoid organs.

Teleosts have a lymphatic circulatory system, described in plaice and reviewed in other teleosts by Wardle (1971), though they do not possess peripheral lymphoid tissues equivalent to the lymph nodes of mammals. It is not until the evolutionary level of the anuran amphibians that rudimentary lymph nodes are found (Manning and Turner, 1976), a group which also displays

the first haemopoietic bone marrow.

The teleost thymus has been morphologically examined by many workers and in the species examined has lymphoid characteristics: Salmo fario = S. trutta (Deansley, 1927); Astyanax mexicanus (Hafter, 1952); Micropterus salmoides, Ameiurus melas, Catostomas commersoni, C. carpio (Finstad et al., 1964); L. macrochirus (Smith et al., 1967); S. gairdneri (McArdle and Roberts, 1974). The thymus was shown to be a paired organ associated with the gill pouches, attached to the branchial epithelium on the dorsal side of the branchial cavity and found directly under the operculum. The organ was found to be surrounded by a connective tissue membrane and divided into an epithelial cortex and medulla containing blast-like thymocytes and a few lymphocytes.

The teleost spleen was found to be a discrete organ with erythropoietic and granulocytopoietic functions which secondarily contains lymphoid structures (Corbel, 1975). Red and white splenic pulp have been observed as have cells similar to mammalian plasma cells in antigenically stimulated fish (Ortiz-Muniz and Sigel, 1971). The production of prominent splenic follicles in response to antigen, as seen in higher vertebrates has not been observed in teleosts.

As has already been noted the pronephric kidney of teleosts has been shown to possess lymphoid activity and it has been suggested that this organ may contain the functional equivalent of the mammalian lymph nodes (Smith et al., 1967; Chiller et al., 1968, 1969a,b; Ortiz-Muniz and Sigel, 1971; Emmrich et al., 1975; Ellis, 1976).

ii. The humoral immune response of teleosts

The humoral immune response against a wide range of particulate and soluble proteins, and haptens has been recorded in the Teleostei with the production of high molecular weight (HMW) antibodies, 600,000 to 700,000 D (13 to 16S), which have

mammalian IgM characteristics. Although an enhanced antibody response has been demonstrated a transition to a low molecular weight (LMW) IgG-like immunoglobulin has not been adequately proven, though modifications of the higher molecular weight immunoglobulin have been observed in the course of the immune response. Confirmation of an IgG-like, LMW immunoglobulin, requires the analysis of amino acid sequences in the polypeptides of the heavy chain components of the molecule (Nisonoff et al., 1975), as yet not fully undertaken by teleost immunologists.

The studies examining the immune response of teleosts are numerous and have been the subject of many reviews (Ridgway, Hodgins, and Klontz, 1966; Clem and Leslie, 1969; Snieszko, 1970; Finn, 1970; Carton, 1973a,b; Corbel, 1975). Serum albumin, gammaglobulins and other soluble factors have been used to raise humoral antibodies successfully by many workers, mainly in salmonid and cyprinid hosts. It is because of the economic importance of the latter two groups that they have received greatest attention when the immune response to particulate antigens, especially disease organisms, have been investigated.

The humoral immune response of S. gairdneri has been shown to produce HMW antibody fraction only. Post (1966a,b) using Aeromonas hydrophila as an antigen found a HMW antibody but one having a wide range of β electrophoretic mobility. Similarly Anderson and Klontz (1970) using A. salmonicida found precipitin activity in the β_2 electrophoretic band. Hodgins et al. (1967) using soluble BSA, BGG and KLH antigens found that the precipitins formed had a sedimentation coefficient of 19 S and were sensitive to 2-ME reduction, a property of mammalian IgM (Svehag and Mandel, 1964). The use of hapten linked DNP-Limulus haemocyanin and the bacteriophage FH_5 by Dorson (1972 a,b) produced precipitin and neutralising activity only in an HMW macroglobulin fraction, but having a sedimentation coefficient of 16S. He also demonstrated heavy and light chain fractions in the macroglobulin having similarities with mammalian antibodies.

Alexander, Wilson and Kershaw (1970) examining the precipitin

activity of S. salar serum to fractions of UDN diseased tissue described two antibodies, a 19S 2-ME sensitive macroglobulin having a β_2 electrophoretic migration properties and also a 7S non-2-ME sensitive and β_1 migrating molecule. It has not been shown, however, that the smaller antibody molecule was different in heavy chain structure or a single molecule of the heavier IgM-like polymer. Evelyn (1971) using salmonid kidney disease bacteria (SKDB) observed precipitin activity in the HMW serum fraction of Oncorhynchus nerka, though he also observed three LMW fractions which adsorbed onto the SKDB. In the coho salmon, Oncorhynchus Kisutch, Cisar and Fryer (1974) found that antibodies to A. salmonicida were 17S and 2-ME sensitive. Electrophoretic analysis of the components of this HMW antibody, and direct electron microscope examination, indicated that the salmonid antibody was a tetrameric macroglobulin similar in structure to the pentameric mammalian IgM.

Uhr, Finkelstein and Franklin (1962) found that prolonged immunisation of C. auratus with diptheria toxin or X174 bacteriophage over a five month period produced a shift from 19S to 7S antibody molecules. Both fractions were, however, 2-ME sensitive. Watson, Paulissen and Yen-Watson (1968) also noted two antibody active fractions, β and γ, separated by electrophoresis, in the serum of C. auratus and Notemigonus crysoleucas against A. liquifaciens and Streptococcus (OX39). With the same antigens Summerfelt (1966) had been unable to find anything but a γ-region in the serum of N. crysoleucas. Marchalonis (1971) also found HMW and 7S components using HGG and Limulus haemocyanin, though in this case only the HMW fraction was active as antibody. The 7S fraction was shown, however, to be antigenically similar to the HMW molecule and was concluded to be a monomeric fragment of that molecule. Trump (1970) in his examination of the response of C. auratus to BSA found no shift to a LMW fraction, though he did demonstrate that the HMW antibody fraction was split into two groups (16.4S and 15.3S) by molecular charge on an ion-exchange chromatography column. This difference was shown to be a function of the molecular charge rather than molecular structure. A similar result has been found for the same species

by Everhart (1971, 1972) using BSA attached to arsanilic or sulphanilic acid haptens, there being two populations of HMW antibody differentiated by their electric charge. Everhart gave only one sedimentation coefficient of 13.2S for the two antibody populations. A single 14.5S antibody to DNP-BSA or SRBC was found for C. carpio by Shelton and Smith (1970), which by electron microscope observation was shown to be a tetrameric molecule. Ambrosius and Frenzel (1972) also found a single HMW antibody to DNP-BGG in C. carpio, but with a sedimentation coefficient of 13S.

The catfish Ictalurus punctatus has only been shown to produce one type of HMW antibody. Acton, Weinheimer, Hall, Niedermeier, Shelton and Bennett (1971b) demonstrated a tetrameric antibody molecule to Salmonella typhose and human erythrocytes by examination of the heavy and light chains. Similarly, with electron microscope verification, Hall, Evans, Dupree, Acton, Weinheimer and Bennett (1973) found a single 14S (610,000D) tetrameric antibody to DNP-human erythrocytes.

Antibodies to HGG in Perca fluviatilis were found to be single HMW fractions, Richter (1968) finding a 14 to 15S molecule with 2-ME sensitivity and Ambrosius, Hemmerling, Richter and Schimke (1970) a 14.5S molecule, which they also found when the perch were inoculated with chicken erythrocytes.

2-ME sensitive HMW antibodies have only been found in the serum of other teleosts using as antigen BSA (Clem and Sigel, 1966), DNP-KLH (Russell, Voss and Sigel, 1970) in L. griseus and human myxovirus (Sigel and Clem, 1965), BSA (Clem and Leslie, 1969), DNP-BGG (Leslie and Clem, 1969) in H. albium. Fletcher and White (1973) found HMW humoral antibody only when they examined the response of P. platessa to Vibrio anquillarum.

In response to parasitic infections humoral antibodies have been shown to be HMW molecules. The chub, Leuciscus cephalus, was found to produce precipitating antibodies against the

acanthocephalon Pomphorhynchus laevi (Harris, 1972), though Kennedy and Walker (1969) could not find an antibody response to the cestode, Caryophyllaeus laticeps, although the chub was found capable of rejecting the parasites in certain cases. Harris and Cottrell (1976) observed natural HMW antibodies to the dogfish parasitic nematode Proleptus obtusus in P. platessa, which they explained as a cross reaction caused by the nematode parasite of the dogfish having similar antigenic determinants to the plaice specific species of nematodes.

iii. Humoral immune response to viral antigens

The sequences of humoral IgM and IgG response to non-replicating virus in mammals is usually similar to that observed for other antigens. Svehag and Mandel (1964) found that the response to poliovirus in rabbits was dependent on the dose given. An 8×10^6 PFU dose of the virus produced a typical primary response after a latent period (inductive period) of 8 to 12h. The production of IgM, which decayed after 10 to 15d, was followed by IgG levels which were maintained for 30 weeks. On the other hand a smaller dose of 3×10^6 PFU only elicited an IgM response which decayed over a period of 30d. Subsequent challenges with the same poliovirus dose induced only IgM once more, though the time for decay and peak titre were shown to increase. Similar results were shown by Uhr and Finkelstein (1963) using X174 bacteriophage in guinea pigs. In human recipients the X174 bacteriophage was found by Peacock, Jones and Gough (1973) to induce IgM only on primary challenge, but IgM was produced on secondary challenge with the same dose.

Research papers dealing specifically with the immune response of fishes to viral antigens have been listed in table 1.

The use of virus, and specifically bacteriophages, as antigens in teleosts has produced variable response. Uhr et al. (1962) were the first to raise antibody to a bacteriophage, X174, in C. auratus. At a holding temperature of $30^{\circ}C$ and an X174 dose of 10^{10} PFU neutralising antibody was first detected

Table 1, The immune response of fishes to viral antigens in the literature

Species	Antigen	Antibody Assay	Author	Comment
Agnatha				
Eptatretus stoutii	T2	k	5,11	No clearance, no antibody. Used multiple inoculations.
	T2,MSP-8	k	13	No antibody. Inoculated at 0,7 & 14d.
Petromyzon marinus	T2	k	5	1° inoculation cleared after 21d, 2° inoculation cleared at 52d, & no antibody formed.
	f2	k	9	One inoculation, 28 out of 37 produced antibody.
	T2,MSP-8	k	13	Clearance 30d, antibody response in 3 out of 10 after 1° inoculation, 4 out of 5 after 2.
Elasmobranchii				
Rhinobatos productus	T2	k	5,13	1° inoculation cleared at 21d, no antibody. 2° cleared at 11d, some antibody.
Triakis semifasciata	T2	k	18	Not as responsive as rabbit & mouse.
Heterodontus francisci	T2	k	5	As for R. productus.
	T2		6	Weak response.
Negaprion brevirostris	Myxoviruses	h	1,16,17	High antibody titres at 40d, 2° response poor. Used multiple inoculations. FCA enhanced the response.
	PR8	h	3	Poor antibody titre, maximum response 24d. Multiple inoculations used. Splenectomy did not affect the titre.
	T2	-	15	2° clearance accelerated.
Ginglymostoma cirratum	Myxoviruses	h	1,16,17	As for N. brevirostris.
	Influenza A	r	4	Small amounts of antibody produced in neonatal sharks.
	T2	k	15	As for N. brevirostris.
Platyrhinoides triseriata	T2	k	19	5 out of 9 of fish produced antibody 16d after single inoculation.
Chondrostei				
Amia calva	T2	k	13	1° clearance at 12d. Clearance and antibody titre enhanced in 2° response.
Polydon spathula	T2	k	5	1° clearance at 21d, weak antibody response. 2° response enhanced.
Lepisosteus platyrhynchus	Myxoviruses	h	16,17	Peak titre 10d after single inoculation. No 2° response observed.

Table 1 continued

Teleostei				
Salmo gairdneri	FH5	k	2	Poor response, 3 out of 5 fish produced antibody.
	VHS	i,SD_{50}	7	Only 1 in 40 fish gave low antibody titre. Monthly doses of antigen given, but virus cleared.
	IPN, SRCD, OSD	r,i	8	No humoral antibodies detected.
	IPN	i	21	13 out of 15 fish produced neutralising antibody.
Salvelinus fontinalis	IPN	i	21	2 fish examined produced antibody.
	IPN	i	22	Antibody level dependent on IPN carrier state of fish.
Oncorhynchus nerka O. tshawytscha	IPN, SRCD, OSD	r,i	8	No humoral antibodies detected.
Haemulon albium	Myxoviruses	h	1,17	Inhibition of 2^{o} antibody response.
Carassius auratus	X174	k	20	Antibody first detected 3 weeks after inoculation, peak at 5 months. Multiple inoculations used.
Ameriurus melas	T2	k	12	1^{o} clearance at 4d, antibody at 14d.
Trichogaster trichopterus	IPN	i	14	Low antibody titres, suppressed by heavy metals.
	IPN	i	23	Low levels of antibody, suppressed by splenectomy.
Ictalurus punctatus	VSV	i	10	Weak response only when FCA added to the inoculum.

Key

k inactivation constant
r radial immunodiffusion
i neutralisation of infective titres
h haemagglutination inhibition

1 Clem and Sigel (1963)
2 Dorson (1972 a,b)
3 Ferren (1967)
4 Fidler, Clem and Small (1969)
5 Finstad and Good (1966)
6 Frommel, Litman, Finstad and Good (1971)
7 Jørgensen (1971)
8 Klontz, Yasutake and Parisot (1965)
9 Marchalonis and Edelman (1968)
10 McGlamery, Dawe and Gratzek (1971)
11 Papermaster and Good (1964)
12 Papermaster, Condie and Good (1962)
13 Papermaster, Condie, Finstad and Good (1964 a)
14 Roales and Perlmutter (1977)
15 Sigel et al (1967)
16 Sigel and Clem (1965)
17 Sigel, Moewus and Clem (1963)
18 Suran, Tarail and Papermaster (1967)
19 Thomas, Sanders and Wiley (1972)
20 Uhr, Finkelstein and Franklin (1962)
21 Wolf and Quimby (1969)
22 Yamamoto (1975)
23 Yu, Sarot, Filazzola and Perlmutter (1969, 1970)

3 weeks after inoculation. The peak of antibody response was observed 5 months later after administration of phage at 3 week intervals.

The response of H. albium to human myxovirus, PR8, was found by Clem and Sigel (1963) and Sigel and Clem (1965) to produce low haemagglutination inhibition titres and on secondary challenge they observed no indication of memory or enhanced response. Inoculations of PR8 were administered under three different time schedules, a single inoculation, a second 30d after the first, or weekly inoculations. Of the three the weekly regime was found to produce the highest titres. A second challenge during the first 3 months after inoculation was found to produce no renewed antibody response, and it was not until a period of 5 months had elapsed that the titre rose to the primary level. This weak response to viral antigen was also shown by McGlamery, Dawe and Gratzec (1971) when they inoculated I. punctatus with 2.5×10^7 PFU of vesicular stomatitis virus (VSV) in Freund's complete adjuvant. There was no enhanced secondary response either.

Klontz, Yasutake and Parisot (1965) inoculated live salmonid disease causing virus (IPN, SRCD, and OSD) into fingerling S. gairdneri, Oncorhynchus tshawytscha and O. nerka at a dose which would cause a 50% stock mortality. No agglutinating antibody was detected and humoral neutralising antibody titres were very low. They did however note lymphopoietic activity in the anterior kidney 4 to 5d after injection of the virus.

Inoculation of infectious doses of IPN together with an equal dose administered orally was found by Wolf and Quimby (1969) to produce neutralising antibody titres in 13 out of 15 S. gairdneri. The two fish having no neutralising antibody in the serum were postulated as being IPN carriers. Two Salvelinus fontinalis inoculated with IPN were also found to produce neutralising antibody after a period of 3 weeks. At this time the IPN had started to replicate and was released in the faeces,

however, 9 months later the IPN virus was found to have been completely cleared from the fish tissues, thus implicating a more active secondary response. Yamamoto (1975) found similar increased levels of neutralising antibody to IPN were related to decreasing levels of the virus in the tissues of S. gairdneri, though again IPN-carrier states were found in fish showing no antibody activity.

Egtved virus (viral haemorrhagic septicaemia - VHS) was inoculated into S. gairdneri by Jørgensen (1971), who also examined naturally infected fish. He found that only one of the inoculated fish had a low but significant neutralising antibody titre, all the other fish showing a negative response.

Dorson (1972a,b) inoculated S. gairdneri with a non-pathogenic virus, FH_5 bacteriophage. Single 10^9, 10^{10}, and 10^{11} PFU intraperitoneal inoculations gave neutralising antibody at the highest phage dose and only 3 out of 5 of the group receiving the highest dose produced a positive response. Peak titre in the responding fish was reached at 60d.

Viral particles are known to be highly antigenic in mammals (Peacock et al. 1973; Burns and Allison, 1975), however, their antigenicity in teleosts has not been so far supported by the slow speed of initiating antibody synthesis, the low titres of antibody produced and the inactive secondary response found in many fish. Dorson (1972a) suggested the need for adjuvants to increase antibody titres in S. gairdneri, however, the use of Freund's complete adjuvant by McGlamery et al. (1971) did not increase the response to VSV in I. punctatus. Klontz et al. (1965) suggested that the temperature of 10 to 13°C used during their experiments on salmonids needed to be increased. The effect of temperature on the immune response will be discussed in a later section.

Teleosts have been shown to produce enhanced secondary responses to non-viral antigens, though the transition to the LMW, IgG-type, antibody molecule has not been demonstrated. Richter (1968) observed an increased secondary response to HGG

in Perca fluviatilis, Papermaster et al. (1964a) found an increased secondary response in M. salmoides using kidney disease bacteria and Evelyn (1971) produced a five-fold increase in secondary titre of O. nerka with the same antigen.

iv. Secretory antibodies

As early as 1935, Nigrelli observed that the mucus of certain fish was toxic to parasites and suggested the presence of antibodies. HMW IgM-like antibodies which had antigen specificity have now been demonstrated in the mucus of Labrax murone, Gadus callarias, Gadus virens, Sebastes marinus (O'Rourke, 1961); P. platessa (Fletcher and Grant, 1969); Tachysurus australis (DiConza and Halliday, 1971) L. griseus (Bradshaw, Richards and Sigel, 1971) and Leuciscus leuciscus (Harris, 1972). These antibodies were found to be induced by antigen and were antigenically similar to humoral antibodies.

The occurrence of a secretory piece as found linking the antibody molecules of mammalian secretory IgA has not been demonstrated in fish, though normal IgM molecules have been shown to be secreted in man (Berger, Ambender, Hopes, Zepp and Herizy, 1967) and pigs (Allen and Porter, 1970; Porter, Noakes and Allen, 1970).

v. Specificity of antibody

Antibody specificity is at the heart of immunology and defines the antibody molecule (Roitt, 1974). Everhart and Shefner (1966) were able to show that the antibody of C. auratus against BSA was as specific as rabbit anti-BSA and possibly more specific if the partial identity of rabbit anti-BSA with sheep serum albumin was taken into consideration. The rabbit antibodies had, however, a higher avidity for antigen than the fish antibody, which also increased with time and in secondary response.

vi. Anaphylaxis (Type I - Immediate hypersensitivity)

In mammalian subjects the reinoculation of antigens 2 to 3 weeks after the primary inoculation may result in the sensitised animal showing signs of asphyxia caused by smooth muscle contraction and capillary dilatation in the lungs. The effect is produced by the antigen reacting with a specific antibody, termed homocytotrophic antibody or reagin, attached to the surface of mast cells or circulating basophils (Roitt, 1974) which then release vasoactive amines.

Dreyer and King (1948) observed stress reactions when a second inoculation of horse serum was injected into perch, goldfish, rock bass and common sunfish, these reactions not being observed in controls inoculated with egg albumin. An anaphylactic response was observed by Szakolczai (1969) when C. carpio sensitised by natural infection to Aeromonas punctata were inoculated with the bacterium. Other workers have since been unable to demonstrate anaphylaxis or the presence of skin sensitising antibodies, which may in part be a result of the lack of IgE or certain IgG subclasses known to be associated with the response in mammals. The teleosts examined were: S. gairdneri (Hodgins, Weiser and Ridgway, 1967); H. albium (Clem and Leslie, 1969); L. leuciscus (Kennedy and Walker, 1969); Harris, 1973b); L. cephalus (Harris, 1973b). There is, however, one exception in which Baldo and Fletcher (1975) demonstrated immediate hypersensitivity in P. platessa to intra-dermal inoculations of fungal extracts, though a similar response was not found in Platichthys flesus. In the latter fish an inoculation of serum from fungal sensitised plaice was found to transfer hypersensitivity, an erythema being produced on subsequent challenge with the fungal extract.

The mode of action of the anaphylactic response in teleosts, when observed, is not known, though mast cells have been described in teleosts by Roberts, Young and Milne (1972) and histamine has been found in the dermis of plaice. Anti-histamines, however, did not inhibit plaice erythema reactions and other mediators such as C-reactive protein may thus be

involved (Baldo and Fletcher, 1975).

vii. Non-specific serum factors

Natural heteroagglutinins and isoagglutinins have been shown to be present in fish sera, these being structurally different from each other as well as structurally different from agglutinins induced by immunisation. This group of non-specific factors has been exhaustively used in the immunogenetic study of blood group polymorphisms, analagous to the human ABO blood group system (Cushing, 1970; Hildemann, 1970).

Natural agglutinins have been considered in certain cases to be indicative of antigen exposure (Fujihara and Tramel, 1968; Janssen and Meyers, 1969; Janssen, 1970), however, Hodgins, Wendling, Braaten and Weiser (1973) have shown natural agglutinins in S. gairdneri for three species of xenogeneic erythrocytes. The agglutinins were found to be of LMW and non-precipitable by 22% sodium sulphate, whereas the antigenically induced agglutinins were all of HMW and were precipitated by 22% sodium sulphate. The possibility does arise, however, of an HMW agglutinating antibody agglutinating two antigen species having the same antigenic determinants (Harris and Cottrell, 1976).

Natural serum factors which haemolysed heterologous erythrocytes were first reported by Liefmann (1911) and in 1945 Cushing characterised a LMW fraction of C. carpio serum which had similarities with guinea pig complement (GPC) activity. Mammalian complement is a series of molecules that work together to lyse erythrocytes and bacteria, in vitro, which have been opsonised with antibody. The similarities of the mammalian and teleost complement system have been reviewed by Cushing (1970) and Corbel (1975).

Luk'yanenko (1965) demonstrated the presence of lysozyme in the sera of fish and Fletcher and Grant (1969) found the enzyme in the epithelial secretions of P. platessa. The amount of epithelial lysozyme secretion was found to be greater in 'stressed' plaice (Murray and Fletcher, 1976). They also

observed lysozyme on the surface of blood neutrophil-like cells and in peritoneal macrophages stimulated with Micrococcus luteus. The biological role of secreted lysozyme was not fully understood, though it was found that the enzyme had bacteriolytic properties against M. luteus.

C-reactive protein (CRP) is another molecule with probable bacteriocidal properties which has been found in the sera of vertebrates following an infection. Watson, Paulissen and Yen-Watson (1968) found CPR-like protein in teleosts and this work was extended by Fletcher and Baldo (1976) to the sera of marine teleosts. The latter two authors even found CPR in the gonads of the lumpsucker (Cyclopterus lumpus).

In the course of many replicating viral infections humoral antibody is formed at too late a stage to be effective (Roitt, 1974). It is during the inductive phase of antibody formation that the replication of the invading virus in the host tissues is inhibited by the rapid production of interferon (Issacs, 1961). Interferon has been shown to be produced in in vitro fish cell cultures (Gravell and Malsberger, 1965; Beasley, Sigel and Clem, 1966; Oie and Loh, 1969) and demonstrated in S. gairdneri experimentally infected with VHS by Kinkeline and Dorson (1973). Amend (1970) also suggested that interferon had been implicated in preventing infectious haematopoietic necrosis (IHN) in S. gairdneri. The protective effect of interferon in the trout was found to build up and break down rapidly, but only occurred at temperatures above 14 to 15°C. Interferon has been implicated also in intracellular suppression of bacteria (Remington and Merigan, 1970; Weinstein, Waitz and Carne, 1970; Gober, Friedman-Kien, Havell and Vilček, 1972), Rickettsiae (Kazar, Krantwurst and Gordon, 1971), Chlamydiae (Hanna, Merigan and Jaweta, 1966), and protozoans (Jahiel, Nussenzweig, Vanderberg and Vilček, 1968; Remington and Merigan, 1968) in mammals.

viii. Sites of antigen inoculation

Fishes have been immunised by many techniques with variable results. Hildemann (1962) recognised possible differences in the route of antigen entry and found that intraperitoneal inoculation of erythrocytes was the most effective way of raising the immune response in C. auratus. Similar results were found by Fujihara and Nakatani (1971) in salmonids. Intraperitoneal inoculation of Chondrococcus columnaris produced maximum titres before equivalent intramuscular inoculations, though with the addition of Freund's complete adjuvant both inoculation routes produced similar titres. Earlier results by Fujihara (1967) had indicated a more active response via a subcutaneous inoculation route. Fletcher and White (1973) found the subcutaneous route more active than intraperitoneal or oral immunisation of P. platessa with V. anguillarum when examining humoral antibody, however, for inducing secretory antibodies in the mucus only oral immunisation was effective.

In many cases there appears to be little difference between subcutaneous, intramuscular and intraperitoneal inoculation routes, this being so for I. punctatus inoculated with C. columnaris (Schachte and Mora, 1973) and S. trutta inoculated with cellular antigens (Ingram and Alexander, 1977).

ix. Oral and other methods of immunisation

The complications of inoculating large stocks of fishes, stress, time and cost, have initiated a large volume of work on oral immunisation. This method of immunisation has been shown to produce antibody titres in teleosts (Duff, 1942; Krantz, Reddecliff and Heist, 1964b; Post, 1966a,b; Fujihara, 1969; Fujihara and Nakatani, 1971; Fletcher and White, 1973; Anderson and Nelson, 1974), though the benefits of this method have not been conclusively demonstrated in the field (Snieszko, 1970; Fryer, Nelson and Garrison, 1972).

Immunisation of O. kisutch by hyperosmotic infiltration of killed V. anguillarum and A. salmonicida was used by Antipa and

Amend (1977) after BSA had been shown to enter S. gairdneri via the lateral line and gills in earlier experiments (Amend and Fender, 1976). Agglutinating titres were found to be formed against V. anguillarum by both the hyperosmotic infiltration and intraperitoneal inoculation routes, the rates of antibody formation and maximum titres being similar, however only intraperitoneal inoculation produced high antibody titres against A. salmonicida. The technique of hyperosmotic infiltration raises interesting speculation on the role of the lateral line, other than its sensory role, it relation to the lymph duct which lies under the line. It may also indicate one of the reasons for the increased disease susceptibility of stressed fish as being the result of increased tissue permeability, already demonstrated by Stevens (1968) for the vascular system of stressed S. gairdneri.

x. Adjuvants

Freund's adjuvants are used widely for enhancing the formation of humoral antibodies against antigens. Incomplete adjuvant oils act as antigen depots releasing the antigen slowly and thus prolonging exposure. Freund's complete adjuvants (FCA) contain an additional mycobacterial component, of which the lipoglycopeptide was found to be the only active fraction in mammals (Hiu, 1977). This fraction was further subdivided into a glycopeptide which stimulated cellular activity and a lipid moiety which stimulated the humoral immune response.

The exact action of FCA has not been demonstrated, though like incomplete adjuvant it will also retain antigen which is then released slowly. The stimulation of B-cell precursors has been envisaged as being antigen concentration dependent, there being a range of B-cell precursors having a range of high to low affinity for antigen. Adjuvant would prolong the effective antigen dose and thus stimulate the low affinity B-cells to produce antibody, a form of 'clonal recruitment' (Siskind and Benacerraf, 1969). This hypothesis was disputed by Civin, Levine, Williamson and Schlossman (1976) in favour of a direct enhancement of the B-cells by prolonged antigen exposure producing 'clonal expansion'. Other effects of FCA are in the expansion of T-cell populations and the stimulation of macrophages to process antigen (Taub,

Krantz and Dresser, 1970; Allison and Davies, 1971; White, 1972; Taussig, 1974, 1977).

FCA is traditionally most effective in increasing antibody titres to thymus dependent antigens, for example SRBC, through T-cell stimulation (Roitt, 1974). Antigens which are independent of T-cell activity in the production of immunity are restricted to a few large molecule polymers, for example pneumococcus polysaccharide and polymerised flagellin (Roitt, 1974). It was thus put forward by Feldmann (1976) and Feldmann et al. (1977) that the regularly repeating determinants required to stimulate the B-cells were assembled on macrophages, known to be essential to the process, by specific antigen receptors (IgT) formed and released from the T-cells. This would also help to explain FCA stimulation of T-cells, B-cells and macrophages as an integrated part of T-dependent antigen stimulation.

Adjuvants have been shown to enhance antibody formation in teleosts against both soluble and particulate antigens. Human myxovirus in adjuvant oil emulsion was shown to produce an enhanced response in H. albium (Sigel and Clem, 1965; Clem and Sigel, 1966) and C. columnaris antibody titres were enhanced by adjuvant in Prosopium williamsoni (Fujihara and Hungate, 1972). Ambrosius and Lehmann (1965) used both aluminium oxide and adjuvant oil and observed an enhanced response to several antigens in Ameiurus nebulosus, the enhancement being more marked than in rabbits given similar treatments.

Another species of catfish, I. punctatus, showed enhancement with the inclusion of FCA in inoculations of VSV and BSA (McGlamery, Dawe and Gratzek, 1971). Sigel, Russel and Bradshaw, (1967) were able to raise an immune response to BSA in species of Lutjanus when the antigen was administered as an alum precipitate, and Fletcher and White (1973) found FCA essential for antibody production when inoculating P. platessa with antigen.

Enhancement of the immune response has been demonstrated

with the use of adjuvants in the salmonids S. trutta and S. gairdneri (Krantz, Reddecliffe and Heist, 1963, 1964a,b; Fujihara, Olson and Nakatani, 1965; Hodgins, Weiser and Ridgway, 1967, Paterson and Fryer, 1974b). Post (1962, 1966a) found, however, that the rate of the antibody response in S. gairdneri to Aeromonas hydrophila was retarded by emulsifying the antigen with FCA. A slower response was also found when non-optimal concentrations of antigen were inoculated with adjuvants (Sigel and Clem, 1966; Evelyn, 1971; Avtalion, Wojdani, Malik, Shahrabani and Duczyminer1973). No enhanced antibody production was found in Anguilla japonica by Muroga and Egusa (1969) when V. anguillarum was inoculated with either Freund's incomplete or chrome alum adjuvants.

At this time no literature has been discovered attempting to explain the differences in response between incomplete and complete Freund's adjuvants in teleosts.

xi. Age and Maturity

The metabolic changes produced by seasonal factors, growth and maturation, as would be expected are found to modify the immune response. Epshtein, Avetikyan, Lavrovskaya, Rogozhnikova and Artemova, (1960) observed a better response in gamma globulin formation of C. auratus inoculated with horse serum and SRBC in the spring than the autumn, this probably being due to the autumnal decrease in temperature and nutritional state of the fish. C. carpio were found by Avtalion et al. (1973) not to produce an immune response before they were 3 months old, and Wolf and Quimby (1969) found that adult female S. gairdneri produced a greater immune response against IPN than male fish.

Barrow (1955) maintained 'shoals' of tench, goldfish and perch which were infected with trypanosomes. He observed antibody formation only in individuals at the top of the 'peck-order' which may have been due to the fitness of the higher social order and also stressing factors causing non-response in the lower orders. Snieszko, Dunbar and Bullock (1959) worked with

four strains of S. fontinalis and observed that certain strains were more resistant to furunculosis and ulcer diseases. They also indicated that it was the slower growing fish strains which could resist infection more easily. Krantz, Reddecliffe and Heist (1963, 1964a,b) observed similar differences in response to A. salmonicida in strains of S. gairdneri.

xii. Temperature

Fishes are poikilothermic and thus the ambient temperature of their environment will interact with their life processes, including those of immune responsiveness. Optimum temperature ranges for cold water fish of 10 to 15°C, and 20 to 30°C for warm water fish, have been put forward by Snieszko (1969), below which the immune response was retarded or absent. A slower rate of antibody production has been shown by various workers for cold water species, such as salmon and trout, when compared with the warm water cyprinids, carp and goldfish (Nybelin, 1935, 1943; Snieszko, 1958; Bisset, 1947; Cushing, 1942; Post, 1963).

The rainbow trout, S. gairdneri, will produce optimum antibody titres at 14 to 15°C (Hodgins et al., 1967; Ridgway, 1962b; and Fujihara and Nakatani, 1971), similarly S. trutta between 14 and 16°C, but not below 9°C (Ingram and Alexander, 1977). Optimum temperatures for antibody formation were found to be 28°C for C. auratus (Cushing, 1942; Trump and Hildemann, 1970) and at values above 12°C for C. carpio (Goncharov, 1962; Fijan and Cvetnić, 1964, 1966). There are, however, examples of antibody titres having been raised at temperatures below 10°C. Ridgway (1962a) produced a response at 5 to 8°C in the sable fish, Anoplopoma fimbria, and Nybelin (1968) a response in Lota lota at 5°C, Paterson and Fryer (1974a) found an antibody response in juvenile coho salmon, O. kisutch at 6.7°C and Harris (1973a) induced antibody synthesis at 2°C in L. leuciscus.

It is now evident that temperature does have a major effect on the latent or inductive period of the teleost antibody response. Harris (1973a) found on examination of haemagglutination titres that the latent period of the primary response

increased with decreasing temperature in L. leuciscus, being 10 d at 18°C and from 20 to 30 d at 2°C. The agglutinating antibody response of Tinca tinca was found by Nybelin (1935) to be initiated at 4d when the temperature was 24°C, but at 20°C the latent period increased to 9d. Similarly, Klontz (1972) states that a decrease of 3°C from the optimum temperature of salmonids, he had determined using 'standard antigens', doubled the induction period of antibody synthesis from 16 to 21d to 35 to 42d. In comparison the induction period of mammals is from 3 to 4d, though at a temperature of 37°C.

Avtalion (1969b), Avtalion, Malik, Lefler and Katz (1970) and Avtalion et al. (1973) have demonstrated that C. carpio, which was unable to produce an antibody response at low temperature (below 12°C), could produce a rising antibody titre at that low temperature if the fish were first inoculated and maintained for 8d at 25°C. Carp inoculated at the lower temperature and producing no subsequent antibody response when raised to the higher temperature again produced antibody. It was thus probable that the sensitisation of the immune system and antibody release were not primarily affected by temperature, but there was some intermediary stage dependent on temperature. Similar results were found for A. japonica at low temperatures by Muroga and Egusa (1969). The functioning of the processes of protein synthesis are established by the very fact of survival at low temperatures and the knowledge that fish species adapt protein synthesis and enzyme systems to their environmental temperature. Certain cold water fish can survive at temperatures down to -2°C and their enzyme systems are not impaired because of evolutionary adaptations in the molecular kinetics of the enzymes (Johnston and Goldspink, 1975).

The effect of temperature on the inflammatory and cellular responses of teleosts have been reviewed by Finn and Nielsen, (1971b), McQueen, MacKenzie, Roberts and Young (1973) and Roberts (1975b). All have reported a decrease in the inflammatory response with lower temperatures, and even a small decrease in temperature, 24°C down to 21°C, in L. macrochirus

doubled the time required to produce an inflammatory response to digenean metacercariae, Uvulifer ambloplitus (Hoffman and Putz, 1965). Finn and Nielsen (1971b) using FCA or Staphylococcus inoculations to produce an inflammatory response in S. gairdneri found little change in the emigrating leucocyte count with temperature, though the 15° to 5°C fall in the temperature produced a 50% delay in the time that the macrophages entered the site of inflammation. They also point out that the possibility of an age, weight or seasonal relationship with inflammatory activity could not be discounted in their experiments. A similar result was found in hypothermic mice, kept at between 20 and 22.5°C for 24h (Svanes, 1964 a,b) in which a general decrease in inflammatory response to a turpentine injection was observed but no change in the infiltration of leucocytes. Janssen and Waaler (1967) examined hibernating hedgehogs, with a body temperature of 6°C, and they found neither an inflammatory response nor production of antibodies.

Covert and Reynolds (1977) have demonstrated that the survival of C. auratus inoculated with A. hydrophila was greatly enhanced by increasing the water temperature. They further observed that carp in the course of an inflammatory response, which were placed in a temperature gradient, were found to move into a zone 4.8°C higher than the acclimation temperature and even maintain their circadian rhythm of preferred temperature around this higher temperature.

Seasonal variations of temperature associated with the incidence of infectious diseases also indicates the importance of temperature and temperature changes in the survival of fish populations. This work has been reviewed by Roberts (1975b) and he indicates the role of temperature as a stressing agent.

xiii. Stress

Stress is a summation of physiological responses by which an animal maintains metabolic equilibrium when subjected to physical or chemical forces and when this metabolic balance is disturbed the chances of survival are reduced.

Infectious organisms cause disease if changes in the environmental conditions in which the host live are optimal. The occurrence of stressing situations will increase the probability of disease outbreaks, though direct evidence for the involvement of stress is small (Snieszko, 1974). An increased interest in fisheries research has indicated many instances of decreased resistance caused by stress factors, and these are frequently encountered in hatchery practice. Many fish pathogens are commonly encountered in water supplies, however, disease problems caused by A. liquefaciens or Pseudomonas fluorescens rarely occur unless the fish stock have been excessively handled or crowded (Bullock, 1963), and similarly myxobacterial gill diseases only tend to be produced in crowded fish stocks (Snieszko, 1964). In the natural environment, however, stress factors will occur as complexes and will probably have additive or synergistic effects.

Wedemeyer (1970a) has reviewed the importance of the physiologically induced changes produced by stressing factors on the disease responses of fishes. The effects of environmental factors, seasonal factors such as temperature, respiratory factors, and the effect of natural and industrial pollutants on infectious diseases have been reviewed by Snieszko (1974).

In mammalian stress conditions adrenal corticosteroid hormones are released and have been associated with decreases in the numbers of circulating lymphocytes, also demonstrated in fish by Slicher (1961), with inhibition of the inflammatory and cellular responses, gamma-globulin production, interferon production and with increases in the proteolytic activity of the plasma (Wedemeyer, 1970a). Levels of 17-hydroxycorticosteroids (17-OHCS) in the plasma of teleosts have been shown to increase with crowding, handling and forced swimming. Robertson, Hane, Wexler and Rinfret (1963) administered levels of 17-OHCS equivalent to those found in spawning Pacific salmon to S. gairdneri which resulted in skin infections and depletion of lymphocytes from the thymus and spleen. Bisset (1949) found that single inoculations of an extract of (mammalian) adrenal

cortex increased the antibody titre to P. fluorescens ten-fold in perch, however, twice the concentration of extract only increased the titre two-fold.

The 'stress' hormones mediate changes in carbohydrate, mineral and protein metabolism, thus the general nutritional health of the organism will be of importance. Ascorbic acid levels in salmonids were found to be depleted by short term stressors (Wedemeyer, 1970a), such a depletion will impair mammalian immune responses (Hartley, 1948; Long, 1950) and ascorbic acid has also been found to be a requirement of antibody synthesis in S. fontinalis (Field, Elvehjem and Juday, 1943; Halver, 1972).

Carp exposed to cold winters have been found subject to large mortalities in spring caused by A. liquefaciens (Schäperclause, 1954) and spring viraemia (Fijan, 1972). At the low winter temperatures the cellular and humoral responses were found to be inactive, though the temperature was also too low for replication of the micro-organisms, thus acute disease symptoms were not detected (Liebmann, Offhause and Riedmüller, 1960). In the warmer water of spring the normal healthy carp were shown to eliminate the disease organisms and those nutritionally debilitated by the winter developed fatal disease symptoms.

B. Heavy metals and toxicity in teleosts

Fishes are used as important test animals for assaying water pollution, though because of the short term tests used to assess acute pollutant responses the role of disease in teleosts cannot easily be observed. A smaller amount of work has been undertaken to examine the effects of exposure of fishes to low levels of pollutants, but this work has shown that chronic levels can harm fish and impair fecundity or survival of the offspring.

The heavy metals zinc, copper, lead, cadmium, chromium

and nickel are commonly found in British industrial effluents. Zinc and copper, along with phenol, cyanide and ammonia were considered by Brown, Shurben and Shaw, (1970) to be the five most important pollutants produced by industry. Much of the heavy metal pollutants were found to be deposited in the primary sludge of sewage works or became attached to the silt of river beds.

The toxicity of the heavy metals to fishes has been found to be related to bicarbonate alkalinity, pH, temperature, oxygen saturation, humic acid complexes and interactions of the pollutants themselves. The problem of heavy metals and other pollutants in our freshwater environment has been emphasised since 1968 by the annual reviews in the Journal of the Water Pollution Control Federation on the effects of pollutants on freshwater fishes and the reviews of the European Inland Fisheries Advisory Commission (EIFAC) on pH (1968), temperature (1969), ammonia (1970), phenol (1972), zinc (1973), copper (1976) and cadmium (1977).

i. Heavy metal background values

In the refining of metal ores it is common to find together all the major heavy metals zinc, copper, lead, iron, cadmium, chromium and nickel, the principal metal present being dependent on the geological area. These metals are leached from wastes, and even natural rocks, into surface waters (Bowen, 1966). They also reach the environment from refining processes, from the use of the metals in industrial processes, agricultural practice and from the burning of fossil fuels (Solbé, 1974).

Natural levels of heavy metals are very variable, as can be seen in table 2, as too are the concentrations of man-made pollutants. The majority of toxicity studies have thus been carried out in order to define minimum standards for the purity of drinking water, overseen by the World Health Organisation, and define levels of pollutants in which freshwater fish will successfully complete all stages of their life cycle, overseen

by EIFAC in Europe.

Table 2, Natural levels of heavy metal in the environment
Compiled from Allen, Grimshaw, Parkinson and Quarmby (1974)

	Zn	Cu	Ni	Cr	Cd	Pb
SOIL $\mu g\ g^{-1}$	1-300	0.1-100	5-500	10-200	0.03-0.3	2-20
TISSUES $\mu g\ g^{-1}$	100-300	10-100	0.1-5	0.01-0.3	0.05-0.5	0.1-3
FRESH WATER $mg\ dm^{-3}$	5-50	$2\text{-}50 \times 10^{-3}$	5-500	0.1-0.5	$1\text{-}50 \times 10^{-3}$	2-20

ii. Direct lethal action of heavy metals

The results of early workers, using heavy metal salts in solution, implicated the precipitation of gill mucus and the subsequent suffocation as having been the cause of death. This was shown by Lloyd (1960) and Mount (1966) not to be the primary cause in S. gairdneri exposed to acute lethal levels of zinc. In these fish the epithelial cells of the gill secondary lamellae had swollen, separated from the pillar cells and in some specimens had sloughed off. Skidmore (1970) and Skidmore and Tovell (1972) observed similar responses in S. gairdneri exposed to zinc solutions, though they described the lifting of the gill epithelium as being typical of an acute inflammatory response. A granulocyte-containing exudate was found to pass from the blood spaces into the intracellular lymphoid spaces. This response was also observed by Abel and Skidmore (1975) for anionic detergents in S. gairdneri and by Abel (1976) in S. trutta. Morgan and Tovell (1973) found the inflammatory reaction to be consistent with the action of other pollutants on the gills and they suggested that

this may be a protective reaction in order to increase the pollutant to blood diffusion distance in the secondary lamellae. Zinc exposed Gasterosteus aculeatus were found by Matthiessen and Brafield (1973) to exhibit cellular disintegration of the gills and an increase in the number of active chloride cells on the secondary lamellae, possibly reflecting the increased permeability caused by the zinc.

The disruption of the gill structure has been found by a number of other workers. Copper salts were found to damage the gills of Pseudopleuronectes americanus (Baker, 1969), cadmium in Fundulus heteroclitus (Gardner and Yevich, 1970), phenols in S. gairdneri (Christie and Battle, 1963; and Mitrovic, Brown, Shurben and Berryman, 1968), detergents in S. gairdneri (Brown, Mitrovic and Stark, 1968), acids in S. fontinalis (Plonka and Neff, 1969) and in S. gairdneri (Smith and Piper, 1972) and ammonia in S. gairdneri (Smart, 1976).

Tissue asphixia was also found to be caused by copper and zinc in S. gairdneri, there being increased concentrations of lactic acid and low levels of pyruvic acid in the tissues of exposed fish (Skidmore, 1970, Burton, Jones and Cairns, 1972).

Gardner and Yevich (1970) found tissue damage other than the tissues of the gills when F. heteroclitus was exposed to cadmium. The number of eosinophilic cells were increased in the blood and there were pathological changes in the kidney and intestinal tract. Histological changes were also observed in the haemopoietic portion of the spleen of F. heteroclitus, and by the same authors in the spleen of cadmium-exposed S. gairdneri. The exposure of P. flesus to cadmium was found by Larsson, Bengtsson and Svanberg,(1976) to produce inflammation of the pancreas, reduce the size of the liver and cause anaemia, these effects being also related to an observed change in the carbohydrate metabolism, an increase in blood sugar and liver glycogen and reduced blood lactate and muscle glycogen. Scardinius erythrophthalmus exposed to sub-lethal levels of zinc was found to have reduced levels of liver fat and glycogen after 6 months

exposure (Ministry of Technology, 1966).

Cadmium has been found to affect many enzyme processes and effect changes in metabolic and ionic balance. Jakim, Hamlin and Sonis (1970) found that the liver enzymes acid phosphatase, xanthine oxidase and catalase were depressed in F. heteroclitus. Oxygen uptake was blocked in in vitro preparations of the liver mitochondria of L. macrochirus by low levels of cadmium (Hiltibran, 1971) and higher levels were found to block plasma lactate dehydrogenase and glutamic oxalacetic transaminase activity in C. commersoni (Christensen, 1971). Christensen (1975) found an increase of the latter two enzymes in cadmium exposed S. fontinalis and at low levels of cadmium he observed a decrease in body weight and an increase in serum protein and acetylcholinesterase levels.

When Larsson et al. (1976) exposed P. flesus to sub-lethal levels of cadmium they observed changes in the ionic balance of the plasma as well as metabolic changes, Na^+, Cl^- and Mg^{2+} were elevated and K^+ and Ca^{2+} levels were reduced. Changes in the water and electrolyte balance of C. auratus exposed to sub-lethal levels of cadmium were also found by McCarty and Houston (1976). In these fish there was a reduction in plasma Na^+ levels and an increase in the K^+ and Cl^- levels in the muscle tissues. Ionic imbalances produced by heavy metals have been thought to cause neuromuscular disturbances, hyperexcitability, convulsions and tetany in L. macrochirus (Cearley and Coleman, 1974; Eaton, 1974), S. fontinalis (Benoit, 1976), Jordanella floridae (Spehar, 1976) and vertebral damage in Phoxinus phoxinus (Bengtsson, 1974; Bengtsson, Carlin, Larsson and Svanberg, 1975).

It must be remembered that as well as being harmful in excess many of the heavy metals are required as micro-nutrients (Phipps, 1976). Zn^{2+} potentiated enzyme systems are numerous, for example carboxypeptidase and carbonic anhydrase, and Zn^{2+} has also been found to enhance wound healing (Wacker, 1976). Both Cd^{2+} and Cu^{2+} will directly interfere with these activities of Zn^{2+}. Cr^{3+} is involved in mammalian glucose metablism, controlling

the clearance of glucose from the blood. Chromium also controls the levels of cholesterol and lipid biosynthesis and is involved in both amino acid and nucleic acid synthesis. Ni^{2+} may be involved in the complex control mechanisms of enzymic reactions and is also connected with RNA and DNA metabolism. All three ionic states of copper are thought to be biochemically active. They are found linked to catalytic proteins, respiratory proteins and oxidase enzymes. Copper deficiency is known to induce anaemia because of its involvement in the maturation of erythrocytes.

Lead and cadmium are not known to have an essential biochemical role in animals. Lead salts have been found to inhibit aminolaevulinic acid dehydrogenase involved in erythrocyte production and has been found associated with neurological disorders in mammals (Hammond, 1977). Cadmium is a much more toxic metal than lead and is known to interfere with iron metabolism. It also has a high affinity for sulphydryl and hydroxyl groups and ligands containing nitrogen the binding of which affects many general, but essential, enzyme systems (Nilsson, 1970).

iii. Heavy metal toxicity and teleosts

Toxicity data have usually been expressed as the lethal concentration of the pollutant which produced 50% mortality and this LD_{50} ($\equiv LC_{50}$) was found to be dependent on the length of time the fish were exposed (Ball, 1967a). Ball (zinc: 1967b ; cadmium: 1967c) found the relationship to be a curvilinear one, the LD_{50} decreasing exponentially with increasing exposure time. The threshold level for a heavy metal was thus found difficult to estimate unless fish were exposed to the toxicant for several months. Similar results have been found for fish exposed to copper (Brown, Shaw and Shurben, 1974) and to cadmium (Pascoe and Mattey, 1977).

Skidmore (1964) in his review of toxicity experiments was able to demonstrate that several environmental factors would modify the toxicity of zinc to fishes, these being water hardness, pH, temperature and dissolved oxygen levels.

a. Toxicity, pH and water hardness

Heavy metals have been shown to be more toxic to fishes in soft than in hard waters: G. aculeatus (Jones, 1938); S. gairdneri (Lloyd, 1960; Brown, 1968; Solbé, 1974; Bilinski and Jonas, 1973); Ctenopharyngodon idella (Tabata, 1969). The calcium ion has been shown to be the active component in water hardness reducing zinc toxicity (Jones, 1938; Afflech, 1952; Sreenevasan and Raj, 1963; Tabata, 1969), though Tabata (1969) also found that the addition of sodium ions reduced zinc toxicity. The rearing of S. gairdneri in hard water was found by Lloyd and Herbert (1962) and Lloyd (1965) to enhance subsequent survival in soft water zinc solutions and this protection was not lost until 5d in the soft water had elapsed. He suggested that the calcium exerted its effect internally and that the zinc toxicity was related to the tissue calcium content.

In natural waters pH and water hardness are closely related to the solubility of the metal ions. Sprague (1964a) found an increased survival time in S. salar parr in zinc treated soft water when the pH was increased above 7.5. Similarly Mount (1966) found a decrease in pH from 7.5 at several values of water hardness increased the toxicity of zinc to Pimephales promelas, as did Solbé (1974) in S. gairdneri.

The toxicity of a metal ion is dependent on its chemical states at equilibrium and these may be modified by water hardness, pH and temperature (Montgomery and Stiff, 1971). For example hexavalent chromium can exist in three forms in a normal range of water pH, the hydrogen chromate anion ($HCrO_4^-$), chromate (CrO_4^{2-}) and dichromate ($Cr_2O_7^{2-}$). $HCrO_4^-$ and CrO_4^{2-} are pH dependent and $Cr_2O_7^{2-}$ and $HCrO_4^-$ are functions of the total hexavalent chromium concentration.

$$CrO_4^{2-} + H^+ \rightleftharpoons HCrO_4^-$$

$$2HCrO_4^- \rightleftharpoons Cr_2O_7^{2-} + H_2O$$

Trama and Benoit (1966) found that different combinations of Cr^{IV} had different toxicities to L. macrochirus, $HCrO_4^-$ being more lethal than CrO_4^{2-}. The cupric ion was found by Shaw and Brown (1974) to complex with carbonate, amino acids and humic substances (nitrilotriacetic acid). The latter was found not to be significantly involved in S. gairdneri toxicity and they implicated the free cupric ion as being the only active form, modified in its concentration by pH and water hardness.

$$2Cu^{2+} + HCO_3^- + 2OH^- \rightleftharpoons Cu_2(OH)_2CO_3 + H^+$$

The chelating of heavy metals by naturally occurring soluble factors such as humic acids, amino acids and polypeptides has also been shown by Grande (1967) and Sprague (1968a) to influence the concentration of active heavy metal ion. The active Zn^{2+} ion has been shown to be similar to the cupric ion, its concentration being controlled again by pH and carbonate levels (Solbé, 1974). Both cadmium and lead have been shown to be relatively insoluble and also dependent on the pH and carbonate content of the water (Pickering and Henderson, 1966).

b. Toxicity and Temperature

Lloyd (1960) demonstrated that increases in water temperature decreased the survival time of S. gairdneri exposed to zinc solutions. A similar result was found by Eisler (1971) for F. heteroclitus exposed to cadmium solutions. Zitko and Carson (1977) observed that S. salar held at 10°C were more susceptible to zinc solutions than they were at their lower ambient temperature. They also demonstrated a seasonal fluctuation in zinc sensitivity of 1+yr fish, an increase in toxicity being found in the summer months which decreased in the following spring. Although factors such as pH and water hardness were not observed to change in the latter study the water

temperature and the maturation changes were not separated in this examination of seasonal toxicity, thus it was not possible to show which was the controlling factor.

c. Toxicity and Oxygen

S. gairdneri unacclimated to low oxygen tensions in the water prior to testing were found by Lloyd (1960) to be more susceptible to zinc poisoning than if they were first acclimated. Cairns and Scheier (1957) also observed an increase in zinc toxicity with decreasing oxygen tension in L. macrochirus.

Jones (1938, 1947) demonstrated that acute toxic concentrations of zinc caused a marked increase in the ventilation rate of G. aculeatus, though oxygen consumption was seen to decrease. Anaethetised S. gairdneri were found by Skidmore (1970) and Burton, Jones and Cairns (1972) not to change their oxygen consumption, even when exposed to high concentrations of zinc. Smart (1975) using the same trout species found that the oxygen consumption increased with the increasing ventilation frequency when exposed to toxic levels of ammonia. Acutely toxic levels of copper were found by O'Hara (1971) to increase the oxygen consumption of L. macrochirus, but after the sixth hour of exposure the consumption declined until death ensued. This was also found by Brafield and Matthiessen (1976) for G. aculeatus exposed to 1mg Zn dm^{-3} in calcium-free water. The oxygen consumption increased erratically at first and then declined before death.

d. Toxicity, maturity and size

Edwards and Brown (1966) found that the fry of S. gairdneri were more vulnerable to heavy metals than the adults, and the eggs were found to be four times more tolerant. Goodman (1951) also reported an increase in the resistance to zinc between 2 and 10 week old rainbow trout, though the acclimation time to the 1mg Zn dm^{-3} in the water supply of the

hatchery may have been partly responsible for the observed change. The eggs of S. salar were reported by Grande (1967) to be more susceptible to zinc than the fry. Bengtsson (1974) found that adult P. phoxinus were more resistant to long term zinc exposure than the juvenile minnows, though Jones (1938) had earlier found no difference in resistance to zinc between juvenile and adult G. aculeatus. Zitko and Carson (1977) have demonstrated a four-fold sensitivity to zinc solutions in 2+yr juvenile S. salar compared with younger 1+yr fish.

e. Toxicity and mixtures of heavy metals

Heavy metals are rarely found as single pollutants but as mixtures. The presence of two metals, or pollutants, has been found in most cases to produce a summation of the two separate toxicities. A summation of toxicities was found by Lloyd (1961) for S. gairdneri exposed to zinc and copper, by Brown and Dalton (1970) with mixtures of zinc, copper and nickel, and by Sprague and Ramsey (1965) for S. salar exposed to zinc and copper. Lloyd (1961) observed, however, that when the trout were in soft water the concentrations of zinc and copper had a synergistic effect.

The addition of cadmium to zinc and copper mixtures has been found to increase the toxicity above that predicted by the summation of individual toxicity results. Eisler and Gardner (1973) found that non-lethal concentrations of cadmium would increase the mortality of F. heteroclitus in saline zinc and copper mixtures, and Eaton (1973) also found non-lethal levels of cadmium added to zinc and copper mixtures to be more toxic to P.promelas than predicted by summation.

The effects of mixtures of heavy metals with other pollutants have been examined in teleosts. Doudoroff, Leduc and Schneider (1966) found that cyanide molecules were more important than zinc ions when calculating the toxicity of mixtures to P. promelas. Ammonium ions, important in many

polluted waters and in intensive fish culture ponds, when mixed with zinc (Herbert and Vandyke, 1964) or copper (Herbert and Shurben, 1964) were found to have an additive effect on S. gairdneri, though at low levels of toxicant the relationships between ammonia and heavy metal were found to be more variable and complicated by heavy metal-ammonium ion complexes. These metal-ammonium ions were found to be as toxic as the free metal ions, in comparison to metal-cyanide complexes which were found to be less toxic than the free ions by Doudoroff (1956). Phenol, an important industrial pollutant, was found by Herbert and Vandyke (1964) to act independently when added to zinc solutions.

Detergents have been shown to be highly toxic in solution to fishes (Abel, 1974). Calamari and Marchetti (1973) found that anionic detergents when mixed with copper or mercury solutions were 'more than additive' in their toxicity to S. gairdneri, where as, non-ionic were found to be 'less than additive' in their effect. An examination by Brown, Mitrovic and Stark (1968) into the toxicity of soft alkyl benzene sulphonate (ABS) in S. gairdneri acclimated to 0.8 mg Zn dm^{-3} found that solutions of ABS and zinc were more toxic than solutions of ABS alone. The gills were also demonstrated to show greater histological damage in the ABS-zinc solution than would have been caused by ABS or zinc alone, this damage being a greater proliferation of cells at the base of the secondary lamellae. No evidence has yet been put forward showing the existence of metal-detergent complexes which may be more toxic than the separate components.

f. Sub-lethal levels of pollutant

The effects of sub-lethal levels of heavy metals are difficult to detect and can only be assessed by long term observations, even through several generations. Brungs (1969) has shown that heavy metals inhibited the reproduction of P. promelas at levels which do not affect survival, growth and maturation. Brown, Shurben and Miller (1976, cited in EIFAC

1977) have examined the effects of non-lethal levels of cadmium, down to 2µg Cd dm^{-3}, and they found that all the concentrations used retarded the development of ova and reduced the survival of artificially-stripped eggs and the fry of S. gairdneri. Spermatogenesis was not found by these workers to be affected, even though very low levels of cadmium have been found very toxic to mammalian spermatozoa (Phipps, 1976). In the same experimental fish Hughes (1976) and Hughes and Perry (1966) found a reduction in the oxygen diffusing capacity of the gills, observed by micro-morphometric examination of the secondary lamellae, though certain fish at the lowest cadmium concentration were found to have an enhanced diffusing capacity.

The behaviour of fishes in a pollutant will indirectly affect their survival. S. salar has been shown to make avoidance reactions at low levels of zinc, 0.053 mg dm^{-3}, and this response was further enhanced to 0.006 mg Zn dm^{-3} when 0.0004 mg Cu dm^{-3} was added to the water (Sprague, 1964b). S. gairdneri has been shown by Sprague (1968b) to make avoidance movements to 0.005 mg Zn dm^{-3}. Avoidance has been demonstrated in the natural environment of S. salar by Saunders and Sprague (1967) who noted that a large proportion of upstream migrating fish returned downstream when the particular stream under observation had become polluted by mine wastes. C. carpio and C. auratus have been found not to be as sensitive as S. gairdneri and other salmonids in heavy metal avoidance tests (Syazuki, 1964; Ishio, 1966).

Pollutants can indirectly affect the wellbeing of fishes by causing deleterious behavioural changes, for example by blocking the chemo-sensory organs. Bardoch, Fujiya and Moll (1965) found that detergents blocked the chemical senses of Ictalurus natalis which were then unable to find food and their growth rate was subsequently reduced. The erratic behaviour produced by such metals as cadmium has also been shown to interfere with the normal activities of fishes: L. macrochirus (Cearley and Coleman, 1974; Eaton, 1974), P. flesus (Larsson

et al., 1976), G. aculeatus (Brafield and Matthiessen, 1976; Pascoe and Mattey, 1977). Bengtsson (1974) found that zinc solutions caused phases of hyper- and hypoactivity in P. phoxinus, further, normal day activity was displaced to night activity. The stone loach, Noemacheilus barbatulus, normally hides under stones during the day, but in cadmium polluted streams Solbé and Flook (1975) observed that the hiding activity had been lost. These changes of behaviour would probably decrease the chances of survival in these fish by increased exposure to predation.

C. Pollutants, diseases and the immune response

i. Pollutants and diseases in teleosts

At this time only a few research projects have made an explicit examination of the effects of pollutants on outbreaks of disease or on the disease mechanisms. Most of the work relating the diseases of fishes to polluted waters has thus been made on circumstantial evidence.

Snieszko (1974) quotes two examples of marine inshore disease epidemics in the highly polluted Chesapeake Bay, USA, in which the bacterium Pasteurella piscicida was found in dying fish of the first epidemic and C. columnaris in the second. The cause of the epidemics was put down to the pollution and the bacteria were considered as being opportunists which had invaded the fish at a late stage. Both bacterial species, however, were found capable of producing disease symptoms, under laboratory conditions similar to those seen in the two epidemics.

Sonstegard (1974) in his summary of the data for white sucker papilloma, a neoplasm having a viral aetiology, occurrence in C. commersoni concluded that the incidence of the disease was related to the degree of pollution in the Great Lakes of North America. He also found that the tumors had the highest incidence on the lips of the fish (Sonstegard, 1975), although

this neoplasm was normally found on all parts of the body. White suckers are benthic feeders, thus Sonstegard suggests there may have been a tumor inducing factor in the sediments of the polluted lakes. A similar increase in the incidence of gonadal tumors in C. auratus and C. carpio since 1950 was observed by Sonstegard (1975) to be correlated with the increasing waste discharge into the Great Lakes. Again the cause of the disease incidence cannot be directly related to the oncogenic virus, environmental pollution, carcinogens or to an interaction of all the possible factors. Brown, Hazdra, Keith, Greenspan, Kwapinski and Breamer (1973) surveyed the unpolluted and polluted waters of Canada and found an increased incidence of fish neoplasms in polluted areas, however, they also found a 97% increase in the parasite fauna associated with the polluted fish.

Pippy and Hare (1969) reported the effects of a surge of copper and zinc containing pollutant down the Miramichi river, Canada, in the summers of 1967 and 1968. S. salar grilse migrating down the river which had survived the pollution were found to be infected with A. liquefaciens. The implication of heavy metals in disease mechanisms has received only a small amount of direct experimental attention. Cadmium was found by Weis and Weis (1976) to inhibit the initial healing and blastema formation when the fins of F. heteroclitus were surgically amputated. The concentration of cadmium used, 10 $\mu g\ dm^{-3}$, was found to be non-lethal only over a 14d period. Hillier and Perlmutter (1971) observed that the amount of IPN causing virus found in tissue cultures of S. gairdneri gonad was significantly increased when cultured with 10 mg Zn dm^{-3} but not with 7.5 mg Zn dm^{-3}. At zinc levels above 10 mg dm^{-3} Rachlin and Perlmutter (1969) had earlier shown that the mitotic index of S. gairdneri gonad cultures was reduced by 70%, thus indicating a substantial weakening of the cells at the higher zinc concentrations, Rødsaether, Olafsen, Raa, Myhre and Steen (1977) found that V. anguillarum was a commensal in the gut of freshwater-adapted Anguilla anguilla and that the bacterium

could not be transmitted because of its efficient destruction when exposed to fresh water. The introduction of a non-toxic dose of copper, 30 to 60 $\mu g\ dm^{-3}$ into the water supply, however, changed the commensal relationship of the bacterium into one of disease with subsequent mortality of the host.

Over the last decade the accumulation of man made insecticides and herbicides in the environment have been implicated in disease outbreaks. Van Valin, Andrews and Eller (1968) observed that the incidence of granuloma was higher in C. auratus held in ponds treated with a commercial insecticide Mirex, though specimens of L. macrochirus were found to be unaffected. The pesticides 'carbaryl' and '2,4-D' were found by Butler (1969) to produce a higher incidence of a myxosporidian parasite of the central nervous system in exposed fish. Shimanda (1972) observed pathological changes in the intestine, spleen, adrenal and thyroid glands of 'guppies and trout' given food containing DDT. These lesions were then found to become secondarily infected with bacteria. Similar lesions were found in the livers of S. gairdneri by Ashley (1967) and in the tissues of F. heteroclitus by Ferraro, Wolke and Yevich (1977) produced by the carcinogen dimethylnitrosamine.

In 1971 Perkins, Gilchrist and Abbot (1972) observed a high incidence of fin damage, epidermal ulcers and lymphocystis in P. platessa and Limanda limanda caught in the Irish Sea. The authors of the report related this to the high levels of industrial and domestic pollution of the northern section of the Irish Sea and in particular to the high levels of polychlorinated biphenyls (PCB) in that area. The same conclusion was reached by Mahoney, Midlige and Devel (1973) who found a high incidence of fin rot diseases in fish from the New York Bight. Couch (1974) examining the effect of a PCB pesticide, 'Arachlor', on the histopathology of Leiostomus xanthurus livers also observed fin rot in his treated fish.

Wedemeyer (1970a) and Snieszko (1974) have outlined the

role of stress factors in fishes and suggest that metabolic responses in such situations can produce symptoms equivalent to those found in the stress diseases of mammals. It is probable that the major action of pollutants is that of a stressing agent. The mediators of stress, the corticosteroids, are elevated by the natural processes of maturation and spawning, and also in the event of handling and crowding of fishes in pisciculture (Robertson et al., 1963). Bromage and Fuchs (1976) found circulating corticosteroid production to be elevated in C. auratus exposed to sodium lauryl sulphate, though such an effect with other pollutants has not been examined.

Stressors in the environment can increase the invasive passage of bacterial organisms and other antigens into a fish (Amend, 1970). The internal organs of fish in polluted waters have been found more likely to harbour bacteria (Collins, 1970) and presumably other invasive organisms such as viral particles. At some point the threshold level of the fish's defence mechanisms is over reached and the invading or commensal pathogens are then able to produce a disease state.

ii. Pollutants and the immune response

As early as 1918 Toyama and Kolmer had produced evidence of the inhibition of antibody formation to SRBC in rabbits by high concentrations of arsphenamine and mercuric chloride, though low levels of the arsenate were found to stimulate antibody formation. It was not until 1971 that the direct observation of the effect of pollutants on mammalian humoral antibody responses was again made (Wassermann, Wassermann, Kedar and Djavaherian, 1971). In the previous year, however, the Russian fish biologists Goncharov and Mikryakov (1970) demonstrated the effect of low levels of phenol on antibody formation in C. carassius.

Wassermann et al. (1971) treated rabbits with p,p'-DDT, in the drinking water, for 18d before inoculating heat killed

Salmonella typhi or SRBC. The antibody titres to the two antigens and total 7S globulins were decreased in the DDT treated animals, though the decrease in the DDT-SRBC group was not significantly different from the control. An examination of the plasma DDT levels showed the concentration in the S. typhi treated rabbits to be significantly higher than in those inoculated with SRBC, and Wassermann et al. (1971) suggested that there was an antigen effect creating a differential absorption of DDT. Rabbits fed PCBs in their diet were also found to produce significantly lower serum-neutralisation antibody titre to pseudorabies virus (Koller and Thigpen, 1973), though total γ- globulin levels were not found to be affected.

Zarkower (1972) exposed mice to carbon dust, sulphur dioxide and a mixture of the two, and followed the antibody response in the lymph nodes, spleen and serum to a nasal inhalation of killed E. coli. An overall suppression of the antibody response was observed over a period of 192d, though an enhanced response was observed in the first 135d in the lymph nodes of the carbon and sulphur dioxide exposed mice.

A small amount of research work has been documented in the literature relating the effects of heavy metals on the mammalian immune response. Koller (1973) investigated the humoral immune response of rabbits to pseudorabies virus when fed levels of lead acetate, cadmium chloride and mercuric chloride in drinking water. In all the heavy metal treated groups the viral neutralisation antibody titre was significantly reduced. A similar result was found for mice given several oral concentrations of lead acetate and using SRBC as the antigen (Koller and Kovacic, 1974). Antibody forming cells in the spleen were assayed and were found to be reduced in number in those mice receiving the lead. The suppression of cells in the 8d of the primary response to SRBC was dependent on the lead concentration and in the secondary response the suppression was greater, though the differences due to the lead concentrations were not as marked. An examination of the

antibodies to SRBC indicated that it was the LMW 7S antibodies that had been preferentially suppressed, which may imply that the IgG producing memory cells had been lost.

Metal ions in certain situations have been shown to produce an immuno-adjuvant response, for example low levels of arsphenamine (Toyama and Kolmer, 1918), cadmium injected into rats 14d before antigen inoculation, (Jones, Williams and Jones, 1971) and selenium (selenite) inoculated into mice (Spallholz, Martin, Gerlach and Heinzerling, 1975).

iii. Pollutants and the immune response of teleosts

Direct observations of the antibody response in teleosts exposed to pollutants are few, only numbering four citations at this time. Goncharov and Mikryakov (1970) using yearling C. carpio inoculated with Aeromonas punctata vaccine (1.5 to 2.2 x 10^8 cells) concluded that exposure to non-toxic levels of phenol, 12.5 mg dm^{-3}, significantly reduced agglutinin titres in both starved and feeding fish. Radioactive waste in water was examined by Strand, Fujihara, Templeton and Tangen (1972) using embryo, juvenile and yearling S. gairdneri and following the agglutinating titres to natural C. columnaris infections. Both juvenile and yearling fish showed a suppression of the antibody titre compared to the controls, though no significant difference was observed between the two concentrations of tritium used, 1.0 and 10.0 μ Ci cm^{-3}. In both the experiments the agglutinating titres were low and the results indicated that the average titre was dependent on the incidence of zero titres, which were found to increase in the pollutant exposed fish, thus those fish producing antibodies had similar titres in both control and treated groups.

The effects of sub-lethal levels of methylmercury, copper and a mixture of the two on the immune response of the blue gourami (Trichogaster trichopterus) were examined by Roales and Perlmutter (1977) using IPN virus and heat killed Proteus vulgaris as antigens. Antibody levels in all the

metal-exposed fish were found to have been decreased. Zero antibody titres to P. vulgaris were recorded in all the methylmercury and copper-exposed fish. Anti-IPN antibodies in the copper and methylmercury-copper exposed groups were zero at 3 weeks after inoculation commencedbut were detectable at 4 weeks. The methylmercury group which had an anti-IPN titre in week 3 was reduced to zero titre in week 4. Again antibody titres were low and because of the all-or-none titre situation a significant comparative antibody response between the two metals and the mixture could not be shown statistically. Roales and Perlmutter (1977) also cited the work of Sarot (1973) who demonstrated a suppression of antibody formation in zebra fish exposed to zinc solutions.

D. Aims of the research

This investigation was initiated to indicate the relationship between the apparently poor ability of teleosts to raise a humoral antibody response to pathogens and the susceptibility of these fishes to disease organisms observed in situations of heavy metal polluted waters. Two freshwater species were chosen as experimental subjects for this purpose, Salmo trutta and Cyprinus carpio, representing respectively a 'sport' fish inhabiting fast flowing and well aerated waters and a 'coarse' fish inhabiting static waters of lower oxygen content. The choice of species was also influenced by their availability as easily cultured teleosts which could be maintained under laboratory conditions.

A simple bacteriophage, MS2, was chosen as an antigen because of the ease of culture and purification, and with the knowledge that similar viral species had shown good antigenicity in mammals without pathogenicity. As the antigen was a bacteriophage an assay of viral numbers could be devised around the ability of the MS2 to produce lytic plaques in a lawn of Echerichia coli which would indicate the presence of neutralisation activity in test sera.

The first objective was to use the plaque neutralisation assay to examine the humoral immune response of teleosts to primary and secondary single intraperitoneal inoculations of antigen, presented at different concentrations and also with the addition of adjuvants as suggested by Dorson (1972a,b). From these preliminary experiments it was expected to ascertain the ability of teleosts to respond to a viral antigen and whether or not quantitative differences would be found between the different antigen presentations.

The second objective was to examine the relationship of the humoral immune response to temperature and examine the reports in the literature that immuno-incompetence was observed in teleosts below species optimum temperatures. The opportunity also arose at this time to study the humoral immune response of a cold water-adapted marine teleost, Notothenia rossii, and compare the response to that of trout and carp.

The third objective was to examine the possible effect of non-lethal levels of waterborne doses of the heavy metals nickel, zinc, copper and chromium upon the primary and secondary humoral immune responses of the trout and carp, and also examine the effect of the two less soluble metals lead and cadmium by inoculating intraperitoneal doses of the metals into MS2 sensitised trout.

From these results it was expected to re-examine the humoral immunity of teleosts as documented in the literature.

II MATERIALS AND METHODS

A. Maintenance of fish

i. Salmo trutta and Cyprinus carpio

Brown trout (Salmo trutta) were obtained from the Calverton Fish Farm, Severn-Trent Water Authority, Calverton, Nottinghamshire.

Mirror carp (Cyprinus carpio) were obtained from two sources, stocks held by the Severn-Trent Water Authority and from Cotswold Carp Farms, Bourton-on-the-Water, Gloucestershire.

The fish were held in the laboratory aquarium in 100 dm^3 and 500 dm^3 polythene tanks with a 0.25 dm^3 min^{-1} through flow of well aerated tap water. The water was passed through activated charcoal (type 206A, Sutcliffe, Speakman and Co) and the temperature thermostatically controlled before entering the tanks. A photoperiod of 12h day: 12h night was maintained throughout the experiments.

All fish were acclimatised in their experimental holding tanks at the required temperature and water flow for a period of 14d before commencement of an experiment. They were hand fed daily on Beta Trout Growers Diet 417, No 5 (BP Nutrition (UK)), surplus food being removed immediately.

The experimental fish were given no pre-laboratory treatment with fungicides, such as malachite green.

ii. Notothenia rossii marmorata (Fischer) (Norman, 1938

Specimens of immature 'fjord-phase' (3+ to 4+ yr) Notothenia rossii caught on the continental shelf of South Georgia, South Atlantic, were maintained by the British Anarctic Survey, Cambridge. The experimental fish were held at 2.0 $\pm$ 0.3°C in the Survey's circulating sea water system under a continuous low-light regime. Individual fish were separated

by perspex partitions in order to stop cannibalism, often observed in this species when kept together in aquaria. The fish were fed with chopped Antarctic krill and squid every two days.

iii. Anaesthetic and handling

Fish were anaesthetised in a 1g:200 dm^3 solution of MS222 (tricaine methanesulphonate, Sandoz, Basle), made up from 2 dm^3 water taken from the experimental tank, until they could be handled without making violent movements. A fresh anaesthetic solution was prepared for each experimental group and the container, a 10dm^3 polythene bucket, well washed with tap water between groups.

When taking blood samples or giving an inoculation the anaesthetised fish were placed ventral side uppermost in a holding box. This was an open topped box of internal dimensions 28cm x 5cm x 5cm, made of 0.5cm 'Perspex' (ICI,UK) and having several 1.0 cm diameter drainage holes in the base. The box was lined with two sheets of 1.0 cm thick expanded polystyrene forming a V-well and giving ridged support to the length of the fish. Prior to handling the fish the V-well was lined with a wet sterile paper towel or sterile cotton cloth which could be wrapped over the head and body.

After being handled the anaesthetised fish were placed in a 50dm^3 tank of well aerated and clean water, at the experimental temperature, and on recovery returned to the experimental aquaria.

iv. Length and Weight

The length and weight of the experimental trout and carp was dependent on seasonal supply from the fish farms.

Fish were anaesthetised and without delay measured from the snout to the inside fork of the caudal fin $\pm$ 0.25 cm. The

body weight was then taken, to $\pm$ 0.25g, using a torsion balance.

v. Tagging

In experiments where groups of trout were mixed in the aquaria opercular tags were used. Anaesthetised fish were laid on their sides on a wet sterile cotton cloth. A micro-punch was then used to make two 0.075 cm diameter holes, 0.2 cm apart and 0.3 cm from the edge of the bony operculum, through which was threaded a length of 0.03 cm diameter nylon fishing line. The tags were made of 0.1 cm lengths of 0.1 cm diameter coloured plastic insulation sleeving. Combinations of 3 out of 5 colours were threaded onto the nylon line, the ends of which were tied with a double knot and trimmed such that the tags lay firmly against the outer surface of the operculum.

The micro-punch was constructed from a pair of 18 cm steel scissor-forceps. The punch head was made from a 0.5 cm length of 21G hypodermic needle soldered into one tip of the forceps so that, when the forceps were closed, the needle passed through a 0.2 cm hole in the opposite tip.

B. Inoculation and blood sampling

i. Inoculation

All inoculations of antigens, heavy metals and control salines were made intraperitoneally. Entry into the body cavity was made one side of the mid-ventral line, halfway between the pectoral fins and anal vent, using a 23G 3 cm disposable hypodermic needle and 1.0 cm^3 syringe (Becton, Dickinson). The hypodermic was held at an acute angle to the body and a slight upward pressure exerted to avoid piercing the internal organs. The inoculum was injected into the body cavity when, after careful lateral movement, the needle was felt to be free of tissue.

ii. MS2 bacteriophage antigen and adjuvants

The bacteriophage antigen MS2 was inoculated as a suspension in teleost saline (Appendix 3) or as water in oil emulsions with incomplete and complete Freund's adjuvants (Bacto adjuvants, Difco, Michigan, USA).

Emulsions were prepared using the double hubbed needle method of Berlin and McKinney (1958) for small volumes. The double-hubbed needle emulsifier was constructed by inserting a 23G 3.0 cm disposable hypodermic needle halfway into the barrel of a 21G 3.5 cm needle. The needles were set into a pre-drilled block of perspex, using araldite resin (Ciba-Geigy, UK), such that only the open ends of the needle hubs were exposed.

Sterile 1.0 cm^3 disposable syringes were filled with 0.5 cm^3 MS2 suspension or 0.5 cm^3 of sterile adjuvant and securely fitted into the ends of the emulsifier. The aqueous phase, MS2 suspension, was first forced through the smaller 23G needle into the syringe containing adjuvant. The contents were then passed through the needles a further 5 to 6 times until all the fluid was milky white. By this method there was no separation of the emulsion within the first 24h. After use the emulsifier was placed in absolute ethanol and then soaked overnight in 5% Decon 75 (Analytical Supplies, Derby). When free of oil the emulsifier was soaked in three changes of deionised water and then dried and stored in a 45°C drying oven.

iii. Blood sampling

Blood was taken by caudal venepuncture from anaesthetised fish using a sterile disposable 23G 3.0 cm hypodermic needle and 1.0 cm^3 syringe.

For trout and carp entry was made through the dermis at an acute angle on the mid-ventral line 0.2 to 0.5 cm posterior

to the anal fin. The needle was then elevated to 45° and inserted through the tail muscles. The puncture of the caudal vein was then achieved by guiding the needle tip between the ventral processes of the caudal vertebrae into the haemal arch.

Blood samples were taken from N. rossii in a similar manner, the site of entry being 1.0 to 2.0 cm from the caudal end of the long anal fin between two fin rays.

Not more than 0.1 cm^3 blood was withdrawn before removing the needle. A small amount of bleeding was permitted from the wound to avoid the possibility of blood clotting in the caudal vein (GC Shearer, MRCVS, University of Salford, personal communication). When serial samples were taken from the same fish subsequent entry was made at a different point, thus permitting the previous wound to heal fully.

The site of entry was dried using absorbant paper tissues before and after blood sampling.

iv. Serum

Blood samples were immediately transferred into sterile labelled 1.5 cm^3 polypropylene reaction tubes (Eppendorf) and left to clot. Trout and carp blood was clotted at 20°C for 3h and the clot allowed to shrink overnight at 1°C. Tubes of N. rossii blood were transported to Nottingham from BAS, Cambridge in crushed salt-ice and maintained at 1°C overnight to clot. The tubes were then spun at 1500g for 5 min (Piccolo, Heraeus Christ, GmbH) and the sera decanted into sterile 1.5 cm^3 polypropylene tubes using a 0.1 cm^3 automatic pipette (Sigma) and disposable tips. Sera were assayed for antibody activity immediately and any residual sera were stored at -20°C.

C. Culture of bacteriophage and host, neutralisation assay and antibody titre

The culture and assay of the bacteriophage were modified

from the procedures of Adams (1959) and Eisenstark (1967), and the serum antibody assay from the work of Stashak, Baker and Roberson (1970 a,b) using the bacteriophage X174. At all times sterile procedures were observed when handling the phages and host bacteria.

i. MS2 bacteriophage

The bacteriophage MS2 is a Picornovirus (group 1) infecting Echerichia coli K12 HfrH (NCIB No. 10235) having the male factor (F^+). The phage can only infect the host via the sex pili of the bacterium. In late log phase and stationary growth the pili tend to be lost, more phenocopies becoming F^-. The use of these stages, for convenience, in the virus assay method to be discussed was, however, not demonstrated to be disadvantageous.

The characteristics of MS2, in which group M12, f2, and R17 are included, have been reviewed by Fraenkel-Conrat and Wagner (1974) and are listed in table 3. These phages were first isolated from sewage and have been found to occur in the normal human intestine.

ii. Culture of the host, E. coli K12 HfrH

Cultures of E. coli were prepared from freeze dried stocks maintained in the Department of Life Sciences, Trent Polytechnic, and originally obtained from the National Collection of Industrial Bacteria. Standard microbiological and sterile techniques were used in the preparation of E. coli cultures.

Single colonies were streaked out onto 10 cm^3 of full strength blood agar base (BAB, Oxoid), poured 1h previously into sterile disposable Petri plates. The inverted plates were incubated at 37°C overnight, then a vigorous single colony was selected and streaked onto five BAB agar slants. The slants were prepared by autoclaving 10 cm^3 aliquots of semi-molten BAB in Universal Bottles and placing them on their sides at an angle

Table 3, Characteristics of the Phages used

(after Fraenkel-Conrat and Wagner, 1974)

	MS2	QB	X174	P22
Type	Picornovirus Group I RNA phage	Group III RNA phage	DNA phage	DNA phage
Molecular Wt	1 strand 1.0×10^6 D	1 strand 1.2×10^6 D	1 strand 1.6×10^6 D	1 strand 2.6×10^6
Total Molecular Wt.	3.6×10^6 D	4.0×10^6 D	6.2×10^6 D	2.6×10^7 D
Sedimentation Coefficient	80-82 S	80 S	114 S	500 S
Shape	Icosahedral	Isometric	Icosahedral	Isometric
Diameter	23 - 25 nm	25 nm	25 nm	60 nm
Capsid protein	4 molecular types	180 molecules	60 molecules	Complex of major, minor head proteins + scaffolding protein
Site of infection	1 molecule A-protein	1 molecule A-protein	12 capsomeres (x3 proteins)	Short 6-pin tail assembly
	4.2×10^4 D	4.4×10^4 D	1.56×10^5 D each	9.9×10^4
Host used	*E. coli* K12	*E. coli* K12	*E. coli* C	*S. typhimurium*

of 20° from the horizontal to cool. After overnight incubation at 37°C the slants were refrigerated at 1°C until required.

E. coli cultures used in MS2 assays and culture were prepared from these agar slants, to which were added 10 cm^3 nutrient broth (Oxoid). After gentle shaking the contents were divided between three empty and sterile Universal bottles. A further 20 cm^3 of nutrient broth was added to each bottle, which after shaking were incubated at 37°C overnight. Cultures not used immediately for viral assay were refrigerated at 1°C for up to five days.

New cultures were prepared from freeze dried ampoules every two months, and every six months a culture, obtained from a single colony sub-cultured from a known MS2 susceptible culture, was freeze dried for future use (Edwards High Vacuum Freeze Drier, Model EF03).

iii. Culture of the bacteriophage MS2

The bacteriophage was obtained from stocks maintained by the Department of Life Sciences, originating from the University of Leicester. As in the case of the host bacterium all the cultures used were obtained from one original colony.

Two methods were employed in the culture of MS2, the plate method of Eisenstark (1967) and the "liquid media method" of Davern (1964).

a. Plate culture method

The use of the soft agar layer over a layer of BAB (Adams, 1959) was found suitable for the preparation of small amounts of phage for inoculation. The techniques used were similar to those used in the assay of the phage titre and in the neutralisation of antibody.

A base layer of full strength BAB was pre-poured into sterile Petri dishes, 10 cm^3 per dish. A soft agar overlay medium (see Appendix 1) was dispensed in 2 cm^3 aliquots into sterile 5 cm^3 glass tissue culture tubes ('Phage Tubes') and maintained at 50°C. To each of the phage tubes was added 0.1 cm^3 of an overnight culture of E. coli. All volumes of solutions 1.0 cm^3 and less were dispensed using sterile polyprópylene tips and automatic pipettes (Finnpipette, Jencons). Non-confluent viral plaques were obtained by diluting 0.05 cm^3 of MS2 stock suspension by 1 x 10^{-10} in sterile deionised water and transferring 0.1 cm^3 of this solution to the phage tubes. After gentle swirling of the tubes the contents were overlayed onto the solid BAB plates. When the soft agar had solidified the Petri dishes were inverted and incubated at 37°C for 8h. A viral plaque was chosen showing good lysis and a distinct perimeter to the plaque. The plaque was then removed, using a sterile wire loop, and placed in a sterile Universal bottle of 10 cm^3 of sterile deionised water containing 0.1 cm^3 chloroform. After homogenising the agar plaque with a sterile glass rod and centrifugation for 10 min at 3000 g 0.1 cm^3 aliquots of the supernatant were transferred to prepared phage tubes containing 2 cm^3 soft agar and 0.1 cm^3 E. coli suspension. Overlaying and incubation were carried out as before.

The greatest efficiency of plating was achieved when there were just enough plaques to be confluent on the E. coli lawn (Eisenstark, 1967).

The lysed overlays were scraped into a covered 100 cm^3 beaker using a sterile spatula. To the beaker were added 1.0 cm^3 buffer A (Appendix 2) and 0.1 cm^3 chloroform per culture plate. The mixture was homogenised with a sterile glass rod and then decanted into sterile Universal bottles. The homogenates were given two bursts of ultrasonic disruption at 20 k hz and an amplitude of 6 nm (MSE 100 Watt Ultrasonic Disintegrator) for 15s to aid further lysis, the bottles being cooled in an ice bath after each burst. The

agar and bacterial debris was deposited by centrifugation for 20 min at 3000 g. The supernatant was decanted into 25 cm^3 MSE ultracentrifugation tubes and spun at 40,000 g for 120 min (MSE SS50) in an 8 x 25 cm^3 angle rotor and at a temperature of 4^oC. The waxy-yellow viral pellet was resuspended in buffer A, 10 cm^3 per pellet, and re-spun for 120 min at 40,000 g. The viral pelletsfrom this centrifugation were resuspended in 1.0 cm^3 teleost saline, see Appendix 3, sonicated at 6 microns for 15 s to disperse the phage particles, and stored in sterile glass bijou bottles in a 1^oC refrigerator.

The viral titres produced by this method were consistently in the 10^{12} plaque forming unit (PFU) range ($2.65 \times 10^{12} \pm 2.4 \times 10^{11}$, n = 6).

b. Liquid medium culture and two-phase separation method

The MS2 bacteriophage was cultured in the minimal growth medium used by Davern (1964) consisting of an autoclavable phosphate buffer medium A and a filter sterilised medium supplement B (Appendix 4). The separation procedure was adapted from Albertsson (1967) and Watanabe and August (1967).

100 cm^3 of medium A were autoclaved in each of 10 x 250 cm^3 shake flasks and 5 cm^3 medium B introduced using a 1.0 cm^3 repeating syringe (AR Horwell) and attached Millipore filter (pore size 0.45 μm). Each flask was incubated with 1.0 cm^3 of an overnight culture of E. coli and incubated at 37^oC in an orbital shaker-incubator. 2.5h after commencing incubation 1.0 cm^3 samples were aseptically removed from the cultures and the optical density (OD) read at 640 nm. On reaching an OD of 0.26 (10^8 cells cm^{-3}) 1.0 cm^3 of an MS2 suspension containing 10^{10} PFU cm^{-3} (a multiplicity of x5) was added to each of the flasks and incubation continued a further 4h.

The cultures were then transferred to a sterile 2 dm^3 separating funnel, to which 100 cm^3 lysing medium (Appendix 5) and the funnel shaken to mix the contents. To the 1 dm^3 of lysate were added 68.1g polyethylene glycol 6000, 1.93g sodium dextran sulphate and 16.9g sodium chloride. The funnel was again gently shaken, until the polymer phases had dissolved, and then left overnight at 4°C to permit the formation of the polymer emulsion containing the phage. This lower turbid layer was run off into a sterile 30 cm^3 polycarbonate centrifuge tube, with 1.0 cm^3 graduations, and spun 15 min at 1000g (MSE 18 at 4°C) to separate the polymer phases. The middle layer of the sandwich was retrieved by removing the upper and lower layers with a sterile pasteur pipette. The sandwich layer was then well mixed with 10.0 cm^3 of 1.0% (w/v) sodium dextran sulphate in sterile teleost saline and then 0.15 cm^3 3M KCl was added per 1.0 cm of suspension. After allowing the dextran sulphate to precipitate out for 2h at 4°C the mixture was centrifuged for 10 min at 1000g. The supernatant was decanted into sterile Universal bottles and stored at 1°C.

The titre of MS2 cultured in this way was $3.3 \times 10^{11} \pm 1.3 \times 10^{10}$ PFU, n = 4.

iv. MS2 viral plaque assay and neutralisation assay for serum antibody

The procedures and techniques used in the assay of phage stocks and antibody titre were similar. Serum antibodies were assayed using a viral neutralisation technique in which the number of surviving phage were counted for a series of serum dilutions. The classical method of assessing antibody titre, that is the calculation of the inactivation constant k described by Adams (1959), was not chosen for the assay of fish sera. Stashak, Baker and Roberson (1970a) concluded from their work with X174 in humans that the 50%

neutralisation dose of serum (SD50) was a more accurate method of estimating antibody titre against the bacteriophage. A smaller volume of serum is required by the method, this being of major significance when working with small animals. The SD_{50} is now the method favoured by clinical immunologists when using bacteriophage antigen (Peacock, Jones and Gough, 1973).

The semi-micro assay procedure used in this study was modified from the Petri dish method of Adams (1959) to aid the processing of large numbers of serum samples within the restricted space of a bench-top sterile hood (LEEC, Nottingham) and to cut the volume of consumables used.

In order to obtain up to 250 non-confluent viral plaques in each of the six 3 cm^3 wells of the tissue culture plates (Flow Laboratories, UK) used, the size of the plaques was reduced by increasing the agar concentrations of the overlays and the concentration of E. coli added to form the bacterial lawn (Fraser and Crum, 1975). No significant difference in plating efficiency was found between Petri and tissue culture plates (Petri 121 $\pm$ 3 PFU, tissue culture 120 $\pm$ 2 PFU, n = 23, t = 1.13, $P > 0.1$).

The assay methodology can be divided into four sections; serum dilution, antibody antigen incubation, agar overlaying and calculation of neutralisation antibody titre. These are diagrammatically summarised in figure 1.

a. Serum dilution and phage titration

Serial double or quarter dilutions were prepared in duplicate from 0.025 cm^3 aliquots of sera. The sterile teleost saline diluent was pre-pipetted into the U-wells of Cooke Microtitre plates (Flow Laboratories, UK) and the dilutions made using 0.025 cm^3 rosette diluters (Cooke Microtitre, Flow Laboratories, UK).

FIGURE 1

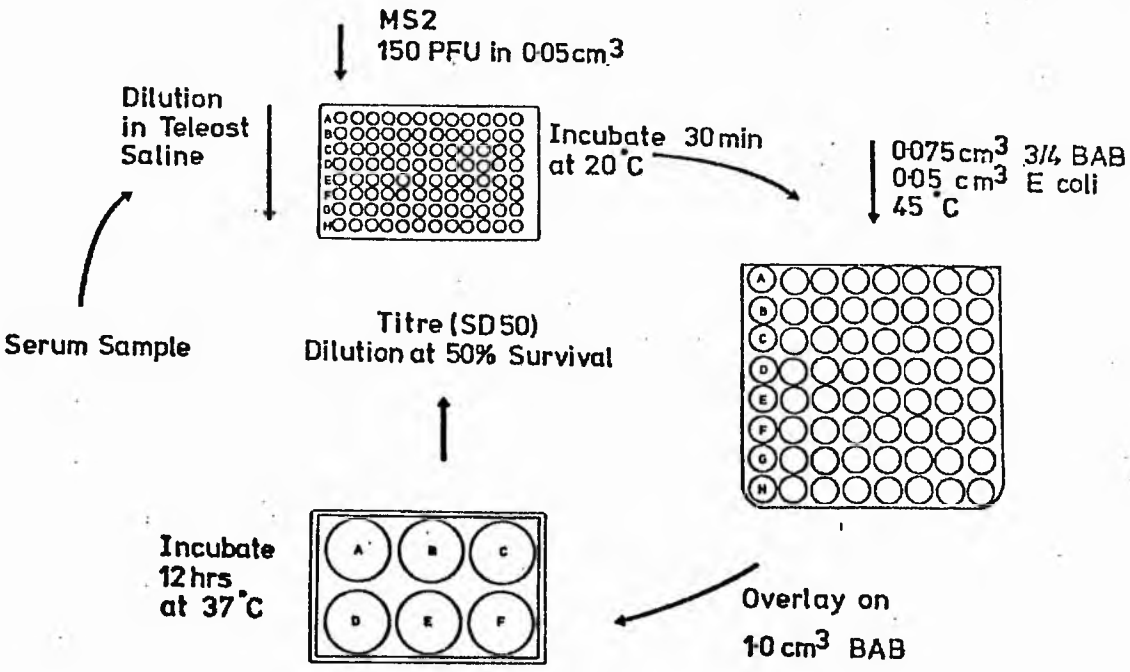
Viral plaque neutralisation assay: Diagrammatic representation

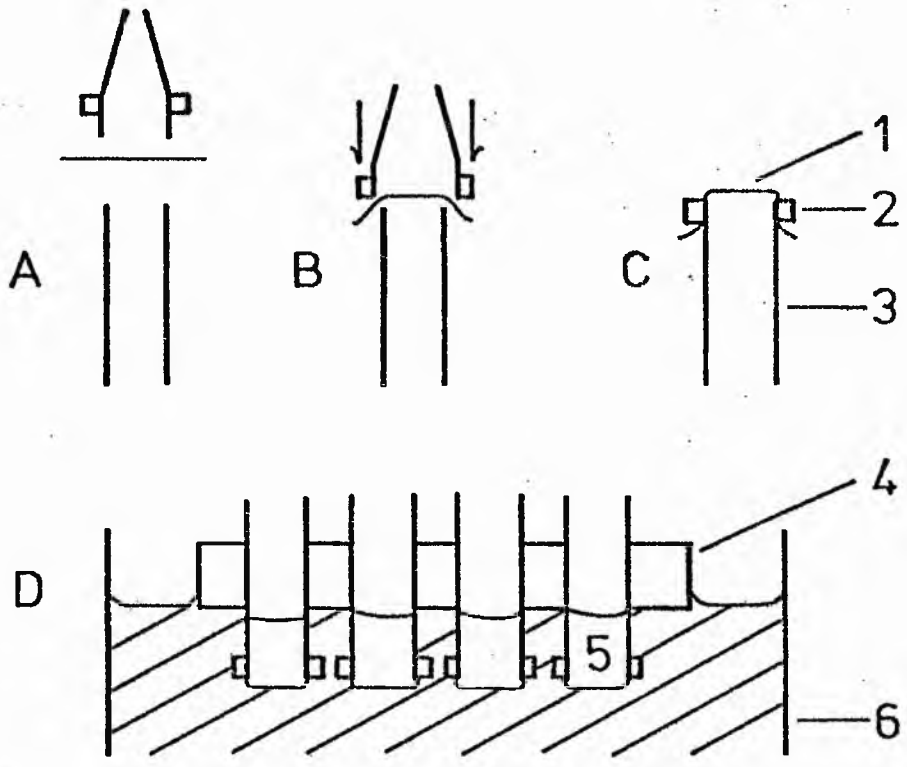
FIGURE 2

Dialysis of small samples

A to B The placement of the Visking membrane (1) over one end of the glass tube (3) using a polypropylene pipette tip to secure the silicone rubber band (2)

D The floatation of tubes containing sera (5) in a beaker of diluent (6) by means of an expanded polystyrene collar (4)


MS2
150 PFU in 0·05cm3
Dilution
in Teleost
Saline
Incubate 30 min
at 20°C
0·075cm3 3/4 BAB
0·05 cm3 E coli
45°C
Serum Sample
Titre (SD 50)
Dilution at 50% Survival
Incubate
12 hrs
at 37°C
Overlay on
1·0 cm3 BAB


A
B
C
1
2
3
D
4
5
6

This same procedure was used for assaying the bacteriophage titre of stock solutions, inocula and serum samples. These dilutions were then transferred direct to the agar overlays.

b. Antibody - antigen incubation

To each of the Microtitre wells were added approximately 150 PFU of bacteriophage, diluted from a phage stock of known titre , in 0.025 cm^3 aliquots using a Cooke Microtitre dropping pipette (Flow Laboratories, UK). Wells containing no serum were prepared as controls, thus giving a maximum phage count or "100% survival".

The effect of incubation time was assessed using a series of $\frac{1}{4}$ dilutions of sera from S. trutta and C. carpio which were of known neutralisation activity. These replicates were then incubated at 1° and $20^{\circ}C$ with aliquots of bacteriophage and overlayed at selected time intervals. The calculated titre values were plotted in figure 3A,B and indicated that the reaction between antibody and antigen was a rapid one. Although little difference was observed between the two incubation temperatures maximum titres were attained more quickly in the trout sera.

The effect of four pre-incubation temperatures, 1°, 20°, 37° and $65^{\circ}C$ for 30 min, on trout and carp serum neutralisation activity was assessed. The titre results in figure 3C,D indicated that the teleost sera were sensitive to temperatures above $20^{\circ}C$, trout being more susceptible than carp.

The incubation times and temperatures selected for subsequent assays were 30 min at $20^{\circ}C$ for S. trutta and 2h at $20^{\circ}C$ for C. carpio. Sera from N. rossii were incubated at $1^{\circ}C$ for 16h.

FIGURE 3

Neutralisation antibody titres: The effects of incubation time and temperature.

Mean antibody titre values (SD_{50}), five replicates from the serum of one fish of known neutralisation activity, are plotted together with $\pm$2SE

A and B The effects of incubation time

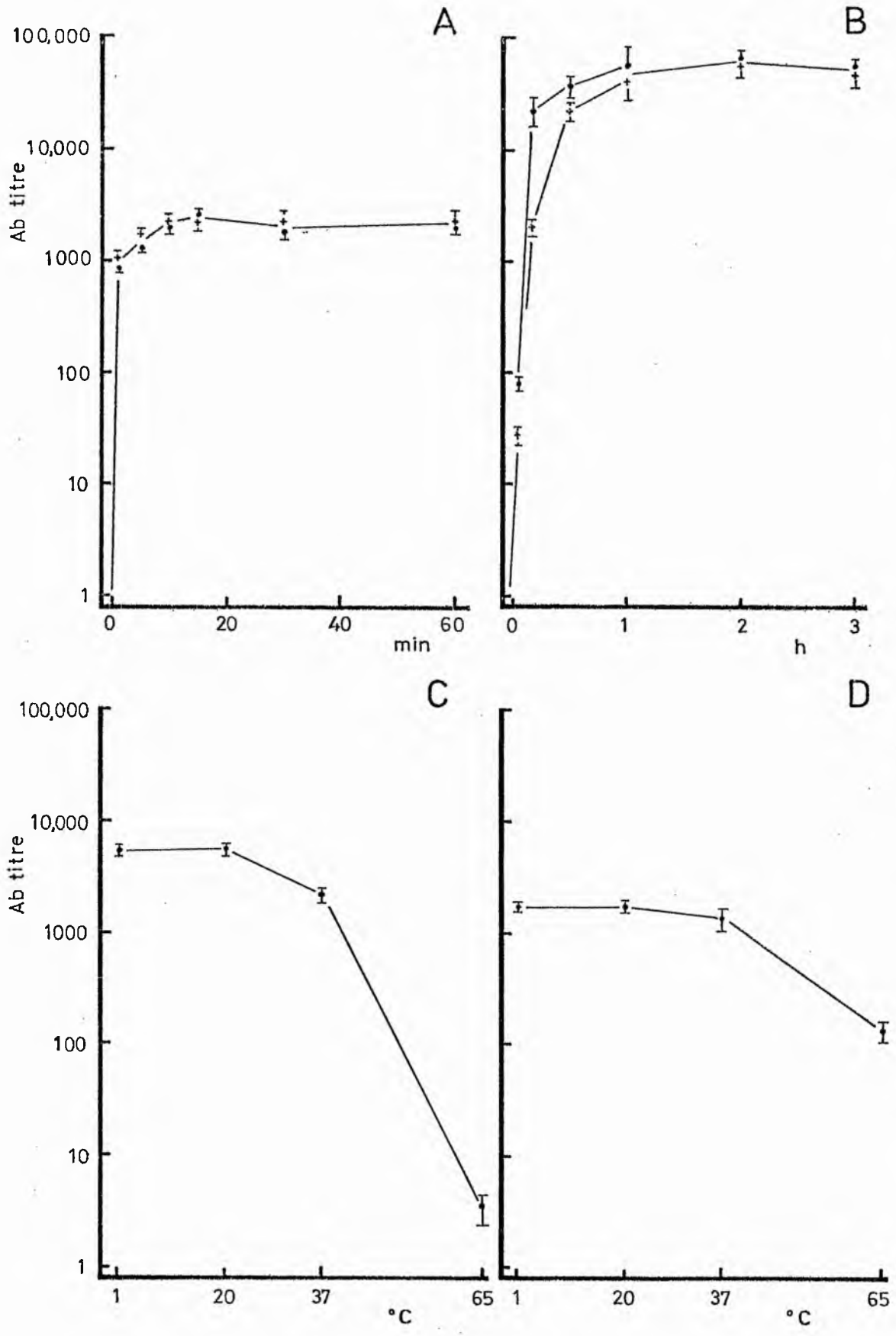
A *S. trutta* serum) • 1°C incubation temperature

B *C. carpio* serum) • 20°C incubation temperature

C and D The effects of four pre-incubation temperatures for 30 min

C *S. trutta* serum

D *C. carpio* serum


A
B
C
D
100,000
10,000
1000
100
10
1
Ab titre
0
20
40
60
min
0
1
2
3
h
1
20
37
65
°C

c. Agar overlay technique

Base layers of full strength BAB were prepared at least 3h before use. 1.0 cm^3 of the agar, freshly autoclaved and cooled to 70°C, was pipetted into the wells of sterile tissue culture plates using an automatic pipetting syringe (1.0 cm^3, AR Horwell). The base layer was layed down with a semi-circular sweep of the syringe around each well, thus, eliminating the need for rotating the plates in order to spread the agar evenly.

The overlay, 0.75 cm^3 aliquots of sterile and molten $\frac{3}{4}$ strength BAB, was pipetted into the walls of WHO macrohaemagglutination plates (Flow Laboratories, UK) using the automatic syringe. The WHO plates were maintained at 50°C over a thermostatted photographic dishwarmer (Model 2, Photax, UK). 0.025 cm^3 of an overnight culture of E. coli K12 was added to each of the molten agar overlay wells just before plating out. The incubated serum dilutions were transferred to the agar wells and immediately overlayed onto the BAB base layers. When the overlays had set they were inverted and placed in a 37°C incubator for a minimum period of 6h.

The serum dilutions and agar overlays were transferred using automatic pipettes with interchangeable(disposable) polypropylene tips.

Pipette tips were place in absolute methanol immediately after use to avoid contamination. They were then boiled in deionised water to remove agar, soaked overnight in 1% Decon 75, rinsed in two changes of deionised water, and then placed to dry in a 45°C oven until reused.

The used WHO plates, microtitre plates and tissue culture plates were sterilised in a solution of Chloros (Analytical Supplies, Derby), cleaned of agar and soaked overnight in 1% Decon 75. The same rinsing and drying procedure

as above was used.

It was found that the washing sequence used gave viral sterility and little bacterial contamination was observed after overnight incubation of the assay plates at 37°C.

d. Bacteriophage and antibody titres

The phage plaques were counted for each dilution in a series and the phage titre or per cent phage survival calculated. The reciprocal of the serum dilution was then plotted on a logarithmic scale against the percentage of PFU surviving and was found to produce a sigmoid curve (figure 4) as predicted by Stashak et al. (1970a).

Two methods were used to calculate the serum dose required to produce 50% inactivation of the bacteriophage (SD_{50}), the first being by construction of the sigmoid curve on semi-logarithmic graph paper, the second using a least mean squares regression programme (Basic, PDP 11). In both cases only serum dilutions producing 20 to 80% PFU survival were taken to construct the regression line, values below or above not being on the straight line portion of the graph. The levels of serum antibody were expressed as a reciprocal of the SD_{50} dilution.

Serum antibody and serum phage titres of more than one individual have been meaned $\pm$ 2 SE (standard errors) from the means of the individual replicates. When plotted against time the titre values have been plotted on a logarithmic ordinate.

e. Antibody titres against X174, QB and P22

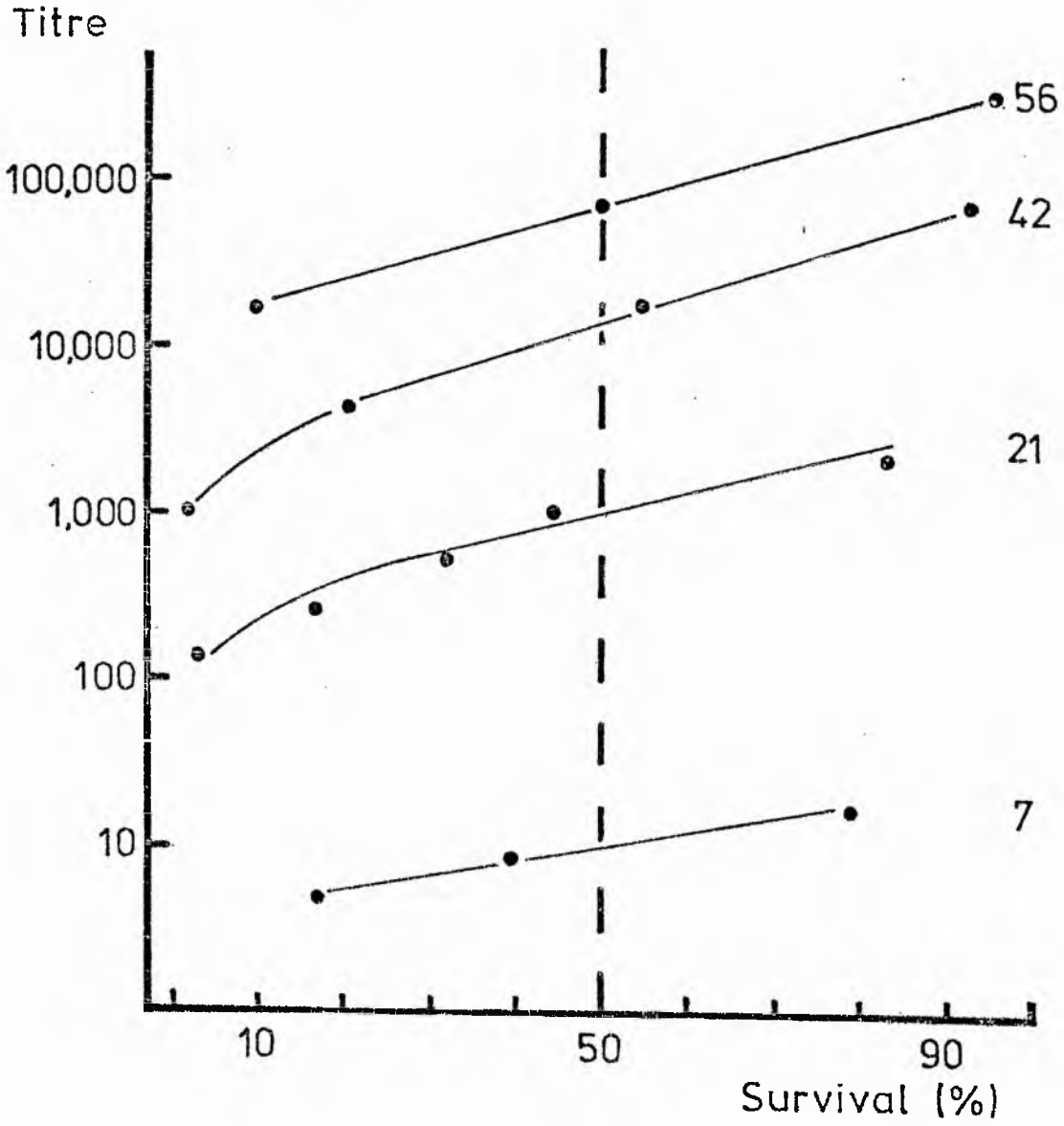
The same techniques were used to culture X174, QB and P22 bacteriphages and to assay anti-viral activity of fish

FIGURE 4

Serum dose and per cent MS2 bacteriophage survival; The calculation of neutralisation titre (SD_{50}) as the serum dose which permits 50% viral survival.

The representative MS2 bacteriophage survival curves are plotted using data from one S. trutta from which sera were obtained 7, 21, 42 and 56 d after inoculation with a primary dose of 10^9 PFU MS2-Freund's Incomplete Adjuvant.

Each point represents the mean of two replicate bacteriophage survival values plotted against the serum titre (1/serum dilution). Between 20 and 80% survival the points fall on a straight line, and the SD_{50} can be read from the 50% survival intersect.


Titre
100,000
10,000
1,000
100
10
56
42
21
7
10
50
90
Survival (%)

sera against these phages. QB and P22 bacteriophages were from cultures maintained by the Department of Life Sciences, Trent Polytechnic, and were grown on E. coli K12 HfrH and Salmonella typhimurium LT_2 Trp C_3 respectively. The bacteriophage X174 was cultured on E. coli C, both being donated by Dr B. Bucknall, Department of Medicine, University of Bristol.

f. Replication of neutralisation antibody titre results and the effect of storage at -20°C

Sera of known neutralisation activity from S. trutta were pooled and sub-divided into replicate aliquots before storage at -20°C. The neutralisation titre of the sera was assayed fresh and at weekly intervals after storage commenced. The results (table 4) indicated that there was no significant difference between replicate assays, that there was no significant difference between the progression of weekly assays and that there was no significant difference between the fresh and stored serum titres over the period examined. The assay procedure used for measuring neutralisation antibody titre was thus demonstrated to be consistent and that for a storage period of 42d at -20°C there had been no effect on titre with time or freezing.

g. Statistical methods

Replicate neutralisation antibody titres, transformed to logarithms, for individual sera were meaned and these means from treatment replicates were then meaned $\pm$ SE using the statistical method described by Bailey (1959) for small samples ($n = < 30$). Meaned titres were then plotted on semi-logarithmic graph paper with $\pm$ 2SE bars.

The examination of neutralisation titres with time and from the same individual fish made the statistical analysis of the data as a total unit invalid (Fisher, 1970). Thus peak titres and secondary response plateau titres were compared using the 't-statistic' only (Bailey, 1959). Peak

Table 4 Replication of antibody titre values and susceptibility to storage at -20°C of pooled S. trutta sera

	Mean SD_{50} value	MS2 $\pm$ SE	n = 5
Fresh Sera	8175	193	
Frozen +7d	8303	130	
+14d	8498	159	
+21d	8280	116	
+28d	8306	179	
+35d	8103	229	
+42d	8071	160	
+49d	8194	206	

ANOVA table (Bailey, 1959)

Source of Variance	Sum of Squares	Degrees of Freedom	Mean Square	F ratio
Between	135245	7	193207	1.38
Within	448538	32	140168	-
	583872	39		

No significant difference between and within means

$P > 0.05$

titres were defined as the first maxima of the neutralistion antibody titre after the primary and secondary inoculations of the bacteriophage. The plateau titres of the secondary response were derived from the mean of secondary response antibody titre values subsequent to the first week after the peak titre of the secondary response. As the number of weeks over which the plateau titre was observed and the number of fish within groups were variable these values may only be used as an indication of differences between groups. However, an examination of the antibody titre plots, with the addition of the $\pm$ 2SE bars, can be used as a valid indicator of significant differences between antibody titre curves (L. Back, Department of Biostatistics, University of Nottingham School of Agriculture, personal communication).

Per cent survival, haematocrits, changes in body weight and length were transformed to arcsines before statistical examination.

D. Characterisation of serum antibodies to MS2

i. Gel Filtration

Antibody rich sera were fractionated on Sephadex G-200 (Pharmacia) using an ascending 50 x 1.5 cm column (Wright Scientific Ltd.) and a buffer head of 10 cm. The Sephadex G-200 was prepared at room temperature, 6.0g being equilibrated in 1.0 cm^3 of buffer (0.1 M Tris-HCl, 0.2 M NaCl, pH 8.0) by stirring overnight in a buchner flask. After degassing the Sephadex under vacuum the column was packed and maintained in a $4^{o}C$ cold room. The column was left overnight to stabilise with buffer running through.

The exclusion volume (void volume) and inclusion volume (total gel volume) were determined by running 1.0 cm^3 of 0.1% blue dextran, 0.1% potassium dichromate in buffer through the column. Samples from the column were

collected using a Shandon-Jeffs automatic fraction collector (type SAD2401) and fraction cutter (type MBJ). Blue dextran was eluted in the exclusion volume and detected spectrophotometrically at 580 nm and the potassium dichromate, indicating the inclusion volume, was detected at 373 nm. The buffer flow rate was 8.6 cm^3 min^{-1} and 15 min fractions were collected.

The elution of sera, up to 0.75 cm^3 samples, was monitored at 280 nm. The fractions were dialysed against sterile water overnight at 4°C and then assayed for antibody activity (Fletcher and White, 1973). A 0.25 cm^3 sample of urease (Sigma, molecular weight 280,000D) was used as a marker protein in a separate run for molecular weight comparison.

On completion of a serum fractionation the buffer flow was reversed for a period of six hours. The descending buffer contained 0.02% sodium azide. The column was washed with azide free buffer in the ascending mode for at least 16 h before re-use.

a. Dialysis of small samples

The ends of 3.0 cm lengths of 0.6 cm diameter pasteur pipette tubing were ground smooth and the tube cleaned by previously described procedures. One end of a tube was covered with 2 cm^2 of pre-soaked dialysis membrane (Visking Dialysis Tubing 18/32, Scientific Instruments Centre) held in place by a 0.2 cm thick band of silicone rubber tubing (internal diameter 0.3 cm, external diameter 0.6 cm). A 1.0 cm^3 disposable polypropylene pipette tip was used as an applicator. Prepared tubes were inserted through 8.0 x 8.0 cm sections of 1.0 cm thick expanded polystyrene (figure 2). The whole was rendered MS2 sterile by placing in a 45°C drying cabinet overnight.

0.2 cm^3 of the samples to be dialysed were pipetted into the tubes which were then floated on 0.5 dm^3 of sterile water, or teleost saline, in a covered 1.0 dm^3 glass beaker.

ii. Mercaptoethanol (2-ME) sensitivity

Pooled antibody rich sera were added in equal volume to 0.1 cm^3 teleost saline containing 0.2 M 2-ME in a sterile 1.5 cm^3 polypropylene reaction tube. After 24h the samples were acidified with 0.01 cm^3 4M HCL and the 2-ME was removed by dialysis against two changes of teleost saline, for 6h and 24h. Control sera were treated in a similar manner without the addition of 2-ME. The treated sera were assayed for antibody activity.

iii. Complement Fixation Test and Haemagglutination

A preliminary examination of complement fixation, as an assay for MS2 antibodies, was made using pooled trout sera of known viral neutralisation titre.

Sheep red blood cells (SRBC, SA01), rabbit haemolytic serum (RHS, VD15) and guinea pig complement (GPC, CT01) were obtained from Wellcome Reagents Ltd. Teleost saline was used as diluent, with the addition of 0.001 M $MgSO_4.7H_2O$.

a. Natural Haemagglutination

Double dilutions of trout serum were prepared in teleost saline using the Cooke microtitre system. To the 0.05 cm^3 serum dilutions were added an equal volume of a 2.5% suspension of SRBC. The microtitre plates were incubated at 20°C for 4h and then examined for agglutination of the red cells under a binocular microscope.

b. Natural Haemolytic Activity and Complement Fixation

The complement fixation test of antibody titre was modified from Bradstreet and Taylor (1962) and the Maltner Antigen Test for syphilis (Wellcome Reagents).

Serial 0.75 cm^3 quarter dilutions of pooled trout serum were made using the Cooke microtitre system. A 100-fold dilution of MS2 stock was added to the serum dilutions in 0,025 cm^3 aliquots. An MS2-free control was made by addition of 0.025 cm^3 sterile teleost saline to a serum dilution series. The microtitre plates were incubated at 20°C for 30 min.

Dilutions, 1/10, 1/20, 1/30, 1/40, 1/50, 1/60, of complement (GPC) were added in 0.05 cm^3 aliquots to replicate series of serum dilutions. In one replicate series and an MS2-free control series per tissue culture plate the complement was replaced by 0.05 cm^3 of sterile teleost saline. To each microtitre well was added 0.05 cm^3 of 2.5% sensitised-SRBC suspension. Sensitised SRBC's were prepared by addition of 1 volume of $\frac{1}{500}$ dilution RHS to 1 volume of 5% SRBC. The plates were then incubated at 37°C for 30 min and examined for complete lysis. The antibody titre was taken as being the highest serum dilution not showing complete lysis.

iv. Antibody-Antigen Precipitation

Several methods of immuno-precipitation were used with MS2-antibody rich sera, but because of the negative results a more complete description of the methods will be restricted to the appendix. The methods were:

a. The double diffusion precipitation technique developed by Ouchterlony (1948, 1970) and the microtechnique of Crowle (1958), modified by Tyrell (1973).
b. The single radial diffusion technique of Grandien & Norrby (1975), the intact MS2 being immobilised in the

agarose gel, and the Mancini technique (Roitt, 1974) in which the MS2-antibody was added to the gel.

c. The countercurrent-immunoelectrophoretic method of Moody (1976).

d. A small scale flocculation test using haematocrit tubes to produce a fluid-fluid boundary layer of MS2 suspension and serum (modified from Smith, 1961).

v. Macrophage migration inhibition test

The method was modified from the description given by Roitt (1974) and Morley, Wolstencroft and Dumonde (1973) for mammalian peritoneal exudate macrophages and Timur (1976) for the blood macrophages of plaice.

Whole blood samples from unsensitised and MS2 secondary sensitised S. trutta, and C. carpio were drawn halfway up into heparinised micro-haematocrit tubes (Gelman, Hawksley) and the dry end flame-sealed. The tubes were spun for 2 min in a micro-haematocrit centrifuge (Heraeus Christ, GmbH) and then rested vertically in a 50 cm^3 beaker of absolute ethanol such that the level was above the buffy coat zone. The tubes were then manipulated with sterile forceps and broken at the buffy coat-plasma interface, using a micro-glass cutter and resting the tubes on a sterile sheet of glass. The ends of the haematocrit tubes containing the buffy coat were placed in sterile 6-well tissue culture plates (unused), a small amount of sterile silicone grease being used to hold the tube in position. To each well 1.0 cm^3 'tissue fluid' (Flow Laboratories, UK, see Appendix 7) was added. To replicate wells were added either 0.05 cm^3 sterile saline, 0.05 cm^3 MS2 stock suspension or 0.05 cm^3 MS2 antibody complex. The latter was a corresponding serum of known antibody titre incubated at 20°C for 30 min with an equal volume of a 10^{-8} dilution of MS2 stock suspension.

The tissue plates were incubated at 20°C and examined at 24h and 48h for migration of the buffy coat.

An inhibition of migration was recorded when the buffy coat did not flow from the cut end of the micro-haematocrit tube.

E. The immune response of Salmo trutta

i. MS2 antigen concentration and the use of adjuvants

Fifty opercular tagged 1+yr, 104 to 176g, S. trutta were maintained in ten polythene tank aquaria at 13.75 ± 0.5°C with a 0.25 dm^3 min^{-1} through flow of water. The trout were inoculated with 0.1 cm^3 combinations of 1.7×10^9, 1.7×10^6 or 1.7×10^3 PFU MS2 in either sterile teleost saline, incomplete or complete Freund's adjuvant. A control group of five fish were each inoculated with 0.1 cm^3 sterile teleost saline. The fish were inoculated such that the ten treatments, five fish per treatment, were randomly dispersed throughout the ten tanks.

A pre-inoculation 0.1 cm^3 blood sample was removed from each fish and thereafter serial blood samples were taken for antibody assay at 7d intervals.

A secondary inoculation of 1.7×10^9 PFU MS2 in a 0.1 cm^3 volume of teleost saline was given to each of the trout, except the controls which were inoculated with sterile saline, after peak antibody titre had been observed. Serial blood sampling was continued for a further 105d at 7d intervals.

ii. Waterborne inoculation of MS2

Three groups of five 1+yr, 112 to 150g, opercular tagged trout were inoculated with 0.1 cm^3 1:1 volumes of teleost saline-Freund's incomplete adjuvant (IA) emulsion, saline-Freund's complete adjuvant (FCA) emulsion or 0.1 cm^3 sterile

teleost saline and then maintained in a 500 dm^3 aquarium. A control group of five trout was completely isolated in a 100 dm^3 aquarium and each fish was inoculated with 0.1 cm^3 sterile teleost saline-complete adjuvant emulsion. Both tanks received a through flow of water, 0.25 dm^3 min^{-1}, at a temperature of 13.75 $\pm$ 0.5°C.

The water supply to the 100 dm^3 tank was stopped and 4.0 cm^3 of stock MS2 suspension added to the water, giving a final MS2 concentration of $5.0 \times 10^4 \pm 1.5 \times 10^3$ PFU cm^{-3}. The water in the tank was left static, except for aeration, for a period of 4h before the water flow was restored. Additional mixing of the MS2 suspension into the water was considered non-essential because of the adequate aeration.

Serum samples were obtained for antibody assay 2h before addition of the MS2 suspension and at 7d intervals thereafter for a period of 42d.

iii. Clearance of MS2 from the serum of S. trutta

Five 1+yr, 127 to 136g, trout, maintained in a 100 dm^3 flowthrough aquarium at 15.5 $\pm$ 0.5°C, were given primary inoculations of $7.7 \times 10^9 \pm 8.6 \times 10^8$ PFU MS2 in 0.1 cm^3 teleost saline. At progressing time intervals over a period of 3d 0.05 cm^3 blood samples were assayed for live MS2 particles and antibody activity.

49d after the complete clearance of the primary inoculum of MS2 a secondary inoculation of $9.2 \times 10^8 \pm 6.2 \times 10^7$ PFU in 0.1 cm^3 teleost saline was given to each fish. Assays for live MS2 and antibody activity in the sera were made over a period of 7d on 0.05 cm^3 blood samples.

iv. A preliminary histological investigation of the tissues of S. trutta after MS2 and carbon inoculations

a. MS2 bacteriophage

Eighteen 0+yr, 45 to 50g, trout were held in a 100 dm^3 through flow aquarium at a temperature of 15.5 $\pm$ 0.5°C. Each fish was given a 0.1 cm^3 primary inoculation of a 10^{-3} dilution of MS2 stock suspension. The fish were then killed quickly in 1:10,000 MS222 in batches of three at 8h, 36h and 128h after inoculation.

A second 0.1 cm^3 inoculation of a 10^{-3} dilution of MS2 stock suspension was given to each of the remaining fish, 49d after the first inoculation. The fish were again killed in batches of three at 0h, 8h and 36h.

Immediately after death sections of the following organs were removed: spleen, liver, small intestine, posterior and anterior kidney and heart, and fixed in 10% formal Cortland fish saline (Appendix 9). The heads of the trout were excised from the body at the junction of the cranium and the vertebral column using a sharp scalpel. The operculum, lower mandible and ventral gill arches were trimmed off before placing in formal saline.

After fixation the tissues were embedded in fibrowax (RA Lamb, London) and sectioned at 5µm. The sections were stained with haematoxylin and eosin.

b. Carbon

In this experiment twelve 0+yr, 45 to 50g trout, held in a 100 dm^3 flow through flow aquarium at 15.5 $\pm$ 0.5°C, were inoculated with 0.1 cm^3 carbon suspension. The carbon suspension was a 1:3 volume dilution of indian ink (Winsor and Newton, London) in teleost saline, such that 0.1 cm^3 contained 4 x 10^{-3}g carbon. The suspension was

autoclaved in a Universal bottle before use.

The fish were killed in 1:10,000 MS222 at 5 min, 4h, 6h and 8h after inoculation in batches of three. The tissues were removed, fixed and processed as described in the last experiment.

The tissues of three untreated trout from the same stock were processed as controls for both the MS2 and the carbon experiments.

F. Temperature and the immune response to MS2 bacteriophage in S. trutta, C. carpio and N. rossii

i. Salmo trutta

Primary and secondary antibody responses were examined in groups of five 1+yr, 104 to 176g, brown trout at 4.5 $\pm$ 0.5°C, 9.0 $\pm$ 0.5°C and 15.5 $\pm$ 0.5°C. The data from the incomplete adjuvant - 10^9 PFU MS2 group of the first trout experiment, at a temperature of 13.75 $\pm$ 0.5°C, was also compared with the results of this experiment.

The three groups were maintained in 100 dm^3 polythene tank aquaria. The 15.5°C group received water from the thermostatically controlled aquarium system. The water fed to the 9.0°C group was at mains water supply temperature and the final group was maintained in an insulated cold room with an Eheim 386 power-filter recirculation system containing activated carbon.

ii. Cyprinus carpio

Groups of 3+yr, 88 to 186g, mirror carp were maintained at 9.0 $\pm$ 0.5°C (6 fish), 15.5 $\pm$ 0.5°C (6 fish) and 22.0 $\pm$ 0.5°C (5 fish) in 100 dm^3 through flow aquaria. The 9.0°C and 15.5°C had the same parent water supply as the trout

experiments above. The 22.0°C group of carp was supplied by water from the 15.5°C thermostatically controlled aquarium supply which had been re-heated by two rheostat controlled 1 kw Vitreosil rod heaters (Analytical Supplies, Derby) before entering the tank.

All the experimental trout and carp were given 0.1 cm^3 inocula of MS2 suspension in FIA emulsion, having a titre of $1.95 \times 10^9 \pm 7.2 \times 10^7$ PFU 0.1 cm^{-3}. Sera were assayed for antibody activity at 7d intervals. After the peak antibody titre had been reached a secondary 0.1 cm^3 inoculum of the same MS2 suspension, diluted in teleost saline only, was administered to each fish and the sera assayed at 7d intervals for secondary antibody response.

iii. Notothenia rossii : The immune response to MS2 bacteriophage and the use of adjuvants at 2.0 ± 0.3°C

Three groups of 3+ to 4+yr, 58 to 64g, N. rossii were inoculated with $1.95 \times 10^9 \pm 7.2 \times 10^7$ PFU MS2 in 0.1 cm^3 teleost saline (6 fish), in FIA emulsion (6 fish), or in FCA emulsion (5 fish). A control group of five fish were each given an inoculum of 0.1 cm^3 sterile saline.

At 14d intervals 0.1 cm^3 blood samples were taken and assayed for antibody activity. After the peak antibody activity of the primary response had been reached a secondary inoculum of 1.95×10^9 PFU MS2 in 0.1 cm^3 of teleost saline was given to each fish, the control fish being re-inoculated with 0.1 cm^3 sterile saline. The secondary antibody response was followed over a period of 112d at 14d intervals.

G. Heavy metals and the immune response to MS2 bacteriophage in Salmo trutta and Cyprinus carpio

i. Heavy metal dosing experiments

The 100 dm^3 aquarium tanks were arranged in two tiers of five pairs. Each pair received a 0.25 dm^3 min^{-1} through flow of thermostically controlled water, 15.5 $\pm$ 0.5°C, of the required heavy metal dilution from an automatic dosing apparatus. Water from the upper tanks of the paired tiers flowed into the lower by gravity, and then to drain, via open T-siphons.

The experimental brown trout were held in the upper tier of aquaria and mirror carp in the lower tier.

a. Automatic dosing apparatus

The automatic dosing apparatus (figure 5) was designed in collaboration with the Water Research Centre, Stevenage, from models used by Abram (1960) and Stark (1967) using many materials normally available in the laboratory.

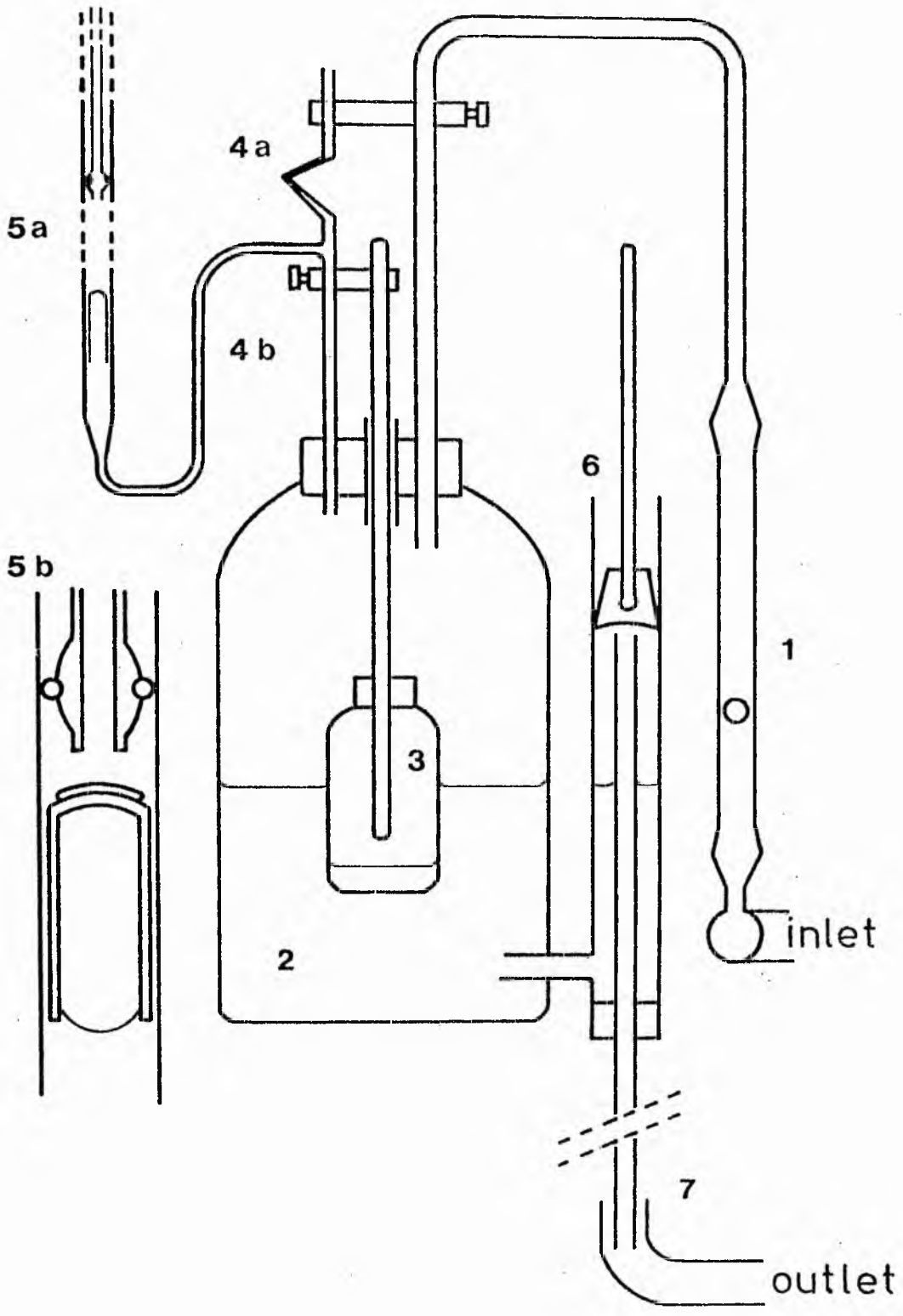
The apparatus consists of a wide necked brown glass 2 dm^3 dilution bottle (2) (BDH Chemicals, 3kg Na Cl), a siphon assembly (6), a float and valve assembly (3,4), and a measuring burette (5).

Thermostatically controlled water from the aquarium header tank entered the dilution bottle continuously, the flow being measured and controlled by a 0 to 1.4 dm^3 min^{-1} flow-meter and valve assembly (1) (B14, Meter Rate). The siphon assembly was sealed (Dow Corning, silicone aquarium sealer) into a pre-drilled hole in the side of the dilution bottle and held rigidly in the vertical position by wood templates. The vertical 90 cm, internal diameter 0.5 cm, glass downfall tube and the rubber bung (6,7), used to control the head of siphoning water, were adjusted to give an outflow of 1.5 dm^3, at an inlet flow of 0.25 dm^3 min^{-1}, each time

FIGURE 5

Dosing apparatus for heavy metal solutions.

1. Water supply inlet regulated by a flow-meter and valve.
2. 2 dm^3 dilution bottle.
3. Polythene float assembly which operates valves 4a, 4b.
4a. "Kinking T-valve" controlling the entry of heavy metal stock solution into the burette 5a.
4b. "Kinking T-valve" controlling the entry of a measured dose of heavy metal from burette 5a into the dilution bottle 2.
5a. Measuring burette. The dose of heavy metal is set by adjusting the central glass capillary rod, and entry of further solution is stopped by the PTFE float blocking the capillary (5b).
6. An overflow siphon regulates the volume of the "make and break" flow of dosed water into the aquaria (7).


4a
5a
4b
6
5b
1
3
inlet
2
7
outlet

the siphon emptied the dilution bottle.

Stock solutions of heavy metals in 100 dm^3 capacity polythene bins were positioned above the upper level of the dosing burettes. The solution was fed to the apparatus under gravity, using a siphon, via the "kinking T-valve" (Abram, 1960). The valves were made from lengths of 'Microwall' 0.3 cm diameter elastomer tubing (HD Symons and Co., London) attached to a glass T-piece, the latter being operated by the rise and fall of the 0.1 dm^3 polythene bottle float (3) in the dilution bottle filling and emptying cycle.

When T-valve 4a was open the heavy metal concentrate entered the burette, a 1.0 m length of precision drawn glass tube, internal diameter 1.0 $\pm$ 0.0002 cm (JA Jobling, UK), raising the hollow machined 5.0 cm PTFE (Xylon, UK) float. On reaching the end of the adjustable 2.0 m capillary tube, sealed inside the burette with a silicone rubber 'O'-ring, the capillary was blocked by a thin layer of silicone sealer on the cap of the PTFE float (5b), thus stopping the entry of further solution into the burette. The opening of valve 4b during the filling cycle of the dilution bottle enabled the fixed volume of heavy metal concentrate to drain into the dilution chamber.

All sections of the apparatus in contact with stock solutions, uncontaminated or dosed water were soaked overnight in 1M HCl and then three changes of deionised water before being used. Where ever possible all tubing and connectors were made of polythene.

b. Heavy metal stock solutions

The heavy metals (Analar reagent grade, BDH Chemicals) were pre-weighed into sterile disposable plastic Universal bottles (Flow Laboratories). These were dissolved prior to use in 100 dm^3 water, from the aquarium supply, in a

polythene bin and transferred to the stock bins using an electric water pump and polythene hose.

c. Heavy metal concentrations in the tanks

The readings observed for the heavy metal analysis between the upper and lower tanks of each of the two tier aquaria were the same at all times.

A test analysis of the zinc concentration flowing from one automatic doser into the aquaria was made. Eight samples taken over consecutive hours gave a mean of 1.056 mg Zn dm^{-3} with SD $\pm$ 0.034 and SE $\pm$ 0.012. This was similar to the results of Stark (1967). He found with a mean of 1.602 mg Zn dm^3 an SD $\pm$ 0.022, and over a period of 168d there was an error of $\pm$ 5% in the volume of water delivered from the siphon.

Over a period of 140d in two experiments to be described with zinc concentrations of a similar level, 1.06 and 1.04 mg Zn dm^{-3}, there were respectively 8.9 and 9.8% fluctuations of Zn concentration about the mean. This can be put down to the changes in the total volume of water delivered in the siphoning cycle, caused by fluctuations of the inflowing water through the valve and flowmeter assembly.

d. Nickel, Zinc, Copper and Chromium and the immune response to MS2 bacteriophage in S. trutta and C. carpio

Four of the two tier aquaria received dilutions of one of the four heavy metals. These were 0.75 mg Ni, 1.06 mg Zn, 0.29 mg Cu and 1.01 mg Cr, all in 1.0 dm^3 and having a SE $\pm$ 0.01 mg dm^{-3}. The levels of heavy metal were estimated as being non-toxic over a 38 week exposure period required to examine the primary and secondary immune responses of trout and carp, taking into consideration the pH (7.83 $\pm$ 0.02), total hardness of the aquarium water (206.9 $\pm$ 1.6 mg dm^{-3} as

$CaCO_3$), temperature (15.5 ± 0.5°C) and also the levels of heavy metal which could be accurately assayed by the available laboratory techniques. The concentration of the more toxic copper was derived from 50% of the 14d LC_{50} of Shaw and Brown (1974) using S. gairdneri at pH 7.25, total hardness 250 mg dm^{-3} as $CaCO_3$ and a temperature of 10°C. The level of zinc chosen for this experiment was 20% of the 4d LC_{50} observed for S. gairdneri by Solbé (1974) which was below the long term toxicity level quoted by this author. Nickel and chromium were also adjusted to values near 1 mg dm^{-3}.

Six 1+yr, 64 to 182g, brown trout were placed in each of the upper tier tanks and five 3+yr, 57 to 190g, mirror carp in each of the lower tier tanks. The fifth tier of tanks contained the control trout and carp, six in each tank, receiving undosed water from an automatic dosing apparatus.

All the fish were given a primary 0.1 cm^3 inoculation of MS2 suspension in FIA emulsion, having a titre of $1.95 \times 10^9 \pm 7.2 \times 10^7$ PFU. Blood samples of 0.1 cm^3 were taken immediately before inoculation and at 7d intervals. After reaching maximum primary antibody titre the fish were inoculated with a secondary dose of the same MS2 suspension diluted in saline only, and the secondary antibody response was followed at 7d intervals.

On the termination of the experiment a 1.5 cm^3 blood sample was taken from each fish. A small portion of this blood was used immediately for replicate micro-haematocrit determinations. Blood was drawn up into heparinised micro-haematocrit tubes and one end flame sealed. The tubes were then spun in a micro-haematocrit centrifuge for 2 min and the % haematocrit read ± 0.5% on an haematocrit reader (Gelman, Hawksley).

The terminal sera were assayed for heavy metal and total protein concentrations. The following tissues were examined

histologically for effects which may have been caused by the heavy metals: gill, skin, spleen, liver, intestine, anterior and posterior kidney. Small sections of tissue were removed from the freshly killed fish and fixed in a large volume of 10% formal Cortland saline. These were then embedded in fibrowax, sections cut at 5μm and stained with haematoxylin and eosin.

e. Zinc concentrations and the immune response to MS2 bacteriophage in S. trutta and C. carpio

In this experiment four of the two tier aquaria were dosed with four concentrations of zinc (mg Zn dm^{-3} $\pm$ SE): 0.14 $\pm$ 0.01, 0.53 $\pm$ 0.005, 1.04 $\pm$ 0.02 and 2.13 $\pm$ 0.02 with a through-flow of 0.25 dm^3 min^{-1}. The fifth tank received undosed water as a control. The water temperature was 15.5 $\pm$ 0.5°C and had a pH of 7.81 $\pm$ 0.02 and a total hardness, as $CaCO_3$, of 198.2 $\pm$ 3.9 mg dm^{-3} which remained constant throughout the experiment.

Six 1+yr, 60 to 129g, brown trout were placed in each of the upper tier tanks and six 3+yr, 73 to 154g, mirror carp in each of the lower tanks. The primary and secondary responses to the MS2 bacteriophage were followed as for the previous experiment, the inoculation titre being 1.58 x 10^9 $\pm$ 2.0 x 10^8 PFU MS2.

Terminal haematocrit, the Zn^{2+} and protein concentration of sera were also measured. Tissues from the freshly killed fish, listed in the last experiment, were examined histologically.

ii. Inoculated lead and cadmium concentrations and the immune response to MS2 bacteriophage in Salmo trutta

For each of the two experiments twenty-five 1+yr brown trout were opercular tagged and held at 15.5°C $\pm$ 0.5°C in two

500 dm^3 polythene aquaria with a through flow of 0.25 dm^3 min^{-1}. All the fish were given a primary inoculation of 10^9 PFU MS2 in 0.1 cm^3 FIA emulsion. 49d later a 0.1 cm^3 inoculation of 10^9 PFU in saline was given and the fish were then left a further 56d. 21d before commencing the experiment a further inoculation of 0.1 cm^3 of 10^9 PFU MS2 in saline was given.

The effect of lead and cadmium on an already raised antibody response was thus examined.

a. Lead

Four groups of five, 98 to 199g, fish were given 0.1 cm^3 intraperitoneal inoculations of 0.01, 0.05, 0.1 or 0.3 mg Pb. Five control fish were given 0.1 cm^3 inoculations of teleost saline adjusted to pH 3 with 4M HNO_3, this being the pH of the 0.3 mg Pb solution. The stock solution of lead used to make the inocula was prepared by dissolving 3.9975 g $Pb(NO_3)_2$ (Analar reagent grade, BDH) in 20 cm^3 1% (v/v) HNO_3 and diluting to 0.5 dm^3 (≡ 5000 mg Pb dm^{-3}).

To follow the progress of the antibody titre 0.1 cm^3 blood samples were taken just before lead inoculation and at 7d intervals thereafter.

b. Cadmium

Four groups of five, 114 to 216g, fish were inoculated with 0.1 cm^3 of cadmium solution containing 0.05, 0.1 or 0.2 mg Cd. Five control fish received 0.1 cm^3 inocula of sterile teleost saline. The stock cadmium solution was prepared by dissolving 1.141 g $3CdSO_4.8H_2O$ (Analar reagent grade, BDH) in 100 cm^3 deionised water (≡ 5000 mg Cd dm^3).

In this experiment 0.1 cm^3 blood samples were taken at -7d and just before inoculation with cadmium and then continued at 7d intervals. At 84d after cadmium inoculation

a fourth inoculum of 10^9 PFU MS2 in 1.0 cm^3 saline was given to the 0.05, 0.1 and 0.2 mg Cd groups. The control group was not reinoculated.

When the lead and cadmium experiments were terminated final haematocrits and serum protein concentrations were determined. Tissues from the freshly killed fish were also examined histologically, as in the previous heavy metal experiments.

iii. Survival of MS2 bacteriophage in heavy metal solutions

The effect of heavy metals on the survival of MS2 bacteriophage was assessed by the incubation of a known number of bacteriophage particles with $\frac{1}{4}$ dilution series of the heavy metals in 0.05 cm^3 of sterile teleost saline in microtitre plates at a temperature of 20°C for 30 min. The per cent survival of the MS2 bacteriophage was calculated by comparing the plaque formation of heavy metal exposed bacteriophage with control aliquots of MS2 incubated with metal-free teleost saline. Replicate per cent survival values, meaned using arcsine transformation, were plotted against the logarithm of heavy metal concentration, and an estimate of the concentrations which produced 50% survival of MS2 bacteriophage was made.

iv. Heavy metal analysis

Concentrations of nickel, zinc, copper and chromium in heavy metal dosed aquarium water and serum samples were assayed using the methods described by Allen, Grimshaw, Parkinson and Quarmby (1974). The methods used are annotated in the appendix 8.

Zinc and copper concentrations were measured by atomic absorption spectrophotometry (AAS) using EEL 240 or Perkin-Elmer 103 spectrophotometers at the instrument settings

recommended by the manufacturers. Nickel and chromium were assayed by colorimetry using a Pye-Unicam SP6oo UV spectrophotometer.

All standards of $NiSO_4.7H_2O$, $ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $K_2Cr_2O_7$ were prepared from 'AAS grade reagents' (BDH Chemicals or Analytical Supplies, Derby), and reagents for the colorimetric assays were of 'Analar' grade.

Water samples were collected in 1 dm^3 polythene bottles, and those reagents which could be stored were refrigerated in 0.5 dm^3 and 0.1 dm^3 polythene bottles. Glass 100 cm^3 and 10 cm^3 volumetric flasks were used for making dilution series from the standard solutions. Reagents were dispensed using disposable polypropylene pipette tips and automatic pipette. For the colorimetric assays capped 5 cm^3 disposable polythene test tubes were used.

All apparatus in contact with solutions was soaked overnight in 1M HCl and given three rinses in deionised water before being used. Deionised water was used at all times for dilutions except in the case of serum samples which were diluted with teleost saline.

Samples of untreated aquarium water and fish food pellets were assayed for heavy metals by Severn Trent Water Authority, Regional Laboratories, Meadow Lane, Nottingham.

v. pH and water hardness

The pH of water samples was measured using a Pye-Unicam 292 pH meter and the total and calcium hardness of the samples using Schwarzenbach water hardness reagents 'CVS' (BDH Chemicals Ltd).

vi. Protein estimations

The protein concentrations of sera and MS2 suspensions were determined using the 260 nm/280nm extinction method of Warburg and Christian described by Dawson, Elliott, Elliott and Jones (1969) and calculated as mg cm^{-3}.

III RESULTS

A. The immune response of teleosts to MS2 bacteriophage

i. MS2 antigen concentration and the use of adjuvants in S. trutta

The experiment was designed to demonstrate the possible relationships of antigen concentrations and the use of adjuvants with the humoral immune response of S. trutta to MS2 bacteriophage. The neutralising antibody titres raised against single inoculations of three antigen concentrations with the addition of incomplete and complete Freund's adjuvants were followed through primary and secondary response and are documented in figure 6 and appendix 10.

All the experimental specimens of S. trutta examined gave positive humoral neutralising antibody titres to both primary and secondary intraperitoneal inoculations of MS2. Further, no immuno-competent individual was observed in these experiments and the antibody titres were in the same order of magnitude as those observed by Peacock, Jones and Gough (1973) in humans sensitised with the bacteriophage X174.

Primary antibody production was initiated within the first 7d after the inoculation of the bacteriophage and peak antibody titres were reached at between 14 to 49d, depending on the treatment given. The rise in antibody titre, or in rate of antibody production, was observed to be similar in all the treatments, the titre then reaching a peak which was dependent on the bacteriophage concentration and presence of adjuvants. The peak antibody titre response was shown to be significantly related to MS2 concentration in the inoculum (table 5), this being especially prominent in the MS2-saline inoculated fish (figure 6A).

Incomplete Freund's adjuvant (FIA) was seen to have a marked effect on the highest concentration of inoculated bacteriophage, the peak titre being significantly greater than the equivalent MS2-saline inoculated fish (figure 6C).

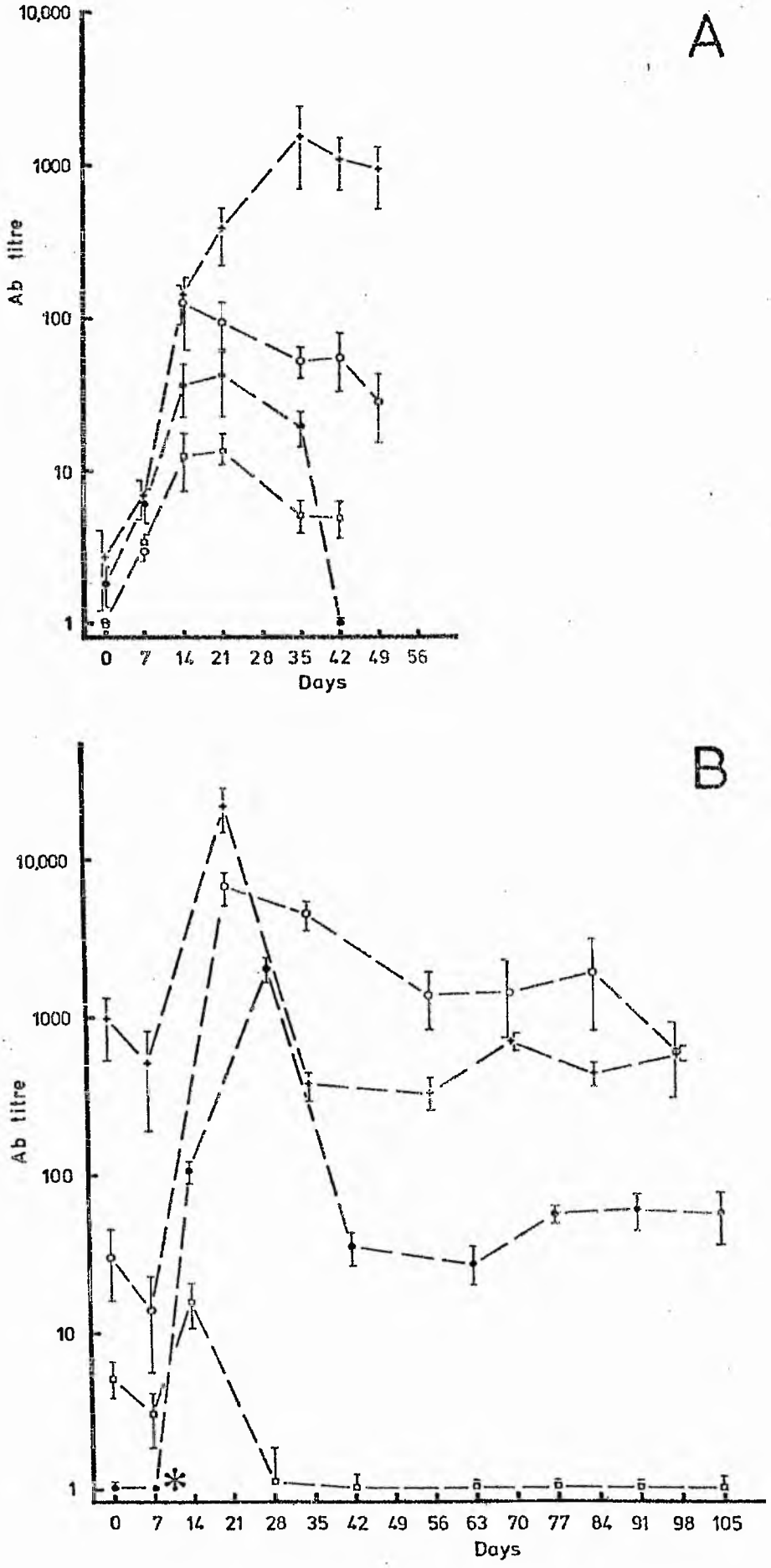
FIGURE 6

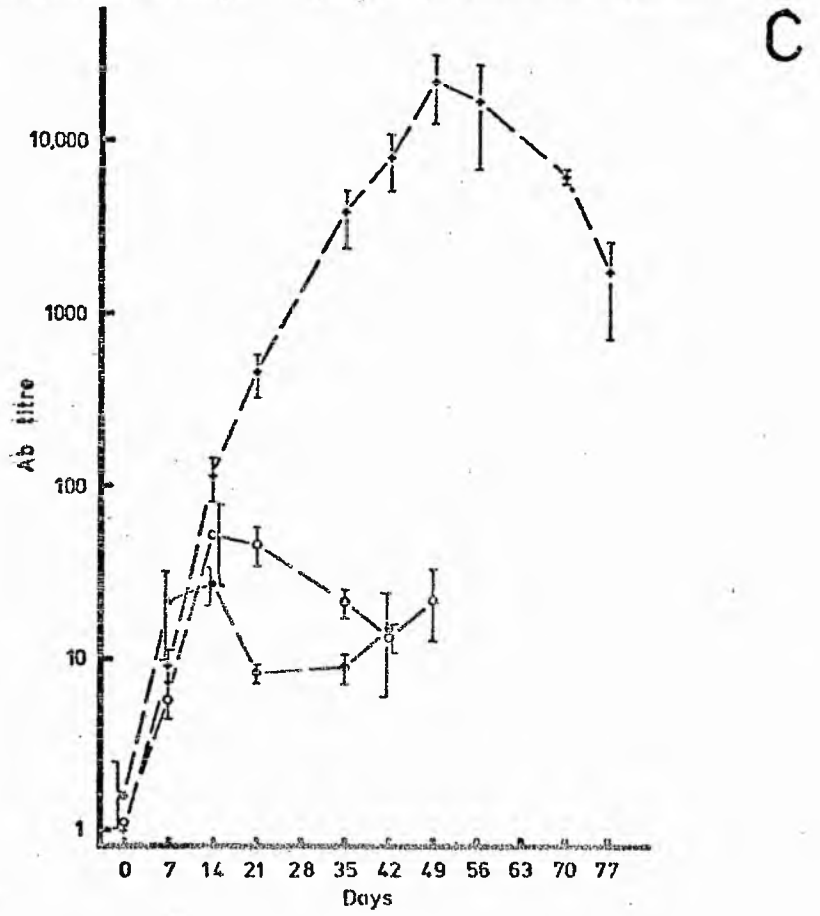
Immune response of *S. trutta* to an intraperitoneal inoculation of *MS2* bacteriophage: The effects of antigen concentration and adjuvants at 13.75± 0.5°C.

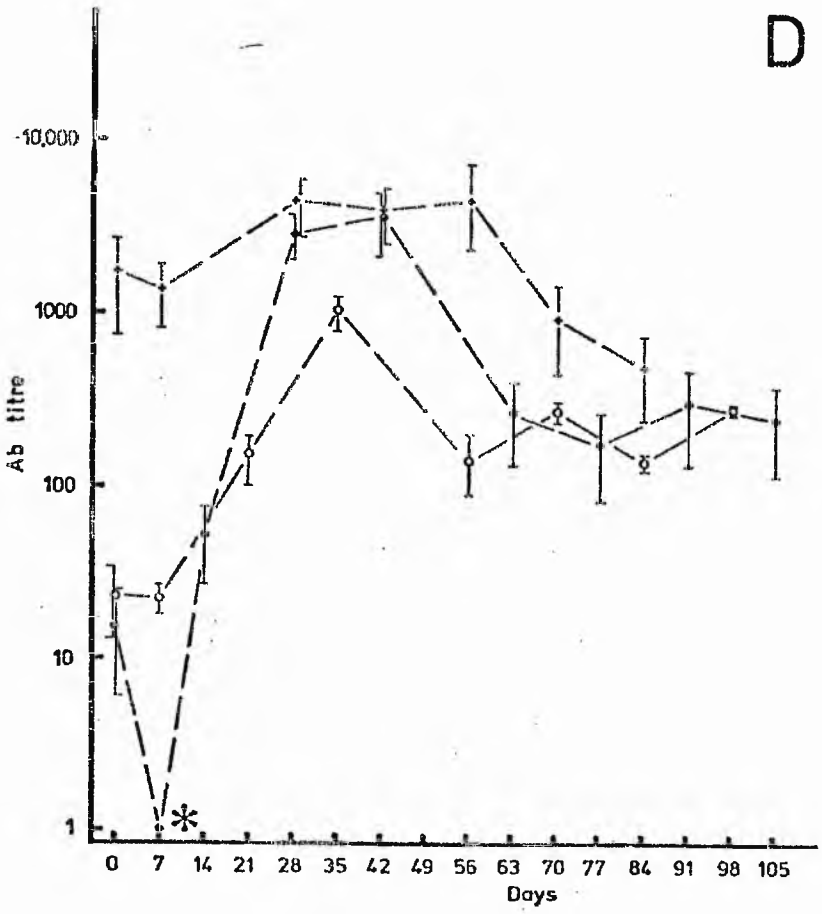
Mean antibody titre values (SD_{50}) from five fish are plotted, together with ± 2SE bars.

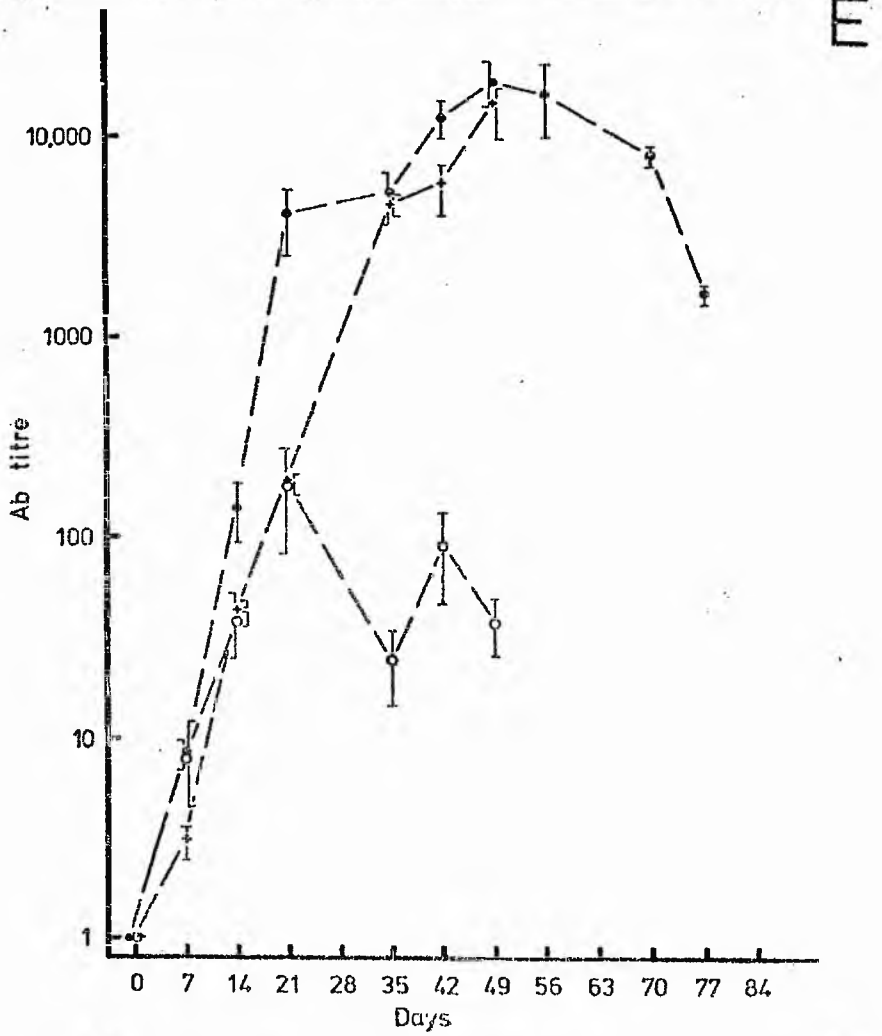
Primary Response Fish inoculated at day 0 with		Secondary Response Fish inoculated at day 0 with
A. ●	1.7×10^3 PFU *MS2*-saline	B. 1.7×10^9 PFU *MS2*-Saline
o	1.7×10^6 PFU *MS2*-saline	
+	1.7×10^9 PFU *MS2*-saline	
□	Control, Saline only	Control, Saline only
C. ●	1.7×10^3 PFU *MS2*-Freund's Incomplete Adjuvant	D. 1.7×10^9 PFU *MS2*-Saline
o	1.7×10^6 PFU *MS2*-Freund's Incomplete Adjuvant	
+	1.7×10^9 PFU *MS2*-Freund's Incomplete Adjuvant	
E. ●	1.7×10^3 PFU *MS2*-Freund's Complete Adjuvant	F. 1.7×10^9 PFU *MS2*-Saline
o	1.7×10^6 PFU *MS2*-Freund's Complete Adjuvant	
⊕	1.7×10^9 PFU *MS2*-Freund's Complete Adjuvant	

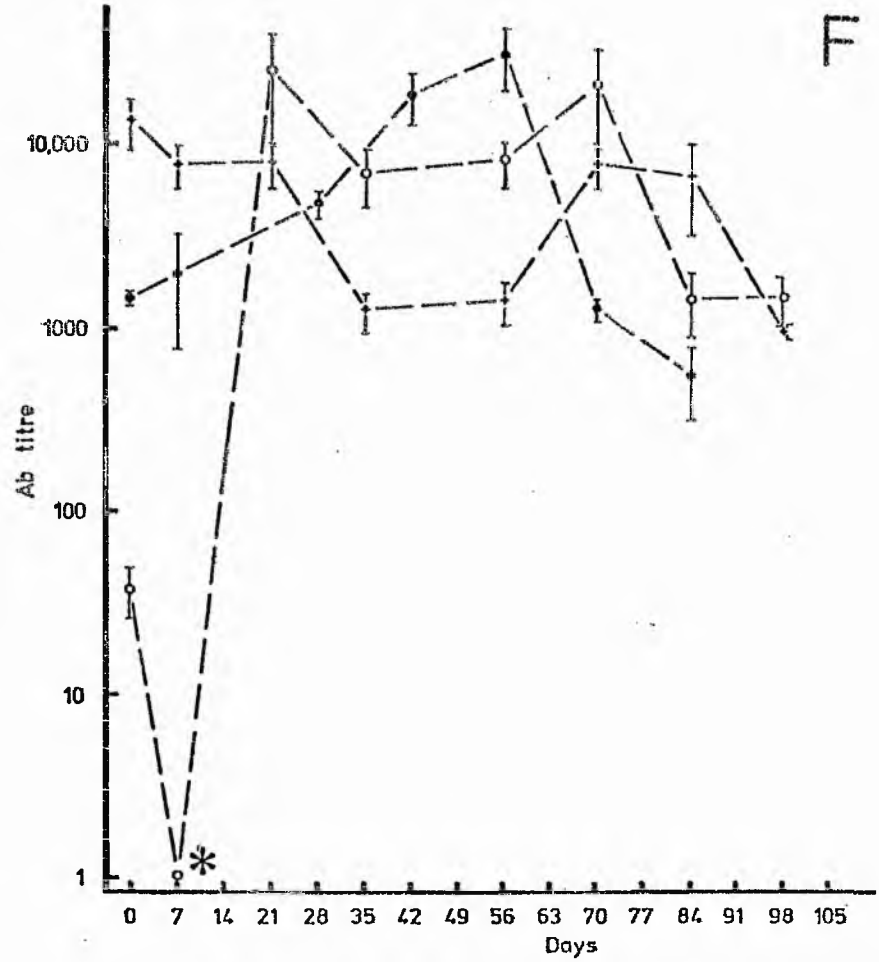
* Live *MS2* bacteriophage in the sera


A
10,000
1000
100
10
1
Ab titre
0 7 14 21 28 35 42 49 56
Days
B
10,000
1000
100
10
1
Ab titre
*
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105
Days


C
10,000
1000
100
10
1
Ab titre
0 7 14 21 28 35 42 49 56 63 70 77
Days


D
10,000
1000
100
10
1
Ab titre
*
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105
Days


E
10,000
1000
100
10
1
Ab titre
0 7 14 21 28 35 42 49 56 63 70 77 84
Days


F
10,000
1000
100
10
1
Ab titre
*
0 7 14 21 28 35 42 49 56 63 70 77 84 91 98 105
Days

The production of neutralisation antibodies in this group of trout appeared to be prolonged, thus enabling high titres to be built up which peaked at 49d and then decreased. At the lower concentrations of inoculated bacteriophage the addition of FIA was observed to have had no effect, though peak titres in the 10^6 PFU inoculated group were significantly lower than in the equivalent MS2-saline inoculated group (table 5A), indicating some form of suppression.

The use of FCA markedly increased the neutralisation antibody titres of the 10^3 and 10^9 PFU inoculated groups, peak titres at 49d being in the same order as those for the 10^9 PFU-FIA group. In the case of the 10^3 PFU-FCA group the higher range of titres observed were reached at an earlier date than in the other groups, 21d onwards, indicating an enhanced rate of antibody formation with the addition of FCA (figure 6E). The intermediate group, inoculated with 10^6 PFU-FCA, produced a similar titre response to that of the saline-MS2 inoculated group, though in secondary antibody response the titres produced by these fish were characteristic of the FCA inoculated group. This suppression cannot be explained adequately by the results presented here.

The response of S. trutta to a second inoculation of 10^9 PFU of MS2 bacteriophage exhibited characteristic and significant increases in the peak neutralisation antibody titres and the rate at which antibody was produced were found in the saline-MS2 inoculated group (figure 6B, table 5A). A similar enhanced response was observed in the low titre antibody responding group which were administered incomplete and complete adjuvants. After an initial peak the titres were found to level out or reach a plateau rather than decay, thus maintaining high levels of neutralisation antibody over several weeks of continued observation. Differences in antibody titre between the secondary response groups were not as marked in response to MS2 concentration as they were in primary response groups, though plateau titres, derived by pooling the titres of sera taken on day 35 and subsequent weeks after secondary inoculation, were increased by the

addition of incomplete adjuvant and were highest in the FCA groups (table 5B). In the adjuvant inoculated trout in which the primary neutralisation antibody response was high the secondary response titre did not surpass the primary peak titres. It may be possible to speculate from these results that in S. trutta the maximum threshold of MS2 bacteriophage neutralisation antibody titres would be within SD_{50} values of 10,000 to 100,000.

The inoculation of the secondary bacteriophage dose in all cases produced little antibody titre increase in the first 7d, in many cases a decrease in titre was observed and in the case of the three groups inoculated with 10^3 PFU or MS2 live bacteriophage was detected in the sera of these fish at day 7 (see Appendix 10). From 7 to 21d antibody titres were observed to rise rapidly. The inhibition observed in the first 7d was further investigated in a later experiment examining the clearance of the MS2 bacteriophage from the serum of S. trutta.

Bacteriophage neutralisation titres were observed in the control group of trout, inoculated with sterile saline, which mirrored both the primary and secondary responses of the bacteriophage sensitised fish. The peak SD_{50} values were low, the levels being in the same order as the 'natural' bacteriophage neutralisation titres which have been observed in this study. After the 'secondary response' peak the neutralisation titres in the control sera fell to non-significant levels (figure 6B).

The control fish in this experiment were randomly mixed with the bacteriophage inoculated groups, thus they were in contact with fish which had been demonstrated to harbour live MS2 bacteriophage for several days. The next experiment was thus devised in order to examine the possible stimulation of serum neutralisation activity by waterborne bacteriophage inoculation routes.

Table 5A The effect of antigen concentration and adjuvants on the immune response of S. trutta to MS2 bacteriophage.

A. Peak titres of primary and secondary response

MS2 inoculum titre PFU	Primary Response			Secondary Response	
	Day of Peak Response	Antibody titre ± SE	P	Day of Peak Response	Antibody titre ± SE
Control	21	14.6 ±1.6		14	15.3 ±2.4
S 10^3	21	42.6 ±9.6	***	28	1989 ±156
S 10^6	14	127 ±33.2	***	21	6466 ±731
S 10^9	35	1559 ±429	***	21	20679 ±3012
A 10^3	14	27.0 ±3.4	***	42	3753 ±816
A 10^6	14	51.6 ±13.1	***	35	1051 ±111
A 10^9	49	22050 ±4977	**	28	4521 ±837
CA 10^3	49	17331 ±2234	**	56	33280 ±6532
CA 10^6	21	180 ±49.6	***	21	25834 ±7851
CA 10^9	49	13337 ±2124		21	8120 ±967

n = 5 fish per group
S = Saline
A = Freund's incomplete adjuvant
CA = Freund's complete adjuvant

P = significant difference between antibody titres of the primary and secondary response.

** P = <0.01, *** P = <0.001

Primary response antibody titres of treatments grouped in ascending order, with a significant difference of P = <0.01 between groups.

(Control) $<$ ($S10^3$, $A10^3$, $A10^6$) $<$ ($S10^6$, $CA10^6$) $<$ ($S10^9$) $<$ ($A10^9$, $CA10^3$, $CA10^9$)

Secondary response antibody titres of treatments grouped in ascending order, with a significant difference of P = <0.001 between groups.

(Control) $<$ ($A10^6$) $<$ ($S10^3$, $A10^3$, $A10^9$, $S10^6$, $CA10^9$) $<$ (S^9, $CA10^6$, $CA10^3$)

Table 5B The effect of antigen concentration and adjuvants on the immune response of S. trutta to MS2 bacteriophage

B. Plateau titres; secondary response titres from day 35 after secondary inoculation, mean ± SE

	MS2 inoculum titre PFU		
Vehicle	10^3	10^6	10^9
Saline	745 ± 81.4	1900 ± 189	468 ± 28.4
	***	***	***
Adjuvant	1181 ± 239	** 435 ± 53.6	*** 2555 ± 392
	***	***	
Complete Adjuvant	13832 ± 1926	** 7292 ± 1186	** 2821 ± 377

All MS2 inoculated fish produced antibody titres significantly greater, $P = < 0.001$, than the control fish inoculated with saline only. Antibody titre of Control fish was < 1.

Significant difference between groups

**P = < 0.01
***P = < 0.001

n = 25, five fish sampled over a period of five weeks.

ii. Waterborne route of MS2 bacteriophage inoculation

Specimens of S. trutta, inoculated with aliquots of sterile saline, incomplete or complete adjuvants, were maintained in a single large aquarium and when the fish had recovered from the initial handling they were exposed to a waterborne dose of 5.0×10^4 PFU cm^{-3} MS2. As the 'natural' serum neutralisation activity of the trout before MS2-exposure was significant and the titres were significantly different between groups (Appendix 11) the SD_{50} values were subtracted from the experimental groups before plotting figure 7.

All three experimental groups produced an elevation of the bacteriophage neutralisation titre in the sera, with peak titres from 14 to 21d. The control fish, isolated from the experimental groups and only inoculated with sterile saline - FCA emulsion, were also observed to produce a significant increase in neutralisation activity. On days 7, 14 and 21 the experimental groups exposed to MS2 did, however, have titres which were elevated above those of the control group (Appendix 11) and the peak titres were also significantly greater than that of the control group (table 6).

Differences were noted in the form of the response between the three groups of exposed fish. The saline inoculated group of trout produced the largest neutralisation antibody response, exceeding that of the FIA group. In the FCA inoculated group the response was observed to be prolonged after the fall of bacteriophage neutralisation activity at 21d in the other groups.

iii. Clearance of MS2 bacteriophage from the serum of S. trutta

A primary dose of 7.7×10^9 PFU MS2 inoculated intraperitoneally into S. trutta was found to enter the serum of the fish very rapidly, at 1 min a titre of $8.4 \times 10^2 \pm 2 \times 10^2$ PFU cm^{-3} of live MS2 bacteriophage was detected. The live MS2

FIGURE 7

Immune response of *S. trutta* to a waterborne infection of *MS2* bacteriophage

Mean antibody titre values (SD_{50}) from five fish are plotted, together with $\pm$ 2SE bars.

□ Control, Freund's Complete Adjuvant inoculated (No *MS2* exposure)

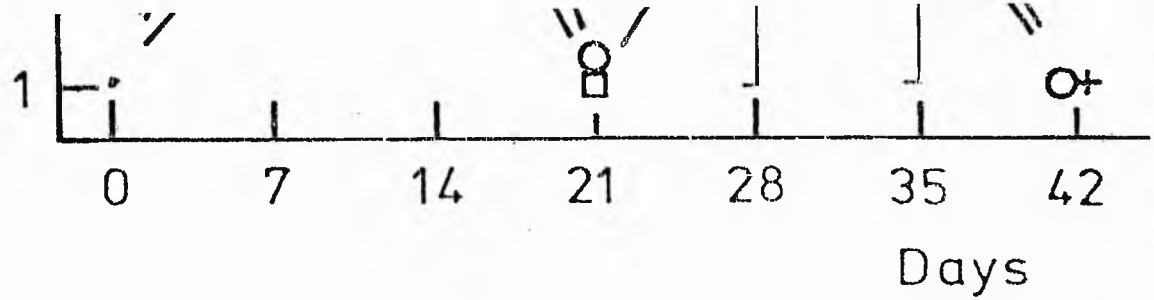
● Saline inoculated

o Freund's Incomplete Adjuvant inoculated

\+ Freund's Complete Adjuvant inoculated

} Waterborne exposure to 5.0×10^{4} PFU cm^{-3} *MS2* at time 0 for a period of 4h.

Water temperature $13.75 \pm 0.5^{\circ}C$


1
0
7
14
21
28
35
42
Days

Ab titre
10
100

Table 6 Waterborne 'inoculation' of MS2; day of peak response and peak titres, mean ± SE, in S. trutta

Inoculum	Day of Peak Response	Antibody titre $\bar{x} \pm$ SE
Control (Complete Adjuvant)	14	6.3 ± 0.9
Saline	14	45.6 ± 2.5
Adjuvant	14	13.9 ± 1.3
Complete Adjuvant	21	21.2 ± 6.7

All fish, except for the controls, were exposed to 5.0×10^4 PFU cm^{-3} of MS2 in the aquarium water.

All titres significantly greater than the control $P = < 0.001$.

n = 5 fish

titre in the sera was then observed to rise rapidly (figure 8), reaching a peak titre of $3.1 \times 10^9 \pm 3.7 \times 10^8$ PFU cm^{-3} at 24h. The latter titre represented a substantial proportion of the inoculated phage, approximately 80%, when the blood volume, 1.5 to 2.0 cm^3, of the 132 ± 4.0g trout used was taken into consideration. The clearance of the live bacteriophage from the serum over the following 24h was also observed to be rapid. At +38h after inoculation one trout had cleared the bacteriophage and at +44h two fish were producing detectable levels of bacteriophage neutralisation activity, which continued to increase. All five fish produced significant neutralisation titres at +62h.

A second inoculation of $9.2 \times 10^8 \pm 6.2 \times 10^7$ PFU MS2 was observed to produce a somewhat different response in the MS2-sensitised trout, which at that time still had detectable levels of neutralisation antibody (48.8 ± 12.3). The increase in the numbers of live bacteriophage which entered the serum was again observed to be rapid and reached a titre of $7.9 \times 10^6 \pm 1.9 \times 10^9$ PFU cm^{-3} + 30 min after inoculation which was only 1% of the original inoculum, however, the clearance of the bacteriophage was found to be slower. The live bacteriophage in the serum decreased gradually with time in an almost linear manner (figure 8) and was not cleared until 144 to 152h after inoculation. This represented an increased clearance time of 3.5d in the secondary inoculated fish. At the time of total bacteriophage clearance, + 152h, positive neutralisation titres were detected in all the five fish and were observed to rise rapidly at + 168h.

The effect of incomplete and complete adjuvants on the uptake and clearance of live bacteriophage in the serum have not been examined.

iv. 'Natural' MS2 bacteriophage neutralisation activity

Low levels of MS2 bacteriophage neutralisation activity have been observed in a proportion of the fish used in

FIGURE 8

Clearance of an intraperitoneal inoculation of MS2 bacteriophage from the serum of S. trutta and subsequent antibody titre formation.

A. Mean antibody titre values (SD_{50}) from five fish are plotted, together with ± 2SE bars.

• Primary Response titres after inoculation of 7.7×10^9 PFU MS2

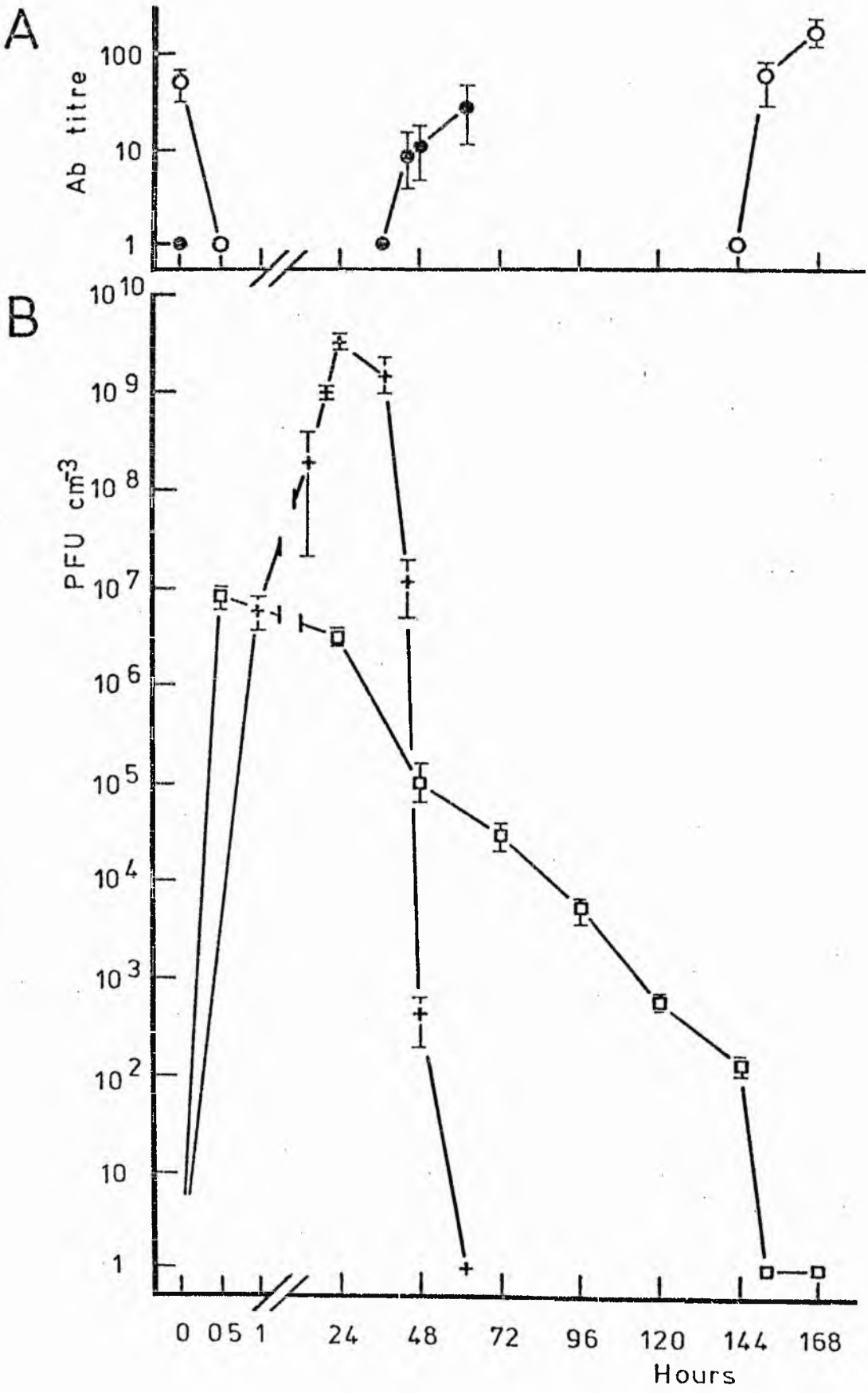
o Secondary Response titres after inoculation of 9.2×10^8 PFU MS2

B. Mean live bacteriophage titre values from the sera of five fish are plotted, together with ± SE bars.

+ Primary Clearance of live bacteriophage after inoculation of 7.7×10^9 PFU MS2

□ Secondary Clearance of live bacteriophage after inoculation of 9.2×10^8 PFU MS2

Water temperature $15.5^\circ \pm 0.5^\circ C$


A
Ab titre
100
10
1
B
PFU cm-3
10 10
10 9
10 8
10 7
10 6
10 5
10 4
10 3
10 2
10
1
0 05 1 24 48 72 96 120 144 168
Hours

experiments prior to their primary sensitisation with MS2. In S. trutta 43% of those fish examined had 'natural' neutralisation titres, one fish having an SD_{50} value as high as 45.7, and in C. carpio a smaller number of sera, 21%, were found to show neutralisation activity. None of the N. rossii examined had detectable levels of 'natural' bacteriophage neutralisation activity. In those groups with detectable neutralisation activity the titres were found to fit a normal Poisson distribution. The titres were also found to be higher in S. trutta (table 7) and in groups of fish examined in July and August. The latter groups also contained a larger proportion of fish with neutralisation titres than those examined in February.

v. Characterisation of MS2 bacteriophage neutralisation antibody

a. G-200 gel separation

The separation of serum fractions on G-200 Sephadex revealed that MS2 neutralisation activity was only found as a fraction of the first peak of three major peaks to be eluted (figure 9). The active fraction was not found in the void volume, however, as has been found by other workers for other species of fish, for example Fletcher and White (1973) and Harris and Cottrell (1976), but in the sieved elution volume. The ascending flow technique used in this study may have been responsible for the retention of the HMW fractions, thus the full G-200 molecular sieving range for proteins specified by Pharmacia (1975) of 5000 to 800,000 D was probably utilised.

The elution volumes of peak MS2 bacteriophage neutralisation antibody activity have been listed in table 8 for the three species of fish examined and the approximate values for the molecular weights of the fractions calculated. In all the sera examined the antibody active fractions were of a larger molecular weight than the 280,000 D of the urease

Table 7 Natural levels of MS2 bacteriophage neutralisation activity

Date	Number of active sera	Total number of fish examined	Neutralisation titre $\bar{x} \pm SE$	Maximum Neutralisation titre
S. trutta				
Feb '76	11	50	0.9 ± 0.3	11.5
July '76	15	20	11.1 ± 2.8**	32.4
Aug '76	15	30	5.4 ± 2.2	45.7
Feb '77	4	10	3.8 ± 2.2	22
Feb '77	13	25	2.5 ± 0.5	9.2
C. carpio				
Aug '76	13	26	2.6 ± 0.8**	20.8
Feb '77	1	25	0.9 ± 0.2	5.6
Feb '77	0	15	-	-
N. rossii				
-	0	17	-	-

** Significantly higher neutralisation titres than in the other groups $P = < 0.01$

FIGURE 9

Serum G-200 Sephadex separation profiles and MS2 bacteriophage neutralisation fractions

Each diagram represents the protein elution profile read at an absorbance of 280nm (-x-) and the antibody neutralisation titres (—●—) of the elution fractions from pooled sera.

S. trutta

A. Primary Response sera (serum load 0.75 cm^3).

B. Secondary Response sera (serum load 0.75 cm^3).

C. carpio

C. Primary Response sera (serum load 0.25 cm^3).

D. Secondary Response sera (serum load 0.75 cm^3).

N. rossii

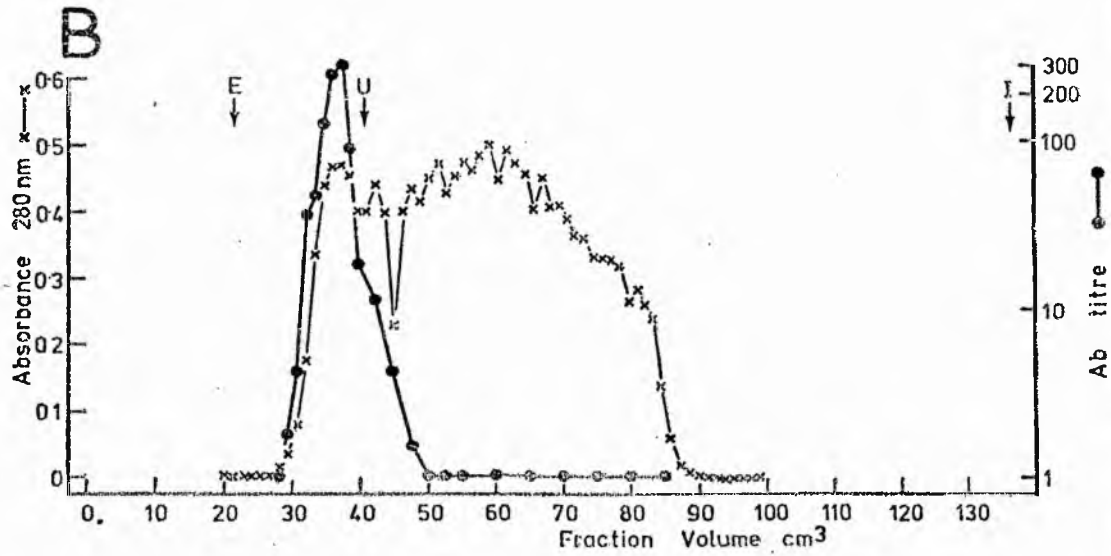
E. Secondary Response sera (serum load 0.7 cm^3)

E = Exclusion volume (void volume)

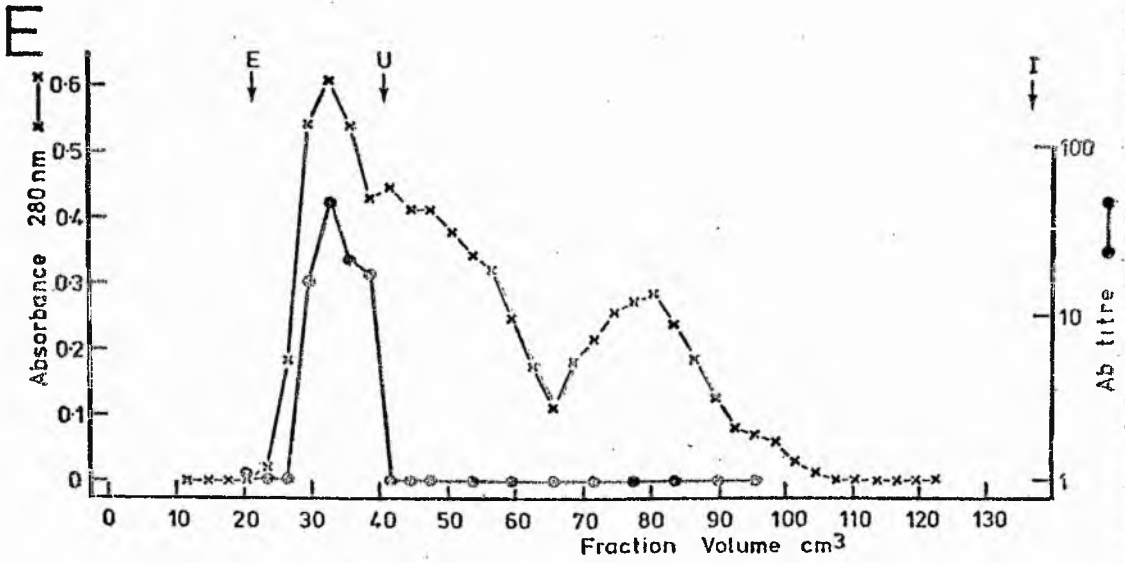
I = Inclusion volume (total gel volume)

U = Urease elution volume, molecular weight 280,000D

Serum separation carried out at 4^oC


B
E
U
I
Absorbance 280 nm
0·6
0·5
0·4
0·3
0·2
0·1
0
300
200
100
10
1
Ab litre
0.
10
20
30
40
50
60
70
80
90
100
110
120
130
Fraction Volume cm3

A
Absorbance 280 nm
0
0·1
0·2
0·3
0·4
0·5
0·6
E
C
I
1
10
100
Ab titre


E
E
U
I
Absorbance 280 nm
0·6
0·5
0·4
0·3
0·2
0·1
0
100
10
1
Ab titre
0
10
20
30
40
50
60
70
80
90
100
110
120
130
Fraction Volume cm3

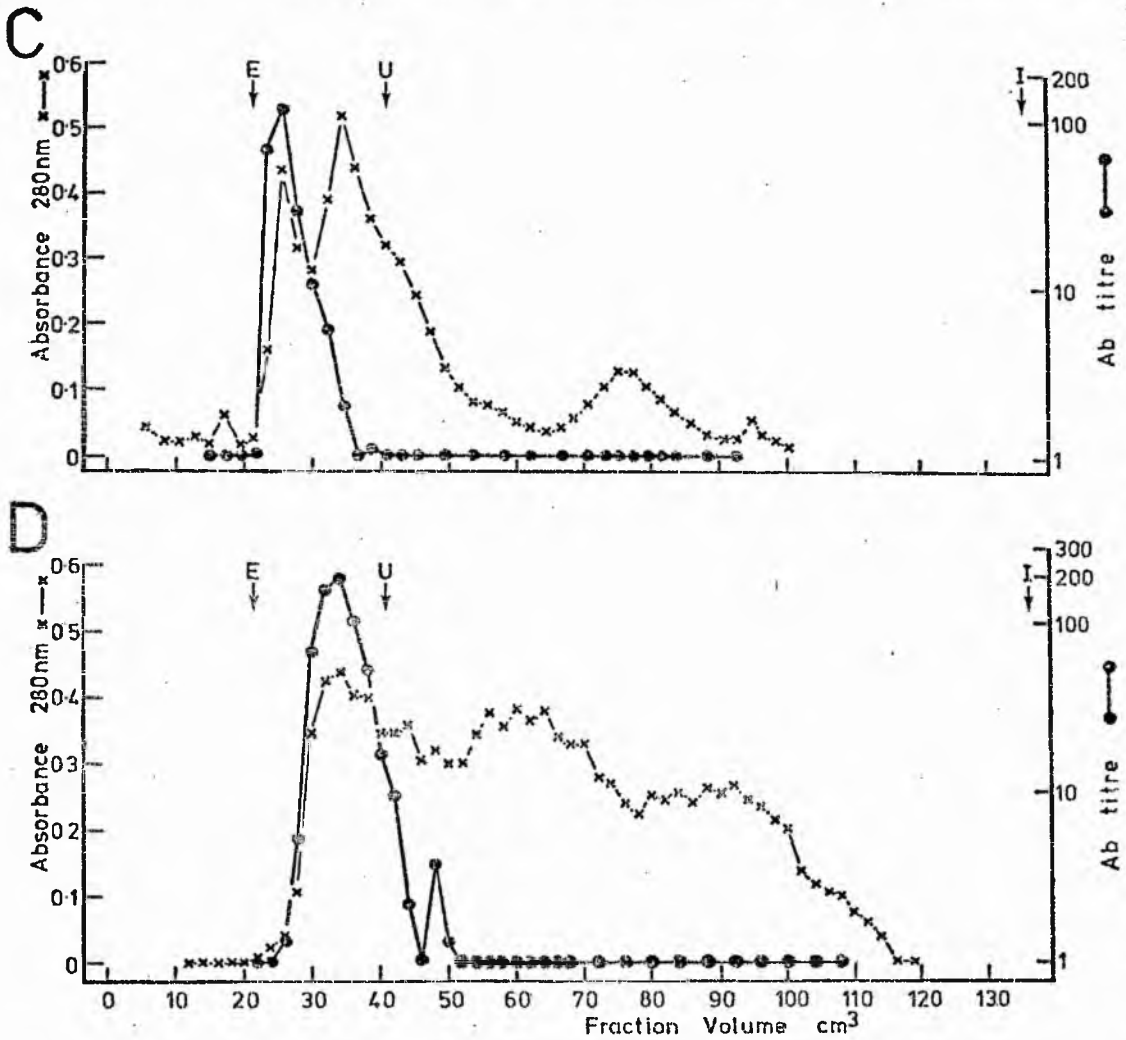

C
D
E
U
I
Absorbance 280nm
Ab titre
0·6
0·5
0·4
0·3
0·2
0·1
0
200
100
10
1
300
0 10 20 30 40 50 60 70 80 90 100 110 120 130
Fraction Volume cm3

Table 8 G200-Sephadex elution volumes for MS2-antibody rich sera

	Elution volume cm^3	V_e/V_0	$\frac{V_e-V_0}{V_t-V_0}$	Molecular weight Daltons
V_0	21.6	1		800,000
V_t	136.8	6.33	1.0	5000
V_e urease	40.8	1.89	0.17	280,000
V_e S.trutta, 1^o	36.0	1.67	0.125	390,000
2^o	37.2	1.72	0.135	360,000
V_e C.carpio, 1^o	25.8	1.19	0.037	600,000
2^o	34.0	1.57	0.108	420,000
V_e N.rossii, 1^o	-	-	-	-
2^o	32.5	1.51	0.095	460,000

V_e = Elution volume - peak of fraction

V_0 = Void volume

V_t = Inclusion volume

marker, thus they were larger than the mammalian IgG (150,000 D, 7S) though smaller than the mammalian IgM (900,000 D, 19S).

The bacteriophage neutralisation fraction of S. trutta sera in both primary and secondary MS2 sensitised fish required larger elution volumes than the active fractions of C. carpio and N. rossii indicating that trout antibody was a smaller molecular weight fraction. In both trout and carp the secondary responding neutralisation fractions were of lower molecular weight than those of the primary responding sera.

b. 2-Mercaptoethanol sensitivity

The MS2 neutralisation activity of all three fish species was significantly reduced when active sera were incubated with 2-ME (table 9) and compared with control sera which had retained neutralisation activity when dialysed against teleost saline. The sera of both S. trutta and C. carpio treated with 2-ME still retained a significant low level of neutralisation activity. The residual activity was highest in the carp sera, though this may have been a reflection of the higher initial neutralisation titre in these sera.

The substantial reduction of the active MS2 neutralisation molecules indicated the breakdown of disulphide bondings in these molecules characteristic of higher vertebrate antibodies and indicated they were of the more sensitive polymeric molecular type (Grubb and Swann, 1958). The residual activity may have indicated that there were 2-ME non-sensitive fractions present in the neutralisation sera of trout and carp, but ineffective 2-ME reduction and the possible presence of active sub-fractions (Green, 1969) could not be discounted.

Table 9 2-Mercaptoethanol sensitivity of MS2-antibody produced in secondary sensitised fish

	Antibody titres $\bar{x} \pm$ SE	
Serum	Control	2-ME
S. trutta (n=4)	952 ± 23.4	15.3 ± 2.3
C. carpio (n=4)	1843 ± 22.1	25.5 ± 3.1
N. rossii (n=4)	221 ± 5.2	<1

All MS2-antibody control titres significantly greater than titres of 2-ME treated sera
P = <0.001

Table 10 Specificity of MS2-antibody produced in secondary response fish

Antigen / Serum	SD_{50} plaque neutralisation titres $\bar{x} \pm$ SE			
	QB	X174	P22	MS2
S. trutta (n=4)	12.9 ±0.8 **	6.9 ±2.3 **	2.4 ±0.6	11670 ±414
C. carpio (n=4)	1.9 ±0.4	<1	<1	8709 ±219
N. rossii (n=4)	<1	<1	<1	323 ±12.7

All MS2-antibody titres significantly greater than neutralisation titres for the other bacteriophage species P = <0.001

Significant differences between QB, X174 and P22
** P = <0.01

c. Specificity of MS2 bacteriophage neutralisation activity

The MS2 neutralisation activity produced after secondary inoculation of the MS2 bacteriophage by all three species of fish examined was significantly more specific in its neutralisation properties for MS2 bacteriophage than for QB, X174 and P22 bacteriophages (table 10). Significant low levels of neutralisation activity against QB, X174 and P22 were found in the sera of S. trutta, though not in the other two fish species. There was also a significant difference between the activity of the trout sera on the three bacteriophages, the order of susceptibility to neutralisation being P22 < X174 < QB.

d. Complement fixation test and haemaglutination in the sera of S. trutta

Trout sera in all the replicate tests made were shown not to agglutinate untreated SRBC, however, when RHS-sensitised SRBC were exposed to trout sera haemolysis was observed up to a maximum titre of 1/4. This natural haemolytic activity was removed from MS2-sensitised fish sera by preincubation with MS2 bacteriophage (table 11), thus the presence of a low level of trout complement, which could be fixed by MS2-antibody complex, was indicated.

The results of the complement fixation test in table 11 using GPC indicated a maximum MS2-antibody titre, which would fix complement, of 1/4096 compared to the plaque neutralisation titre SD_{50} value of 50,474 for the pooled trout sera used in this study.

e. Antibody- antigen precipitation

Sera of known MS2-neutralisation activity, from all three species of fish, were found not to form antibody-antigen precipitates which could be detected by the four immuno-precipitation methods documented in this study. (See Appendix 8).

Table 11, Complement fixation test on S. trutta serum

Antibody titre of trout serum by plaque neutralisation assay SD_{50} was 50474 ± 7793

GPC dilution factor	Serum dilution factor	1	4	16	64	256	1024	4096	16384	65536	262144
Control serum (no antigen)	NONE	+	+	0	0	0	0	0	0	0	0
Antibody-antigen Serum	NONE	0	0	0	0	0	0	0	0	0	0
	10	+	+	+	+	+	+	+	+	+	+
	20	0	0	0	+	+	+	+	+	+	+
	30	0	0	0	0	0	+	+	+	+	+
	40	0	0	0	0	0	0	0	+	+	+
	50	0	0	0	0	0	0	+/0	+	+	+
	60	0	0	0	0	0	0	+	+	+	+

Maximum titre - 1/4096 n = 6 complement fixation tests

\+ = > 50% complete lysis of replicates

0 = > 50% Inhibition of lysis

+/0 = 50% complete lysis of replicates

vi. Macrophage migration inhibition test

The buffy coats, or white cell layers, separated in microhaematocrit tubes from the whole bloods of C. carpio and S. trutta were found to migrate into the 'tissue fluid' medium in the typical fan like manner described by Roitt (1974) and Timur (1976) and illustrated in figure 30. The addition of antigen and antibody-antigen complex from MS2-sensitised trout and carp to the tissue fluid, was found to inhibit migration of the buffy coats (table 12). No change in the state of migration was observed in the tubes between 24h and 48h of incubation at 20°C.

The migration of the buffy coats from trout and carp not previously sensitised to MS2 bacteriophage was not actively inhibited by addition of antigen or antibody-antigen complex, except for two out of the five trout exposed to antibody-antigen complex which were inhibited.

B. A preliminary histological investigation of the lymphoid tissues of S. trutta

i. Thymus

This bilaterally paired organ was found to lie dorsally in the gill chambers and be intimately associated with the pharyngeal epithelium of that area and with the gills (figure 31). The thymus was not visually apparent on external examination of S. trutta as was the case for S. gairdneri which displays a distinct white thymic body through the epithelium.

The thymus was found to be organised into a surrounding cortex of epithelial cells (McArdle and Roberts, 1974) which penetrated as cords of cells into the loosely structured medulla of large nucleated and deep haematoxylin staining thymocytes (figure 32) and a smaller number of lighter staining lymphocytes. Dispersed in the medulla were observed 'rosette-like' accumulations of epithelial and mucus cells

Table 12 *S. trutta* and *C. carpio* macrophage migration inhibition test

White cells	Control	+ Antigen	+ Antibody-antigen
T_{MS2}	-----	-++++	+++++
T_O	-----	-----	---++
C_{MS2}	-----	-++++	-++++
C_O	-----	-----	-----

T_{MS2} and C_{MS2} = *S. trutta* and *C. carpio* respectively sensitised with MS2 bacteriophage

T_O and C_O = Unsensitised fish

\+ = Inhibition of migration

\- = Normal migration

Results for individual fish presented, n = 5

similar to those found by McArdle and Roberts (1974), large spherical and highly vacuolated mucus cells, and a blood vascular system (figure 33). The structures resembling Hassal's corpuscles observed by McArdle and Roberts (1974) in S. gairdneri were not seen in S. trutta.

ii. Spleen

The spleen was also found as a discrete organ, lying in the abdominal cavity on the surface of the gut folds in trout but found to be hidden within the folds of the intestine in C. carpio. This organ was bounded by a single layer of epithelial cells and appeared to have no gross organisation of red and white pulp into distinct areas (figure 34). Mature erythrocytes were found to be the commonest cellular element of the spleen. The white cells were found to be clustered and surrounded by plugs of splenic mesoderm. Lymphocytes were also found clustered around capillaries and ellipsoids. The ellipsoids of the trout spleen were similar to those observed by Ellis, Munro and Roberts (1976). The lumen of the ellipsoid was found to be narrow, often only one erythrocyte being observed, and surrounded by a flattened endothelium. The arteriole was surrounded by a layer of large macrophage-like cells, usually a single but overlapping layer of cells, and held together by a distinct boundary sheath (figure 35). Ellipsoids were also observed in C. carpio though these were found to be larger than those of the trout (figure 36).

Melano-macrophages, as defined by Roberts (1974), were found in very small numbers in the normal trout spleen in comparison to the aggregations of larger cells observed in the carp (figure 37). These cells were found to be rounded and composed of a large volume of clear yellow-brown cytoplasm.

iii. Kidney

The teleost kidney can be divided into two histologically distinct regions, the anterior pronephros and posterior opisthonephros. In the adult fish the pronephric tubules are lost leaving this part of the kidney as an haemopoietic structure, though the opisthonephros also possesses a similar haemopoietic parenchyma in the inter-tubular spaces.

The trout kidney was found along the ventral side of the vertebral column and covered by a tough connective tissue septum. The pronephros was found to consist of a network of blood vessels and sinuses (figure 38) formed from reticulo-endothelial cells (Ellis et al., 1976). Large aggregations of tightly packed melano-macrophage-like cells were observed surrounded by kidney white pulp cells (figure 39), distinct from the aggregations of the large rounded melano-macrophages of the carp (figure 37). A large proportion of erythrocytic red pulp was found in the kidney, though areas of red and white pulp were not always discrete.

iv. Uptake of intraperitoneally inoculated MS2 bacteriophage and carbon in S. trutta

a. MS2

The intraperitoneal inoculations of MS2 bacteriophage produced no observable changes in any of the organs examined other than the spleen of S. trutta. At +8h after primary inoculation little change was observed in the spleen. Aggregations of 'melano-macrophages' containing large amounts of pale yellow-brown cytoplasm were observed at +36h (figure 40) and at +7d the aggregations had expanded and vacuolation of the cells was observed (figure 41). A similar aggregation and expansion of the 'melano-macrophages' was observed at +8h and +36h after a secondary inoculation of MS2 bacteriophage.

b. Carbon

Unlike the bacteriophage, carbon particles are visible under the light microscope and were thus used to observe the distribution of inoculated particles from the peritoneal cavity. As early as 5 min after inoculation free carbon particles were observed in the blood vessels and sinuses of all the tissues of S. trutta and at +4h carbon was seen to be trapped in the blood spaces of the gill secondary lamellae, kidney, pericardium, cardiac muscle and a small amount in the dermis and intestine. At no time after inoculation was carbon found within the tissues of the thymus or the liver, except those particles of carbon phagocytosed by cells of the blood and retained in the blood vessels of those tissues.

A large amount of carbon was found to be intracellularly trapped in the spleen at +4, +6 and +8h. At +4h carbon was intracellularly associated with the macrophages of the ellipsoid sheaths (figure 42), though at +8h macrophages containing carbon were observed generally throughout the white pulp of the spleen unassociated with the ellipsoids.

At 6h the amount of free carbon in the atrial and ventricular spaces of the heart had decreased and a large amount of carbon was observed to be in the cytoplasm of elongated reticulo-endothelial cells of the atrial muscle (figure 43). Free macrophages and rounded reticulo-endothelial cells full of carbon were observed in atrial sections of +8h fish.

The amount of carbon in both pronephric and opisthonephric kidney was found to increase at +6h, the majority of the carbon being intracellular in the cells of the white pulp, though some free carbon particles were observed to be attached to the basement membranes of kidney tubules (figure 44).

Although the fate of the inoculated carbon was not examined for a sufficient period to observe its clearance, the uptake and distribution of carbon from the peritoneal cavity was observed to be rapid, and the major organs of entrappment to be the reticulo-endothelial cells, spleen and kidney.

C. Temperature and the immune response to MS2 bacteriophage

i. S. trutta

S. trutta was found to produce humoral neutralisation antibody at all the four experimental temperatures examined when challenged with 10^9PFU MS2 in FIA, though induction time and peak titres were found to be modified (figure 10, appendix 12). The onset of primary response was found to be similar at the two higher temperatures used, 13.75 and 15.5°C, which both showed an induction period of under 7d, though at 9.0°C an induction period of 14d was observed, increasing to between 21 and 28d at 4.5°C. In the latter group live bacteriophage was not cleared from the serum until 21d after inoculation. On the initiation of humoral antibody formation the rate of increase in titre was found to be similar at all the temperatures examined. In comparison to the trout held at 15.5°C the 13.75°C group indicated a significant slowing down of the rate of antibody formation, although the lower peak titre in the latter group was not significantly different.

The primary peak titres (table 13A) were found to decrease with decreasing temperature and also the time to reach peak titre increased. A peak titre at 35d in the 15.5°C group of trout was increased to 77d at 4.5°C.

All the experimental temperature groups were found to respond to a second inoculation of 10^9 PFU MS2 in saline in a similar manner to the 15.5°C group of trout. The bacteriophage was cleared immediately within the first 7d in the 4.5°C trout which indicated an enhanced response in comparison

Table 13 The effects of water temperature on the immune response of S. trutta to MS2 bacteriophage

A. Day of peak response and peak titres of primary and secondary response, mean ± SE

Water temperature °C	Primary Response		Secondary Response	
	Day of Peak titre	Antibody titre $\bar{x}$ ± SE	Day of Peak titre	Antibody titre $\bar{x}$ ± SE
4.5	77	394 ± 68.3	14	446 ± 111
		* *		*
9.0	42	2227 ± 207 * *	28	922 ± 153
		* * *		* * *
13.75	49	22049 ± 4977 * *	28	4521 ± 837
				* * *
15.5	35	30448 ± 6035	21	14651 ± 6251
9.0 group raised to 16.0			84	1595 ± 307

n = 5 fish

B. Plateau titres; secondary response titres from day 35 onwards after secondary inoculation, mean ± SE

	Water temperature °C							
	4.5		9.0		13.75		15.5	
Antibody titre $\bar{x}$ ± SE	222 ±19.7	***	500 ±52.9	***	2689 ±393	**	6312 ±704	
n serum samples used from 5 fish	25		25		20		35	

Significant differences between group titres

* P = <0.05
** P = <0.01
*** P = <0.001

FIGURE 10

Immune response of S. trutta to MS2 bacteriophage: The effects of water temperature.

Mean antibody titre values (SD_{50}) from five fish are plotted, together with ± 2SE.

A. Primary Response. 1.95×10^9 PFU MS2-Freund's incomplete adjuvant inoculated on day 0 (13.75°C group inoculated with 1.7×10^9 PFU MS2-Freund's Incomplete Adjuvant).

B. Secondary Response. 1.95×10^9 PFU MS2-Saline inoculated on day 0 (13.75°C group inoculated with 1.7×10^9 PFU MS2-Saline).

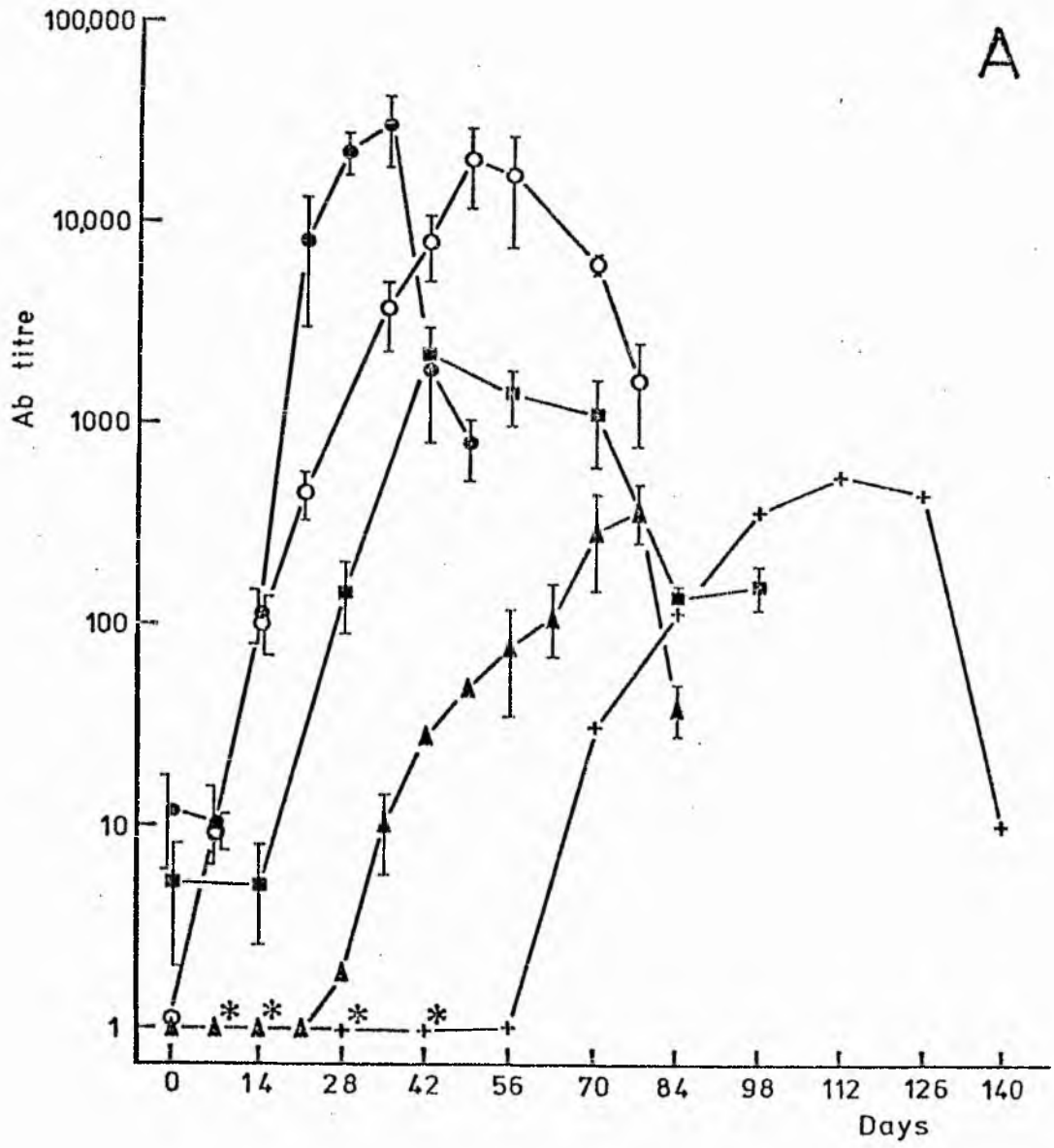
- ● 15.5 ± 0.5°C
- o 13.75 ± 0.5°C
- ■ 9.0 ± 0.5°C[1]
- ▲ 4.5 ± 0.5°C

} S. trutta

\+ 2.0 ± 0.3°C N. rossii, results for 10^9 PFU MS2-Freund's Complete Adjuvant inoculated fish (see figure 12)

* Live MS2 bacteriophage in the sera

■[1] In secondary response the 9.0°C group of S. trutta experienced a temperature increase. The water temperatures are inset for this group.


A
100,000
10,000
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100
10
1
Ab titre
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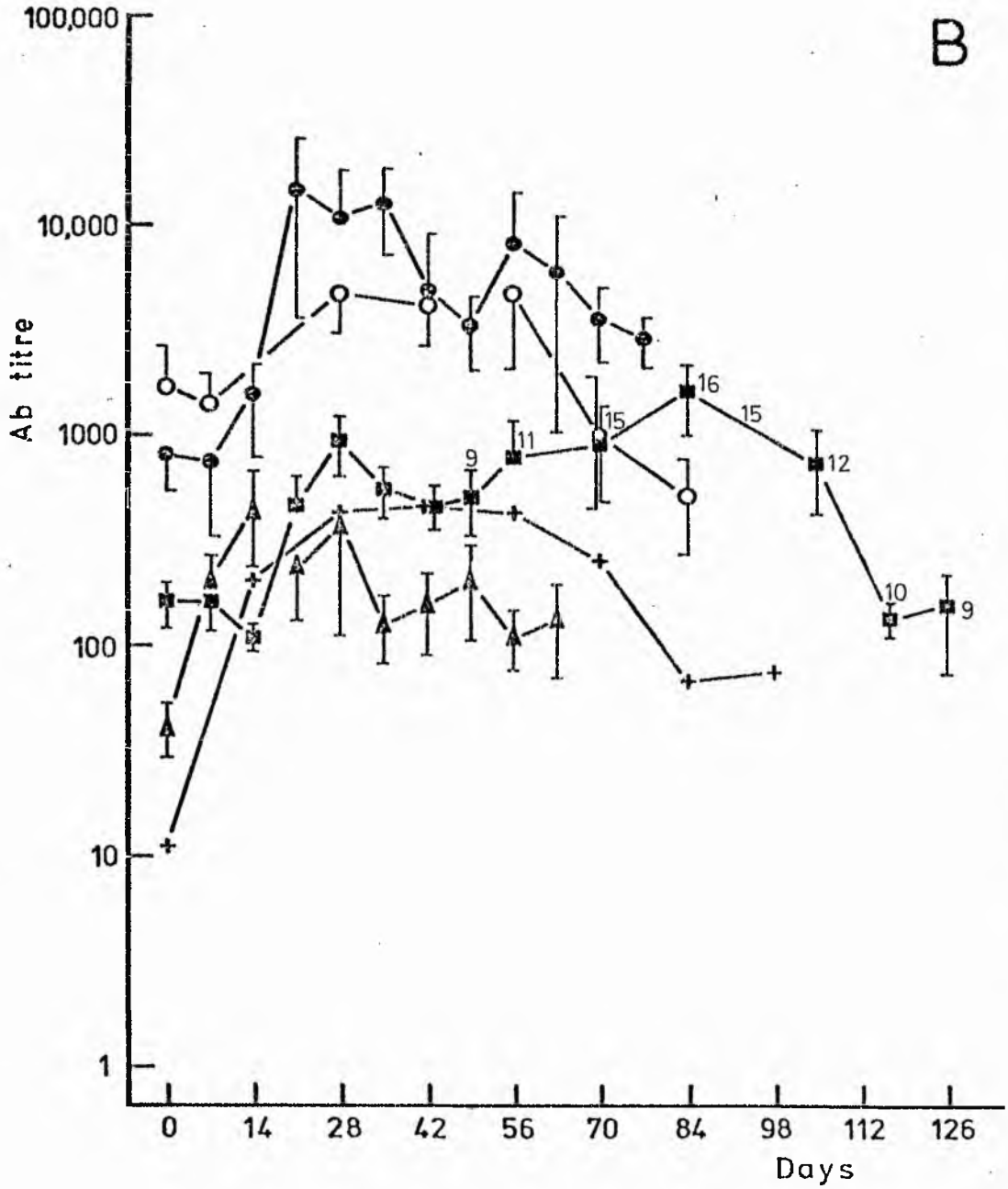
14
28
42
56
70
84
98
112
126
140
Days


B
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Ab titre
0
14
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42
56
70
84
98
112
126
Days
16
15
15
12
11
9
10
9

FIGURE 11

Immune response of C. carpio to MS2 bacteriophage: The effects of water temperature.

Mean antibody titre values (SD_{50}) from n fish are plotted, together with ± 2SE bars

A. Primary Response 1.95×10^9 PFU MS2-Freund's Incomplete Adjuvant inoculated on day 0.

B. Secondary Response 1.95×10^9 PFU MS2-Saline inoculated on day 0.

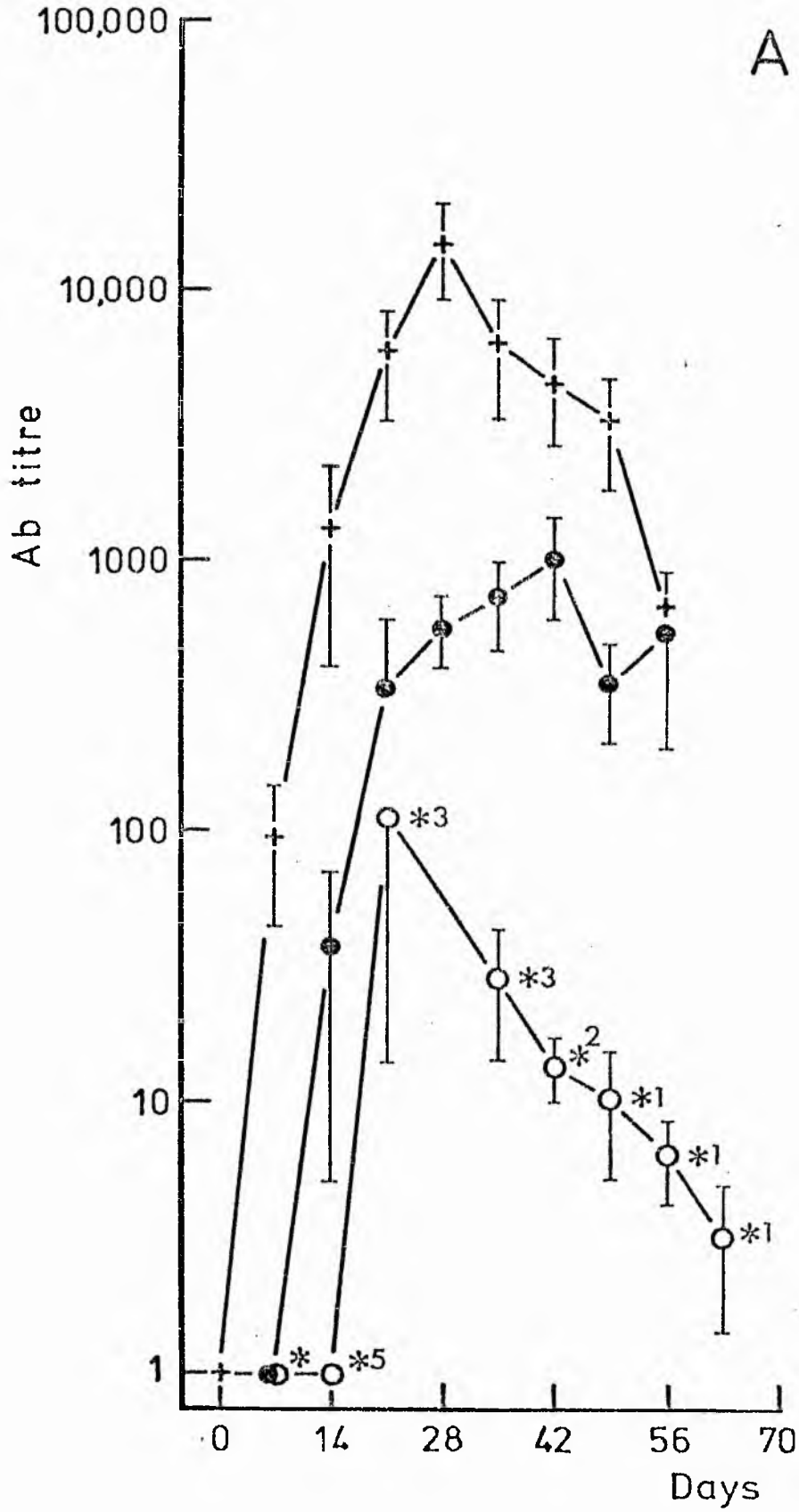
+ $22.0 \pm 0.5^{\circ}C$ n = 5

• $15.5 \pm 0.5^{\circ}C$ n = 6

o $9.0 \pm 0.5^{\circ}C$ n = 6

*[1] Live MS2 bacteriophage in the sera. Inset numerals indicate the number of sera containing live MS2.

t↓ Water temperature drop to $15.5^{\circ}C$ for a short period of time between day 28 and 29 after secondary inoculation in the $22.0^{\circ}C$ group.


A
100,000
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100
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Ab titre
*3
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*2
*1
*1
*1
*
*5
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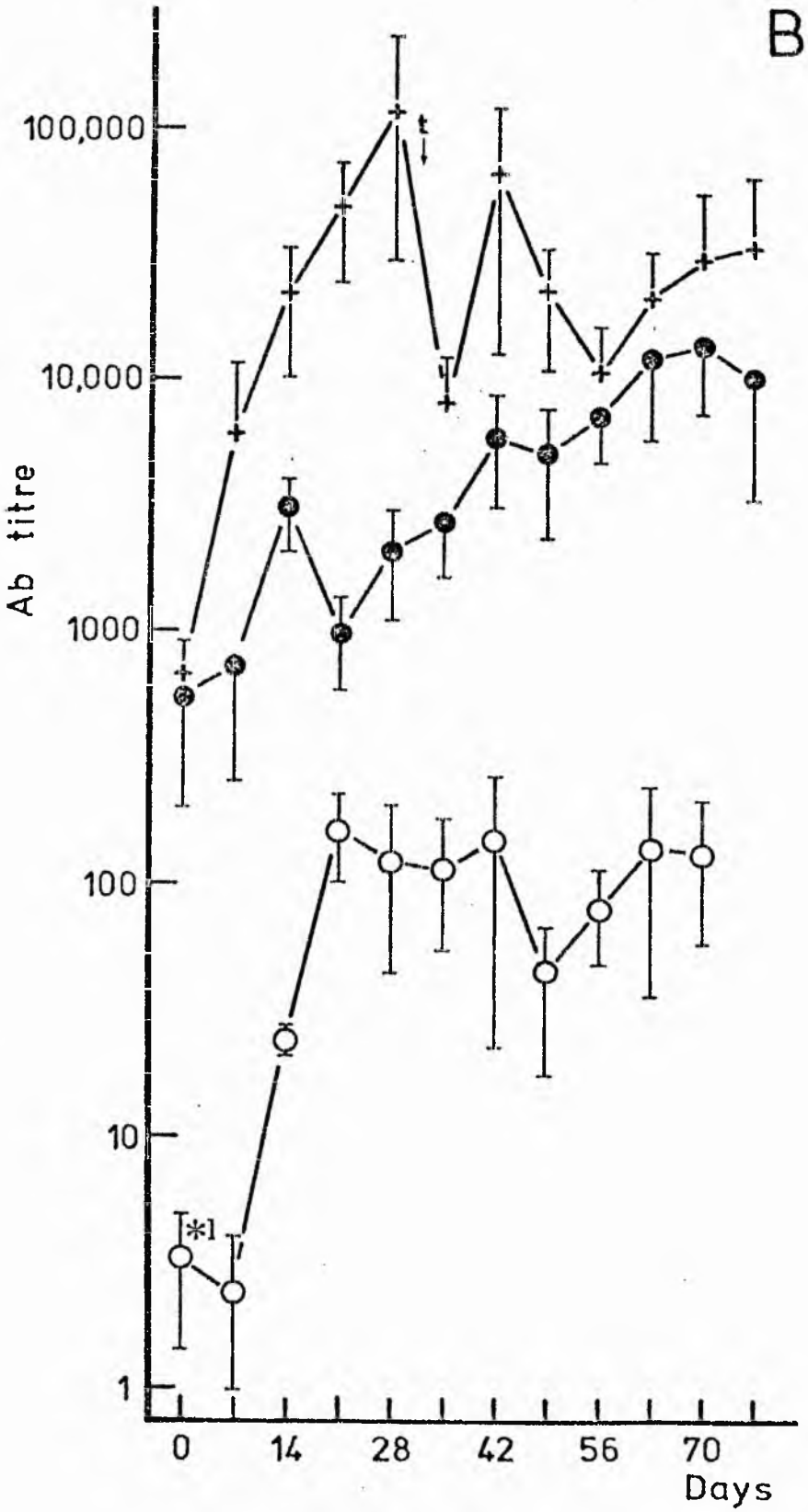
14
28
42
56
70
Days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Ab titre
*1
t
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14
28
42
56
70
Days

to that observed in primary response.

The secondary response peak antibody titres and plateau titres, in which titres were pooled from day 35, were found to be significantly lowered by decreasing the temperature (table 13). No enhancement of antibody titre was observed, a result expected from the first series of experiments examined using the FIA inoculation regime. The time required to reach peak titre was, however, shorter in the secondary response fish being between 14 and 28d for all the experimental temperatures.

The group of trout maintained at 9.0°C was subjected to a gradual increase in ambient temperature from day 56 after secondary inoculation which reached a maximum of 16.0°C. At this latter temperature a second peak in the antibody titre was observed in this group at 84d which was higher than that of the first secondary peak titre response at 9°C. The subsequent fall in ambient temperature back to 9°C was followed by a decrease in antibody titre.

ii. C. carpio

All the specimens of C. carpio used in these experiments were observed to produce humoral neutralisation antibodies in response to intraperitoneal challenge with MS2 bacteriophage. The groups of carp maintained at 22.0, 15.5 and 9.0°C were found to respond to a primary inoculation of 10^9 PFU MS2 in FIA and a secondary inoculation of MS2 in saline in a similar manner to the trout. These fish produced rapid primary and secondary peak antibody titres which in secondary response were maintained for the duration of the experiment (figure 11, appendix 13).

Again temperature was found to modify peak titre responses and the induction time of the primary response. At 22°C the antibody induction period and rate of antibody production were found to be similar to those of trout at

15.5°C. The response at the lower temperatures was marked by increased induction times and significantly lower peak antibody titres (table 14A). The carp maintained at 15.5°C were found not to clear the inoculated MS2 bacteriophage from the serum within the first 7d, the initiation of antibody formation being from day 7 to 14. Though the rate of antibody formation was initially similar to that of the 22°C group of fish the peak titre was significantly lower and the time of peak titre was delayed by 14d. The induction period of the 9°C carp was further extended and 5 out of 6 fish retained live MS2 in their sera at 14d. Even when the meaned peak antibody titre was recorded for responding fish three of the carp still retained levels of live MS2 in the sera and in one of these fish the bacteriophage was retained 70d at which time a secondary inoculation was given. Thus a temperature of 9°C was substantially more effective in reducing the total immune response to MS2 bacteriophage in carp than in the equivalent trout group.

The three groups of carp responded to the secondary inoculation of MS2 bacteriophage in an enhanced manner (figure 11B). No live bacteriophage was detected in the sera of the inoculated carp after 7d and even the 9°C group of carp, which had retained live bacteriophage for 70d after primary inoculation, cleared the secondary inoculation within 7d. As observed in secondary response trout antibody formation was not very significantly increased within the first 7d, though titres then rapidly rose and reached a peak at from 14 to 28d after inoculation (table 14A) as was observed for trout. These titres were also higher than the primary peak titres, the difference being significant for the 15.5 and 22°C groups. The 9 and 22°C groups of carp were found to maintain an elevated plateau titre. The antibody titre of the 15.5°C group continued to increase after the first peak, though total meaned plateau titres, derived from pooled titres subsequently to day 35, still remained significantly different and increased sequentially with temperature (table 14B).

Table 14 The effects of water temperature on the immune response of C. carpio to MS2 bacteriophage

A. Day of peak response and peak titres of primary and secondary response, mean ± SE

Water temperature °C	Primary Response		Secondary Response		n fish
	Day of Peak titre	Antibody titre $\bar{x}$ ± SE	Day of Peak titre	Antibody titre $\bar{x}$ ± SE	
9.0	21	119±51.7	21	165±31.9	6
		*		* * *	
15.5	42	1026±205	14	3153±502	6
		* * *		* *	
22.0	28	14669±3028	28	116727±43530	5

B. Plateau titres; secondary response titres from day 35 onwards after secondary inoculation, mean ± SE

	Water temperature °C				
	9.0		15.5		22.0
Antibody titre $\bar{x}$ ± SE	115 ±14.4	***	6868 ±749	***	27219 ±4165
n serum samples used	36		42		35
n fish	6		6		5

Significant differences between group titres

* P = <0.05
** P = <0.01
*** P = <0.001

At an unknown time during the 28th day after secondary inoculation a power failure in the subsidiary water heating unit to the 22°C group of carp decreased the water temperature to 15.5°C for a short period until+29d. The subsequent antibody titre of this group of fish at +35d was found to be significantly lower ($P < 0.001$), though at +42d the titre rose once more to the level of the preceding titre (figure 11B). It must be noted also that this temperature disturbance may have affected the peak antibody titre response of the 22°C carp group.

iii. N. rossii

The general form of the humoral antibody response to MS2 bacteriophage in N. rossii was again similar to that found in trout and carp, there being a primary response peak which decayed and an enhanced secondary response showing a maintained titre (figure 12 appendix 14).

The clearance of a primary challenge of MS2 from the sera of N. rossii was considerably extended at 2.0°C. The antibody titre response of FIA-MS2 inoculated fish has been compared with the response in trout in figure 10A. The bacteriophage was first cleared from the sera of the FCA-MS2 inoculated group of fish between +42 to +56d after inoculation, from the sera of FIA-MS2 inoculated fish at 56d and from the saline-MS2 inoculated group between +56 and +70d. On clearing the bacteriophage, antibody titres were found to rise quickly and reached a peak from +98 to 112d. No significant differences were observed between the saline and adjuvant treatments with regard to primary peak antibody titres (table 15A).

A secondary inoculation of MS2 bacteriophage in saline was cleared from all the fish within 14d and antibody titres reached a peak between 14 and 28d after inoculation. Peak antibody titres were significantly increased in the secondary response saline-MS2 and FCA-MS2 inoculated groups, with the

FIGURE 12

Immune response of *N. rossii* to *MS2* bacteriophage and the use of adjuvants at 2.0 ± 0.3°C.

Mean antibody titre values (SD_{50}) from n fish are plotted, together with ± 2SE bars.

A. Primary Response
Fish inoculated at day 0 with

o 1.95×10^9 PFU *MS2*-Saline n=6

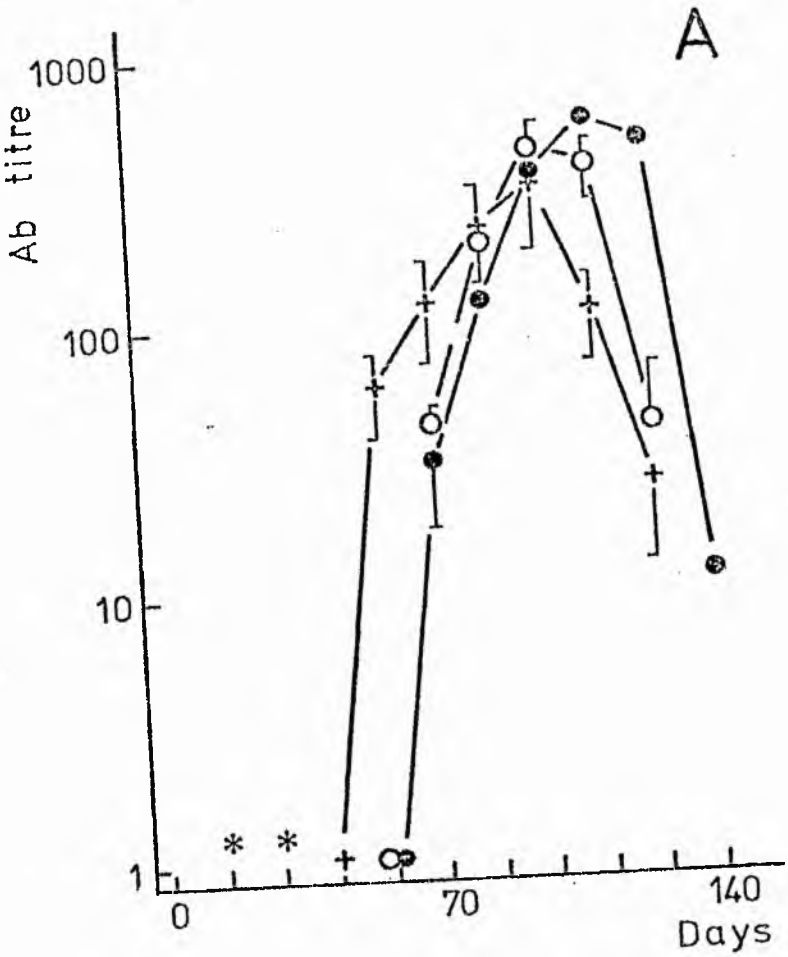
● 1.95×10^9 PFU *MS2*-Freund's Incomplete Adjuvant n=6 (n = 1 from day 84)

\+ 1.95×10^9 PFU *MS2*-Freund's Complete Adjuvant n=6

B. Secondary Response
Fish inoculated at day 0 with

1.95×10^9 PFU *MS2*-Saline

* Live bacteriophage in the sera.


A
1000
100
10
1
Ab titre
0
70
140
Days

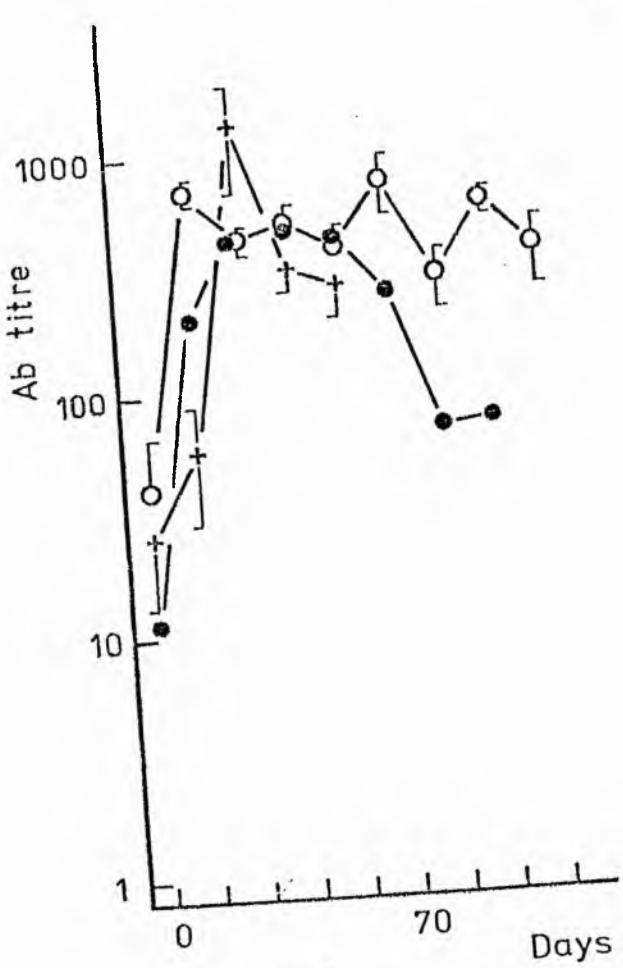

B
1000
100
10
1
Ab titre
0
70
Days

Table 15 The effects of adjuvants on the immune response of N. rossii to MS2 bacteriophage at 2.0 ± 0.3°C

A. Day of peak response and peak titres of primary and secondary response, mean ± SE

Inoculum vehicle & MS2 PFU	Primary Response		Secondary Response		n fish
	Day of Peak titre	Antibody titre $\bar{x}$ ± SE	Day of Peak titre	Antibody titre $\bar{x}$ ± SE	
Control (Saline)	-	<1	-	<1	6
Saline-10^9	98	443 ± 52.1 *	14	708 ± 41.9	6
Incomplete Adjuvant-10^9	112	562	28	437	1
Complete Adjuvant-10^9	98	345 ± 84.0 ***	28	1318 ± 320	6

All treatments show significantly greater antibody titres than controls $P = <0.001$

B. Plateau titres; secondary response titres from day 28 onwards after secondary inoculation, mean ± SE

	10^9 PFU MS2 in inoculum vehicle		
	Saline	Incomplete Adjuvant	Complete Adjuvant
Antibody titre $\bar{x}$ ± SE	451 ±33.8	400 ±19.3	672 ±124
n serum samples used	42	4	18
n fish	6	1	6

Significant differences between group titres

* $P = <0.05$
*** $P = <0.001$

latter group having the highest peak secondary response titres ($P < 0.05$). Differences between the three groups in secondary plateau titres, pooled titres from day 28, were concluded not to be significant (table 15A).

The secondary response of N. rossii has thus been shown to be similar in response time to that of trout (figure 12B) and carp, and equivalent in antibody titre response to trout maintained at 4.5 and 9°C and carp at 9°C.

From day 84 after primary inoculation the FIA-MS2 group of N. rossii contained only a single specimen due to accidental mortality. Though the results from this fish could not be used to make valid comparisons with the other two groups of fish it was noted that the response of this fish followed closely the pattern of response shown by the other fish.

iv. Clearance of MS2 bacteriophage

Figures 13 and 14 were constructed from data of live MS2 bacteriophage titres observed in the sera of S. trutta, C. carpio and N. rossii from the previous experiments. It was noted that the presence of adjuvants modified the clearance of MS2 from the sera of N. rossii, the retention of virus being greatest in the MS2-saline inoculated group. Bacteriophage clearance was enhanced by the addition of FIA and FCA, though it was observed that the final clearance of MS2 in the MS2-saline group was very rapid, falling from 2.9×10^6 to 0 PFU cm^{-3} within 14d.

From an examination of the clearance data for a primary inoculation of MS2 bacteriophage given to the three species of fish at their various experimental temperatures (figure 14) it was concluded that S. trutta was more able to clear the bacteriophages at 15.5°C and below when compared to C. carpio. At the lower temperature at which

FIGURE 13

Survival of MS2 bacteriophage in the serum of N. rossii after primary inoculation.

Mean live MS2 bacteriophage titre values from the sera of six fish are plotted, together with ± SE bars

Fish inoculated on day 0 with

- o 1.95×10^9 PFU MS2-Saline
- • 1.95×10^9 PFU MS2 Freund's Incomplete Adjuvant
- + 1.95×10^9 PFU MS2-Freund's Complete Adjuvant

* Maximum serum titre value if all bacteriophage in the sera.

Water temperature $2.0 \pm 0.3^{\circ}C$

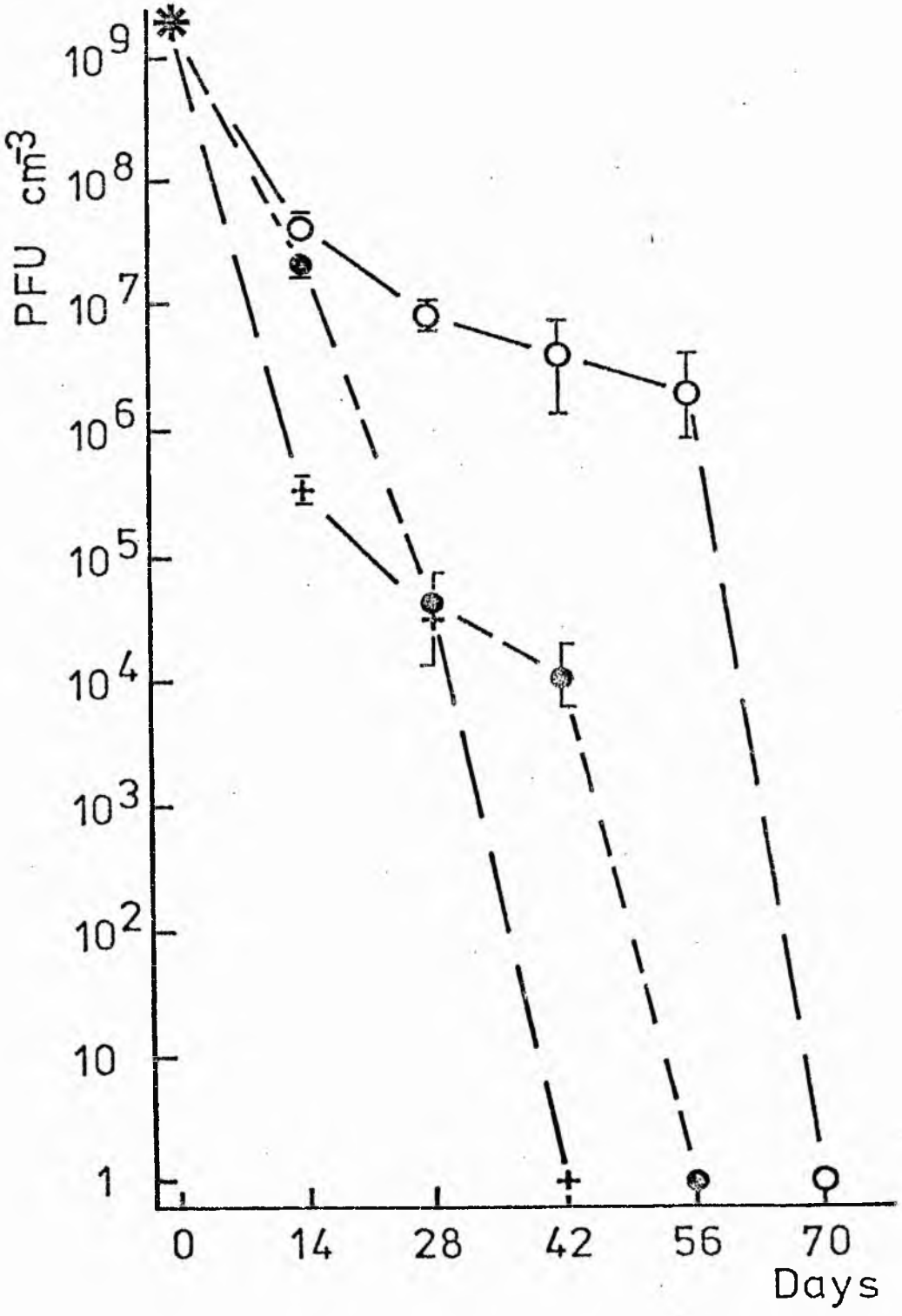

PFU cm⁻³
10⁹
10⁸
10⁷
10⁶
10⁵
10⁴
10³
10²
10
1
0
14
28
42
56
70
Days

FIGURE 14

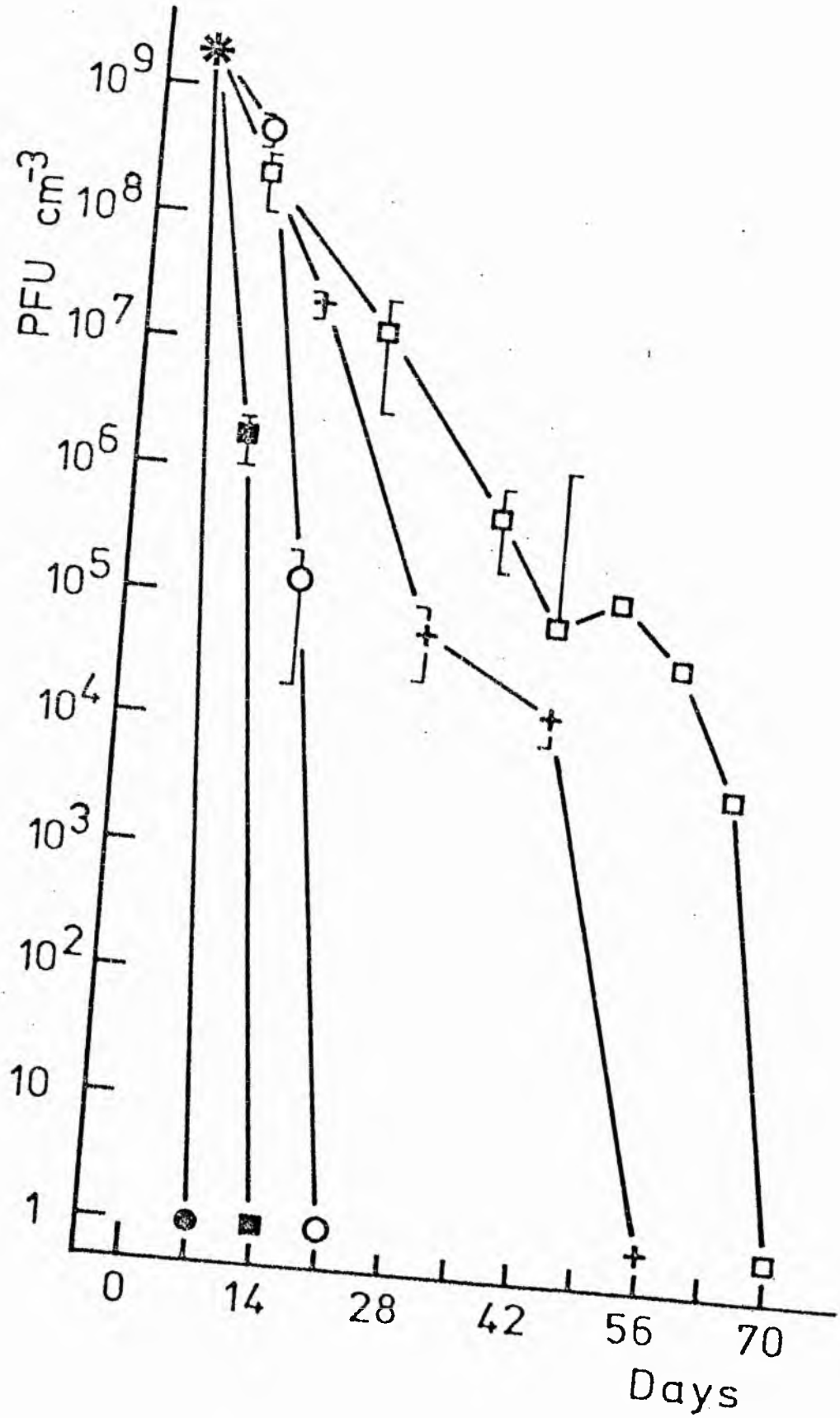
Survival of MS2 bacteriophage in serum after primary inoculation: The effects of species and water temperature.

Mean live MS2 bacteriophage titre values from the sera of n fish are plotted, together with ± SE bars.

Fish inoculated on day 0 with 1.95×10^9 PFU MS2-Freund's Incomplete Adjuvant.

	Species	Water Temperature	
+	N. rossii	2.0 ± 0.3°C	n = 6
o	S. trutta	4.5 ± 0.5°C	n = 5
□	C. carpio	9.0 ± 0.5°C	n = 6
■	C. carpio	15.5 ± 0.5°C	n = 6
●	C. carpio	22.0 ± 0.5°C	n = 5
●	S. trutta	15.5 ± 0.5°C and 9.0 ± 0.5°C	n = 5

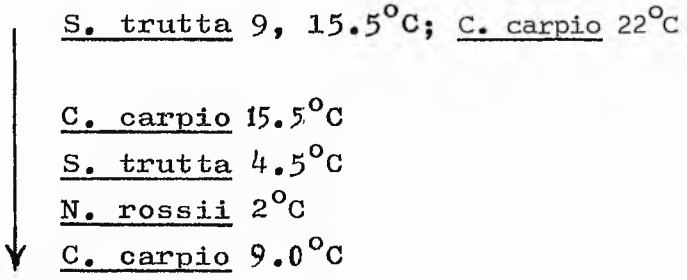
* Maximum serum titre value if all bacteriophage in the sera.


PFU cm-3
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10
1
0
14
28
42
56
70
Days

carp were exposed MS2 clearance was shown to be slower than for trout at 4.5°C and even N. rossii at 2°C.

The clearance times of MS2 bacteriophage followed the sequence -

Increasing time of MS2 clearance



v. Haematocrits and serum protein levels

The haematocrit values recorded at the termination of the experiment were found to be significantly related to the temperature in S. trutta, decreasing with decreased temperature (figure 15). In C. carpio a significantly higher haematocrit was only found in the fish maintained at 15.5°C.

Total protein levels in the sera of both trout and carp were found to decrease with decreasing temperature. All three temperature groups of trout examined were found to show significant differences, though only the carp held at the lowest temperature, 9°C, had a significantly lower serum protein level.

FIGURE 15

Effects of water temperature: Haematocrits and serum protein levels of S. trutta and C. carpio.

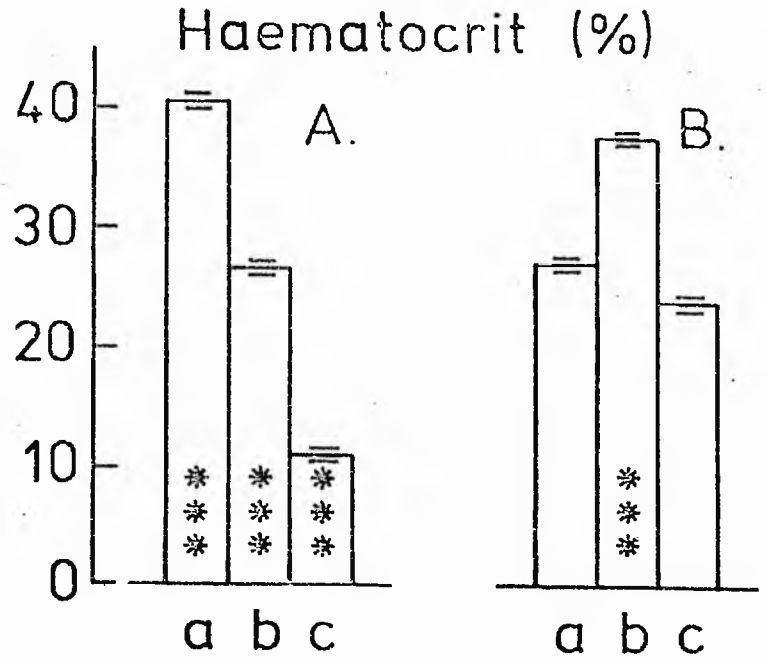
Mean values ± SE from n fish are plotted.

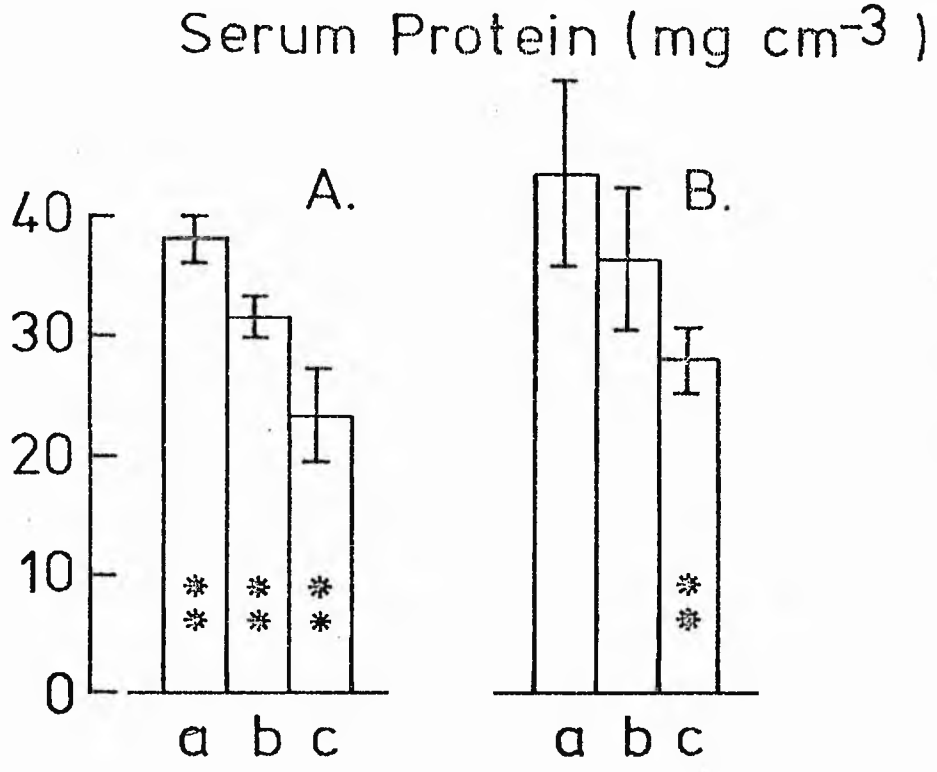
A.	S. trutta	a	15.5 ± 0.5°C	n = 5
		b	9.0 ± 0.5°C	n = 5
		c	4.5 ± 0.5°C	n = 5
B.	C. Carpio	a	22.0 ± 0.5°C	n = 5
		b	15.5 ± 0.5°C	n = 6
		c	9.0 ± 0.5°C	n = 6

** $P = <0.01$

*** $P = <0.001$

Level of significant difference between mean values.


Haematocrit (%)
40
30
20
10
0
A.
B.
a b c
a b c


Serum Protein (mg cm-3)
40
30
20
10
0
A.
B.
a b c
a b c

D. Heavy metals and the immune response to MS2 bacteriophage

i. Nickel, zinc, copper and chromium

The effect of exposure to four heavy metal solutions (0.75 mg Ni dm^{-3}, 1.06 mg Zn dm^{-3}, 0.29 mg Cu dm^{-3} and 1.01 mg Cr dm^{-3}) on the humoral immune response of S. trutta and C. carpio, maintained at 15.5°C was examined initially. The pH of the water, 7.83 ± 0.02, and the total hardness as $CaCO_3$, 206.9 ± 1.6 mg dm^{-3}, were stable throughout the experiment. The unexposed control groups of fish were found to produce primary and secondary immune responses equivalent to those observed earlier using the 10^9 PFU MS2-FIA and secondary 10^9 PFU MS2-saline regime of inoculation.

The time course of the humoral antibody response of each heavy metal group is compared to the control response in figures 16, 17 and Appendix 15, the SD_{50} values of antibody being plotted against time in days after primary inoculation. Peak primary and secondary antibody titres and plateau titres, derived by pooling titres subsequent to day 28, have been documented in table 16.

a. S. trutta

Except in the case of zinc-exposed trout (figure 16B) the other three heavy metal groups were found to retain live MS2 bacteriophage in their sera 7d after primary inoculation and in the case of the chromium exposed fish live bacteriophage was also detected 7d after secondary inoculation. The initial rates of antibody formation were found not to be depressed though primary peak titres were reduced significantly in all the heavy metal exposed groups except the copper exposed trout (table 16C). In the latter the humoral antibody response was found to be delayed by a period of 7d and remained out of phase with the control response even after the control peak antibody titre had been reached and had started to fall.

FIGURE 16

Immune response of *S. trutta* to *MS2* bacteriophage in the presence of dosed heavy metals.

Mean antibody titre values (SD_{50}) for six fish are plotted, together with $\pm$2SE bars.

A. □ 0.75 - 0.01 mg Ni dm^{-3} Nickel-exposed fish

B. ▲ 1.06 - 0.01 mg Zn dm^{-3} Zinc-exposed fish

C. + 0.29 - 0.01 mg Cu dm^{-3} Copper-exposed fish

D. ■ 1.01 - 0.01 mg Cr dm^{-3} Chromium-exposed fish

• Control fish unexposed to heavy metal (plotted in each diagram)

$+^{1}$ Inset numerals indicate the number of mortalities within that week.

* Live bacteriophage in the sera

All fish were inoculated on day 0 with 1.95×10^{9} PFU *MS2*-Freund's Incomplete Adjuvant. A secondary inoculation of 1.95×10^{9} *MS2*-Saline was administered on day 49 (↑)

Water temperature $15.5 \pm 0.5^{\circ}C$

Water hardness (total) 206.9 ± 1.6 mg dm^{-3} as $CaCO_3$

Water pH 7.83 ± 0.02

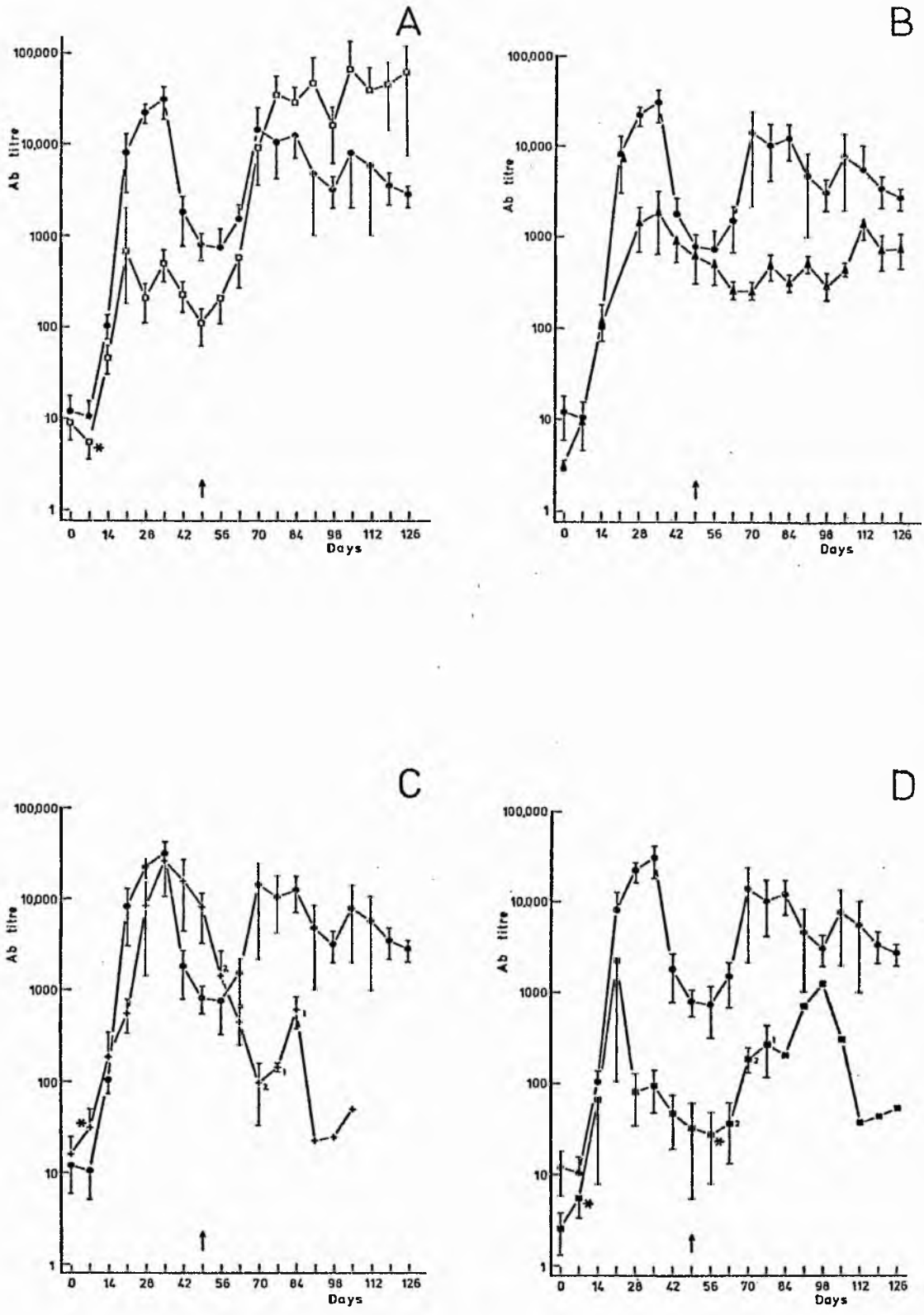

A
B
C
D
Ab titre
100,000
10,000
1000
100
10
1
0
14
28
42
56
70
84
98
112
126
Days

FIGURE 17

Immune response of *C. carpio* to *MS2* bacteriophage in the presence of dosed heavy metals

Mean antibody titre values (SD_{50}) for n fish are plotted, together with $\pm$2SE bars

A. □ 0.75 $\pm$ 0.01 mg Ni dm^{-3} Nickel-exposed fish n = 5

B. ▲ 1.06 $\pm$ 0.01 mg Zn dm^{-3} Zinc-exposed fish n = 5

C. + 0.29 $\pm$ 0.01 mg Cu dm^{-3} Copper-exposed fish n = 5

■ 1.01 $\pm$ 0.01 mg Cr dm^{-3} Chromium-exposed fish n = 5

● Control fish unexposed to heavy metal (plotted in each diagram) n = 6

$+^{1}$ Inset numerals indicate the number of mortalities within that week

* Live bacteriophage in the sera

All fish were inoculated on day 0 with 1.95×10^{9} PFU *MS2*-Freund's Incomplete Adjuvant. A secondary inoculation of 1.95×10^{9} PFU *MS2*-Saline was administered on day 56 (↑)

Water temperature 15.5 $\pm$ 0.5°C

Water hardness (total) 206.9 $\pm$ 1.6 mg dm^{-3} as $CaCO_3$

Water pH 7.83 $\pm$ 0.02

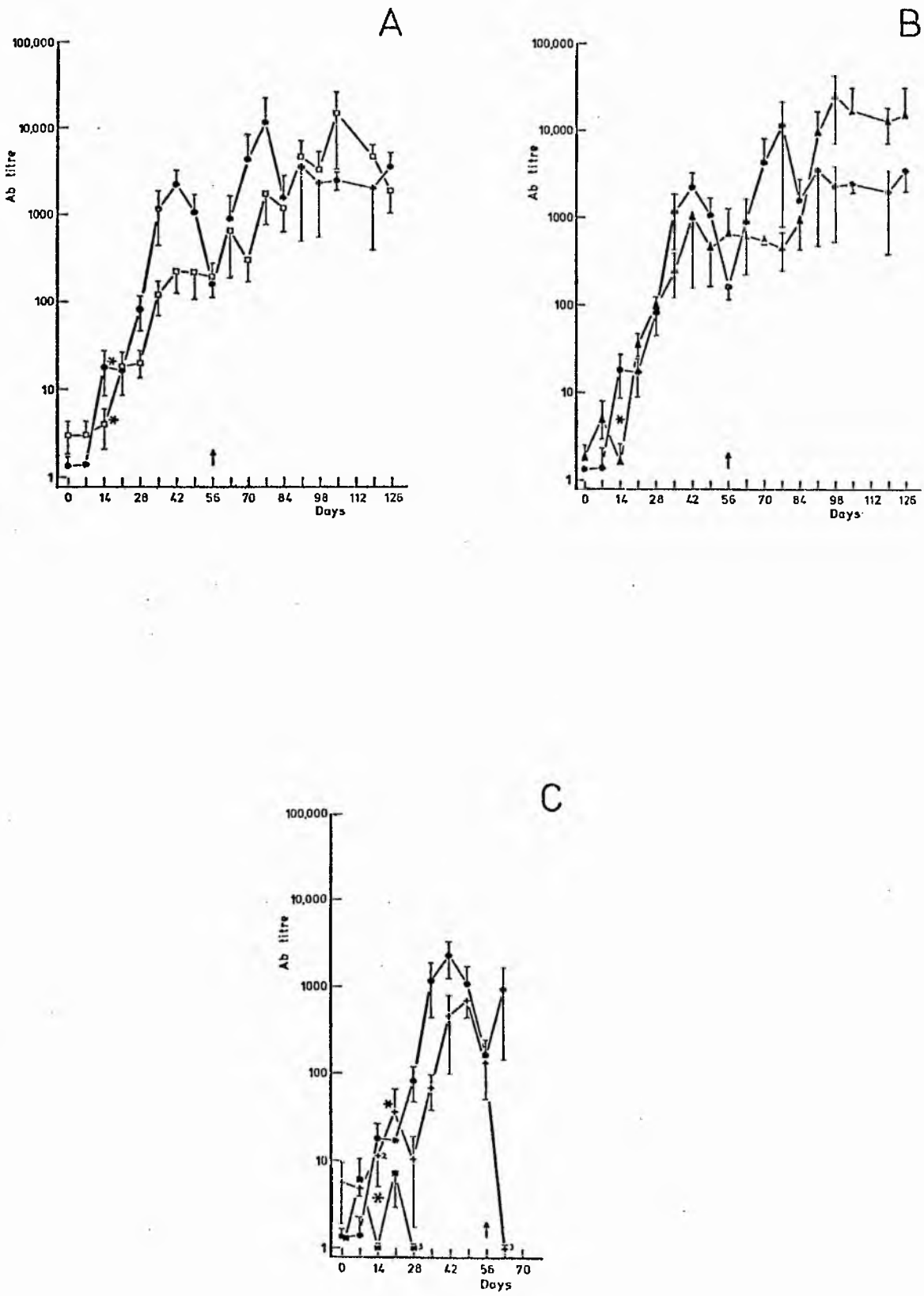

A
B
C
Ab titre
Days
100,000
10,000
1000
100
10
1
0
14
28
42
56
70
84
98
112
126

Table 16 The effects of heavy metals on the immune response to MS2 bacteriophage:

Day of peak response and peak titres of primary and secondary response, mean ± SE. Plateau titres; secondary response titres from day 28 onwards after secondary inoculation, mean ± SE.

A. S. trutta (n = 6 fish, per treatment except where indicated for secondary response).

Heavy metal $mg\ dm^{-3}$	Primary Response: Day of Peak titre	Primary Response: Antibody titre $\bar{x} \pm SE$		Secondary Response: Day of Peak titre	Secondary Response: Antibody titre $\bar{x} \pm SE$		Plateau titres $\bar{x} \pm SE$	n serum samples used
Control (nil)	35	30448 ±6035		21	14652 ±6230		6559 ±651	42
0.75 Ni	21	692 ••• ± 257	***	28	35554 ±11124		46288 •• ±3762	42
1.06 Zn	21	8168 ••• ±2594	**	28	513 ••• ±73.1		686 ••• ±80.5	42
0.29 Cu	35	26201 ±7932	***	35	618 •• ±122	(n=2)	32.9 ••• ±4.4	4
1.01 Cr	21	2284 ••• ±1089	**	28	284 ••• ±82.5	(n=2)	396 ••• ±90.5	7

B. C. carpio

Heavy Metal $mg\ dm^{-3}$	Primary Response: Day of Peak titre	Primary Response: Antibody titre $\bar{x} \pm SE$		Secondary Response: Day of Peak titre	Secondary Response: Antibody titre $\bar{x} \pm SE$	Plateau titres $\bar{x} \pm SE$	n serum samples used	n fish
Control (nil)	42	2215 ±505	**	21	11598 ±5394	2647 ±176	36	6
0.75 Ni	42	229 •• ±51.1	***	21	1753 ±393	5234 • ±1055	30	5
1.06 Zn	42	1085 ±364	**	42	26273 ±9383	15877 ••• ±590	30	5
0.29 Cu	49	571 •• ± 66.9		-	-	-	-	3
1.01 Cr	21	7.2 •• ±2.2		-	-	-	-	5

Significant difference between primary and secondary titre responses

** P = <0.01 ***P = <0.001

Significance differences from control titre values

•P = <0.05 ••P = <0.01 •••P = <0.001

In the secondary response fish all but the nickel-exposed trout (figure 16A) showed a statistically significant depression of antibody response (table 16A). The nickel-exposed fish produced an antibody titre response in step with the controls (figure 16A) rising above the primary peak titre values and also rising slightly above the peak titre of the secondary responding controls. The plateau titre of the nickel-exposed group was significantly higher than the control (table 16A) though peak titres were not significantly different.

The zinc-exposed trout did not produce an increase in secondary response titre, though they did retain a steady level of antibody titre which became slightly elevated from day 63 after secondary inoculation. The plateau titres of the zinc exposed trout were found to be significantly lower than those of the control group (table 16A).

Both the chromium and copper-exposed groups (figures 16 C, D) of trout experienced mortalities during the secondary response and demonstrated symptoms typical of heavy metal toxicity. In the early stage of toxicosis, about one week before death, the fish assumed a 'nose-down' posture and were unable to swim in a straight line. The fish were often observed to circle with the head as the axis of the movement, a form of slow whirling. One to two days before death the fish lost the ability to co-ordinate activity, and, although active muscular movements were observed, the fish were unable to float upright and were thus found floating on their sides. At this stage the fish were removed from the aquaria, blood samples were taken and portions of the organs fixed in formal saline. Those fish which had stopped respiring before examination were found to be rigid and the muscle tissues turgid.

The trout exposed to chromium (figure 16D) produced an initial increase in secondary antibody titre which reached, at +28d after inoculation, a peak that was

substantially lower than in the controls (figure 16A). At this time four of the six chromium-exposed trout had been removed with symptoms of toxicity and another fish was already showing early stage symptoms. One fish, however, did survive to the end of the experiment and produced an increasing antibody titre which reached a peak at +49d after secondary inoculation and then fell.

The copper-exposed trout were found to give no immediate response to the secondary inoculation of bacteriophage and the titre levels produced in the primary response continued to fall. Four trout which demonstrated toxicity symptoms were removed within the first 21d after secondary inoculation and the remaining two fish produced a small rise in antibody titre at 28 to 35d into the secondary response (figure 16C). All the copper-exposed fish developed toxicity symptoms before the termination of the experiment. One trout survived for 56d after secondary inoculation, a total of 119d of copper exposure.

b. C. carpio

Live MS2 bacteriophage was found to be retained in the sera of C. carpio for a longer period of time after primary inoculation when compared to S. trutta. In this experiment the bacteriophage was retained in the serum of one carp from each treatment at +14d, which in the case of the copper treated fish was not eliminated until +21d to +28d after primary inoculation. All the surviving fish cleared the secondary inoculation of bacteriophage within the first 7d (figure 17, appendix 15).

The copper and chromium levels used proved to be more toxic to the carp than the trout, though similar symptoms of toxicity were observed. Only three out of the five copper-exposed carp survived to reach a peak antibody titre which was 7d after the control peak and which was significantly lower (table 16B). The remaining fish of the copper

group died 9d after secondary inoculation and although the +7d secondary sera contained no live bacteriophage no antibody activity could be detected in these fish.

Carp exposed to chromium showed symptoms of toxicity 2d after the +28d primary sera were taken. The response to the primary inoculation of bacteriophage was erratic, alternately rising and falling, with a mean maximum titre value of 7.2. The final sera taken at +28d had no detectable neutralisation activity.

The zinc-exposed carp produced a similar response to the nickel-exposed trout (figure 17B) though the primary peak titre was not significantly lower (table 16B). In the secondary response fish a rise in antibody titre was retarded for 28d but the subsequent titre rose above the peak control value, although not significantly different from it. The secondary response plateau titres were, however, significantly higher than the control.

The peak primary response titre of the nickel-exposed carp was significantly suppressed (figure 17A, table 16B) and the secondary response followed a similar pattern to the zinc-exposed group, in which the initial titre was retarded and then rose above the control titre values. The secondary titres of the nickel-exposed fish did not rise as rapidly as the controls, they were significantly lower at 21d post-inoculation. However, the titre continued to rise and reached a maximum mean titre 56d after inoculation which was significantly higher than the primary response titre but not significantly different from the peak secondary response of the control. The secondary response plateau titre of the nickel-exposed fish was just significantly greater than that of the control (table 16B).

c. Weight and length

The experimental fish were observed to feed well throughout the experiment, except in those fish in which toxicity symptoms were observed. Significant differences from control values were observed in the total percentage changes in weight andllength throughout the experiment (figure 18). The controls, and the nickel- and zinc-exposed fish were observed to have increased in weight, the increase being significantly greater in the nickel- and zinc-exposed trout. A larger weight increase was also observed in the nickel-exposed carp but the zinc-exposed group produced only a small and non-significant weight increase. The exposure to copper and chromium was found to stop growth and produce a decrease in weight which was significant in both trout and carp. The weight results were reflected in the changes in length observed in trout. The increase in length of the zinc-exposed trout was not significantly different from the control but that of the nickel-exposed group was significant and correlated with the weight change of this group.

Similarly the decrease in weight observed in copper- and chromium-exposed fish was related to a significantly lower length in the chromium group and a decrease in length of the copper group. No significant length change was observed in the control group of carp though the trend in the heavy metal exposed groups was for a decrease in length. The decreases in length were significant in the zinc- and chromium-exposed groups of carp.

d. Haematocrit

Haematocrit values were significantly lower in copper- and chromium-exposed fish (figure 18) and a smaller depression was observed in the nickel-exposed fish. The carp exposed to zinc also had a significantly depressed haematocrit when compared to the control group.

FIGURE 18

Heavy metal exposure experiments: Body weight and length changes, haematocrits, serum protein and serum heavy metal levels.

Mean values ± SE from n fish are plotted

A. S. trutta n = 6

B. C. carpio n = 5 (Control fish n = 6)

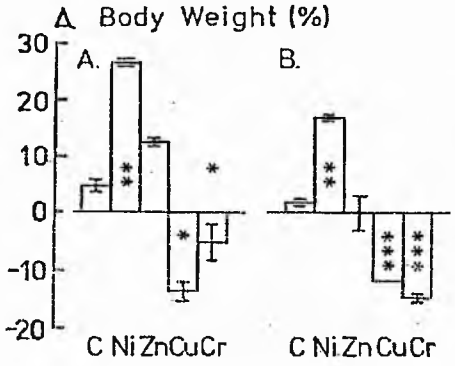
Ni 0.75 ± 0.01 mg Ni dm^{-3} Nickel-exposed fish
Zn 1.06 ± 0.01 mg Zn dm^{-3} Zinc-exposed fish
Cu 0.29 ± 0.01 mg Cu dm^{-3} Copper-exposed fish
Cr 1.01 ± 0.01 mg Cr dm^{-3} Chromium-exposed fish
C Control fish unexposed to heavy metals

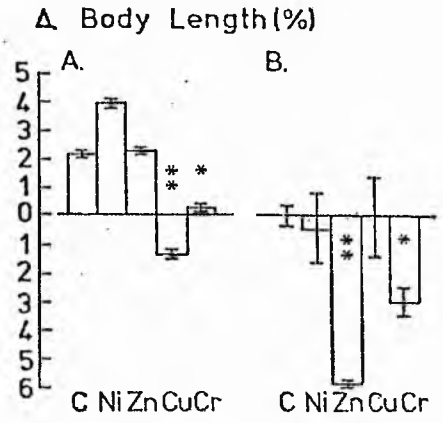
 Control fish serum heavy metal values

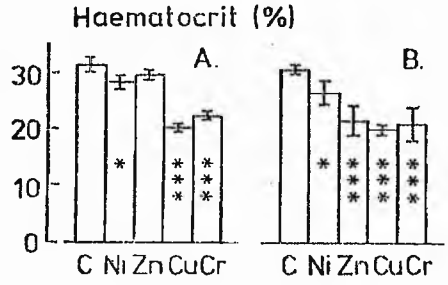
* P = <0.05

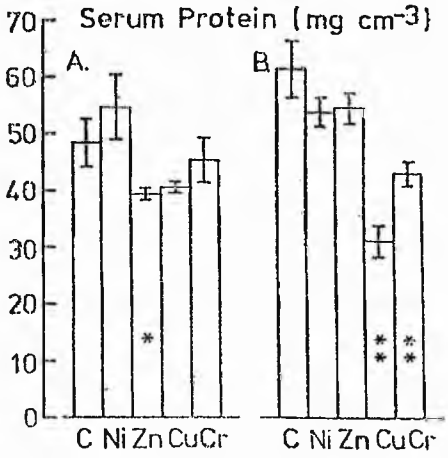
** P = <0.01 Level of significant difference from the control values

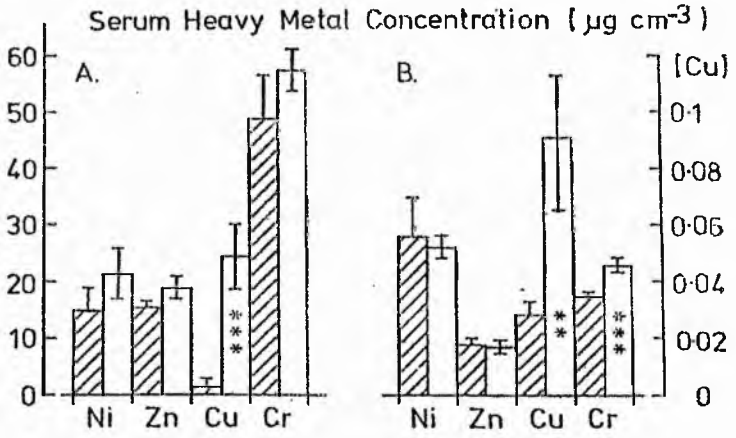
*** P = <0.001


Δ Body Weight (%)
A.
B.
30
20
10
0
-10
-20
C Ni Zn Cu Cr
C Ni Zn Cu Cr


Δ Body Length (%)
A.
B.
5
4
3
2
1
0
1
2
3
4
5
6
C Ni Zn Cu Cr
C Ni Zn Cu Cr


Haematocrit (%)
A.
B.
30
20
10
0
C Ni Zn Cu Cr
C Ni Zn Cu Cr


Serum Protein (mg cm-3)
A.
B.
70
60
50
40
30
20
10
0
C Ni Zn Cu Cr
C Ni Zn Cu Cr


Serum Heavy Metal Concentration (μg cm-3)
A.
B.
60
50
40
30
20
10
0
[Cu]
0·1
0·08
0·06
0·04
0·02
0
Ni Zn Cu Cr
Ni Zn Cu Cr

e. Serum protein

Total serum protein was found to be depleted in zinc- and copper-exposed trout and copper- and chromium-exposed carp (figure 18)

f. Serum heavy metal

Serum levels of all four of the heavy metals were found to be elevated above the control values in trout although this was statistically significant only for the copper-exposed fish. Serum copper was also significantly elevated in the copper-exposed carp as was chromium in the chromium-exposed group (figure 18).

g. Histology

No gross morphological differences of the internal tissues of trout and carp were observed by light microscopy between the heavy metal exposed fish and the controls. Changes were observed, however, in the epidermis and gills of certain groups. The epidermis of trout exposed to copper was found to be thinner than that of the control group (figure 45) and in chromium-exposed trout an increase in the number of epidermal mucous glands was observed (figure 46).

Structural changes were observed in the gills of trout exposed to copper characterised by a detachment of the epithelial layer of the secondary lamellae. In copper-exposed carp a thickening and fusion of the epithelium at the basis of the secondary lamellae was observed (figure 47). A similar thickening and fusion was observed in carp exposed to nickel, though in these fish there was also a protozoan infection associated with the gills (figure 48).

A fungal infection was noted in the kidney of one trout specimen exposed to chromium which had destroyed a large area

of the kidney pulp and had initiated leucocyte infiltration into the infected area. (figure 49)

ii. Zinc levels and the immune response

A series of four concentrations of zinc solution (0.14,0.53, 1.04 and 2.13 mg Zn dm^{-3}) were used for a more extensive examination of the effects of heavy metal concentration on the humoral immune response of S. trutta and C. carpio, maintained at 15.5°C. As in the previous experiment the water pH, 7.81 ± 0.02, and the total hardness as $CaCO_3$, 198.2 ± 3.9mg dm^{-3}, were stable throughout the experiment. Zinc was chosen for its positive action on the immune response at the non-toxic level used in the previous experiment and the lack of observed morphological change in the tissues examined. The levels of zinc used in the experiment were found not to produce symptoms of toxicity in the course of primary and secondary response. However, mortality of carp exposed to 0.14 mg Zn dm^{-3} occurred 35d after the primary inoculation of MS2 bacteriophage. The latter fish were found on examination to have a heavy infection of the ciliate Chilodonella sp. in the mucus of the gills and skin. The ciliate protozoan was also found in very small numbers in the mucus of the 0.53 mg Zn dm^{-3} group of carp but produced no apparent effect.

The humoral antibody response SD_{50} values for trout and carp were plotted with time and each level of zinc concentration was compared with the response of control fish in figures 19 and 20 (Appendix 17). The response of the control fish was again comparable with the MS2-FIA inoculated fish of the previous experiments. Peak primary and secondary antibody titres were tabulated in table 17 along with mean secondary plateau titres, derived from pooled titres subsequent to day 28.

FIGURE 19

Immune response of *S. trutta* to *MS2* bacteriophage in the presence of dosed zinc concentrations

Mean antibody titre values (SD_{50}) for six fish are plotted, together with $\pm$2SE bars

A. o 0.14 ± 0.01 mg Zn dm^{-3})
B. + 0.53 ± 0.005 mg Zn dm^{-3}) Zinc-exposed fish
C. ■ 1.04 ± 0.02 mg Zn dm^{-3})
D. ▲ 2.13 ± 0.02 mg Zn dm^{-3})

● Control fish unexposed to zinc

All fish were inoculated on day 0 with 1.58×10^9 PFU *MS2*-Freund's Incomplete Adjuvant. A secondary inoculation of 1.58×10^9 PFU *MS2*-Saline was administered on day 49 (↑)

Water temperature $15.5 \pm 0.5^{\circ}C$

Water hardness (total) 198.2 ± 3.9 mg dm^{-3} as $CaCO_3$

Water pH 7.81 ± 0.02

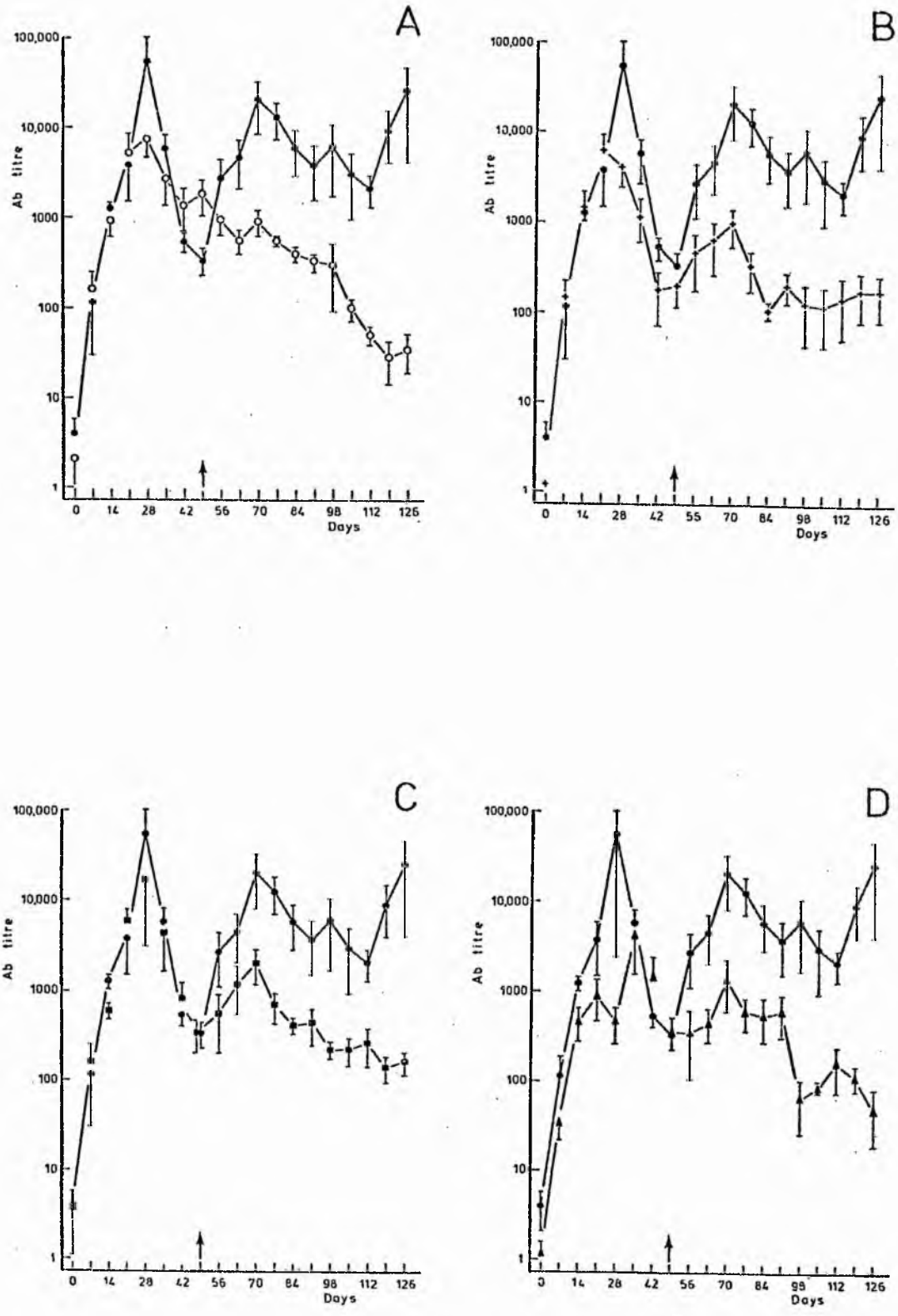

A
B
C
D
Ab titre
100,000
10,000
1000
100
10
1
0
14
28
42
56
70
84
98
112
126
Days

FIGURE 20

Immune response of C. carpio to MS2 bacteriophage in the presence of dosed zinc concentrations

Mean antibody titre values (SD_{50}) for six fish are plotted, together with $\pm$2SE bars

A. o 0.14 $\pm$ 0.01 mg Zn dm^{-3}

\+ 0.53 $\pm$ 0.005 mg Zn dm^{-3}

B. ■ 1.04 $\pm$ 0.02 mg Zn dm^{-3}

C. ▲ 2.13 $\pm$ 0.02 mg Zn dm^{-3}

} Zinc-exposed fish

● Control fish unexposed to zinc

$+^{1}$ Inset numeral indicates the number of mortalities within that week

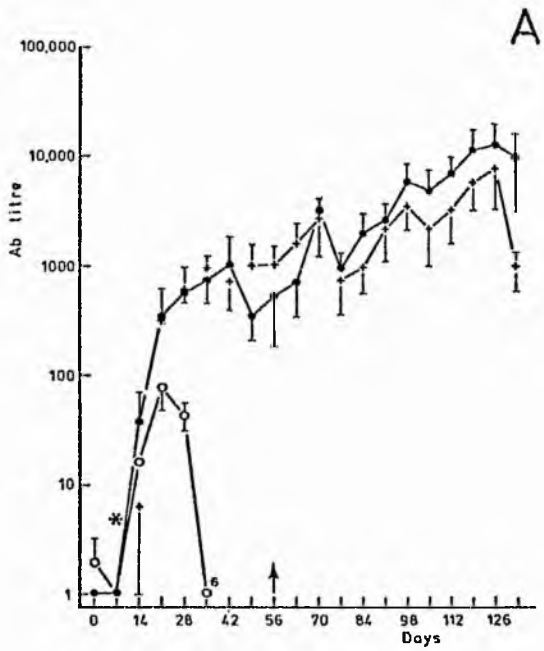
* Live bacteriophage in the sera

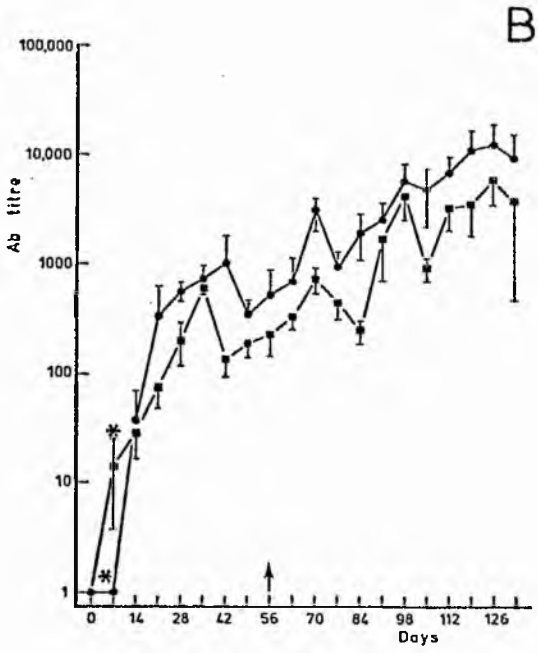
All fish were inoculated on day 0 with 1.58×10^{9} PFU MS2-Freund's Incomplete Adjuvant. A secondary inoculation of 1.58×10^{9} PFU MS2-Saline was administered on day 56 (↑).

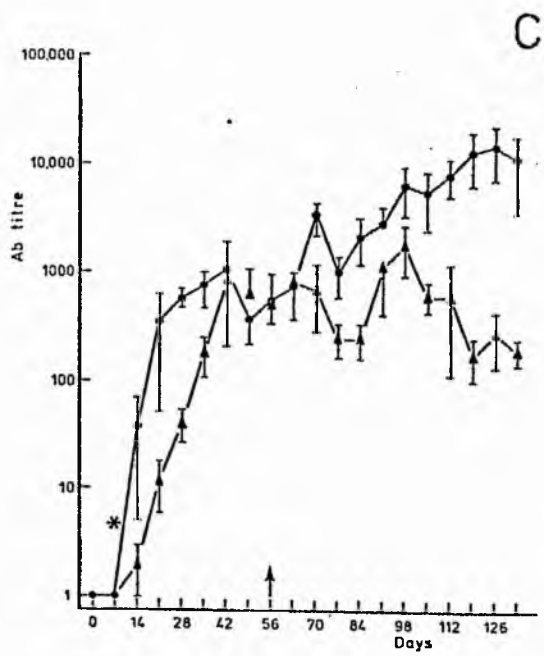
Water temperature 15.5 $\pm$ 0.5°C

Water hardness (total) 198.2 $\pm$ 3.9 mg dm^{-3} as $CaCO_3$

Water pH 7.81 $\pm$ 0.02


A
100,000
10,000
1000
100
10
1
Ab titre
0
14
28
42
56
70
84
98
112
126
Days


B
100,000
10,000
1000
100
10
1
Ab titre
0
14
28
42
56
70
84
98
112
126
Days


C
100,000
10,000
1000
100
10
1
Ab titre
0
14
28
42
56
70
84
98
112
126
Days

a. S. trutta

The humoral immune response of S. trutta was found to be similar over the range of zinc concentrations used. The treated groups followed the same initial rate of primary antibody formation as found in the control group. The highest concentration of zinc, 2.13 mg dm^{-3}, was found to produce an early suppression of the immune response at days 14 to 28 after bacteriophage inoculation and a maximum peak titre at +35d, 7d after the other groups. With the exception of the 1.04 mg Zn dm^{-3} exposed group of trout the peak primary titres were significantly lower than the control value, though there was no significant difference observed between these three titre values.

After secondary inoculation the zinc treated trout produced a small elevation in antibody titre 21d after inoculation. These titres were significantly lower than the primary peak titres and the control secondary peak titre. The differences between the peak titres of all the four zinc treated groups were again not significant. From 21d after secondary inoculation the antibody titres of the zinc treated fish gradually decreased and the plateau titres were thus significantly lower than the control (table 17A), though there was no significant difference or progression observed between the four zinc-exposed groups.

b. C. carpio

In the primary responding C. carpio no significant difference was observed between the control fish and the 0.53 to 1.04 mg Zn dm^{-3} exposed groups (table 17B). The Chilodonella infected carp exposed to 0.14 mg Zn dm^{-3} ceased to increase the antibody titre at 21d and this titre value was significantly below the primary peak titre of the control. The antibody titre of the infected carp declined and was reduced to non-significant levels at +35d, just before death. The highest concentration of zinc exposure

Table 17 The effects of zinc on the immune response to MS2 bacteriophage: Day of peak response and peak titres of primary and secondary response, mean ± SE. Plateau titres; secondary response titres from day 28 onwards after secondary inoculation, mean ± SE.

A. S. trutta (n = 6 fish per treatment)

Zinc mg dm^{-3}	Primary Response			Secondary Response			Plateau titres $\bar{x} \pm SE$	n serum samples used
	Day of Peak titre	Antibody titre $\bar{x} \pm SE$		Day of Peak titre	Antibody titre $\bar{x} \pm SE$			
Control (nil)	28	54356 ±25376		21	20010 ±6339		8271 ±1289	42
0.14 Zn	28	7226$^{\bullet\bullet}$ ±1363	**	21	892$^{\bullet\bullet\bullet}$ ±141		221$^{\bullet\bullet\bullet}$ ±35.5	42
0.53 Zn	21	6141$^{\bullet\bullet}$ ±1677	**	21	951$^{\bullet\bullet\bullet}$ ±216		172$^{\bullet\bullet\bullet}$ ±12.5	42
1.04 Zn	28	16937 ±6935	**	21	1991$^{\bullet\bullet}$ ±409		320$^{\bullet\bullet\bullet}$ ±32.2	42
2.13 Zn	35	4345$^{\bullet\bullet}$ ±1413		21	1408$^{\bullet\bullet}$ ±404		282$^{\bullet\bullet\bullet}$ ±35.3	42

B. C. carpio (n = 6 fish per treatment)

Zinc mg dm^{-3}	Primary Response			Secondary Response			Plateau titres $\bar{x} \pm SE$	n serum samples used
	Day of Peak titre	Antibody titre $\bar{x} \pm SE$		Day of Peak titres	Antibody titre $\bar{x} \pm SE$			
Control (nil)	42	1026 ±354	***	14	3153 ±502		6868 ±787	48
0.14 Zn	21	79.1$^{\bullet}$ ±15.2		-	-		-	-
0.53 Zn	35	971 ±121	*	14	2868 ±803		3310$^{\bullet\bullet\bullet}$ ±437	48
1.04 Zn	35	625 ±25.6		14	783$^{\bullet\bullet\bullet}$ ±93.9		2928$^{\bullet\bullet\bullet}$ ±352	48
2.13 Zn	42	867 ±334		14	713$^{\bullet\bullet\bullet}$ ±221	***	580$^{\bullet\bullet\bullet}$ ±90.5	48

Significant differences between primary and secondary titre response

*P = <0.05 **P = <0.01 ***P = <0.001

Significant differences from control titre values

$^{\bullet}$P = <0.05 $^{\bullet\bullet}$P = <0.01 $^{\bullet\bullet\bullet}$P = <0.001

used, 2.13 mg Zn dm^{-3}, produced no significant depression of the primary peak antibody titre, though the initial rate of antibody formation and the general rise in antibody titre were delayed.

The response to a secondary inoculation of MS2 bacteriophage in the control carp was found to produce a normal enhanced peak antibody titre, however, the subsequent maintained response differed from the previous experiments by continuing to rise in titre , reaching a peak 70d after inoculation. The carp exposed to the high levels of zinc, 1.04 and 2.13 mg Zn dm^{-3}, were found to have significantly lower peak secondary response titres than the control group. The subsequent titres rose once more in phase with the control but maintained the lower titre level. Antibody titres following the secondary peak response were significantly lower in all the zinc-exposed fish and the meaned plateau titre was found to be depressed with increasing zinc concentration (table 17B). Only the plateau titre of the highest zinc concentration group, 2.13 mg Zn dm^{-3}, was found to be significantly lower than the other two zinc exposed groups.

c. Weight and length

In the zinc-exposed trout the per cent change in body weight was observed to be significantly lower than that of the control trout (figure 21). The trout exposed to 2.13 mg Zn dm^{-3} were found to produce the smallest mean body weight increase. The body weight changes did not show any further relationship to zinc concentration however.

Two groups of zinc-exposed carp, 0.53 and 2.13 mg Zn dm^{-3}, demonstrated body weight increases slightly higher than that of the controls. Only the 1.04 mg Zn dm^{-3} exposed group indicated a significantly depressed increase in weight.

FIGURE 21

Zinc experiments: Body weight and length changes, haematocrits, serum protein and serum zinc levels.

Mean value ± SE from six fish are plotted

A. S. trutta

B. C. carpio

a 0.14 ± 0.01 mg Zn dm^{-3}
b 0.53 ± 0.005 mg Zn dm^{-3}
c 1.04 ± 0.02 mg Zn dm^{-3}
d 2.13 ± 0.02 mg Zn dm^{-3}

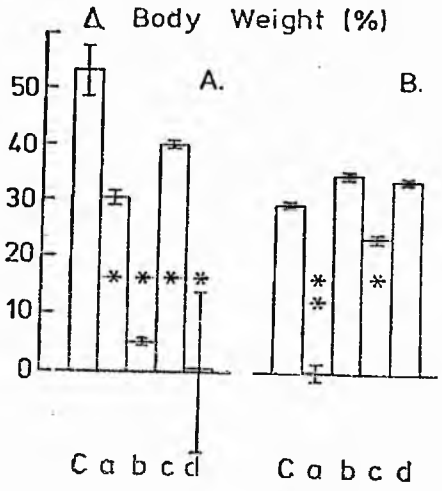
} Zinc-exposed fish

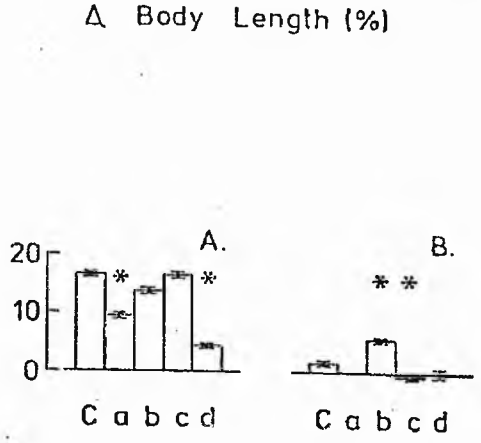
Control fish unexposed to zinc

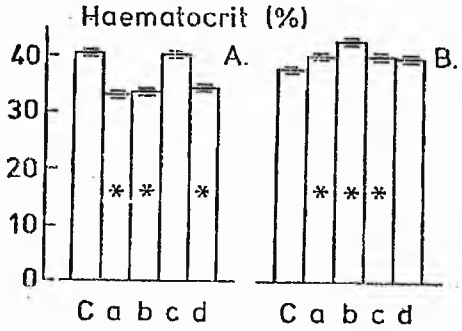
* P = < 0.05

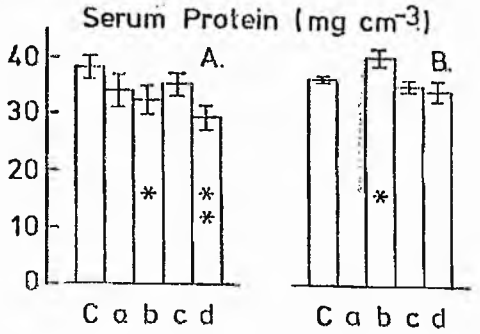
** P = < 0.01

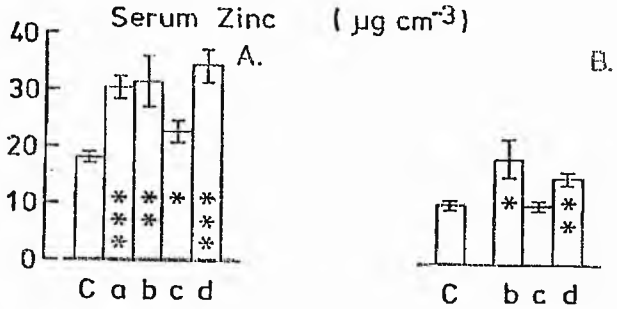
*** P = < 0.001


Δ Body Weight (%)
A.
B.
50
40
30
20
10
0
C a b c d
C a b c d


Δ Body Length (%)
A.
B.
20
10
0
C a b c d
C a b c d


Haematocrit (%)
A.
B.
40
30
20
10
0
C a b c d
C a b c d


Serum Protein (mg cm-3)
A.
B.
40
30
20
10
0
C a b c d
C a b c d


Serum Zinc (μg cm-3)
A.
B.
40
30
20
10
0
C a b c d
C b c d

The percentage changes observed in body length were likewise found to show no significant trend with zinc concentration, though again the 0.14 and 2.13 mg Zn dm^{-3} exposed trout groups showed significantly lower increases in body length, as did the 1.04 mg Zn dm^{-3} exposed carp.

No differences in feeding behaviour were observed between the experimental groups of trout and carp.

d. Haematocrit

Final haematocrits were found to be significantly reduced in zinc-exposed trout, with the exception of the 1.04 mg Zn dm^{-3} exposed group which showed no significant difference from the control (figure 21). The converse result was observed in the zinc-exposed carp with an increased haematocrit which was significantly different from the control value except in the case of the 2.13 mg Zn dm^{-3} exposed group.

e. Serum protein

Total serum protein levels were found to be depressed in zinc-exposed trout, though these lower levels were only significant in the 0.53 and 2.13 mg Zn dm^{-3} exposed groups (figure 21). The zinc-exposed carp on the other hand showed no significant decrease in protein levels and the 0.53 mg Zn dm^{-3} exposed carp were even found to show a significant elevation of the serum protein concentration.

f. Serum zinc

Although the levels of serum zinc were elevated significantly in the majority of the zinc-exposed fish those fish which were exposed to the higher concentrations of zinc did not show significantly higher serum zinc levels (figure 21). Levels of serum zinc were found to be lowest in the 1.04 mg Zn dm^{-3} exposed fish, significantly higher

than the control level in trout but not so in carp.

g. Histology

As predicted by the results of the previous experiment no gross histological changes were observed in the fish exposed to 1.04 mg Zn dm^{-3} and lower levels. In trout exposed to 2.13 mg Zn dm^{-3} an increase in the number and size of the mucous glands in epidermal sections was observed. Although no epidermal changes were observed in the carp exposed to zinc at the highest concentration changes were noted in the sections of the gill. A proliferation of tissue at the basis of the secondary lamellae and a loosening of the epithelium was observed, as already seen in the copper-exposed trout (figure 47).

Of the internal tissues only the trout kidney of the 2.13 mg Zn dm^{-3} exposed group was seen to be damaged. The columnar epithelial cells of a number of kidney tubules were observed in sections from these fish to have started to break down at the border of the lumen (figure 50).

iii. Inoculated lead and cadmium and the immune response of S. trutta

The inoculation of a tertiary dose of MS2 bacteriophage was found to produce consistently high levels of neutralisation antibody in S. trutta which were maintained over the experimental period of 119d and were significantly higher than secondary response plateaus. The effects of intraperitoneally inoculated concentrations of lead and cadmium on the tertiary immune response have been plotted against time in figures 22 and 23. These results have also been tabulated in tables 18 and 19. In the case of the lead inoculated trout, table 18, the antibody titres for three consecutive sampling weeks have been pooled and the means calculated. Results from the cadmium

experiment, table 19, have been used to compare the pooled minimum antibody titre values of each treatment with values obtained before cadmium inoculation. In the latter table pooled antibody titre values produced after a booster inoculation of 10^9 PFU MS2-Saline have been examined. The number of weeks over which antibody titre values were pooled are indicated in table 19.

a. Lead

Significant differences were observed between experimental groups in the antibody titres of sera taken just before lead inoculation (table 18). The control and 0.3 mg Pb groups of trout were found to contain fish all producing very high responses and the 0.05 and 0.01 mg Pb groups were all low responding fish. In the cadmium experiment antibody titres were thus examined 7d before cadmium inoculation in order that high and low responding fish could be mixed within the experimental group. The humoral antibody titres of the control fish remained high throughout the experiment and showed no detectable suppression after the inoculation of sterile saline at pH 3. Moreover an increase in titre was observed throughout the duration of the experiment.

A rapid fall in antibody titre was observed over a period of 21d in the trout inoculation with 0.3 mg Pb, after which mortality of the fish with very low humoral antibody titres gave the impression of a small titre rise in the surviving trout. The titre of the two surviving trout just before death at +49d was found not to be significant.

The group of trout inoculated with 0.1 mg Pb showed no decrease in antibody titre until 21d after the lead had been inoculated after which time the titre began to fall significantly until +49d. A recovery in antibody titre was then observed, a peak titre being reached at

FIGURE 22

Inoculated lead concentrations and the tertiary immune response of *S. trutta* to *MS2* bacteriophage

Mean antibody titre values (SD_{50}) for five fish are plotted, together with $\pm$2SE bars

Concentrations of lead inoculated on day 0

▲ 0.01 mg Pb 100 g^{-1}

□ 0.05 mg Pb 100 g^{-1}

+ 0.1 mg Pb 100 g^{-1}

o 0.3 mg Pb 100 g^{-1}

● Control fish, pH 3.0 Saline inoculated only

$+^{1}$ Inset numerals indicate the number of mortalities within that week

All fish were given primary and secondary inoculations of 10^9 PFU *MS2*. A tertiary inoculation of 10^9 PFU *MS2*-Saline was administered 21d before lead inoculation.

Water temperature $15.5 \pm 0.5^{o}C$

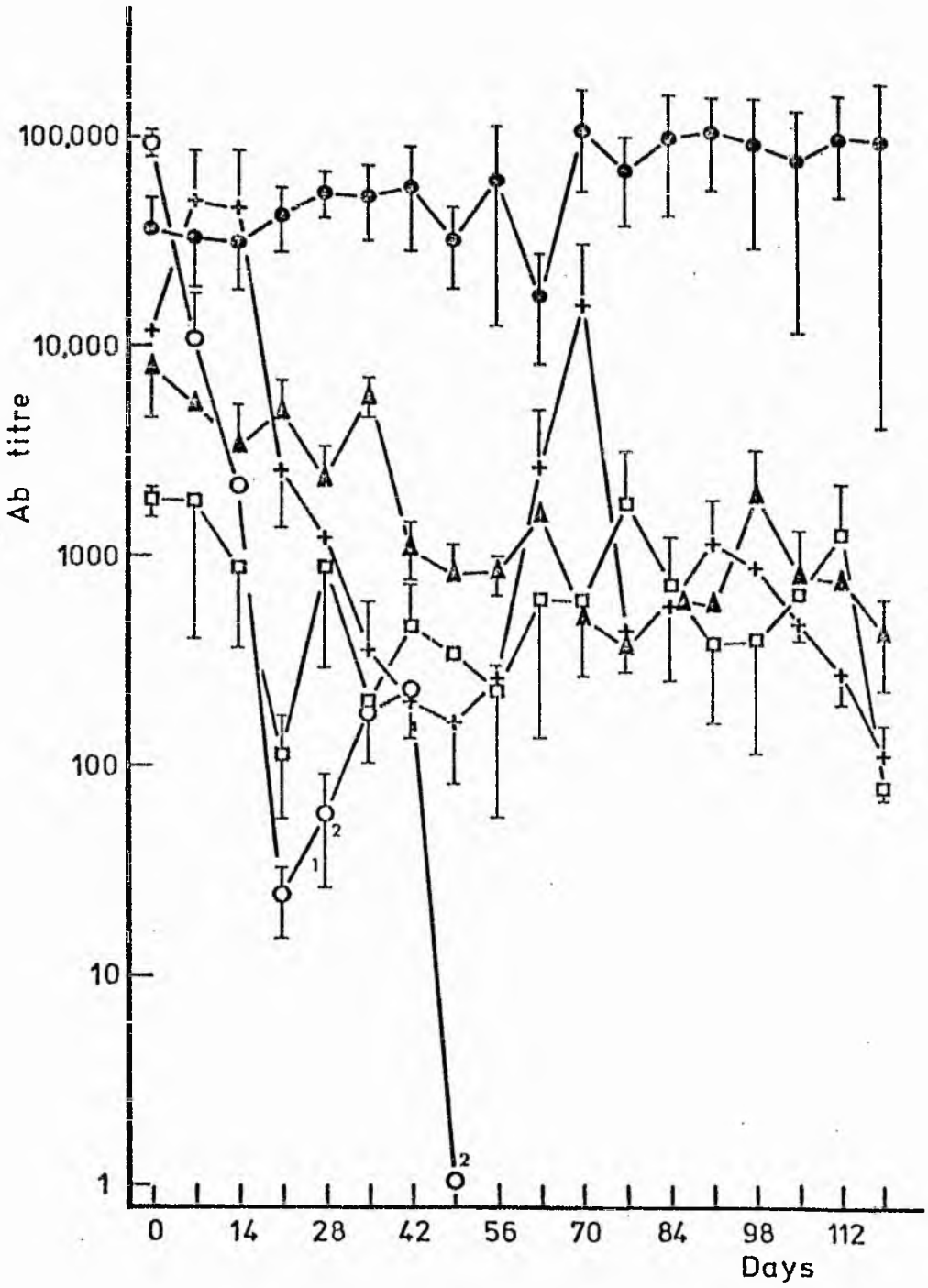

100,000
10,000
1000
100
10
1
Ab titre
0
14
28
42
56
70
84
98
112
Days

Table 18 The effects of inoculated lead on the tertiary immune response to MS2 bacteriophage in S. trutta

Days after Lead inoculation	Antibody titre $\bar{x} \pm SE$				
	Control	Lead inoculated mg Pb $100g^{-1}$ 0.3	0.1	0.05	0.01
0 to 14	33561 ±6676	52496 ±15957	68847 ±17334	1451*** ±254	5983*** ±791
21 to 35	49788 ±8193	46.4*** 8.1	43347 ±20488	403* ±107	4362 ±445
42 to 56	51237 ±8212	-	184*** ±16.9	343* ±66.5	908** ±80.9
63 to 77	67225 ±11625	-	6233** ±2547	863 ±249	819** ±124
84 to 98	98949 ±14897	-	885*** ±172	490* ±99.5	1084** ±218
105 to 119	92774 ±16835	-	229*** ±39.4	817 ±225	684** ±119

$\bar{n}$ = 15, 5 fish sampled for three consecutive weeks

Significant differences between 0 to 14d titres of Pb inoculated fish and control titres *** P = <0.001

Significant differences between titres of 0 to 14d groups and the following time groups for each dose level

* P = <0.05
** P = <0.01
*** P = <0.001

+70d and after which there was again a significant fall in titre.

Trout inoculated with 0.01 and 0.05 mg Pb were observed to show a declining antibody titre over the time course of the experiment, though the decline was not as marked as in the groups receiving higher concentrations of lead.

b. Cadmium

All three concentrations of inoculated cadmium were found to produce a significant depression in the antibody titre of trout which was related to cadmium concentration (figure 23, table 19). The highest dose, 0.2 mg Cd, significantly reduced the humoral antibody titre to a low level in 7d and to non-significant levels at death, 14d after inoculation of the cadmium.

No mortalities occurred in the other two groups inoculated with cadmium. The antibody titres of the 0.1 mg Cd inoculated trout were depressed immediately after inoculation and were significantly lower by +21d. From +35d the depression of the antibody titre ceased and a low level titre was maintained.

The depression of antibody titre in the 0.05 mg Cd inoculated trout was not immediate and in the initial stage after inoculation an increase in titre was observed before the depression commenced. By +70d a significant fall in antibody titre had been observed which reached a minimum level at +84d and was of a similar titre level to that observed in the 0.1 mg Cd inoculated fish.

An intraperitoneal inoculation of 10^9 PFU MS2 in saline which was administered to the 0.05 and 0.1 mg Cd groups of trout was found to stimulate an increase in antibody response (figure 23). In the 0.5 mg Cd group

FIGURE 23

Inoculated cadmium concentrations and the tertiary immune response of C. carpio to MS2 bacteriophage

Mean antibody titre values (SD_{50}) for five fish are plotted, together with $\pm$2SE bars

Concentration of cadmium inoculated on day 0 (↑)

- □ 0.05 mg Cd 100 g^{-1}
- \+ 0.1 mg Cd 100 g^{-1}
- o 0.2 mg Cd 100 g^{-1}
- ● Control fish, Saline inoculated only
- $+^{1}$ Inset numerals indicate the number of mortalities within that week
- ∀ Control fish removed from the shared aquarium to a new tank

All fish were given primary and secondary inoculations of 10^9 PFU MS2. A tertiary inoculation of 10^9 PFU MS2-Saline was administered 21d before cadmium inoculation A booster inoculation of 10^9 PFU MS2-Saline was administered to cadmium inoculated fish only (↑).

Water temperature 15.5 $\pm$ 0.5°C

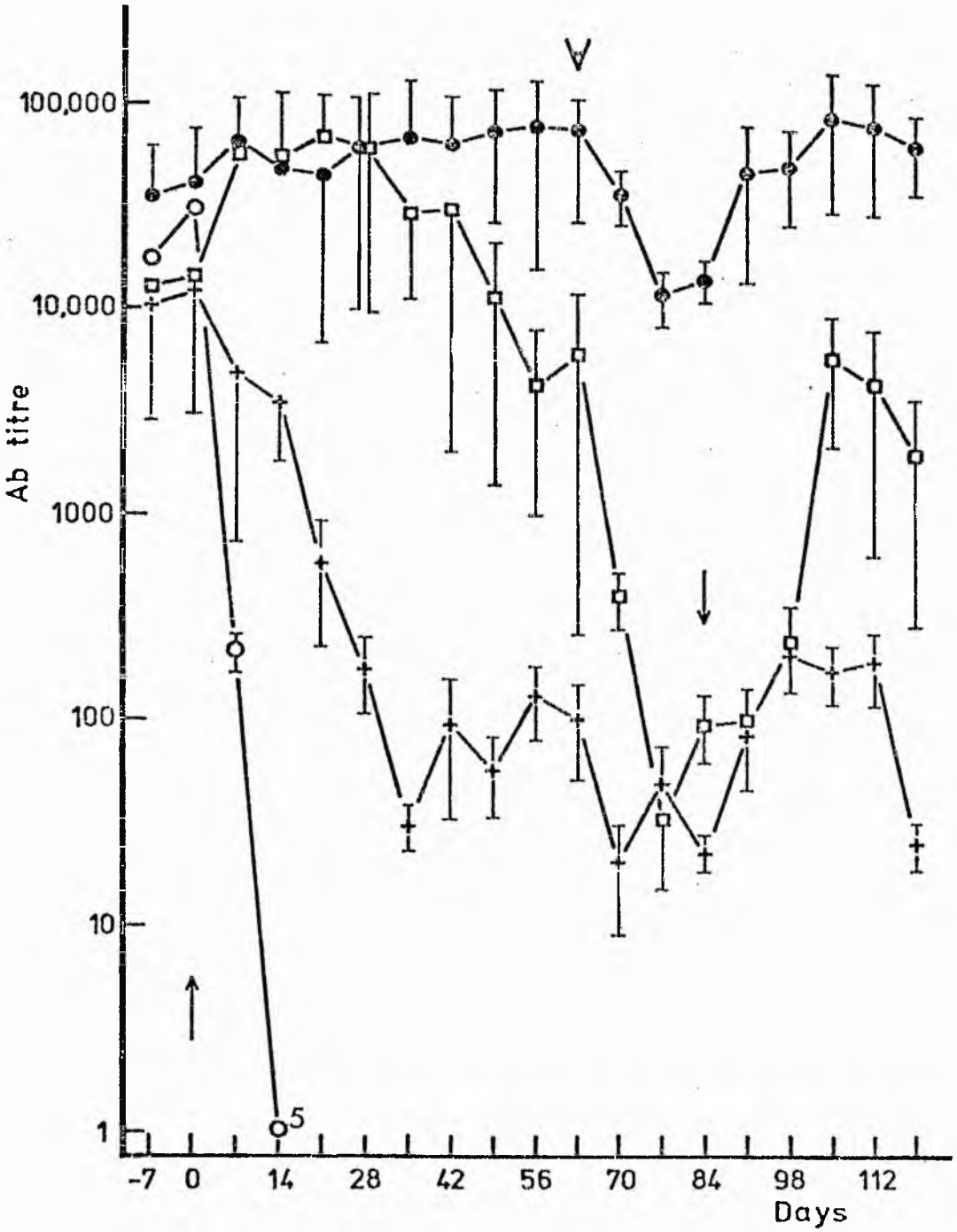

100,000
10,000
1000
100
10
1
Ab titre
-7
0
14
28
42
56
70
84
98
112
Days
5

Table 19 The effects of inoculated cadmium on the tertiary immune response to MS2 bacteriophage in S. trutta

The periods over which weekly antibody titre results have been meaned ± SE are indicated within the brackets

	Antibody titre $\bar{x} \pm$ SE			
	Control	Cadmium inoculated mg Cd $100g^{-1}$ 0.2	0.1	0.05
Pre-cadmium inoculation titres (-7 and +0d)	37354 ±10321	24359 ±7273	11183 ±2663	13769 ±3126
		***	***	***
Minimum titres after cadmium inoculation	26749 ±8486 *** (+28 to +70d)	215 ±22.1 *** (+7d)	60.3*** ±6.3 (+35 to +84d)	81.4*** ±9.3 (+77 to 91d)
4^{o} Peak titres after a booster inoculation of MS2, except for control fish (+98 to+119d)	** 69129 ±7171	-	*** 169*** ±16.3	*** 3057*** ±678

n = 5 fish per treatment

Significant differences between cadmium treated and control titres

*** P = < 0.001

Significant differences between treatments

**P = <0.01 ***P = <0.001

the increase was very marked and at 21d after the MS2 inoculation a significantly increased peak titre had been reached, though significantly lower than the initial titres before cadmium inoculation (table 19). The titres of the two groups of trout were then observed to fall once more.

At +63d the control fish were transferred to a separate tank and in the following 21d the antibody titre of this group was observed to fall significantly before returning to its original level. The effect of having removed the control fish on the cadmium inoculated groups could not be assessed and may have been a contributory factor in the observed titre fall in these fish.

c. Weight and length

Trout inoculated with lead were found to have a much reduced percentage change in body weight during the course of the experiment (figure 24). The weight increase of these fish became smaller with increasing concentrations of inoculated lead and at the highest lead level of 0.3 mg Pb a decrease in weight and length was observed. This corresponded with a significantly reduced change in per cent body length.

In the cadmium inoculated trout the observed weight changes were not as marked which may have been related to the smaller per cent weight increase of the controls as compared to the control fish of the lead experiment. A significant decrease in per cent change in weight was found in the 0.2 mg Cd inoculated fish, the latter group showing only a very small increase in weight over the experimental period. A small non-significant weight increase was observed in the 0.1 mg Cd inoculated trout. A decrease in per cent body length change was found in all the cadmium inoculated groups with increasing cadmium concentration, the lower length increases being significant for the 0.1 and

FIGURE 24

Lead and cadmium experiments: Body weight and length changes, haematocrits and serum protein levels.

Mean values $\pm$SE from five fish are plotted.

A. Lead inoculated S. trutta

a 0.01 mg Pb $100g^{-1}$

b 0.05 mg Pb $100g^{-1}$

c 0.1 mg Pb $100g^{-1}$

d 0.3 mg Pb $100g^{-1}$

C Control fish, pH 3.0 Saline inoculated

B. Cadmium inoculated S. trutta

a 0.05 mg Cd $100g^{-1}$

b 0.1 mg Cd $100g^{-1}$

c 0.2 mg Cd $100g^{-1}$

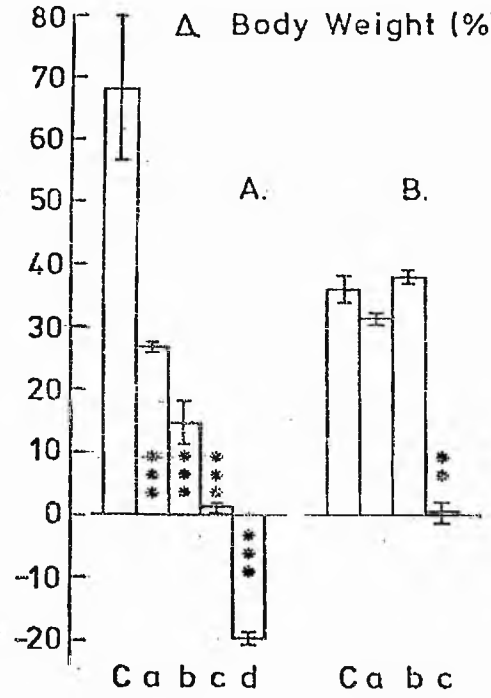
C Control fish, Saline inoculated only

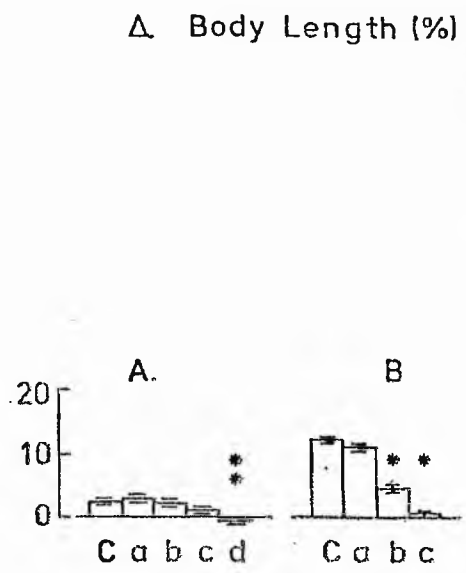
* $P = <0.05$

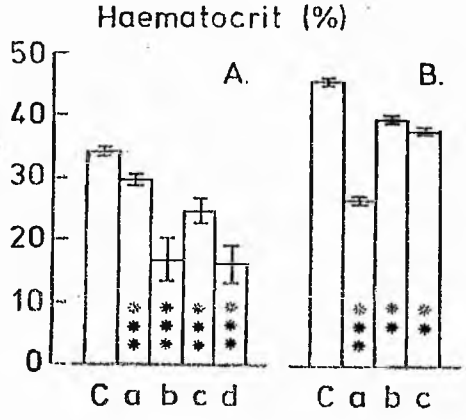
** $P = <0.01$

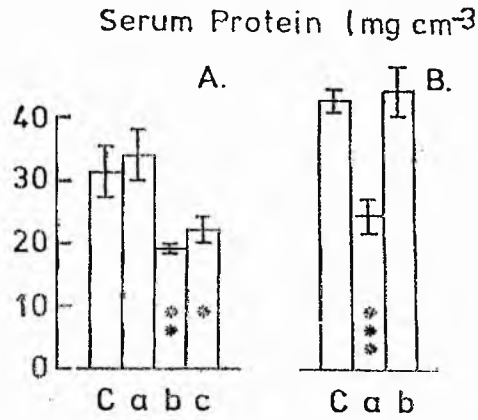
*** $P = <0.001$

Level of significant difference from the control values


Δ Body Weight (%)
80
70
60
50
40
30
20
10
0
-10
-20
A.
B.
C a b c d
C a b c


Δ Body Length (%)
20
10
0
A.
B
C a b c d
C a b c


Haematocrit (%)
50
40
30
20
10
0
A.
B.
C a b c d
C a b c


Serum Protein (mg cm-3)
40
30
20
10
0
A.
B.
C a b c
C a b

0.2 mg Cd inoculated trout when compared with the control value.

d. Haematocrit

Haematocrits were significantly depressed in all the lead and cadmium inoculated groups of trout, though the changes did not indicate a direct relationship with metal concentration (figure 24). In the lead inoculated fish the 0.05 and 0.2 mg Pb groups were found to show the lowest haematocrits and in the cadmium inoculated fish the lowest haematocrits were found in the 0.05 mg Cd group.

e. Serum Protein

Trout inoculated with 0.05 mg Cd or Pb and 0.1 mg Pb were found to have significantly lower total serum protein levels than the controls (figure 24). However, 0.01 mg Pb and 0.1 mg Cd inoculated fish produced small but non-significant rises in serum protein levels.

f. Histology

No gross morphological changes were observed in sections of tissues taken from lead and cadmium inoculated trout.

The trout inoculated with lead were observed to be darker skinned than the controls and from +91d after inoculation with 0.1 mg Pb the epidermis was observed to lift away from the dermis producing bubbles 1 to 2 cm in diameter and filled with a clear fluid. Histological examination of skin sections from these fish revealed no additional damage, other than the parting of the epidermis from the dermis, or the inclusion of bacteria in the fluid of the swelling. The fluid from one fish lesion was removed using a syringe and 23G hypodermic needle and was found to have a low level of MS2 neutralisation activity, with an SD_{50} value of 54.0 ± 16.3.

iv. MS2 bacteriophage survival in heavy metal solutions

The bacteriophage MS2 was found to be sensitive to heavy metals producing the sigmoid dose response curves plotted in figure 25. Estimates of the heavy metal dose permitting 50% survival of the bacteriophage have also been noted in the figure. The toxicity of the heavy metals to MS2, using the 50% survival criterion, was found to be

$$Cd, Cr > Cu > Pb > Zn > Ni$$

v. Heavy metal content of the mains water source and the fish food

The heavy metal values for the mains water supply and the food used in these experiments are listed below in table 20. The values for the water supply were towards the limits of detection by the methods used, thus only the maximum values observed have been listed. Of these zinc, copper and cadmium were at or just above the levels of heavy metal recommended for the culture of S. gairdneri by EIFAC (1973; 1976; 1977).

The Beta Trout Growers Diet used throughout the experiments was found to contain significant levels of heavy metal, of which zinc, copper and iron were high.

Table 20 Heavy metal content of mains water and fish food

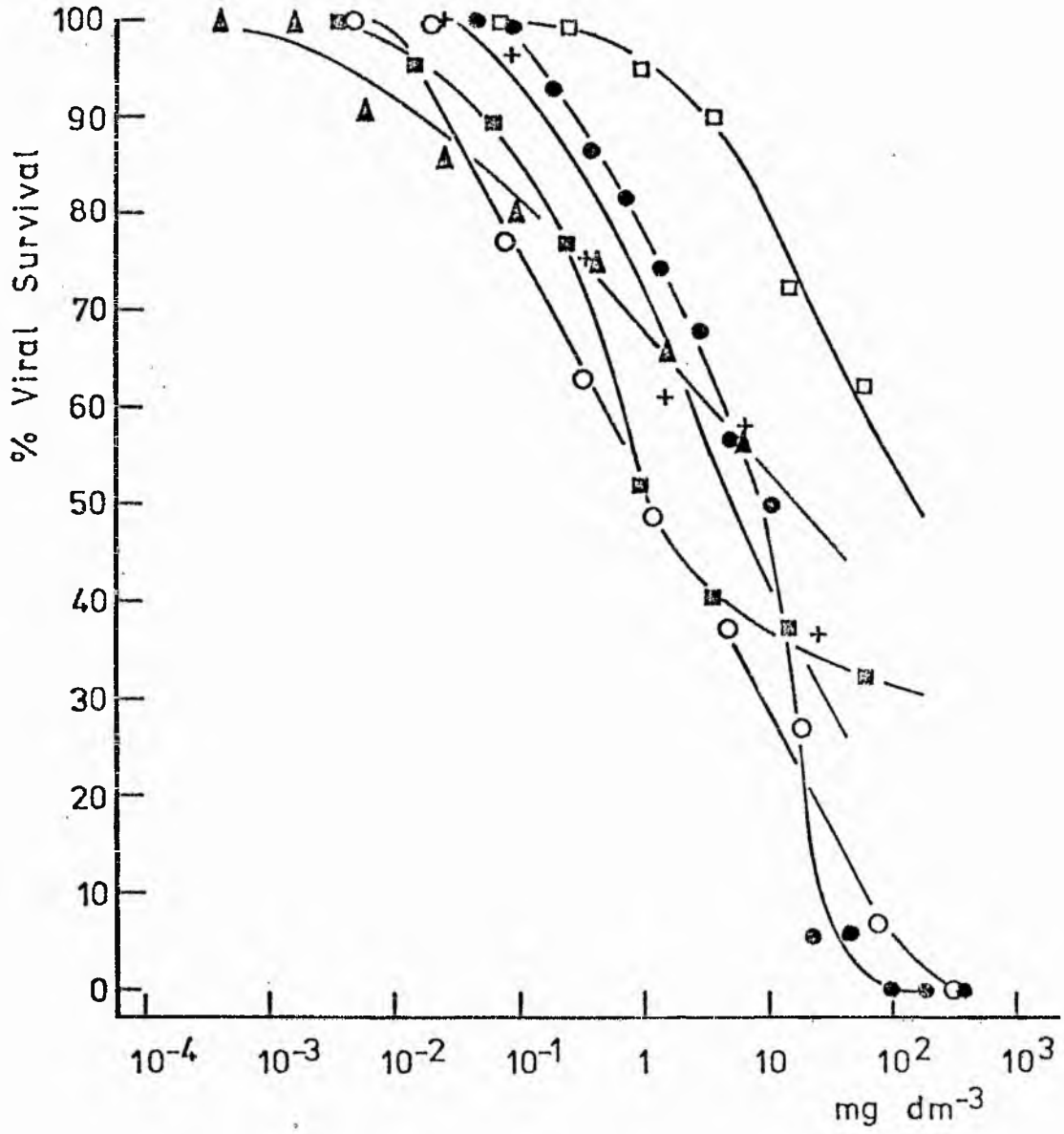
Heavy Metal	Mains Water mg dm^{-3}	Food mg Kg^{-1}
Iron (Fe)	Nil	2750.0
Manganese (Mn)	0.02	-
Cadmium (Cd)	0.002	1.0
Copper (Cu)	0.03	15.0
Lead (Pb)	0.026	6.0
Zinc (Zn)	0.05	83.0
Nickel (Ni)	0.006	4,0
Chromium (Cr)	0.014	3.0

FIGURE 25

Survival curves of MS2 bacteriophage in heavy metal solutions.

Each point represents the mean of four replicate titres, after incubation at 20°C for 30 min.

Metal Solution		Approximate concentration of heavy metal permitting 50% viral survival mg dm^{-3}
▲	Zinc	20
◻	Nickel	160
+	Copper	8
■	Chromium	1
●	Lead	10
o	Cadmium	1


% Viral Survival
100
90
80
70
60
50
40
30
20
10
0
10^{-4}
10^{-3}
10^{-2}
10^{-1}
1
10
10^{2}
10^{3}
mg dm^{-3}

IV DISCUSSION

The humoral immune response of the teleosts examined, S. trutta, C. carpio and N. rossii, has been found to be typical in form to those responses described in higher vertebrates sensitised with viral particles and in particular bacteriophages. Fish which had been sensitised with a primary challenge of MS2 bacteriophage were found to produce an enhanced secondary response and to maintain levels of antibody production which indicated the possession of immune memory.

The poikilothermic nature of teleosts has naturally been shown to be an important factor in the time course and level of humoral neutralisation antibody production. Trout and carp maintained at their optimum temperatures, 15° and 22°C respectively, were found capable, however, of producing an immune response of similar time course and level of antibody production as has been found for mammals by Svehag and Mandel (1964) and even for humans by Peacock, Jones and Gough (1973). The neutralisation antibody produced in response to MS2 bacteriophage in teleosts was found to be of an HMW type, though no change to a LMW form was detected as has been commonly found in the mammalian humoral immune response.

Under conditions of constant temperature and water quality the exposure of trout and carp to externally administered levels of heavy metals and the exposure of trout to inoculated concentrations of cadmium and lead have shown the suppression of the humoral immune mechanisms raised against MS2 bacteriophage. It was possible in certain cases to relate this suppression quantitatively to the level of heavy metal exposure.

A. The humoral immune response of teleosts

All the specimens of S. trutta, C. carpio and N. rossii sensitised with MS2 bacteriophage were observed to produce active neutralisation antibody, thus demonstrating the bacteriophage to be highly immunogenic in teleosts, and closely comparable to the highly immunogenic response to viral particles found in mammals (Burns and Allison, 1975). The immunogenicity of bacteriophages and viral particles has been found by other workers to be low in fishes, with the production of low titres, and in many cases, the observation of non-responding fish (table 1).

i. The use of the SD_{50} value to measure neutralisation titre

The small scale plaque neutralisation assay has been shown in this study to be a sensitive and reproducible technique for measuring MS2 neutralisation antibody in the sera of teleosts and also the compact nature of the assay permitted large numbers of sera to be assayed at one time.

In previous studies of humoral antibody production to bacteriophages the viral inactivation constant k has been used as a measure of antibody activity. This method first described by Adams (1959) has been used by many clinical workers (Ochs, Davies and Wedgwood, 1971) and also by workers studying the immune response of fishes to bacteriophages (table 1). A critical study made by Haimovich and Sela (1969) using haptenated T_4 bacteriophage found that the inactivation constant method of measuring antibody levels was unreliable in detecting antibody concentration because of the differing affinities of mammalian IgM and IgG antibodies for antigen, affinities which have been found to change with the course of the mammalian response within both IgM (Webster, 1968) and IgG groups (Svehag, 1965; Finkelstein and Uhr, 1966). Blank, Leslie and Clem (1972) formed a similar conclusion on examination of the affinity

and valence (the number of combining sites) of the antibody molecules formed in man, rabbit and the "grouper" (Epinephelus sp.) that the neutralisation rate constant k was not measuring directly the reaction of the antibody binding to T_2 bacteriophage. A further disadvantage was revealed by Witte and Slobin (1972) when they demonstrated that the reaction between f2 bacteriophage and its antibody was not of the first order kinetics required to calculate the inactivation constant of Adams (1959). In the present study an examination of the association of antibody and bacteriophage indicated that the initial rate of combination (figure 3) was too fast to quantify accurately.

Stashak, Baker and Roberson (1970 a,b) and Peacock et al. (1973) found that the SD_{50} method of measuring antibody levels to X174 bacteriophage overcame the problems of the activation constant method and gave a linear relationship with the antibody content of human anti-sera.

Witte and Slobin (1972) in their examination of haptenated f2 bacteriophage and antibody interactions proposed a simple steric model of bacteriophage neutralisation by the antibody in which a single molecule of the antibody interacted with a single critical site on the f2 bacteriophage. This critical site they considered to have been on, or in the vicinity of the A-protein which is important in the infection of the host E. coli. Rolfe and Sinsheimer (1965) had earlier speculated on the existence of a single neutralisation site for a single molecule of antibody when they examined antigenic fractions of X174 bacteriophage.

The similarities of MS2 and X174 and f2 bacteriophages would make the neutralisation of one MS2 particle by one antibody molecule a similar possibility. Thus in the assay the neutralisation of 150 viral plaques would imply the action of at least 150 molecules of antibody, though this would certainly underestimate the number of

molecules required. The infection and lysis of one host E. coli bacterium requires a multiplicity of MS2 particle attachments to the sex pilus; a multiplicity of 5 MS2 bacteriophages was found by Strauss and Sinsheimer (1963) and Chakrabarti and Gorini (1975) to be the most efficient infective dose. The neutralisation of a plaque in the plaque neutralisation assay would therefore be achieved by more than one antibody molecule. Nevertheless the viral plaque neutralisation assay has been shown to be a sensitive assay of antibody titre by many workers (Peacock, et al., 1973) and also in the present study. Furthermore, the examination of plaque neutralisation was probably assessing the response of one specific antibody, namely that antibody which was determined specifically for the infection site of MS2 bacteriophage or even the A-protein itself.

A comparison of the SD_{50} values for serum antibody and the titre detected by the complement fixation test clearly showed that the plaque neutralisation assay was ten times more sensitive in detecting antibody titres to MS2 bacteriophage. The complement fixation test is in its own right a sensitive assay for antigens and antibody, for a single attachment of complement to an antibody-antigen complex will produce an amplifying complement cascade (Ruddy, Gigli and Austen, 1972). In the context of the present study the latter complement cascade was blocked by 'fixing' the C1 fraction of GPC to the trout antibody-MS2 bacteriophage complex, thus stopping the lysis of the sensitised SRBC. This 'fixation' of C1, however, is modified by the type of antibody present. In mammals only IgM and IgG can fix C1, IgM has been found to be the more active of the two and IgG subclasses have been found to show a range of activity (Roitt, 1974). However, the complement fixation test should have had the advantage of detecting all the antibodies which would have been formed to the four antigenically distinct proteins of the MS2 bacteriophage capsid rather than just the single protein unit of the infection site.

ii. MS2 bacteriophage neutralisation antibody

The three species of teleost examined have all been found to produce humoral antibody. That is, the MS2 bacteriophage neutralisation activity observed in these fish was stimulated by the antigen and the active serum fraction was a HMW molecule, which above all showed specificity in its neutralisation.

The molecular size of the HMW teleost antibodies have been shown in the literature to be variable, ranging from 500,000D (Ambrosius and Frenzel, 1972; Everhart, 1971, 1972) to 900,000D (Harris and Cottrell, 1976), though it has generally been agreed that the structure of teleost antibody is similar to mammalian IgM. However, the teleost antibody is a tetrameric polymer, though composed of two heavy and two light chains per sub unit (Shelton and Smith, 1970; Acton, Weinheimer, Hall, Neidermeir, Shelton and Bennett, 1971; Dorson, 1972a,b; Hall, Evans, Dupree, Acton, Weinheimer and Bennett, 1973).

In this study the estimated molecular weight of the neutralisation active fraction separated on G-200 sephadex was found to range from 360,000D in S. trutta to 600,000D in C. carpio. The result for the mirror carp was thus similar to that found by Ambrosius and Frenzel (1972) for this species and by Everhart (1971, 1972) for C. auratus, but the estimates for the trout, N. rossii and secondary response carp sera are lower than published results. It is possible that fragmentation of the antibody molecules may have occurred in the separation processes, as was found by Marchalonis (1971) who found a non-active 7S LMW molecule which was antigenically similar to the HMW antibody molecule. In the present study no active LMW sub-fraction was detected in the primary sera and neither was there a shift to a LMW antibody detected in the secondary sera, thus other methods would be required to detect non-active sub-

fractions should fragmentation have occurred. The antibody active fractions of the sera of secondary response trout and carp were observed, however, to have a slightly lower molecular weight than the active fractions of the primary response. The HMW antibody of C. auratus to BSA was found by Trump (1970) to be of two types, 16.4S and 15.3S, though this division also observed by Everhart (1971, 1972) was thought to have been caused by molecular charge and not molecular weight. A similar change in molecular charge may have occurred in the antibodies to MS2 bacteriophage in the trout and carp, but this would need to be confirmed by sucrose density gradient ultracentrifugation, electrophoresis, and by the determination of antigenic similarities.

Further confirmation of an IgM-type structure for the MS2 antibody was demonstrated by the 2-ME sensitivity of the neutralisation active fraction of the secondary response sera from all three species of teleost examined. Neutralisation activity was completely reduced in N. rossii, though in trout and carp residual activity was observed. This does not, however, indicate the presence of a non-2-ME sensitive IgG-like antibody (Green, 1969) but may only be the result of incomplete 2-ME reduction, or of the presence of active heavy and light fractions as observed by Green (1969) using the electron microscope or may even have been a manifestation of the 'natural' neutralisation activity that has been observed in these fish. As the residual neutralisation activity was found to be proportional to the initial MS2 neutralisation activity an incomplete reduction of the antibody molecules would seem to be indicated here.

"Multivalent antigens when mixed with bivalent antibodies in solution combine to form three dimensional lattices which aggregate and precipitate"(Roitt, 1974). The precipitation of the complex does, however, rely on the combination of optimal proportions of antibody and antigen

which in excess of either component may stop precipitin formation. The precipitin test has been used extensively with bacterial antigens and to some extent with viral antigens (Cowan, 1973). One of the greatest problems found with the use of viral antigen has been the production of an adequate concentration of the purified virus.

With the methods employed in this study the neutralisation antibodies produced by the three teleost species against MS2 bacteriophage gave no observable precipitin reaction. All the methods were so designed to produce optimal antibody and antigen concentrations by diffusion, thus the initial concentrations of the antibody or antigen could have been too low or the properties of the antibody binding sites on the MS2 particle were such that cross linking of the bacteriophage by more than one antibody molecule was not possible.

The presence of complement in the sera of teleosts has been well documented (Cushing, 1970). In the examination of MS2 neutralisation antibody production in S. trutta it was found that both homologous and heterologous complement activity was fixed by the MS2-antibody complex. The complement activity of the pooled trout sera was not large, only demonstrating activity upto a $\frac{1}{4}$-dilution. However, the active response in lysing RHS-sensitised SRBC and not unsensitised SRBC demonstrated that the complement fraction of trout serum was capable of complexing with mammalian antibody and was thus probably similar to higher vertebrate complement. Chiller, Hodgins and Weiser (1969b) found that SRBC sensitised with S. gairdneri antibody could only be lysed by complement from this species, heterologous complement from mammals, birds and amphibia were found to be inactive. It would thus seem that the antibody attachment site on mammalian complement, although it can attach to and fix trout antibody, is more specific in its action of initiating the complement cascade than trout complement.

The ability of trout antibody to fix complement again indicates that the antibody molecule is of a type similar to mammalian IgM or IgG antibody. The antibody produced by MS2 sensitised fish was found to be specific in its neutralisation activity, thus defining one of the major principles of antibody activity. In C. carpio and N. rossii sera containing MS2 neutralisation antibody was found not to neutralise the closely related bacteriophages QB, X174 and P22. Significant levels of neutralisation activity against the three bacteriophages were found in the sera of S. trutta, though these levels were not significant in comparison to the MS2 neutralisation activity in the trout sera. The non-specific neutralisation activity appeared to be related to the degree of similarity between the bacteriophages and indicated a progression of affinity with MS2, QB being the closest and P22 the least similar. Similarities of the antigenic protein grouping could produce such a situation, and one such example has been described by Harris and Cottrell (1976) for the 'natural' antibody of P. platessa against a parasitic nematode which was specific for another species.

iii. 'Natural' neutralisation activity

The MS2 bacteriophage was chosen as an antigen because of the probability that the experimental fish would not have been previously exposed thus enabling a true primary response to be observed. MS2 was first isolated from sewage and occurs in the normal healthy human intestine, even so, it would not be expected to contaminate fish hatchery waters and especially not the flowing bore hole water used to culture the trout used in this study. 'Natural' levels of MS2 bacteriophage neutralisation activity were observed, however, in certain trout and carp before MS2 sensitisation and, although these levels were low in the majority of fish, one trout did have an SD_{50} value of 45.7. No neutralisation activity was found in any of the specimens

of N. rossii examined and in this case the exposure to MS2 in the marine environment would be very unlikely. It was not established in the present study if the 'natural' neutralisation activity observed in the trout and carp was an antibody, though 'natural' HMW precipitins and agglutinins have been described for teleosts. However, there has been a dispute in the literature as to whether these molecules were true induced antibodies. Many authors have considered them as true antibodies and have said that they indicate previous exposure to antigen (Fujihara and Tramel, 1968; Janssen and Meyers, 1968; Janssen, 1970). However, Hodgins, Wendling, Braaten and Weiser (1973) found natural agglutinins in S. gairdneri against xenogeneic erythrocytes which were not likely to have been induced, and they were also found to be LMW molecules distinct from HMW antibody.

Low levels of X174 bacteriophage neutralisation activity were found also by Stashak et al. (1970a, b) in both conventionally reared and germ-free unsensitised mice, and observed by Peacock, et al. (1973) in humans. The latter authors did find, however, that the response to bacteriophage sensitisation was a genuine primary response, although they could not rule out possible infection from the intestine and transmission of a secretory IgA type response.

The sensitisation of the experimental fish from environmental waterborne sources can not be ruled out completely, although this would be unlikely because of the flowing water source used in trout and carp culture and the poor survival of the bacteriophage in water. However, Janssen and Meyers (1968) detected specific antibodies to several human pathogenic bacteria in P. fluviatilis found in waterways of heavily populated areas and waterborne MS2 'infection' has been demonstrated in the present study to occur under experimental conditions with trout held in static water.

An increase in MS2 neutralisation activity was observed in the sera of trout inoculated with sterile saline and maintained in aquaria with MS2 inoculated fish. It was because of the possibility of bacteriophage escaping from the inoculated trout, confirmed by Hutchinson (1977, unpublished) in this laboratory, that groups of trout were exposed to waterborne MS2 bacteriophage, though at comparatively high titres. Hutchinson found that 6.9×10^{7} PFU of the 8.4×10^{10} PFU MS2 (approximately 0.1%) inoculated intraperitoneally into 0+yr S. trutta had escaped after 36h into the surrounding water.

The waterborne MS2 bacteriophage experiments demonstrated that humoral antibody levels could be raised in this manner, the bacteriophage entering the trout possibly via the lateral line (Amend and Fender, 1976), the gills or the gut. Methods of providing protective immunity other than by parenteral means were first demonstrated in S. clarkii (≡ S. gairdneri) by Duff (1942) and several workers have since demonstrated the technical possibility of non-parenteral routes of immunisation in fishes (Snieszko, 1970; Amend and Fender, 1976; Antipa and Amend, 1977). The presence of live MS2 bacteriophage within the tissues of the fish exposed to waterborne bacteriophage particles has yet to be conclusively demonstrated, though preliminary examination of sera from exposed trout, undertaken in this laboratory (Saunders, 1977, unpublished), has shown the ability of live MS2 to enter the blood vascular system from the external environment. At this time the routes and mechanisms via which bacteriophage was lost from inoculated S. trutta or taken up by unsensitised fish have not been examined. Thus an examination of the permeability of the gills, gut and general epithelial surfaces to viral particles and even an examination of the suggestion of Avetikyan (1956) that antigen was picked up from the gut of fish by migrating leucocytes would be valuable.

Control trout isolated from MS2 sensitised fish and inoculated with sterile saline-FCA emulsion were also found to produce a small rise in neutralisation activity. The inoculation of sterile saline or saline-FCA may have acted as a stressful event which had elevated levels of natural non-specific neutralisation factors. Such factors are known to exist in fishes: natural agglutinins and precipitins (Hodgins, 1973), lysozymes (Luk'yanenko, 1965; Fletcher and Grant, 1969; Murray and Fletcher, 1976), C-reactive proteins (Fletcher and Baldo, 1976), and interferon (Gravell and Malsberger, 1965; Beasley, Sigel and Clem, 1966; Oie and Loh, 1969; Kinkelin and Dorson, 1973), though normally intracellular, has been found to be released into the interstitial fluids of mammals where it acts as a 'germicide'.

iv. Primary immune response to MS2 bacteriophage

In fish inoculated with a single primary dose of MS2 bacteriophage, and maintained at 15.5°C for S. trutta and 22°C for C. carpio, the form and time course of the humoral antibody response were found to be similar to that of mammals, though peak titres were reached at a slightly later time. The humoral antibody response to MS2 was activated within the first week after inoculation, at between day 2 to 3 in trout, and has been demonstrated to be faster than the 8d required to produce anti-BSA antibodies in C. carpio at 25°C which was observed by Avtalion et al. (1973).

On the initiation of the primary antibody response titres rose rapidly and reached a peak at 14 to 35d, after which titres dropped. Svehag and Mandel (1964b) found the response of rabbits to poliovirus to be faster than this with the production of peak titres being within 4 to 5d after inoculation, these titres then fell over a period of three weeks. Although this was a faster response than observed

in the fish the peak titres raised to 10^6 PFU poliovirus were of the same order of SD_{50}, 100 to 1000, as those raised in trout to an equivalent PFU dose of MS2. This was similarly so for peak titre values in primary X174 bacteriophage inoculated mice (Stashak et al., 1970, a,b) and humans (Peacock et al., 1973), though with this bacteriophage the peak titres were reached 6 to 20d after inoculation and thus neared the peak response times observed in the present study for trout and carp at their optimum temperature. The time of peak titre in these teleosts may also be reflected in the comparative immunogenicity of the viral antigen used, as was shown by the mammalian response to poliovirus and X174 bacteriophage.

The response to MS2 bacteriophage was found in S. trutta to be dependent on antigen concentration, a factor which has already been demonstrated in teleosts for viral antigens by Sigel and Clem (1965) and bacterial antigens by Evelyn (1971) and Paterson and Freyer (1974a), though Dorson (1972a,b) was unable to show any concentration dependence in S. gairdneri with FH_5 bacteriophage and similarly Avtalion et al. (1973) found no significant difference in response between groups of C. carpio inoculated with different concentrations of BSA. In humans Peacock et al. (1973) were only able to detect very small changes of titre with different concentrations of X174, though Uhr and Finkelstein (1963) had shown a concentration dependent response to this bacteriophage in guinea pigs, though at higher concentrations a threshold of antibody production was observed.

High concentrations of antigens when inoculated into mammals have been found to produce a mixed primary response with more emphasis being placed on IgG synthesis (Burns and Allison, 1975) levels of which were maintained for long periods of time. Svehag and Mandel (1964b) found that only a small increase in inoculated poliovirus concentration from

6×10^6 to 8×10^6 PFU shifted the primary response from a totally IgM response to a mixed IgM-IgG response. In all the three species of fish examined only a HMW antibody response was found though threshold levels of antibody production were observed and later a true secondary immune response was formed.

The experimental doses of inoculated MS2 bacteriophage were calculated as particle numbers or PFU, however in terms of total protein the amount of antigen inoculated was small. Thus the 0.1 cm^3 inoculated doses of 10^3, 10^6 and 10^9 PFU used in the initial experiments with S. trutta represented 5.6×10^{-16}, 5.6×10^{-13} and 5.6×10^{-10} g protein respectively. Even at the lowest dose of bacteriophage challenge significant titres of antibody were produced in primary response trout. When soluble and large particulate antigens have been used relatively large doses, in the mg range, have been inoculated in order to stimulate antibody production in fishes as it has been assumed that fish require a high threshold of antigenic stimulation (Luk'yanenko, 1966). Repeated or large doses of antigenic stimulation have not produced, however, the desired response in teleosts and may even have produced immunogenic tolerance. The latter has been recorded in mammals (Mitchison, 1965; Dresser and Mitchison, 1968; Gershon and Kondo, 1971, Asherson, Ferluga and Janossy, 1973; Weigle, 1973) and has been observed in C. carpio inoculated with 20 to 50 mg of BSA by Avtalion et al. (1973) and in S. gairdneri inoculated with repeated 1.0 mg doses of A. salmonicida by Ellis (personal communication). Although a threshold level of MS2 bacteriophage stimulation may have been reached in the present study no evidence of tolerance was observed, thus higher doses of the bacteriophage need to be examined for tolerogenic response.

v. Secondary immune response to MS2 bacteriophage

An initial suppression of the humoral response was observed following the secondary inoculation of MS2 bacteriophage in all three of the teleost species examined. The rate of bacteriophage clearance was found to be much reduced in comparison to primary response clearance (figure 8) and renewed humoral antibody synthesis was not detected until 6d after inoculation of S. trutta at 15.5°C, 3.5d longer than for primary response. Those fish which showed low titre primary responses were found to retain live MS2 bacteriophage for longer than 7d, and, even in those fish which had relatively high humoral neutralisation titres, a fall in titre was observed in the 7d period after secondary inoculation. Such a decrease in antibody titre was found in secondary sensitised C. carpio by Ambrosius and Shaßker (1964) using porcine serum, by Avtalion (1969b) with BSA, and by Richter (1971) using HGG and BGG. A suppression in antibody response was also observed by Peacock et al. (1973) in humans responding to a secondary inoculation of X174 bacteriophage, though an enhanced clearance and early antibody synthesis have been found more usual in mammals (Farber, 1969). An increased clearance rate was found by Nelstrop, Taylor and Callard (1968) in C. auratus intravenously inoculated with T_2 bacteriophage, however, no neutralisation antibodies were detected after the clearance of the bacteriophage.

The observed suppression in the initial secondary response was followed by a rapid rise in antibody titres which reached a peak 14 to 21d after inoculation. As the rate of humoral antibody formation and peak titres were higher than those of the primary response and taking into account the upper titre threshold limit it was probable that memory and enhancement, characteristics of the mammalian secondary response, were observed in all three species of teleost examined. Humans in secondary response

were found by Peacock et al. (1973) to produce X174 antibody titres slightly earlier than observed in the fish at 7 to 10d, though peak titres in S. trutta and C. carpio were found to have similar SD_{50} values to the human subjects in the region of 10,000.

In the few reports in the literature which have examined the secondary response of teleosts an anamnestic response has been observed (Ambrosius and Shaëker, 1964; Ambrosius and Frenzell, 1972; Avtalion, 1969a,b; Richter, 1971).

A true secondary response produces antibody titres which are maintained over a long period of time and for viral antigens this may be months or even several years (Burns and Allison, 1975). Although the neutralisation antibodies produced in the secondary response to MS2 bacteriophage did not show a shift to a LMW antibody in teleosts the levels of antibody produced were found to be maintained for the full two months over which the experiments were extended after secondary peak titres had been reached. The maintenance of high antibody titres was further demonstrated in trout which received a tertiary inoculation of MS2 and in which antibody titres remained constant over a period of four months. The ability of teleosts to maintain these high levels of humoral antibody over much longer periods of time has not been examined here, though such a study would be important in an examination of the long term immunity of fishes to diseases.

The dose of the primary inoculation of MS2 was found to have an effect on the humoral antibody titres of secondary response trout, the dose of the secondary inoculation of bacteriophage being the same for all the fish. In the fish not inoculated with adjuvants the maintained plateau titres of secondary response were found to be directly related to the dose of the primary inoculation of bacteriophage. The

effect of low secondary doses of MS2 bacteriophage was not investigated thus the production of an enhanced immune response to a second low dose of MS2 is speculative. Thus the potential to continue raising true primary responses in fish, as observed by Svehag and Mandel (1964b) in mice, and the relationship of antigen concentration to the transition to a true secondary response require further study. This is especially interesting in the case of the teleosts where the HMW antibody is retained in the true secondary immune response in comparison to mammals in which there is a transition to the LMW IgG antibody molecule.

vi. Immune suppression

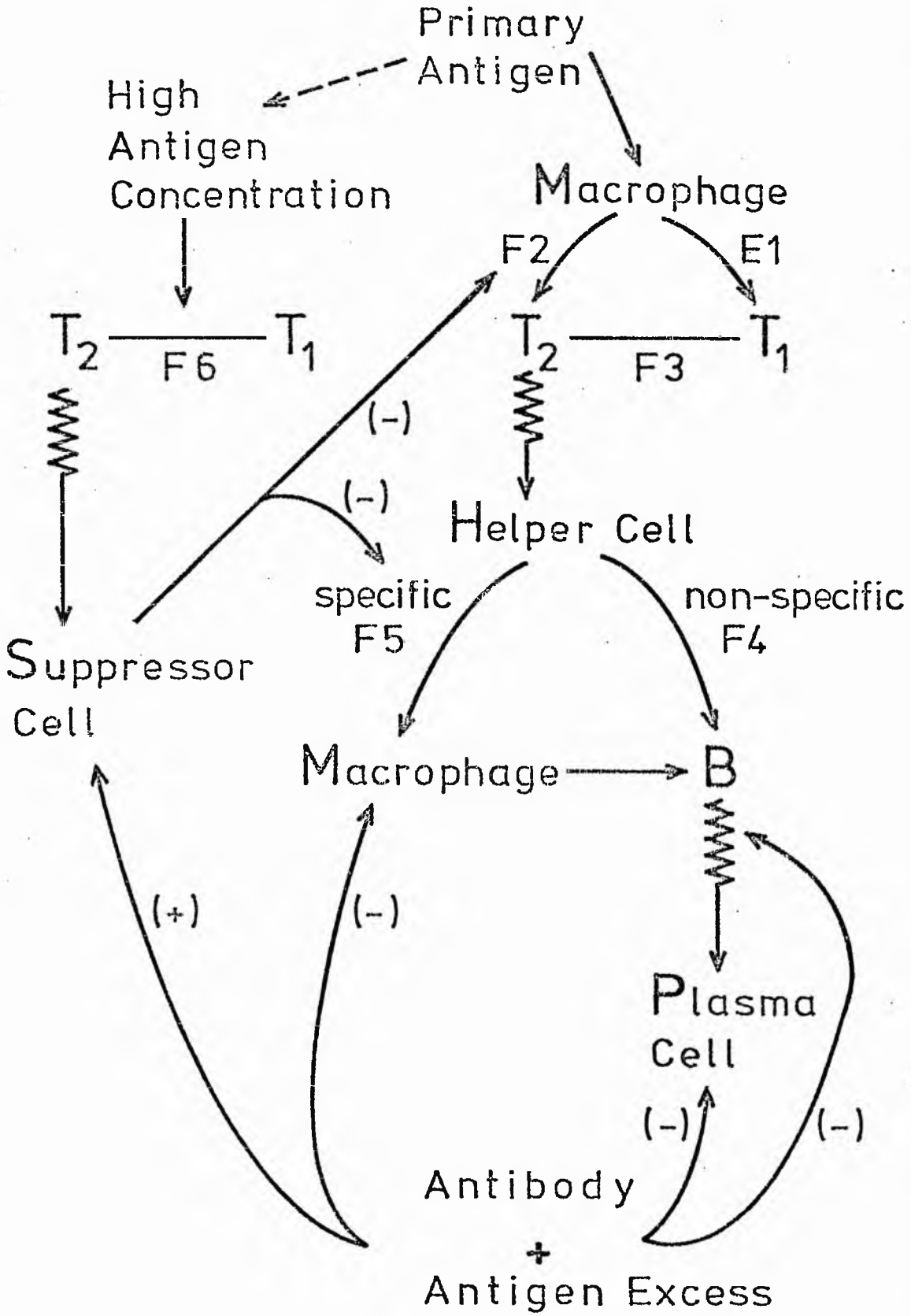
The humoral immune responses of teleosts, and of higher vertebrates, have been shown to be suppressed by excess of antigen, to be suppressed in their humoral response to a secondary challenge of antigen, and to regulate their humoral antibody titre levels. The mechanisms involved in such feedback processes are complex in mammals and a great many of these mechanisms still have to be conclusively elucidated. They are based on a local interaction of lymphocyte and macrophage populations and are mediated by soluble factors, as reviewed by Feldmann et al. (1977) and summarised in figure 26.

In mammals a primary challenge of a high antigen concentration will directly interact with sub-populations of T-cells to activate suppressor cell activity (Coutino, Gronoweiz, Bullock and Möller, 1974; Coutino and Möller, 1975). This suppression, which can be transferred to non-suppressed individuals using suppressor cells from the suppressed animals, was demonstrated by Gershon and Kondo (1971) for mice hyperimmunised with SRBC. Strayer, Consenza, Lee, Rowley and Kohler (1974) and Kohler, Kaplan and Strayer (1974) also found that the suppression of humoral immunity could be transferred by the hyperimmune sera of the

FIGURE 26

Interactions of lymphoid cells in higher vertebrates: A diagrammatic representation (after Feldmann et al., 1977).

T_1	Short lived T-cells
T_2	Long lived T-cells
B	B-cell
Plasma cell	B-cell releasing antibody
F_1 to F_6	Serum soluble factors
⟿	Maturation of cell into ...
— or →	Interaction of cells or factors
(-) (+)	Negative or positive feedback controls


Primary
Antigen
High
Antigen
Concentration
Macrophage
F2
E1
T2
F6
T1
T2
F3
T1
(-)
(-)
Helper Cell
specific
F5
non-specific
F4
Suppressor
Cell
Macrophage
B
(+)
(-)
Plasma
Cell
(-)
(-)
Antibody
+
Antigen Excess

mice when administered to neonatal mice thus indicating a non-cellular factor.

The inoculation of specific antibodies into mammals (Uhr and Möller, 1968) and birds (Seto, 1976) has been shown to suppress the primary immune response to the antigen, possibly by the formation of inhibitory antibody-antigen complexes. However, circulating antibody levels have been found to regulate themselves directly. This was shown by experimentally absorbing antibody from the circulation (Weigle, 1975) which stimulated an increase in the antibody level, and conversely, the addition of antibody was found to suppress the level. Again these regulatory mechanisms were found to be mediated through T-cell suppressor and helper activity which regulated the division of sensitised B-cells to form antibody producing plasma cells. Möller, Britton and Möller (1971) observed 4 to 5d cyclic fluctuations of 19s antibody in mice sensitised with bacterial antigen which they suggested were produced by an antibody blockade of antigenic sites thus blocking the sensitisation of immunocompetent cells, but after catabolism of the antibody the antigen was once more free to stimulate the cells. Seto (1976) suggested that there may also be a direct action of antibody on the antibody forming cells which would form a feedback mechanism. Indeed fluctuations in the numbers of splenic antibody producing cells have been observed in mammals which correspond to the humoral antibody fluctuations (Weigle, 1975).

The humoral immune feedback mechanisms in mammals have been found to be more active in suppressing the primary immune response and the IgM producing cells, the secondary response was found less sensitive to suppression (Uhr and Möller, 1968) possibly because of the formation of memory cells and the shift to IgG producing cells found in the mammalian response.

The suppressive mechanisms of antigen and antibody feedback controls are diverse. They are probably mediated

through the T-cell populations as summarised by Feldmann et al. (1977), but the possibility does exist of there being a direct action on the B-cells (Seto, 1976) and even on the associated macrophages (Haughton, 1974). The role of such mechanisms in teleosts can only be speculative at this time. Excess antigen-produced suppression could be produced by T-like suppressor cells and the suppression of secondary antigen clearance may be produced by antibody-antigen complexes which activate similar T-cells or act directly upon the antibody producing cells and macrophages. The greater suppression of secondary clearance observed in the teleosts in comparison to mammals may be due to the continued IgM-type response which was found to be more susceptible to suppression in mammals. It is interesting that the enhanced secondary antigen clearance which was observed by Nelstrop et al. (1968) in C. auratus was accompanied by no detectable antibody formation and may thus be attributable to a lack of antibody-antigen complex formation.

The exact mechanisms and reasons for humoral antibody control are not understood though the immune system must have a means of maintaining antibody levels and a capability to react to antigen challenges of different magnitude. Weigle (1975) suggested that the major reason for feedback suppression was to stop the exhaustive differentiation of competent lymphocytes and thus conserve a stock of memory cells for future use. A great deal of further information would seem to be required with regard to the cellular mechanisms of antibody control and also their intracellular switching mechanisms. This would be especially true in the case of teleosts in which antigens, such as MS2 bacteriophage, have been observed to remain free in the blood vascular system for long periods of time and in natural situations where pathogens may be continuously present in the aquatic environment (Wedemeyer, 1970a).

vii. Adjuvants

Dorson (1972a,b) suggested that the lack of adjuvants in inoculations of FH_5 bacteriophage was the possible cause of the poor antibody response he observed in S. gairdneri. Adjuvants have indeed been shown to increase the neutralisation antibody response to MS2 bacteriophage of S. trutta in this present study. The work reported in the literature, although sparse, has demonstrated the enhancement of the immune response of the teleosts, by the use of adjuvants, to viral antigens (Sigel and Clem, 1965; Clem and Sigel, 1966), bacterial antigens (Krantz, Reddecliff and Heist, 1963, Krantz, 1964a,b; Fujihara, Olson and Nakatani, 1965; Fujihara and Hungate, 1972; Fletcher and White, 1973; Paterson and Freyer, 1974b), and soluble antigens (Ambrosius and Lehmann, 1965; Sigel, Russel and Bradshaw, 1967; Hodgins, Weiser and Ridgway, 1967).

The classical responses to FCA and FIA were observed in the humoral immune response of S. trutta to MS2 bacteriophage, of which FCA produced the greatest overall enhancement. The primary response antibody titres were not increased in rate of formation but were found to be extended in time thus producing a larger peak titre. This was in line with the theories of prolongation of the effective antigen dose, whether by clonal recruitment (Siskind and Benacerraf, 1969) or by the now more favoured clonal expansion (Civin, Levine, Williamson and Schlossman, 1976) of B-cell-like lymphocytes. Primary antibody response titres were found to be enhanced only in the highest MS2 bacteriophage doses inoculated when combined with FIC, there being no observed effect in the lower doses. However, in the FCA inoculated trout the lowest dose of 10^3 PFU MS2 as well as the highest dose was found to produce a very marked enhancement of humoral response, with possibly a small increase in the rate of the response. The enhancement produced by FCA would be indicative in mammals of a thymus dependent antigen and T-cell stimulation. Thus the two forms

of adjuvant stimulation observed in the trout may indicate the existence and participation of two types of lymphocyte, the T- and B-like cells, in the humoral immune response of teleosts. As yet there is little evidence of two major functional populations of lymphocyte in fishes (Ellis, 1977) though Etlinger, Hodgins and Chiller (1975, 1976) with the use of mitogens found that in S. gairdneri only mammalian T-stimulating mitogen (Concanavalin A) stimulated the thymocytes and B-stimulating mitogen (lipopolysaccharides) stimulated only the pronephric lymphocytes. It is thus probable that further work will demonstrate the presence of T- and B-cells in fishes.

The addition of FIA to the lower inoculated doses of MS2 bacteriophage and FCA to the intermediate 10^6 PFU MS2 dose were observed to have no significant effect, though in the secondary response these groups of trout produced a response typical in form to those groups which had been stimulated by the adjuvants. In the literature not all the reports on the effects of adjuvants in teleosts have demonstrated enhancement of the immune response. Muroga and Egusa (1969) found no increase in antibody response with FIC and chrome alum, while McGlamery, Dawe and Gratzek (1971) only observed a weak response with FCA, and Post (1962, 1966a) found that adjuvants retarded the immune response. Certain combinations of adjuvant and antigen concentration were found by Sigel and Clem (1966), Evelyn (1971) and Avtalion et al. (1973) not to have an effect on antibody formation in teleosts, although other combinations produced enhancement, thus they all suggested that optimal proportions of antigen and adjuvant were required for successful stimulation of the humoral immune response. Such an explanation may account for the all-or-none effect of the adjuvants observed in the trout in the present study. The adjuvants may possibly have modified the concentration of antigen presented to the immune system, this modified dose then falling to within zones of immunological responsiveness or paralysis as was

demonstrated by Mitchinson (1965) for mammals.

The enhancement produced by the adjuvants in S. trutta was found to be carried into the secondary response although the peak titres were not higher than those of the primary response. However, the secondary plateau titres of the adjuvant inoculated fish were higher than those of the equivalent saline-MS2 inoculated groups. A possible increase in the number of memory cell lymphocytes may have been indicated here in response to adjuvant administration.

The clearance of MS2 bacteriophage from the serum of trout in relation to the administration of adjuvants, was not examined in this study, however, the observation of live bacteriophage and suppressed antibody titres 7d after secondary inoculation of adjuvant treated trout suggested that no enhancement of secondary bacteriophage clearance had taken place. One of the properties of FCA observed in mammals has been found to be the stimulation of macrophages to process antigen (Taub, Krantz, and Dresser, 1970; Allison and Davies, 1971; Unanue, 1972, White, 1972; Taussig, 1974), it would thus be valuable to separate the phagocytic and humoral antibody components of bacteriophage clearance from the sera of fish in the presence of FCA, in order to detect possible phagocytic enhancement in primary response and the possibility of phagocytic memory. Evidence has been found that migrating white cells, probably macrophages, from MS2-sensitised trout and carp had the ability to recognise MS2 antigen and stop migration. In the mammalian system this recognition is initiated by antigen specific lymphocytes in the presence of antigen releasing a migration inhibition factor (MIF) which stops the migration of macrophages thus placing them in the vicinity of the antigen (Roitt, 1974). The possible presence of MIF-like reactions were also observed in the plaice, P. platessa, by Timur (1975). The co-operation between the phagocytic macrophages and the lymphocytes, or more probably subclasses of lymphocytes, has not been

examined in teleosts and indeed much of our knowledge in the mammalian field is speculative (Feldmann, et al., 1977).

With the present results it would also be difficult to predict the involvement of humoral antibody in the clearance of MS2 from the sera of the fish examined, though in the clearance of the secondary inoculum of MS2 examined in trout, the prolonged presence of the bacteriophage in the sera and the final high rate of antibody formation are not consistent with an antigen clearance produced by the formation of antibody-antigen complexes alone. Further more, the presence of increased levels of antibody-antigen complexes have been found to produce a negative feedback in the antibody producing system (Uhr and Möller, 1968; Weigle, 1973, 1975; Seto, 1976).

B. Temperature and the immune response to MS2 bacteriophage

Environmental temperature is probably one of the most significant external factors involved in the normal life processes of poikilothermic fishes, thus there have been many observations on the dependent effects of temperature on the immune system of teleosts. The results of this study are no exception.

The three species of teleost examined each have a different optimum temperature requirement, for S. trutta this was in the region of 15°C and for C. carpio just over 20°C, while N. rossii in the natural environment of the South Atlantic experiences only small changes of temperature from 0° to 2°C. At the optimum temperatures, the maximum temperatures used in the present study, the humoral immune response to MS2 bacteriophage was quickly initiated and was vigorous in the production of neutralisation antibody. Reduction of the ambient temperature from the optimum produced two effects, firstly an increase in the clearance time of bacteriophage from the sera and the length of the inductive phase, as found by Nybelin (1968), Klontz (1972)

and Harris (1973), and secondly a decrease in the primary peak titres, though little change in the rate of antibody production was observed once initiated. The difference between species' optimum temperatures was here illustrated by the fact that the equivalent antibody titre response in carp was 5°C above the temperature necessary to produce a similar response in trout, this was thus in line with Snieszko's (1970) classification of optimum temperatures for these two species. Further, in N. rossii the humoral antibody titre response was equivalent to that of the trout at 5°C and the carp at 10°C again indicating species adaptation, though in the case of N. rossii antigen clearance time and antibody induction time were increased by 3 to 4 weeks. An extension of this work would provide useful information on species adaptation, should the opportunity arise to examine the immune response of N. rossii at temperatures above those experienced in its natural habitat and the responses of trout and carp below 5°C.

MS2 - sensitised fish at different temperatures responded to a secondary challenge with an antibody production which was dependent on temperature, however, the clearance of antigen and the induction of antibody synthesis were achieved 7d after antigen inoculation at all temperatures and in all three species of fish. The ability to produce enhanced antibody titres, in comparison with primary titres, was also retained at all the lower temperatures.

The effect of temperature changes on the already raised secondary response has not been examined. However, one group of trout held at 9°C did provide some circumstantial observations on the effect of temperature change. From 56d post inoculation the ambient temperature of the water supply in which this group was maintained rose and reached a peak at 84d of 16°C which corresponded to a significant elevation in the antibody titre. Secondary titres have been observed to fluctuate and may be analogous to the cyclical fluctuation

in 19S antibody observed by Möller, Britton and Möller (1971) in their examination of bacterial antigen response in mice, which was thought to be a feed-back inhibition produced by antibody. Thus the correlation of temperature and titre in this one isolated case must be regarded with caution. Nevertheless the effect of temperature fluctuations on already raised immune responses will be of extreme importance to fish in their natural habitat and should thus be given a more extensive examination.

The synthesis and release of humoral MS2 neutralisation antibody levels have been confirmed to be dependent on temperature, as has been shown by other workers using different antigens and fish species. The need for a period of elevated temperature on primary antigen challenge of fish maintained at temperatures below the species optimum, as observed by Muroga and Egusa (1969) and Avtalion et al. (1973), has been shown to be unnecessary for the production of an immune response. Harris (1973) found that L. leuciscus was immunocompetent at 2°C, though the latent or antibody induction period was found to be increased by 10 to 20d. It was this induction period after primary challenge that was found to be temperature dependent in trout, carp and N. rossii.

An examination of MS2 bacteriophage clearance over the primary antibody induction period showed that the live bacteriophage could be removed from the sera at all the temperatures examined, though at lower temperatures the bacteriophage remained in the sera for longer periods of time and was gradually removed (figure 14). Ferguson (1975) also reported a decrease in the clearance time of radiolabelled bacteria from the blood stream of P. platessa at lower temperatures which he related to possible metabolic effects in the process of bacterial endocytosis. With these results it is difficult to envisage, as Bisset (1947, 1948) and Avtalion et al. (1973) have done, a discrete temperature sensitive event which occurs after the process of phagocytosis

and antigen processing. Antigen clearance and presumably phagocytosis were, in the present study, found to be temperature dependent with regard to primary MS2 challenge, however, clearance and antigen processing on secondary challenge, in an immune system with a demonstrated memory, was not found to be temperature dependent. There is thus a phase in the primary sensitisation of the immune mechanisms which is related both to time and temperature (figure 27).

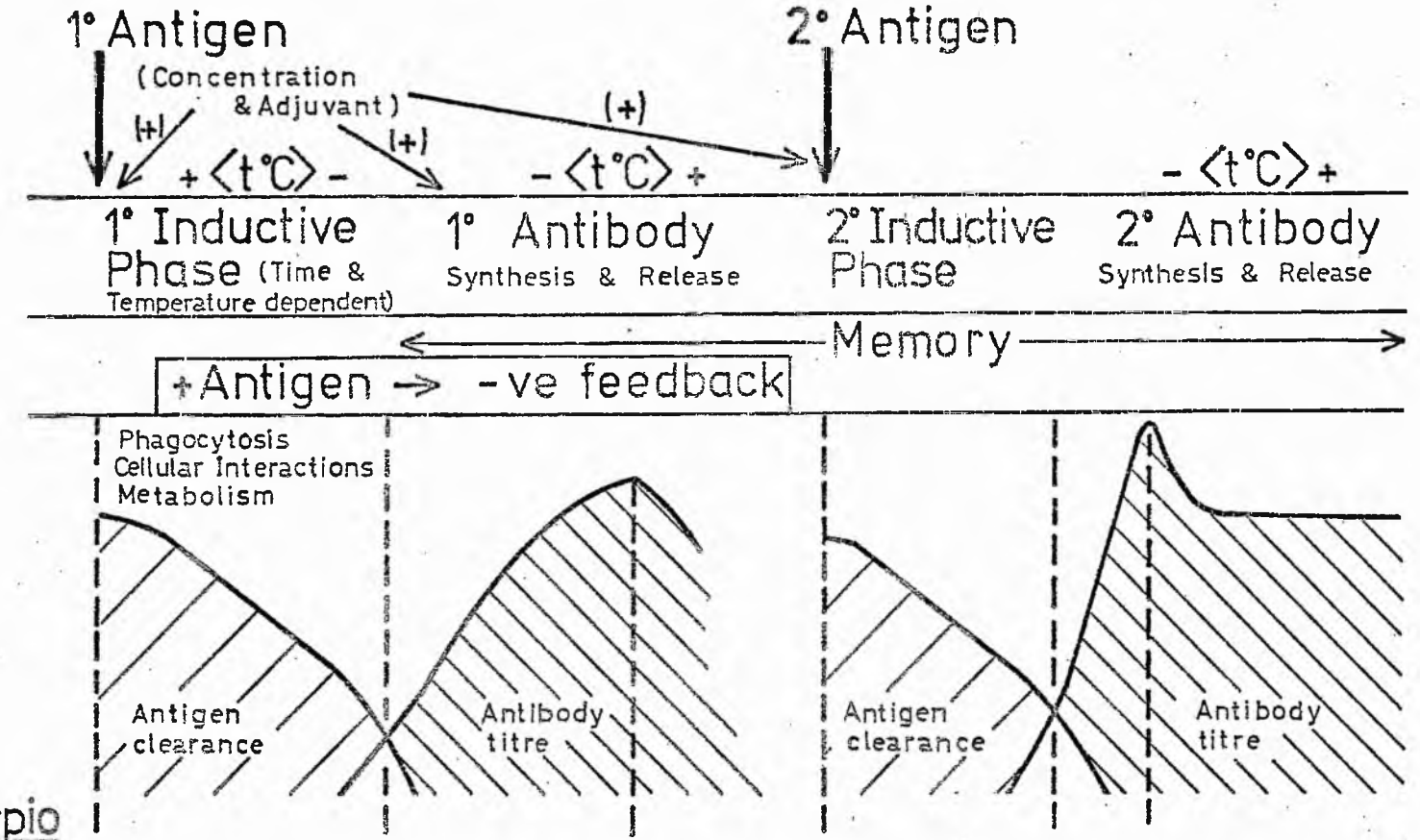
The bodily functions of fishes are depressed by decreasing temperature: feeding slows (Keast, 1968), the processes of digestion, metabolism and growth decrease (Ouchi, 1969). Cellular multiplication slows, and direct effects have been demonstrated in the replication of antibody producing cells in teleosts (Ortiz-Muniz and Sigel, 1971), and also the inflammatory and cellular immune processes slow down (Finn and Nielson, 1971b; McQueen,MacKenzie, Roberts and Young, 1973; Ferguson, 1975; Roberts, 1975b). However, at low temperatures the bodily functions of fishes are not totally inhibited as acclimation and adaptation of enzymatic processes occur with temperature changes (Johnston and Goldspink, 1975). This must also be extended to the immune mechanisms.

Furthermore, the activity of the pathogens at low temperatures must be considered as their activity will be also proportionally affected by temperature, thus a rapid immune response at low temperatures may not be critical. However, the growth of micro-organisms is not usually completely inhibited at low temperatures and even psychrophilic organisms are known (Levy, Campbell and Blackburn, 1973). It would thus seem necessary that the rate of growth of a pathogen and the ability of the host fish to raise an immune response at low temperatures must be resolved, especially in temperate fresh waters where seasonal low temperatures are observed and acclimation of the humoral immune response apparently does not occur.

FIGURE 27

Antigen clearance, humoral antibody induction and release in teleosts: A diagrammatic representation of the time sequences after primary and secondary inoculations of antigen.

+/-	Positive or negative effect on the rate of the indicated phase of the immune response
<t°C>	Temperature decrease and increase
Opt. Temp.	Optimum water temperature for S. trutta (15.5°C) and C. carpio (22.0°C) used in this study



S.trutta C.carpio Opt. Temp.	0	2·5	28 TO 49	0	-	6 TO 7 14 TO 28 days
" " -5°C	0	7 TO 14	42 TO 56	0		6 TO 7 14 TO 28
" " -10°C	0	28 TO 42	77	0		6 TO 7 14 TO 28
N. rossii 2°C	0	42 TO 70	98 TO 112	0		6 TO 7 14 TO 28

C. Cellular aspects of the immune response

i. Uptake and distribution of particulate antigens

Antigens once they have gained entry into the fish have been found to distribute themselves very rapidly. Intraperitoneally inoculated MS2 bacteriophage was found to be rapidly taken up into the sera of S. trutta, there being significant levels of live bacteriophage detected 1 min after inoculation which reached a peak at +24h. At this stage approximately 80% of the inoculated phage was estimated to have entered the trout sera. In a continuation of this work, Hutchinson (1977, unpublished) examined the internal organs of S. trutta after bacteriophage inoculation, and found that from +30 min to +1h after inoculation high levels of live bacteriophage were found in all the major organs of the abdominal cavity. Bacteriophage titres remained high in all the tissues with only a small amount of clearance over a 32h period (figure 28).

Carbon particles were also found to be taken up from the peritoneal cavity of the trout very rapidly and at 5 min after inoculation free carbon particles were observed in the blood vascular systems of all the organs examined. Ellis, Munro and Roberts (1976) observed intraperitoneal inoculations of carbon to cause a leucocyte active inflammatory response in P. platessa and by +48h most of the peritoneal macrophages were full of carbon. Snieszko (1970) had earlier in his review of immunisation found indications from the literature that leucocytes transported antigens to the antibody producing tissues. In the present study free carbon particles and live bacteriophage were observed in the blood vascular system for up to 4h in the case of carbon and 2.5d for MS2 in S. trutta at 15.5°C, thus a large proportion of the foreign particulate matter entered the blood vascular system without the aid of host leucocytes. Mackmull and Michels (1932) believed that particulate matter entered the mesenteric blood vessels from the peritoneal fluid in

FIGURE 28

Distribution of an intraperitoneal inoculation of 4.6×10^{10} PFU MS2 bacteriophage in the tissues of S. trutta with time (from Hutchinson, 1977 unpublished). Mean PFU values are plotted for four replicate fish.

A • Peritoneal cavity PFU per fish

\+ Serum PFU cm^{-3}

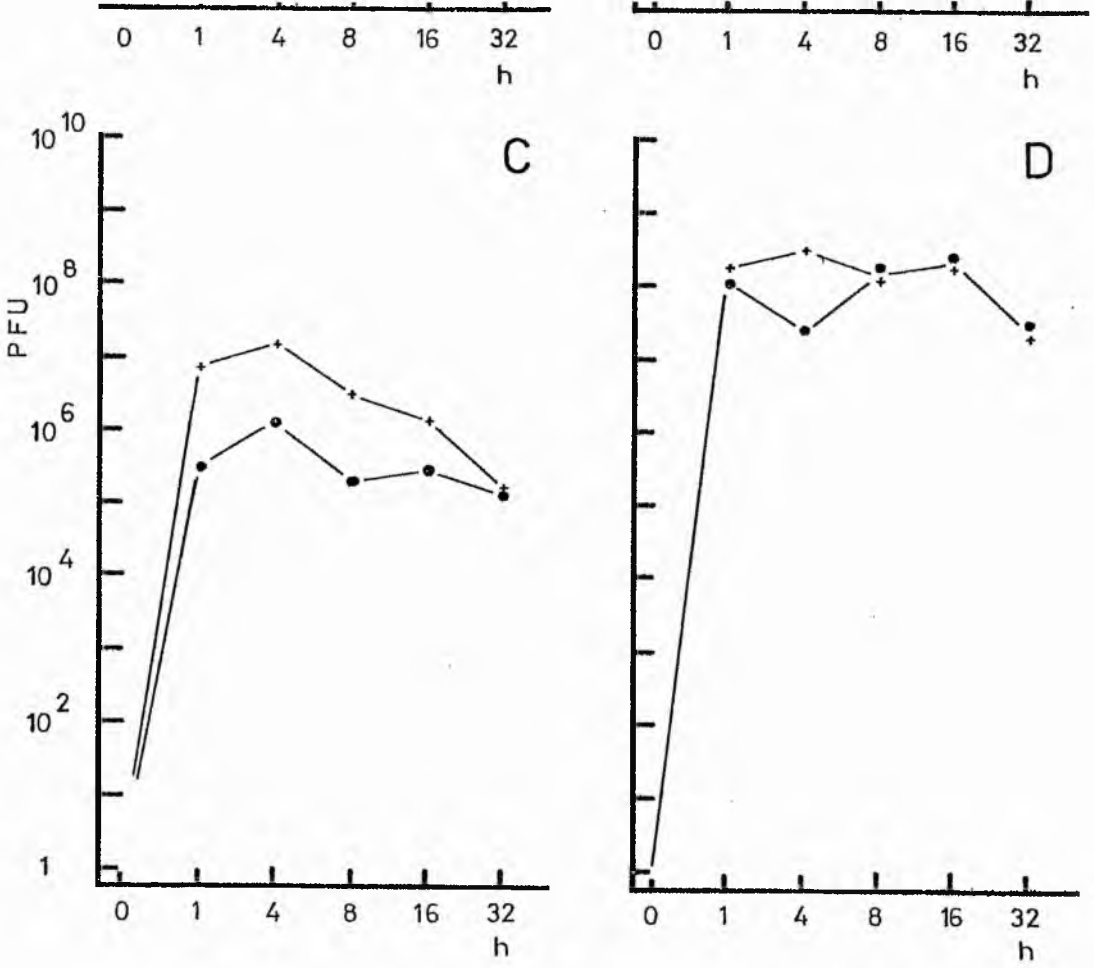
B • Spleen PFU g^{-1}

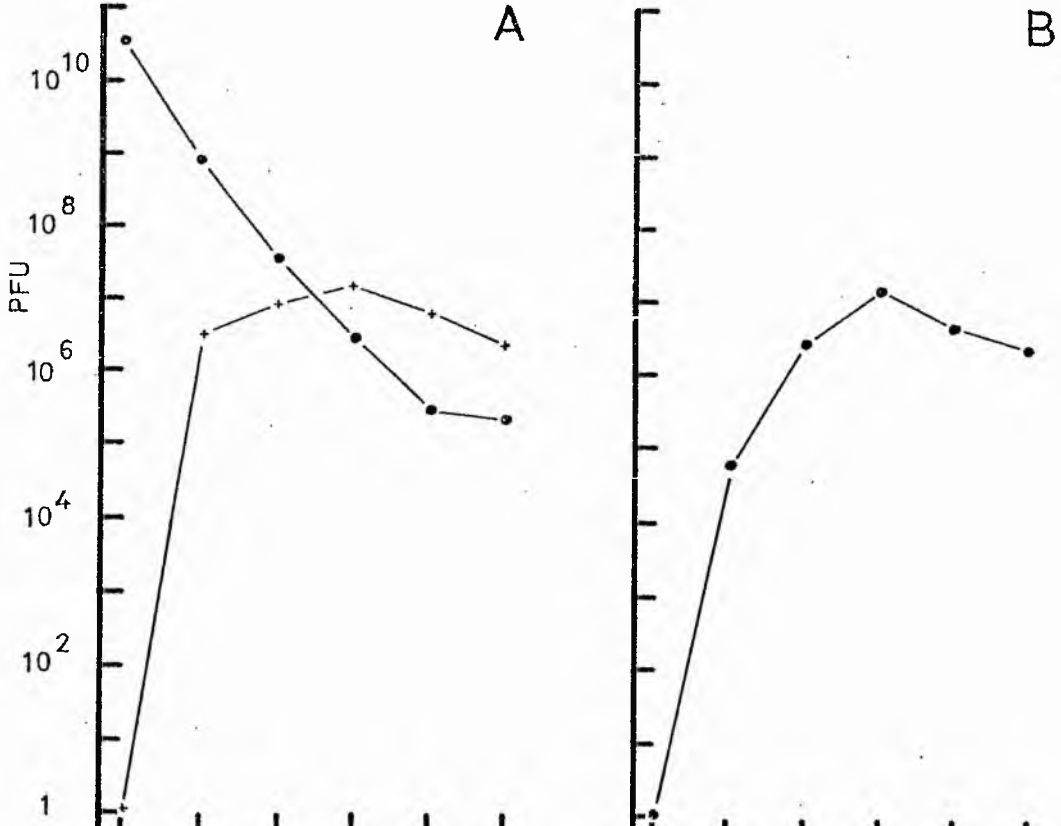
C • Anterior kidney PFU g^{-1}

\+ Posterior kidney PFU g^{-1}

D • Liver PFU g^{-1}

\+ Stomach and intestine PFU g^{-1}


0 1 4 8 16 32
h
C
D
PFU
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
1


A
B
PFU
10^{10}
10^{8}
10^{6}
10^{4}
10^{2}
1

T. adspersus, a theory also put forward by Ellis et al. (1976) to explain the uptake of free carbon particles into the blood vascular system. Carbon filled phagocytic cells were observed, however, quite early on in the blood vascular system 4 to 8h after inoculation.

The entrappment of carbon particles from the blood circulation was observed to occur chiefly in the spleen and kidney of the trout, and also to some extent in the reticulo-endothelial (RE) system of the heart, as observed by Ferguson (1975) and Ellis et al. (1976).

ii. Spleen

The structure and function of the spleen of the trout, and by inference from the structure of the carp spleen, were found to be similar to those described by White (1969) in chickens and by Ellis et al. (1976) in P. platessa. Carbon particles were first trapped from the arterial blood in the walls of the trout spleen ellipsoids and at +4h carbon was intracellularly associated with the wall of macrophage-like cells. At +8h macrophages containing carbon were observed to be free in the white pulp, however, observation was not continued long enough to see the possible aggregation of these macrophages with melano-macrophage aggregates as described by Ellis et al. (1976). Clusters or 'follicles' of white cells, which resemble the germinal follicles described by White, Henderson, Eslami and Nielsen (1975) for the chicken spleen, were not observed in the spleens of MS2-stimulated trout and carp. Neither have germinal follicles been described in the literature for other fish species.

Although it was not possible to observe directly intracellular MS2 particles in macrophages associated with the melano-macrophage centres of the trout spleen there was an observed increase in the size of these aggregations at

+36h and 7d after inoculation, and also at +8 and +36h after secondary inoculation. Ellis and de Sousa (1974) found that a proportion of radiolabelled small lymphocytes reinoculated into the donor P. platessa migrated from the blood circulation into the white pulp surrounding the melano-macrophage centres and these small lymphocytes were able to synthesis RNA. This would indicate a similar mechanism to that in chickens described by White et al. (1975) in which dendritic macrophages on the periphery of the ellipsoids collected antigen in association with antibody and then migrated with immunocompetent lymphocytes to form a germinal centre. The movement of phagoyctic cells from the ellipsoids to the melano-macrophage centres may have represented a similar function to that of the germinal centres of higher vertebrates, thus bringing together antigen and sensitised lymphocytes and then enabling the cellular proliferation of antibody producing cells. Ellis et al. (1976) have also speculated that the antigen trapping macrophages, melanocytes and melano-macrophage centres of the fish spleen may be related to the germinal follicle-producing mechanisms found in higher vertebrates by the same evolutionary precursor.

The trout melano-macrophages at the time of antigen induced aggregation were also observed to be vacuolated and were presumably in the process of active synthesis, whether for extracellular release or the internal digestion of possibly phagocytosed MS2 particles was unknown. The role of melano-macrophages, described in fishes by Roberts (1974), is not understood though they have been associated with injured tissues (Mawdesley-Thomas and Young, 1967), parasitic cysts (McQueen, et al., 1973) and in the septicaemias of trout (Thorpe and Roberts, 1972). These cells contain fine particles of melanin which gives them the observed light yellow-brown colouration. Melanin was found by Edelstein (1971) to oxidise NADH to produce hydrogen peroxide, a substance used extensively by polymorph leucocytes for breaking down ingested bacteria (El Maallem and Fletcher,

1976) and this process may also account for the observed intracellular activity of the trout melano-macrophages.

Parrott and de Sousa (1971) described a differentiation of the mammalian spleen into thymus dependent (T) and thymus independent (B) zones of antigen and lymphocyte localisation, thus the T-zones show responsibility for cellular immune response and the B-zones for humoral immunity. This differentiation may also exist in teleosts, for Ellis and de Sousa (1974) found that radiolabelled lymphocytes of P. platessa, apart from localising in the melano-macrophage centres, localised in the red pulp of the spleen and in the kidney. Unlike the small lymphocytes found in the melano-macrophage centres the other populations of lymphocytes did not actively synthesize RNA and may thus represent populations of cells which might possess T-zone activity.

iii. Kidney

Initial levels of carbon were observed to be low in the trout kidney immediately after inoculation, an observation also made by Ellis et al. (1976) for the plaice kidney, and large amounts of phagocytosed carbon were not found until +6 and +8h after inoculation. The phagocytic cells in the plaice kidney were described by Ellis et al. (1976) as RE cells which "rounded up" at 24 to 72h after carbon inoculation and uptake, though they did observe the occasional large phagocytic cell which was similar to a blood monocyte. The larger number of phagocytic cells that were observed in the plaice opisthonephros were not observed in the trout, though small amounts of free carbon were found associated with basement membranes of the kidney tubules of the trout examined.

The aggregation of carbon containing macrophages into melano-macrophage centres had not been observed after 8h

in the trout. This was undoubtedly because of the short time period of the experiment, initially designed to investigate the distribution of intraperitoneally inoculated carbon. The localisation of carbon carrying macrophages in the melano-macrophage centres of the plaice kidney was not observed until 72h to 25d after carbon inoculation by Ellis et al. (1976). No increase in the activity of the kidney melano-macrophage centres was observed in trout inoculated with MS2 bacteriophage which was comparable to the activity found in the spleen, even in secondary MS2 inoculated fish.

iv. Blood vascular system and atrium

The active phagocytosis of carbon particles by RE cells of the atrium of P. platessa demonstrated by Ferguson (1975) and Ellis et al. (1976) was also observed in the carbon inoculated trout. The intramuscular blood spaces of the trout atrium and ventricle were observed to be ideal structures for the trapping and retention of carbon particles, this would also be true for bacteria (Ferguson, 1975) but for viral particles this has yet to be assessed. The activity of the atrial RE in phagocytosing carbon and the process of "rounding up" of these cells to form free macrophages observed in the trout and in the plaice would appear to be related to the efficient sieving properties of the cardiac muscle. The ease with which micro-organisms can enter the blood vascular system of fish and be trapped in the heart makes this organ vulnerable to disease organisms. An examination of young cultured salmonids and flatfish made by Ferguson (1975) revealed that they were susceptible to acute septicaemias caused by aeromonad and vibrio bacteria. The RE cells of these fish were found to be sloughed off from the atria and there was evidence of subsequent myocardial necrosis.

The blood spaces of the gill secondary lamellae were

also observed to trap carbon readily in the trout, however, there was no evidence of endothelial cells in the gills phagocytosing carbon particles. This would seem surprising when consideration of the proximity of the environment and the blood vascular system in the gills is taken into account.

v. Liver

The tissues of the trout liver were found not to contain carbon in contrast to the high phagocytic activity of the Kupffer cells found in the mammalian liver and also the phagocytic activity of the elasmobranch liver found by Hoskins and Hoskins (1918). However, examination of the liver of other teleosts has revealed no phagocytic activity (Mackmull and Michels, 1932; Ellis et al., 1976). No accumulation of carbon particles or phagocytic activity was found in the gut wall, white muscle or dermis of the inoculated trout except for a few blood-borne phagocytic cells containing carbon.

vi. Thymus

No trace of carbon was found within the thymic tissues of the trout examined, neither was there any observed stimulation of thymic activity by MS2 bacteriophage. Similar results were observed by Ferguson (1975) and Ellis et al. (1976) in the plaice. Ellis and de Sousa (1974) in their examination of the migration of intravenously reinoculated and uridine-labelled lymphocytes found the labelled cells only in the spleen and kidney of the plaice, and no cells were found to migrate to the thymus. This was also consistent with the view that the mammalian thymus is a primary lymphoid organ in which lymphocytes proliferate independently of antigens and then emigrate. However, the origin of the thymic lymphocytes has yet to be found for fishes. In mammals the thymic stem cells originate and migrate into the thymus from the embryonic yolk sac and later from the bone marrow and liver (Moore and Owen, 1967). Many early fish histologists

believed, however, that the thymocytes and certain of the lymphocytes of fishes originated from the embryonic thymic bud (Maurer, 1886; Beard, 1894; Deansley, 1927; Lele, 1933; Hafter, 1952) while other workers believed that stem cells migrated into the thymic buds (Hammar, 1909; Maximow, 1912; Hill, 1935). Although no work has been done to elucidate this question in the fishes the work of Turpen, Volpe and Cohen (1973) and Volpe and Turpen (1975) using diploid and triploid chromosomally marked frog embryos demonstrated that the thymic lymphocytes of R. pipiens arose from the thymic bud and not from blood-borne stem cells migrating into the thymus as in mammals. They also found that the majority of the lymphocytes in the spleen, kidney and bone marrow of the frog were derived from the thymic cells, however, there was a small proportion of lymphocytes which were not derived from the thymus and which may have represented a population of T-independent cells. It may be that the group of fish anatomists who thought that the fish thymocyte and lymphocyte originated from the thymic bud is correct in its observations, though plausible this has yet to be conclusively demonstrated. At the moment there is still controversy over the immunological properties of the teleost thymocytes. Emmrich, Richter and Ambrosius (1975) found surface immunoglobulin on leucocytes in the carp thymus and Ellis (1976) reported that he had found immunoglobulin on the surface of lymphocytes in the plaice thymus. The surface immunoglobulin present on the thymic leucocytes was claimed by Emmrich et al. (1975) to represent B-like cells which were primed for future antigen stimulation and antibody production. Chicken T-cells were found by Hudson, Thantrey and Roitt (1975) to possess surface immunoglobulins when they increased the sensitivity of the labelling technique, though they found the surface immunoglobulin to be dependent on B-cell activity and that it was probably synthesised by the B-cells. The possibility thus exists that the thymic leucocytes of the carp were labelled by exogenous immunoglobulin which was detected by the more sensitive technique that Emmrich et al. (1975) had used.

The thymus in teleosts may thus be, as in young mammals, a site for initiating the surface receptors which confer immunocompetence on lymphocytes, possibly T-like, before they emigrate to the sites of immune sensitisation, replication and antibody production.

The thymus in all vertebrates has been found to undergo a process of involution usually at the onset of maturity in which the lymphoid tissue degenerates and is replaced by connective tissue cells. Deansley (1927) observed involution in S. fario (S. trutta) which started at sexual maturity and was nearly complete by +2.5 yr. In the plaice Lele (1933) did not observe involution until the fish were approximately 7+yr, several years after sexual maturity, and McArdle and Roberts (1974) described a 3+yr specimen of S. gairdneri, which though showing bilateral hyperplasia, retained the structure of a normal thymus. In all the thymus tissues examined in the trout there was no apparent loss of lymphoid tissue.

The significance of the loss of the thymic tissue in teleosts is not known and no attempt has been made to examine the immune response of fish lacking thymic tissues either by natural involution or by thymectomy of embryonic or adult tissues. Similarly, only two attempts have been made to examine the effect of splenectomy in fishes with negative results when soluble BSA antigen was used (Ferren, 1967), but positive results were found when IPN infections were examined (Yu, Sarot, Filazzola and Perlmutter, 1970). This would again indicate that the spleen of teleosts is important in processing particulate antigens although all antibody production cannot be initiated in this organ, and indeed antibody synthesis and release does occur in the teleost kidney (Chiller, Hodgins, Chambers and Weiser, 1969a; Smith, Wivel and Potter, 1970; Ellis, 1976).

The teleost fishes have been demonstrated to actively process foreign particulate matter in a similar fashion to the higher vertebrates, though they lack the lymphatic ducts and nodes and also the phagocytic activity of the mammalian liver. The phagocytic activity of the RE system, particularly active in the heart, kidney and spleen of fish, and the venous blood filtration capacity of the kidney for trapping blood-borne phagocytes may be systems which forerun the evolutionary development of the peripheral lymph nodes found in higher vertebrates. In mammals the RE system of the heart only exists as an embryonal ability for haematopoiesis which is lost in the adult (Jarplid, 1964).

The association of antigen, macrophages and lymphocytes required for the production of the sensitised lymphocytes of cellular and humoral immunity exists in the spleen and kidney of fishes. The presence of T and B lymphocytes, probably derived in the lower vertebrates entirely from the thymus, with distinct cellular and humoral functions of immunity would now seem to be a reasonable hypothesis in fishes and also the presence of T- and B-zones of antigen localisation and cellular proliferation, divided between the spleen and kidney, would seem probable.

The existence of T- and B-like lymphocyte activity and their association with other immunocompetent cells are thus speculative. A knowledge of the mechanisms of cellular co-operation and control of the adaptive immune responses, as gained by Feldmann et al. (1977) for mammals, will be essential in the fishes for assessing the limitations of their defence mechanisms to disease.

D. Heavy metals and the humoral immune response of teleosts

In general it has been observed that the exposure to the heavy metal levels examined in this present study has had a detrimental effect on the humoral neutralisation antibody production of S. trutta and C. carpio. At the same time,

however, it must be noted that the sub-lethal levels of the heavy metals examined did not produce total immuno-incompetence in the experimental fish. It was thus evident that these results confirmed the expected results of toxicant exposure observed in the literature for fishes (Goncharov and Mikryakov, 1970; Roales and Perlmutter, 1977; Sarot, 1973) and mammals (Toyama and Kolmer, 1918; Wassermann, Wassermann, Kedar and Djavaherian, 1971; Zarkower, 1972; Koller, 1973; Koller and Kovacic, 1974). The results of this study have been summarised in table 21.

The levels of dosed heavy metals were found not to stop the initiation of humoral antibody formation in trout and carp, except in the case of the 1.01 mg Cr dm^{-3} exposed carp. In the latter group of fish no true antibody response was raised to the MS2 bacteriophage in the 28d before death. The greater susceptibility of the carp to the chromium was surprising since the trout held in the same concentration of chromium survived the period of primary response without mortality. A possibly greater susceptibility was also observed in the 0.29 mg Cu dm^{-3} exposed carp. However, EIFAC (1973, 1977) concluded that C. carpio was equivalent to S. gairdneri in its susceptibility to zinc and cadmium and that these fish species were twice as sensitive as S. trutta, thus similar results may possibly be expected for copper and chromium.

The heavy metal exposed trout were observed to be retarded in their ability to remove a primary inoculation of bacteriophage from the serum, with the exception of the zinc exposed fish even at the highest concentration 2.13 mg Zn dm^{-3}. However, in the secondary response the bacteriophage was cleared within 7d of the inoculation in all the heavy metal exposed fish. An impaired hepatic phagocytic activity was observed by Trejo, Di Luzio, Loose and Hoffman (1972) in rats fed lead acetate though in this case they found no suppression of the immune response as was observed by Koller (1973) in rabbits. As an examination of the effects of heavy metals on

TABLE 21

Summary of heavy metal experiments

0	=	not significantly different from the control value
0- 0+	=	not significantly different but below or above the control value
-	=	significantly lower than the control value
+	=	significantly higher than the control value
✓	=	immune response to inoculated antigen observed
↑↓	=	secondary response plateau increases or decreases with time

Table 21 Summary of heavy metal experiments

Metal mg dm^{-3}	Inductive Phase	Antibody Response 1°	2°	Plateau	Growth	Ht %	Serum Protein	Serum Metal
Cr 1.01	-	✓-	✓-	-↓	-	-	0-	0+
Cu 0.29	-	✓-	-	-↓	-	-	-	+
Ni 0.75	-	✓-	✓+	+↑	+	-	0+	0+
Zn 0.14	0	✓-	✓-	-↓	-	-	0-	+
0.53	0	✓-	✓-	-↓	-	-	-	+
1.04	0	✓0	✓-	-↓	-	0	0-	+
1.06	0	✓0	-	+↑	+	0	-	0+
2.13	0	✓-	✓-	-↓	-	-	-	+
Cr 1.01	0	✓-	D	D	-	-	-	+
Cu 0.29	0	✓-	D	D	-	-	-	+
Ni 0.75	0	✓-	✓-	+	+	-	0-	0+
Zn 0.14	0	✓-	D	D	-	+		
0.53	0	✓0	✓0	-↑	0+	+	+	+
1.04	0	✓0	✓-	-↑	-	+	0-	0
1.06	0	✓0-	✓-	+↑	0	-	0-	0
2.13	0	✓0	-	-↓	0+	0+	0-	+
		3° and 4° Responses						
Pb 0.01		-	-		-	-	0+	
0.05		-			-	-	-	
0.1		-			-	-	-	
0.3		-			-	-		
Cd 0.05		-	✓-		-	-	-	
0.1		-	✓-		0+	-	0+	
0.2		-			-	-	-	

the phagocytic and antibody inductive phases of the primary immune response in vertebrates has not been widely reported in the literature a further investigation of the increased clearance times observed, and its relation to phagocytosis in trout, would give valuable information about these initial processes of antigen assimilation which are important in primary infection by disease organisms.

In the course of raising the humoral immune response immune suppression was observed for all the dosed heavy metals, though the pattern of response did vary. All the four heavy metals, nickel, zinc, copper and chromium, were found to suppress the primary response although in the copper-exposed fish this took the form of a delay in reaching the peak titre rather than a fall in the maximum titre. The zinc-exposed fish were not greatly suppressed in peak titre and only the lowest 0.14 mg Zn dm^{-3} dose produced a significcantly lowered response in carp, however, these were the fish which were killed by an outbreak of a Chilodonella infection.

The secondary humoral antibody titres were more consistent in their response with all but the nickel-exposed trout demonstrating a significant suppression of titre. The latter trout were observed to produce an enhanced secondary response antibody titre which remained significantly higher than the control value throughout the maintained secondary response. A similar enhancement of antibody titre was observed in the nickel-exposed carp and in the trout of the first 1.06 mg Zn dm^{-3} experiment, after the initial retarded secondary response.

An enhancement of the immune response has been observed by other workers when examining the effects of metals in mammals (Toyama and Kolmer, 1918; Jones, Williams and Jones, 1971; Spallholz, Martin, Gerlach and Heinzerling, 1975) and was put down to an adjuvant-like activity of the metals when administered at the right concentration. This should not be

confused with the common use of iron and aluminium oxides as adjuvants (Warren, Kende and Takano, 1969) which bind small particulate antigens such as virus particles in a similar manner to adjuvant oil and thus enhance phagocytosis. The adjuvant activity of the metals observed was probably due to the stimulation of the cellular and enzymic responses involved in the immune response. Selenium was found by Spallholz et al. (1975) to have an adjuvant effect on the immune response of mice to SRBC and they suggested that the activity was correlated with vitamin E activity which acted together with selenium to enhance antibody synthesis. This activity was noted, however, only within a narrow range of selenium concentrations and may possibly explain the non-enhancement of the immune response by the other concentrations of zinc examined.

The mechanisms by which enhancement was produced by the waterborne nickel and zinc are uncertain in the present experiments, although enzymic enhancement would be possible with consideration of the known biological activity of nickel and zinc (Phipps, 1976). The fact that the enhanced response was not observed until the secondary response would suggest that a build-up of metal ion was required within the body and that the metal was involved, as an essential micro-nutrient, in the enzymic processes which stimulated activity when presented at the right concentration. Many metals are essential as micro-nutrients but their action may also be indirect. Low levels of cadmium were observed by Hughes (1976) to have a beneficial effect on respiratory parameters of the gill secondary lamellae and may also have conferred an inhibitory effect on the commensal microorganisms and pathogens of these fish. However, as was observed in the carp infected with Chilodonella, the balance between the inhibition of the potential pathogen and the effect of the heavy metal on the fish was a delicate one.

The effect of single inoculations of lead and cadmium concentrations as expected significantly depressed the already

raised tertiary immune response of the trout. The greatest depression was observed with the higher concentrations of heavy metal, though only with the highest concentrations used, 0.2 mg Cd and 0.3 mg Pb, was the antibody titre completely suppressed and mortality consequent. All the other concentrations of cadmium and lead maintained a depleted but fluctuating level of antibody in the trout and the cadmium inoculated groups retained the ability to respond to an MS2 bacteriophage challenge, although the response in the group receiving the higher 0.1 mg Cd dose was significantly lower than that of the 0.05 mg Cd group. The effect of the inoculated heavy metal was thus evident for 119d after inoculation which would suggest the heavy metal was still actively suppressing antibody synthesis or that a long term cellular response had been suppressed. In mammals lymphocytes have been found with life spans of 4 to 5d and over 90d (Everett and Tyler, 1967), the short lived cells being B-cells and the long lived cells T-cells, though these life spans have been found to overlap for the two types of lymphocyte (Parrott and de Sousa, 1971). Thus in the trout the initial suppression of long lived cells which synthesised antibody would not be consistent with the life span of mammalian B-cells and may indicate the presence of a long-lived control of the antibody producing cells by cells analagous to T-helper cells which may fix the level of antibody response. Fluctuations with significant increases of antibody titre were observed, especially in the lead inoculated groups, which would suggest that the suppression caused by the metals was not entirely within the antibody producing cells and may indicate the renewal of short-lived and antibody producing B-like lymphocytes. A consideration of the life span of antibody molecules, which for human IgM the half-life was found to be approximately 5d (Waldmann and Strober, 1976), though a longer half-life of 23d was found for IgG, would also indicate that continued synthesis of antibody was necessary to maintain the titres observed in the metal-inoculated fish.

For the zinc dosed fish the effect of metal concentration was not directly reflected in the level of secondary response antibody titre suppression. A similar reduction of the differential antibody titre was observed in secondary response mice orally dosed with concentrations of lead acetate by Koller and Kovacic, (1974). The secondary peak titres were depressed and the maintained titres fell in the zinc exposed trout, but no significant difference was observed between the different levels of exposure. Although in the carp a greater suppression was observed in the fish exposed to the highest zinc concentration compared to the lowest. This lack of response between concentrations may have been related to the observed similarity of the serum zinc levels in these fish. The serum zinc levels were significantly elevated but similar in concentration at all the different levels of zinc exposure. This would indicate that the trout and carp had some control of zinc uptake from water containing non-lethal levels when the serum zinc reached a threshold level, or, that this level was maintained by removal of the zinc from the serum and the excess was stored or excreted. Indeed, certain species of teleost have been demonstrated to control body zinc levels by actively secreting the metal back into the environment (Matthiessen and Brafield, 1977).

The importance of fish in the human food chain has initiated work on the accumulation of heavy metals such as zinc (Matthiessen and Brafield, 1977), cadmium (Badsha and Sainsbury, 1977), mercury (MacLeod and Pessah, 1973) and multiple species of heavy metal (Romeril and Davis, 1976) in the tissues of fishes. Heavy metal accumulation from contaminated waters has been found dependent on the size and species of the fish and seasonal factors (Badsha and Sainsbury, 1977). The latter authors also found the levels of cadmium in Merlangus merlangus to fall in the months of November and December with the lower water temperatures. Temperature has been found to modify metabolic rate, diffusion and active transport across membranes and will also govern

the ventilation rates of fishes which in turn will modify the total concentration of dissolved metal exposed to the gills. MacLeod and Pessah (1973) found that S. gairdneri when held in 0.1 mg Hg dm^{-3} doubled their rate of mercury accumulation for every $5^{o}C$ rise in water temperature.

The use of radioactive tracers, zinc-65 (Saiki and Mori, 1955; Lloyd, 1960; Slater, 1961) and caesium-137 (Hewett and Jefferies, 1976) have indicated that the gills of fishes are the most important site of waterborne heavy metal entry, though substantial concentrations of metal are probably also taken in with the food and absorbed across the gut (Chipman, Rice and Price, 1958). However, the fate of the heavy metals and their action on the metabolic processes of fishes has not been well documented in the literature. Such studies may well be important when considering the high loads of heavy metals found in the fish food used in these experiments or even in the natural foods of teleosts.

Many of the heavy metals are readily chelated by proteins and are found dispersed through out the body tissues. Pentreath (1973) found that zinc accumulated in the skin and gonad of P. platessa, Chipman et al.(1958) found the kidney in salmonids to be the major organ of zinc accumulation. The kidney probably processes many other heavy metals. Lead has been found to produce inclusion bodies in the cytoplasm and nucleus of the renal tubules of the rat (Choie and Richter, 1972; Goyer, 1973; Moore and Goyer, 1974) and cadmium and mercury have been shown to produce necrosis of teleost renal tubules (Gardner and Yevich, 1970; Tafanelli and Summerfelt, 1975; Trump, Jones and Sahaphong, 1975). Necrosis of kidney tubules was observed in the trout exposed to 2.13 mg Zn dm^{-3}, however, as Trump et al. (1975) point out, changes which cannot be observed under the light microscope do occur at lower concentrations of heavy metal exposure or before necrosis has been observed in the renal cells.

The kidney has not been found the only target organ of heavy metals. Pathological changes in the haemopoietic portion of the spleen of cadmium-exposed F. heteroclitus were observed by Gardner and Yevich (1970), and similar effects have been observed in the haemopoietic organs caused by copper (Baker, 1969) and methyl mercury exposure (Rucker and Amend, 1969; Suzuki, Miyama, and Toyama, 1973). Although gross morphological changes were not observed in the spleens of heavy metal exposed fish in this present study the haematocrits of these fish were found to be lower than control values with the exception of zinc-exposed carp which showed increased haematocrits. An anaemic condition has often been found to be the result of heavy metal exposure and has been well documented for cadmium in the higher vertebrates, in birds (Freeland and Cousins, 1973), mammals (Friberg, Piscator and Nordberg, 1971) including man (Nilson, 1970) and in teleosts (Larsson, Bengtsson and Svanberg, 1976). Although a disturbance of the electrolyte balance has been observed in teleosts exposed to heavy metals (Larsson et al., 1976; McCarty and Houston, 1976) only a small shrinkage of the erythrocytes was observed by Larsson et al. and the anaemia was suggested to have been caused either by a reduced production or an increased destruction of erythrocytes. However, Schiffman and Fromm (1959) observed an increase in the haematocrit of S. gairdneri exposed to sub-lethal levels of chromium as was found in the zinc-exposed carp, which may have been caused by a swelling of erythrocytes or their release from haemopoietic organs. It was thus evident that the erythrocyte population was affected by heavy metal exposure, the response to which was dependent on the extent of the exposure, and that the sites of action were probably the haemopoetic centres which are also important in processing immuno-competent cells. An assessment of the total haematology of fishes has been found to be related to a wide range of factors, physiological stress and disease as well as the effects of toxicants (Blaxhall, 1972), and may well be used to indicate such situations but not their causes.

The liver, a major target and storage organ for metals such as cadmium in mammals (Flick, Kraybill and DiMitroff, 1971; Friberg et al., 1971) and similarly in fishes (Ministry of Technology, 1966; Eisler, 1974; Tafenelli and Summerfelt, 1975; Larsson et al., 1976) has been found to be readily affected by heavy metals. These effects have been shown in teleosts to be related to carbohydrate metabolism (Ministry of Technology, 1966; Larsson, et al., 1976) and numerous enzymic processes (Jackim, Hamlin and Sonis, 1970; Hiltibran, 1971; Christensen, 1971, 1975). Such interference in the metabolic processes will undoubtedly affect the growth of fishes (Christensen, 1975) as has been observed in this study. Except in the case of the nickel-exposed fish all the other heavy metal exposed fish were found to be retarded in their growth and this was very marked in the trout which were treated with inoculated concentrations of lead. The direct effects of metabolism and growth on the immune response of a teleost has been observed by Goncharov and Mikryakov (1970) in starved C. carpio. In the starved fish the antibody titre response to A. punctata antigen was found to be significantly reduced, thus a general depletion of metabolic activity observed in the present study may have been partially responsible for antibody suppression.

The involvement of heavy metals in the cellular metabolism and particular functions of organs may affect the immune responses of teleosts through the haemopoietic centres, possibly by direct action on the antibody producing and other immunocompetent cells. Indeed, Koller and Kovacic (1974) found that in immuno-suppressed mice, dosed with lead acetate, the numbers of antibody producing cells in the spleen were reduced with increasing lead concentration and that the suppression of antibody forming cells was greater in the secondary response mice. The presence of heavy metal has been found to suppress and even stop mitosis in cultured fish cells (Rachlin and Perlmutter, 1968, 1969) possibly by binding to the active polypeptides formed by the nucleolus of the cells (Studzinski, 1965). Rachlin and Perlmutter (1969) found

that 10 mg Zn dm^{-3} added to S. gairdneri gonadal cell cultures did not affect the mitotic index, but at 18 mg Zn dm^{-3} the index was reduced by 70%. Although the serum zinc levels of the control carp were found to be in the order of 10 mg dm^{-3} those of the control trout in the present study ranged from 10 to 20 mg dm^{-3} and the zinc exposed fish showed serum zinc levels up to 10 to 15 mg dm^{-3} higher than the control values. The control values indicated that there must have been some species control over serum zinc levels and the sensitivity of cells. Indeed Rachlin and Perlmutter (1968) found that P. promelas epithelial cell cultures were ten times more sensitive than the gonadal cells of S. gairdneri. The rises in zinc levels, and in those of the other heavy metals, in exposed fish may have been sufficient to affect cellular division but the sensitivity of the haemopoietic cells and immune competent cells has yet to be determined. A large amount of the heavy metals entering the fish tissues will be protein bound and thus relatively inactive. This was demonstrated by the neutralisation of MS2 bacteriophage by free metal ions but not by control serum samples which contained heavy metal levels which in the free state would have produced 50% mortality in the bacteriophage.

The heavy metals bind readily to proteins. The ability of calcium ions to block or compete for the protein binding sites may possibly be the reason for the decreased toxicity of heavy metals to fish in hard waters.

This is especially true in the case of zinc where the bivalent calcium can actively compete for protein binding sites because the linkage is reversible (Matthiessen and Brafield, 1977). The heavy metal-protein binding ability will cover a wide range of biologically active molecules, especially enzymes, and including antibody molecules. Williams, Caraway and deYoung (1954) found that lead bound to antibody in vitro and Jones, Williams and Jones (1971) speculated that cadmium was capable of breaking down the

essential structural configuration of antibodies thus blocking their mode of action. It has also been suggested that complement activity can be suppressed in a similar manner by lead ions (Hemphill, Kaeberle and Buck, 1971). The readjustment of the tertiary structure of proteins by the attachment of heavy metals is a wide spread mechanism (Phipps, 1976) and can be beneficial in the activation of enzymes but conversely the readjustment of molecules which rely on a steric configuration to function actively, such as enzymes, antibodies and complement, will drastically affect their function.

Specific metal binding proteins, metallothioneins, have been observed in vertebrates which have the ability to"mop up" heavy metals (Shaikh and Lucis, 1970; Piotrowski, Trojanowska, Wisniewska-Knypl and Balanowska, 1973). Olafson and Thompson (1974) found that these low molecular weight proteins (10,000 D) could be induced by cadmium exposure in fur seals and in the teleost Sebastodes caurinus. This may have accounted for the observed increase in resistance to toxic levels of zinc conferred on trout species by previous acclimation to low levels of zinc (Goodman, 1951; Affleck, 1952; Lloyd, 1960; Edwards and Brown, 1966).

The metallothioneins which were induced in the livers of rats were found by Shaikh and Lucis (1970) to be rich in cysteine and sulphydryl groupings to which heavy metals readily bound. The production of sulphur groupings appears to be ubiquitous throughout living organisms. Brown (1977) found high levels of sulphur formation in an isopod, Asellus meridianus, exposed to cadmium and lead. Non-protein sulphydryl groups were found to be formed in heavy metal tolerant Aspergillus niger (Ashworth and Amin, 1964; Antonovics, Bradshaw and Turner, 1971), and in copper tolerant yeasts heavy metal sulphide precipitates were found to be related to an excessive cysteineproduction (Ashida, 1965).

The affinity of heavy metals to sulphydryl linkages would explain the observed blockade of antibody and complement activity reported in the literature as these molecules possess such sulphydryl linkages as an essential part of their functional structure. A similar effect would be observed also in enzymatic activity (Wada, Toyokawa, Suzuki, Suzuki, Yano and Nakao, 1969; Nilsson, 1970) and protein synthesis (Ellis and Fang, 1971). However, the degree to which such bindings may have occurred with the antibody and antibody synthesising processes in the heavy metal exposed fish of the present experiments is not known.

The effects of heavy metals whether at the sub-cellular, cellular or organ level of organisation are numerous, and it would thus not be surprising if the reactions of exposed fish are involved in stress mechanisms, as Bromage and Fuchs (1976) found in C. auratus exposed to sodium lauryl sulphate. These stress mechanisms have also been implicated in the diseases of fishes (Meyer, 1970; Wedemeyer, 1970a; Snieszko, 1974). Heavy metals have been shown to increase the incidence of disease in mammals experimentally exposed to heavy metals and administered infectious agents (Koller and Kovacic, 1974) and have been shown to be directly involved in the suppression of antibody titres and antibody producing cells, though the mechanisms by which this occurs are not precisely known. The literature reviewed would also seem to imply such a relationship in pollutant-exposed teleosts and indeed direct suppression of humoral immunity has been demonstrated in trout and carp used in this study.

Although the effects of heavy metals on trout and carp in the terms of stress reactions were not quantifiable the effects of stress processes may have been observed in two instances. The first instance was observed when the control trout of the cadmium experiment were transferred to a new tank which resulted in a fall in antibody titre and the second was the inability of Chilodonella infected carp to produce an immune response. The latter case was an interesting one in that the infection

produced mortality and antibody suppression only in the carp dosed with the lowest concentration of zinc; the controls and fish exposed to higher concentrations of zinc were unaffected although the ciliate was present as a commensal. It is probable that the ciliate would have been suppressed in its own activity by the higher zinc concentrations, and it may thus have been that an interaction between the stressor effect of the low concentration of zinc on the carp and the tolerance of the ciliate produced the right conditions for symptoms of disease to appear. However, further evidence of such a relationship between commensals and potential pathogens is required as in this case replicate tanks were not available at the various dose concentrations.

Diseases are the end-point of an interaction between a "noxious-stimulus" and a biological system (Mawdeseley-Thomas, 1972b) and the onset of these diseases is influenced by the appropriate relationship of the physiology of the fish, the pathogen and the environment acting together (Snieszko, 1974). The balance between the potential pathogen and disease in teleosts would appear to be a delicate one and in itself is a complex series of interactions. In the natural situation of the wild or hatchery environment the host fish and potential pathogens mix freely together, thus the relationship of these interactions on subsequent disease processes and the immune mechanisms is in itself an area requiring further study.

The relationship of heavy metals or other pollutants to diseases in teleosts cannot be examined as an effect on the immune responses solely, though an effect on the humoral immune responses of teleosts has been shown. In the dosed and inoculated heavy metal experiments the action of these metals was undoubtedly varied and at the concentrations used may have produced stress reactions, interacting directly with antibody producing cells, antibody and complement mechanisms, general enzymic processes or even the ionic balance of the fish. It was possibly surprising that the regular application of the anaesthetic used in these experiments, MS222, considered by

Wedemeyer (1970b) and Soivio, Nyholm and Huhti (1977) as a stressing agent, because of the changes produced in the ionic balance of S. gairdneri, did not make any apparent modification in the humoral immune response of the teleosts examined.

V. CONCLUSIONS

All specimens of the three teleost species examined, S. trutta, C. carpio and N. rossii, produced humoral neutralisation antibodies in response to single inoculations of MS2 bacteriophage. The bacteriophage was found to be very antigenic as only very small amounts of antigenic protein (5.6×10^{-16} g) were required to induce neutralisation activity. The assay for neutralisation titre was found to be a sensitive one which gave a simple, accurate and reliable method of indicating the antibody-producing capacity of the teleosts examined.

The basic investigation of the humoral immune response of trout, carp and N. rossii revealed that the response in teleosts possessed similar features to that of mammals and humans responding to non-replicating viral antigens. The antibody produced by all three species of teleost showed classical characteristics of IgM-like identity, an HMW fraction sensitive to 2-ME reduction. Other properties defining the neutralisation activity as that of an antibody were the complement fixation properties and the specificity of the immune sera, though no precipitin activity was observed which may be consistent with single antigenic attachment sites on MS2 bacteriophage. Although no transition from HMW to LMW IgG-like antibody was observed, typical primary and enhanced secondary humoral immune responses showing memory were observed in the teleosts. When the trout and carp were held at their species' optimum temperatures the peak antibody titres and induction phases of the immune response were both quantitatively and temporally similar to those observed in mammals.

The humoral immune response of teleosts to MS2 bacteriophage has been found to be dependent on antigen presentation. Increased concentrations of bacteriophage and the

addition of adjuvants have been found to increase the total amount of antibody production and the degree of memory, whether by clonal recruitment or enhancement of B-like cells has yet to be discovered. Further, specialisation of immunocompetent cell populations may also be indicated by the FCA-enhanced effect on low doses of MS2 antigen, characteristic of T-lymphocyte stimulation. Although the existence of T-and B-lymphocyte homologues in teleosts would appear to be likely from circumstantial evidence (Ellis, 1977) the presence of two functional populations of lymphocytes has yet to be conclusively demonstrated.

Temperature was probably the most important modifier of humoral immunity in the three teleosts, as has also been recognised by many reports in the literature, and was found to be especially important in the primary challenge by an antigen. None of the fish examined at low temperatures were found to be immuno-incompetent to MS2 bacteriophage stimulation, though further work at the lower temperature limits of trout and carp and the upper limit of N. rossii would yield more critical information on this topic. Although low temperatures reduced the level of antibody production this may not be as detrimental as was first thought if the replication and growth of possible pathogenic organisms at these temperatures were also to be considered. It was also noted that species adapted to colder waters possessed some adaptation of the humoral immune response. Thus trout and carp retained the $5^{o}C$ difference, related to their optimum temperatures, when held at lower temperatures and N. rossii at $2^{o}C$ was able to produce a titre response equivalent to trout at $5^{o}C$ and carp at $10^{o}C$. However, the most crucial aspect of low temperatures may be their influence on the processing of antigen and on the antibody induction period after the primary challenge of the antigen. It is interesting that the processing of antigen and the mechanisms of immune memory were unaffected on secondary challenge, so that the onset of humoral antibody production in the secondary response was independent of

temperature, and was found to be of the same time period in all the three species of teleost. In these fish an all-or-none effect on the immune response produced by low temperatures cannot be accepted under conditions of normal antigen challenge, that is, at doses or under other conditions which would not normally cause immune paralysis.

The processing of antigen and the induction of the primary immune response has thus been considered as a timed sequence of events of which one or many temperature sensitive components may be slowed by decreased environmental temperature. The exact mechanisms are not known though undoubtedly low temperatures will slow biochemical reactions and the phagocytic activity of cells, however, these processes in secondary response fish were seemingly unaffected. An investigation of the immediate clearance of a secondary challenge of MS2 bacteriophage in teleosts held at different temperatures would indicate more exactly the effect of temperature on the phagocytosis and processing of antigen.

The ability to produce an enhanced secondary antibody titre has been noted in the teleosts examined, however, a more intensive examination of the secondary inductive period of trout held at 15.5°C showed that MS2 clearance was delayed by a period of 3.5d when compared to the time required for primary clearance. This delay was also evident in the antibody titres of other fish 7d after secondary inoculation, even live phage was found to be present in the sera on some occasions. Feedback mechanisms have been discussed and it is probable that in the latter case antigen or antibody-antigen complexes had activated a negative feedback in the cellular processes of secondary immunity. It is thus suggested that such mechanisms may also be involved in the low immunoresponsiveness of teleosts, which has been found by many workers, when challenged with large or repeated doses of an antigen.

The relationship of natural infections and potential

pathogens found commensal on, or in many fish to their presentation to the immune processes as antigen and the possible effects of long term exposure to potential antigens need to be examined. It has been demonstrated that water-borne MS2 can stimulate a humoral immune response in trout, though in this case the exposure time was short, 4h, thus the possibility of long term non-infective antigen exposure suppressing the humoral antibody response may occur if negative feedback mechanisms exist. Should antigen penetrate the integument of teleosts there exists an active system of phagocytic cells and non-specific factors such as lysozyme, C-reactive protein, interferon and natural agglutinins, but it is not known if these systems remove antigen from the pathways of immune stimulation. There may also be a separate response of teleosts to antigens which cannot normally penetrate the healthy fish, and, as found by Fletcher and White (1973) this process may result in only secretory antibody being formed. Whether this process is related to, or is independent of the processes of humoral immunity in fish is not known.

The relationship between the presentation of antigen and the effects on the immune response of teleosts may be affected by the presence of other species of antigen, as would be found in many natural situations. The use of an independent antigen such as MS2 bacteriophage, which in itself is not pathogenic in its activity, may be useful in future studies of fish-pathogen interactions in answering some of these questions.

A wide knowledge of the function of leucocytes and the part they play in the defence processes of vertebrates has been gained over the last decade. Although the study of teleost leucocytes has been biased by morphological similarities with mammalian cells (Ellis, 1977), without regard to their actual function, the identification of immunocompetent lymphocytes in teleosts is well established. However, the interactions and stimulation of lymphocytes with

other immunocompetent cells are not yet clear.

The teleost has the ability to trap and phagocytose free antigen entering the body and blood vascular system, an essential process if the ease with which particles such as carbon and MS2 can enter the blood vascular system is noted. Trout inoculated with carbon were found to trap the foreign particles at three major sites, the atrium, the splenic ellipsoids and in the kidney white pulp. Carbon was phagocytosed at these sites mainly by reticulo-endothelial cells which then 'rounded up' and migrated into the white pulp of the spleen, and kidney, presumably to aggregate around the observed aggregations of melano-macrophages as other workers have documented. It is evident that these aggregations of cells, along with a specialised population of lymphocytes, interact and stimulate the synthesis and release of antibody, and are possibly equivalent to the B-zones of stimulation or germinal centres found in the spleen and lymph nodes of mammals.

The existence of lymphocyte sub-populations has not been clearly elucidated in teleosts, though the evidence now available would indicate their presence. The use of cellular interactions and control mechanisms in immunocompetent teleosts, similar to those found in mammals, would not be unreasonable in the circumstances. In mammals these mechanisms are complex and are mediated by immunocompetent cells requiring the interaction of various T-cell populations, B-cells and macrophage populations. It is at this level that various enhancement and suppressor feedback mechanisms control both the humoral and cell mediated responses of immunity (figure 26). Feedback suppressor mechanisms appear to function in teleosts as well as the various forms of enhancement of the humoral immunity. An increased knowledge of such mechanisms would advance the understanding of the evolution of adaptive immune responsiveness and the disease processes of teleosts.

Non-lethal levels of heavy metals were found to suppress

the humoral antibody response of trout and carp to MS2 bacteriophage. In the dosed metal experiments it was found that mechanisms of antigen clearance were delayed, but the ability to initiate an immune response was not lost. The effect of both inoculated and dosed levels of heavy metals was to lower the level of antibody production. The concentration of the heavy metal exposure was in general proportional to the observed suppression in antibody titre. This was not distinct in the zinc-exposed trout, probably because of the mechanisms of zinc uptake and control which produced a threshold level of zinc activity in the sera of these fish, but the concentration effect was evident in lead and cadmium inoculated trout and in fish which were exposed to lethal doses of heavy metal. In the latter case antibody levels were reduced to undetectable levels before death.

Enhancement of the humoral antibody titres was observed in secondary response fish exposed to nickel and in one group of carp exposed to 1.06 mg Zn dm^{-3}. An adjuvant-like activity of these metals on the immunocompetent cells has been suggested. Many of the heavy metals used in this study, apart from cadmium and lead, are essential micro-nutrients in biological systems, and low levels of these metals above those found in the normal environment may have to be considered beneficial in the short term. Very low levels of cadmium were found by Hughes (1976) to enhance the respiratory efficiency of S. gairdneri and a biocidal effect of zinc may have been observed in carp infected with a ciliate pathogen, though in the latter case the lowest zinc-exposure dose may not have been sufficient to keep the pathogen growth in check.

The possible mechanisms by which the heavy metals may affect humoral antibody synthesis in teleosts are numerous, and they are probably a product of numerous interactions. There are the direct effects of metal binding to proteins, whether with enzymes, antibody, complement or non-specific proteins which produce steric changes and block molecular activity. There are known effects of heavy metals on the

haemopoietic organs of teleosts which will also directly effect the cellular division of lymphocytes and thus affect the numbers of memory and control cells. Indirectly the immune mechanisms may be affected by the nutritional and metabolic state of the teleost, and even by the triggering of stress mechanisms.

The susceptibility of teleosts to disease organisms and the progression to a disease state is indeed as Snieszko (1974) suggested a complex interaction of the host, environment and pathogens. Much is known about the effects of the complexities of environmental factors such as temperature, pH and water hardness on the toxicity of single heavy metals or pollutant complexes. The immune mechanisms, and probably the interactions between pathogens, must also be considered in a complex of interactions on, or in the host fish which will also be influenced by changing environmental factors. In this study antigen concentration, temperature and sub-lethal levels of heavy metals have been found singly to affect directly or indirectly the humoral immune response of teleosts and must be so considered when relating these results to the natural situation.

Although low levels of pollutant metal may not be lethal to teleosts such exposure may result in a decrease in the resistance of the fishes to pathogens, pathogens which may be obligate, commensal or free living are found in the normal environment of fishes especially in polluted waters. In the long term, that is over several generations, even the smallest suppression of an organism's efficiency and ability to mediate with the environment will decrease the probability of that organism's survival. As was pointed out by Larsson et al. (1976) these insidious changes of an animal's physiological processes must be considered a more serious threat than an easily observed 'fish kill', which has too often been used to define a pollutant event. Perhaps a more serious aspect may be that the increasing background levels of various pollutants will not kill fishes but that they will adapt or man will

learn to adapt them to live with the pollutants. Levels of zinc in the bones of Gadus morhua have been increasing since 1865 in line with increasing background levels of zinc (Scott, 1977), and it may be that mechanisms such as the induction of metallothioneins have already started to adapt to minute increases in environmental heavy metal levels.

VI. SUMMARY

1. The humoral neutralisation antibody titre response to single intraperitoneal inoculations of MS2 bacteriophage was followed in three species of teleost Salmo trutta, Cyprinus carpio and Notothenia rossii.

2. The neutralisation activity of sera was measured as a 50% bacteriophage neutralisation titre (SD_{50}) and the technique was found to be a sensitive assay of neutralisation antibody.

3. Neutralisation titres to MS2 bacteriophage were found in the sera of 43% of the trout and 21% of the carp examined before immunisation. No neutralisation activity was observed in unsensitised N. rossii. An experimental exposure of trout to waterborne MS2 bacteriophage demonstrated that humoral neutralisation antibody could be induced without antigen inoculation. Waterborne infection by antigens and the presence of natural neutralisation factors in the serum have been discussed.

4. The humoral immune response to primary and secondary inoculations of MS2 bacteriophage was similar in form to that observed in mammals and humans and was quantitatively modified by antigen concentration, adjuvants and temperature. A true primary response and a secondary response which showed enhancement and immune memory were observed in all the three species of teleost examined.

a. Peak primary antibody titres were found to increase with increased primary antigen concentration which was also found to influence secondary response plateau titres.

b. The addition of Freund's incomplete adjuvant to the antigen inoculum was found to enhance the peak primary titre of 10^9 PFU MS2 inoculated trout and to enhance

secondary response plateau titres. The antigen concentration and adjuvant effects have been suggested as representing an increased priming of clonal lymphocyte populations, possibly B-like cells, and the formation of increased numbers of memory cells.

c. The addition of Freund's complete adjuvant was found to enhance the peak primary titres to both the highest and lowest concentration of inoculated MS2 bacteriophage, though the non-response of the intermediate 10^6 PFU inoculated group may have indicated zones of responsiveness necessitating the use of correct proportions of adjuvant and antigen. The secondary response plateau titres of all the three antigen concentration groups were further enhanced by the complete adjuvant. The response to Freund's complete adjuvant has been suggested as evidence of the involvement of two populations of lymphocyte in the humoral immune response of teleosts should the classical action of complete adjuvant in stimulating T-cells be taken into account.

d. The effect of temperature has been shown to be an important factor in the quantitative aspects of the humoral immune response, but low temperatures have not been found to produce immuno-incompetence in the teleosts examined. Two effects of decreased temperature have been demonstrated, firstly a depression of the peak antibody titre responses and secondary response plateau titres and secondly an increase in the latent or inductive period of antibody formation in the primary response. Once initiated the rate of antibody titre rise was comparable at all the temperatures examined and the induction of the secondary response was found to be unaffected by temperature or species. Although the induction of the humoral immune response was dependent on temperature an all-or-none temperature-related trigger mechanism was not observed but rather a time sequence of events which was modified by

temperature has been suggested.

e. A species adaptation of humoral immunity was observed in the three species of teleost. In trout and carp the 5°C difference between their established optimum temperatures was found to be retained in the quantitative response of humoral immunity at lower temperatures. There was no apparent evidence of acclimation of low temperatures in the two species. The titre response of N. rossii at 2°C was similar to that of trout at 5°C and carp at 10°C.

5a. The clearance of primary inoculations of MS2 bacteriophage from the sera of teleosts was found to be dependent on temperature and in N. rossii was also influenced by the presence of adjuvants. Trout maintained at 15.5°C were found to clear bacteriophage from their sera 2 to 3d after inoculation and carp at 22°C within the first 7d. A decrease in temperature from these optima increased the time of bacteriophage clearance and antibody induction period to 7 to 14d with a 5°C decrease and 28 to 42d with a 10° decrease. N. rossii at 2°C was found to have a 42 to 70d induction period and bacteriophage clearance was found to be faster in the adjuvant inoculated fish.

b. The clearance of a secondary inoculation of MS2 bacteriophage was found to be independent of antigen concentration or the adjuvants used in the primary inoculum and was also independent of temperature. In all three species of teleost the bacteriophage was cleared 6 to 7d after inoculation thus indicating that the sensitised cellular activity and memory were independent of temperature.

c. The bacteriophage clearance and inductive phase of secondary response were found to be 3.5d longer than in

primary response trout at 15.5°C, and an initial suppression of secondary antibody titres was also observed in the other species. Feedback mechanisms, immune suppression and paralysis have been discussed in this context.

6a. MS2 bacteriophage and carbon particles were intraperitoneally inoculated into trout to examine their uptake and distribution within the tissues. Both MS2 and carbon were detected in the blood vascular system directly after their inoculation. MS2 was detected in sera 1 min after inoculation and reached a peak titre 24h later which represented approximately 80% of the inoculated particles being taken into the blood vascular system.

b. The phagocytic activity of reticulo-endothelial cells in taking up carbon particles was observed in sections of trout atria, splenic ellipsoids and kidney 4h after inoculation and these organs have been considered the major organs of particulate antigen entrappment. No carbon was found within the tissues of the liver and the thymus.

c. The structures of the three major lymphoid organs, thymus, spleen and kidney have been described for the trout and have been discussed in relation to phagocytic activity and melano-macrophage aggregation.

7. Only the HMW fraction of the MS2 sensitised teleost sera was found to have neutralisation activity in both primary and secondary response fish and no transition to a LMW molecule was observed. The neutralisation fraction was found to be 2-mercaptoethanol sensitive, specific for MS2 bacteriophage and it fixed both homologous and heterologous complement, though no precipitin activity was observed. It was thus concluded

that the neutralisation activity was produced by a true antibody which was possibly similar to the tetrameric IgM-like molecules found in other teleost species.

8. Trout and carp were continuously exposed to levels of waterborne heavy metals, 0.75 mg Ni dm^{-3}, 0.14 to 2.13 mg Zn dm^{-3}, 0.29 mg Cu dm^{-3} and 1.01 mg Cr dm^{-3}, of which only the nickel and zinc concentrations were found to be non-lethal for the duration of the experiment.

a. In general a suppression of the humoral immune response was observed in the heavy metal exposed fish. However, the response was not totally suppressed except in those fish which died from heavy metal toxicosis before the experiment was terminated.

b. A decrease in the rate of clearance of live MS2 bacteriophage from the serum was observed for trout exposed to heavy metals, with the exception of the zinc-exposed group.

c. The nickel-exposed fish exhibited enhanced secondary response antibody titres, as did carp exposed to 1.06 mg Zn dm^{-3} after the initially suppressed secondary response. An adjuvant effect of certain metals has been discussed.

d. The effect of the different concentrations of zinc was found to be slight except for the secondary plateau titres of carp which were significantly lower at the highest concentrations of zinc. The similarity of the increased serum zinc levels in fish exposed to the different zinc levels was suggested as a possible cause and the control of heavy metal levels discussed.

9a. Intraperitoneally inoculated concentrations of 0.01 to

0.3 mg Pb and 0.05 to 0.2 mg Cd per 100g body weight were found to suppress an already raised tertiary immune response. The suppression was dependent on metal concentration, though again complete suppression was observed only in the 0.3 mg Pb and 0.2 mg Cd inoculated trout just before death.

b. Cadmium inoculated trout were challenged with a fourth dose of MS2 bacteriophage and a humoral immune response was observed. The latter was significantly decreased in the fish receiving the higher concentration of inoculated cadmium.

10. The suppressive effects of heavy metals have been discussed in relation to their possible mode of action, stressor activity and with reference to disease situations.

VII P L A T E S

(Figure plates 29 to 50)

FIGURE 29

Viral plaque assay plate: The clear plaques produced by MS2 bacteriophage can be seen in a 'lawn' of E. coli. The wells, 1 to 6, represent increasing dilutions, 1/4 to 1/4096, of a serum taken from S. trutta x $\frac{2}{3}$ actual size.

1 'Lawn' of E. coli, no viral plaques

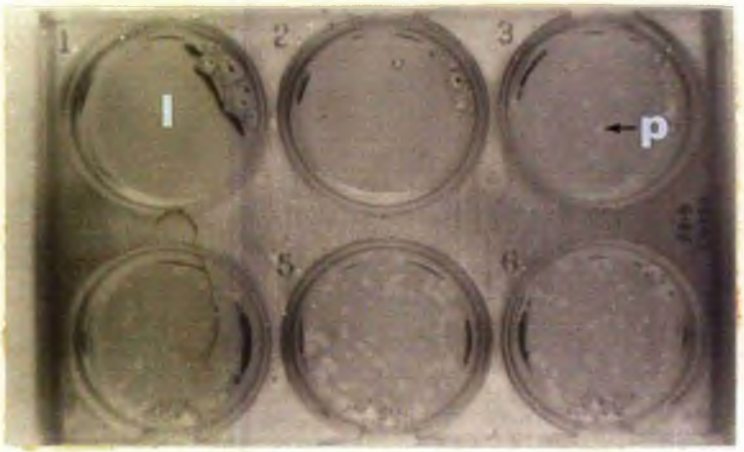
p Clear plaque formed by the bacteriophage MS2

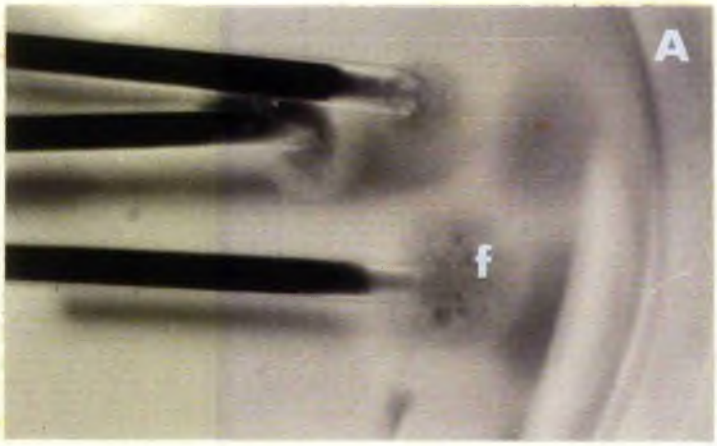
FIGURE 30

In vitro macrophage migration inhibition test using blood macrophages in the 'buffy coat' of a micro-haematocrit taken from an MS2-stimulated S. trutta.
A. The macrophages in the 'buffy coat' are migrating out of the micro-haematocrit tube in the absence of MS2 antigen to produce the characteristic 'fan'.
B. In the presence of MS2 antigen migration is inhibited and no characteristic 'fan' produced.

Both photographs x 5.

f. 'Fan' produced by the migrating 'buffy coat'.


1
2
3
I
p
5
6


A
f

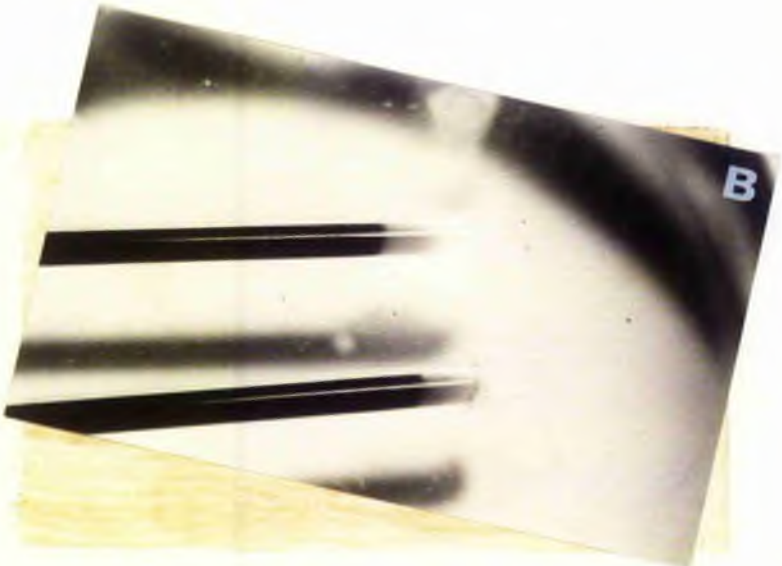

B

FIGURE 31

Transverse section through the hind-gill region of S. trutta showing the paired thymus. H & E, bar = 1mm

bc	Buccal cavity
g	Gills
oc	Opercular cavity
th	Thymus

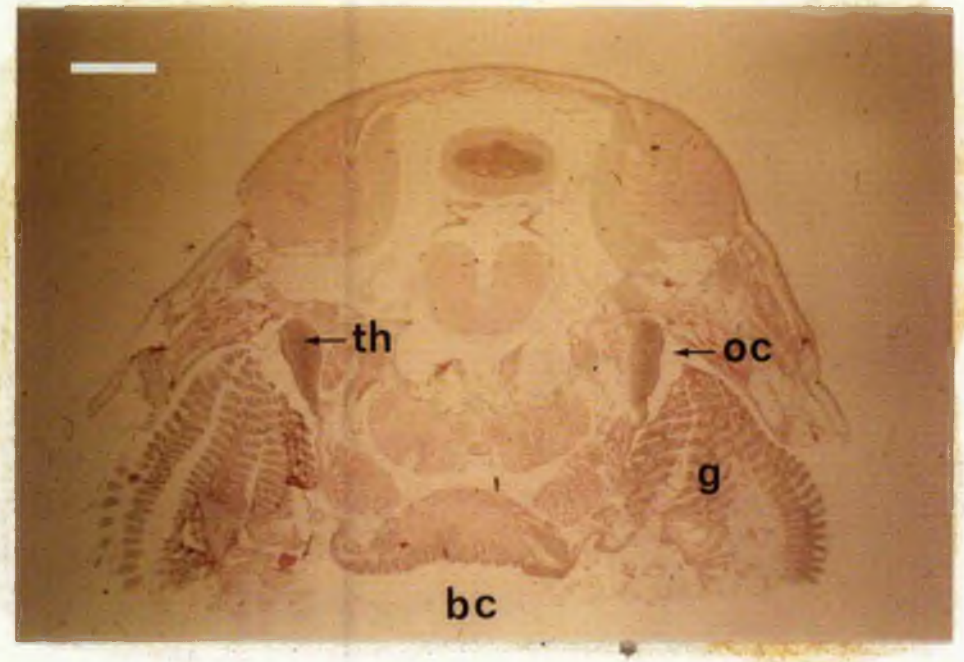

th
oc
g
bc

FIGURE 32

Thymus of S. trutta: A section through the thymus showing the epithelial cortex and cords which surround the thymocytes.

H & E, A. bar = 100 µm
B. bar = 20 µm

ec Cord of epithelial cells
ic Inner epithelial cortex
oc Outer epithelial cortex
t Thymocytes

FIGURE 33

Thymus of S. trutta: A. A section through the thymus showing a 'rosette' of epithelial and mucus cells. H & E, bar = 20 µm.

B. A section through the thymus showing a large round 'mucus' cell. H & E, bar = 10 µm.

mc 'Mucous' cell
r 'Rosette' of epithelial and mucous cells.
t Thymocytes

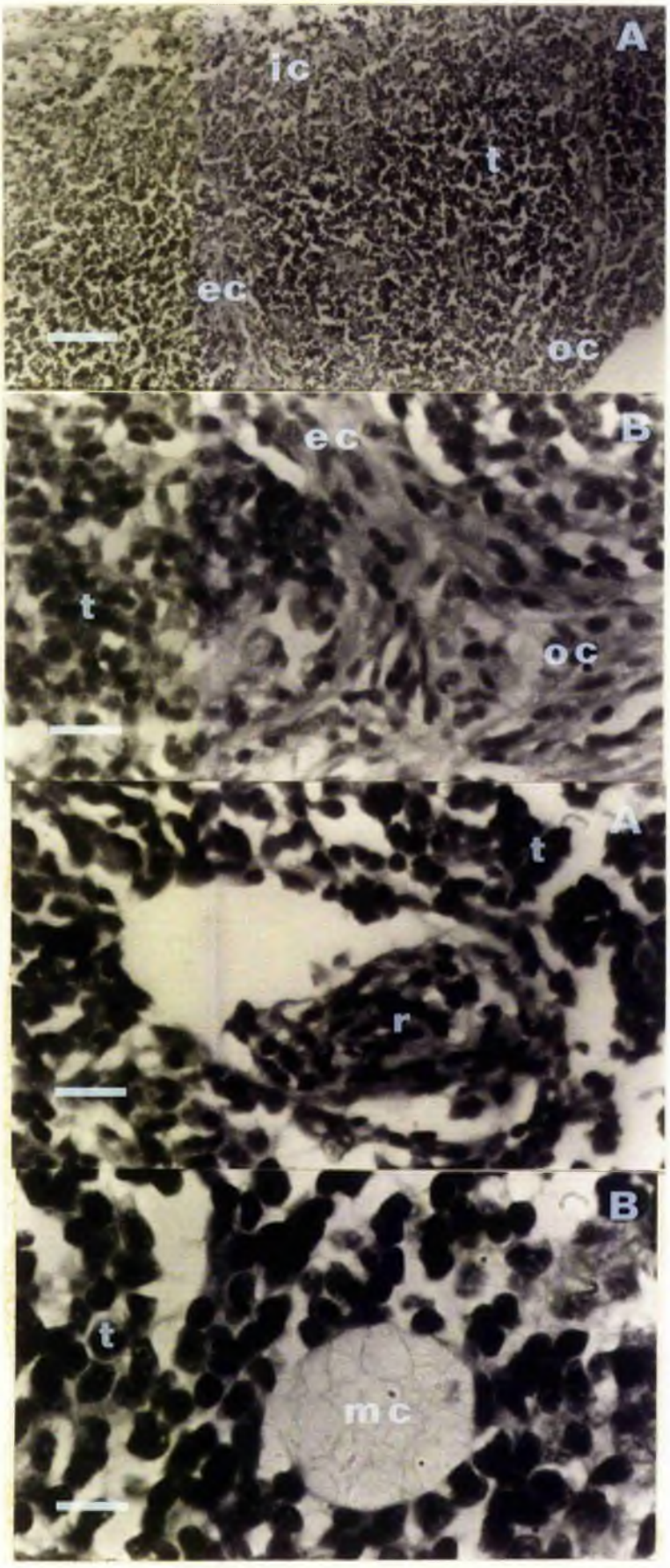

A
ic
t
ec
oc
B
ec
t
oc
A
t
r
B
t
mc

FIGURE 34

Spleen of S. trutta: A section through the outer edge of the spleen which has an even distribution of red and white pulp.

H & E, bar = 20 μm

FIGURE 35

Spleen of S. trutta: A section showing ellipsoids in the splenic pulp. H & E, bar = 10 μm.

en Endothelial cell of the ellipsoid sheath
er Erythrocyte in the lumen of the ellipsoid
m Macrophage of the ellipsoid sheath

FIGURE 36

Spleen of C. carpio: A section showing an ellipsoid.

H & E, bar = 10 μm.

en Endothelial cell of the ellipsoid sheath
er Erythrocyte in the lumen of the ellipsoid
m Macrophage of the ellipsoid sheath
rp Red pulp, an area of erythrocyte aggregation

FIGURE 37

Spleen of C. carpio: A section showing an aggregation of 'melano-macrophages', large round cells containing fine granules which gave the cytoplasm a yellowish colouration. H & E, bar = 10 μm.

m-m Melano-macrophage

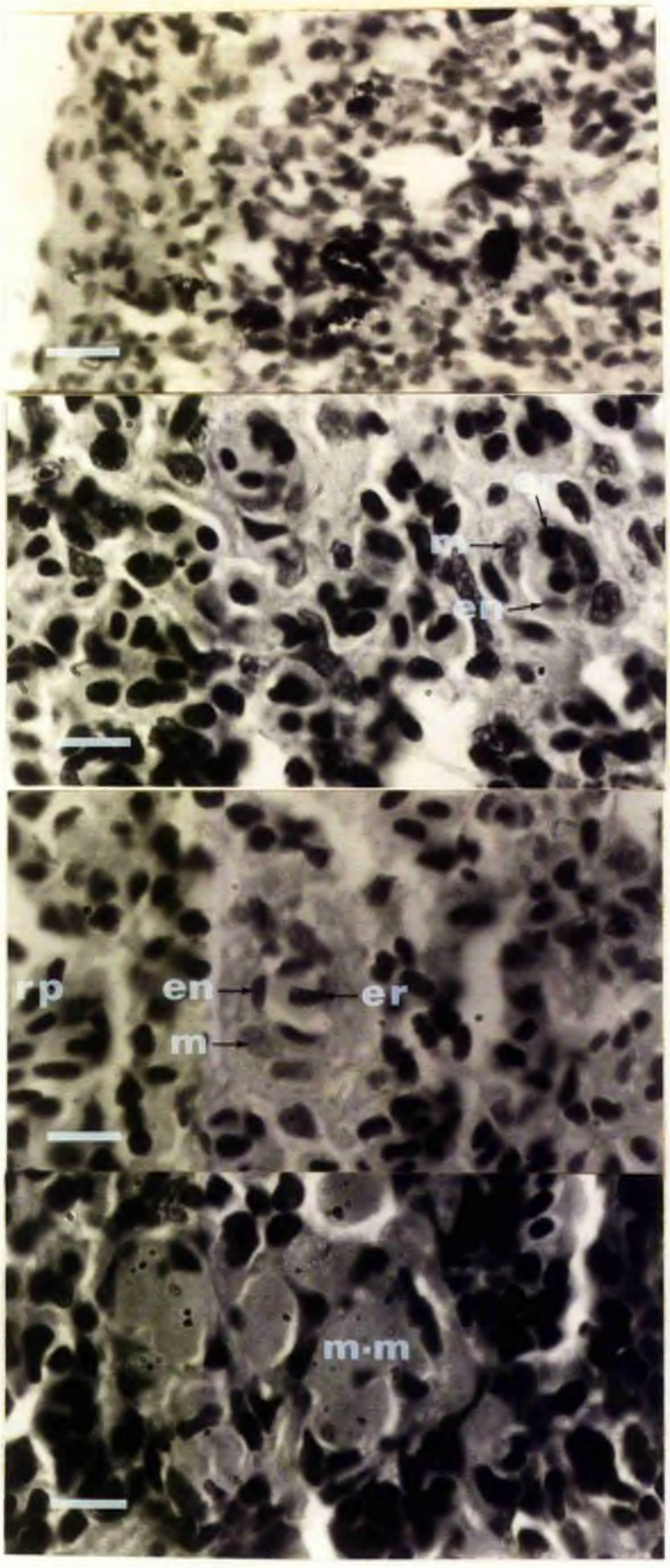

m
en
rp
en
er
m
m·m

FIGURE 38

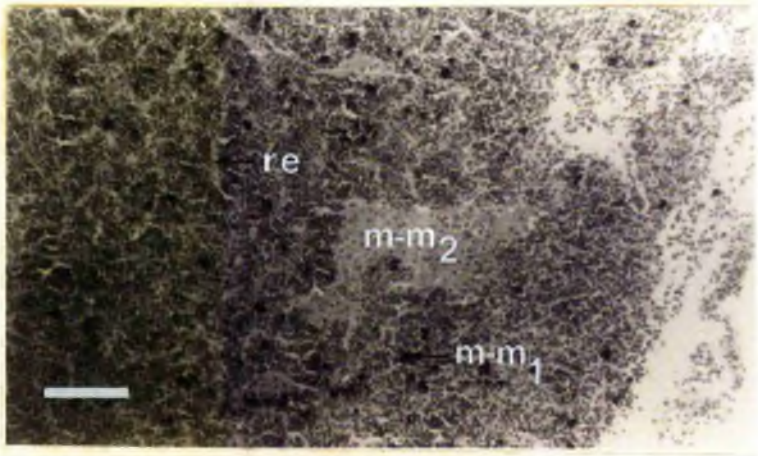
Pronephric kidney of S. trutta: Sections showing the vascular sinuses formed from the reticulo-endothelial system. H & E, A. bar = 100 μm
B. bar = 20 μm

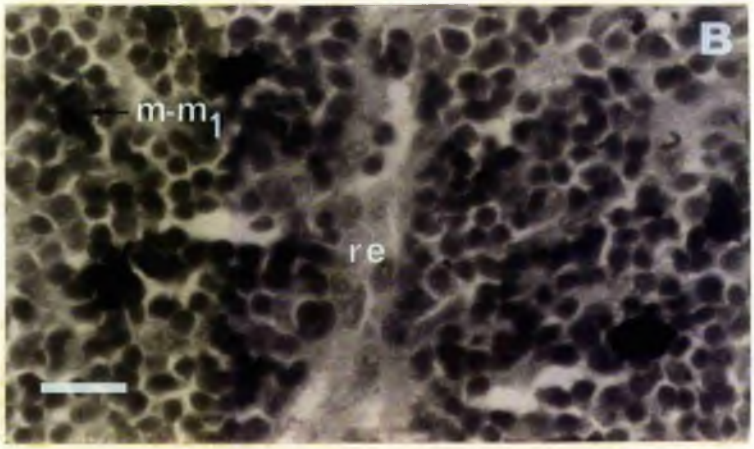
$m\text{-}m_1$	Melano-macrophages containing large granules of melanin
$m\text{-}m_2$	Large cells containing fine granules which gave the cytoplasm a light-yellow colour, possibly 'melano-macrophage' aggregations or endocrine.
re	Reticulo-endothelial sinus

FIGURE 39

Pronephric kidney of S. trutta: A section showing a compact aggregation of cells, containing fine granules of a light-yellow pigment, which may be melano-macrophages or endocrine cells. H & E, bar = 20μm

$m\text{-}m_1$	Melano-macrophages containing large granules of melanin
$m\text{-}m_2$	Large light-yellow pigmented cells


re
m-m2
m-m1


B
m-m1
re

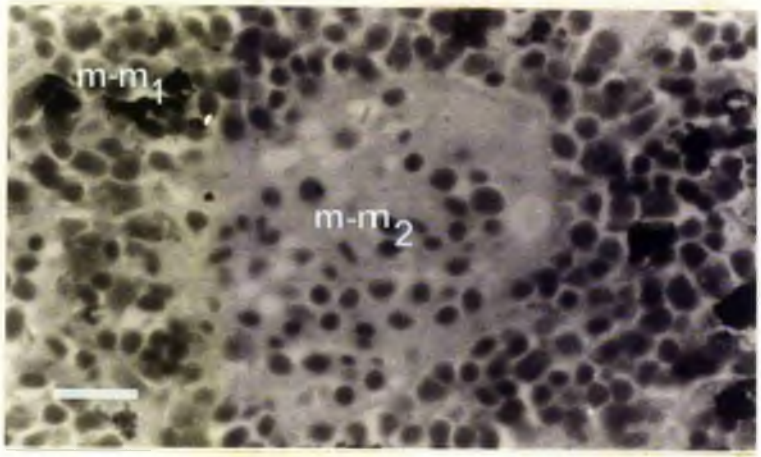
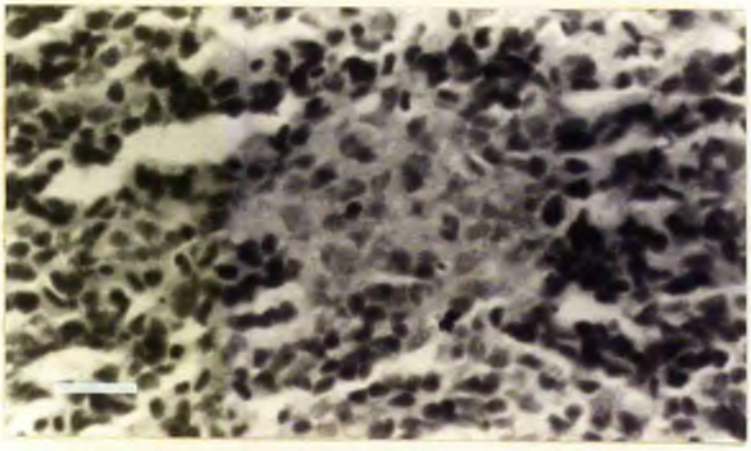

m-m1
m-m2

FIGURE 40

Spleen of S. trutta: A section showing the aggregation of cells which contained fine granules of light-yellow pigmentation, possibly melano-macrophages, +36h after the inoculation of MS2 bacteriophage. H & E, bar = 20μm

FIGURE 41

Spleen of S. trutta: A section showing vacuolation of a possible melano-macrophage aggregation +7d after the inoculation of MS2 bacteriophage. H & E, bar = 20μm.



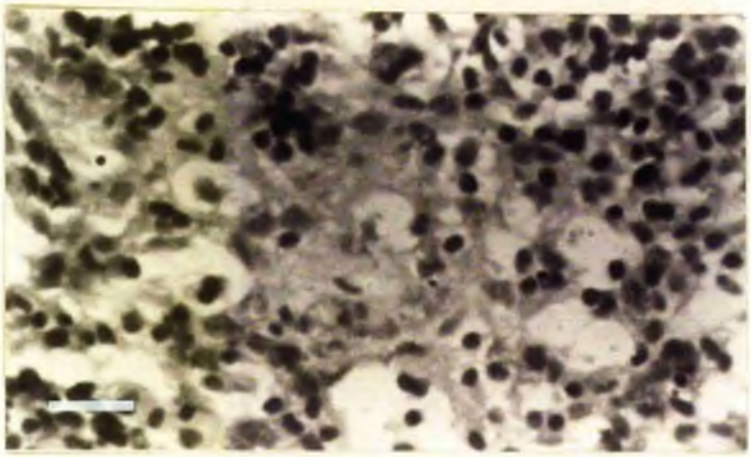


FIGURE 42

Spleen of S. trutta: A section showing carbon around and in cells of the ellipsoid sheaths +4h after inoculation. H & E, A. bar = 20 µm
B. bar = 10 µm

c Carbon around an ellipsoid
cm A macrophage of the ellipsoid sheath containing carbon.

FIGURE 43

Atrium of S. trutta: A section showing carbon in atrial reticulo-endothelial cells +6h after inoculation. Reticulo-endothelial cells can be seen to have 'rounded up' and become free carbon macrophages. H & E, bar = 10µm.

m RE cell replete with carbon 'rounding up'.
re RE cell containing carbon.

FIGURE 44

Opisthonephric kidney of S. trutta: A section showing carbon around the basement membrane of kidney tubules and within reticulo-endothelial cells of the nephric pulp, +4h after inoculation. H & E, bar = 20µm.

c Carbon attached to the basement membrane of a kidney tubule
m Phagocytic cell containing carbon, probably reticulo-endothelium.

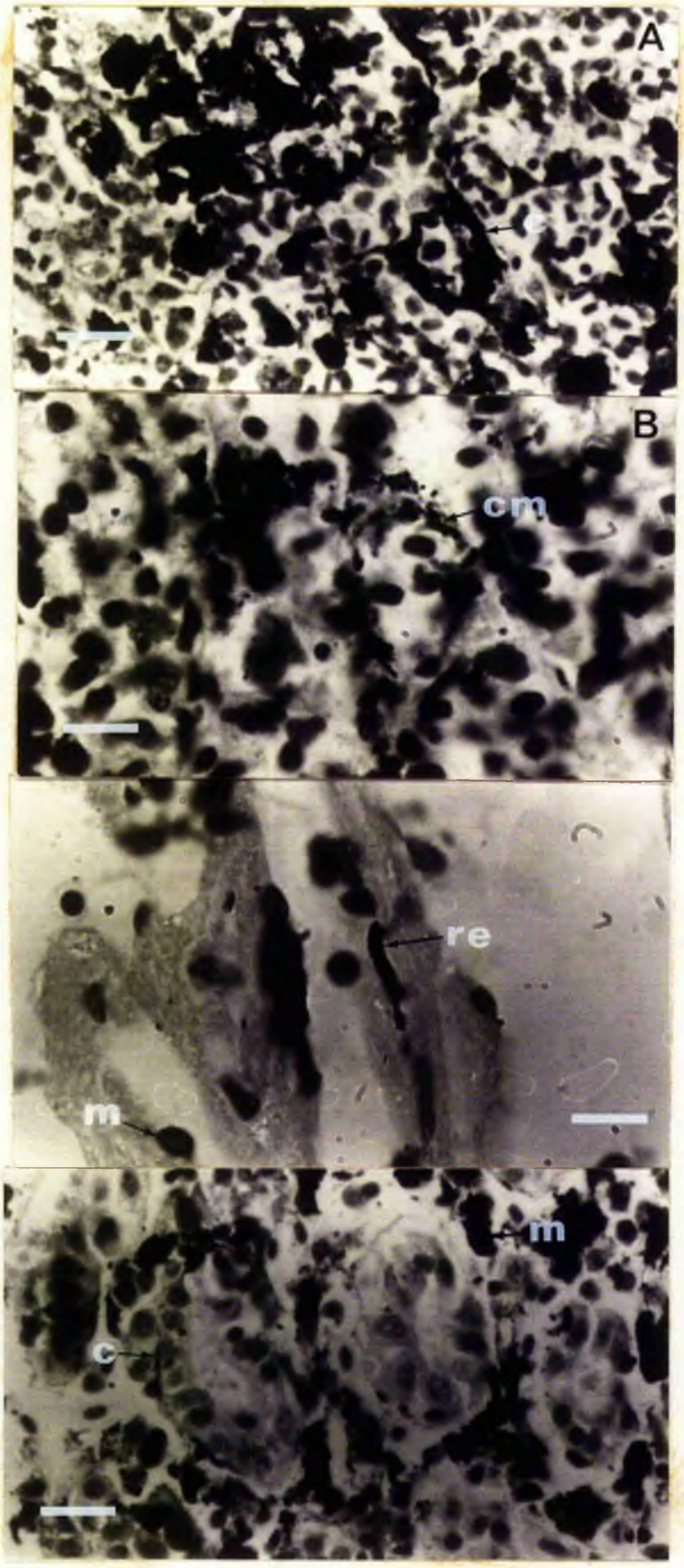

A
B
cm
re
m
m
c

FIGURE 45

Epidermis of S. trutta: A. A section of epidermis taken from a control fish unexposed to heavy metals. H & E, bar = 20μm

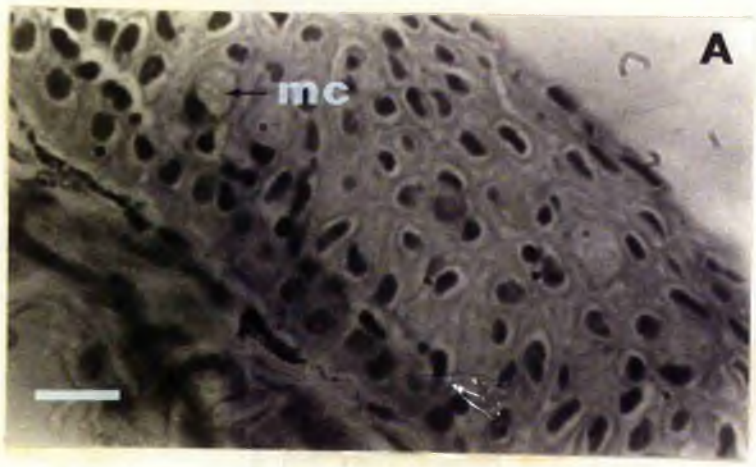
B. A section showing the thinner epidermis observed in 0.29 mg dm^{-3} copper-exposed fish. H & E, bar = 20μm.

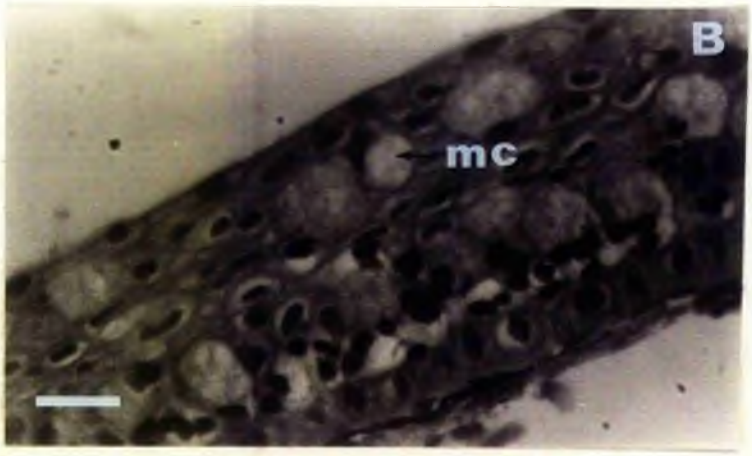
mc Mucous cell.

FIGURE 46

Epidermis of S. trutta: A section showing the large mucous cells and their rupture in 1.01 mg dm^{-3} chromium-exposed fish. H & E, bar = 20 μm.

mc Mucous cell.


A
mc


B
mc

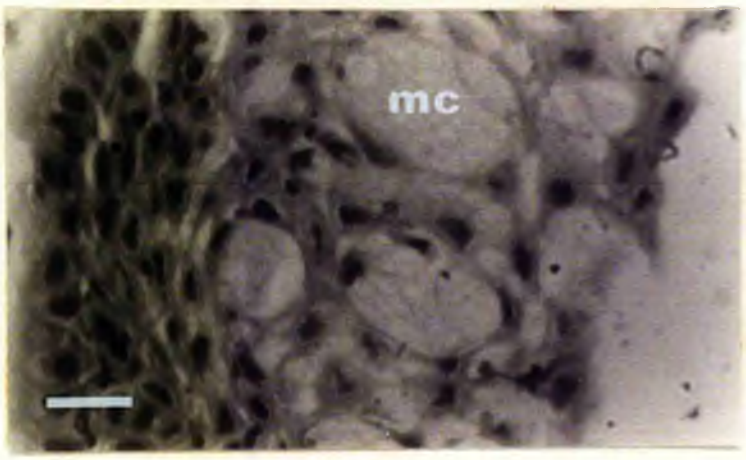

mc

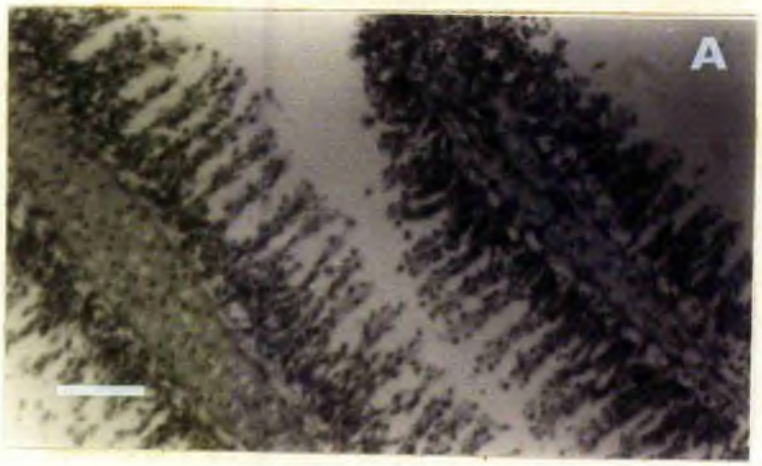
FIGURE 47

Gill filaments of 0.29 mg dm^{-3} copper-exposed fish:
A. S. trutta, a section showing the breakdown of the secondary lamellae. H & E, bar = 100µm.
B. C. carpio, a section showing the thickening of the basis of the secondary lamellae. H & E, bar = 100 µm.

FIGURE 48

Gill filaments of 0.75 mg dm^{-3} nickel-exposed C. carpio: A section showing a ciliate protozoan infection which may have been the cause of the observed thickening of the secondary lamellar basis. H & E, bar = 20µm.

p Ciliate protozoan.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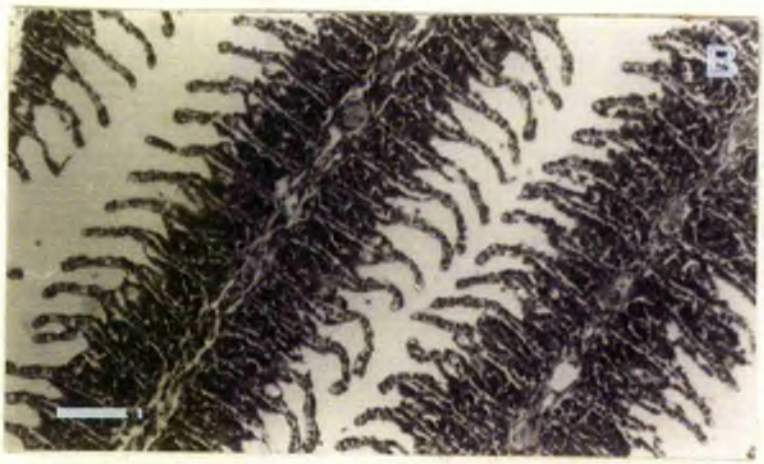
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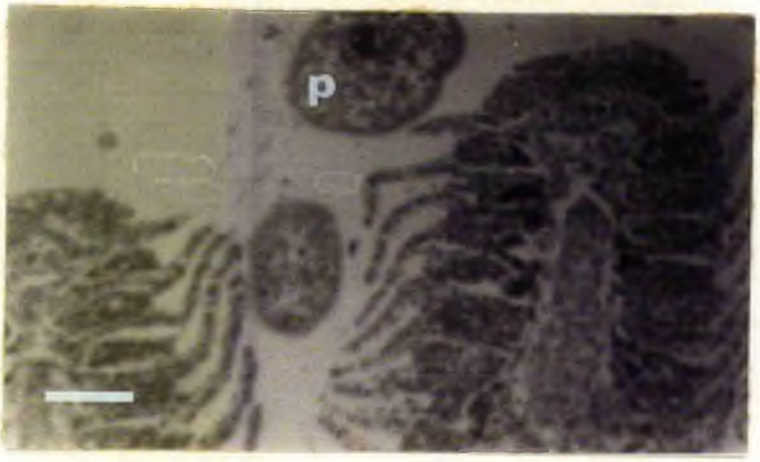

P

FIGURE 49

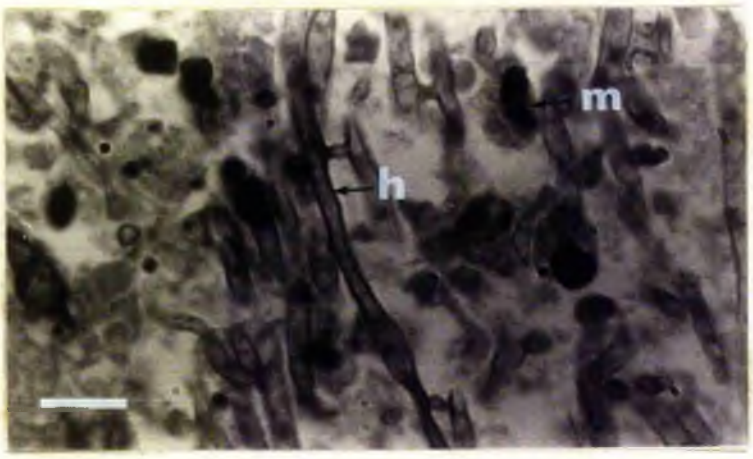
Opisthonephric kidney of S. trutta: A section showing fungal hyphae, probably Saprolegnia sp., found in one 1.01 mg dm^{-3} chromium-exposed fish. A loss of nephric structure and the infiltration of phagocytic cells were observed. H & E, bar = 10 µm.

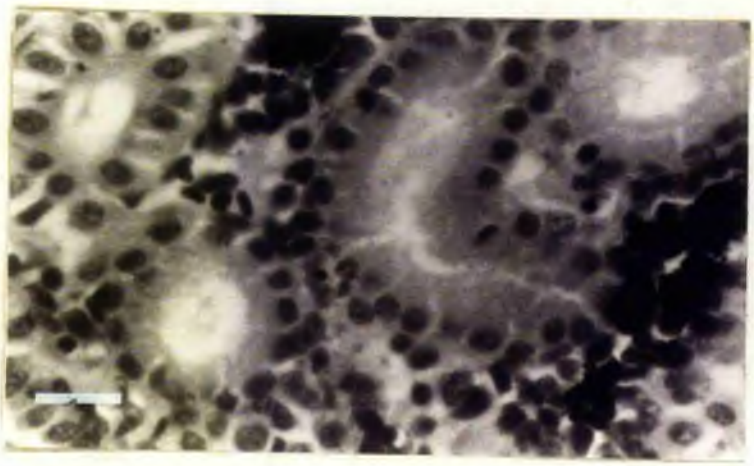
h Fungal hyphae

m Phagocytic cells in association with the fungal hyphae

FIGURE 50

Opisthonephric kidney of S. trutta: A section showing the breakdown of the nephric tubules in 2.13 mg dm^{-3} zinc-exposed fish. H & E, bar = 20 µm.


m
h



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IX. APPENDICES

1. Soft agar overlay medium.

2. Buffer A.

3. Teleost saline

4. a. Medium A.
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5. Lysing medium.

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10. The immune response of S. trutta: MS2 bacteriophage concentration and adjuvants.

11. Waterborne inoculation of MS2: Antibody titre response of S. trutta.

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14. N. rossii: Antibody titre response and the use of adjuvants.

15. Heavy metals: Antibody titre response in S. trutta and C. carpio.

16. Heavy metals: Water quality.

17. Zinc concentrations: Antibody titre response in S. trutta and C. carpio.

18. Zinc concentrations: Water quality.

19. Inoculated lead: Tertiary antibody titre response in *S. trutta*.

20. Inoculated cadmium: Tertiary antibody titre response in *S. trutta*.

APPENDICES.

1. Soft agar overlay medium.

Oxoid agar No 3	25 g
Bacto peptone	0.5g
Deionised water	0.5 dm^3

2. Buffer A.

Hydroxymethyl aminomethane (TRIS)	6.05 g
NaCl	5.84 g
EDTA sodium salt	3.72 g
Deionised water	1.0 dm^3

Adjusted to pH 7.6 using 4M HCl.

3. Teleost saline.

NaCl	6.75 g
KCl	0.32 g
CaCl	0.36 g
$NaHCO_3$	0.15 g
$MgSO_4.7H_2O$	0.23 g
Deionised water	1.0 dm^3

Adjusted to pH 7.6 using 4M HCl.

4. a. Medium A.

Na_2HPO_4	7.0 g
KH_2PO_4	3.0 g
NH_4Cl	0.5 g
Deionised water	1.0 dm^3

b. Medium supplement B.

$MgSO_4$	1.23 g
$CaCl_2$	0.11 g
Casamino acids	6.5 g
Glycerol	3.0 cm^3
Deionised water	60.0 cm^3

5. Lysing medium.

Lysozyme (Sigma)	0.01 g
EDTA sodium salt	0.37 g
TRIS	12.1 g
Chloroform	1.0 cm^3
Deionised water	0.1 dm^3

Adjusted to pH 8.0 using 4M HCl.

6. Antibody-antigen precipitation tests

a. Double-diffusion precipitation techniques.

i. Ouchterlony (1948, 1970).

Ion agar was prepared in 20 cm^3 batches in Universal bottles, autoclaved then centrifuged at 500 g for 5 min before 8 cm^3 aliquots were pipetted into disposable Petri dishes. When the agar had set a central well and six peripheral wells were cut using a template and cutter (Shandon Scientific). The wells were 0.3 cm in diameter and 0.3 cm apart. The central wells were filled with dilutions of a purified stock of MS2 bacteriophage, 1,10^{-3},10^{-6}or 10^{-9}, using a 0.01 cm^3 automatic pipette and the outer wells were filled with 1, 1/4, 1/16, 1/64, 1/256, or 1/1024 dilutions of sera. The Petri dish lids were replaced and the dishes placed in a polythene bag which was then sealed. After 7d at 20°C in an incubator the agar wells were 'topped up' with the appropriate dilution of bacteriophage or sera and left a further 7d.

Ion agar.

Bacto agar (Difco,Michigan,USA)	4.0 g
Sodium azide	0.3 g
0.1 M KH_2PO_4	28.0 cm^3
0.1 M Na_2HPO_4	72.0 cm^3
Deionised water	0.3 dm^3

ii. Microtechnique of Crowle (1958) modified by Tyrell (1973).

Standard microscope slides were immersed in concentrated sulphuric acid containing 1% potassium permanganate, rinsed in deionised water and air dried. The slides were then evenly coated with 0.1 cm^3 of 1.6% agarose (BDH, electrophoretic grade) made up in deionised water containing 0.1% sodium azide and then left to dry at 40°C. Five turns of 1cm wide 'Sellotape' were wound around the ends of the slides such that a chamber 2 cm wide was formed and into which 0.05 cm^3 of 0.6% agarose containing 0.1% sodium azide was evenly spread and allowed to

set.

'Perspex' templates 2.5 x 2.5 x 0.3 cm were drilled with a central 0.15 cm diameter hole and six peripheral holes of the same diameter 0.4 cm apart. Each well was then countersunk on the upper surface to form a reservoir 0.3 cm in diameter and 0.25 cm deep. After cleaning in 1% Decon, rinsing in deionised water and drying, the lower surface of the template was thinly coated with a mixture of equal parts petroleum jelly and silicone grease. It was then carefully placed over the agarose chamber using the bands of tape as supports.

The wells were filled with the same dilutions of bacteriophage and sera as were used in the Ouchterlony technique with the aid of a 0.01 cm^3 automatic pipette. The slides were then incubated at 20°C in a sealed plastic box containing moist tissues for 2d. The wells were 'topped up' and incubation continued a further 7d after which the templates were carefully lifted off.

b. Single radial diffusion.

Ion agar was prepared in a similar manner to that used in the Ouchterlony technique. In the first of the single radial diffusion techniques (Grandien and Norby, 1975) MS2 bacteriophage was incorporated into the agar at a temperature of 40°C such that there was a dilution of the stock bacteriophage by 10^{-3}, 10^{-6} and 10^{-9}, and 1 cm^3 aliquots were dispensed into the wells of tissue culture plates. When the agar had set a central well was cut in it, using a 0.3 cm diameter cutter, which was filled with 1, 1/4, 1/16, 1/64, 1/256 or 1/1024 dilutions of sera using a 0.01 cm^3 automatic pipette. In the second Mancini technique (Roitt, 1974) sera of known high neutralisation activity were incorporated into the agar such that there was a 1/16, 1/64 or 1/256 dilution of the sera, and 1, 10^{-3}, 10^{-6} and 10^{-9} dilutions of bacteriophage stock were placed in the central wells. The

plates were then sealed inside polythene bags and incubated for 7d at 20°C. The wells were then refilled with the appropriate dilution of bacteriophage or sera and incubated for a further 7d.

c. Countercurrent-immunoelectrophoresis (Moody, 1970).

A Shandon Scientific gel pattern for immunoelectrophoresis, containing prewashed 7.6 x 2.5 cm microscope slides, was filled with 0.75% agarose made up in veronal buffer pH 8.2 containing 0.1% sodium azide. When the agarose was set three 0.3 cm diameter wells were cut out 0.3 cm apart such that the wells were in line with the intended electrical poles. The central wells were filled with 1, 10^{-3}, 10^{-6} or 10^{-9} dilutions of bacteriophage stock using a 0.01 cm^3 automatic pipette and the two outer wells were filled with one of the 1, 1/4, 1/16, 1/256 or 1/1024 dilutions of sera. Electrophoresis was carried out using horizontal slab electrophoretic equipment with water cooling (Shandon Southern) and veronal buffer. A constant current of 2 mA cm^{-1} was applied for 2h.

Veronal buffer.

Diethyl barbituric acid	1.38 g
Sodium veronal	8.76 g
Sodium azide	1.0 g
Deionised water	1.0 dm^3

Adjusted to pH 8.2 using 4M HCl.

Staining of gels.

As no precipitates were observed using the above procedures the ion agar and agarose gels were stained. The gels were placed in teleost saline adjusted to pH 7.2 using 4M HCl and left overnight to elute unprecipitated proteins. They were then stained for 8h in 1% thiozine red (Gurr, London) made up in deionised water and then rinsed in deionised water. The gels were then fixed overnight in 1% glacial acetic acid before examination.

d. Micro-flocculation test (Smith, 1961).

Dilutions of MS2 bacteriophage stock and sera made up for the Ouchterlony technique were used. Serum dilutions were drawn halfway up micro-haematocrit tubes (Gelman Hawksley) and then an equal volume of bacteriophage dilution was drawn into the tube. The serum end of the tubes were then sealed with plastic clay (Gelman Hawksley) and the tubes fixed vertically into a block of plasticine. The tubes were incubated at 20°C and examined at 7d intervals.

7. Tissue fluid.

i. Earle's Balanced Salt Solution 10.0 cm^3
Minimal Essential Medium-amino acids 2.0 cm^3
Sodium bicarbonate 2.0 cm^3

(All above supplied sterile by Flow Laboratories, UK.)

Deionised water, sterile and cold 0.1 dm^3

ii. Glutamine 0.5 cm^3
Foetal Bovine Serum 5.0 cm^3
(Above supplied sterile by Flow Laboratories, UK)

iii. 1.0 cm^3 of (ii) was added to 6.0 cm^3 of (i) just before use.

8. Heavy metal analysis.

a. Atomic absorption spectrophotometry : Zinc and copper.

	Zinc	Copper
Stock solution	$ZnSO_4.7H_2O$ 0.4398 g dm^{-3} ≡ 100 mg Zn dm^{-3}	$CuSO_4.5H_2O$ 0.393 g dm^{-3} ≡ 100 mg Cu dm^{-3}
Standards	i. 10.0 cm^3 stock in 100.0 cm^3 ≡ 10.0 mg dm^{-3} (A) Zn or Cu. ii. 0.05 cm^3 A to 1.0 cm^3 A in 10.0 cm^3 ≡ 0.05 mg dm^{-3} to 1.0 mg dm^{-3} of Zn or Cu.	
Wavelength	213.8 nm	324.7 nm
Flame	Air-acetylene (lean-blue flame)	

b. Diphenylcarbazide photometric method for chromium.

Stock solution.

$K_2Cr_2O_7$ 2.928 g dm^{-3} ≡ 1000 mg Cr dm^{-3}

Standards.

i. 1.0 cm^3 stock in 100.0 cm^3 ≡ 10.0 mg Cr dm^{-3} (A)

ii. 0.05 cm^3 A to 1.0 cm^3 A in 10.0 cm^3 ≡ 0.05 mg Cr dm^{-3} to 1.0 mg Cr dm^{-3}.

Reagents.

i. 1.0 cm^3 sample or standard.

ii. 1.0 cm^3 0.2% diphenylcarbazide w/v in acetone (made just before use).

iii. 0.05 cm^3 concentrated H_2SO4.

Shake reagents together in a capped tube.

Read optical density at 540 nm immediately, using distilled water as a blank.

c. Dimethylglyoxime photometric method for nickel.

Stock solution.

$NiSO_4.7H20$ o.4784 g dm^{-3} ≡ 100 mg Ni dm^{-3}.

Standards.

i. 10.0 cm^3 stock in 100.0 cm^3 ≡ 10.0 mg Ni dm^{-3}(A)

ii. 0.05 cm^3 A to 1.0 cm^3 A 10.0 cm^3 ≡ 0.05 mg Ni dm^{-3} to 1.0 mg Ni dm^{-3}.

Reagents.

i. 1.0 cm^3 HCl (1.0 cm^3 in 30.0 cm^3 deionised water).
ii. 1.0 cm^3 sample or standard or deionised water blank.
iii. 0.2 cm^3 sodium tartrate (20% w/v in deionised water).
iv. 1.0 cm^3 potassium persulphate (4% w/v in deionised water).
v. 0.06 cm^3 dimethylglyoxime (1% w/v in ethanol, made just before use).
vi. 0.25 cm^3 5M NaOH.

Shake reagents together in a capped tube.

Leave 30 min at room temperature and read optical density at 465 nm.

9. Formal Cortland fish saline.

NaCl	7.25 g
$CaCl.6H_2O$	0.33 g
KCl	0.38 g
NaH_2PO_4	0.41 g
Na_2HCO_3	1.0 g
$MgSO4.7H_2O$	0.23 g
Glucose	1.0 g
Formalin	0.25 dm^3
Deionised water	1.0 dm^3

Adjusted to pH 8.0 using 4M NaOH.

10 The immune response of S. trutta: MS2 bacteriophage concentration and adjuvants

i. Primary antibody titre response, mean ± 2SE

Day \ Concentration	Control	Saline 10^3	Saline 10^6	Saline 10^9	FIA 10^3	FIA 10^6	FIA 10^9	FCA 10^3	FCA 10^6	FCA 10^9
0	0	1.9 ±0.6	0	2.7 ±1.5	1.6 ±1.0	1.1 ±0.5	0	0	0	0
7	3.4 ±0.4	6.1 ±0.5	3.1 ±0.4	6.9 ±1.9	20.9 ±11.5	5.9 ±1.5	9.3 ±1.9	8.6 ±1.3	8.5 ±3.9	3.0 ±0.6
14	12.9 ±5.5	36.9 ±14.3	127 ±66.6	127 ±37.2	27.0 ±6.7	51.6 ±26.2	113 ±34.2	139 ±45.9	38.5 ±13.5	42.5 ±5.6
21	14.6 ±3.3	42.6 ±19.2	97.0 ±34.4	390 ±164	8.4 ±1.0	46.7 ±11.5	452 ±128	3957 ±1343	180 ±99.3	190 ±3.4
35	5.2 ±1.3	19.8 ±5.3	52.9 ±12.2	1559 ±858	9.1 ±1.9	21.6 ±4.5	3777 ±1433	4859 ±1410	24.2 ±9.8	4258 ±432
42	5.0 ±1.4	1.0	58.3 ± 25.2	1120 ±425	15.2 ±8.9	13.5 ±2.5	8047 ±2909	11493 ±2368	89.8 ±43.2	5309 ±1587
49			29.7 ± 14.1	993 ±273		23.3 ±10.6	22049 ±9955	17330 ±4468	37.7 ±12.1	13337 ±4248
56							17248 ±10552	14904 ±6036		
70							6337 ±3.9	7121 ±666		
77							1699 ±985	1489 ±91.6		

ii. Secondary antibody titre response, mean ± 2SE (Live MS2 bacteriophage titres in sera are tabulated in the square brackets, mean PFU ± 2SE)

Day \ MS2 Concentration	Control	Saline 10^3	Saline 10^6	Saline 10^9	FIA 10^3	FIA 10^6	FIA 10^9	FCA 10^3	FCA 10^6	FCA 10^9
0	5.0 ±1.4	1.0	29.7 ±14.1	993 ±273	15.2 ±8.9	23.3 ±10.6	1699 ±985	1489 ±91.6	37.7 ±12.1	13337 ±4248
7	2.9 ±1.1	0	13.9 ±8.5	501 ±320	0	22.9 ±4.4	1387 ±575	2013 ±1245	0	7642 ±1959
		[2.5×10^5 ±1.8×10^5]	[1.2×10^5 ±9.7×10^4]		[2.7×10^5 ±2.3×10^5]				[4.1×10^5 ±2.4×10^5]	
14	15.3 ±4.8	103 ±14.5			52.9 ±25.4					
21			6467 ±1463	20679 ±6025		131 ±27.6			25834 ±15703	8121 ±1935
28	1.0	1989 ±313			2955 ±856		4521 ±1675	4776 ±767		
35			4230 ±808	357 ±44.7		1051 ±221			7108 ±2532	1261 ±316
42	1.0	336 ±74.1			3753 ±1633		4019 ±1414	18999 ±6093		
56			1270 ±517	311 ±68.0		152 ±58.6	4711 ±2884	33280 ±13065	8479 ±2401	1469 ±393
63	0	262 ±66.4			274 ±142					
70			1322 ±543	659 ±42.7		284 ±30.7	988 ±535	1306 ±150	23033 ±12439	7975 ±2048
77	0	532 ±53.2			180 ±101					
84			1747 ±100	398 ±61.5		142 ±13.1	511 ±264	576 ±258	1533 ±592	7301 ±3873
91		561 ±142			317 ±179					
98			556 ±275	525 ±21.9		306 ±20.5			1585 ±497	1038 ±68.8
105	0	527 ±197			262 ±145					

iii.

	Control	Saline 10^3	Saline 10^6	Saline 10^9	FIA 10^3	FIA 10^6	FIA 10^9	FCA 10^3	FCA 10^6	FCA 10^9
Weight ±SE (g)	151.0 ±4.25	136.5 ±6.5	137.25 ±4.75	145.25 ±8.5	133.5 ±7.5	147.0 ±5.75	137.25 ±11.0	138.5 ±8.25	137.5 ±7.75	138.25 ±10.75
Length ±SE (cms)	23.0 ±0.25	22.0 ±0.25	22.25 ±0.25	22.75 ±0.5	21.75 ±0.5	22.0 ±0.5	22.25 ±0.5	22.0 ±0.25	22.0 ±0.25	21.25 ±0.5
Male: Female	4:1	3:2	5:0	2:3	4:1	3:2	4:1	4:1	3:2	2:3
n	5	5	5	5	5	5	5	5	5	5

11. Waterborne inoculation of MS2: Antibody titre response of S. trutta mean ± 2SE

Inoculated group / Days	Control	Saline	FIA	FCA
'Natural' titre (subtracted)	2.4 ±1.2	6.9*** ±2.6	9.4 ±3.9	16.9*** ±6.8
0	-	-	-	-
7	3.1 ±1.3	28.7*** ±2.9	5.9** ±1.6	10.1*** ±2.1
14	6.3 ±1.8	45.6*** ±5.0	13.9*** ±2.5	18.5*** ±5.9
21	0	4.0 ±4.2	1.0	21.2*** ±13.5
28	4.3 ±2.1	10.3** ±2.7	2.6 ±2.3	15.8*** ±7.4
35	4.8 ±1.1	5.3 ±2.6	3.4 ±2.4	4.5 ±5.9
42	6.0 ±3.7	15.3** ±6.0	0	0
Weight ±SE(g)	122.0 ±3.0	122.0 ±5.25	128.5 ±3.5	133.0 ±4.5
Length ±SE(cm)	17.25 ±0.25	17.0 ±0.25	17.5 ±0.25	17.5 ±0.25
n	5	5	5	5

Significantly different from control values

**P = < 0.01
***P = < 0.001

12. Temperature: Antibody titre response of S. trutta, mean ± 2SE

(Live MS2 bacteriophage titres in the sera are tabulated in the square brackets, mean PFU ± 2SE)

i. Primary response

Temp °C	4.5	9.0	13.75	15.5
Day				
0	0	3.7 ± 1.7	1	12.1 ± 6.3
7	[4.2×10^{8} ± 5.8×10^{7}]		9.3 ± 1.9	10.6 ± 5.4
14	[1.4×10^{5} ± 8.6×10^{4}]	5.1 ± 3.2	113 ± 34.2	103 ± 36.6
21	0		452 ± 128	8394 ± 5199
28	1.9	140 ± 65.3		22456 ± 5342
35	10.8 ± 4.9		3777 ± 1433	30448 ± 12071
42	28.2 ± 3.2	2227 ± 814	8047 ± 2909	1813 ± 1006
49	50.2 ± 2.9		22049 ± 9955	818 ± 282
56	77.4 ± 45.8	1460 ± 486	17248 ± 10552	
63	111 ± 46.7			
70	303 ± 153	1165 ± 536	6337 ± 10.9	
77	394 ± 137		1699 ± 986	
84	41.2 ± 10.6	139 ± 13.1		
98		163 ± 40.8		

ii. Secondary Response

Temp °C	4.5	9.0	13.75	15.5
Day				
0	41.2 ± 10.6	163 ± 40.8	1699 ± 986	818 ± 282
7	205 ± 66.7	163 ± 45.7	1387 ± 575	767 ± 448
14	446 ± 223	108 ± 16.9		1539 ± 880
21	233 ± 107	452 ± 186		14652 ± 12503
28	386 ± 284	922 ± 306	4521 ± 1675	10881 ± 7751
35	126 ± 44.1	550 ± 149		12833 ± 5460
42	153 ± 65.3	468 ± 118	4018 ± 1414	4796 ± 3921
49	202 ± 96.6	499 ± 173		3488 ± 1223
56	109 ± 33.9	776 ± 397 (11°C)	4711 ± 2885	8023 ± 6874
63	134 ± 63.8			5914 ± 5014
70		925 ± 484 (14°C)	988 ± 535	3607 ± 1432
77				2931 ± 731
84		1595 ± 615 (16°C)	511 ± 264	
91				
105		740 ± 315 (12°C)		
116		138 ± 24.8 (10°C)		
126		159 ± 87.1 (9°C)		
Weight ±SE (g)	108.5 ±6.25	108.0 ±4.0	137.25 ±11.0	152.5 ±11.0
Length ±SE (cm)	21.25 ±0.25	21.0 ±0.25	22.25 ±0.5	24.5 ±0.5
Male: Fomale	6:0	3:2	4:1	4:2
n	6	5	5	6

13. Temperature: Antibody titre response of C. carpio, mean ±2SE (Live MS2 bacteriophage titres in the sera are tabulated in the square brackets, mean PFU±2SE)

i. Primary response

Temp °C	9.0	15.5	22.0
Day			
0	0	0	0
7	$[2.1\times10^{8} \pm 1.1\times10^{6}]$	$[1.7\times10^{6} \pm 7.8\times10^{5}]$	97.5 ± 51.2
14		38.1 ± 32.9	1313 ± 934
21	117 ± 104 $[1.4\times10^{7} \pm 1.1\times10^{7}]$	342 ± 292	5895 ± 2477
28		564 ± 186	14669 ± 6056
35	29.4 ± 14.9 $[5.8\times10^{5} \pm 3.7\times10^{5}]$	740 ± 267	6292 ± 2998
42	13.6 ± 3.8 $[7.6\times10^{4} \pm 7.5\times10^{4}]$	1026 ± 411	4545 ± 1972
49	10.5 ± 5.3 $[1.5\times10^{5} \pm 1.0\times10^{5}]$	352 ± 144	3255 ± 1408
56	6.5 ± 2.3 $[4.3\times10^{4} \pm 2.9\times10^{4}]$	547 ± 365	679 ± 376
63	3.2 ± 1.8 $[4.3\times10^{3} \pm 4.3\times10^{3}]$		

ii. Secondary response

Temp °C	9.0	15.5	22.0
Day			
0	3.2 ± 1.8 [4.3×10^3 ± 4.3×10^3]	547 ± 365	679 ± 376
7	2.4 ± 1.6	728 ± 477	6030 ± 5539
14	24.7 ± 3.5	3153 ± 1005	23450 ± 13675
21	165 ± 63.9	979 ± 401	48926 ± 24796
28	126 ± 80.3	2094 ± 953	116727 ± 87041
35	119 ± 63.1	2743 ± 1111	7907 ± 4641
42	142 ± 129	6042 ± 2925	66322 ± 53948
49	43.7 ± 26.7	5193 ± 2832	22858 ± 11436
56	83.0 ± 34.3	7582 ± 2777	10909 ± 5582
63	141 ± 106	12659 ± 6727	21525 ± 11440
70	139 ± 79.4	14019 ± 7471	31660 ± 26868
77		10503 ± 7186	34627 ± 32410
Weight ±SE(g)	147.25 ± 29.0	111.75 ± 12.0	151.9 ± 34.25
Length ±SE(cm)	16.25 ± 1.25	15.5 ± 0.75	17.0 ± 1.25
n	6	6	5

14. N. rossii: Antibody titre response, mean ±2SE (Live MS2 bacteriophage titres in the sera are tabulated in the square brackets, mean PFU ±2SE) and the use of adjuvants at 2.0°C

1. Primary response

Inoculated group / Day	Saline	FIA	FCA
0	0	0	0
14	$[4.0\times10^{7}\pm6.8\times10^{4}]$	$[3.6\times10^{7}\pm4.9\times10^{3}]$	$[3.4\times10^{5}\pm3.3\times10^{4}]$
28	$[8.7\times10^{6}\pm2.7\times10^{6}]$	$[4.3\times10^{4}\pm3.2\times10^{4}]$	$[3.1\times10^{4}\pm2.6\times10^{4}]$
42	$[3.8\times10^{6}\pm3.3\times10^{6}]$	$[1.9\times10^{4}\pm1.8\times10^{4}]$	1
56	$[2.9\times10^{6}\pm2.6\times10^{6}]$	1	58.0 ± 19.6
70	43.1 ± 6.4	31.8 ± 14.8	122 ± 50.5
84	194 ± 80.5	120	227 ± 93.1
98	442 ± 104	363	345 ± 168
112	391 ± 103	562	110 ± 41.5
126	41.2 ± 28.5	468	25.8 ± 13.9
140		11.2	

ii. Secondary response

Inoculated group / Day	Saline	FIA	FCA
0	41.2 ± 28.5	11.2	25.8 ± 13.9
14	708 ± 83.9	204	57.7 ± 32.0
28	445 ± 44.3	437	1318 ± 641
42	498 ± 112	468	325 ± 62.7
56	393 ± 86.1	407	273 ± 76.7
70	726 ± 218	250	
84	192 ± 82.3	69.2	
98	589 ± 13.7	74.1	
112	369 ± 121	75.8	

	Control	Saline	FIA	FCA
Weight ±SE(g)	61.5±0.8	95.3±10.6	60.0±0.7	116.1±16.6
Length ±SE(cm)	19.5±0.5	19.4±0.7	18.8±0.5	21.5±1.1
n	5	6	6 From 1° response day 84 n = 1	6

All uninoculated control fish had no detectable neutralisation activity throughout the experiment.

15 Heavy metals: Antibody titre response, mean ± 2SE (Live MS2 bacteriophage titres in the sera are tabulated in square brackets, mean PFU ± 2SE)

i. Primary response: S. trutta

Metal / Day	Control	Ni	Zn	Cu	Cr
0	12.1 ± 6.3	9.1 ± 7.4	3.2 ± 0.8	16.7 ± 8.5	2.7 ± 1.3
7	10.6 ± 5.4	5.8 ± 2.3 [$3.5\times10^{4}\pm2.9\times10^{4}$]	10.0 ± 5.4 [$6.3\times10^{5}\pm6.4\times10^{4}$]	31.5 ± 18.6 [$2.0\times10^{4}\pm2.0\times10^{4}$]	5.7 ± 2.4 [$3.1\times10^{4}\pm3.0\times10^{4}$]
14	104 ± 36.6	47.2 ± 16.3	122 ± 63.1	189 ± 138	67.1 ± 60.9
21	8394 ± 5199	691 ± 514	8168 ± 5188	549 ± 233	2284 ± 2180
28	22456 ± 5342	223 ± 96.2	1421 ± 769	8340 ± 6943	81.7 ± 47.2
35	30448 ± 12071	503 ± 198	1969 ± 1326	26201 ± 15865	96.1 ± 48.3
42	1813 ± 1006	228 ± 83.3	937 ± 409	15793 ± 11508	47.7 ± 28.2
49	818 ± 282	111 ± 49.1	624 ± 311	8049 ± 4870	33.3 ± 27.8

ii. Secondary response: S. trutta († indicates the death of a fish on or after the day of serum sampling)

Metal / Day	Control	Ni	Zn	Cu	Cr
0	818±282	111±49.1	624±311	8049±4870	33.3 ± 27.8
7	766±448	215±105	515±208	1433±1282 ††	28.4± 20.6 [$6.0\times10^{4}\pm1.6\times10^{4}$]
14	1539±880	590±324	275±64.2	449±200	37.9±25.2 ††
21	14652±12502	9157±5712	271±56.8	97.8±64.2 ††	190.9 ± 65.1 ††
28	10881±7751	35554±22248	513±146	146±5.1	283 ± 163 †
35	12833±5459	29207±13083	336±71.3	618±243 †	234
42	4796±3921	49809±42767	536±116	22.4	750
49	3488±1223	16015±10937	312±103	25.7	1318
56	8023±6874	72677±65334	474±76.8	50.7 †	324
63	5914±5014	40781±33748	1541±566		38.9
70	3607±1432	48627±34529	780±334		46.7
77	2931±731	66903±59421	823±363		57.5
Weight ±SE (g)	152.5±11.0	127.5±11.5	145.5±14.0	140.5±11.5	131.5±9.0
Length ±SE (cm)	24.5±0.5	23.0±0.5	24.0±0.5	24.0±0.5	23.25±0.5
Male: Female	4:2	4:2	4:2	3:3	4:2
n	6	6	6	6	6

iii. Primary response: C. carpio († indicates the death of a fish on or after the day of serum sampling)

Day \ Metal	Control	Ni	Zn	Cu	Cr
0	1.4±0.4	3.0±1.2	1.8±0.7	5.7±3.8	1.3±0.2
7	1.4±0.9 [$7.8\times10^5\pm6.3\times10^5$]	3.0±1.3 [$6.8\times10^5\pm5.0\times10^5$]	5.0±2.1 [$4.2\times10^5\pm3.1\times10^5$]	4.9±1.0 [$5.3\times10^5\pm4.3\times10^5$]	6.0±4.3 [$1.4\times10^6\pm1.3\times10^6$]
14	18.3±9.6 [$1.7\times10^5\pm1.6\times10^5$]	4.0±1.9 [$4.2\times10^4\pm4.2\times10^4$]	1.6±1.0 [$1.1\times10^5\pm8.7\times10^4$]	11.4±6.5 [$1.5\times10^5\pm9.0\times10^4$]	1 [$2.2\times10^4\pm2.2\times10^4$]
21	17.0±8.2	17.4±10.3	37.0±11.4	36.9±29.6 [$3.3\times10^4\pm1.1\times10^4$]	7.2±4.3
28	82.2±36.4	20.7±7.0	90.0±37.6	10.5±8.9	0 † † † † †
35	1144±723	121±51.2	262±138	67.2±29.2	
42	2215±1010	228±102	1085±930	456±320	
49	1048±730	229±121	479±315	571±134	
56	162±83.9	198±90.5	667±624	130±80.9	

iv. Secondary response: C. carpio

Metal / Day	Control	Ni	Zn	Cu	Cr
0	162±83.9	198±90.5	667±624	130±80.9	-
7	906±768	662±475	649±424	0	
14	4337±3990	308±135	544±20.7	-	
21	11598±10798	1753±985	476±224		
28	1583±1311	1214±565	998±565		
35	3619±3138	4756±2477	10157±7382		
42	2328±1800	3369±1944	26273±18966		
49	2589±541	15353±12053	17521±16427		
63	2039±1642	4808±1884	13586±5920		
70	3723±1574	1902±726	16524±15734		
Weight ±SE (g)	108.8±28.5	106.5±23.0	114.0±15.0	101.0±19.0	93.0±20.0
Length ±SE (g)	17.0±1.5	16.5±0.9	18.0±1.0	17.0±1.3	16.0±1.0
Male: Female	6:0	5:0	5:0	5:0	5:0
n	6	5	5	5	5

16. Heavy metals: Water quality

		mean		± SE
i.	Temperature	15.5	±	0.5°C
	pH	7.83	±	0.2
	Total water hardness	206.9	±	1.6 mg dm^{-3}
	Calcium hardness	88.7	±	0.5 mg dm^{-3}
	Magnesium hardness	117.1	±	0.9 mg dm^{-3}

ii. Experimental heavy metal concentrations

Nickel (Ni)	0.75	±	0.01 mg dm^{-3}
Zinc (Zn)	1.06	±	0.01 mg dm^{-3}
Copper (Cu)	0.29	±	0.01 mg dm^{-3}
Chromium (Cr)	1.009	±	0.014 mg dm^{-3}

17. Zinc concentrations: Antibody titre response, mean ± 2SE (Live MS2 bacteriophage titres are tabulated in the square brackets, mean PFU ± 2SE)

1. Primary response: S. trutta

Day \ Zn Concentration mg dm-3	Control	0.1	0.5	1.0	2.1
0	4.0±1.9	2.1±1.7	1.2±1.2	3.8±1.7	1.1±0.4
7	110±82.1	165±90	146±78.3	161±93.8	34.5±12.3
14	1255±195	919±308	1368±844	599±114	476±198
21	3386±2691	5084±3449	6141±3355	5497±2418	916±437
28	54356±51952	7225±2723	4007±1565	16927±13871	468±203
35	5361±2749	2692±1375	1182±622	4954±3325	4345±2826
42	538±135	1351±662	179±107	805±409	1499±965
49	339±111	1086±766	207±85.7	337±139	348±176

ii. Secondary response: S. trutta

Zn Concentration mg dm^{-3}	Control	0.1	0.5	1.0	2.1
Day					
0	339±111	1086±766	201±85.7	337±139	348±176
7	2652±1683	972±294	452±285	548±239	360±258
14	4486±2596	548±163	623±366	1178±665	455±188
21	20010±12678	893±283	951±432	1991±818	1409±808
28	12432±5413	543±65.6	328±146	687±215	608±232
35	5796±3141	391±71.9	109±22.8	416±82.3	554±278
42	3664±2241	329±55.4	204±75.4	433±196	605±300
49	6138±4435	292±209	123±81.3	223±49.2	67.2±41.6
56	2872±2072	95.5±26.5	116±75.5	227±71.4	89.0±13.1
63	2048±760	49.6±11.0	147±95.4	262±114	161±89.2
70	8995±5910	28.0±14.1	175±95.4	140±47.5	117±37.9
77	24224±20362	34.4±17.3	170±91.8	165±40.5	50.9±31.3
Weight ±SE (g)	67.0±3.0	96.75±15.5	91.75±15.0	67.76±3.75	67.5±4.5
Length ±SE (cm)	15.5±0.25	18.25±1.25	17.75±1.25	16.0±0.25	16.25±0.25
Male: Female	3:3	6:0	3:3	5:1	5:1
n	6	6	6	6	6

iii. Primary response: C. carpio († indicates the death of a fish on or after the day of serum sampling)

Zn Concentration mg dm^{-3}	Control	0.1	0.5	1.0	2.1
Day					
0	0	1.9 ± 1.3	0	0	0
7	0	0	0	14.4 ± 10.7	0
	$\left[\begin{matrix}1.7 \times 10^{6} \pm \\ 8.4 \times 10^{5}\end{matrix}\right]$	$\left[\begin{matrix}1.0 \times 10^{7} \pm \\ 8.1 \times 10^{6}\end{matrix}\right]$	$\left[\begin{matrix}2.1 \times 10^{6} \pm \\ 6.4 \times 10^{5}\end{matrix}\right]$	$\left[\begin{matrix}3.9 \times 10^{6} \pm \\ 2.8 \times 10^{6}\end{matrix}\right]$	$\left[\begin{matrix}1.5 \times 10^{6} \pm \\ 6.9 \times 10^{5}\end{matrix}\right]$
14	38.1 ± 32.9	16.0 ± 4.9	6.4 ± 1.6	29.7 ± 13.3	1.9 ± 0.9
21	342 ± 292	79.1 ± 30.4	332 ± 183	75.9 ± 27.7	11.9 ± 6.1
28	565 ± 83.4	43.7 ± 12.6	618 ± 385	208 ± 90.8	40.2 ± 13.6
35	740 ± 267	0	971 ± 244	625 ± 51.7	178 ± 70.2
42	1026 ± 920	† † † † † -	633 ± 235	140 ± 45.3	
49	352 ± 144		1016 ± 607	199 ± 58.7	602 ± 452
56	547 ± 365		1073 ± 514	240 ± 91.1	525 ± 198

63	12659±6727		6470±2887	2939±1988	169±71.4
70	14019±7471		8653±5147	6572±2755	276±139
77	10504±7186		1036±390	4117±3610	185±58.7

Weight ±SE (g)	111.75±12.0	130.0±6.0	119.0±8.25	133.5±5.0	139.25±4.5
Length ±SE (cm)	15.5±0.75	16.0	16.0±0.5	17.25±0.25	17.5±0.25
Male: Female	6:0	6:0	6:0	6:0	6:0
n	6	6	6	6	6

iv. Secondary response: C. carpio

Day \ Zn Concentration mg dm-3	Control	0.1	0.5	1.0	2.1
0	547±365	-	1073±514	240±91.1	525±198
7	528±477		1693±832	345±83.2	768±205
14	3153±1005		2868±1606	783±188	713±441
21	979±401		775±408	469±159	244±81.9
28	2094±952		1026±433	261±64.1	244±90.6
35	2743±1111		2349±1203	1854±1113	1119±832
42	6042±2926		3705±1544	4575±1851	1767±878
49	5193±2832		2299±1239	978±237	594±183
56	7582±2777		3472±1742	3584±1373	621±516

18 Zinc Concentrations: Water quality

		mean	±	SE
i.	Temperature	15.5	±	0.5°C
	pH	7.81	±	0.02
	Total water hardness	198.2	±	3.9 mg dm^{-3}
	Calcium hardness	91.2	±	3.6 mg dm^{-3}
	Magnesium hardness	107	±	1.8 mg dm^{-3}

ii. Experimental zinc concentrations

	mean	±	SE
1	0.14	±	0.01 mg Zn dm^{-3}
2	0.53	±	0.005 mg Zn dm^{-3}
3	1.04	±	0.02 mg Zn dm^{-3}
4	2.13	±	0.02 mg Zn dm^{-3}

19 Inoculated Lead: Tertiary antibody titre response ±2SE in S. trutta († indicates the death of a fish on or after the day of serum sampling)

Pb Concentration (mg) / Day	Control	0.01	0.05	0.1	0.3
0	36005±14987	7749±3237	1879±240	11489±9706	93807±6171
7	33250±14150	5234±3128	1866±1479	49980±35261	10955±6784
14	31430±13199	3381±1711	887±519	45138±41689	2166±1426
21	42450±13816	4869±1897	114±58	2512±1136	24.0±9.0 †
28	54088±12920	2364±862	901±609	1214±844	59.0±33.0 ††
35	52828±20842	5726±1109	193±118	353±257	184±82.0
42	58665±30322	1101±333	462±294	202±65.0	232±99.0
49	32411±13636	800±360	342±265	161±78.0	0 ††
56	62636±50599	822±167	225±168	260±39.0	—
63	17669±9561	1613±567	631±494	2656±2264	
70	113816±58291	429±164	616±480	15599±15354	
77	68677±31226	416±133	1819±1414	445±294	
84	101227±60451	679±355	684±536	607±349	
91	105179±48883	600±202	383±222	1154±696	
98	90471±60670	1964±1243	404±286	893±807	
105	75520±62358	824±520	699±621	483±68.0	
112	106318±53785	790±521	1282±975	283±77.0	
119	97722±93691	438±204	82±9.0	114±40.0	
Weight ±SE(g)	149.0±14.0	158.0±15.0	121.75±14.5	148.5±20.5	149.0±14.0
Length ±SE(cm)	26.0±1.5	25.0±1.0	23.5±1.0	23.5±1.0	26.0±1.5
Male: Female	4:1	5:0	4:1	3:2	4:1
n	5	5	5	5	5

.20 Inoculated Cadmium: Tertiary antibody titre response ±2SE in *S. trutta*. († indicates the death of a fish on or after the day of serum sampling)

Cd Concentration (mg)	Control	0.05	0.1	0.2
Day				
-7	34458 ± 27743	12826 ± 9862	10115 ± 7266	17720 ± 14912
0	40250 ± 34833	14711 ± 8893	12606 ± 9557	30999 ± 27127
+7	56494 ± 51431	55593 ± 51686	4079 ± 3378	215 ± 44.2
14	47676 ± 42798	53826 ± 58229	3596 ± 1746	0
21	44759 ± 38174	68205 ± 47427	573 ± 344	0
28	60402 ± 50527	59756 ± 50667	178 ± 71.6	††††††
35	69526 ± 64224	29282 ± 17336	30.2 ± 7.2	
42	62230 ± 50034	29793 ± 27873	95.3 ± 62.2	
49	73590 ± 48295	11135 ± 9777	57.9 ± 24.1	
56	77033 ± 61833	4109 ± 3961	131 ± 54.3	
63	70353 ± 34049	5916 ± 5664	101 ± 49.3	
70	35727 ± 10821	397 ± 123	20.3 ± 11.3	
77	11180 ± 3671	49.6 ± 23.7	33.8 ± 18.3	
84	13928 ± 2995	96.4 ± 34.8	22.4 ± 4.7	
		[re-inoculated with MS2 on day 84]		
91	46161 ± 33089	98.3 ± 41.3	86.4 ± 39.9	
98	49847 ± 25042	231 ± 124	224 ± 89.2	
105	87757 ± 58714	5557 ± 3481	173 ± 52.0	
112	77381 ± 48956	4270 ± 3667	191 ± 74.1	
119	63026 ± 26307	1948 ± 1659	25.2 ± 6.4	
Weight ±SE (g)	156.25 ± 29.5	124.25 ± 10.0	165.25 ± 32.35	181.0 ± 32.25
Length ±SE (cm)	20.75 ± 1.5	20.5 ± 0.75	22.25 ± 1.5	22.5 ± 1.5
Male: Female	3:2	4:1	5:0	3:2
n	5	5	5	5