MODULATORY EFFECTS OF CADMIUM AND COPPER ON THE SUSCEPTIBILITY AND IMMUNE RESPONSE OF COMMON CARP, CYPRINUS CARPIO (L) TO SELECTED PATHOGENS.

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TO

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DECLARATION

I hereby declare that the thesis has been compiled by myself and is the result of my own investigations. It has neither been accepted, nor is being submitted for any other degree. All the sources of information have been duly acknowledged

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ABSTRACT

Pollution of the aquatic environment has received considerable attention in recent years. The fear that pollutants might predispose fish to disease is a cause of great concern. Recent evidence has shown that, in the case of some pollutants, the fear was well-founded. The lack of detailed studies in this field prompted the present study.

A comprehensive approach was made towards understanding the effects of two common aquatic pollutants, the metals cadmium and copper, at sublethal levels, on the disease resistance and immune response mechanisms of common carp, *Cyprinus carpio* L. Two pathogens were used as models, which differ from each other with respect to the nature of the disease processes and their cellular requirements for optimal immune response. *Ichthyophthirius multifiliis* (Fouquet), the causative agent of white spot disease and *Aeromonas hydrophila* which causes bacterial haemorrhagic septicaemia were employed to examine the immunomodulatory effects of the metals. A third model, Sheep Red Blood Cell, with known cellular requirements and immunokinetics, was used as a reference antigen. The *in vitro* and *in vivo* effects of the metals on lymphocyte proliferation induced by putative T and B-cell mitogens were also evaluated. The ability of the metals to induce a stress response in carp was assessed by monitoring the kinetics of plasma cortisol. Subtle structural changes produced by the metals in the primary barriers and haematopoietic organs were also examined histologically.

Exposure of naive carp for 10 days to sublethal levels (50 μgl^{-1}) of cadmium and copper significantly increased their susceptibility to *I. multifiliis* as measured by mean parasite intensity. Previously immunized carp with established immunity and high "tomite agglutination titre" could not mount a protective immune response following 10 days exposure to 25 and 50 μgl^{-1} cadmium and copper. Concurrent exposure of carp to either cadmium or copper with a series of controlled low level immunization exposures to the parasite, did not alter the kinetics nor the magnitude of the "anti-ich" humoral antibody titre. Control carp acquired complete resistance earlier than metal exposed ones.

Exposure of carp to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) for 40 days with the primary immunization given 10 days after the commencement of metal exposure did not reduce the primary antibody response, but significantly reduced the magnitude of the secondary response. Similar experimental conditions also revealed the significant suppressive effects of these metals on the number of both Rosette Forming and Plaque Forming Cells. Long term exposure (30 days) to cadmium before the primary immunization did not impair the kinetics and magnitude of the primary and secondary humoral response, but, in contrast, copper significantly suppressed both the primary and secondary response. Exposure of carp to the metals 18 days after the primary immunization with SRBC significantly reduced the magnitude of the secondary humoral response. Primary immunization given concurrently with the commencement of metal exposure, significantly reduced the magnitude of both the primary and secondary response.

Carp exposed to cadmium and copper for 10 days were more susceptible to *A. hydrophila* than unexposed controls. Previously immunized carp could not mount an effective protective immunity following 10 days exposure. In contrast to *I. multifiliis*, the decrease in the protective immunity was associated with a significant drop in the humoral bacterial agglutination titre. Exposure to the metals for 10 days before immunization with *A. hydrophila* heat killed bacterin reduced the magnitude of the humoral response significantly. Immunization carried out simultaneously with the commencement of metal exposure suppressed the magnitude of both the primary and secondary humoral antibody response.

Lymphocytes collected from carp exposed to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) showed a reduced blastogenic response to the mitogens concanavalin (Con A) and lipopolysaccharide (LPS). A significant suppression was observed in lymphocytes collected after 6 to 9 days of exposure to the respective metals. Both the metals *in vitro* showed similar suppressive effects. Concentrations of 10^{-7} M (copper) or 10^{-6} M (cadmium) and greater, suppressed the mitogenic response to both the mitogens significantly.

Exposure to both the metals induced a stress response, characterized by elevation of plasma cortisol. The elevation in cadmium exposed carp was transitory in nature, the

values returning to near basal values and persisting at that level till the end of the experimental duration. In the copper exposed carp, the cortisol decreased gradually from the maximum seen following 24 hours until day 9 before showing a secondary elevation which persisted. Both the metals produced leucopenia. Cadmium exposure did not have any effect on the haematocrit, whilst copper caused an elevation. The pathological effects on the structure of the primary barriers were considerable at the concentrations tested. The pathological changes in the haematopoietic organs were not so severe, but included multifocal necrosis of the haematopoietic tissue and indications of fragmentation of malanomacrophage centres in the kidney and spleen.

Both cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) did not completely abrogate the immune response of carp. Studies evaluating the immunotoxicity of aquatic pollutants should give more emphasis to the magnitude of suppression rather than looking for a complete abrogation of immune response. This study has clearly shown that a reduction in the magnitude of the immune response is sufficient to predispose fish to disease. These findings are significant when assessing the impact of these pollutants and when determining the maximum limits tolerable in the natural environment.

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CHAPTER 1

GENERAL INTRODUCTION

INTRODUCTION

Over the last two decades, studies on the impact of pollution on fish health has been intensified. Environmental contaminants, especially chemical toxicants, have been associated with outbreaks of disease in fish in natural populations and, as such, have received considerable attention (Pippy and Hare, 1969; Snieszko, 1974; Brown *et al.*, 1977; 1979; Mearns and Sherwood, 1977; Overstreet and Howse, 1977; Sinderman, 1979; Murchelano, 1982; Moller, 1985). Most of the evidence relating disease to pollutants is circumstantial, coming from field studies which have attempted to show increased frequency of disease conditions such as ulcers, neoplasms, fin erosion, skeletal anomalies and lymphocystis, in fish inhabiting heavily polluted environments.

Sublethal levels of toxic agents in mammals (Sharma, 1984) and fish (Zeeman, 1986) have been shown to have effects upon immune system structures and/or functions that may ultimately be almost as harmful as the direct toxic effects. Alterations of the immune response are usually reflected by changes in the susceptibility to disease agents, latent viral infections and even tumour formations. There is growing evidence that the immune surveillance mechanisms are important in the survival of altered cells, and immune suppression can favour the existence and growth of tumour cells. Thus the increased incidence of integumental tumours such as epidermal papillomas and stomatopapilloma in fish from highly polluted waters as reviewed by Sinderman (1979) may possibly indicate the immunosuppressive effects of several pollutants.

The ability of aquatic pollutants to alter the disease resistance and immune response mechanisms in fish could have grave consequences on wild fish populations in

general, and aquaculture in particular. It is very well established from studies of epidemiology, that fish disease occurs when susceptible fish are exposed to virulent pathogens under certain environmental stress conditions. The interactions between the host, pathogen and the environment are fundamental determinants in the outbreak of disease (Snieszko, 1974).

The role of stress in the host's resistance to disease has been summed up by Dubos (1955) as follows "There are many situations in which the pathogen is a constant and ubiquitous component of the environment, but causes a disease only when some weakening of the patient by another factor allows infection to proceed unrestrained, at least for a while". The role of stress in altering disease resistance of fishes has been well documented (Wedemeyer, 1970; Snieszko, 1974; Wedemeyer *et al.*, 1976).

The concept of "stress" and the response of fish to "stressors" (stimuli) has evoked much concern and interest in the recent past. A host of agents including toxic chemicals can "stress" fish and act through Selye's general adaptation syndrome (Selye, 1950) to cause interactions between fish endocrinological, haematological and immunological systems (Wedemeyer *et al.*, 1976; Mazeaud *et al.*, 1977; Donaldson, 1981; Pickering, 1981). Whether such a toxicant acts directly upon the fish immune system or indirectly via a general adaptation syndrome, is a very delicate question. Partitioning the immunosuppressive effects of environmental toxicants between stress-mediated or direct toxicant-mediated effects is very difficult in any study. On the other hand, it is vital to understand the stress response of fish to environmental contaminants, as components of the stress response are thought to be immunosuppressive (Ellis, 1981).

1.1 Immunomodulatory Effects of Stress-induced Cortisol

Fish under stressful conditions are known to produce elevated levels of corticosteroids from the stimulated interrenal cells (Mazeaud *et al.*, 1977; Pickering, 1981). Several studies aimed at elucidating the mechanism (s) which will explain the relationship between plasma corticosteroids and increased disease susceptibility in fish have implicated immunosuppression as a major underlying effect. The following brief review will reveal the serious nature of stress effects in fish and highlight the increased attention being devoted to this area of research over recent years.

"Stressors" commonly encountered in intensive aquaculture practices such as handling, crowding, netting, transportation, poor water quality, anoxia and social interaction are known to elevate plasma corticosteroids in fish (Wedemeyer and McLeay; 1981; Donaldson, 1981; Robertson *et al.*, 1987; Pickering and Pottinger, 1987).

Elevated levels of endogenous plasma corticosteroids resulting from such acute stressors have been associated with leucopenia (Angelidis *et al.*, 1987), increased susceptibility to disease (Perlmutter *et al.*, 1973; Walters and Plumb, 1980; Angelidis *et al.*, 1987; Maule *et al.*, 1987; Peters *et al.*, 1988), reduced phagocytic activity of macrophages (Peters and Schwarzer, 1985; Ellsaesser and Clem, 1986; Angelidis *et al.*, 1987), impaired mitogenic response of lymphocytes (Ellsasser and Clem, 1986; Maule *et al.*, 1987), reduced antibody production (Perlmutter *et al.*, 1973; Miller and Tripp, 1982; Maule *et al*, 1987), damage to lymphoid organs (Peters and Schwarzer, 1985) and suppression of cellular responses such as natural cytotoxic cell activity (Ghoneum *et al.*, 1988).

Several studies have utilized exogenous corticosteroid implantation or administration

to achieve corticosteroid levels in fish near to physiological levels (Pickering and Duston, 1983; Barton *et al.*, 1987). These studies have observed several of the effects listed above and have shown the immunosuppressive properties of elevated cortisol in fish.

Exogenous corticosteroids have increased the susceptibility of fishes to bacterial, viral and parasitic infections (Pickering and Duston, 1983; Mushiake *et al.*, 1984; Pickering and Pottinger, 1985; Woo *et al.*, 1987; Wechsler *et al.*, 1986), suppressed the protective immunity (Houghton and Matthews, 1986), and humoral antibody response (Anderson *et al.*, 1982; Thomas and Lewis, 1987) and interfered with lymphocyte proliferation (Grimm, 1985; Ralph *et al.*, 1987).

The potential ability of several environmental contaminants including heavy metals to elevate plasma corticosteroid levels in fish (Donaldson and Dye, 1975; Schreck and Lorz, 1978; Donaldson, 1981; Bennett and Wolke, 1987a) and the possibility of low levels of these contaminants producing protracted elevation of cortisol in fish, clearly highlights the need for better understanding of pollution-disease interactions in fish and the underlying mechanisms.

1.2 Immunomudulatory Effects of Heavy Metals

Heavy metals have long been recognised as potential environmental contaminants with serious toxic effects on aquatic life. Their high toxicity, persistence in the environment, ability to accumulate in animal tissue and magnify along the food chain, makes them one of the most serious class of pollutants in the aquatic environment. The major concern in the past has been primarily with direct toxicity, with much of the research oriented towards ascertaining the mechanisms of toxicity and the physiological effects. Until recently, little attention had been given to

indirect toxicity and long-term chronic effects. Recent evidence with mammals (Koller, 1980) and fish (Zeeman and Brindely, 1981; Anderson *et al.*, 1984) has shown that chronic, low level exposure to certain metal pollutants may induce subtle changes within a host, including alteration of the immune system and resistance to diseases.

A substantial amount of progress has been made in the field of mammalian immunotoxicology. Research of this nature in lower vertebrates, including fish, appears to have taken the cue from mammalian research findings. The following brief overview of the immunomotoxicology of heavy metals in mammals brings to light the serious nature of this problem. Much of the published work is largely confined to such metals as cadmium, mercury and lead, possibly because of their very high toxicity to human beings. The immunomodulatory effects of metals in mammals is well established and extensively reviewed by Koller (1979), (1980), Koller and Vos (1981), Sharma (1984) and Blakely and Tomar (1986).

1.2.1 Mammals

Cadmium exposure has been associated with increased susceptibility of homiotherms to bacterial (Cook *et al.*, 1975), viral (Gainer, 1977) and protozoal (Exon *et al.*, 1975) infections. On the other hand cadmium has also been shown to increase the resistance to bacterial (Hill, 1979) and viral (Exon *et al.*, 1979) diseases. Differences in species, route of metal administration, dosage and duration of exposure and virulence of the pathogen are some of the explanations given for the conflicting observations (Koller *et al.*, 1980).

The evidence for effects of cadmium on humoral antibody production in homiotherms is again contradictory. Several studies have observed impaired antibody

synthesis (Jones *et al.*, 1971; Koller, 1973; Koller *et al.*, 1975; Bozelka *et al.*, 1978; Blakely, 1985; Blakley and Tomar, 1986), whilst others have reported enhanced antibody synthesis (Jones *et al.*, 1971; Koller *et al.*, 1976). Cadmium has been known to repress the cell-mediated immune response in mammals (Garwoski and Sharma, 1978; Muller *et al.*, 1979; Fujimaki *et al.*, 1983), and such responses as the delayed type hypersensitivity reactions, which require T-lymphocyte participation, are severely suppressed.

Looking at the effects of cadmium on antibody response to antigens with different cellular requirements, several findings have been shown to be consistent. Cadmium significantly suppresses antibody synthesis to thymus dependent and macrophage dependent antigens such as Sheep red blood cells (Blakley and Tomar, 1986) and the effect as it relates to humoral immunity appears to be largely associated with T-lymphocyte dependent responses (Koller *et al.*, 1976; Kawamura *et al.*, 1983; Blakley, 1985; Blakley and Tomar, 1986). On the contrary, antibody synthesis to both thymus independent, macrophage dependent and thymus independent, macrophage independent antigens were shown to be enhanced (Stelzer and Pazdernik, 1983; Fujimaki, 1985; Blakley and Tomar, 1986).

Analogous results have been obtained with lymphocyte blastogenesis. B-lymphocyte blastogenesis is enhanced by cadmium while it had no significant effect on T-lymphocyte blastogenesis (Garworski and Sharma, 1978; Koller *et al.*, 1979; Muller *et al.*, 1979; Blakley, 1985). On the other hand, complement receptor activity of B-lymphocytes (Koller and Brauner, 1977) were shown to be seriously effected by cadmium.

With macrophages, cadmium has been found to either stimulate (Cook *et al.*, 1974;

Koller and Roan, 1977) or suppress (Loose *et al.*, 1978a) phagocytosis. In addition, cadmium is known to impair Fc receptor (Loose *et al.*, 1978b) and complement receptor (Cook *et al.*, 1984) activity of macrophages. Other functions of macrophages such as Tumour-killing ability (Nelson *et al.*, 1982), lymphokine production and macrophage mobility (Kiremidjian-Schumacher *et al.*, 1981) are also affected by cadmium.

The effects of lead on the mammlian immune system is not so controversial and it has been shown to consistently suppress various segments of the immune system. Susceptibility to parasitic, bacterial and viral diseases is increased by exposure to lead (Hemphil *et al.*, 1971; Cook *et al.*, 1975; Exon *et al.*, 1975; Gainer, 1977). Lead also appears to promote the growth of viral and chemical induced neoplasms (Gainer, 1973). Lead interferes with the functioning of reticuloendothelial systems (Trejo *et al.*, 1972), components of natural immunity (DeBruin, 1971; Ward *et al.*, 1975) and complement receptor activity on B-lymphocytes (Koller and Brauner, 1977).

Humoral antibody production (Koller, 1973; Koller and Kovacic, 1974; Blakley *et al.*, 1980) and even memory formation (Koller and Kovacic, 1974) are significantly suppressed by lead. Cell-mediated immunity is also affected by exposure to lead (Muller *et al.*, 1977; Faith *et al.*, 1979). The effects of lead on lymphocyte proliferation is, however, controversial. Lead has produced both suppressive and enhancing effects on lymphocyte blastogenesis induced by T and B-cell mitogens (Shenker *et al.*, 1977; Gaworski and Sharma, 1978; Koller *et al.*, 1979).

The immunosuppressive effects of mercury at subtoxic levels is well documented in mammals. Both organic (Koller, 1975) and inorganic (Gainer, 1977) forms of

mercury are capable of increasing the susceptibility to diseases. Suppression of both the primary and secondary humoral antibody response, including memory formation, is associated with mercury (Koller *et al.*, 1977; Blakley *et al.*, 1980). The organic form of mercury, methyl mercury, does not alter phagocytic properties of peritoneal macrophages or the Fc receptors of B-lymphocytes (Koller, 1979). Lymphocyte blastogenesis induced by T-cell mitogens is known to be inhibited (Garworski and Sharma, 1978).

Selenium in mammals is found to be unique in that it potentiates the immune response rather than suppressing it (Sheffy and Schultz, 1978; Koller *et al.*, 1979; Desowitz and Barnwell, 1980).

In mammals essential metals such as zinc have been found to affect the immune respose both at low and high doses. Phagocytic ability of peritoneal macrophages is inhibited by low and high doses of zinc (Karl *et al.*, 1973). Zinc deficiency has been reported to induce marked atrophy of the thymus and reduced antibody synthesis (Fraker *et al.*, 1977; Fernandes *et al.*, 1979). The mitogenic response of lymphocytes (Mulhern, 1980) and cell-mediated responses (Koller, 1980) are unaltered in animals fed zinc deficient or zinc supplemented diet. High levels of zinc decreases tumour incidence (Mulhern, 1980) while zinc deficient diets depress T-killer cell activity (Fernandes *et al.*, 1979).

Copper is an important environmental contaminant but surprisingly there is very little information available on its immunotoxic properties in mammals. Other metals such as nickel, iron and arsenic are also known to compromise various components of non-specific and specific parts of the immune system in mammals (Gainer, 1977; Weinberg, 1978; Adkins *et al.*, 1979; Blakley *et al.*, 1980).

From this review it is evident that many of the non-essential toxic metals compromise the immune system of mammals. Damage may occur to a particular cell (B or T-lymphocyte or macrophage) or may involve more than one cell which regulates the proliferation and differentiation of other cells responsible for normal functioning of the immune system.

In summary, lead appears to consistently suppress most of the segments of the immune system whilst cadmium has produced mixed results. The effects of cadmium as it relates to humoral immunity is largely related to its effects on T-lymphocyte dependent processes. Mercury in both its organic and inorganic form suppresses the humoral immune response, whilst selenium has been shown to enhance humoral immunity. Zinc deficiency results in atrophy of lymphoid organs like thymus with a subsequent reduction in the humoral immune capacity. Various other metals may also compromise the components of the immune system.

1.2.2 Fish

Research dealing with the immunotoxicological effects of metals in fish is still in its infancy. The available findings are patchy, very general, and largely confined to disease susceptibility and humoral immune response studies. As it will become clear from the following review, the majority of the findings are based on studies which have looked at one or two aspects of the immune system. There is very little possibility of drawing any inferences as to the effects of these metals on specific components of the immune system of fish. This is made even more complicated by the lack of comprehensive studies which have looked into the effects of any single metal on various components of the immune system.

1.2.2.1 Susceptibility to Disease

Heavy metals have been implicated in outbreaks of disease in fish (Pippy and Hare, 1969; Sniezko, 1974). Recently, controlled laboratory infections have been used to more precisely demonstrate this interaction. Most of the studies available are confined to the effects of copper on the disease resistance of salmonids to bacterial pathogens. The section to follow will also show the possible influence of factors such as the method of challenge infection, the type of the pathogen and the timing between metal exposure and challenge infection on the results obtained.

Several studies have demonstrated the ability of copper to lower the disease resistance in fish. Eels, *Anguilla anguilla* L. and coho salmon, *Oncorhynchus kisutch* (Walbaum) held in copper contaminated waters (18 to 60 μgl^{-1}) were found to die of vibriosis, caused by the naturally occurring *Vibrio anguillarum* (Rodsaether *et al.*, 1977; Stevens, 1977) suggesting the possibility that the copper was predisposing the fish to these naturally occurring bacterial pathogens. Exposure to low levels of copper for a week was reported to increase the susceptibility of rainbow trout, *Salmo gairdneri* (Richardson) to viral (IHNV) infection (Hetrick *et al.*, 1979).

Rainbow trout were found to be increasingly susceptible to *Yersinia ruckeri* following 96 hours exposure to copper concentrations of 7 and 10 µgl-*1* (Knittel, 1981). Similarly, Baker *et al.* (1983) observed copper exposed rainbow trout and chinook salmon, *Onchorynchus tahawytsch* (Walbaum), to be more susceptible to *V. anguillarum*. In both these studies immersion was used as a challege method and, surprisingly, it was observed that relatively low concentrations of copper were required to produce early maximum mortality, whilst fish exposed to higher concentrations were less susceptible than those exposed to lower concentrations.

On the other hand, long-term, 30 day, exposure of rainbow trout to copper

concentrations of 10.9 and 21.5 μgl^{-1} was found not to affect their susceptibility to *Aeromonas hydrophila* challenge by injection (Sanarski, 1982). Results such as this might indicate the ability of fish to acclimate to low levels of copper, in view of the finding that short term exposure to similar levels of copper does increase the susceptibility to bacterial disease (Baker *et al.*, 1983).

The differential effects of copper in altering the susceptibility to disease in the Japanese eel, *Anguilla japonica* (Temminck & Sehlegal) in relation to the pathogens used was observed by Mushiake *et al.* (1984). They found exposure of Japanese eel to higher concentrations of copper, such as 100 and 250 μgl^{-1}, to increase their susceptibility to *Edwardsiella tarda* and *Pseudomonas anguilliseptica*, but not to *V. anguillarum*.

The suggestion of MacFarlane *et al.* (1986) that copper and cadmium offer protection against *Flexibacter columnaris* infection in the striped bass, *Morone saxatilis* (Walbaum) is very exciting. However, upon close examination of the experimental protocol the reasons for this are obvious. The experimental procedure involved exposing the fish to metals for a day followed by a two minute contact (dip) infection and then continuing the metal exposure for another seven days. The nature of the bacterium chosen and the exposure protocol followed appears to have contributed to their observation.

Of the other metals investigated, chromium at very high levels of 500 μgl^{-1}) was found to increase the susceptibility of coho salmon to *V. anguillarum* infection (Sugatt, 1980). Lead and selenium were found to have no apparent effect in altering the susceptibility of striped bass, *M. saxatilis* to *F. columnaris* infection, whilst arsenic was found to have an enhancing effect (MacFarlane *et al.*, 1986).

Parasitic challenge infections are very rarely used to demonstrate the link between heavy metals and disease susceptibility in fishes. The work of Ewing *et al.* (1982) is the only study in this direction. Channel catfish, *Ictalurus punctatus* (Rafinesque), exposed to copper (25 to 3200 μgl^{-1}) were found to be more susceptible to the protozoan ciliate, *Ichthyophthirius multifiliis*, challenge infection. They also found a weak, but statistically significant positive correlation between exposure concentration and susceptibility to the parasite.

1.2.2.2 Humoral Antibody Response

The metals copper, cadmium, lead, methyl mercury, chromium, zinc and manganese have been evaluated for their immunosuppressive effects in fish. It is evident from the literature that the results appear to depend to a certain extent on the experimental protocols followed and factors such as the type of the antigen chosen and the temporal relation between metal exposure and immunization.

Essential metals such as zinc and manganese have produced contrasting effects on the humoral antibody response. Zinc suppresed the serum antibody response of the zebra fish, *Brachydanio rerio* (Hamilton), to *Proteus vulgaris* heat killed vaccine, but did not affect the response against infectious pancreatic necrosis virus (Sarot and Perlmutter 1976). The modulatory effect of zinc appears to be different with different antigens. On the other hand long term exposure of carp, *Cyprinus carpio* L. to very high concentration (50 mgl^{-1}) of manganese was found not to suppress the antibody response to *Yersinia ruckeri* antigen administered 7 days after the commencement of the metal exposure (Cossarini-Dunier *et al.*, 1988).

The humoral immune response of blue gouramis, *Trichogaster trichopterus* L. to *Proteus vulgaris* was found to be decreased following exposure to methyl mercury

(9 μgl^{-1}) and copper (9 μgl^{-1}) individually, whilst the two metals in a mixture did not have any additive effect (Roales and Pelmutter, 1977; 1980). The immunization injection given concurrently with or 1 week after the commencement of metal exposure did not make any difference in the observed suppressive effects. Sugatt (1980) found that the antibody response of coho salmon, *O. kisutch* to *V. anguillarum* to be suppressed by exposure to high levels of chromium for 2 weeks with the bacterin injection being given before the commencement of metal exposure. The observed suppression in this case was confusing in that the antibody response was suppressed at 6 weeks, but not at 2 or 4 weeks post-immunization.

Reports on the total suppression of the humoral antibody production is often associated with high concentrations of toxic metals. Such a response was observed in brown trout, *Salmo trutta* L. and carp, *C. carpio* exposed to very high concentrations of copper (290 μgl^{-1}) and chromium (1001 μgl^{-1}) over a long term (38 weeks), with the MS2 bacteriophage immunization given 2 weeks after the commencement of the metal exposure (O'Neill, 1981a). The ability of toxic metals such as cadmium and lead to substantially lower the amount of antibody, already in circulation in previously immunized fish was demonstrated by O'Neill, (1981b). Intraperitoneal injection of high levels of cadmium (200 μgl^{-1}) and lead (300 μgl^{-1}) to previously immunized brown trout, *S. trutta* decreased the level of circulating antibody to MS2 bacteriophage.

In a four month exposure study, Viale and Calamari (1984) found cadmium at low levels (1.0 and 10 μgl^{-1}) to slightly reduce the humoral immune response whilst copper (30 and 100 μgl^{-1}) and chromium (50 and 200 μgl^{-1}) at relatively high levels had no apparent effect on rainbow trout, *S. gairdneri* which is a very sensitive species to copper toxicity. The immunization schedule in their study which included

five injections of the antigen human red blood cell (hRBC) over a two month period, with the first being carried out one month after the commencement of the metal exposure, may to some extent explain the findings.

Non-essential toxic metals such as cadmium at very low levels have been shown to enhance the humoral antibody response in fish (Thuvander 1989). This interesting observation was made in rainbow trout, *S. gairdneri* which was exposed to cadmium (3.6 μgl^{-1}) for 12 weeks and *V. anguillarum* O-antigen was injected 5 weeks after the beginning of metal exposure. This result may suggest the process of acclimation in fish to very low levels of toxic metals.

Paradoxical effects of very high concentrations of cadmium on the antibacterial antibody response to *Bacillus cereus* of two marine fish species was reported by Robohm (1986). Exposure to 12 mgl^{-1} cadmium caused significant inhibition of serum antibody titres in cunners, *Tautogolabrus adsperus* (Walbaum) while the antibody response in striped bass, *M. saxatilis* exposed to cadmium (10 mgl^{-1}) was enhanced six fold. The concentrations employed in this study is well above the lethal limit of many fresh water fish species. The stimulating effect of cadmium on the antibody production in *M. saxatilis* may suggest the relative resistance of this species to cadmium toxicity.

1.2.2.3 Macrophage Activity

Phagocytosis has been regarded as a major line of defence against invading pathogens (Ellis *et al*,, 1976). Some studies have selected this line of approach in attempts at evaluating the immunosuppressive effects of chemical pollutants. In the interesting studies of Weeks and Warinner (1984) and Weeks *et al.* (1986), the phagocytic activity and chaemotactic response of macrophages collected from fish

inhabiting polluted environments was observed to be significantly reduced when compared to those collected from fish in unpolluted waters. There is very limited information on the effects of metal pollutants on the phagocytic activity of fish macrophages and the available information is often conflicting.

Robohm and Nitkowski (1974) observed cadmium exposure (12 mgl^{-1}) in cunners to cause a greater clearance rate, but a lower killing rate, of intra-cardially injected bacteria by the macrophages. The process of blood clearance of the primary MS2 bacteriophage was noted to be suppressed by nickel, copper and chromium (O'Neill, 1981a). This process in brown trout, took more than 7 days compared to less than 3 days in controls, whilst in carp exposed to copper, it took more than 2 weeks. On the other hand manganese in the range of 6.25 to 50 mgl^{-1} had a strong enhancing effect on phagocytosis of *Y. ruckeri* in carp (Cossarani-Dunier *et al.*, 1988).

Chemiluminiscence (CL) assays have been used to delineate the effects of the metals copper and cadmium on phagocytic activity of rainbow trout macrophages (Elsasser *et al.*, 1986). Copper at low levels (10 μgl^{-1}) suppressed the CL response of macrophages *in vitro* but the effects with cadmium (1 μgl^{-1}) were found to be variable. A significant increase in the CL was observed when cadmium was added one hour prior to, or immediately before, the assay, but following a 24 hour exposure a decreasing effect was seen.

1.2.2.4 The Mitogenic Response of Lymphocytes

Lymphocyte proliferation studies have been used extensively as tools to evaluate the immunosuppressive effects of metal toxicants in mammals. In recent years this *in vitro* technique has also been used in fish research to demonstrate the immunosuppressive effects of stressors (Ellsaesser and Clem, 1986), antibiotics

(Grondel *et al.*, 1985) and heavy metals (Ghanmi *et al.*, 1989).

Most of the available findings are based on *in vitro* studies carried out using essential metals. Cenini and Turner (1982) found zinc (10^{-7} to 10^{-3} M) *in vitro* to suppress the proliferative response of carp lymphoid cells induced by phytohaemagglutinin (PHA), While Ghanmi *et al.* (1989) found zinc (10^{-7} to 10^{-3} M) to enhance ^{3}H-thymidine incorporation in carp lymphoid cells stimulated by concanavalin (Con A), PHA and lipopolysaccharide (LPS). Similarly, manganese (10^{-7} to 10^{-3} M) *in vitro* was not suppressive but mitogenic by itself (Ghanmi *et al.*, 1989).

It is clear from the above review that several of the toxic metals have induced alterations in the disease susceptibility and immune response of fish. However, there is very little information on the effects of these toxic metals on the mitogenic response of lymphocytes. Lymphoid cells collected from rainbow trout following 5 and 9 weeks of exposure to low levels of cadmium (0.7 and 3.6 μgl^{-1}) showed a normal proliferative response to mitogens Con A and LPS (Thuvander, 1989). This finding might indicate the ability of rainbow trout to acclimate to low levels of metal pollutants such as cadmium over a long term.

1.2.2.5 Problems and Scope of Heavy metal Immunotoxicology in Fish

Of importance is the concentration of metals used in some of the published studies. A wide range of concentrations have been utilized, with copper 10 to 290 μgl^{-1}, and cadmium 10 to 12000 μgl^{-1}, to demonstrate their effects on the fish immune system. When these values are compared with the acute toxicity values of cadmium and copper summarized in Table 1.1 and 1.2 for some of the fresh water fish species, it appears that the concentrations used in some of the studies with salmonids were

high. There are a number of extensive reviews on the toxicity of cadmium and copper to fresh water fish (Nriagu, 1980; 1981; Alabaster and Lloyd, 1980; DeMayo and Taylor, 1981; Mance *et al.*, 1984; Mance, 1987). Regarding the relative sensitivity of different fish species, it is generally agreed that salmonid species are approximately ten times more sensitive than the non-salmonids (Mance, 1987).

From the above review on the effects of heavy metals on disease resistance and the immune response of fish, the following points emerge. The studies on the immunomodulatory effects of heavy metals are largely confined to salmonid fish species. In the vast majority of these studies, bacterial pathogens have been used to demonstrate heavy metal induced alterations in disease susceptibility of fish. The pathogenicity of the bacteria chosen, and the infection method adopted, appears to have had an influence on the results obtained. Copper has consistently increased the susceptibility of fish, especially salmonids, wherever virulent pathogens such as *V. anguillarum* and *Y. ruckeri* were used as models.

The observed effects of cadmium and copper on the humoral immune response of fish is conflicting. Differences in the cellular requirements for optimal expression of humoral immnity to various model antigens used, makes it difficult to attribute the observed effects to cadmium and copper. The temporal relationship between immunization and exposure to the metals also adds to the confusion. Having an established level of antibody may not mean effectiveness of the immune system. The execution of a protective immune response requires the cooperation and interaction of humoral, cellular and nonspecific components of the immune system. There is scarcely any information on the possible effects of metals on the protective immune response of fish.

There is very little information on the *in vitro* and *in vivo* effects of cadmium and copper on lymphocyte proliferation. Most of the published work on other metals is based on *in vitro* effects and no attempts have been made to correlate them with *in vivo* effects.

Several of the observed immunomodulatory effects of metals have been speculated to have resulted partly from the stress mediated response of the fish. However, there is no information regarding the cortisol response of fish at any of these metal concentrations which have induced perturbations on the immune system.

There is a need for a comprehensive approach to evaluate the effects of a single toxicant on several of the closely related aspects of the immune system in order to delineate the immunotoxic properties of the toxicant concerned. The complexity of the immune system makes it difficult to evaluate the effects of chemicals on the immune system in any simplistic way.

1.3 Objectives of the Present Study

Cadmium and copper are amongst some of the important metal pollutants of the aquatic environment. The ability of both these metals to modulate the immune response of fish has come to light in recent years. Copper has very often been shown to predispose fish to infection, but the underlying mechanisms are largely unknown. On the other hand cadmium has so often produced contradictory results in mammalian immunotoxicology and from the studies carried out so far in fish immunotoxicology it appears to behave similarly. By evaluating the effects of these two metals on different aspects of the immune system making use of different models, it was hoped to arrive at a comprehensive account of the immunomodulatory effects of these two metals at sublethal levels.

The concentrations of cadmium and copper utilized in the present study were selected to be sublethal based on preliminary toxicity trials. Within the sublethal level a range of concentrations were evaluated to find the concentration which would induce significant effects. Three levels of cadmium 10, 25 and 50 μgl^{-1}, and copper 10, 25 and 50 μgl^{-1}, were evaluated for their effects on the immune response, protective immunity and susceptibility of carp, *C. carpio* to *I. multifiliis*. From this experiment it was hoped to chose a single concentration of the respective metal for the other experiments to follow.

The present study used three different models to evaluate the immunotoxic properties of sublethal levels of cadmium and copper in common carp, *C. carpio*. The two pathogens selected were the ciliate protozoan, *I. multifiliis* and the bacterial pathogen, *A. hydrophila*. These two pathogen models were used to evaluate the effects of cadmium and copper on aspects such as susceptibility to disease, protective immunity and antibody response of carp. Both of these pathogens are known to cause serious problems in freshwater fish.

I. multifiliis is a parasite of the integument and does not penetrate beyond the basement membrane of the epithelium. Carps develop immunity to this parasite (Hines and Spira, 1974a; Houghton, 1987) and alterations in the kinetics of the immune response and the protective immunity can be quantified. On the other hand, *A. hydrophila* is a systemic bacterial pathogen and the mechanisms of non-specific and specific immunity, and even the mechanisms of protective immunity, may be different from that against *I. multifiliis*.

Despite the fact that carp become immune, the cellular requirements for optimal expression of immunity to *I. multifiliis* is uncertain. On the other hand *A. hydrophila*

is regarded to have both thymus-dependent (TD) and thymus-independent (TI) components (Lamers *et al.*, 1985). The differences between these two pathogens from the point of view of the disease and the immune response was hoped to reveal any of the possible immunotoxic effects that cadmium and copper would have in carp.

It is often difficult to explain the underlying mechanism of general immunosuppression produced by chemical toxicants. However, use of standard antigens of known cellular requirements and immunokinetics has enabled mammalian immunotoxicologists to overcome this problem to some extent. Sheep red blood cell (SRBC) is a putative thymus dependent antigen in fish. Optimal expression of humoral immunity to this putative thymus dependent antigen would require the cooperation and interaction of B and T-like lymphocytes and macrophages. Any interference by the metals in the immune response of fish to this antigen would implicate the involvement of any one or all the three cell components required for the optimal response to this antigen. By using this antigen, it was hoped to gain better insight into the mechanism on any of the possible immunosuppressive effects these metals would have in fish.

Lymphocytes are central to the immune response in vertebrates. *In vitro* lymphocyte proliferation assays are ideal tools to evaluate the effects of chemical pollutants and this is being successfully used in screening of toxic chemicals in mammalian immunotoxicolgy. Evaluating the functional ability of lymphocytes collected from fish exposed to metals would give a better correlate of the *in vivo* effects. By evaluating the *in vitro* and *in vivo* effects of cadmium and copper on the proliferative ability of lymphocytes it was hoped to gain a better understanding of any of the possible effects these metals could have on the fish immune system.

Most chemical toxicants which induce immunosuppression are thought to operate at least partially through the components of the stress response such as corticosteroids. Examining the stress response (cortisol) of carp to cadmium and copper would give indications on processess such as acclimation and exhaustion and would help to understand the relationship between pollutant induced stress response and any of the possible associated effects such as altered disease resistance and immune response.

In taking this approach it is hoped to understand the effects of sublethal levels of cadmium and copper on the disease resistance and immune response of carp more precisely.

Table 1.1 Summary of representative lethal toxicity values of cadmium for different fish species

Fish	Hardness (mgl^{-1} $Caco_3$)	Duration (Days)	LC50 (μgl^{-1})	Reference
fhm	200	4	7200	Pickering and Gast, 1972
bg	200	4	20,400	Eaton, 1974
bg	18	4	2300	Bishop and McIntosh, 1981
zf	160	2	4200	Canton and Slooff, 1982
carp	55	4	240	Rhewoldt *et al.*, 1972
carp		4	480	Abel and Papoutsoglou, 1986
gf	soft	4	2130	McCarty *et al.*, 1977
gf	hard	4	46,800	McCarty *et al.*, 1977
bt	47	4	5080	Holcombe *et al.*, 1983
rbt	125	7	300	Roch and Maly, 1979
rbt	125	10	30-100	Roch and Maly, 1979
rbt	82	4	32	Majewski and Giles, 1981
rbt	82	9	30	Majewski and Giles, 1981

As=Atlantic salmon (*Salmo salar* L.),

bc=brook char (*Salvelinus fontinalis* Mitchill),

bg=bluegills (*Lepomis macrochirus* Rafinesque),

carp=common carp (*Cyprinus carpio* L.),

fhm=fathead minnow (*Pimephales promelas* Rafinesque),

gf=goldfish (*Carassius auratus* L.),

rbt=rainbow trout (*Salmo gairdneri* Richardson),

zf=zebra fish (*Brachydanio rerio*),

Table 1.2 Summary of representative lethal toxicity values of copper for different fish species

Fish	Hardness (mgl^{-1} $Caco_3$)	Duration (Days)	LC50 (μgl^{-1})	References
bg	45	4	1100	Benoit, 1975
fhm	200	4	460	Pickering *et al.*, 1977
fhm	200	4	430	Mount, 1968
fhm	31	4	75	Mount and Stephan, 1969
zf	130	4	240	Anderson *et al.*, 1978
zf	300	4	670	Anderson *et al.*, 1978
carp	53	2	1000	Rehwoldt *et al.*, 1971
carp	53	4	810	Rehwoldt *et al.*, 1971
bc	45	4	100	McKim and Benoit, 1971
rbt	374	6	340	Dixon and Sprague, 1981
rbt	360	4	720	Howarth and Sprague, 1978
As	14	4	25	Zitco *et al.*, 1973

CHAPTER 2

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

The common materials used and methods followed during the course of the present study in different experiments are described below. Materials and methods specific to individual experiments are outlined under the relevant chapters.

2.1 Fish

Common carp, *Cyprinus carpio* L. was used during the course of this investigation and were procured from carp hatcheries in England. The fish were normally quarantined for two weeks during which time they were screened for any parasitic or bacterial disease. Fish which were considered as healthy were transferred to the holding system in the Tropical aquarium facility of the Institute of Aquaculture for maintenance. Fish to be used for experiments with *Ichthyophthirius multifiliis* were screened specifically to make sure that they were not previously exposed to the parasite in question. This was achieved either by challenging a representative group with a low level of infection and/or by measuring the tomite immobilization titre in the serum of the fish. Batches of fish found to be resistant to *I. multifiliis* infection were not used for any parasite-metal interaction studies. As a normal practice, always small fish of 40-60 mm were acquired from the suppliers and were later grown to the required size in the Tropical aquarium facility of the Institute of Aquaculture.

2.2 Maintenance System

The fish were maintained in the recirculatory system in the tropical aquarium facility of the Institute of Aquaculture. The recirculatory-system comprised of large square fish holding tanks (250l), which individually, received water at a rate of 2l/min through delivery pipes from the header tank. The water quality was

maintained by an extensive system of biofiltration. The outflows from individual tanks were chaneled to sump tanks through a series of settling tanks, interdigitated with biofilter tanks which contained biofilter rings. A submersible pump was used to pump the water from the sump tank to the header tank from which water was fed into the fish holding tanks by gravity. The efficency of the biofilters was regularly monitored by measuring NH_3-N and No_2-N in the tank water. The system was cleaned and fresh water added at regular intervals. The water temperature was regulated using thermostatically controlled heaters in the header tank. The temperature was always maintained between 25.5 and 27°C. A 12 hr light and dark regime was maintained using electronically preset lights. Individual fish holding tanks were aerated using diffusers. Additional aeration was provided in the header and sump tanks to cope with the oxygen demand generated by the bacterial activity in the biofilters. The oxygen level in the tanks was always maintained above 6 mgl-1. The fish were fed daily with trout pellet (Ewos Bakers Omega, no.3; protein content-47%)

2.3 Design and Layout of Experimental System

The experimental system was a simple flow-through system (Figure 1.1) which was built in the Tropical aquarium facility of the Institute of Aquaculture. Sixteen glass tanks (20l) were set up in two rows of 8 each. Water from the header tank was taken by a main delivery pipe running in between the two rows of tanks. The outlets from individual tanks were connected to external stand-pipes, which were made use to maintain the minimum water level in tanks, normally 15l. The water flow to each tank ranged between 100 and 150 ml/min, depending on the nature of the experiment and the concentration of the toxicant used.

Cold water from the mains was collected in a reservoir tank, which was connected

in turn to the sump tank. The water from the sump tank was pumped to the header tank using a submersible pump. The overflow from the header tank was fed back to the sump tank. Maintaining a continuous overflow from the header tank ensured a constant head in the header tank to enable even water flow to all the tanks. Inflow from the mains to the reservoir tank was always adjusted to slightly above the total volume of water flowing out through all the experimental tanks at any one given time. The temperature of the incoming water from the mains normally ranged from 5 to 8°C, this was raised to 26±1°C using 10-15 thermostatically regulated immersion heaters.

The water flow through the system was always maintained to achieve the recommended flow rate of 2l/g fish/day. By maintaining a flow rate of 100-150 ml/min, 90% replacement time in individual tanks was always within 7-8 hr. This was very much within the recommended replacement time in a flow-through toxicity system (Sprague, 1976).

2.4 Metal Dosing System

Metal solution was delivered to individual experimental tanks using a 16-channel fixed speed Watson-Marlow Peristaltic pump. Siliconized tubings of known diameter connected to individual channels were used to deliver the exact volume of the toxicant. These short tubings were placed in such a fashion in the individual channel frames, such that, during the rotation of the 8 rollers of the pump, the tubing would be pressed in between the roller and the channel frame at regular intervals. The speed of the pump and the diameter of the tubing would determine the flow rate. The free end of the tubing from one side was connected to the toxicant source and the other side of the tubing to the individual tank using long flexible tubings. The connections were secured with small plastic connectors and nipples. The outlet from

the pump carrying the toxicant was connected to the water inlet pipe of the individual tank in such a fashion that the toxicant would mix with the inflowing water just before entering the tank. This ensured even mixing of the toxicant. A toxicant flow rate of between 1 and 1.5 ml/min was maintained to achieve the required nominal concentration in the tanks. The layout of inlet and outlet in the tanks, aeration and the movement of fish within the tank ensured that the toxicant was mixed evenly and there were no hot or cold spots.

Chloride salts of the metals ($CdCl_2$ $2\frac{1}{2}H_20$ and $CuCl_2$ $2H_20$; BDH Chemicals Ltd.) were used to prepare the metal stock solution. The stock solution of required concentration was made up in distilled water. Normally 10l of stock solution was made at a time. Plastic buckets (10l volume) were used to keep the stock solution.

2.5 Monitoring Metal Concentration

The nominal concentrations of the metals used in the present study were maintained as precisely as possible by regulating the concentration of the metal in the stock solution, toxicant flow rate per minute into individual tank and the water flow rate per minute. The actual concentration of the metal in the tank was monitored regularly with each experiment. Samples of water taken at regular intervals from the individual tanks during each experiment were acidified (1% HNo_3) and analysed using an atomic absorption spectrophotometry. The actual concentration of the metal in the water was calculated from a standard curve. The actual concentrations of the respective metals in the tanks did not differ significantly from the nominal concentrations. The actual concentrations of the metal in the tanks were always within 10% of the nominal concentrations. The nominal and the actual recorded concentrations of the metals in the tanks under each experiment is given in Appendix 1 (cadmium) and Appendix 2 (copper). The concentrations referred to in

individual chapters represent the nominal concentration of the particular metal.

2.6 Monitoring Water Quality Parameters

The water quality parameters temperature, oxygen, pH, alkalinity and hardness were monitored regularly using the standard methods. The mean values of these parameters during the course of this investigation are given in the Appendix 3. Parameters such as NH_3-N, No_3-N, No_2-N were not analysed regularly, primarily because the system was a flow-through system and there was little possibility of any accumulation of metabolites.

2.7 Acclimatization of Experimental Fish

Fish were normally removed from the recirculatory maintenance system, weighed, measured and transferred to the experimental flow-through system where they were held for a minimum of 7 days before the commencement of any experiment. This procedure of acclimatization remained the same throughout the study.

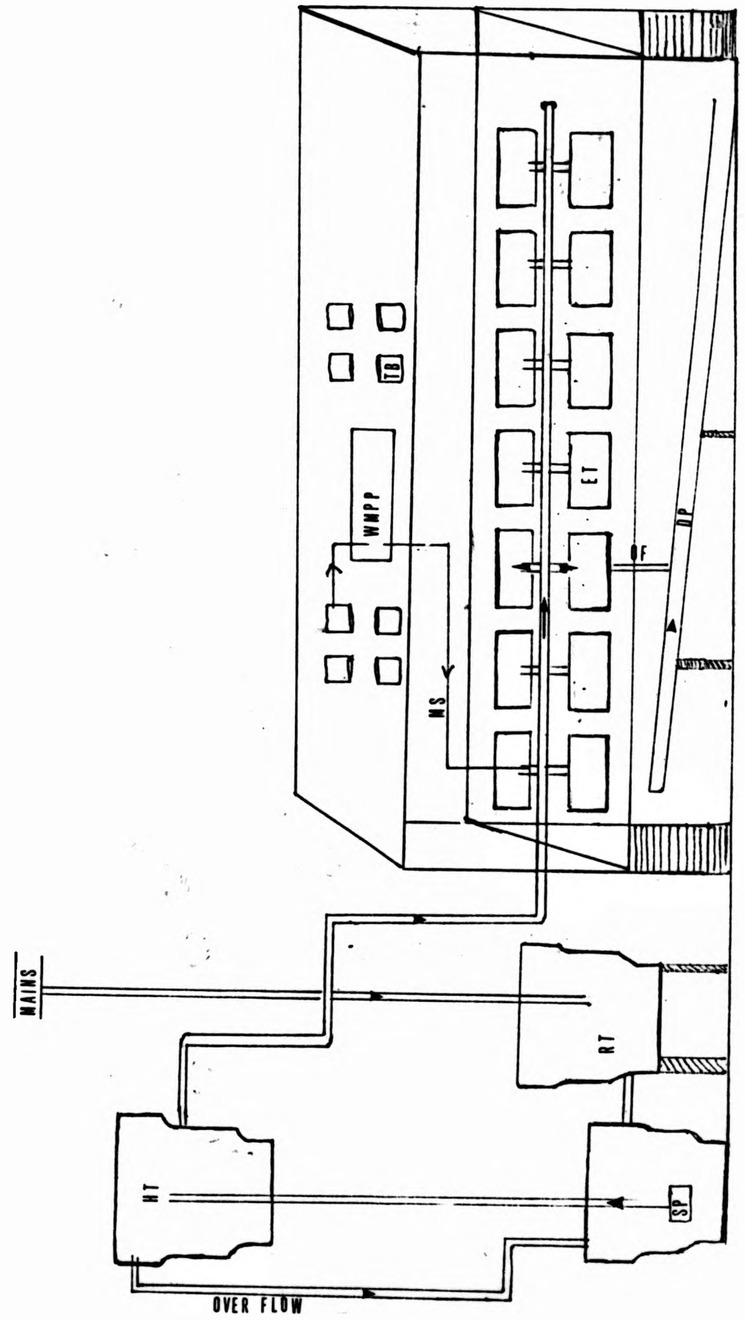
2.8 Statistical Analysis

The results were analysed using Minitab on a VAX mainframe. All the data were analysed by Student's t-test for differences between duplicate tanks. One way Analysis of Variance was used for group comparisons. Two-way Analysis of Variance was used wherever necessary to find the interaction effect. The Two-sample t-test was used to compare the means between two treatments. If the $P<0.05$, the difference was considered to be significant.

The descriptive statistics were estimated from untransformed values. Proportional data such as percent mortality were routinely transformed to arc-sin equivalents and the comparisons were made on the transformed data.

Antibody titres were normally recorded as the reciprocal of the highest dilution giving a positive agglutination. The antibody titres were expressed as $-\log^2$ titre + 1.

Figure 2.1 Schematic diagram of the flow-through system (not to scale)



RT= Reservoir Tank

SP= Submersible Pump

HT= Header Tank

ET= Experimental Tank

WMPP= Watson-Marlow Peristaltic Pump

TB=Toxicant Bucket

MS=Metal Solution Delivery Tubes

OF=Out Flow

DP=Drain Pipe

CHAPTER 3

EFFECTS OF CADMIUM AND COPPER, AT SUBLETHAL LEVELS, ON THE SUSCEPTIBILITY, PROTECTIVE IMMUNITY AND IMMUNE RESPONSE OF COMMON CARP, *Cyprinus carpio* TO THE CILIATE PROTOZOAN, *Ichthyophthirius multifiliis* (FOUQUET, 1876).

3.1 INTRODUCTION

The potential ability of various toxic agents, especially heavy metals, to alter the susceptibility to disease and modulate the immune response in fish, has been reviewed and discussed in Chapter 1. Much of the laboratory evidence relating heavy metals to altered disease susceptibility and immune response in fish, comes from studies using bacterial or viral pathogens (Rodsaether *et al.*, 1977; Hetric *et al.*, 1979; Knittel, 1981; Snarski, 1982; Baker *et al.*, 1983; MacFarlane *et al.*, 1986). The altered disease susceptibility of fish is very often attributed to the immunosuppressive effects of the metals concerned. The ready availability of standardised and reproducible infection and immunization procedures with certain bacterial and viral pathogens, is the most probable explanation for the majority of studies being confined to these pathogens.

Apart from a few field observations, there is very little information available on the effects of pollutants on the disease susceptibility and immune response of fish to parasitic infections. There could be several reasons for the lack of laboratory studies in this area. The maintenance and culture of the majority of fish parasites poses several practical problems, since many have indirect life cycles or life cycles involving various stages in different environments. Standardising reproducible infection procedures, has been sucessful with only a few parasites. In addition, monitoring alterations in the immune response of fish to parasites has not been easy as the immune response of fish to parasites has not been well documented.

The pollution-parasite interrelationship is very interesting because of its nature. The relationship is not simple, and in essence involves a double edged phenomenon. Pollutant stress may result in an increase or in some instances, a decrease in the

prevalence of certain parasites, or alternatively parasites may decrease the host resistance to toxicants.

From the toxicological view point, some studies have been carried out on the influence of parasitisation on the host resistance to pollutants. Perevozchenko and Davydov (1974) found juvenile carp parasitised by the intestinal cestode, *Bothriocephalus gowkongensis*, to be more susceptible to DDT poisoning than non-parasitised individuals. Boyce and Yamada (1977) observed sock-eye salmon (*Onchorhynchus nerka*) smolts with pre-existing parasitisation by the intestinal cestode, *Eubothrium salvelini*, to be more susceptible to zinc toxicity than uninfected smolts. Pascoe and Cram (1977) made similar observations on the stickleback, *Gasterosteus aculeatus*, infected with the larval cestode, *Schistocephalus solidus*, exposed to cadmium. In all these studies, only the parasite load in the host fish was taken as the criterion, to assess the influence of the pollutants on the host resistance.

Much of the evidence relating the influence of toxicants on parasite infection, comes from field studies and circumstantial evidence. Increased prevalence of *Epistylis* in large mouth bass, *Micropterus cylindratus* (Eure and Esch, 1974) and larval trematodes in centrarchid fish (Aho *et al.*, 1976) have been attributed to thermal pollution. Dabrowska (1974) implicated river pollution in increased parasite prevalence in fish in Poland. In an interesting study, Sakanari *et al.* (1984) have looked into the sublethal effects of zinc and benzene on striped bass, *Morone saxatilis*, infected with larval anisakis and indicated the differential effects of pollutants and parasites on the various physiological parameters of the fish.

The available information on the altered susceptibility of fish under stress to parasitic infection, based on laboratory infection, comes from two studies. The work

of Ewing *et al.* (1982) was the first in this direction. They examined the susceptibility of channel catfish, *Ictalurus punctatus* to *I.multifiliis* infection following exposure to copper, and reported a weak, though statistically significant correlation between exposure concentration (32 to 3200 $\mu g l^{-1}$) and infection. Chronic exposure to PCB's was shown to enhance the susceptibility of juvenile bass to infection by the protozoan, *I. multifiliis*, but the increase was not in a dose-dependent manner (Johansen *et al.*, 1985). Recently, Woo *et al.* (1987) have investigated the effects of implanted cortisol, on the susceptibility of rainbow trout, *Salmo gairdneri*, to *Cryptobia salmonisitica* in experimental infections. They observed cortisol implanted fish (70, 140 and 210 $\mu g\ g^{-1}$ body weight) to have significantly higher parasitemias and lower antibody titres compared with the controls.

Stress related immunosuppression in fish to parasites has only recently come under study. Houghton and Matthews (1986) and Houghton (1987) have demonstrated immunosuppression following intraperitoneal administration of corticosteroid (triamcinolone acetonide) and hydrocortisone (21-hemisuccinate) in juvenile carp to *I. multifiliis* infection. Interestingly, the observed immunosuppression was not associated with any significant decline in the "anti-ich" antibody titre.

It is clear that the available information in this area of pollution-parasite interaction is limited. There are several gaps in our knowledge on the intricate relationship between pollution stress and parasitic infection and, in turn, its effects on the host in terms of susceptibility, immune response, acquired immunity and the sensitivity of parasitised host to pollutants. It is also evident from the literature, that there are no specific concerted studies, directed towards looking into the various possible interactions which would be involved in a single toxicant-parasite interaction on a

fish host.

The present study was aimed at looking into the effects of cadmium and copper at sublethal levels on the disease susceptibility, protective immunity and immune response of carp to the protozoan ciliate, *Ichthyophthirius multifiliis*. *I. multifiliis*, the causative agent of white spot disease in fresh water fish was selected as the model to examine the effects of cadmium and copper in common carp *C. carpio*. The reasons for selecting this parasite are outlined below.

I. multifiliis is a pathogenic, holotrichous ciliate which invades the epithelium of skin and gills of fresh water fish and is considered by some to be one of the most serious pathogenic parasites of fresh water fish (Bauer, 1958; Meyer, 1969; Pearson, 1970; Hines and Spira, 1973a). It is cosmopolitan in distribution and accounts for high mortality and economic loss in wild, cultured and ornamental fish species (Chappel and Owen, 1969; Paperna, 1972; Nigrelli *et al.*, 1976; Hoffman, 1978).

The life-cycle of the parasite is simple, direct and the duration of it is dependent on temperature. It takes only around 4-6 days to complete the life cycle at 27°C. Free swimming, invasive tomites penetrate the epithelium of the gill and skin quickly and feed during a period of development the duration of which is dependent on temperature, on the fish host. At maturation the adult trophont leaves the host and drops off into the surrounding environment where it secretes a cyst wall and undergoes repeated mitotic division, resulting in a new generation of tomites. The approximate time spent by the different stages in different environments is shown in the life-cycle diagram (Figure 3.1).

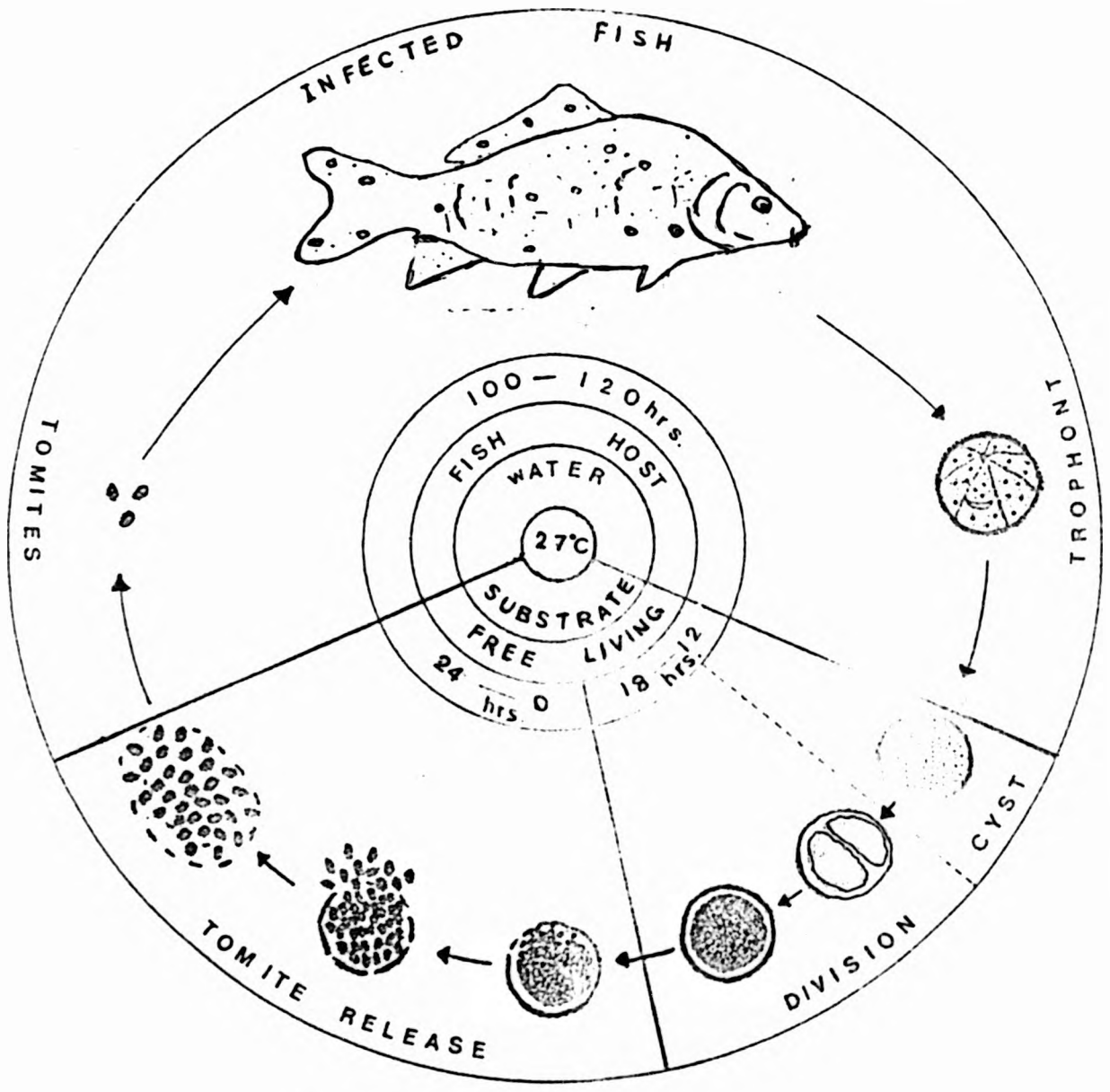


Figure 3.1 The life cycle of *Ichthyophthirius multifiliis*. (Modified from Subashinge, 1986 based on authors personal observations).

Over the years, experimental infection procedures, have been modified and improved. Addition of infected fish to a population of noninfected fish (Hines and Spira, 1973a; Goven *et al.*, 1980), addition of a known number of trophonts to a population of fish (Areerat, 1974; Subasinghe, 1982), and the exposure of test fish to the tomites released by a known number of trophonts (Beckert, 1976), were some of the earlier infection procedures used. In order to overcome some of the shortcomings of the previous infection procedures, new methods have been developed and are being followed by current workers (Dickerson *et al.*, 1981; Houghton and Matthews, 1986; Subasinghe and Sommerville, 1986; Mohan and Sommerville, 1989a). In all these methods, the fish are exposed to a known number of tomites (the invasive stage of the parasite), which are obtained by incubating mature trophonts under controlled conditions.

The ability of fish to acquire protective immunity to *I. multifiliis* has been demonstrated in a wide range of fish species; including rainbow trout (Wahli and Meier, 1985), carp (Hines and Spira, 1974b; Houghton, 1987), catfishes (Beckert and Allison, 1964) and tilapias (Subasinghe, 1986). The possibility of quantifying the immune response of fish to *I. multifiliis* was reported by Hines and Spira (1974b), Wahli and Meier (1985) and Subasinghe (1986) by demonstrating the ability of immune fish serum to immobilize the trophont stage of the parasite. Recently, sera from immune fish have been shown to induce tomite agglutination or immobilization. This tomite agglutination has been shown to be specific, in carp (Houghton, 1977; Mohan and Sommerville, 1989b) and in catfish (Clark *et al.*, 1987; 1988).

Further studies on the immune response of fish to *I. multifiliis* have shown the possibility of involvement of both the cellular and humoral factors. Graves *et al.*

(1985a; b) have demonstrated mobilization and activation of nonspecific cytotoxic cells (NCC) in channel catfish infected with *I. multifiliis*. They have also reported that these NCC's are capable of killing the closely related *Tetrahymena pyriformis*. Clark *et al.* (1988) have recently reported that cilia are the major antigens of *I. multifiliis* and have revealed the presence of anti-ciliary antibodies in a number of infected fish. Cross and Matthews (1989a) on the basis of their observation made on localised leucocyte response in carp infected with *I. multifiliis*, have suggested the involvement of the cellular immune response.

These features make *I. multifiliis* an ideal parasite model to investigate the possible pollution-parasite interactions on a host fish's immune system. To summarize;

1. The parasite has a short life cycle of 4-6 days at 27°C.
2. The infection procedure can be standardised and reproducible results can be obtained between successive infections.
3. Fish can be immunized to *I. multifiliis*.
4. Alterations in the immune response can be easily monitored by challenge infections or by measuring serum tomite agglutination titre.

Different experiments were designed with the aim to gain insight into the possible toxicant-parasite interactions on a host fish's immune system, with the following questions in mind:

1. Would exposure of naive carp to sublethal levels of cadmium or copper for 10 days prior to experimental infection have any effect on the susceptibility and infection pattern?
2. What effects would exposure to sublethal levels of cadmium and copper have on

the ability of immune fish to mount a protective response to a challenge infection?

3. Will carp held continuously in a low infectious environment in the presence of metals, be able to acquire resistance to the parasite?

4. Can sublethal levels of metals influence or modulate the tomite agglutination titre dynamics, when natural immunization and metal exposure are simultaneous?

5. The final objective was to examine the interaction from a reverse view point and find out to what extent parasitisation can influence the resistance of fish to metal toxicity?

3.2 MATERIALS AND METHODS

3.2.1 Maintenance of the Parasite, *Ichthophthirius multifiliis*

An infected ornamental gold fish (*Carassius auratus* L.) obtained from a local aquarist was initially used as the source of the parasite. The infection was passed on to small carp by direct exposure. From this initial parasite stock, a single trophont was isolated and used to propogate the parasite by infecting small fish by direct exposure, and maintained by continued exposure of fish to fish which were already infected. In this way the parasite was maintained for as long as 10 months. By closely monitoring the life cycle, the parasite was maintained easily in a flow through system. This was achieved by turning the water flow off for 18 to 24 hours to coincide with the reproductive division stage of the parasite, during which time the tomite number was maximum in the water. New fish were introduced during this time to enable infection to take place. This way the risk of accumulation of metabolites in the tank was minimised.

3.2.2 Collection of Tomites

The procedure used to produce tomites in the laboratory was as follows: Heavily infected fish with mature parasites were held in a hand net placed inside a circular transparent perspex tank containing dechlorinated water. The subsequent movement of the fish released hundreds of mature trophonts and this process normally took less than two minutes. The trophonts were then carefully collected using a Pasteur pipette, transferred to a measuring cylinder, wherein, after some time, the trophonts would settle down at the bottom. The supernatant water was siphoned off and the trophonts were transferred to small petri dishes with a known volume of well aerated, dechlorinated water and then incubated at 27°C for 18 to 24 hr for tomites to develop. Incubating large numbers of trophonts in a relatively small volume of

water (usually 10ml) ensured easy collection of the tomites which were found at a very high density. The resulting tomite suspension was then transferred to a graduated cylinder and debris allowed to settle. After 30 minutes the swimming tomites were removed by siphoning. The number of tomites per ml was computed by counting 5 aliquots using a Sedgewick-Rafter plankton counting chamber. Neutral red (1%) was used to stain the tomites. This process of trophont collection, incubation, tomite harvesting and estimation of tomite number per ml, remained the same throughout the present study.

3.2.3 Infection Procedure

Experimental infections were always carried in separate tanks designated as infection tanks. The type of tank and the volume of water used for infection depended mainly on the number and size of the fish to be infected. Fish from all the treatments within a single trial were marked by fin clipping and exposed to tomites for 30 min to 1 hr in a single tank. The exposure duration schedule was based on preliminary trials. This ensured an even exposure to tomites of fishes from all the treatments, as opposed to infection in separate tanks. Fish were removed to their respective tanks in the flow-through system after tomite challenge.

Based on preliminary trials, a strategy was adopted in which mild to moderate challenge levels in all the experimental infections were used. This was done for two reasons; using higher challenge levels would result in very heavy infection, especially in naive fish, making it almost impossible to enumerate accurately the parasite load. Secondly, in all the experiments, alterations, if any, in susceptibility and immune response were quantified by means of parasite intensity rather than parasite induced mortality.

3.2.4 Enumeration the Parasite Intensity

Fish were sacrificed on the 3rd and 4th day after the infection. Parasites present on all the fins, body surface and gills (4 gills) from one side were counted. All the fins were excised and placed on clean glass slides and the parasites present on the different fins were counted using a stereo dissecting microscope. In the case of gills, the parasites were carefully scraped from individual gill filaments and counted under a stereo-dissecting microscope. By choosing to count on day 3 and 4 after the infection, the chance of parasites emerging from the host epithelium and dropping off from the fish was eliminated. The results were expressed as Mean Parasite Intensity per fish. This was obtained by dividing the total number of parasites in a particular site (such as gills, body surface, fins) by the number of infected fish.

3.2.5 Immunization Procedure

Fish were immunized by exposure to tomites at sublethal levels of 10-15 tomites ml^{-1} (approximately 1000-2000 tomites per fish) on three separate occasions at 7 day intervals. From days 4-7 following each exposure, natural reinfection was prevented by transferring the fish to aquaria with clean water at 12-18 hr intervals. Fish were sampled randomly during the course of the immunization schedule and their serum agglutination titre followed routinely. Twenty one days after the initial tomite exposure, fish were challenged with a high level of approximately 10,000 tomites per fish at a concentration of 100 tomites ml^{-1} to determine whether immunity had been established. At each immunization exposure stage, a batch of 5 naive fish were also exposed to the tomites to check for the viability of tomites. Naive fish were also used to confirm the potentially lethal dose on final challenge and, at the same time, to check the viability of tomites. Experimental fish (immunized) were found to be resistant to the final challenge. Naive controls, which had not been exposed previously, were heavily infected.

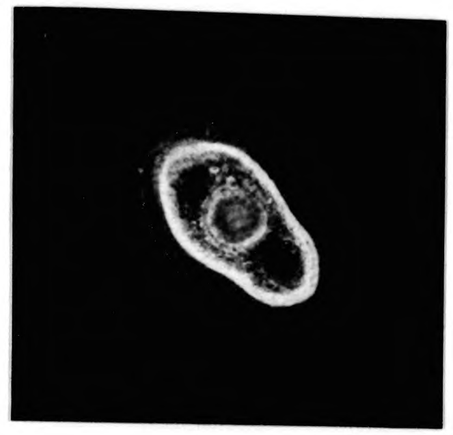
3.2.6 Agglutination /Immobilization Assay

Fish were anaesthetised individually with 100 ppm Benzocaine (ethyl P-aminobenzoate, Sigma) and blood from the caudal vein was collected in heparinized 1ml plastic syringes with 27 gauge needle. It was allowed to clot for an hour at room temperature and left at 4°C overnight for clot retraction. Serum was separated after spinning the blood at 6500 rpm for 5 minutes. Serum was heat inactivated at 45-50°C for 30 mins prior to being used for antibody titrations.

Doubling dilutions of serum was made in sterile phosphate buffered saline (PBS) in 96-well microtitre plates (Flow Laboratories, Scotland). Each well had 100 µl of diluted serum. Approximately 1000-2000 tomites in a volume of 10-20 µl were then added to each well and the plates were incubated at room temperature. The plates were read after 2 and 4 hours for agglutination using a stereo dissecting microscope. Tomite clumping/agglutination usually occurred in less than two hours. The various stages involved in the process of agglutination are shown in Plates 3.1a-f. The stage shown in Plate 3.1f was taken as the end point. With each assay a batch of sera collected from carp which had not been exposed to the parasite previously was used to check for non-specific agglutination. The titres were recorded as the reciprocal of the highest dilution giving positive agglutination. The results were expressed as Mean ± Standard Error of ($-\log_2$ titre + 1). This meant that undiluted serum giving a positive titre had a value of 1 (but not 0).

3.2.7 Preparation of "Ich" Antigen

Tomite suspensions of high density were collected from a series of incubation systems into 15 ml sterile plastic centrifuge tubes. The tomites were killed by adding 0.1% formalin and this was allowed to stand in a refrigerator at 4°C for 1 hr to accelarate the sedimentation process. The tomites were pelleted by spinning the



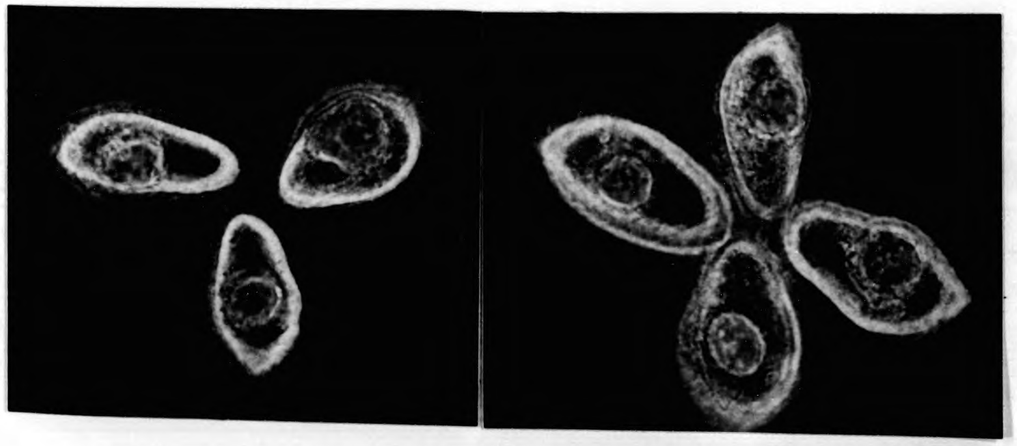
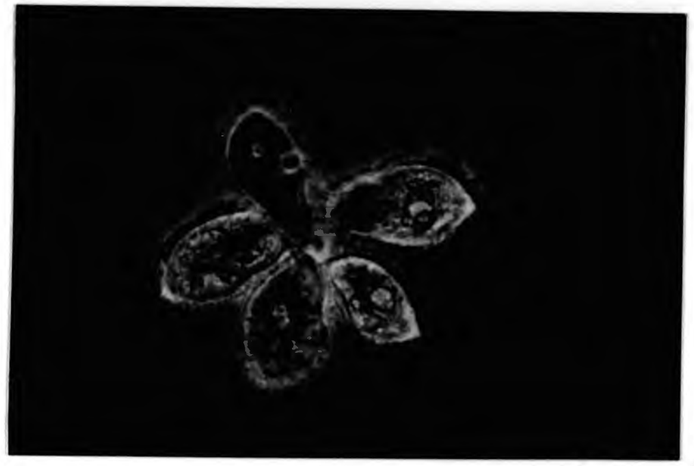
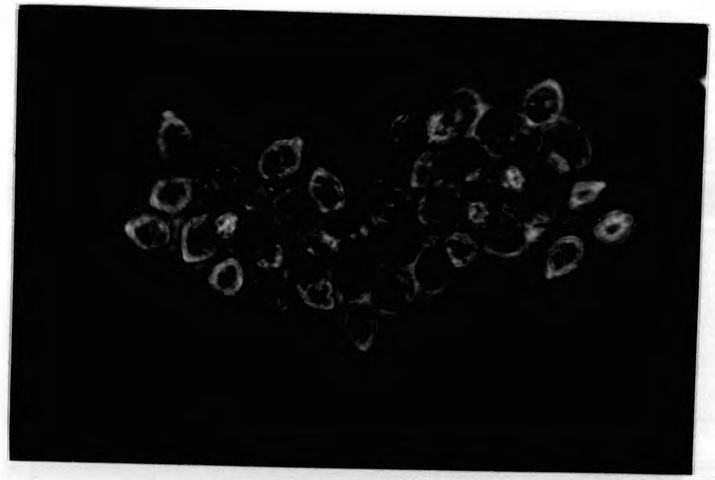
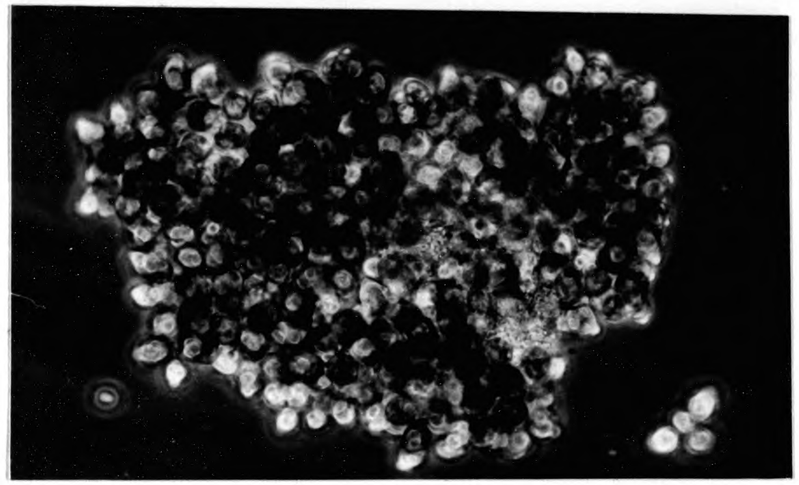
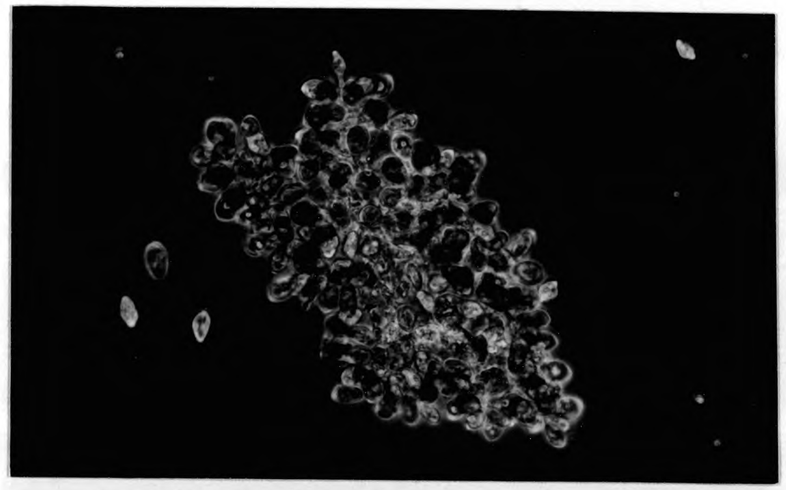


Plate 3.1 Photomicrographs showing the sequential stages in the tomite agglutination process. (A-G)= agglutination of tomites in immune fish serum, H= agglutination of tomite in rabbit antiserum. (A-E, 400X; E, 200X; F-G, 100X).









centrifuge tubes at 2000 rpm for 10 min. Killed tomites were usually found to sediment as white pellets. The supernatant water was discarded and the resulting pellet washed twice in sterile phosphate buffered saline (PBS). After the final wash the pellets were resuspended in 1ml sterile PBS in small plastic vials and stored at -70°C till required for use.

Pooled tomite pellets were resuspended in 5 ml sterile PBS and ultrasonicated for 2 min using an Ultrasonicator. This crude sonicated tomite preparation was used to raise rabbit antisera to "ich".

3.2.8 Rabbit Immunization

New Zealand white rabbits weighing approximately 2kg were used to raise antisera to "ich". Primary injection of "ich" antigen was given with Freund's Complete Adjuant (FCA) in the ratio of 1:1 (0.5ml "ich" antigen with 0.5 ml FCA). The volume injected was 1 ml, having equal volumes of well mixed "ich" antigen and FCA. The antigen was adminstered subcutaneously. Secondary injection of the same volume but with Freund's Incomplete Adjuant was given 4 weeks later. The rabbit was test bled 1 week following the secondary injection and tested for "ich" antibody titre. As the titre was low, an intravenous booster of antigen was given in saline. The rabbit was bled 2 weeks after the booster.

The rabbit antisera was used to cross check the specificity of tomite agglutination. The type of tomite agglutination observed with the rabbit antiserum (Plate 3.1g) was very similar to that seen with immune fish serum.

3.2.9 Experimental Protocol

Five types of experiments were designed to investigate the various possible

interactions between *I. multifiliis* infection and sublethal metal toxicity (cadmium and copper) on carp, *C. carpio*. Only the individual effects of the metals were studied in the present investigation. Cadmium and copper experiments were run at different times. The experimental protocol and procedure used were the same for both the metals.

Experiment 1 <u>The effects of metal exposure on the susceptibility of naive carp to infection</u>

This experiment was designed to look into the effects of exposure of naive carp, *C. carpio* to sublethal levels of either cadmium or copper on the susceptibility to experimental infection with *I. multifiliis*. Ten fish (11.05 ± 1.10 cm; 19.79 ± 3.67 g) per tank per treatment, in duplicate, were exposed for 10 days to one of the metals in a flow through system as described in Chapter 2 (Section 2.4). The nominal cadmium concentrations employed were 0 (control), 10, 25 and 50 μgl^{-1}. The same concentrations were used in copper experiments also. At the end of the exposure period the fish from the different treatment tanks were marked and removed to a single tank for challenge infection (6000 tomites/fish, 30 tomites ml^{-1} for 60 mins). Fish from the duplicate tanks were infected in a duplicate infecting tank. Fish were returned to their respective tanks after the infection. Dosing of metals to the system was discontinued after the infection. Fish were sacrificed on day 3 and 4 after infection and the parasite number in different parts of the body was enumerated.

Representative gill samples were taken on day 3 after the infection for histological studies. The samples were fixed in 10% neutral buffered formalin. The processing, sectioning and staining procedures followed are given in Appendix 4, 5 and 6.

Experiment 2 <u>The effects of metal exposure on the protective immunity of previously immunized carp</u>

Immunized fish with confirmed immunity to *I. multifiliis* according to the standard immunization exposure procedure described in Section 3.2.5 were used in this series of experiments. Random blood samples were collected from 10 immunized fish to measure the tomite agglutination titre before exposure to the metals. The experiments were designed basically to investigate the ability of immune fish to mount a protective immune response following exposure to sublethal levels of cadmium or copper. Immune fish (11.32 ± 0.99 cm; 19.29 ± 3.17 g) were divided randomly into four groups of 10 each. Three groups were exposed to 3 sublethal concentrations of cadmium or copper and the remaining group was treated as a control. Duplicate sets of experiments were run side by side. The levels of metal used were the same as those in Experiment 1. After 10 days of exposure of immune fish to metals in the flow-through system, the fish were marked and removed to a single tank for the challenge infection (8000 tomites/fish; 40 tomites ml^{-1} for 60 mins). At this stage, an additional batch of 10 naive fish were also introduced to the infection tank to check the viability of tomites. After the infection the fish were returned to their tanks and the delivery of metals discontinued. The parasite intensity was enumerated on day 3 and 4 of infection as described in Section 3.2.4.

On day 3 following the infection blood samples were also collected from 3 fish per treatment from duplicate tanks to measure the tomite agglutination titre, before sacrificing the fish for parasite enumeration.

Gill tissue samples were taken from the immune fish before exposure to the metals, and on day 3 after the infection to asses the histopathological changes. The samples

were fixed and processed according the methods given in the Appendix 4.

Experiment 3 <u>The effect of metals on the ability of carp to acquire resistance to *I. multifiliis*</u>

In this experiment, infection and exposure to metals was simultaneous. Four groups of 10 fish (6.09 ± 0.59 cm; 4.13 ± 0.85 g) each were infected (1500 tomites/fish; 15 tomites ml^{-1} for 30 mins) five times starting from day 1 and later at regular intervals of 7 days and exposed continuously, starting from day 1, to either cadmium or copper for a duration of 35 days. Once again the metal concentrations used were the same as in the previous experiments. In this particular experiment there was a slight deviation in the infection procedure. Fish were removed from their metal exposure tanks for an hour to carry out the infection and later returned to their respective tanks. The challenge levels used were low compared to the previous 2 experiments. Reinfection in the control tanks was prevented by transferring the fish to clean aquaria at 12-18 hr intervals between days 5 and 7 following each infection. Reinfection was not taking place in metal treatment tanks since the trophonts could not encyst and divide.

The aim of this study was to see whether cadmium or copper would have any influence on the ability of carp to acquire resistance when held continuously in an infectious environment. At regular intervals following each experimental infection, fish from all the treatments were checked with the aid of either a magnifying glass or stereo dissecting microscope for the percentage prevalence and intensity of infection. The intensity of infection was assessed and scores given on an arbitary scale, ranging from 0 (no infection), through 1 (mild), 2 (moderate), 3 (heavy), to 4 (very severe). At each infection stage between 5-10 non-immune fish, were also

exposed to the tomites to confirm the viability of tomites.

Experiment 4 The effect of metals on the kinetics of the tomite agglutination titre in carp

The design of this experiment was based on the results of the previous experiment (Experiment 3). The infection and metal exposure protocol followed was similar to Experiment 3. Four groups of 12 fish each (10.91 ± 0.92 cm; 20.49 ± 2.87 g) in duplicate, were used in this experiment. The infection level used in this experiment was 2000 tomites/fish (15 tomites ml^{-1}) for 30 min. The objective of the present experiment was to follow the development of immunity by monitoring the kinetics of serum agglutination titre. To this effect 3 fish per treatment were sampled (as described in Section 3.2.6) at regular intervals of 7 days starting from day 7 after the first infection. Some fish had to be resampled for samples on days 28 and 35 as there were only 12 fish per treatment. There was at least 2 weeks time gap maintained between samplings in the case of those used for resampling. Serum agglutination titre of each sample was assayed on the following day of sampling according to the method detailed in Section 3.2.6. For statistical analysis, values from duplicate experiments were pooled.

Experiment 5 The influence of parasitic infection on the toxicity of metals to carp

The last study in this series looked at the interaction from a slightly different perspective. The aim here was to highlight the role of parasitic burden in determining the toxicity of cadmium or copper. The experiment differed from the previous experiments in that the concentrations of metals used were higher in addition to higher infection levels (tomites/fish).

Sixty fish (11.04 ± 1.02 cm; 19.10 ± 2.58 g) were infected (10000 tomites/fish; 60 tomites ml^{-1} for 30 min) and later divided into 6 groups of 10 each, and allocated to 6 tanks. Five groups were exposed to 5 different concentrations of either cadmium or copper and 1 group served as the control. Reinfection in control tanks was prevented by regularly moving the fish at 12-18 hr intervals between days 5 and 7. A second infection (same infection level as the first) was carried out on day 8. The fish were removed from their tanks for an hour for the infection. The mortality pattern was followed over a duration of 15 days, starting from the first day following first infection. The concentrations of metals were 0 (control), 10, 25, 50, 75, 100 μgl^{-1}. A separate control experiment with 5 fish per treatment was also run with just the metals for 15 days and the mortalities followed over that period. The results of the duplicate experiments were combined for the presentation of figures.

Gill samples were taken after infection at different time intervals so as to cover all the different phases of the infection cycle. This included samples just after infection (6-10 hr), day 2, day 5 and on day 9 (moribund fish after 2nd infection). The fixation, processing, sectioning and staining methods followed are found in Appendix 4.

3.3 RESULTS

The results presented here demonstrate the effects of sublethal levels of cadmium and copper individually, in influencing the susceptibility and modulating the immune response of carp, *C. carpio* to the protozoan ciliate, *I. multifiliis*. Cadmium and copper experiments were carried out separately, but in order to avoid repetition, the results of both the metals studied are presented together under the respective experimental headings. The nominal concentrations of metals chosen were sublethal and arrived at from preliminary trails carried out. The actual concentrations of both cadmium and copper in the experimental tanks as measured by atomic absorption spectrophotometry are given in the Appendix 1 and 2. For all presentation purposes, the nominal concentrations are used.

3.3.1 Experiment 1

The results presented here summarize the effects of exposure to sublethal levels of cadmium or copper on the susceptibility and infection pattern of naive carp to *I. multifiliis* experimental infection.

3.3.1.1 Cadmium

The mean parasite intensity (± SD) and the distribution pattern of the parasite in fish exposed to varying concentrations of cadmium for 10 days, prior to infection are illustrated in Figure 3.2. The high variation in parasite intensity, between fish within a treatment, are clearly reflected by the high standard deviation. In spite of this high variation, a very clear trend was evident from the intensity and site distribution pattern of the parasite in infected fish.

Considering the fins, a relationship between the surface area and parasite intensity

seems to exist (Figure 3.2a). Caudal fins in all the treatments had higher parasite number, followed by the dorsal fin. Parasite burden on the 3 paired fins, was relatively less and did not differ significantly ($P< 0.05$) between treatments. However, fish exposed to 50 μgl^{-1} cadmium had a significantly higher ($P< 0.01$) parasite number on the caudal and dorsal fins compared to the other treatments(Figure 3.2a). Fish exposed to lower levels (10 and 25 μgl^{-1}) of cadmium did not differ statistically from the controls. When all the fins are considered together, the 50 μgl^{-1} exposed fish had significantly ($P< 0.01$) higher parasite intensity than the rest of the treatments.

Of the different sites chosen for enumerating the parasite intensity, gills proved to be the most suitable (Figure 3.2b). Firstly, because the parasite number was always high and secondly, there was more uniformity in parasite numbers between fish within a treatment. Gills were found to be either more susceptible or the most preferred site in all the treatments, based on the very high parasite intensity recorded. Gills of fish exposed to 50 μgl^{-1} cadmium showed very high parasite intensity which was significantly different ($P< 0.001$) from the remaining treatments. However, fish exposed to lower levels of cadmium did not differ statistically from the controls. As can be seen from the Figure 3.2b gills were more susceptible, in the case of fish exposed to higher cadmium levels than other areas like body surface or fins in naive fish.

The picture was the same with parasite intensity on the body surface. Only fish exposed to 50 μgl^{-1} cadmium had a statistically higher parasite intensity than the controls and the two lower levels of cadmium exposed groups (Figure 3.2b).

The parasite success was calculated as the percentage of the tomites established on

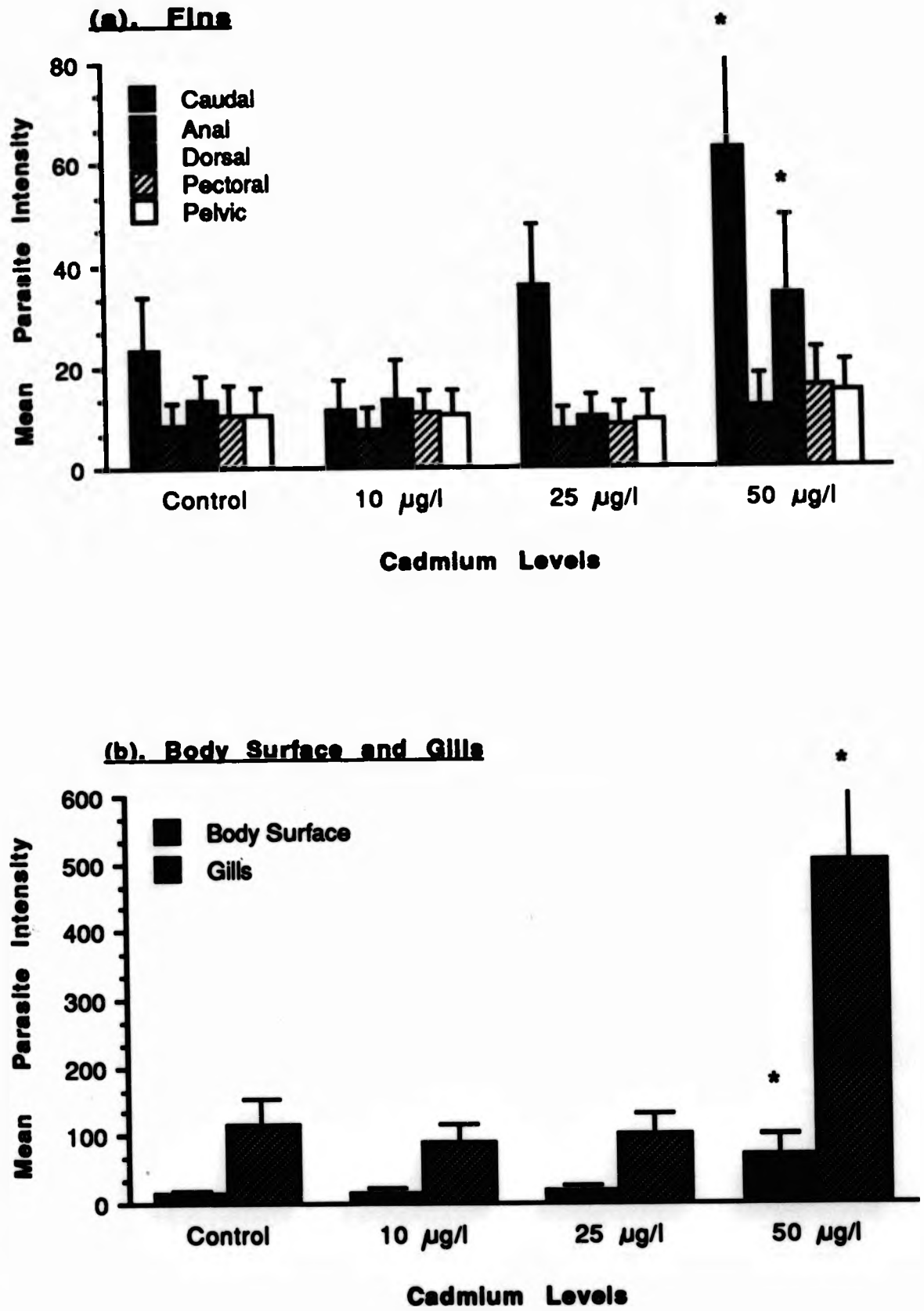


Figure 3.2 Mean parasite intensity (± S.D; n=10) on (a) the fins and (b) body surface and gills of naive carp exposed to different concentrations of cadmium for 10 days prior to experimental challenge infection (6000 tomites/fish, 30 tomites ml^{-1} for 60 min). * denotes significant difference from the control ($P<0.05$).

the fish in relation to the infection dose. In fish exposed to 10 μgl^{-1}, 2.6% of the tomites established while at 50 μgl^{-1} exposure level 11.92% of the tomites established. The percentage of successful establishment of the tomites was significantly higher in fish exposed to 50 µgl-1.

3.3.1.2 Copper

The mean parasite intensity on the fins and gills of carp exposed to copper for 10 days before challenge infection is shown in Figure 3.3. The calculated percentage establishment rate of tomites in the different treatment concentrations were as follows: control (4.1%), 10 μgl^{-1} (3.47%), 25 μgl^{-1} (6.54%) and 50 μgl^{-1} (13.75%). Comparing just the parasite intensity with the previous Experiment (3.3.1.1) it is obvious that the total parasite number on the gills and fins were higher in this experiment.

The overall result was the same as that observed in the cadmium experiment. Only the fish exposed to higher copper levels showed high parasite intensity on the fins (Figure 3.3a). Considering the fins, the caudal fins in all the treatments had more parasites and the total number differed significantly ($P< 0.05$) between fishes exposed to 50 μgl^{-1} copper and the other treaments. Unlike, the cadmium experiment however, the pectoral fins had the second highest number of parasites compared to the other fins. The pectoral, pelvic and dorsal fins of fish exposed to 50 μgl^{-1} copper differed significantly ($P<0.01$) from the control and 10 μgl^{-1} exposed fish by having a higher parasite intensity. Fish exposed to lower levels of copper did not differ statistically from the controls in terms of parasite intensity.

In this experiment, the gills again appeared to be the preferred site for infection, based on the high parasite number recorded, in all the 4 treatments (Figure 3.3b).

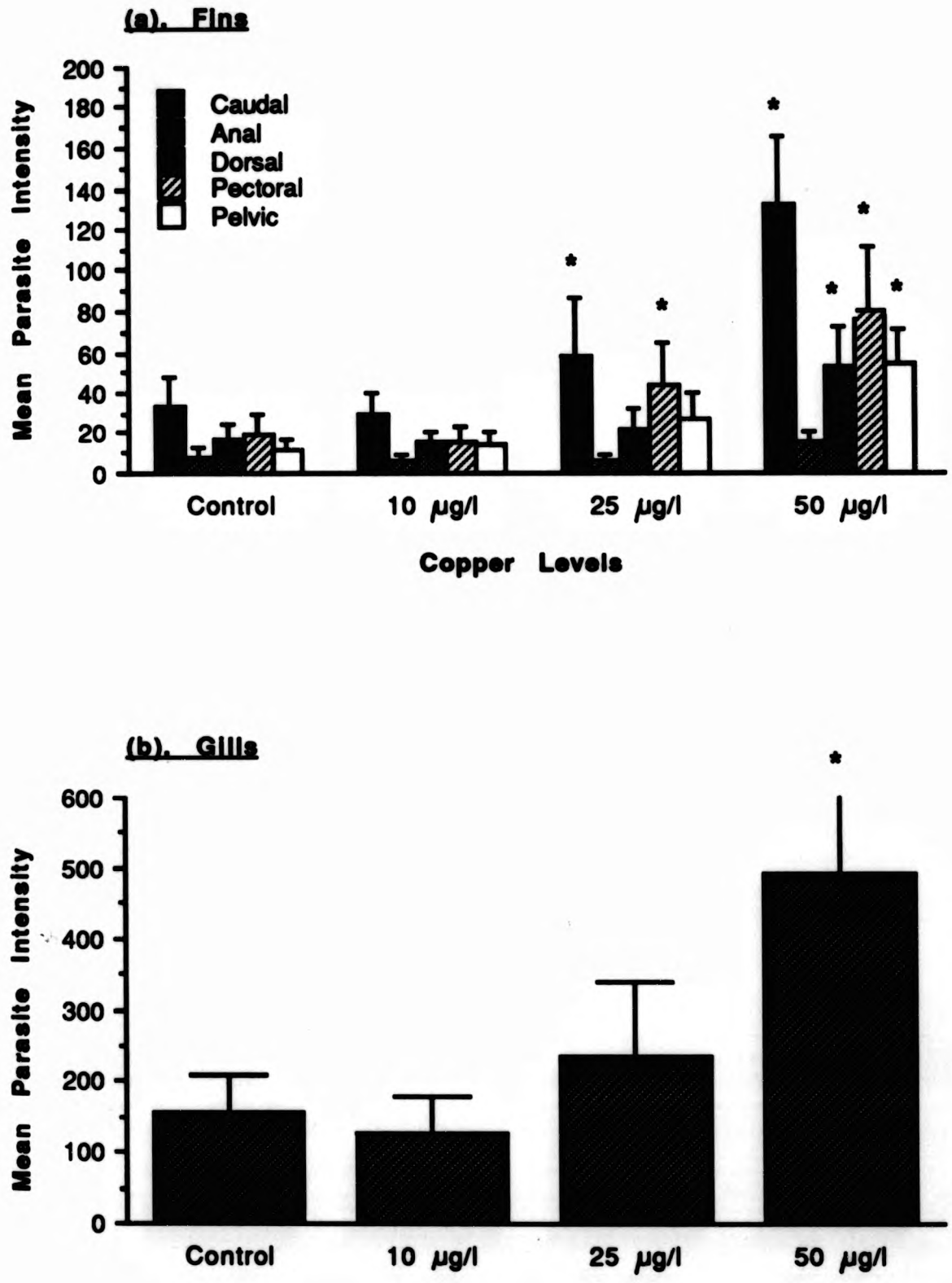


Figure 3.3 Mean parasite intensity (± S.D; n=10) on (a) the fins and (b) gills of naive carp exposed to different concentrations of copper for 10 days prior to experimental challenge infection (6000 tomites/fish, 30 tomites ml^{-1} for 60 min). * denotes significant difference from the control ($P<0.05$).

However, only the fish exposed to 50 μgl^{-1} copper had significantly ($P< 0.001$) higher parasite intensity on the gills than the other treatments, including controls.

3.3.1.3 Histopathology

The pathology described here is from fish sampled after 10 days metal exposure, and from representative samples on day three and four following "ich" infection from all the treatments.

Sublethal levels of cadmium were found to bring about many cellular changes in the gill epithelium. However, the changes noticed were not so severe as to cause mortality. Gross pathological changes were evident in fish exposed to cadmium and copper concentrations of 25 μgl^{-1} and above. The changes in cadmium exposed gills included hypertrophy and hyperplasia of gill epithelial cells and there were signs of necrosis of epithelial and chloride cells (Plate 3.2). The changes were somewhat similar in copper exposed fish, but the severity of the changes appeared to be greater after copper exposure. There was large scale interlamellar hyperplasia of gill epithelial cells and there was a great deal of cell debris as a result of necrosis and sloughing of the epithelial cells. Infiltration of eosinophilic granular cells (EGC's) into the secondary lamellae was much in evidence (Plate 3.3).

Gills of infected fish from metal exposed and control groups revealed some interesting features. Mild hypertrophy and hyperplasia were the common changes observed in the infected controls, the feeding stages of the parasite were embedded within the host epithelium and there were no noticeable changes in the surrounding epithelium. Comparatively, the average size of the parasite in the infected control gills appeared to be smaller and the food vacuoles inside the parasite were not

engorged with host cells and cell debris (Plate 3.4). In both the cadmium and copper exposed (25 and 50 μgl^{-1}) gills, much in evidence were the hypertrophic, hyperplastic and necrotic cells in the gill epithelium and even in the epithelial cells surrounding the parasite (Plate 3.5 and 3.6). The parasites appeared to be larger in the metal treated fish gills and the food vacuoles were filled with intact cells as well as necrotic cell debris (Plate 3.7).

3.3.2 Experiment 2

The results compiled in this section demonstrate the effects of exposure of immune carp to cadmium or copper for 10 days at sublethal levels on the protective immunity and the humoral "anti-ich" antibody response to *I. multifiliis*.. Table 3.1 shows the results of the final challenge after immunization for the immunized fish used in this experiment. The immunity was confirmed by a final challenge infection and also by measuring the tomite agglutination titre. The parasite prevalence on the final challenge was less than <10% and the parasite intensity was almost negligible with very few parasites confined to the periphery of the fins and none at all on the gills. In both the cadmium and copper experiments, batches of naive fish not exposed to any of the metals were used as negative controls in the experimental infection.

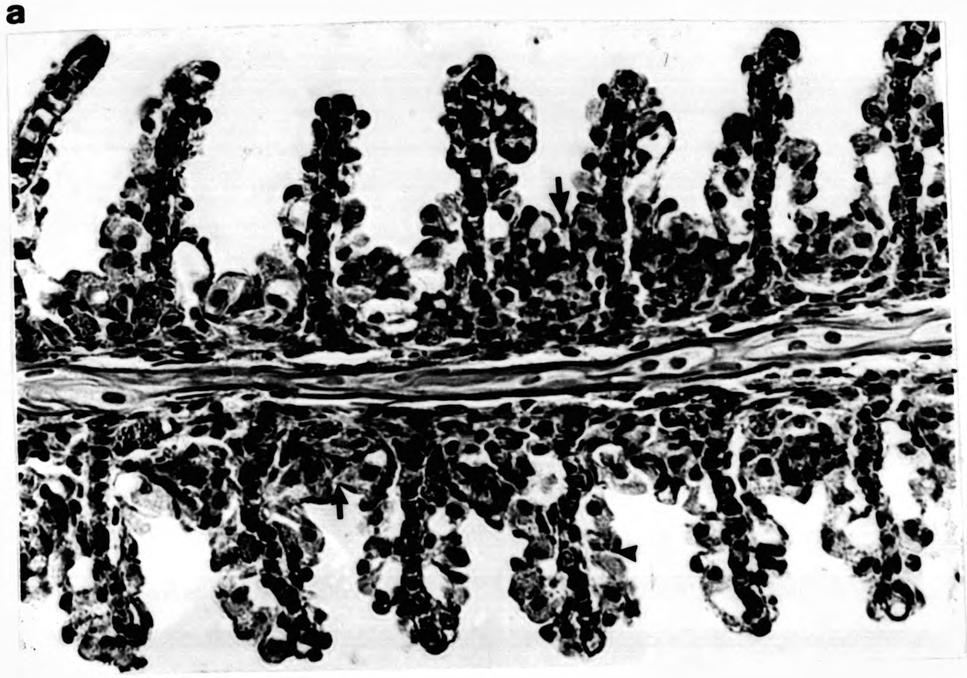
3.3.2.1 Cadmium

The parasite intensity and the distribution pattern of "ich" trophonts on different external surfaces (fins, gills and body surface) in the immune carp exposed to cadmium for 10 days prior to experimental infection are shown in Figure 3.4. All the naive fish, which were not exposed to the metal were infected and, on the whole, the fins had a greater parasite intensity than the gills or the body surface (Figure 3.4a). The fact that the naive controls were infected, shows that the tomites

Plate 3.2 Photomicrographs showing the pathological changes in the gills of carp exposed to 50 μgl^{-1} cadmium for 10 days. Note (a) interlamellar hyperplasia (arrows), vacuolation and initial signs of necrosis (arrow heads).

(a) (H&E, 600X); (b) (H&E, 1500X).

a



b

Plate 3.3 Photomicrographs showing the pathological changes in the gills of carp exposed to 50 μgl^{-1} copper for 10 days. Note (a) interlamellar hyperplasia (arrows) and hypertrophy of epithelial cells, (b) necrosis of lamellar epithelium (arrow head).

(a) (H&E, 600x); (b) (H&E, 1500X).

a

b

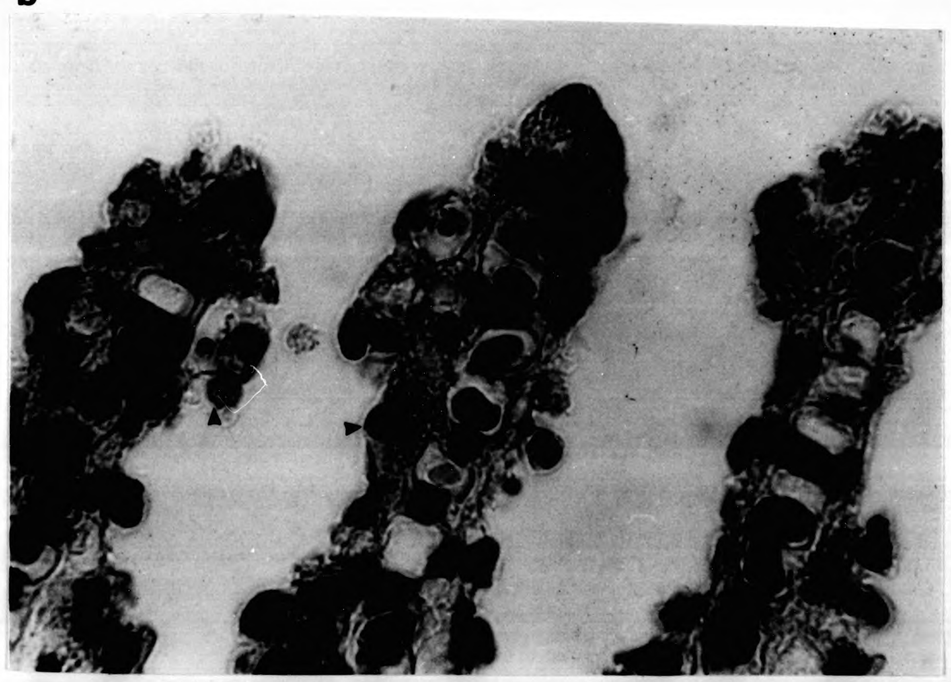


Plate 3.4 Photomicrograph showing feeding stage of *I. multifiliis*, in a control gill on the third day post infection. Note the presence of host cells (arrow) within the cytoplasam of the parasite and the absence of any cellular changes in the surrounding gill epithelium. (H&E, 600X)

Plate 3.5 Photomicrograph showing the feeding stage of the parasite in the gill of a cadmium exposed carp. Note the large number of host cells within the parasite cytoplasm (arrow) and the mild cellular changes in the gill epithelium eg hypertrophy (arrow head). (H&E, 600X).

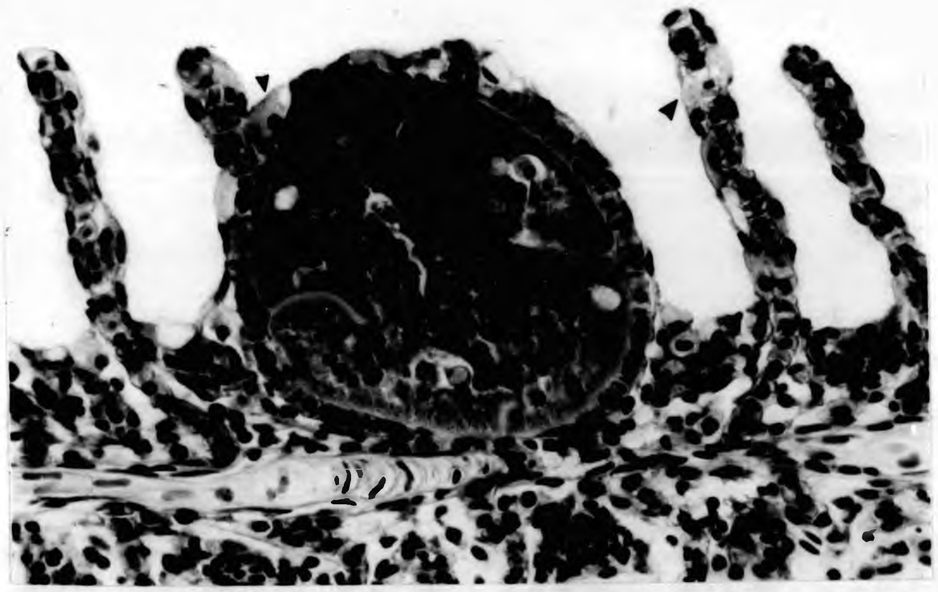
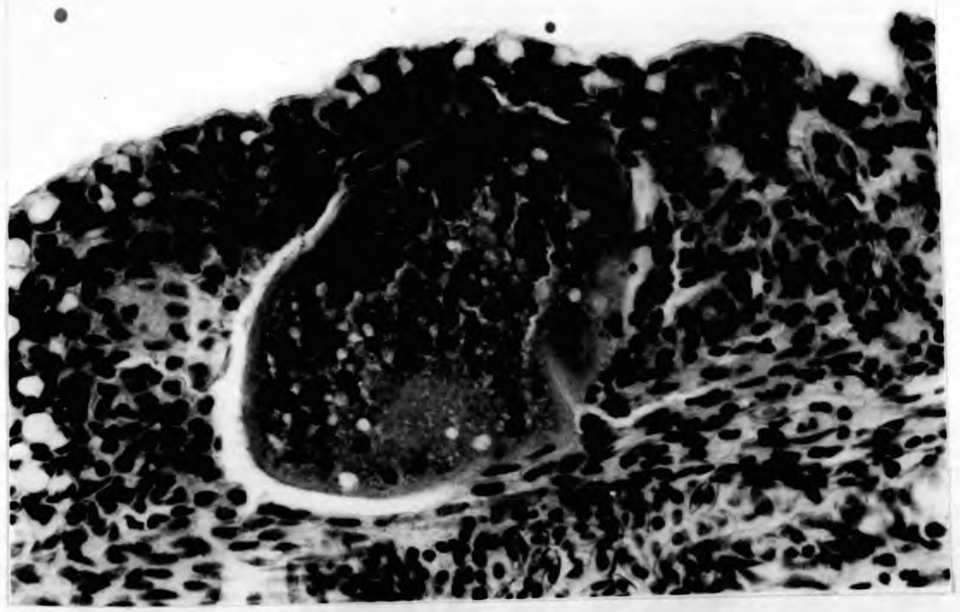


Plate 3.6 Photomicrograph showing the feeding stage of the parasite in the gill of a copper exposed carp on the third day following the infection. Note the cellular changes (arrow head) in the gill epithelium and the food vacuoles of the parasite (arrow). (H&E, 600X).

Plate 3.7 Photomicrograph showing the feeding stage of the parasite on the fourth day following infection in the tip of the primary lamella of a copper exposed fish gill. The parasite has increased in size and note the host cell debris within the cytoplasm (arrow head). (H&E, 600X).

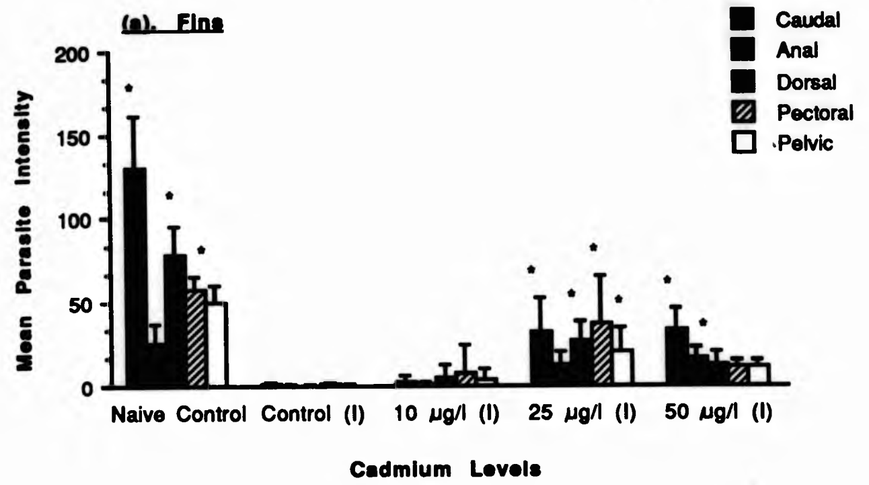


were viable and were not impaired in the infecting tank, because of the mucus released from the immune fish. The tomite establishment levels calculated were; naive control (7.9%), immune control (0.06%), immune 10 μgl^{-1} (0.28%), immune 25 μgl^{-1} (8.24%) and immune 50 μgl^{-1} (7.33%).

Immune fish that were unexposed (control) and that exposed to 10 µgl-1 cadmium for 10 days were resistant to the experimental challenge infection (Figure 3.4). In the immune controls the parasite prevalence was only 20% and the intensity was almost negligible and never exceeded three in any fish from all the 4 gill filaments (3.4b). The picture was similar in the immune fish exposed to 10 μgl^{-1} cadmium. Although, 100% of the fish were infected (Presence/absence of the parasite) the parasite intensity was very low. In 40% of the fishes the gills were totally free of the parasites. Wherever infection was present, it was invariably on the fins, usually concentrated along the peripheral margins (Figure 3.4a).

In contrast, the picture was totally different in the immune fish exposed to 25 and 50 μgl^{-1} cadmium for 10 days. In both the groups the fish were not resistant to the subsequent challenge infection (Figure 3.4). The prevalence was 100% and the gills of all the fish in these two treatments were heavily infected (Figure 3.4b). The parasite intensity on the gills of fish from the two treatments (25 and 50 μgl^{-1}) were significantly higher ($P<0.001$) than the immune controls and the 10 μgl^{-1} exposed group, in addition they were significantly higher ($P< 0.05$) than even the naive controls (Figure 3.4b). On the fins all together, fish exposed to 25 and 50 μgl^{-1} cadmium had significantly higher parasite intensity than the immune controls and the 10 μgl^{-1} exposed immune fish.

From the results it becomes clear that immune fish exposed to levels of cadmium



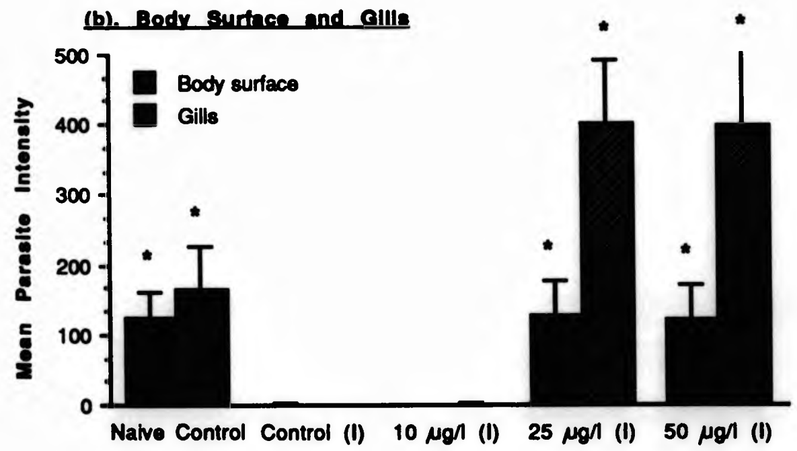


Figure 3.4 Mean parasite intensity (± S.D; n=10) on (a) the fins and (b) body surface and gills of immunized (I) carp exposed to different concentrations of cadmium for 10 days prior to experimental challenge infection (8000 tomites/fish, 40 tomites ml^{-1} for 60 min). Naive controls were introduced during challenge infection to check the viability of tomites. * denotes significant difference from the (I) control ($P<0.05$).

higher than 25 μgl^{-1} for the duration of 10 days significantly reduces the ability of the fish to mount the protective immune response to "ich". Immune controls and those exposed to 10 μgl^{-1} cadmium were able to mount the protective response and prevent any significant infection. Normally, parasites which manage to establish on immune fish do so usually only on the peripheral margins of fins but not on the gills.

3.3.2.2 Copper

Fish from all the treatments exposed to copper, developed the infection but the parasite intensity was considerably lower than in the naive controls (Figure 3.5). The calculated tomite establishment levels were; naive control (5.12%), immune control (0.03%), immune 10 μgl^{-1} (0.83%), immune 25 μgl^{-1} (0.57%) and immune 50 μgl^{-1} (1.12%). The mean parasite intensity and the distribution pattern on the fins, the body surface and gills are illustrated in Figure 3.5.

The immune control fish were resistant to experimental challenge infection. The prevalence (presence/absence of parasite) was 30% in the immune control fish. In 70% of the fish the gills were totally free of parasites, and even in those infected, the highest number was only three.

The parasite prevalence was 100% in all the three groups of immune fish exposed to copper for 10 days. In all the groups, the fins and body surface had more parasite number than the gills. Fish exposed to 50 μgl^{-1} copper, had significantly ($P < 0.001$) higher parasite number on all the three sites compared to the immune controls (Figure 3.5). Only the fins and body surface of fish exposed to 10 and 25 μgl^{-1} copper had significantly higher parasite intensity than the immune controls. There was no statistical difference in the parasite intensity between the three

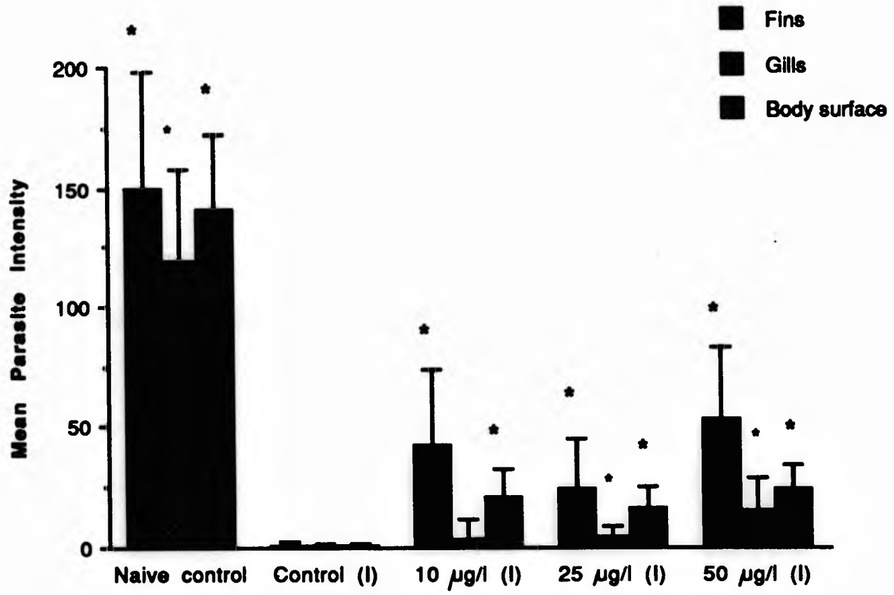


Figure 3.5 Mean parasite intensity (± S.D; n=10) on the fins, body surface and gills of immunized (I) carp exposed to different concentrations of copper for 10 days prior to experimental challenge infection (8000 tomites/fish, 40 tomites ml^{-1} for 60 min). Naive controls were introduced during challenge infection to check the viabilty of tomites. * denotes significant difference from the (I) control ($P<0.05$).

concentrations of copper (Figure 3.5). As could be seen from the results, immune fish exposed to copper for 10 days could not effectively mount a protective immune response and prevent the establishment of the parasites, albeit at low level of infection.

The parasite establishment pattern on different sites on the fish surface differed from those in the cadmium experiment in which the gills of fish exposed to 25 and 50 μgl^{-1} had higher parasite number than even the naive controls. The parasite establishment pattern in the copper experiment showed the normal trend which would be expected in "ich" immune fish, i.e. gills being more resistant than fins and body surface. Although immune fish exposed to copper were all infected, they differred considerably from cadmium exposed groups in terms of site specific susceptibilty. Comparing the naive controls of the respective experiments, the most susceptible sites (gills) of cadmium exposed groups (25 and 50 μgl^{-1}) had around 230% parasite load of naive controls, whilst the copper exposed ones at the same dose level had only between 4 and 13% of the naive controls.

3.3.2.3 Humoral Immune Response

The mean tomite agglutination titres in the immune carp before exposure to either cadmium or copper, and on day three after infection following 10 days exposure to the metals, are illustrated in Figure 3.6. Fish from all the treatments had relatively high tomite agglutination titre (-log2+1 = 7 to 9), following 10 days exposure to cadmium and copper. Interestingly, the titre did not differ statisticlly between treatments within an experiment or between initial titre before exposure to the metal and after 10 days exposure (Figure 3.6). Thus it appeared that immune fish exposed to higher levels of cadmium and copper could not mount a protective response to prevent the establishment of the parasite in spite of having a relatively high serum

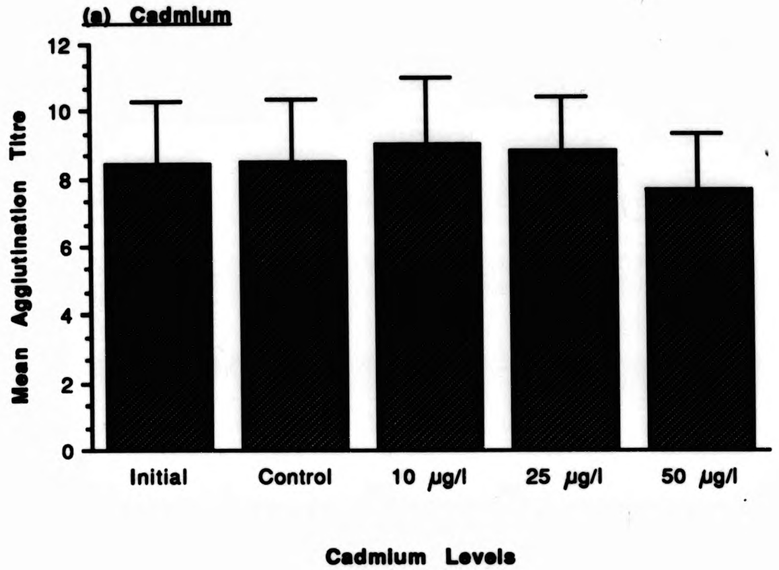


Figure 3.6 Mean humoral "anti-ich" antibody titre (± S.D; n=6) in immunized carp before exposure (initial) to the metals and on day 3 after challenge infection following 10 days exposure to (a) cadmium and (b) copper

tomite agglutination titre.

3.3.2.4 Pathology

The relevant pathological feature observed during this experiment under both cadmium and copper are presented in this section.

The gills of the immune fish used in this series of experiments had massive numbers of intact, round, PAS positive eosinophilic granular cells (EGC). They were mainly concentrated along the base and septum of the gill filaments, along the axis of the primary lamellae especially on the tip, but very few or none at all in the secondary lamellae (Plate 3.8, A-D). The picture regarding EGC's after exposing the fish to metals for 10 days and infecting with "ich", was very interesting. The gills of immune controls had very few round and intact EGC's and the majority of them were more flat, vacuolated and less densely granulated. A significant number of immune control fish gills, which were refractory to the experimental infection, showed a similar picture (Plate 3.9 A-D). The situation was totally different in previously immunized fish exposed to the higher levels of cadmium (25 and 50 μgl^{-1}) or copper (25 and 50 μgl^{-1}) for 10 days, followed by experimental infection. The gills of fish which were infected had large numbers of round, densly granulated and intact EGC's (Plate 3.10 and 3.11). These EGC's appeared very similar to those in immunized fish before the experimental infection. Surprisingly, there was some evidence of trophonts feeding on the EGC's.

3.3.3 Experiment 3

The results documented here demonstrate the effects of metals at sublethal levels on the process of the development of natural resistance to "ich" in a constantly infectious environment. Fish in this experiment were infected at regular intervals

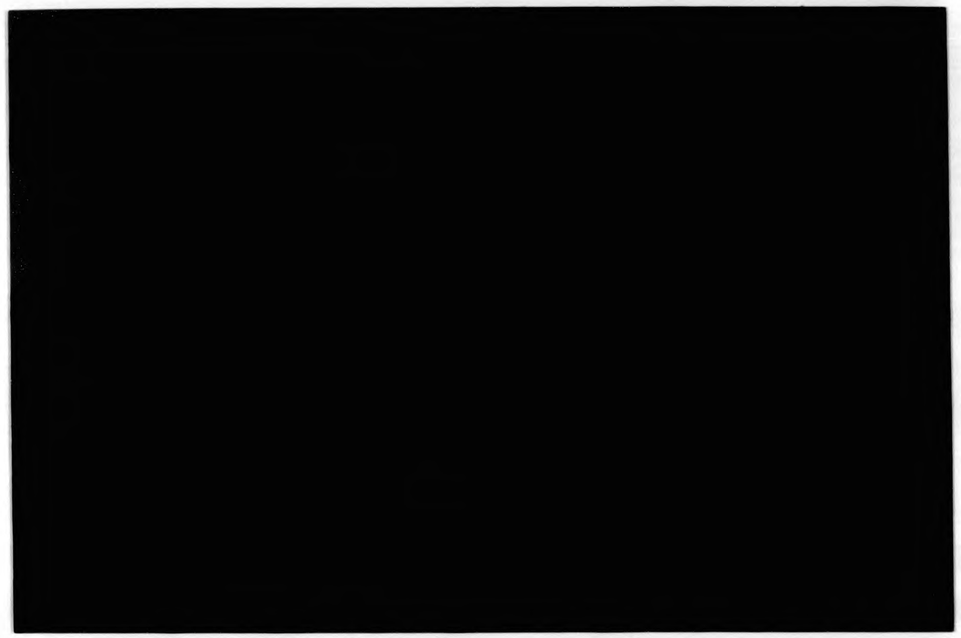
Plate 3.8 Photomicrographs showing the eosinophilc granular cells in the gill of immunized carp. Note the nature and condition of the EGC's (arrow head). EGC's are round and intact.

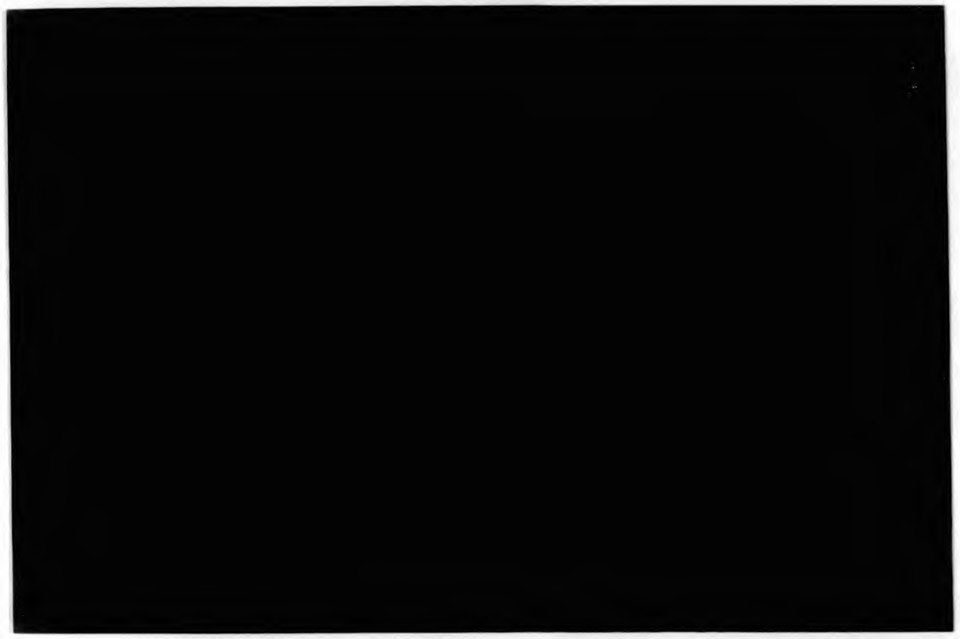
(A) EGC's along the primary lamella (PAS, 375X)

(B) EGC's confined to primary lamella with no infiltration into the secondary lamellae (PAS, 600X)

(C) Showing the density of the EGC's in the primary lamella (PAS, 600X)

(D) EGC's in the gill septal region (PAS,1500X)





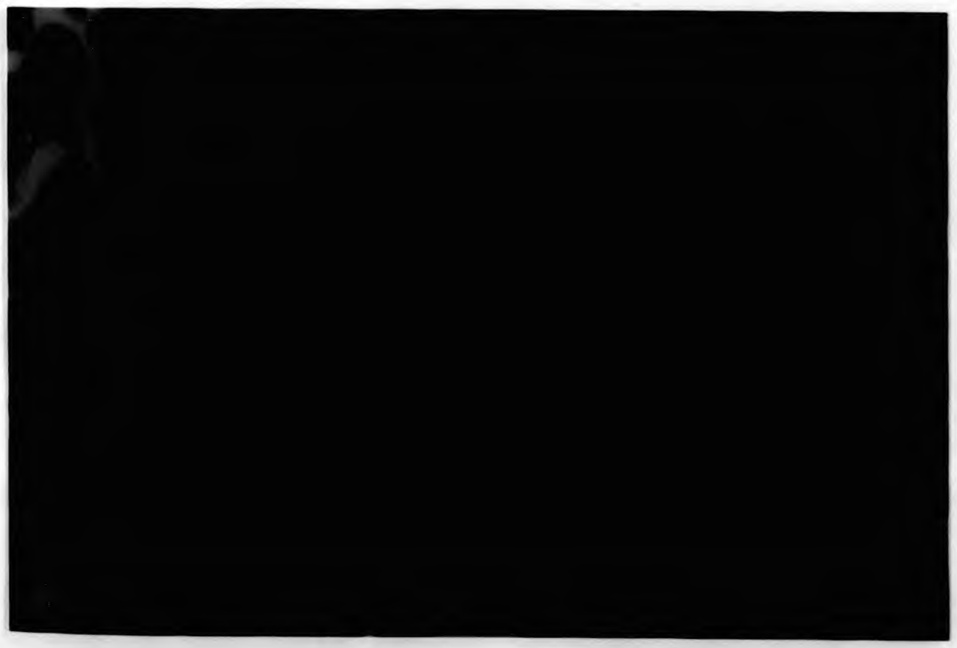


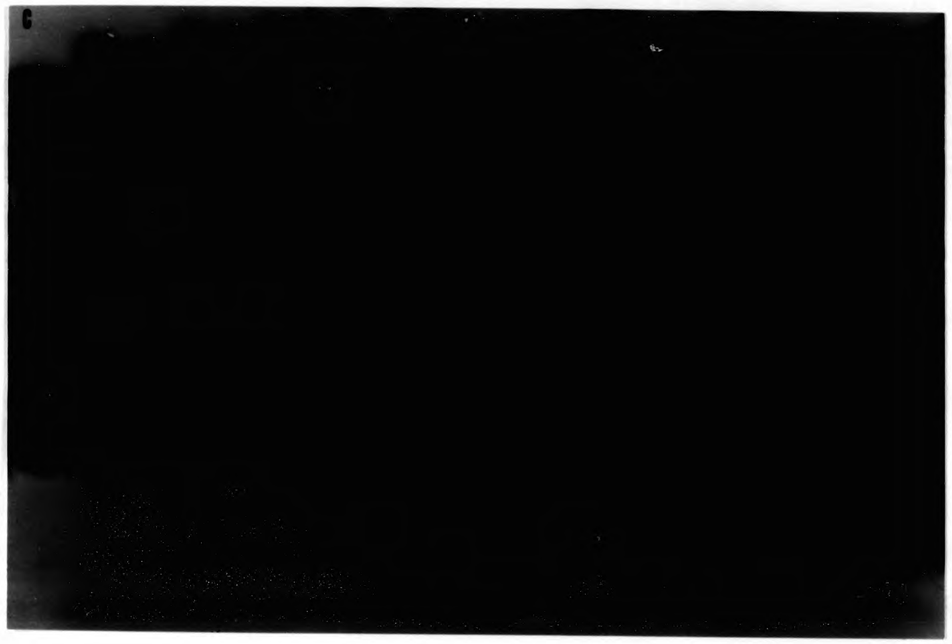
Plate 3.9 Photomicrographs showing the condition of the EGC's in immune control fish following a challenge infection. These fish were refractory to challenge. The EGC's appear flat, vacuolated and show the signs of degenerating cells (arrow head).

(A) EGC's in the gill septal region (PAS, 600X)

(B) Showing details of condition of the EGC's (PAS, 1500X)

(C) EGC's along the primary lamella (PAS, 600X)

(D) EGC's along the primary lamella (PAS, 1500X).



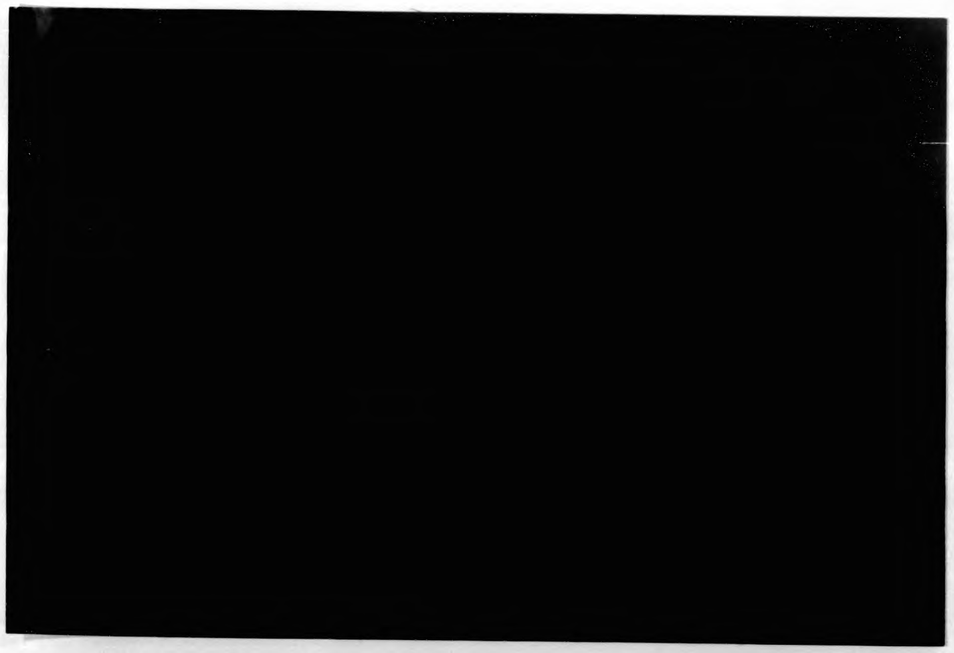


Plate 3.10 Photomicrographs showing the gill of a previously immunized carp exposed to cadmium (50 μgl^{-1}) for 10 days and then challenged with infection.

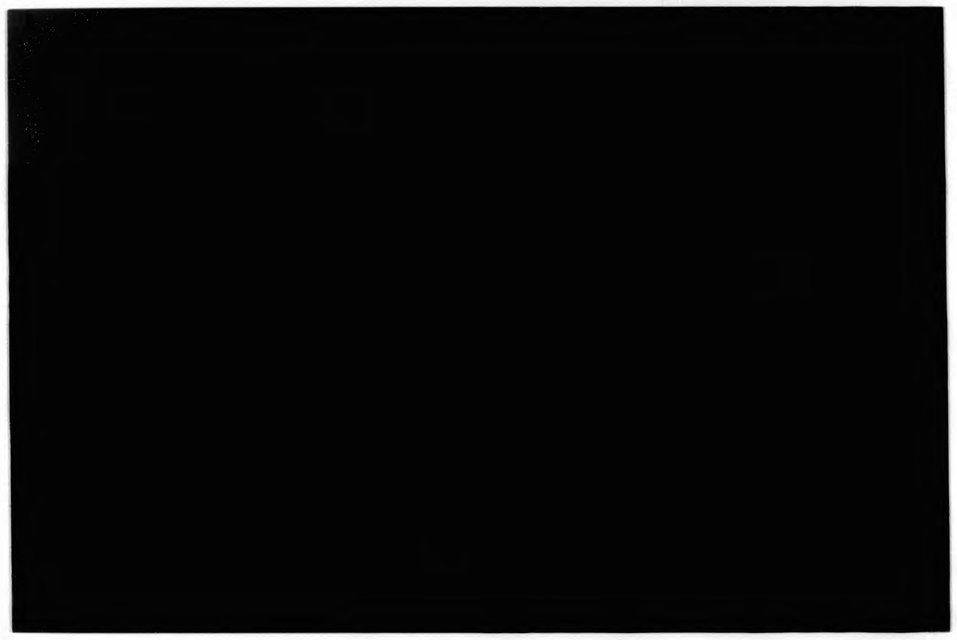
(A). Note the condition of the EGC's in these fish which were not refractory to challenge. The EGC's are round and intact (arrow head). Parasite in the gill septal region (arrow). (PAS, 375X).

(B). Fourth day following infection. EGC's were found within the cytoplasm of the parasite (arrow). (PAS, 600X).

Plate 3.11 Photomicrographs showing the gill of a previously immunized carp exposed to copper (50 μgl^{-1}) for 10 days and then challenged with infection.

(A). Note the condition of the EGC's in these fish which were not refractory to infection (arrow head). Parasite (Arrow) in the interlamellar region. (PAS, 600X).

(B). Parasite (arrow) in the tip of the primary lamella. (PAS, 600X).



with low infection levels and exposed continuously to cadmium or copper starting from day one of infection. At regular intervals following each infection the prevalence (proportion of infected fish within a treatment) and intensity of infection was assessed. Counts of individual parasites were not taken but the intensity was assessed by eye and scores given on an arbitary scale ranging from 0 to 4 as described in Section 3.2.9.3.

In order to check the viability of tomites and for confirmation of the immune status of the experimental fish in the cadmium and copper experiments, naive fish were used. The naive fish were introduced at each challenge infection and the results of the infections of these naive fish are shown in Table 3.2.

3.3.3.1 Cadmium

Figure 3.7(a-d) illustrate the prevalence and intensity of infection in cadmium exposed and control groups of carp over a duration of 35 days. A very close relationship was observed between the number of infections and the percentage prevalence and intensity of infection. Intensity and prevalence decreased rapidly after successive infections in controls and 10 μgl^{-1} exposed fish as they became immune, unlike groups exposed to higher cadmium levels, where the decrease was gradual (Figure 3.7a-d). Control fish had become resistant by the end of the third infection with prevalence being only 20% and the intensity level was very mild. Fish exposed to 10 μgl^{-1} cadmium showed a similar picture, and did not develop the 4th infection. However, the groups exposed to 25 and 50 μgl^{-1} cadmium, appeared to have developed the resistance only by the end of 4th and 5th infections respectively. In these groups, the intensity of infection remained high till the end of 3rd infection.

As can be seen from the Figure (3.7a-d), fish from all the treatments appeared to

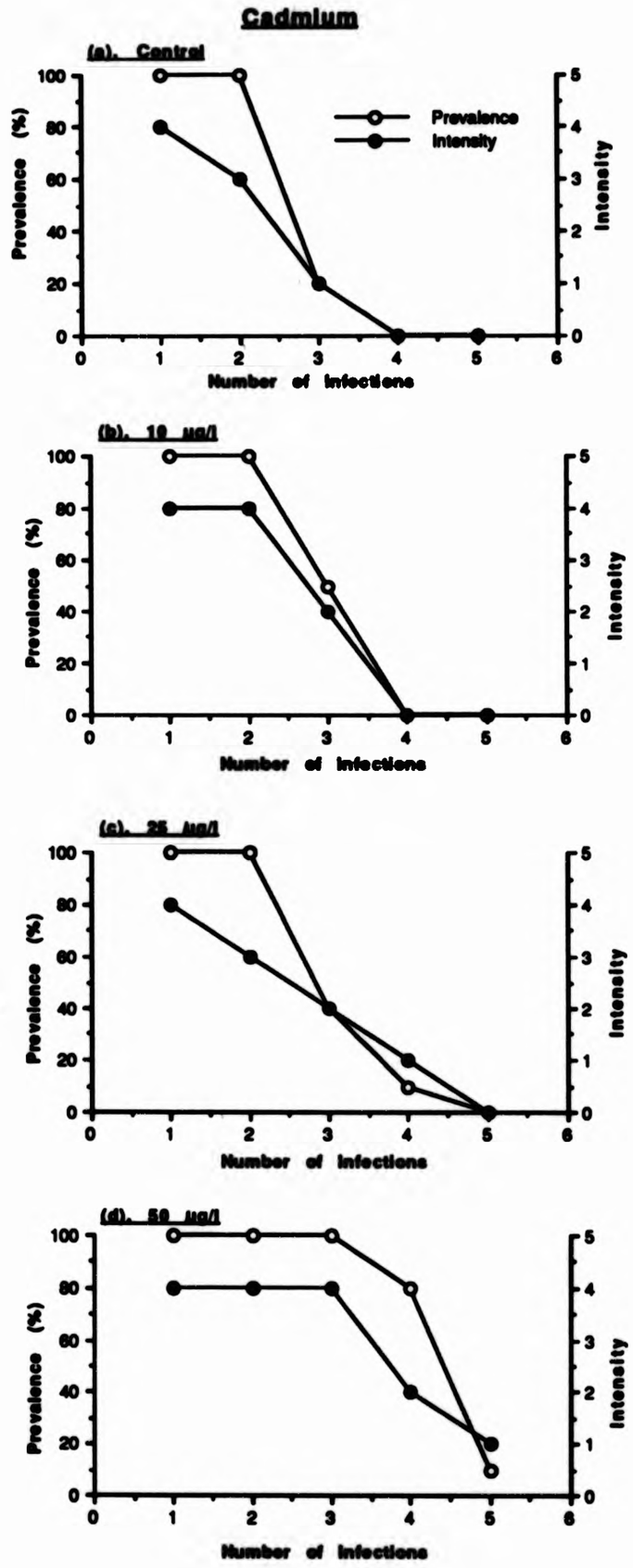


Figure 3.7 The prevalence (%) and intensity of infection following five serial infections (1500 tomites/fish, 15 tomites ml^{-1} for 30 min) at 7 day intervals in carp exposed continuously for 35 days to different concentrations of cadmium, starting from the day of first infection.

have developed resistance and became refractory to infection at various stages during the course of 5 infections over 35 days. The interesting difference between the treatments was the time taken to acquire this resistance. From the results it appears that fish exposed to 25 and 50 μgl^{-1} cadmium, take approximately 7 to 14 days, longer than the controls, respectively to become refractory to infection.

3.3.3.2 Copper

The prevalence and intensity of infection after each experimental challenge in three groups of carp exposed to copper and a control is shown in Figure 3.8. Here again, a very clear relationship between the time taken to develop resistance and the number of infections was seen in all the 4 groups of fish. The relationship is illustrated in Figure 3.8a-d. Fish exposed to the higher copper levels were not totally resistant till almost towards the end of the 5th infection. Even though the intensity decreased with number of infections, the percentage prevalence remained high till the end of 5th infection in fish exposed to 25 and 50 μgl^{-1} copper(Figure 3.8c-d). Control fish appeared to have become refractory to infection by the end of 3rd challenge. Compared to cadmium exposed fish, copper exposed groups appeared to have taken relatively more time to become resistant to infection.

It is clear from the results that fish exposed continuously to low infection could develop resistance, and that, even in the presence of low levels of cadmium and copper, this response was not totally impaired.

3.3.4 Experiment 4

This experiment was very similar to the previous one in terms of metal exposure and infection protocol. The results recorded here demonstrate the effects of cadmium or copper on the kinetics of humoral "tomite-agglutinating" antibody response in

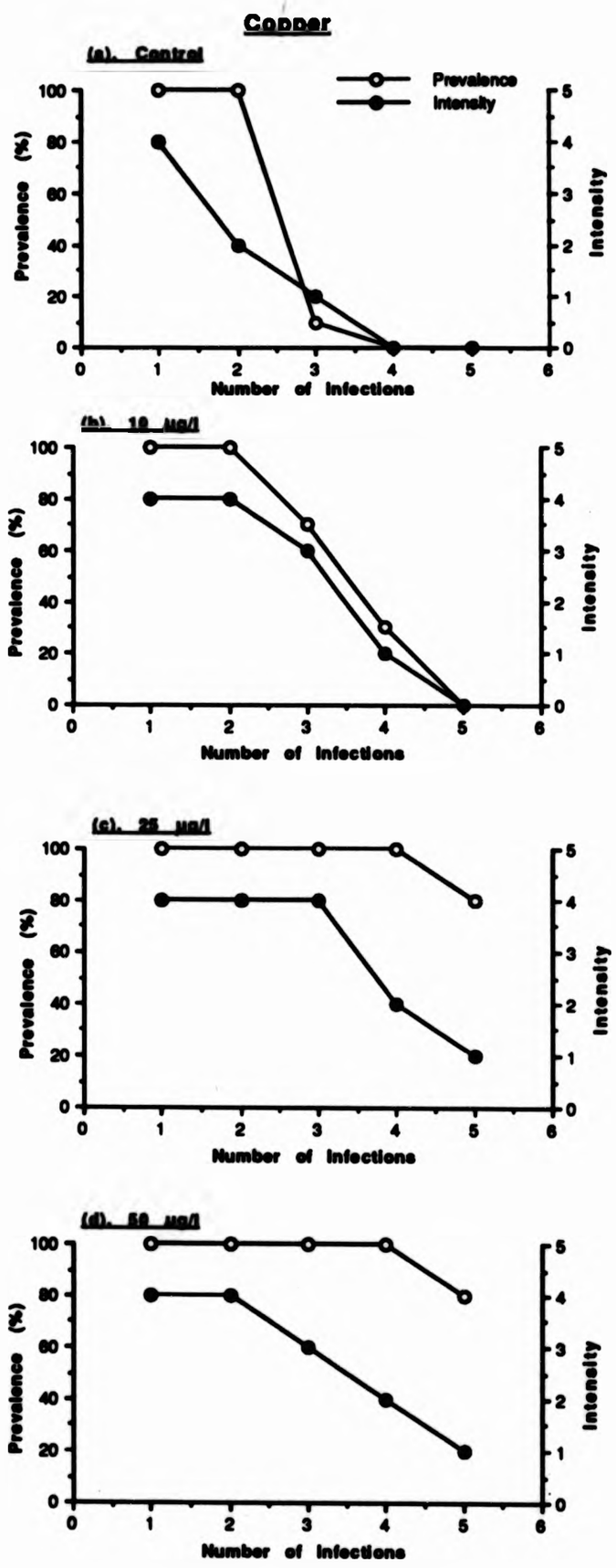


Figure 3.8 The prevalence (%) and intensity of infection following five serial infections (1500 tomites/fish, 15 tomites ml^{-1} for 30 min) at 7 day intervals in carp exposed continuously for 35 days to different concentrations of copper, starting from the day of first infection.

carp to *I. multifiliis*. Table 3.3 gives the prevalence and intensity of infection in the naive carp exposed to the tomites at each of the 5 immunization exposures.

3.3.4.1 Cadmium

The mean tomite agglutination titre with standard deviation in 4 groups of carp at different sampling points are presented in Figure 3.9a. As can be seen from the Figure 3.9a, the agglutination titre had a close relationship with time and the number of immunization exposures, in all the four groups. The titre had reached a peak of around 8 (1:256), by the end of the fourth immunization exposure, in all the groups. Controls and 10 μgl^{-1} cadmium exposed fish had attained high titres by the end of the third immunization exposure (Figure 3.9a). The dynamics of the antibody titres showed a very close relationship with the parasite prevalence and intensity observed, in the present and the previous experiment (Figure 3.7a-d). However, there was no statistical difference in titres between the 4 treatments at any given single sampling point. It should be noted that, in spite of there being no difference in titre between groups, there was a considerable difference with respect to prevalence and intensity of parasite infection.

Controls developed high titres by the end of the third immunization exposure (21 days), while groups exposed to high cadmium levels (25 and 50 μgl^{-1}), took relatively more time to attain the same titre level (28 to 35 days). In all the groups, the titre appears to peak by the end of the fourth infection (day 28) and thereafter appears to plateau. The kinetics of titre is the same in all the groups, with the only difference being slightly lower titres in fish exposed to high (25 and 50 μgl^{-1}) cadmium levels.

3.3.4.2 Copper

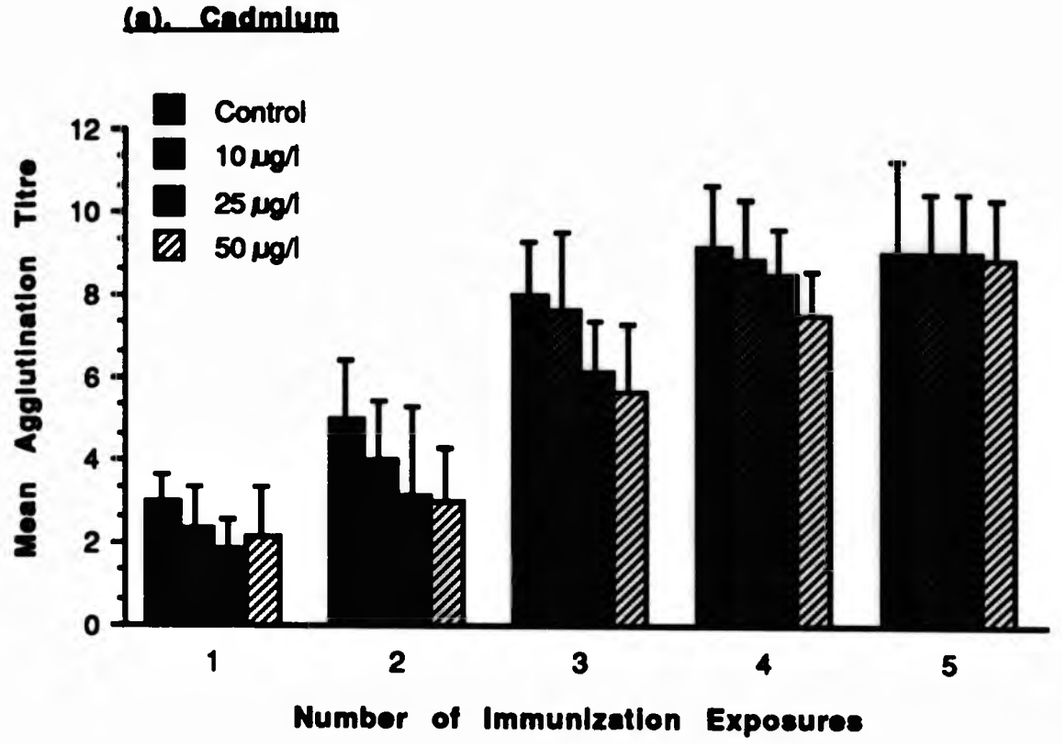
Figure 3.9b shows the mean agglutination titre in 4 groups of carp exposed to copper for 35 days and infected at regular intervals of 7 days, starting from day 1 of exposure. The serum agglutination titre increased with time, and had close relationship with the number of immunization exposures in all the four groups (Figure 3.9b). Controls had attained relatively high titres (9 = 1:512) by the end of the 3rd infection, whilst in those exposed to copper, high titres comparable to the controls were evident only by the end of the fourth immunization exposure. The progression of agglutination titre was once again clearly related to the prevalence and intensity of infection observed in the Experiment 3.3.3.2 (Figure 3.7a-d). The fact that there was no statistical difference in the titre between treatment levels, clearly shows that copper, at these concentrations, did not affect the development of tomite agglutination titre, but only lowered the magnitude of the responses and delayed the process compared to the controls.

3.3.5 Experiment 5

This experiment was different from all the previous ones. The results presented here highlight the effect of a well-established parasite infection and reinfection with *I. multifiliis* on the resistance of carp to cadmium or copper toxicity. The concentrations of cadmium and copper used were high (10 to 100 µgl-1) and the infection levels used was also higher than all the previous experiments. The fish were exposed to the metals over a duration of 15 days, with two experimental infections being carried out (Day one and Day eight).

3.3.5.1 Cadmium

The cumulative percentage mortality in carp exposed to concentrations ranging from 10 to 100 μgl^{-1}, without accompanying infection, is documented in Table 3.4. A mortality of as high as 50% was recorded only in the highest concentration (100



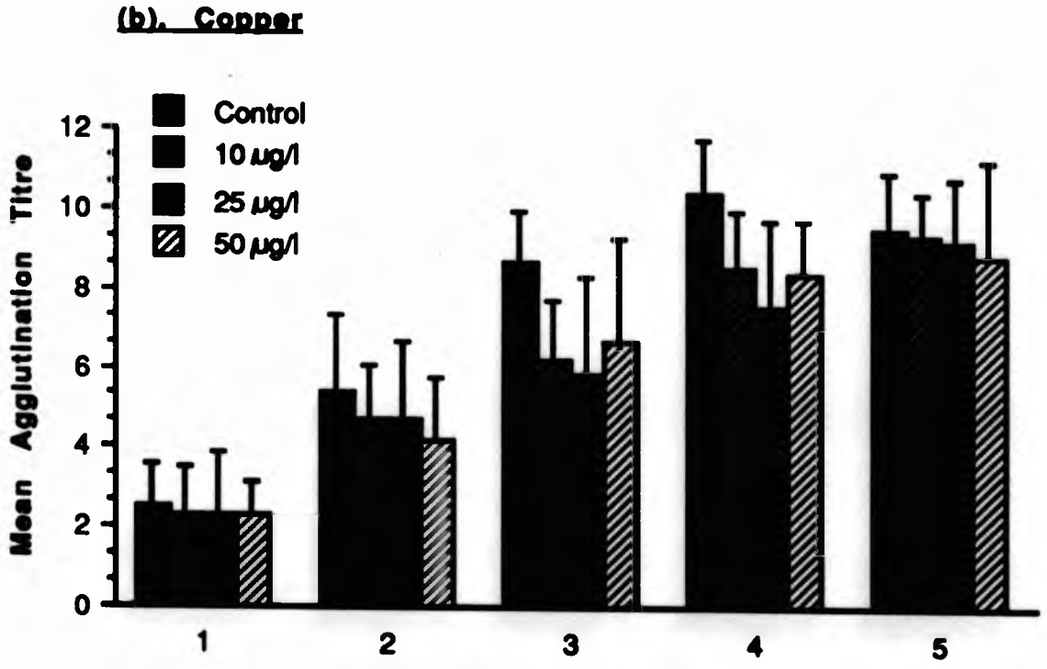


Figure 3.9 The kinetics of humoral "anti-ich" antibody titre (Mean ± S.D; n=6) following each of the five immunization exposures (2000 tomites/fish, 15 tomites ml^{-1} for 30 min) at 7 day intervals in carp exposed to different concentrations of (a) cadmium and (b) copper for 35 days, beginning from the day of first immunization exposure.

μgl^{-1}) after 11 days of exposure. Concentrations upto 50 μgl^{-1} did not bring about any mortality over 15 days.

The cumulative percentage mortality in carp exposed to varying levels of cadmium and during 2 cycles of infection is documented in Figure 3.10a. Mortalities were observed in all the treatments. In the controls, the cumulative mortality reached 35% by the end of day 15. Exposure to higher levels of cadmium (75 and 100 μgl^{-1}) resulted in 100% moratality by the end of the 14th and the 9th day respectively. Even the lower levels of 25 and 50 μgl^{-1} brought about 90% mortality by the end of the 15th day. As would be expected, the mortality pattern showed a close relationship with the metal concentration. However, the most interesting observation, was of the actual timing of the occurrence of maximum mortality. The mortalities increased rapidly between days 6 and 9 which coincided with the emergence of trophonts from the fish host epithelium and the second round of infection.

Figure 3.10b very clearly demonstrates the relationship between daily total mortality over 15 days and the phase of infection. The 3 peaks in mortality, seen from the Figure 3.10b were very closely related to the different phases involved in the life cycle and infection pattern of *I. multifiliis*. The first and the last minor peaks in mortality occurred between days 5-7 and 13-15 respectively. These were associated with the emergence of adults from the fish epithelium and the subsequent damage brought about in the skin and gill tissues. The central major peak in mortality occurred between days 8 and 10 and was related to the 2nd infection (tomite challenge) carried out on day 8. Mortalities observed on days 6, 9 and 14 were significantly higher than those recorded on other days. Examination of the pattern of mortality therfore reveals that the phase of infection was more influential than the actual parasite load.

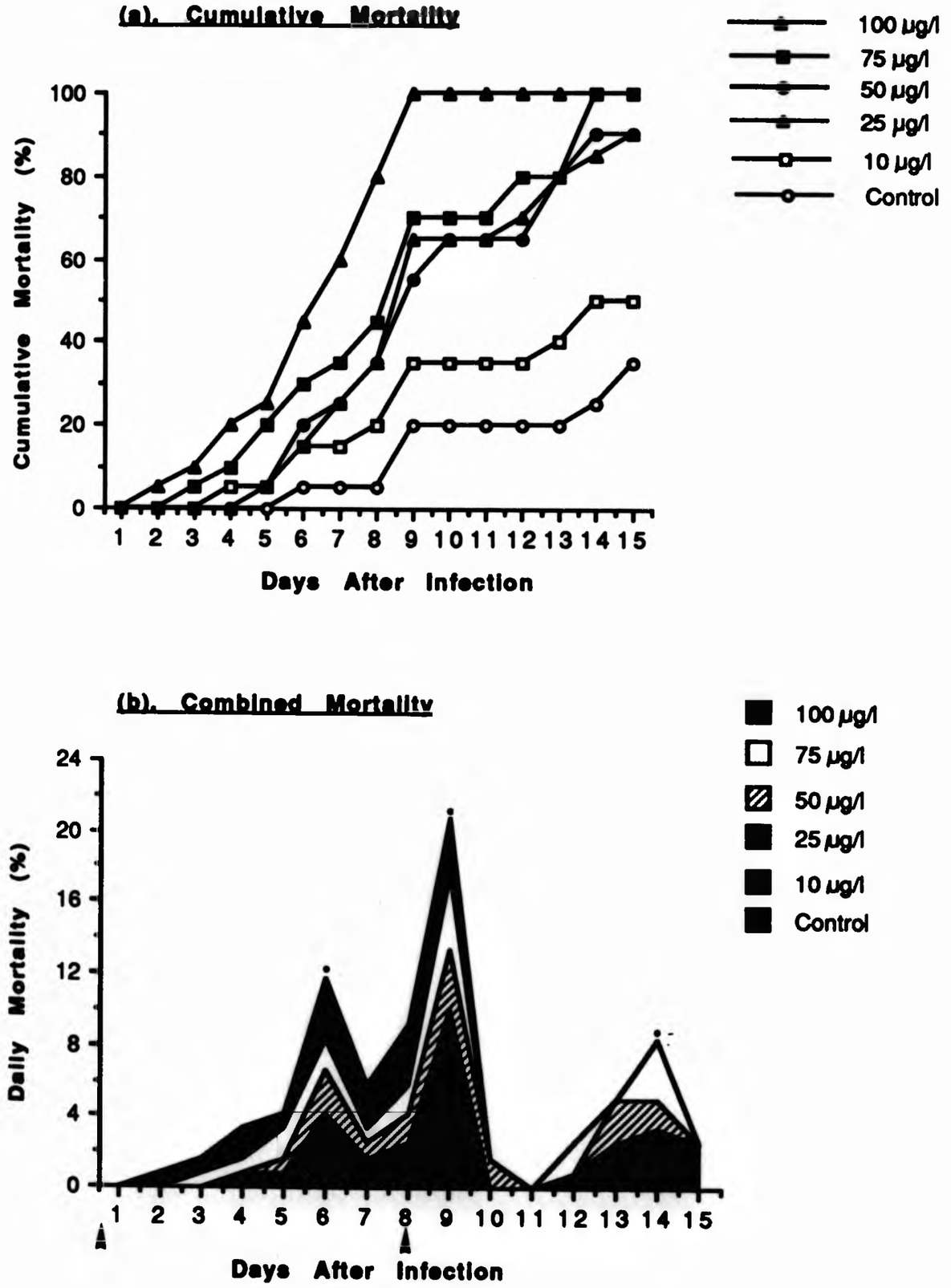


Figure 3.10 The mortality pattern in carp infected twice (10,000 tomites/fish, 60 tomites ml^{-1} for 60 min) over 15 days and exposed simultaneously to different concentrations of cadmium from the day of first infection. (a) Cumulative mortality (%) with concentration (b) total mortality with time. Arrows denote the time of infection. * denotes significant difference from the rest of the sampling points ($P<0.05$).

3.3.5.2 Copper

Table 3.5 gives the cumulative percentage mortality in carp exposed to 5 concentrations of copper ranging from 10 to 100 μgl^{-1}, without infection, over a duration of 15 days. Concentrations of below 50 μgl^{-1} did not bring about any significant mortality, however, copper levels of 75 and 100 μgl^{-1}, resulted in 100% mortality within 13 and 11 days of exposure, respectively.

The cumulative percentage mortalities recorded over 15 days in carp, exposed to copper and two challenge infections are presented in Figure 3.11a. In the highest concentration tested (100 μgl^{-1}), 100% of the fishes died, even before the second infection. Again the close relationship between mortality and copper concentration is evident (Figure 3.11a). Concentrations above 50 μgl^{-1}, resulted in 100% mortality within the experimental period. The onset and progression of mortality was very rapid compared to the controls and the groups exposed to lower levels of copper. A significant proportion of the mortality occurred between days 3 and 9, though it was not restricted to certain days, as was observed in the cadmium experiment, but more widespread throught that period. As can be seen from the Figure 3.11a, the infection levels used resulted in 40% cumulative mortality in the unexposed controls.

Figure 3.11b illustrates the relationship between the total mortality recorded daily and the phase of infection over the 15 days of the experiment. In all the 6 treatments, the maximum mortality occurred between days 3 and 9, with peaks on days 4, 9 and 14. As in cadmium experiment, the peak mortality observed here appears to be related to the phase of the infection cycle.

It appears therefore, from this experiment that the time and concentration of cadmium and copper required to bring about mortality is significantly reduced when

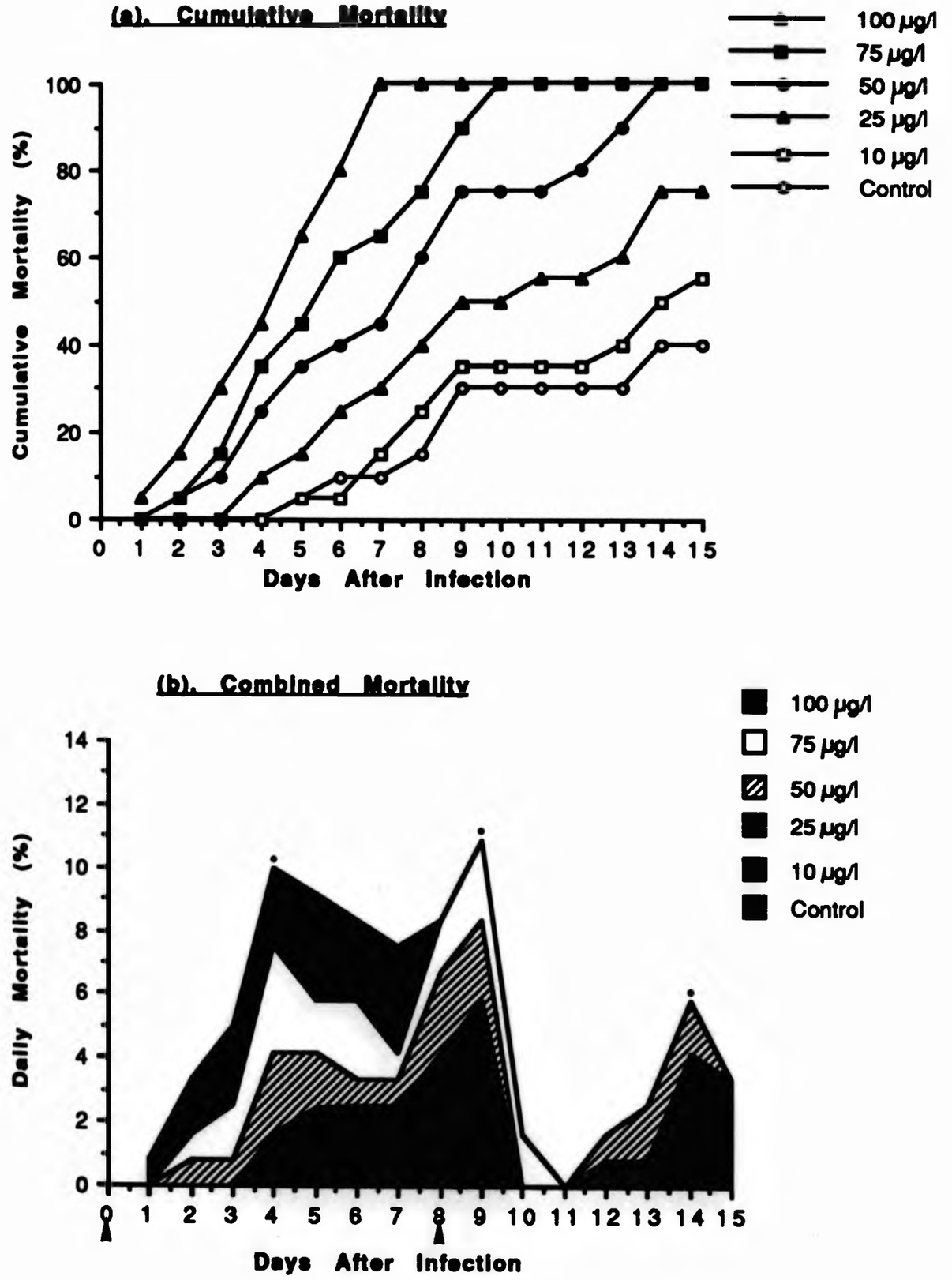


Figure 3.11 The mortality pattern in carp infected twice (10,000 tomites/fish, 60 tomites ml^{-1} for 60 min) over 15 days and exposed simultaneously to different concentrations of copper from the day of first infection. (a) Cumulative mortality (%) with concentration (b) total mortality with time. Arrows denote the time of infection. * denotes significant difference from the rest of the sampling points ($P<0.05$).

accompanied by "ich" infection. The most obvious feature to note is the actual time of the occurrence of the mortality and its relation to the phase of the infection cycle.

3.3.5.3 Pathology

The most interesting pathological changes observed in the gills of carp during different phases of infection in this experiment are presented here.

Tomite penetration appears to severly damage the host epithelium. The damage inflicted by the invasion of the tomites during the second infection during the first 8 hr was very acute (Plate 3.12). The tomites did not normally settle, immediately after penetration, but would continue to migrate within the epithelium. This burrowing or tunneling activity in the host tissue epithelium following successful penetration was a characteristic feature of the tomite invasion. This activity had the effect of leaving spaces in the epithelium. The spaces left by the migrating tomites were infiltrated by different types of granular cells, including EGC's (Plate 3.13). Under heavy infection the tomites were seen amidst cellular debris of hydropic, vacuolated and necrotic cells. The tomites caused large scale damage which resulted in the lifting of small areas of the epithelium from the basement membrane and necrosis of epithelial cells in the immediate vicinity of the tomite. Despite their considerable abilty to penetrate, the tomites were never seen to gain entry through the basement membrane (Plate 3.14). The tomites appeared to settle only after approximately 8 to 12 hours following the infection/penetration.

In samples taken 24 hr after the first infection there was no evidence of this early damage caused by the tomite as described above. Once the tomites settled down they are enclosed within the host epithelium and there appeared to be very little

subsequent tissue damage taking place (Plate 3.15). The constant rotatory movement of the now feeding stages of the parasite (trophont) within the host epithelium, did not appear to produce much damage. As the feeding stages grew and attained maturity, they increased in size significantly. On average they grew from between 30-40 µm at the tomite stage up to 400-600 µm at maturity, in just a matter of 3 to 4 days at 27℃ (Plate 3.16).

After attaining maturity within the host epithelium, the mature trophonts emerged from the epithelium, to encyst and divide on the substrate in the environment. The tissue damage inflicated at this stage during the process of trophont emergence was often severe and the seriousness of the damage depended on the size and number of the trophonts as well as the location from which they emerged. Emergence from the tips of the primary lamellae appear to result in less serious damage than if they were located in the secondary interlamellar region. During this phase the trophonts ruptured the epithelium surrounding them, and in this process, caused large scale lifting of the epithelial layer which then sloughed away (Plate 3.17).

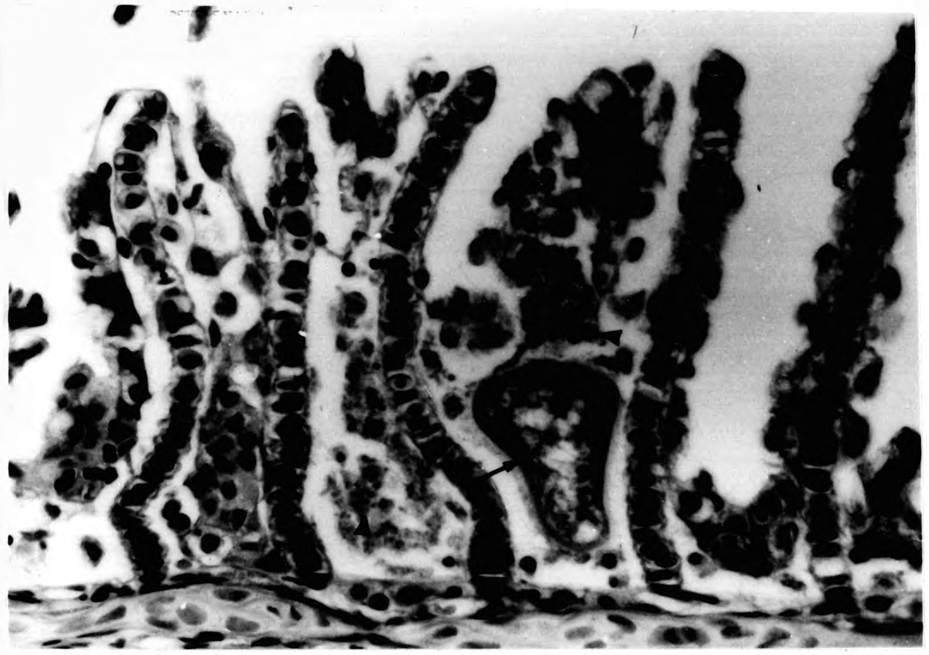
The pathological changes observed in groups infected and exposed to lower concentrations of the metal were very similar to those seen in the control gills. The main difference was that the gills were also showing additional metal-induced pathological changes. These were hypertrophy, hyperplasia, cell necrosis and even sloughing of epithelial cells (Plate 3.18). Moribund fish sampled from higher metal exposure tanks after the second infection showed a high degree of epithelial cell proliferation and extensive necrosis of gill epithelium (Plate 3.19). Such large scale pathological changes appears to have contributed to the mortalty.

Plate 3.12 Photomicrographs showing the initial tissue damage caused by the tomite (arrow) in the process of penetration and migration within the first 8 hours of infection. Note the cellular debris in the interlamellar region (arrow head).

(a) Gill of a control fish (H&E, 600X).

(b) Similar changes in the gill of cadmium exposed fish (H&E, 600X).

a



b

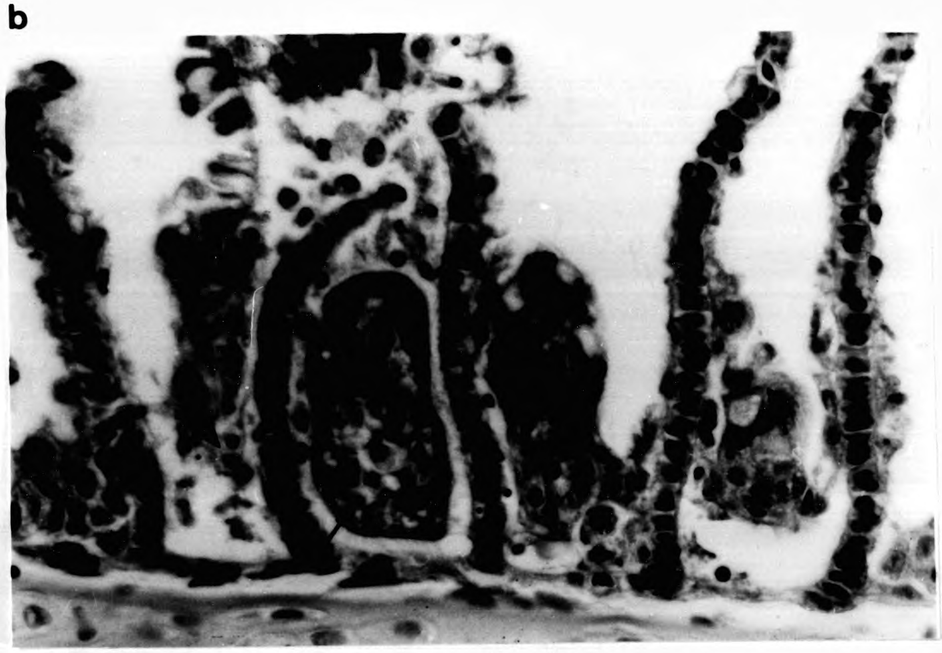


Plate 3.13 Photomicrographs showing (a) the space (arrow head) left by the migrating tomite in the gill tissue and (b) the infiltration of such spaces by the EGC's and other leucocytes (arrow). (H&E, 600X).

a

b

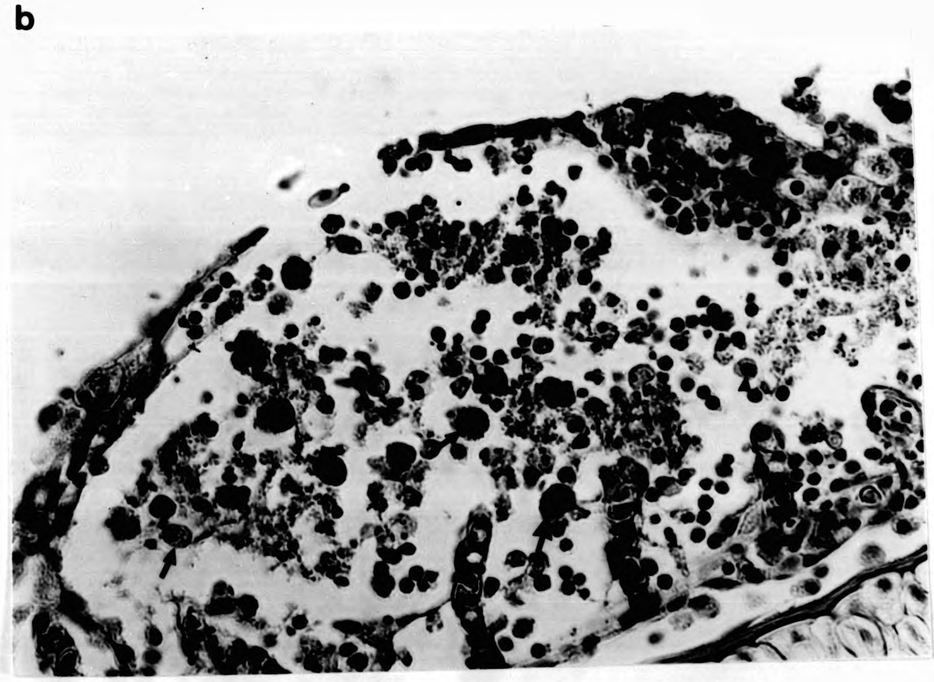


Plate 3.14 Photomicrographs showing the parasite (arrow) within the epithelium following 24 hours of infection. Note the parasite has not penetrated beyond the basement membrane. (H&E, 1500X).

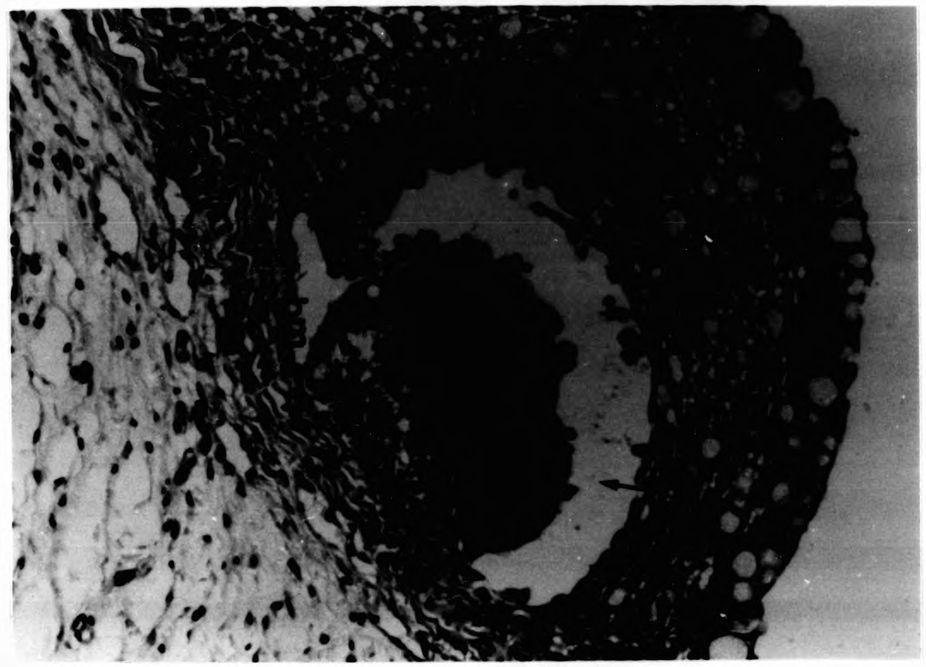


Plate 3.15 Photomicrographs showing the parasites (arrow) enclosed within the epithelium following 24 hours of infection. The associated tissue damage is less recognisable at this stage than at the tomite penetration stage. (H&E, 600X).

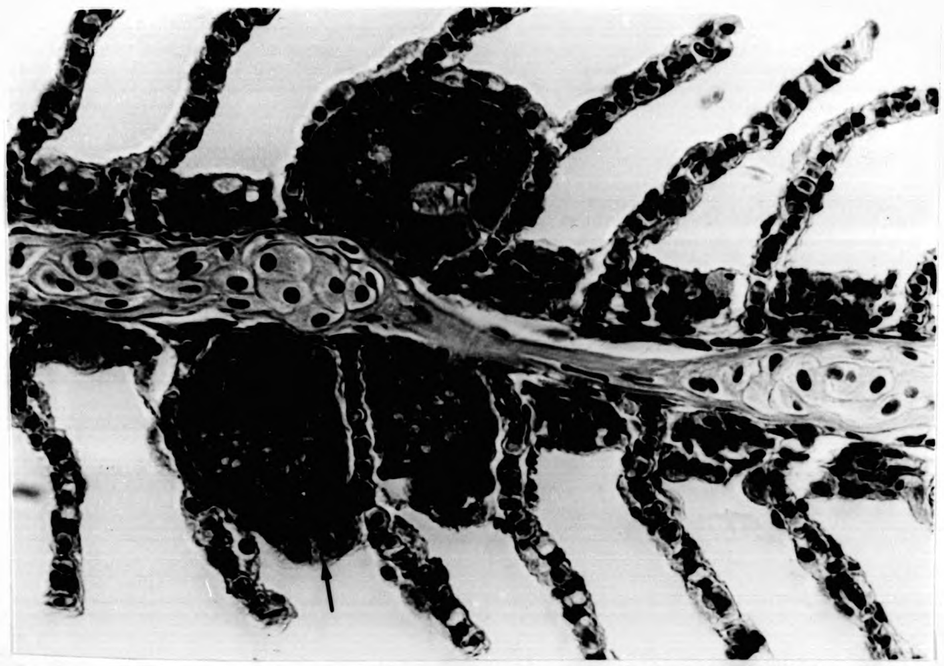


Plate 3.16 Photomicrograph showing a mature parasite (arrow) in the gill of a control fish prior to emergence. Note the lifting of the epidermis due to the large size of the parasite (H&E, 600X)

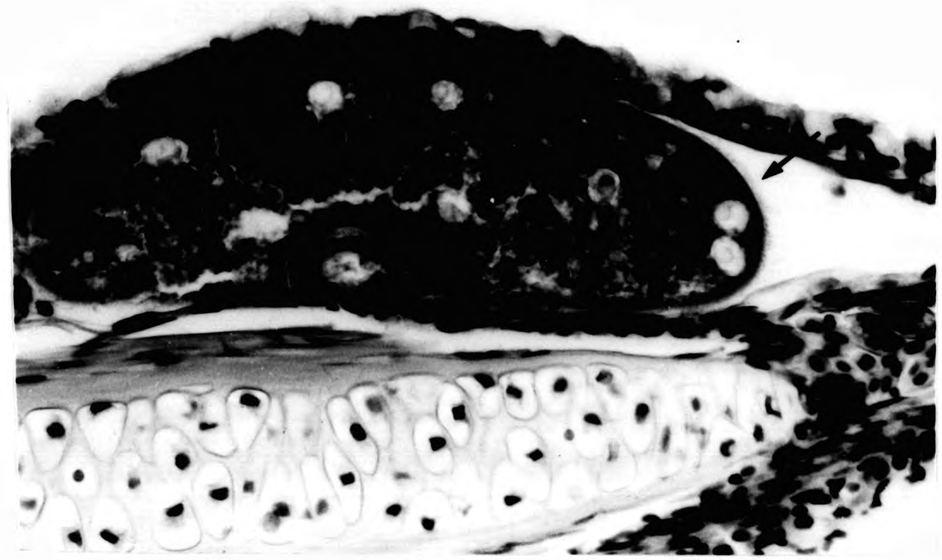
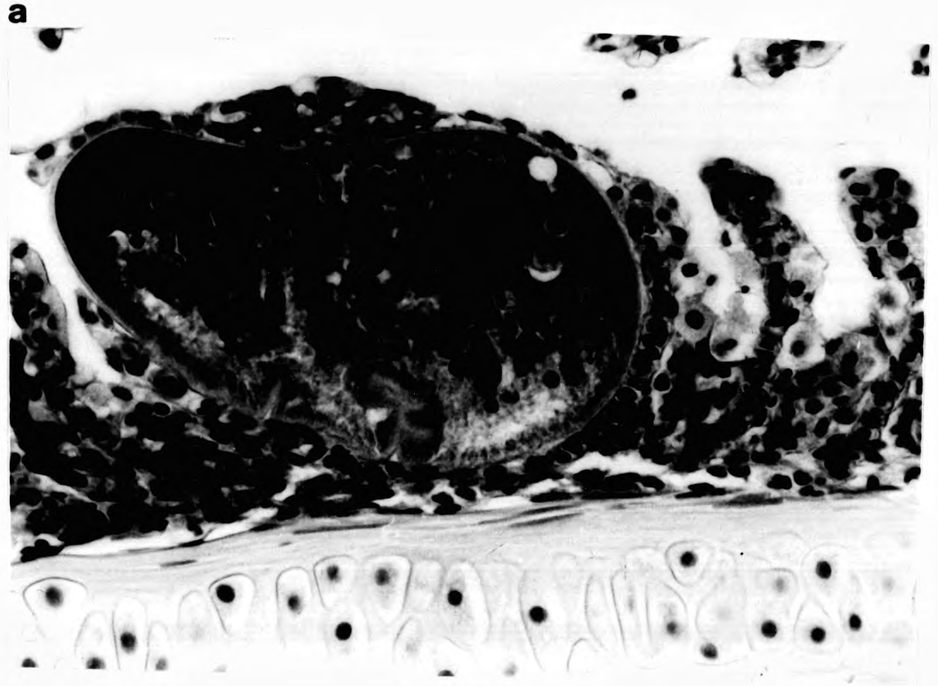


Plate 3.17 Photomicrographs showing the mature parasites in the process of emerging from the gill epithelium of carp exposed to the metals.
(a) 50 μgl^{-1} cadmium. (b) 50 μgl^{-1} copper. (H&E, 600X).

a



b

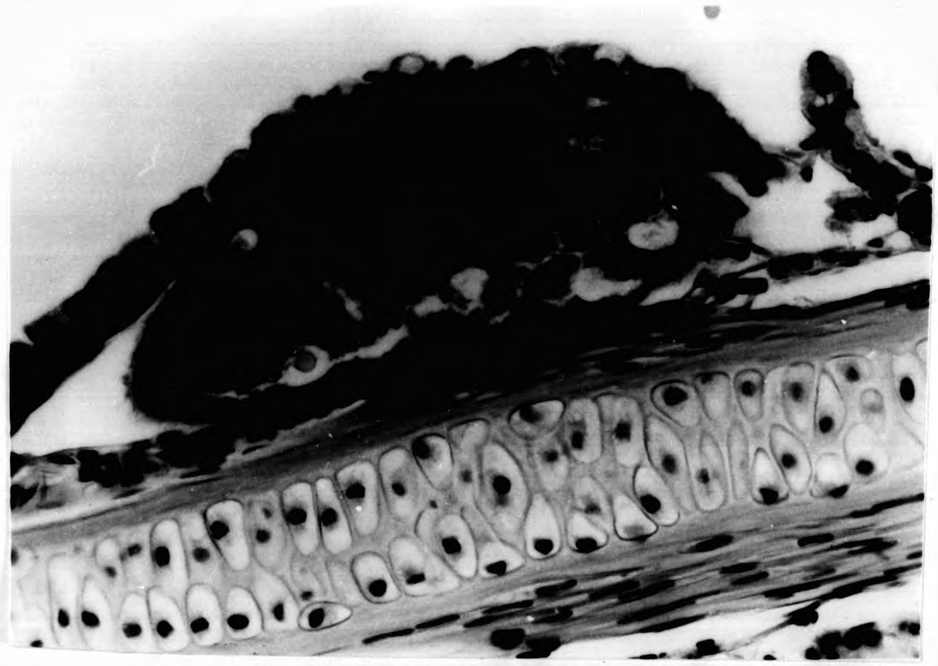
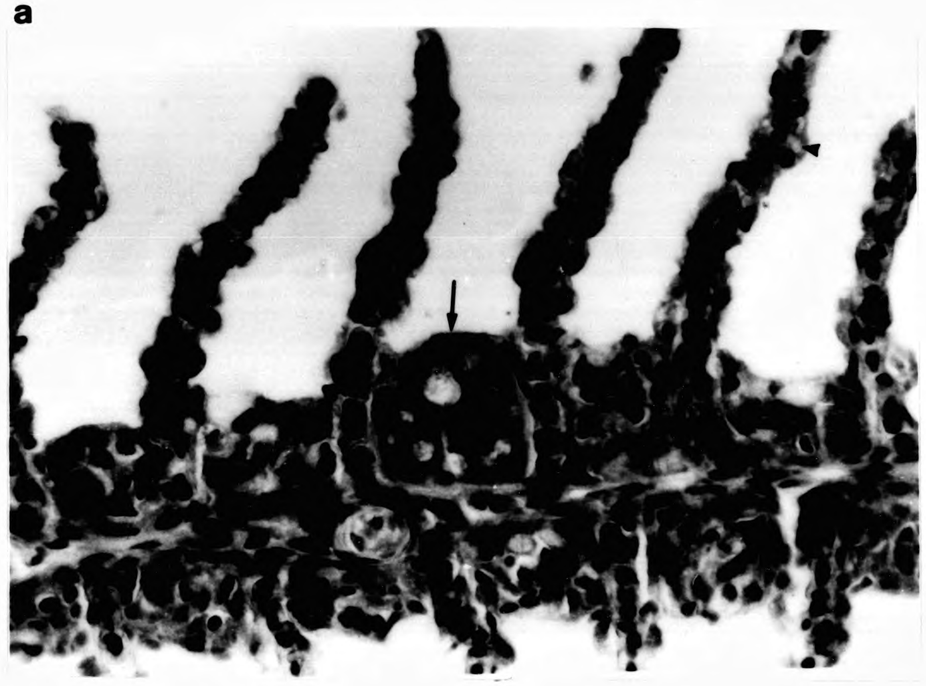


Plate 3.18 Photomicrographs showing the parasite (arrows) in the gills of carop 24 hours after the first infection exposed concurrently to the metals (Expt. 3.3.5). Note the associated cellular changes (arrow heads).

(a) 50 μgl^{-1} cadmium. (b) 50 μgl^{-1} copper. (H&E, 600X).

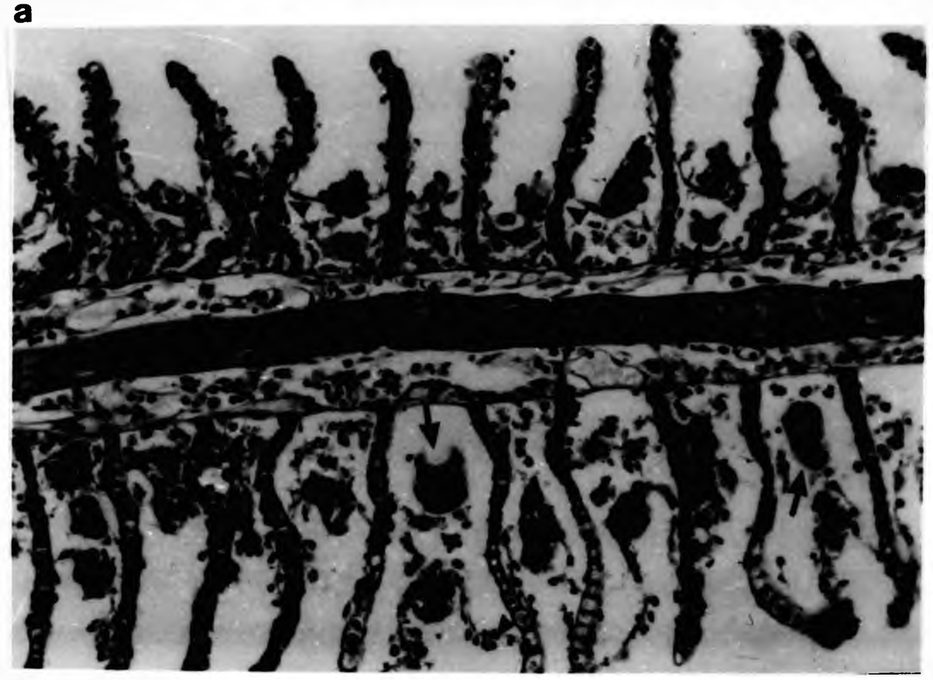
a



b

Plate 3.19 Photomicrographs showing the extensive lifting (arrow head) of the epithelial layer caused by the penetrating tomites (arrow) immediately following the second infection in the gills of (a) cadmium 50 (μgl^{-1}) and (b) copper (50 μgl^{-1}) exposed groups. (H&E, 600X).

a



b

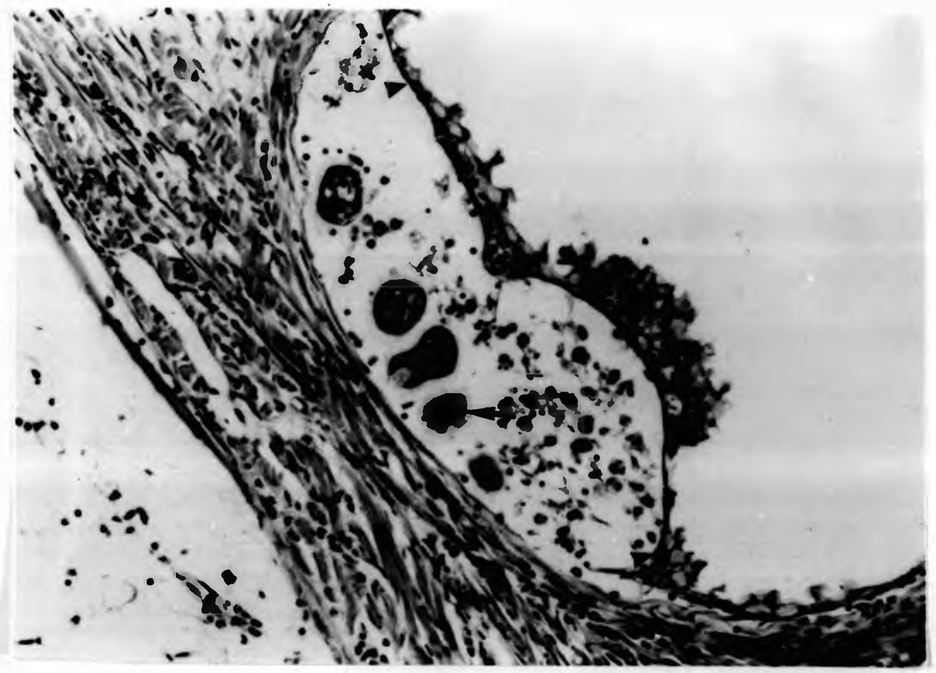


Table 3.1 Prevalence (%) and intensity of infection following final challenge infection in immunized (3 immunization exposures at 7 day intervals) and naive control fish.

Number of fish	Treatment	Prevalence	Intensity
160	immunized	< 10%	Very mild, confined to the margins of fins with none at all in gills
20	naive	100%	Heavy and produced mortality (20 to 30%).

Table 3.2 Prevalence (%) and intensity of infection in naive fish exposed to tomites during each infection in Experiment 3.3.3

Number of fish (Cadmium)	(copper)	Immunization exposure (Number)	(Days)	Prevalence	Intensity
5	5	First	0	100%	Heavy
5	5	Second	7	100%	Heavy
7	5	Third	14	100%	Heavy
7	6	Fourth	21	100%	Heavy
5	7	Fifth	28	100%	Heavy

Table 3.3 Prevalence (%) and intensity of infection in naive fish exposed to tomites during each immunization exposure followed in Experiment 3.3.4.

Number of fish (Cadmium)	(Copper)	Immunization exposure (Number)	(Days)	Prevalence	Intensity
5	5	First	0	100%	Moderate
7	5	Second	7	100%	Heavy
5	5	Third	14	100%	Heavy
6	5	Fourth	21	100%	Heavy
7	5	Fifth	28	100%	Heavy

Table 3.4 Cumulative percentage mortality in carp exposed to varying concentrations of cadmium over 15 days duration, with no accompanying "ich" infection.

	Treatment Levels					
Days	Control	$10\mu gl^{-1}$	$25\mu gl^{-1}$	$50\mu gl^{-1}$	$75\mu gl^{-1}$	$100\mu gl^{-1}$
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	10
6	0	0	0	0	0	10
7	0	0	0	0	10	10
8	0	0	0	0	10	30
9	0	0	0	0	10	30
10	0	0	0	10	10	40
11	0	0	0	10	10	60
12	0	0	0	10	20	60
13	0	0	10	10	20	60
14	0	0	10	10	40	70
15	0	0	10	10	40	70

Table 3.5 Cumulative percentage mortality in carp exposed to varying concentrations of copper for 15 days, without accompanying "ich" infection.

Time (Days)	Control	$10\mu gl^{-1}$	$25\mu gl^{-1}$	$50\mu gl^{-1}$	$75\mu gl^{-1}$	$100\mu gl^{-1}$
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	10
4	0	0	0	0	10	20
5	0	0	0	0	10	30
6	0	0	0	5	25	45
7	0	0	0	5	45	55
8	0	0	0	5	45	65
9	0	0	0	5	55	80
10	0	0	0	10	70	100
11	0	0	5	10	80	-
12	0	0	5	10	90	-
13	0	0	5	10	100	-
14	0	0	5	10	-	-
15	0	0	5	20	-	-

3.4 DISCUSSION

Standardised infection procedures are essential in studies of the present nature, in order to provide a reproducible quantification of disease susceptibility. Over the years, the experimental infection procedures available with *I. multifiliis* have been modified and standardised (Dickerson *et al.*, 1981; Houghton and Matthews, 1986; Houghton, 1987; Clayton and Price, 1988). Basically, this involves exposing the test fish to a known number of invasive tomites. Variables such as the ratio of tomites to fish, density of tomites per ml of the infecting media and the time of contact between the tomite and fish are all given due consideration. However, many of these factors do vary between different workers and different experimental conditions depending on the size and species of fish used and the nature of the experiment.

Preliminary trials in the present study showed minor variations in parasite intensity between fish infected in separate infecting tanks despite the fact that they were under similar conditions. This was the case especially when the original tomite suspension was diluted and divided into aliquots and used for infections in different tanks. To reduce this variation, the present study needed an infection procedure, wherein fishes from all the treatments were exposed evenly to the tomites with minimal or no difference in exposure between different treatment groups. To this end, it was decided to mark the fish from different treatments and expose all of them to the tomites in a single infecting tank with a large water volume. In this way it was hoped to reduce the risk of uneven exposure to tomites of fish from different treatments.

Fish disease studies in general categorize an infected individual as dead /alive or

infected /uninfected, which is several steps removed from the actual process of susceptibility /resistance to infection (Price, 1985). In the present study, the relative susceptibility of carp from different treatments to *I. multifiliis* was quantified in terms of mean parasite intensity per fish. Such a measure of susceptibility to parasitic infection allows for an accurate enumeration of parasite intensity in fish from different treatments.

Previous workers have expressed the parasite intensity per unit area by choosing to count the number of parasites on certain selected sites on the body surface or on certain fins (Hines and Spira, 1973a; Ewing *et al.*, 1982; McCullum, 1985). McCullum (1985) indicated that the average trophont densities do not differ significantly on different parts of the host's body surface. In contrast, Hines ans Spira (1973a), Kozel (1976) and Bone (1983), have consistently observed significant differences in parasite intensity between different parts of the host's body surface. Recently, Price and Bone (1985) have taken into consideration a larger surface area instead of confining the count to selected regions of known area.

Preliminary trials were carried out prior to the present study which showed that there were significant variations in parasite densities on different parts of the body surface and as well as on fins. Interestingly, in the case of the gills the parasite intensity did not differ significantly between the 4 different gill filaments within the gill cavity or between gills from two sides of the fish. Based on this consistent observation, it was decided to count all the parasites on the body surface, the fins and gills from one side instead of only enumerating from selected sites or representative surfaces of known area as other authors have done. To reduce the possible inconsistency resulting from differences in total surface areas between fish,

fish of uniform size were selected for any given experiment.

Since the completion of the present work, Clayton and Price (1988) have published a paper on standardization of the infection-response model in *Ameca splendens* using *I. multifiliis*. They have reported that the number of parasites per square centimeter is significantly higher on the fins compared to the body surface. Because of the significant variation they found in the parasite distribution in different parts of the host's body, they too have concluded that there is a need to observe most, if not all, of the body and fin area to obtain a value representative of the total infection level.

The results of the experiments clearly justified the need to count all the parasites on the various external surfaces in order to provide an accurate quantification, which is essential wherever parasite intensity is used as an index of susceptibility to parasitic infection. The same counts from the individual regions revealed a greater consistency in the parasite distribution in the gills of naive or "immunocompromised" fish than in the fins or body surface. These findings strongly support the choice of the gills as the main site for parasite enumeration, especially, in studies looking at alterations in the immune response. Other infection studies of "ich" in immune fish (Houghton and Matthews, 1986; Houghton, 1987) have observed that, if infection is present, it is invariably confined to the periphery of fins. The interesting feature which most workers have not reported is the near total absence of parasites in the gills of immune fish. These features make gills an ideal site for monitoring, especially in studies of the present nature.

In naive fish infected with *I. multifiliis* it was generally observed that gills had the largest number of parasites followed by fins and body surface. Similar observations

have been made by Hines and Spira (1973a) and Clayton and Price (1988). From the consistent observations on the parasite distribution pattern in appears that the gills were either the preferred site, or a greater number of parasites were able to successfully penetrate and establish in the gills compared to the body surface and fins in naive fish.

Naive fish exposed to lower levels (10 and 25 μgl^{-1}) of both metals did not differ significantly from the controls in terms of mean parasite intensity. However, under both the metal experiments, fish exposed to the higher metal levels (50 μgl^{-1}) for 10 days had a significantly higher parasite burden than both the controls and those exposed to lower levels of metals. In considering the relative susceptibility of different sites, gills showed very conspicuous difference between treatment groups. The gills of fish exposed to higher (50 μgl^{-1}) metal levels had nearly 3 to 4 times more parasites than the controls.

It appears from the results, that exposure of naive carp to sublethal levels of cadmium and copper can alter the relative susceptibility to experimental *I. multifiliis* infection. If susceptibility to parasitic infection were to be used as an index for the effects of metals at sublethal levels, then it appears that cadmium has a more pronounced effect on the gills than on the body surface or fins. In contrast, copper at higher levels also has serious effects on both the gills and body surface.

The reasons for the observed significant increase in the susceptibility of naive carp exposed to cadmium and copper could be several. Some of the most likely explanations are discussed below.

Both the metals at higher levels were shown to bring about several pathological changes in the epidermis and gill epithelium, which included hyperplasia,

hypertrophy, vacuolation, and even necrosis, of primarily the epithelial cells. When such fish are challenged, higher numbers of tomites may be more successful in penetrating and establishing within the damaged host epithelium compared to tomites trying to negotiate the intact primary barriers of defence, the skin and mucus layer in an healthy fish.

In many parasite species, invasion of the host and subsequent successful establishment are critical periods for survival in the parasite's life history (Ewing *et al.*, 1986). Under experimental conditions McCullum (1985) found only 20% of the tomites found a host and grew to maturity. It was seen in the present study that only a proportion of the tomites used in the challenge infection found a host and established and, it is interesting that the proportion of successful establishment was significantly higher in fish that were exposed to the higher metal levels. Houghton (1987) did not find a direct linear relationship between challenge levels and the resulting infection. Estimates such as this, which are based on initial dose levels and the mean intensity of established parasites makes comparison with other studies very difficult because of the variation involved in the infection conditions, such as the duration of challenge and tomite number per fish. However, in all cases, only a proportion of the tomites used in the challenge infection find a host and establish to maturity.

Ewing *et al.* (1986) from their study on the development of *I. multifiliis*, recognised three periods critical for survival of the parasite. The first occurred upon establishment within the host, 0 to 10 minutes post-exposure, when the parasite population which had gained entrance declined by 50%; survival from 10 to 45 minutes post-exposure was constant, but the second critical period came after the parasite left the host and during the free swimming stage. The third occurred during

reproduction. In their opinion, more than half of the mortality of the invasive stage which McCallum (1985) reported probably occurred during the first period of establishment.

Considerable reserves are likely to be spent by the tomite in entering and moving among epithelial cells. Availability of food in the form of cell debris and active feeding by the parasite, therefore, appears to have a major role in the successful establishment of the parasite. Organelles such as ribosomes and crystalline mucocytes within the parasite cytoplasm were found to be less abundant 5 minutes postexposure, than either prior to invasion, or after establishment according to Ewing *et al.* (1985). Organelle abundance is re-established by 45 minutes postexposure, which may indicate active feeding by the parasite on the debris of the disrupted host cells very soon after establishment. Histological observations of the parasite in the present study supports the findings of Ewing *et al.* (1985).

Based on the above observations, two possible explanations for higher parasite intensity in metal exposed fish can be made. Invading tomites may spend comparatively less energy penetrating a fish which has badly damaged primary skin and gill barriers and, as a consequence, more tomites may successfully establish in such a situation. The other possibility is the greater availbility of food immediately after successful invasion, in the form of cell debris, in the case of the fish exposed to higher metal levels. Spending less reserves in invading the host epithelium and getting ready access to food in the form of cell debris, may enhance the successful establishment rate of tomites in fish exposed to higher metal levels.

The argument that hyperplasia is related to increased parasite burden is often debated. There are several convincing observations from previous studies, as well as

in the present, which lend some support to this argument.

The proliferation of epithelial cells brought about by cadmium and copper at higher levels increased the thickness of the epithelium. The extent to which this increase has contributed to the parasite establishment cannot be quantified. In addition to the proliferation, there were other changes such as epithelial cell necrosis, lifting of epithelial layers, sloughing of cells and even vacuolation of mucus cells. These changes result in a breach of the primary skin and gill barriers in spite of proliferation and thickening. Therefore any increase in thickness of epidermis or epithelial layer may not jeopardize the ability of invasive tomites to penetrate, contrary to the opinion of Ventura and Paperna (1985).

Increased surface area due to hyperplasia following copper exposure was believed by Ewing *et al.* (1982) to offer more accessible and suitable sites especially in the gill tissue for the parasites to settle and successfully establish. It is generally believed that hyperplasia results in a decrease in overall surface area. This was also suggested by Hines and Spira (1973a) to explain the rapid increase in parasite number on gills during the second generation infection which they attributed to epithelial proliferation of the gill tissue as a result of first infection. During sexual maturation in salmonids, the epidermis and the underlying dermal tissue of both sexes increase in thickness in response to steroid hormones secreted by the gonads and the number of mucus secreting goblet cells decrease in the males (Pickering 1978; Pottinger and Pickering 1985a, b). Very interestingly coincident with these changes was an increase in the incidence of fungal infections (Richards and Pickering, 1978) and infestation by *I. multifiliis* and *Scyphidia sp.* and by the monogenetic trematode *Gyrodactylus sp* (Pickering and Christie, 1980). However, other physiological changes occuring due to gonad development and spawning in particular may have a greater influence on susceptibility to ectoparasitic infection.

Irrespective of other mechanisms involved in the susceptibility of fish to ectoparasites, these studies apparently lend support to the argument that hyperplastic tissue may have an influence on the ectoparasite densities on fish.

The skin mucus of fish is believed to play a very vital role in the natural defence against parasites and pathogenic microorganisms (Fletcher, 1978; 1981; Ingram, 1980; Pickering and Richards, 1980; Roberts 1989). The defence may be purely mechanical, due to the continuous production of the mucus (Pickering, 1974). However, the presence of lysozymes (Fletcher and Grant, 1968; Fletcher and White, 1973), complement components (Nelson and Gigli, 1968; Harrel *et al.*, 1976) and antibodies (Fletcher and Grant, 1969; DiConza and Halliday, 1971; Harris *et al.*, 1973) of the immunoglobulin IgM type (Bradshaw *et al.*, 1971; Ourth, 1980) suggest that skin mucus takes an active part in the defence system of fish. Stressors which affect mucus production will obviously interfere with its protective role (Fletcher, 1981; Ellis, 1981).

Mucus cells respond to external irritation caused by a wide variety of agents including heavy metals. Proliferation and copious secretion of mucus would be the normal initial response. However, if the irritant/stressor is persistent (chronic), the chemical nature and the amount of mucus secreted may change. Both cadmium and copper exposure for 10 days brought about changes in the mucus cells. The most common change observed was the vacuolation of mucus cells but higher metal levels were even seen to cause necrosis of mucus cells. These changes would certainly have a direct effect on the amount of mucus produced. Changes in the chemical nature of the mucus under stress have been documented by Fletcher *et al.* (1976) who exposed plaice to 2 ppm cadmium; there were no changes in the sulphated glycoprotein of the skin mucus but the proportion of acid glycoprotein in

the gill filaments increased from 48 to 88%. Such changes are believed to affect the viscosity of the mucus which may have an indirect effect on the protective ability of the mucus.

In the light of this information, the observed effects of cadmium and copper on mucus cells could definitely have contributed to the increased susceptibility of naive carp to *I. multifiliis* infection in these experiments. However, Pickering and Christie (1980) did not find any significant relation between demucification and ectoparasitic infection in male salmonids, but they are of the opinion that loss of goblet cells may exacerbate the existing parasitic infection.

Increased susceptibility, especially, to bacterial and viral diseases has frequently been attributed to altered immune respose (O'Neill, 1981b; Baker *et al.*, 1983; Peters *et al.*, 1988). A certain amount of caution has to be exercised before choosing this line of explanation in the present study because of the nature of *I. multifiliis* and its interaction with the host. Immunity is an important physiological mechanism in animals for protection against infection and disease. It can be either a non-specific immunity, which is an innate defence mechanism rendering the hosts resistant to infection, or an acquired, specific process which is induced in response to a foreign agent (Ingram, 1980; Ellis, 1988).

Heavy metals have been shown to affect the phagocytic ability of fish macrophages (Robhom and Nitkiwoski, 1974; O'Neill, 1981a; Elsasser *et al.*, 1986) and even reduce the chemotactic migration of inflammatory cells. Effects like these could result in the loss of ability of phagocytic cells to clear the pathogens and may have contributed to the increased susceptibilitiy to systemic bacterial pathogens observed by several workers in metal exposed fish (Sugatt, 1980; Knittel, 1981; Baker *et al.*,

1983). There is no evidence for macrophage involvement in phagocytosis of either live and/or lysed tomites of *I. multifiliis* in a naive fish and, moreover, the size difference between the macrophages and the invasive tomite stage is such that they are unlikely to be effective. It seems unlikley therefore that cadmium and copper influence the susceptibility of carp to *I. multifiliis* infection by affecting the phagocytic ability of macrophages.

Graves *et al.* (1985b) have demonstrated the possibility of activation and mobilization of non-specific cytotoxic cells (NCC) from the head kidney of "ich" infected channel catfish. They have suggested the involvement of these cells in antibody mediated cytotoxicity in lysing the invading tomite stages in the integument and gill epithelium. These NCC's require contact between effector and target cells to produce lysis and, in addition, optimal killing requires a target cell immobilization and a cytotoxicity period of 10 hours or greater. From this it appears that NCC's may play a more active role in killing the tomites in a fish which is immune to "ich", rather than in a naive fish. Although NCC's have been indentified in carp, there is no recorded evidence of their involvement in "ich" infected fish.

To date, there is no recorded histological evidence of either dead or lysed parasites in the epithelium of a naive host fish. Moreover, there is no evidence for the presence of any mechanism(s), either specific or non-specific, in a naive fish to kill the invading stages of the parasite outside or inside the host epithelium. Therefore it is stongly felt that the increased susceptibility observed in naive carp exposed to higher levels of cadmium and copper was a direct repurcussion of the structural damages brought about by these metals.

The inability of previously immunized carp to mount a protective immune response

following ten days exposure to the metals however throws light on the immune response of carp to *I. multifiliis* in relation to protective immunity, and demonstrates the immunosuppressive effects of cadmium and copper at sublethal levels.

The fish used in the present study were immunized by 3 successive immunization exposures at 7 day intervals and fish were confirmed immune by a final challenge infection. This immunization schedule differed from that of Houghton (1987) with regard to the time interval between successive immunization exposures. However, the immunization results indicate that carp can respond immunologically to the antigenic stimulus administered in the form of relatively low infection doses, by eliciting an immune response which protects them against subsequent infection. This observation is in accordance with previous findings (Hines and Spira, 1974b; Houghton, 1987). Immune fish did occasionlly have a few parasites following an infection, but these parasites were normally confined to the periphery of the fins and, interestingly, the gills would be free of parasites. The relative proximity of the sites to the vascular system has been suggested as the reason for this type of parasite distribution in an immune fish (Hines and Spira, 1974b; Houghton, 1987).

All the naive fish introduced as negative controls during the challenge infection became infected, illustrating that carrying out infections where both naive and immune fish are exposed to the tomites together does not have any effect on the ability of tomites to infect a naive fish. If immune fish had been releasing any anti-parasitic factors in the mucus, then it would have impaired the tomites in the infecting medium.

Cadmium and copper exposure for 10 days had a significant effect in bringing about suppression of the protective immune response in carp to *I. multifiliis* infection.

Immune fish exposed to higher cadmium levels (25 and 50 μgl^{-1}) developed heavy infection and gills appeared to be the worst effected. Controls, and those exposed to 10 μgl^{-1} cadmium, showed little effect. Surprisingly, immune fish exposed to higher cadmium levels had significantly higher parasite intensity than even the naive controls. In the copper experiment, however, the overall parasite intensity was significantly lower than naive controls. Immune fish exposed to all the three copper concentrations had developed the infection, but the intensity was very low. Moreover, fins and body surface had more parasites than gills, a feature consistent with "ich" immune fish. The reasons for this difference between cadmium and copper treatment is not evident.

In the light of this result, an unexpected finding was that cadmium and copper exposure did not have any significant effect in suppressing the serum tomite agglutination titre. The titre was relatively high in all the treatments and, interestingly, the titre did not differ statistically before and after exposure to the metals. The contrasting picture of immune carp exposed to metals becoming susceptible to infection in spite of having high serum tomite agglutination titre, strongly suggests that humoral immunity may not be directly involved in the execution of the protective immune response of carp to *I.multifiliis*. These observation appear to be important in the light of the findings of Houghton (1987) who administered exogenous corticosteroids and found suppression of the protective immunity of juvenile carp to *I. multifiliis* without impairing the humoral immune respose. These results suggest that the components of the cell mediated immune response may play a more important role than thought by earlier workers (Hines and Spira, 1974; Wahli and Meier, 1985) in the immune response of fish to *I. multifiliis*.

It is difficult to precisely pinpoint the reasons for and explain the mechanisms involved in the observed immunosuppressive action of cadmium and copper. The immune response of fish to *I. multifiliis* is still poorly understood. The actual mechanism by which an immune fish is able to either prevent the tomites from gaining entry into the epithelium or stop its establishment after invasion is still not very clear. Based on indirect evidence and drawing inferences from published literature, some possible mechanisms for the observed immunosuppression can be postulated.

The arguments presented earlier in an attempt to explain the increased susceptibility to *I. multifiliis* infection of naive carp exposed to cadmium and copper could also have played a very vital role in this situation. However, the important question of interest is, how could the metals have impaired the execution of the protective response, in spite of the fish having a high tomite agglutination titre in their serum?

The immune response of fish to *I. multifiliis* is very exciting from the immunological viewpoint because of the unique relationship between the parasite and its host. The parasite resides exclusively in the epithelial layer of the fish and has never been reported to penetrate the basement membrane (Ventura and Paperna, 1985; Ewing and Kocan, 1986). A parasite residing in the integument does not come into contact with the vascular system, but infiltrating leucocytes or granulocytes may come in contact with the parasite in response to the injury. On the other hand, parasites in the gill epithelium lie in very close proximity to the vascular system (Ewing and Kocan, 1986). The mechanism by which the immune system of the fish recognises and responds to the parasite are still unknown. All the evidence regarding this is indirect.

The leucocyte response of carp to *I. multifiliis* infection involves an initial but temporary drop in lymphocytes with a concurrent increase in neutrophil and fine reticular cell population early in infection (Hines and Spira, 1973b). The serum proteins found in the mucus of carp after infection was thought by Hines and Spira (1974b) to render the immune fish refractory to infection. But, because of the complete lack of infective stages and developing parasites in an immune fish the same authors (Hines and Spira, 1974b) suggested that the barrier to infection operates, at least partially, in the mucus. Support for the involvement of humoral antibodies also came from Goven *et al.* (1980) who postulated that immunization stimulates the production of agglutinating antibodies which are concentrated in the external mucus, and on contact with such mucus, the tomites are immobilized and prevented from penetrating the immune host.

The sera from "ich" immune fish specifically agglutinates the invasive live tomite stage of *I. multifiliis* (Houghton and Matthews, 1986; Houghton, 1987; Clark *et al.*, 1987, 1988; Mohan and Sommerville, 1989a). Anti-ich sera raised in rabbits (Clark *et al.*, 1987, 1988; the present study) agglutinates the live tomites in a manner very similar to that observed in fish "immune sera" and this strongly suggests that there is an interaction between serum antibodies and the parasite surface.

The work of Graves *et al.* (1985a, b) has thrown some light into the possible mechanisms which may be involved in an immune fish by which it prevents infection. The mobilization and activation of NCC's from the head kidney of "ich" infected channel catfish and the ability of NCC's to cause lysis of *T. pyriformis* and *I. multifiliis in vitro*, has lent support to their hypothesis that NCC's migrate to sites of infection through the vascular system, or the precursor cells present in the peripheral circulation become activated, and are mobilized in the integument during an infection. From their suggestion it would appear that the invading tomites are

immobilized by the antibody allowing the NCC's to bring about lysis. NCC's have been documented in the head kidney, spleen and peripheral blood of several fish species such as carp (Hinuma *et al.*, 1980), channel catfish (Evans *et al.*, 1984a, b; Graves *et al.*, 1984), and rainbow trout (Moody *et al.*, 1985).

Controversy still surrounds the issue as to whether tomites penetrate the immune fish or not. The present study, did not find any histological evidence for the presence of any whole or dead (lysed) tomites in the gill epithelium of immune control carp. However, Houghton (1987) and recently Cross and Matthews (1989b) have reported observing the tomites penetrating the epidermis of immune juvenile carp. Their observation lends support to the argument for the presence of some special cellular mechanisms in the immune fish, where cytotoxic cells in the integument and the gill epithelium, in association with serum antibodies, may bring about lysis of tomites.

In the light of these observations, it can be said that cadmium and copper possibly suppress aspects of the cellular response required for protection against *I. multifiliis* in immunized carp . Further studies in this direction may help to pinpoint the suppressive effects of metals and at the same time clarify doubts as to whether tomites are being prevented from invading the fish host or whether they are lysed following invasion into an immune fish.

From the changes in the population of tissue eosinophilic granular cells (EGC) in the gill epithelium of "ich" infected and immune fish observed in the present study, it is tempting to suggest that there is a relationship between these cells and the response of fish to *I. multifiliis* infection. Uninfected control fish had a normal population of EGC confined to the base of the gill filaments in the gill septal

region. These cells were observed to proliferate and migrate along the axis of the primary lamellae in response to either "ich" infection or metal exposure. The EGC's appeared round, intact and densely granulated in an immune fish. In histological observations one-two days after challenge of immune fish, the cells appeared vacuolated, had lost their integrity, were less densely granulated and gave the appearance of a degenerating cell. The granules could be seen outwith the EGC's, amongst the epithelial cells. However, there is no evidence to indicate that the observed phenomenon was a result of degranulation in an immune fish in response to infection.

The changes and features observed in the immune fish exposed to metals for 10 days and subsequently infected were very interesting. Immune controls and fish exposed to the lower levels of metals which were refractory to the infection challenge, had large numbers of vacuolated, lightly granulated and the degenerating EGC's in addition to several intact round cells. Fish exposed to higher levels of cadmium which were infected in spite of having high serum agglutination titre, had numerous EGC's but the majority of them were round, intact, densely granulated, with no sign of degeneration or degranulation.

The literature concerning eosinophils in fish, especially eosinophilic granular cells of the tissue, is contradictory and confusing (Ellis, 1982). These cells normally were round to oval with a marginally placed nucleus, densely packed with highly eosinophilic granules and staining strongly positive with PAS, indicating a predominace of mucopolysaccharides and a glycoprotein content in the granules. They also stained negatively with Alcian blue, indicating the absence of acid mucopolysaccharides. Increased proliferation and infiltration of EGC to sites of injury (Roberts, 1972; Roberts *et al.*, 1974) and their association with ectoparasitic

infections (Hornich and Tomanek, 1983) indicate that they may play a vital role in protecting vulnerable areas of the fish from the damage of ionic, osmotic and pathogenic agents.

The EGC's in the stratum granulosum of the rainbow trout intestine has been considered as analogues of mammalian mast cells (Ellis, 1985). Following injection of *A. salmonicida* toxins into rainbow trout, Ellis (1985) found a coincidental decrease in the histamine content of the gut and a degranulation of the EGC. In the mammalian mast cell, degranulation is mediated by various mechanisms, including specific sensitization with the immunoglobulin E (IgE) and non-specific mediation by activated complement components. Little is known regarding the mechanisms of degranulation of EGC's in fish (Ellis, 1989). Whatever their role, the morphological differences in the EGC's seen in the control and the metal exposed fish in the present study suggest that cadmium has some effect. This study does not confirm whether metals interfere with the process of activation and/or degranulation of these cells in response to infection in an immune fish, however, it would seem to be an interesting aspect to pursue.

Mucus cells on their own, and possibly as outlets for the serum antibodies, to the exterior were thought to be important in the protective response of immune fish to *I. multifiliis* by Hines and Spira (1974b) and Graves *et al.* (1985a, b). Serum and mucus of rainbow trout were found to have different levels of "ich" immobilizing antibodies by Wahli and Meier (1985) and the anti-parasitic activity of the mucus was greatest during an infection and was least following the disappearance of the parasite. In the serum it was the reverse i.e highest after the disappearance of the parasite and lowest during the acute infection. As to why different levels of antibody are present in mucus and serum during different phases of the infection is

not known. Houghton (1987) looked at the proliferative response in the lymphoid organs during different phases of the infection and failed to find any relationship between proliferative activity in the kidney and immunization. This lends support to the argument of localized antibody production by certain cells in the integument and epidermis. Pursuing this line of approach may be rewarding, in view of the present finding regarding the non-involvement of humoral antibodiy in the protective immune response of carp.

The secretion of antibody in the skin mucus (Fletcher and Grant, 1969; Bradshaw *et al.*, 1971; DiConza and Halliday, 1971; Harris *et al.*,1973; Harrel *et al.*, 1976; Ourth, 1980) strongly suggests a defensive role for mucus. A localised production of antibody from specialised, stimulated antibody producing cells within the epidermis, is the likely explanation for the origin of antibody in mucus. The demonstartion of antibody producing cells in the epidermis of rainbow trout (St.Louis-Cormier *et al.*, 1984; Pelletero and Richards, 1985) the presence of lymphocytes in the skin of carp (Hines and Spira, 1974a; Ventura and Paperna, 1985), rainbow trout (Pickering and Richard, 1980), channel catfish (Ourth, 1980) and in other fish species support the view of localised antibody production in the epidermis.

Exogenous corticosteroids have been shown to induce immunosuppression in carp to *I. mutifiliis* (Houghton and Matthews, 1986) and *Trypanoplasma borreli* (Steinhagen *et al.*, 1989), in rainbow trout to *Cryptobia salmositica* (Woo *et al.*, 1987) and to the PKD causative organism (Kent and Hendrick, 1987). Enhanced reproduction of parasites in immunosuppressed fish was observed by Woo *et al.* (1987) and Kent and Hendrick (1987). This observation is very interesting in view of the recent report on the ability of *I. multifiliis* to reproduce within the host tissue (Ewing *et al.*, 1988).

The suppression of the humoral immune response may, to a certain extent, depend on the time interval between antigen administration and exposure to the stressor in question. In this study, fish with well established humoral antibodies were exposed to the metals and this did not have any effect in lowering the antibody titre. This is similar to the results obtained when corticosteroids were administered 6 days after vaccination of trout with *A. salmonicida* (Chen *et al.*, 1983). A time gap of less than 24 hr between antigen exposure and corticosteroid administration did however suppress the humoral immune response to protozoan (Woo *et al.*, 1987), bacterial (Anderson *et al.*, 1982) and viral (Wechsler *et al.*, 1986) antigens. It appears that, when sufficient time is given for lymphocyte activation and proliferation to occur following immunization, the humoral response may not be suppressed by subsequent external stressors.

The results of the present study clearly demonstrate that caution must be exercised in drawing firm conclusions from experiments based on one facet of the immune response.

From the foregoing discussion it is strongly felt that the type of immunosuppression observed in the present study could be the combined result of structural damages, impairment of the cellular immune response and the possible interference of metals with the execution of the protective response which requires the cooperation of both cellular and humoral factors. From an immunological point of view, the effect of metals on cells such as NCC's, EGC's and mucus cells deserves further attention.

The ability of sublethal levels of cadmium and copper to modulate the acquired immunity and the kinetics of the humoral antibody production to *I. multifiliis* in carp was demonstrated in the present study. The results demonstrate that carp

infected three to five times at regular intervals with low doses of tomites acquire resistance and this degree of resistance was closely related to the level of serum tomite agglutination titre. McCallum (1986) observed partial resistance in black mollies after a single infection with *I. multifiliis*, and did not find any relation between the degree of resistance and the intensity of first infection. The ability of sera to agglutinate live tomites presumably reflects an interaction of serum antibodies with components of the parasite's surface. However, the role of anti-ciliary antibodies in protective immunity remains unclear (Clark *et al.*, 1988).

In the control fish and those exposed to the lowest concentration of both cadmium and copper, the prevalence and the intensity of infection decreased rapidly with successive immunization exposures and the fish were completely refractory to the 4th infection. Relatively high serum tomite agglutination titres were seen by the end of 3rd infection. In view of the finding a lack of protection in fish with high serum titre under metal exposure it is difficult to comment on the nature of the possible relationship between serum tomite agglutination titre and the acquired resistance.

As early as 1953, Bauer found that fish exposed to only one parasite generation acquired partial resistance, but still developed parasite burdens of around one tenth of the first. The kinetics of humoral antibody titre, the development of acquired resistance and the duration of the immune respose in juvenile carp at 22°C have been investigated in greater detail by Houghton (1987) using standard immunization exposure regimes. She used 3 immunization exposures at 14 day intervals and a final challenge 28 days after the last exposure. From her work the serum tomite agglutination titre appear to be high even after 3 infections. Thus the kinetics of the tomite agglutination titre observed in the controls in the present study, is in agreement with the findings of Houghton (1987), inspite of differences in the

immunization regime, fish size and temperature. Higher temperature, bigger fish size and continuous antigen stimulation, appear to be the main contributory factors in the higher titres observed in the present study which achieved immunization after only 2 or 3 exposures administered at 7 day intervals.

Hines and Spira (1974b) found mirror carp to be totally refractory to infection after 21 days. Since the fish in their experiments were maintained continuously in an infectious environment, it is difficult to determine the number of times they would have been infected. As their experiments were run at 20-23°C, it can be estimated that the fish would have been exposed at least to 3 or 4 infections within that 21 days. This, therefore, would appear to confirm the present findings.

This investigation has shown that carp can acquire total resistance after three successive, low level infections. However, the interval between infections, temperature and the level of immunization infection may have an influence on the duration of acquired resistance.

The present study did not attempt to define the duration of resistance. Immune mirror carp were found to remain refractory to infection for as long as 8 months when maintained under continuous infectious environment. In contrast, it lasted for only 35 days when maintained in parasite free conditions (Hines and Spira, 1974b). Houghton (1987) found antibody levels to be sustained for at least 12 weeks, however, she found that the acquired protective immunity declined after 2 months.

Higher concentrations of both cadmium and copper (25 and 50 $\mu g l^{-1}$) delayed the ability of carp to acquire resistance and develop humoral tomite agglutinating antibody. The results revealed that carp can acquire, from partial to complete

resistance to *I. multifiliis* infection and also produce relatively high serum tomite agglutinating antibody in the presence of sublethal levels of cadmium or copper. The most interesting observation is the time taken to acquire this immunity. Fish exposed to cadmium and copper did not become totally refractory to the infection until almost towards the end of 4th and 5th immunization infections. Thus, metal exposure appears to have delayed certain stages in the immune response, thereby causing the fish to require a longer time than controls to acquire the resistance. As with the controls, the number of immunization exposures had a direct relationship to the increase in the serum tomite agglutination titre and the acquired resistance. Unlike controls, the prevalence of infection and parasite intensity in cadmium and copper (25 and 50 μgl^{-1}) exposed groups remained high till the end of the 3rd infection and only started to decline thereafter.

In considering the tomite agglutinating antibody response, it becomes clear that continuous exposure to the metals concurrently with the series of immunization exposures did not alter the kinetics of humoral tomite agglutinating titre in carp but caused a delay in the response. It was also evident that cadmium and copper exposure of previously immunized carp, did not lower the established anti-ich antibody titre.

Carp maintained continuously in an infectious environment in the presence of cadmium and copper acquired partial to complete resistance, but took a longer time compared to the controls. The higher levels of cadmium and copper suppressed the protective immune response of previously immunized carp, possibly by affecting the cellular responses. The findings here suggest that exposure to low levels of metals over a long period may not inhibit the development of partial or total resistance provided the fish is continuously exposed to a low level of infection.

Metals have been shown to induce perturbations at various stages of the immune response (Ellis, 1981; Zeeman and Brindley, 1981; Anderson *et al.*, 1984) which in general includes recognition and processing of antigenic material, activation and proliferation of both B and T-like lymphocytes, antibody production, and finally the execution of the protective response. Very little is known about the cellular requirements for optimal expression of humoral immune response to *I. multifiliis*. The relationship between humoral "anti-ich" antibody and the protective immune response in fish is still confusing and contradictory. From the high serum antibody titres observed in the present study one may infer, at this stage, that cadmium and copper at the levels tested are not affecting the process of the humoral immune response of carp to *I. multifiliis*.

The final execution of the defence mechanism in response to infection requires the cooperation of both humoral and cellular comoponents of immunity. As discussed in the previous sections, the actual mechanism as to how immune fish prevent the invasion of tomites or kill the tomites after invasion, is still unclear. In summary, then, the evidence here strongly suggests that elicitation of such a response against invading *I. multifiliis* tomites requires not only the cooperation of cellular and humoral factors but also non-specific components of the defence system, such as mucus cells, and possibly other specialized cells such as EGC's and NCC's in the integument and gill epithelium.

From the direct and indirect evidence, it is tempting to conclude that cadmium and copper interfere either with cellular components of the immune response or with an array of non-specific defence components, and thereby prevent an efficient execution of the protective mechanism. On the other hand, carp maintained continuously in an infectious environment in the presence of cadmium and copper do develop partial to

complete resistance which is delayed in those fish exposed to higher levels of metals.

Further studies are required to elucidate several of the questions raised in this series of experiments: (i) how do metals suppress the protective immune response? (ii) how is the fish able to acquire resistance to this parasite in the presence of the metals? (iii) what is the relationship between humoral antibody titre and protective immunity? (iv) what is the role of specialized cells such as NCC's and EGC's in relation to the immune response of fish to *I. multifiliis* infection?

In the final experiment, the role of pre-existing parasitisation on the resistance of the host to cadmium and copper was evaluated by monitoring the mortality produced as a result of the interaction. To this effect, higher concentrations of metals and challenge levels were used.

The results clearly illustrated that pre-existing parasitisation can have a significant effect in lowering the resistance of carp to both cadmium and copper toxicity. As would be expected, the mortalities were significantly higher in fish which were both infected and exposed to the metals, compared to fish exposed to either metals or parasites alone. The most interesting feature was the actual time of occurrence of the highest mortality. In both the metal experiments, specific stages of the parasite life cycle had an overriding influence in bringing about mortality in conjunction with the metals.

The peaks in mortality in all the concentrations in both cadmium and copper experiments were very closely related to 2 distinct stages of the parasite; namely emergence of the trophonts from the host epithelium and the tomite invasion of the

second round of infection (simulated reinfection). The time and concentration of metals required to produce mortality were shown to be dramatically reduced during these critical periods of infection.

The severe pathological changes produced by the invading tomites in the process of penetration and subsequent movement in the host tissue epithelium before settling, together with the damage brought about by the emerging adults were crucial from the host's viewpoint. The study clearly showed that during these stages of infection, the fish was very vulnerable, to low levels of metals, and would be expected to be similarly vulnerable to any other pollutant or adverse environmental condition.

Few studies have documented the role of pre-existing parasitisation on the resistance of fish to pollutants. However, in all these studies, only the the presence/absence of parasites or parasite load/density was taken into consideration. The increased susceptibility of parasitizied fishes to DDT (Perevozchenko and Davydov, 1974), zinc (Boyce and Yamada, 1975) and cadmium (Pascoe and Cram, 1971) was attributed to the lowered health status of the fish due to the effects of the parasite. In all these cases fish were parasitised by larval cestodes. The present study differs considerably from the published work in that here, a parasite with a short, direct life cycle was used and the role of the different stages in the parasite development was evaluated in relation to their effect on the resistance of carp to metal pollutants.

The interpretation of results from the present study must also take into account the effects of metals on the different stages of the parasite and their development, especially the stages outside the fish host. The metals were not observed to have any effect on the established parasites on the fish host. However, low levels of cadmium and copper did kill the free swimming stages of the parasite.

CHAPTER 4

MODULATORY EFFECTS OF CADMIUM AND COPPER ON THE PRIMARY AND SECONDARY HUMORAL IMMUNE RESPONSE OF CARP, *C.carpio* TO SHEEP RED BLOOD CELLS (SRBC).

4.1 INTRODUCTION

Heavy metals are known to interfere with the mechanisms of disease resistance in mammals (Koller, 1980) and fish (Zeeman and Brindley, 1981; Anderson *et al.*, 1984). Many of the heavy metals which compromise the immune system of mammals may do so by damaging a particular cell type such as B and T-lymphocytes or macrophages, or by interfering with cell(s) which regulate the proliferation and differentition of other cells responsible for the normal functioning of the immune system (Koller, 1984).

Heavy metals have been reported to alter the susceptibility of fish to bacterial (Rodsaether, *et al.*, 1977; Hetrick, *et al.*, 1979; Sugatt, 1980; Knittel, 1981; Baker, *et al.*, 1983; MacFarlane, *et al.*, 1986), viral (Hetrick, *et al.*, 1979) and parasitic (Ewing *et al.*, 1982) diseases. Immunosuppression induced by the metals has often been postulated as the responsible factor in such increased susceptibility to disease. Heavy metals can also compromise the non-specific components of the body's defence system and contribute to increased disease susceptibility in naive fish as suggested in the previous experiments (Chapter 3). It has also been shown that metals induce immunomodulation to bacterial (Roales and Perlmutter, 1977; Sugatt, 1980; Cossarini-Dunier *et al.*, 1988; Thuvander, 1989), viral (O'Neill, 1981a, b), and other antigens (Viale and Calamari, 1984) in fish.

Upon close examination of the literature it becomes apparent that comparison of the results in Chapter 3 with other work is difficult because of the different immunization and metal exposure protocols various authors have followed. Some of the protocols followed included immunizing and exposing the fish to metals

simultaneously (Roales and Perlmutter, 1980; Sugatt, 1980), short or long-term exposure to the metal prior to immunization and continuing the metal exposure for varying duration (O'Neill, 1981a; Viale and Calamari, 1984; Cossarini-Dunier *et al.*, 1988; Thuvander, 1989) or exposure to the metal after three immunization inoculations (O'Neill, 1981b). The different heavy metals used in these studies in conjunction with a wide variety of antigens, reduces the possibility of satisfactorily attributing the suppression of immune response to a particular effect of the metal concerned. The number of times the antigen was administered also varies in these studies. Some studies have administered the antigen twice (primary and secondary injection) while others have given three to five injections in the course of the study.

Uncertainty surrounds several areas of fish immunology, especially with regard to the heterogeneity of lymphocytes and the cellular requirements for optimal immune response to most antigens. The immunomodulatory effects of heavy metals or any other toxicants, therefore, cannot be attributed with certainty to a particular effect on cell(s) required for the immune response. A brief comparison of the immune system of fish with mammals is, therefore, discussed below to highlight the various stages involved in the immune response and to point out the likely stages where toxicants and/or stressors such as cadmium and copper could exert their detrimental effect.

The immune system of fish is complex as it is with other animals. Optimal expression of the immune system requires the cooperation of humoral, cell mediated and non-specific components. The B-lymphocyte is the cell directly responsible for humoral immunity by differentiation to antibody producing plasma cells after antigen stimulation (Anderson *et al.*, 1984).

Introduction of antigen into a host results in direct stimulation of lymphocytes or

antigen may be intially phagocytized by macrophages which transfer the antigenic determinants to virgin lymphocytes. Lymphocytes are capable of recognising the antigen through specific antigen receptors on their surface. The antigen binds to the surface antibody of lymphocytes and stimulates the small lymphocytes to divide and differentiate. These specific sensitive lymphocytes constitute a clone (Ellis, 1988). On stimulation such clones proliferate and differentiate into daughter cells with a specific function, depending on the population to which the clone belongs (Ellis, 1988).

There are two main populations of lymphocytes in mammals, the thymus derived T-cells and the bone-marrow (bursa of Fabricius in birds) derived B-cells. Uncertainty still surrounds the heterogeneity of lymphocytes in fish. However, evidence from studies based on the presence or absence of surface immunoglobulin (DeLuca *et al.*, 1983), surface markers (Cuchen and Clem, 1977), mitogen responsiveness (Warr and Simon, 1983) and hapten-carrier effect (Miller *et al.*, 1985) provides strong evidence for the presence of both B-like and T-like lymphocytes in fish (Ellis, 1989).

On primary exposure to an antigen the lymphocytes proliferate. Sensitized B-lymphocytes progress through a series of transformations into large lymphocytes and finally lymphoblasts, which differentiate either into antibody producing plasma cells or memory cells. The memory cells possess surface immunoglobulin receptors similar to the original lymphocyte while plasma cells synthesize and secrete circulating antibody. Antibody response to many antigens (Thymus dependent) require cooperation between B-cells and T-cells for optimal expression. T-lymphocytes on stimulation proliferate and differentiate into different types of daughter cells each having a specific function. There are four types of T-cells recognised in mammals: cytotoxic, helper, amplifier and suppressor T-cells. All but

cytotoxic lymphocytes function in regulating the humoral immune response. The T-helper cell is essential in the original cooperation required for the stimulation of B-cells to many antigens. Amplifier cells regulate the proliferation of activated B-cells while suppressor cells suppress antibody synthesis. In general B-cell activity is regulated by a fine balance of helper and suppressor T-cells. T-helper cells in addition to cooperating in the original stimulation are also thought to proliferate into long lived helper memory cells which are required to cooperate with increased number of B-memory cells on subsequent exposure to the same antigen (Ellis, 1988).

Macrophages are a vital component in humoral immunity. In mammals macrophages have receptors for complement, and the Fc portion of immunoglobulin (IgG). They function to localize the antigen and present it to antibody producing cells. Macrophages are also known to process the antigen and make it more immunogenic before presentation to antibody producing cells.

The cooperation between the three components of the immune system, the macrophages (Antigen recognition, processing and presentation), T-lymphocytes (helper, suppressor and amplifier activity) and B-lymphocytes (antibody production) may involve direct cell to cell contact or through soluble factors such as lymphokines produced by stimulated T-cells. Lymphokines and cytokines are communicators between macrophages and the T and B lymphocytes and these factors have been reported to occur in fish (Smith and Braun-Nesje, 1982).

In mammals the stimulated B-lymphocytes transform through a proliferative phase (the pyroninophilic cells) to plasma cells (antibody producing cells). Such a response has been observed in carp following immunization (Secombes *et al.*, 1982a, b). It is

thought that such pyrininophilic cell clusters eventually develop into melanomacrophage centres (MMC) in fish. The MMC in spleen and anterior kidney are areas where macrophages assemble in discrete groups and where phagocytosed material is catabolized, remobilized or deposited (Agius, 1985). The presence of antigens, antigen-antibody complexes and homing of small lymphocytes in the MMC of spleen and kidney suggest that they are actively involved in the humoral immune response and are regarded as phylogenetic precursors of germinal centres in higher vertebrates (Ellis, 1989).

The initiation and control of a specific antibody response to an antigen also depends on whether it is thymus dependent (TD) or thymus independent (TI). In mammals the antibody response to TD antigens require the cooperation of B lymphocytes, T helper cells and macrophages while for TI antigens, only B lymphocytes and macrophages are required (Roitt *et al.*, 1986). Thymus dependency of antigens in fish is not clear. However, putative mammalian TD and TI antigens have elicited comparable antibody response in fish and have provided indications for functional heterogeneity of lymphocytes in fish. A memory immune response to TD antigens has been described in fish (Rijkers *et al.*, 1980b). Miller *et al.* (1985), using an *in vitro* microsystem of channel catfish, have demonstrated that the response to TI antigens required the presence of B-cells with Surface Immunoglobulin (SIg) and macrophages, whereas the response to TD antigens required B-cells, macrophages and SIg negative cells (T-helper cells).

Stressors and or toxicants which induce immunosuppresssion appear to do so by interfering at any one or several stages in this intricate pathway of antibody production. Total suppression of antibody production could suggest the blockade of the afferent immune system involving antigen recognition, uptake, processing and

presentation. From the above information, it is certain that, unless several immunization and metal exposure regimes are followed, using an antigen of known cellular requirements and immunokinetics in conjunction with a single toxicant, it is hard to ascertain the sublethal effect of the toxicant in question on the immune response.

The results discussed in Chapter 3 demonstrated the ability of cadmium and copper to suppress the protective immune response of carp to *I.multifiliis* infection without in fact reducing the antibody titre. Such a response would suggest that the metals interfere with the cellular immune response responsible for the execution of protective mechanism against *I. multifiliis*. The antigenic nature of the parasite and the cellular requirements (thymus dependency) for optimal expression of immune response are uncertain. This prompted the present series of experiments aimed at evaluating the effects of cadmium and copper on the humoral antibody response to antigen of known cellular requirements and immunokinetics.

Sheep red blood cells (SRBC) are regarded as TD antigens in fish. Optimal expression of the humoral immune response to this antigen requires the co-operation and interaction of B and T-like lymphocytes and macrophages. The kinetics of the primary and secondary immune response in carp to SRBC has been well documented (Rijkers *et al.*, 1980b) thus making this a useful model for detecting the influence of metals in question.

With the aforementioned points in view, five different experiments were designed to assess the immunomodulatory effects of both cadmium and copper (individually) on the kinetics and magnitude of the primary and the secondary humoral antibody response to SRBC antigens.

1. Fish were exposed to sublethal levels of metals for 40 days. The primary immunization was administered 10 days after the commencement of metal exposure. The secondary immunization in all the experiments was given 21 days after the primary injection. This experiment aimed to establish whether carp, after short term exposure to metals, would be able to initiate the immune system and proceed with the normal kinetics in the presence of metals.

2. This experiment was similar to the first but the response was monitored by following the kinetics and magnitude of the primary and the secondary plaque forming cell (PFC) and rosette forming cell (RFC) numbers.

3. This experiment was aimed at looking into the long-term effects of sublethal levels of metals on the ability of fish to respond to immunization. Fish were exposed to the metals for 30 days prior to the primary immunization. Metal exposure was discontinued after the primary injection.

4. This experiment was designed to examine the effects of metals specifically on the secondary response. Fish were exposed to the metals 18 days after the primary injection and continued till the end of the experiment.

5. The last experiment in this series looked into the possible effects of metal exposure and immunization administered simultaneously and the metal exposure continued throughout.

4.2 MATERIALS AND METHODS

4.2.1 Sheep Red Blood Cell (SRBC) Preparation

SRBC was obtained as 50% whole blood in Alsever's solution from the Scottish Antibody Production Unit (SAPU, Scotland) and stored at 4°C. Fresh cells not older than a month from the day of bleeding were used in all the experiments. The cells were washed three times in phosphate buffered saline (PBS) by spinning one volume of SRBC with three volumes of PBS at 450g for 10 mins. The washed pellets were resuspended to the required final density in PBS before being used either for immunization or for agglutination assays.

4.2.2 Immunization Procedure

For both the primary and the secondary immunization, washed SRBC pellets were resuspended to a final 10% suspension in PBS. Fish were anaesthetized with 100 ppm Benzocaine, lifted out of the anaesthetic bath individually and placed on their side on a wet sponge mat. Using disposable 1ml plastic syringes with 25 gauge needles the fish were injected with 0.1 to 0.15 ml 10% SRBC intramuscularly and allowed to recover in a well-aerated tank before being transferred to the experimental system. In the immunization schedule followed in the present study, the secondary injection was given 21 days after the primary injection. The procedure of immunization lasted no more than 30 seconds for each fish. With very few exceptions, fish behaved normally after the injection.

4.2.3 Collection of Blood

Fish were anaesthetized, and approximately 500 µl of blood was withdrawn from the caudal vein, using an heparinized disposable 1ml plastic syringe with a 27 gauge hypodermic needle. The fish were allowed to recover in tanks with well-aerated

water before being returned to the experimental system. The blood was transferred to labelled non-heparinized Ependorf vials (1.5ml), allowed to clot at room temperature and kept at 4°C overnight for clot retraction. Serum was separated by centrifuging at 12000 rpm for 5 minutes, and stored at -70°C until required for use.

4.2.4 Agglutination Assay

Agglutination assays were done on serum inactivated by heating at 45°C for 30 min. Serial two-fold dilutions of sera from 1:2 to 1:2048 were set up in PBS in 96-well microtitre plates (Flow Laboratories, Scotland) and 100 µl of 1% PBS-washed SRBC was added to each well. The plates were gently agitated and incubated overnight at 4°C. The lowest dilution to give aggregates of SRBC was taken as the effective serum titre. The antibody titres were recorded as the reciprocal of these dilutions. Data were reported as the mean ± standard error of (- $\log_2$ titre) + 1. This meant that a positive titre for undiluted serum has a value of 1 (but not 0).

4.2.5 Collection and Preparation of Head Kidney and Spleen cells

Fish were anaesthetized and bled completely before dissecting kidney and spleen in order to avoid red blood cell contamination. The organs were dissected carefully under aseptic conditions and transferred to 5ml Eagles minimal essential medium (EMEM), supplemented with 10% foetal calf serum. Cell suspensions were prepared by gently forcing small pieces of the organ through a graded series of hypodermic needles (from 19 down to 27 gauge). The suspension thus prepared was allowed to sediment in centrifuge tubes for 2 min, and the resulting cell clumps removed. The cells remaining in suspension were centrifuged at 6000 rpm in a chill spin and the pellet resuspended in EMEM.

4.2.6 Lymphocyte Separation

Lymphocytes were separated from the cell suspension using the density gradient lymphocyte separation medium (Flow Laboratories, Scotland). Four ml of lymphocyte separation medium was dispensed into a 10ml centrifuge tube and, 2ml of cell suspension was carefully layered on top of the separation medium. These tubes were centrifuged for 20 minutes at 400g. Lymphocytes usually form a grey to white colored layer at the interface of the EMEM and the separation medium. Using a Pasteur pipette, the supernatant down to the upper surface of the lymphocyte layer was aspirated, care being taken not to disturb the lymphocytes. Then, the layer of lymphocytes was aspirated using a clean Pasteur pipette. This was transferred to a centrifuge tube containing at least three times the volume of EMEM. The lymphocyte suspension was centrifuged at 100g for 10 minutes. The supernatant was discarded and the cell pellet was resuspended in fresh balanced salt solution. All procedures involving collection and separation of lymphocytes were carried out on ice.

The viability of lymphocytes was estimated by the Trypan Blue exclusion method. Briefly, 0.1 ml from diluted aliquots of lymphocyte suspension were mixed with 0.1 ml of 0.4% Trypan Blue stain. After a brief mixing on a vortex mixer, the suspension was allowed to stand for 5-10 minutes at room temperature. The number of viable lymphocytes was counted using an haemocytometer.

4.2.7 Plaque Forming Cell (PFC) Assay.

Heterologus red blood cells (SRBC) were used both as immunogen and indicator. Sensitized lymphocytes were incubated with indicator cells in the presence of complement. The antigen-antibody complexes at the surface of red blood cells initiate lytic reactions when the complement is added, thus revealing a central plaque forming cell. In the present study, monolayer plaque assay slides were

produced according to the technique of Rijkers *et al.* (1980a) followed for carp.

Lymphocyte cell suspension (0.1ml) was mixed with 0.1ml of SRBC ($5x10^8$/ml) and 10 µl of complement was added to this mixture. Pooled carp serum was used as the source of complement. The suspension was mixed in a vertical rotor and incubated between two glass slides (Cunningham chambers), which holds about 200 µl. The open ends of the chamber were sealed with vaseline. The chambers were incubated at 25°C for 4 hours. Plaques were scored using a low power dissecting microscope with a dark field. The results were expressed as the number of plaques per 10^6lymphocytes.

4.2.8 Rosette Forming Cell (RFC) Assay

The RFC assay was carried out following the procedure of Blazer *et al.* (1984). Sensitized lymphoid cells form rosettes by attaching red blood cells to their surface after *in vitro* incubation. The assay involved mixing of approximately $5x10^6$ lymphocytes and $5x10^8$ SRBC and later adjusted to a volume of 1ml. EMEM supplemented with 10% FBS and 1% antibiotic was used as the medium. The mixed cell suspension was incubated overnight at 4°C and subsequently rotated for 10 minutes in a multi-purpose rotor. Aliquots of the mixture were loaded on to an haemocytometer and the rosettes were counted under a Phase Contrast objective. The results were expressed as number of RFC/10^6 lymphocytes

4.2.9 Experimental Protocol

A series of metal exposure and immunization schedules were followed to understand the effects of cadmium and copper individually on the primary and secondary immune response of carp to SRBC antigens. Throughout this series of experiments only a single concentration of the metal was chosen. The decision to expose the fish

to only a single concentration of the metal, either cadmium or copper, was based on the results observed in Chapter 3. The concentration choosen (50 μgl^{-1} for cadmium and 30 μgl^{-1} for copper) had significantly affected the susceptibility and immune response of carp, *C. carpio* to *I. multifiliis*. The line of approach in this series of experiments was to some extent similar to that followed in Chapter 3. As all the experiments were run over a long term (60 days), fish were fed minimal feed once in two days.

Experiment 1 The effects of metal exposure prior to the primary immunization on the subsequent humoral immune response

Unlike the other experiments to follow, cadmium and copper treatments were run at different times in this experiment. With each metal treatment, 2 groups of 20 fish each, in duplicate were used. One group was exposed to the metal either cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) for 10 days, while the other group served as the control. In effect, 2 controls were run along with the 2 metal treatments. The mean length and weight of the fishes used for cadmium and copper treatment groups were (8.95 ± 0.65 cm; 12.59 ± 2.04 g: n=80) and (10.43 ± 1.54 cm; 19.29 ± 4.62 g: n=80) respectively.

The primary immunization was administered 10 days after the commencement of exposure to the metals. Metal exposure was continued till day 40. Secondary immunization was carried out 21 days after the primary. This experiment was designed to see whether the metals at sublethal levels have any effect in influencing the process of antigen handling and the subsequent development of immunity. This would also give a measure of the ability of a stressed fish to respond to immunization. The response was measured by monitoring the agglutination titres at

regular intervals after the primary and secondary immunization.

A total of 15 samplings (3 fish per treatment in duplicate at any sampling point) starting from day 6 after the primary immunization to day 30 after the secondary immunization at regular intervals of 3 days were carried out to monitor the serum agglutination titre to SRBC antigens. Fishes were resampled from 7th sampling onwards but precaution was taken not to sample the same fish twice within a period of 2 weeks. To do this, fishes sampled at every sampling point had to be marked by fin clipping. The changes in the lymphoid organs was also followed histologically using kidney and spleen samples taken after the primary and secondary immunization. The samples were fixed in 10% buffered formalin and processed according the procedure given in the Appendix 4.

Experiment 2 The effects of metal exposure prior to the primary immunization on the plaque forming and rosette forming cell numbers

This experiment was very similar to the previous one with regard to the metal exposure and immunization protocol. In all the experiments to follow, the two metal treatments were run together with a single control. The immune response was monitored by assaying the PFC and RFC of the head kidney and spleen. Fifteen fish (12.25 ± 1.03 cm; 23.45 ± 3.78 g; n=90) per treatment in duplicate were used in this experiment.

At each sampling point 2 fish per treatment from duplicate tanks were sacrificed to collect and process the head kidney and spleen cells as described (Section 5.2.5). A total of 7 samplings, starting from day 4 after the primary immunization to day 12 after the secondary immunization were carried out at regular intervals. A cell suspensions obtained from kidney or spleen of a single fish was used to set up

triplicate plaque forming and rosette forming cell assays. The results of duplicate experiments with no significant difference between them were combined for the purpose of statistical analysis between treatments. At each sampling point, kidney and spleen tissues were fixed in 10% buffered formalin to follow the histological changes in the lymphoid organs.

Experiment 3 The effects of long term exposure to metals prior to the primary immunization on the subsequent humoral immune response

This experiment was aimed at looking into the long-term effects of sublethal levels of metals on the immune response of carp to SRBC antigens. The possibility of the acclimation of the fish to very low levels of metals in the long term was examined by assessing the ability of such fish to develop an immune response. Three groups of 15 fish each (10.51 ± 1.24 cm; 18.41 ± 5.57g; n=90), in duplicate were used in this experiment. Two groups were exposed to the respective metals, either cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) for a duration of 30 days. One group which received no metal served as the control. The primary injection was administered after the 30-day metal exposure period. Metal exposure was discontinued after the primary injection. The secondary injection of SRBC was given 21 days after the primary. Antibody production was followed from day 6 after the primary injection to day 30 after secondary injection by sampling 3 fish at a time per treatment. A total of 11 samples per treatment were carried out at regular intervals. Fishes from every treatment had to be resampled from 6th sampling onwards. Care was taken to ensure that no fish was sampled twice within a 2 week period.

Experiment 4 The effects of exposure to the metals following the primary immunization on the secondary humoral immune response

The objective of this experiment was to initiate the process of immunity development and later expose the fish to metals to see if metals would interfere with the later stages of the immune response such as the enhanced secondary antibody production. A group of 60 fish (10.64 ± 1.17 cm; 19.06 ± 5.48 g; n=60) were administered a primary immunization and held in a flow-through system without metals for 18 days. At days 6, 9, 12, and 18 after the primary injection, 3 fish at random were sampled for blood. After 18 days, the fish were randomly divided into 3 groups of 10 each in duplicate and allocated to 6 tanks in the flow-through system. Two groups were exposed to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}), while the third group served as the control. Metal exposure was commenced 18 days after the primary injection. The secondary injection was given 21 days after the primary. Metal exposure was then continued throughout the experimental period. Seven samplings were carried out, starting from day 4 to day 32 after the secondary injection. The fishes had to be resampled from 4th sampling onwards, but care was taken not to resample the same fish twice in less than 15 days.

Experiment 5 The effects of simultaneous exposure to the metals and administration of the primary immunization on the humoral immune response

In the last experiment in this series, an attempt was made to see the effects of metals when both immunization and exposure to metals are concurrent. A group of 60 fish (9.7 ± 0.72 cm; 14.32 ± 2.2 g; n=60) were given a primary injection on day 1 and on the same day they were randomly divided into 3 batches of 20 each and allocated to 3 tanks. A duplicate set of experiment was carried out side by side.

Metal exposure was started on the same day. Two groups received either cadmium(50 μgl^{-1}) or copper(30 μgl^{-1}), while the third received no metal (control). The second injection was given 21 days after the first. Metal exposure was continued throughout the experimental period. The aim here was to see whether metals would directly interfere with in the process of immune development and, since the exposure to metals was concurrent with the primary immunization the problems at the initial stages would be highlighted. A total of 16 samplings per treatment, involving 3 fish per treatment at a time, were carried out in the course of this experiment. The samplings were done at regular intervals of 3 days starting from day 6 after the primary immunization to day 33 after the secondary immunization. Whenever fish were resampled, they were done so, not earlier than 15 days after the previous sampling.

Samples from lymphoid organs were taken during the time of peak primary and secondary response to follow the histological changes. The samples were fixed and processed according to the procedure described in the Appendix 4.

4.3 RESULTS

4.3.1 Experiment 1

The kinetics of the primary and the secondary immune response to SRBC in carp exposed to cadmium or copper for 40 days is presented here. The primary immunization was administered 10 days after exposure to the respective metals and the metal exposure was continued till day 40.

4.3.1.1 Cadmium

The primary and secondary antibody titre recorded in the control group and those exposed to 50 μgl^{-1} cadmium are illustrated in the Figure 4.1a. At the first sampling point (6th day) following the primary injection antibodies were detected in both the groups. In controls the titre increased with time from eg 5.33 on day 12 the following primary injection, and reached a peak value of 5.83 on day 18. In the cadmium exposed groups, the titre remained slightly lower than the controls till day 18 and peaked to a titre of 6.17 on day 21. Statistically there was no difference in the titres between the two groups following the primary injection.

Following the secondary injection on day 21 the titre increased significantly ($P<0.001$) in both the groups (Figure 4.1a). In the controls the titre increased rapidly between day 21 and 30, and remained high (9.5 - 11) till day 45 before decreasing slightly. In the cadmium exposed groups the antibody titre (4.0 on day 24) showed an initial decline immediately following the secondary injection. From day 27 onwards the antibody titre rose and persisted at higher level (7.5-9.0) till day 42 from where it started to decline gradually. Both the mean anti-SRBC titre at several sampling points and the peak secondary antibody titre in the cadmium exposed group following the secondary injection were significantly lower ($P<0.05$ to

P<0.001) than the controls (Figure 4.1a).

4.3.1.2 Copper

The kinetics of anti-SRBC titre following the primary and secondary immunization for carp exposed to copper together with controls are illustrated in Figure 4.1b. In both test and control groups, low levels of antibodies were detected at the first sampling point, 6 days following the primary injection. In the copper exposed group, the titre increased rather sharply from day 6 and peaked on day 18 (6.5) following the primary injection before declining slightly on day 21. Although there was no statistical difference in the peak titre following primary injection between the treatment and control, the titres of the copper exposed group remained lower than controls at all the sampling points following the primary injection.

After the secondary injection on day 21 the antibody titre in controls increased rapidly and peaked on day 27 (10.17), then declined gradually till day 36 (6.33) and increased again to peak on day 45 (10.17). As can be seen from the Figure 4.1b, the antibody titre of the copper exposed group followed an identical trend to the control, but with relatively lower titres throughout. The titre peaked to 8.83 on day 30 and declined sharply to 4.5 on day 36 and peaked again to 8.67 on day 45. The titres in the copper exposed group, after the injection were significantly lower than controls at three sampling points as can be seen from the Figure 4.1b. In addition the peak titre in the copper group following the secondary injection was significantly (P<0.001) lower than that of control. The difference in the copper exposure fish in relation to the controls was not so marked as with cadmium.

4.3.2 Experiment 2

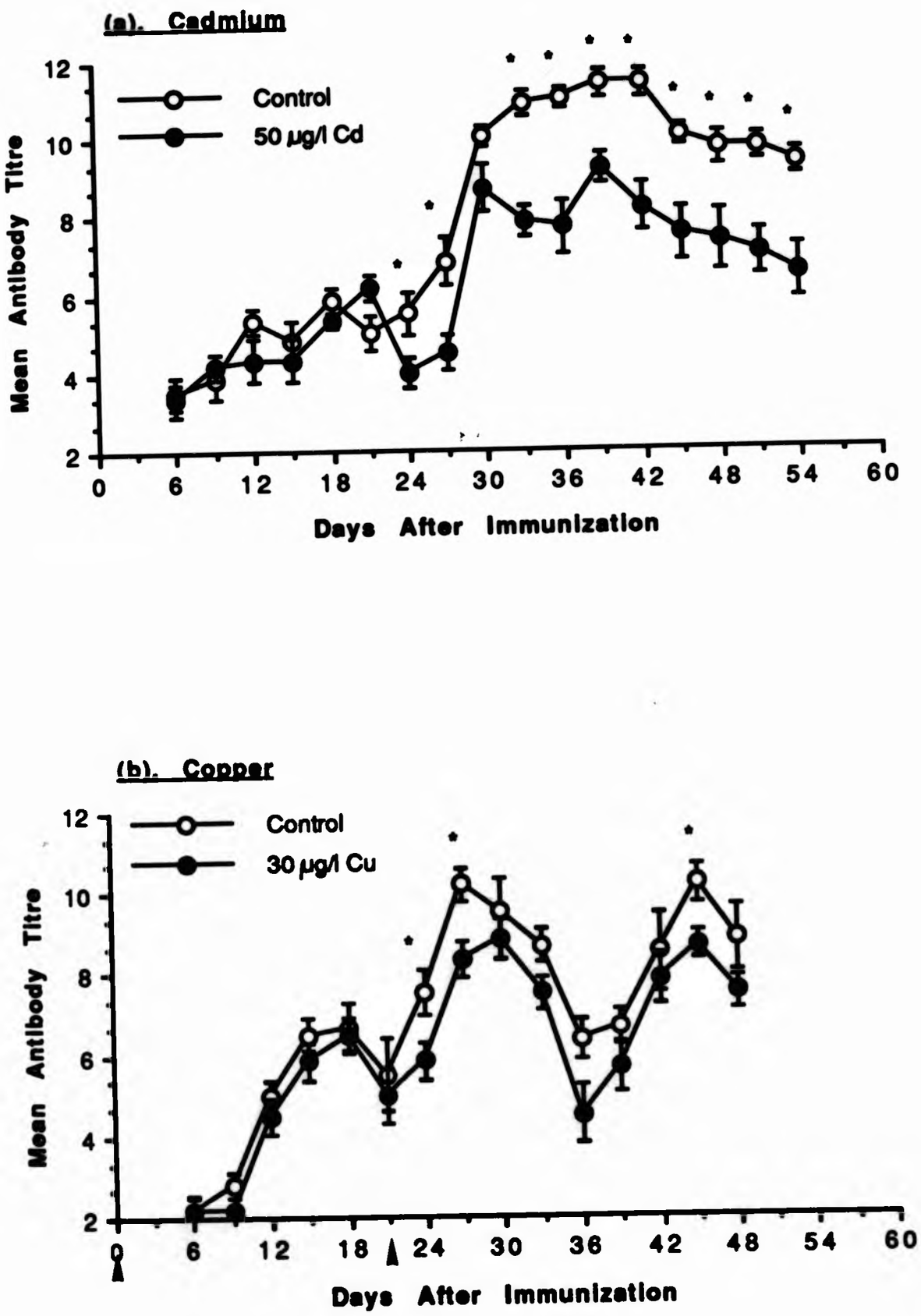


Figure 4.1 The primary and secondary humoral anti-SRBC antibody titre (Mean ± S.E; n=6) in carp exposed to (a) cadmium and (b) copper for 40 days. The primary immunization was administered 10 days after the commencement of metal exposure and the secondary immunization was carried out 21 days after the primary. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

The kinetics of the primary and secondary PFC and RFC response to SRBC immunization in carp exposed to cadmium or copper for 40 days are described here. Primary injection was given 10 days after the commencement of metal exposure.

4.3.2.1 Plaque Forming Cell (PFC) Response

The mean number of PFC/10^6 lymphocytes and their kinetics in the pronephros and spleen of fish exposed to the metals are illustrated in Figure 4.2.

4.3.2.1.1 Pronephros

PFC were detected 4 days after primary injection in the pronephros. The highest number of PFC in the pronephros following the primary injection were recorded in all the treatments on day 9. The PFC in pronephros peaked to 67 in the controls which was significantly higher ($P<0.01$) than the corresponding peaks of 35 and 29 recorded in the cadmium and copper exposed groups respectively (Figure 4.2a-b). The pattern of the response remained the same in all the treatments.

The secondary PFC response in pronephros increased significantly in all the treatment groups and reached a peak between days 6 and 9 following the second injection (Figure 4.2a-b). The response was significantly higher ($P<0.01$) in the controls on days 9, 12 and 15 following the second injection. But for the generally lower numbers of PFC, the kinetics of the secondary response remained the same in the cadmium and copper exposed groups. The peak secondary response was nearly 15 times more than the peak primary response in controls and copper, whilst it was 13 times more in cadmium.

4.3.2.1.2 Spleen

The kinetics of the response in splenic lymphocytes was very similar to that

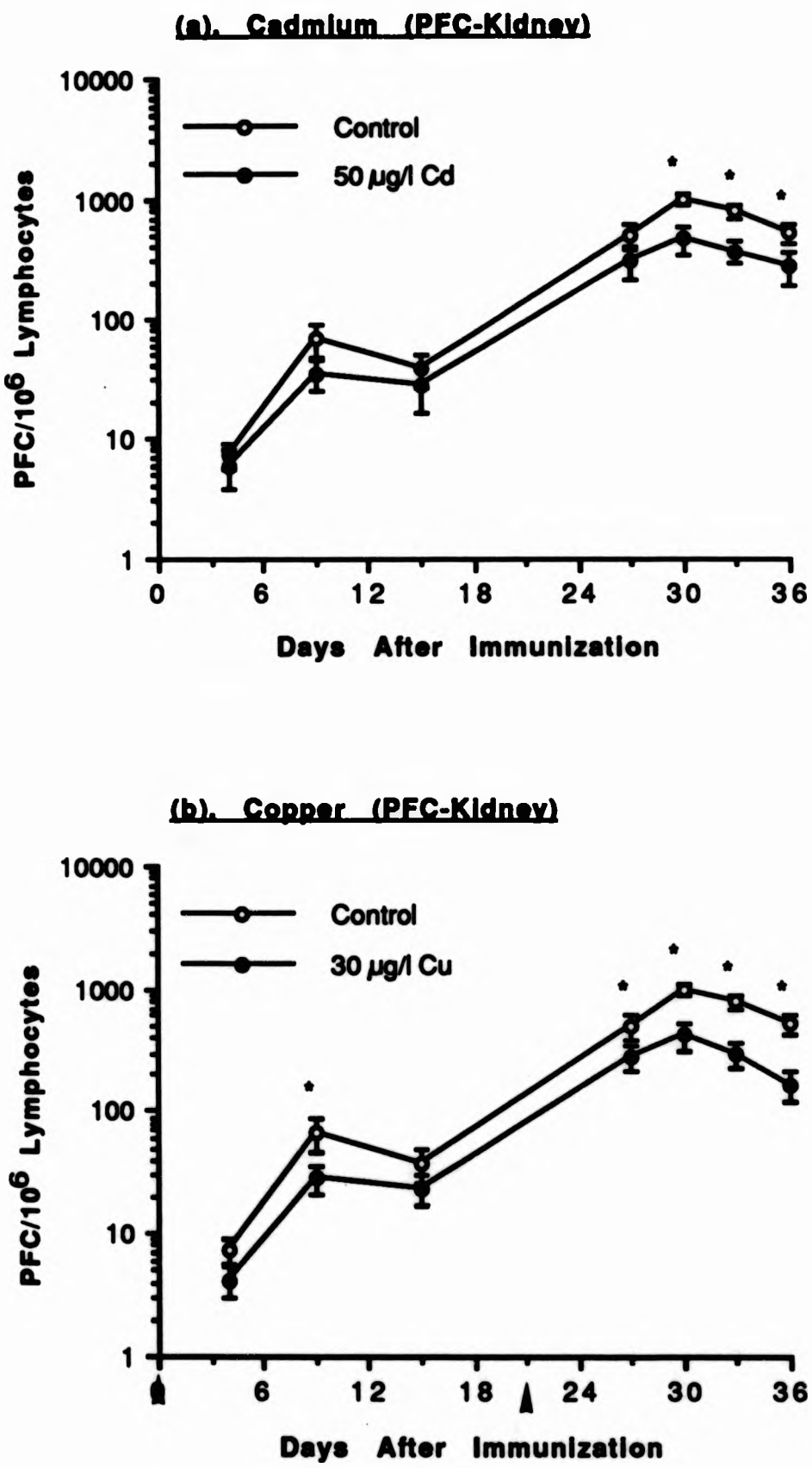
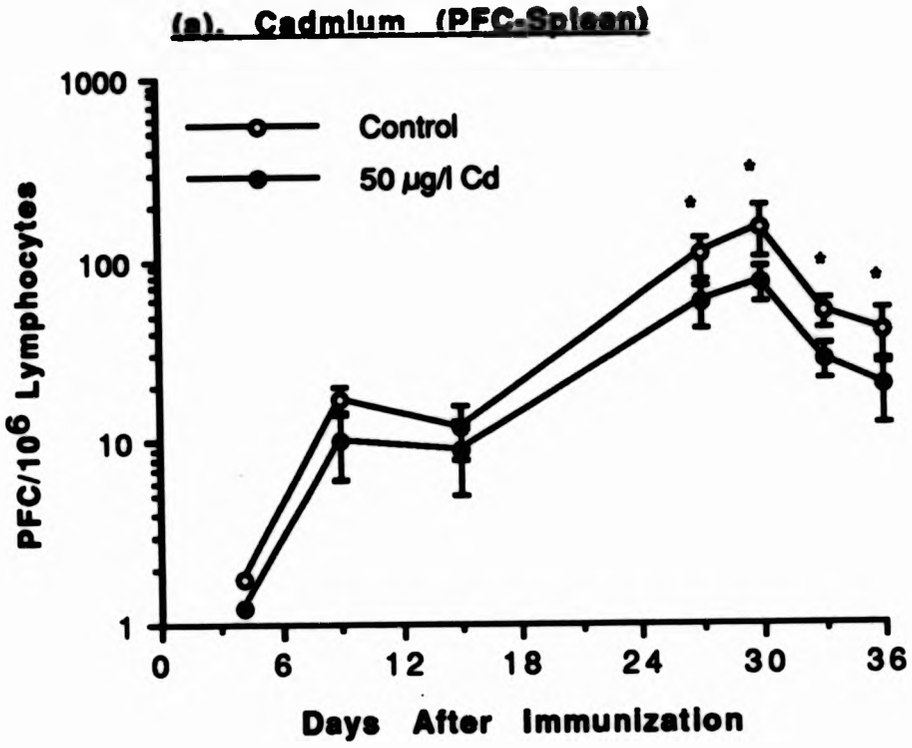


Figure 4.2 The number of PFC/10^6 lymphocytes (Mean ± S.E; n=4) in the pronephros of carp exposed to (a) cadmium and (b) copper for 40 days. The primary SRBC immunization was administered 10 days following the commencement of metal exposure and the secondary immunization was given 21 days after the first. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).



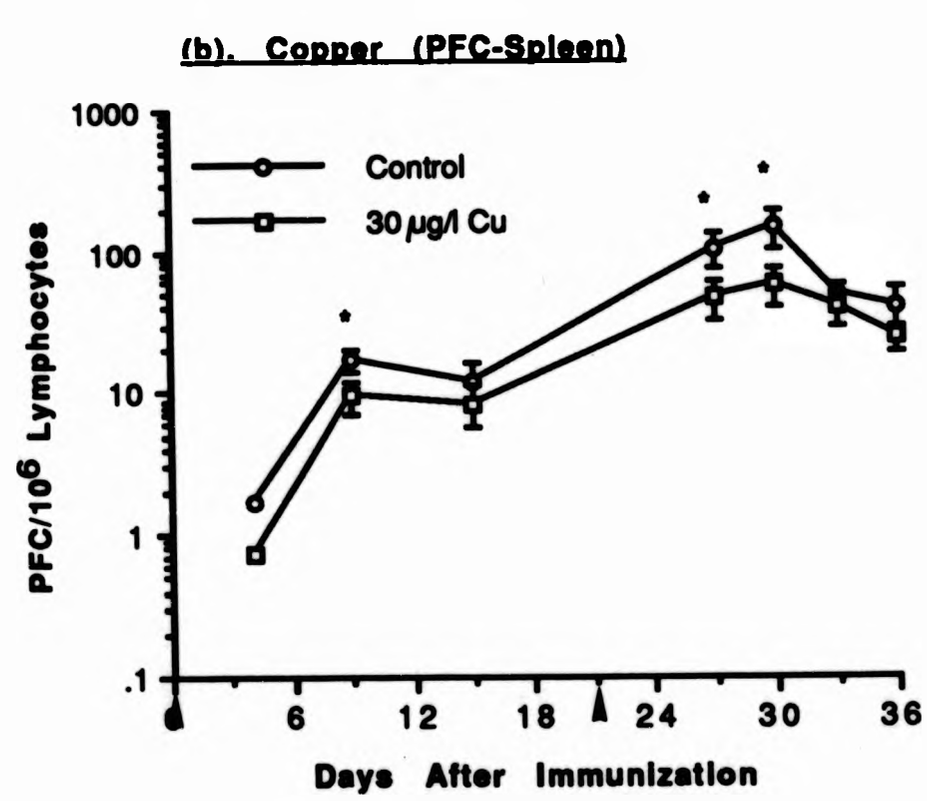


Figure 4.3 The number of PFC/10^6 lymphocytes (Mean ± S.E; n=4) in the spleen of carp exposed to (a) cadmium and (b) copper for 40 days. The immunization schedule same as in Figure 4.2. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

observed in pronephros in all the treatments (Figure 4.3a-b). However, the number of PFC in all the treatments were significantly lower in the spleen than in the pronephros on all corresponding sampling points. The peak secondary response in spleen was 7 (control) and 6 (Cadmium and copper) times lower than pronephros.

The primary response in controls peaked on day 9 and was significantly higher than copper treatment while it did not differ from the cadmium treatment. The secondary response was significantly higher in all the three treatments and attained peak values between days 9 and 12 following the second injection. The increased secondary response was 8 fold in controls while it was 7 and 6 fold in cadmium and copper treatments respectively. The kinetics of the response were similar in all the groups but the peak PFC response in cadmium and copper treatments were significantly lower than controls.

4.3.2.2 Rosette Forming Cell (RFC) Response

The number of RFC per 10^6 lymphocytes in the pronephros and spleen following the primary and secondary injection along with the dynamics of the response are shown in Figure 4.4 and 4.5. The overall kinetics of the response was very similar to that of PFC response. At corresponding sampling points the number of RFC were slightly higher than the number of PFC recorded.

4.3.2.2.1 Pronephros

Pronephros had significantly higher numbers of RFC in all the treatments compared to spleen. The peak primary response in pronephros was recorded on day 9 after the primary injection in all the groups and the control response was significantly higher (Figure 4.4 a-b). The secondary response was considerably elevated in all the groups and the peak response was recorded between days 6 and 9 after the second

injection. The secondary response in the control on days 9, 12 and 15 following secondary injection was significantly higher than the corresponding cadmium and copper exposed groups.

4.3.2.2.2 Spleen

The progression of response was similar in splenic lymphocytes (Figure 4.5a-b). RFC were detected on day 4 after primary injection and peaked on day 9 in the controls and copper, and on day 15 in cadmium treatment. There was no significant difference in the peak primary response observed between treatments following primary injection. The peak secondary response was seen on day 6 after the second injection in both control and copper while it was seen 3 days later in the cadmium treatment. The peak secondary response in the controls was significantly higher than the other treatments. The number of RFC on all the days following the second injection were higher in controls compared to cadmium and copper treatments.

4.3.3 Experiment 3

The results described in this section demonstrate the effects of long term exposure to cadmium or copper on the kinetics of the primary and secondary antibody response to SRBC in carp. The primary injection was given following 30 days exposure to the respective metals and the second injection 21 days following the first. Metal exposure was discontinued after the primary immunization. Both the metal experiments were run at the same time using one unexposed group as controls.

4.3.3.1 Cadmium

The progression of the primary and the secondary antibody response to SRBC in untreated and fish exposed to cadmium is illustrated in the Figure 4.6a. After the

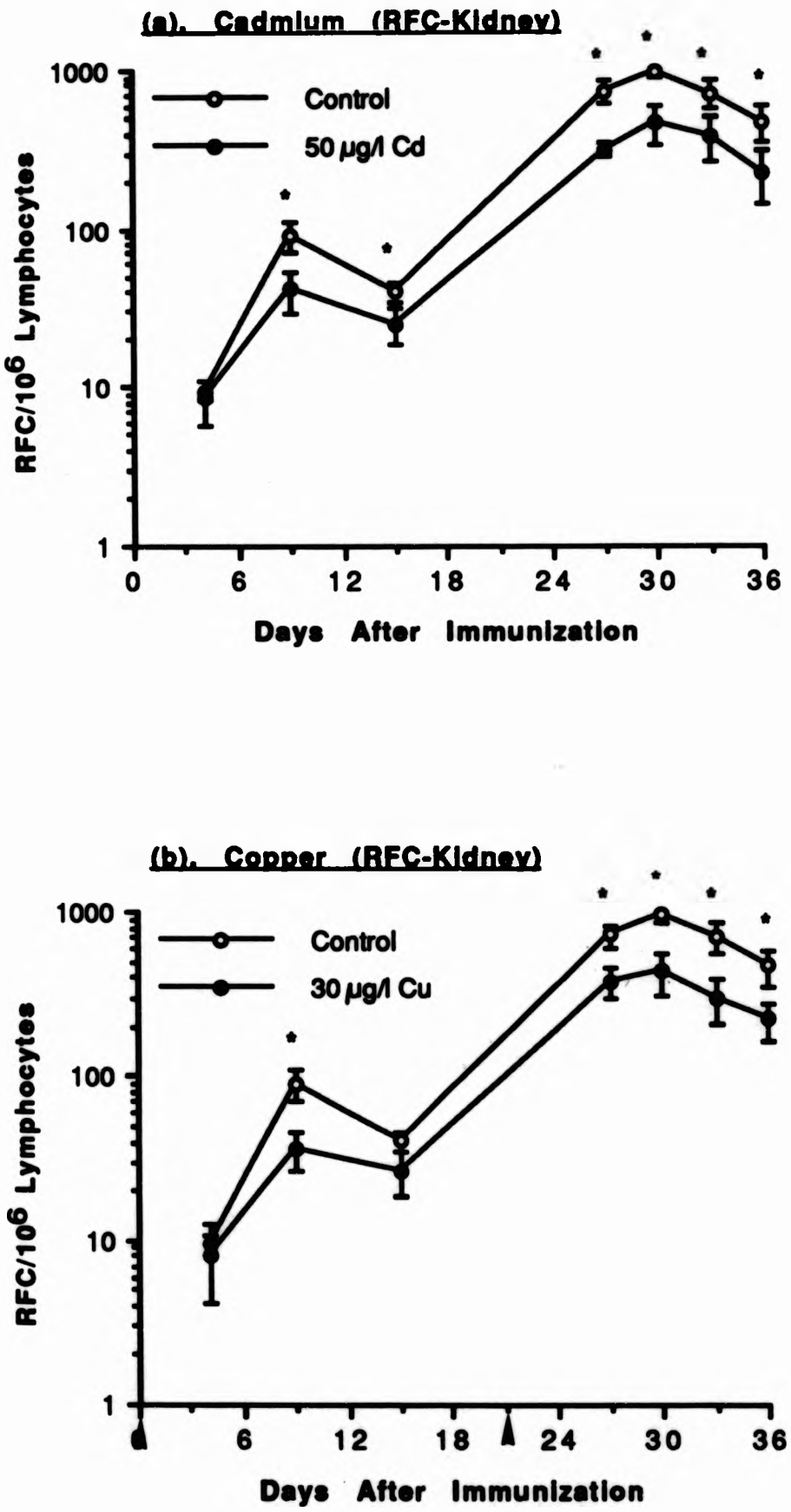


Figure 4.4 The number of RFC/10^6 lymphocytes (Mean ± S.E; n=4) in the pronephros of carp exposed to (a) cadmium and (b) copper for 40 days. The immunization schedule same as in Figure 4.2. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

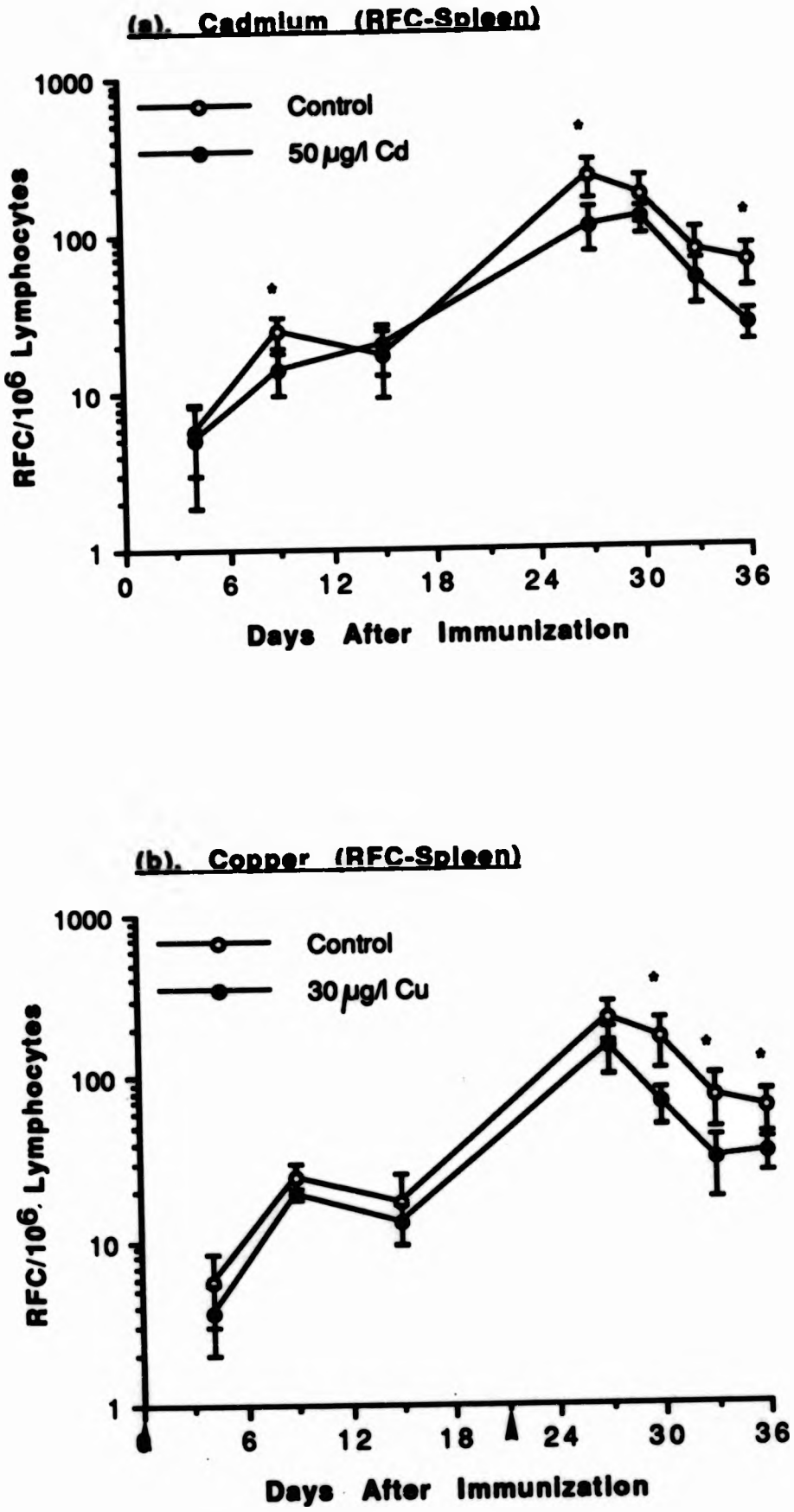


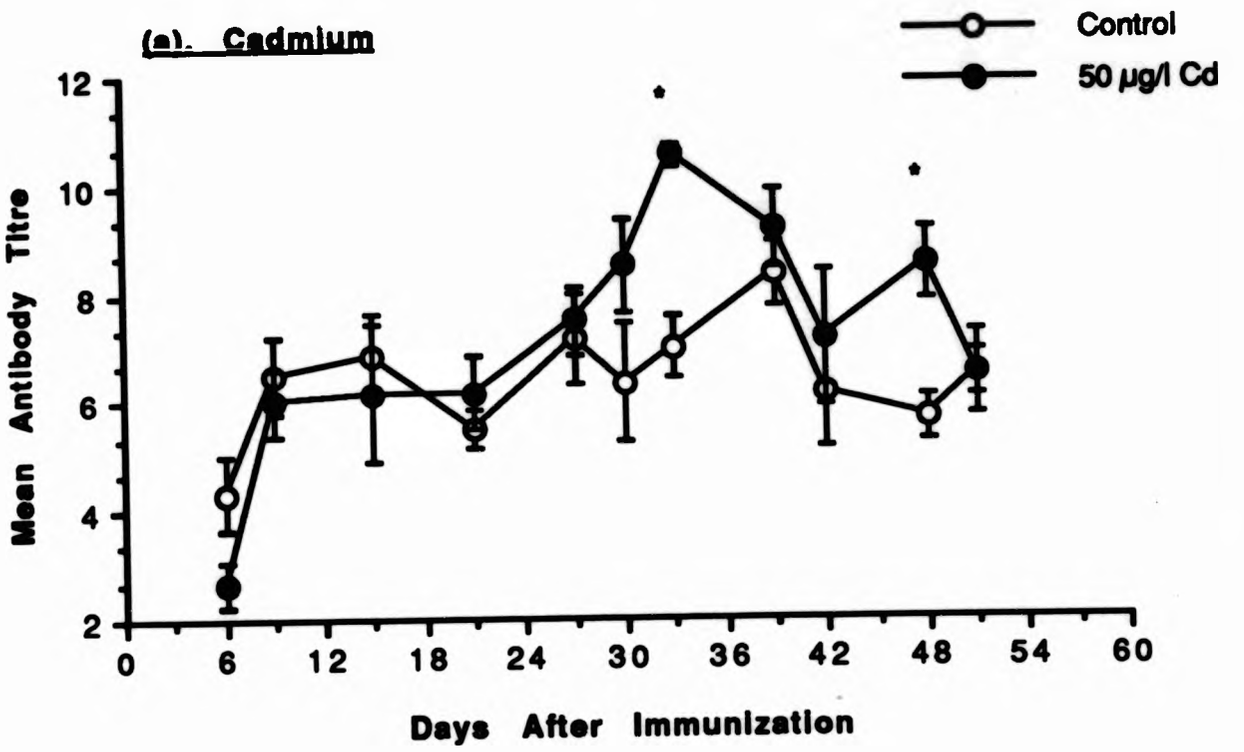
Figure 4.5 The number of RFC/10^6 lymphocytes (Mean ± S.E; n=4) in the spleen of carp exposed to (a) cadmium and (b) copper for 40 days. The immunization schedule same as in Figure 4.2. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

primary injection, the titres in the controls increased suddenly and peaked to 6.8 on day 15. The mean titre of the cadmium exposed group followed a similar trend to that of control following primary injection and peaked to 6.2 on day 15. There were no statistical differences in titre between groups post-primary injection. The variation in the response was very wide in the early stages of the primary response in both the groups. However, prior to the secondary immunization both the groups had almost the same level of antibody.

The kinetics of the secondary antibody response in the controls was not clear cut. The titres peaked and declined without showing any trend, though the titres remained high after the secondary injection till day 42, reaching a peak of 8.8 on day 39. Surprisingly, the titres of the cadmium exposed group remained higher than controls at all the sampling points after the secondary injection as can be seen from the Figure 4.6a. In this group, the titres increased gradually, peaking at day 33 (10.5) and thereafter declining. On days 30, 33 and 48 the titres of controls were significantly lower ($P<0.05$) than that of corresponding titres of the cadmium exposed groups.

4.3.3.1.2 Copper

The anti-SRBC antibody response of controls and fish exposed to copper following the primary and the secondary injections are presented in the Figure 4.6b. It is clear from the Figure 4.6b that both the primary and the secondary antibody response of the copper exposed group were significantly lower ($P<0.001$) than that of controls. The titre in the copper group remained lower than the controls on all the sampling days following the primary injection reaching only as high as 4.7 on day 9. The primary response in the copper group was significantly ($P<0.01$) lower on days 6, 9 and 15 following the primary injection.



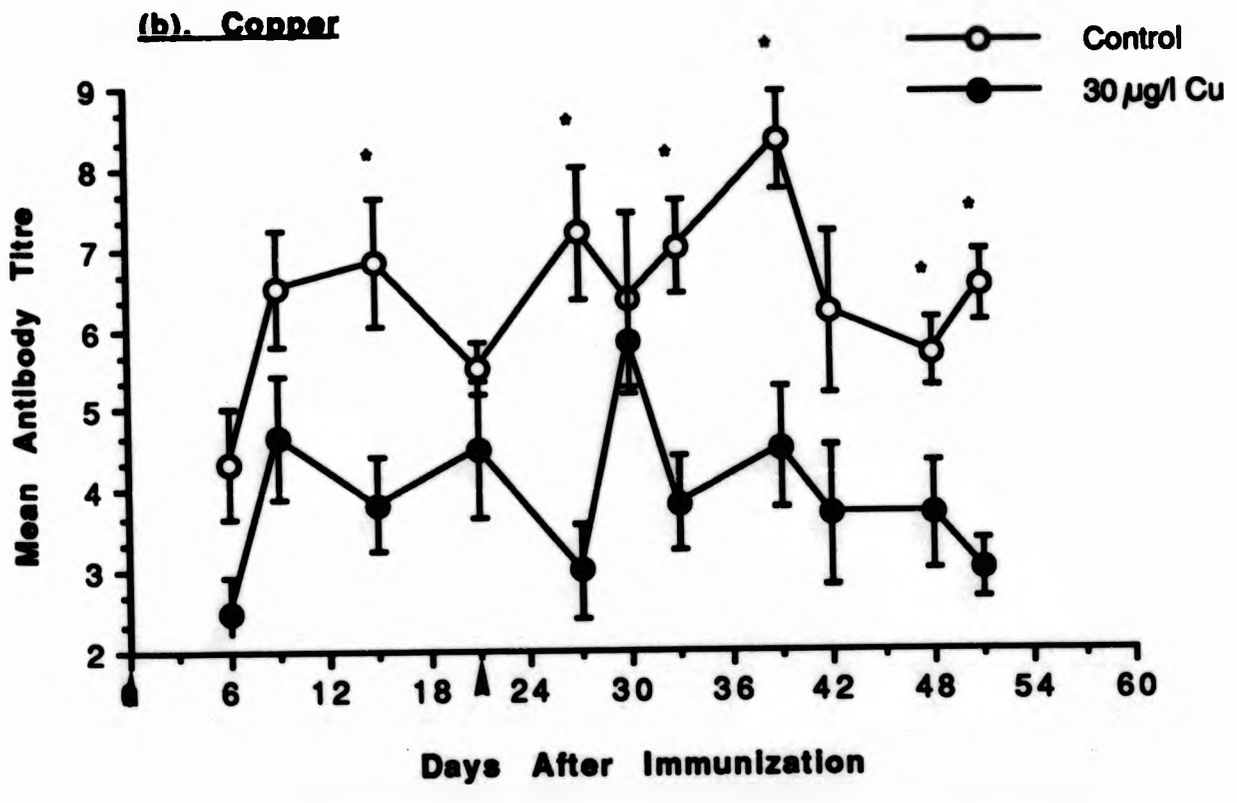


Figure 4.6 The primary and secondary humoral anti-SRBC antibody titre (Mean ± S.E; n=6) in carp exposed to (a) cadmium and (b) copper for 30 days before the primary immunization. The secondary immunization was carried out 21 days after the primary. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

After the second injection the titre in the copper exposed group did not change significantly from the earlier primary response. The highest titre of 5.83 was recorded on day 30, but for this, the titres in all the sampling points after the second injection remained very low (< 4.5). With the exception of day 30, the anti-SRBC titres of the copper exposed groups in all the sampling days were significantly ($P<0.001$) lower than the controls.

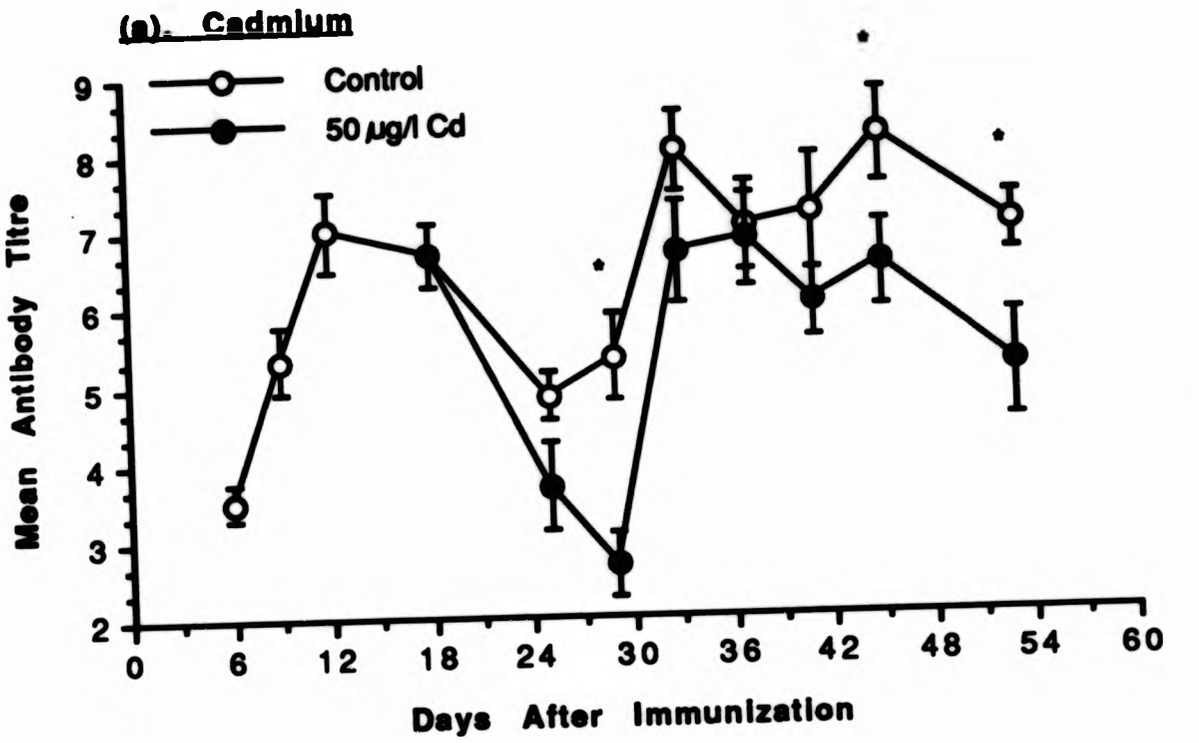
4.3.4 Experiment 4

The kinetics of the secondary antibody response to SRBC and leucocrit response, in carp exposed to either cadmium or copper, 18 days after the primary immunization are presented in this section. Metal exposure was continued till the end of the experiment.

4.3.4.1 Cadmium

Figure 4.7a show the mean antibody titre to SRBC in the controls and fish exposed to 50 μgl^{-1} cadmium. The primary response in the controls was detectable on day 6, which rose sharply peaking (7.0) on day 12, following the primary injection. After the second injection on day 21, the titres increased in the controls reaching a peak titre of 8.0 on day 33. The titres in the controls remained relatively high (7-8) till the end of the experimental duration (53 days). The peak titre (8.0) after the second injection in the controls was significantly higher than the primary peak (7.0), but the titres recorded on other days following the second injection did not differ considerably from the peak primary titre.

Fish exposed to cadmium 18 days after the primary injection produced considerably lower titres following the second injection given on day 21. Following the second injection there was a marked drop in the antibody titre of the cadmium exposed



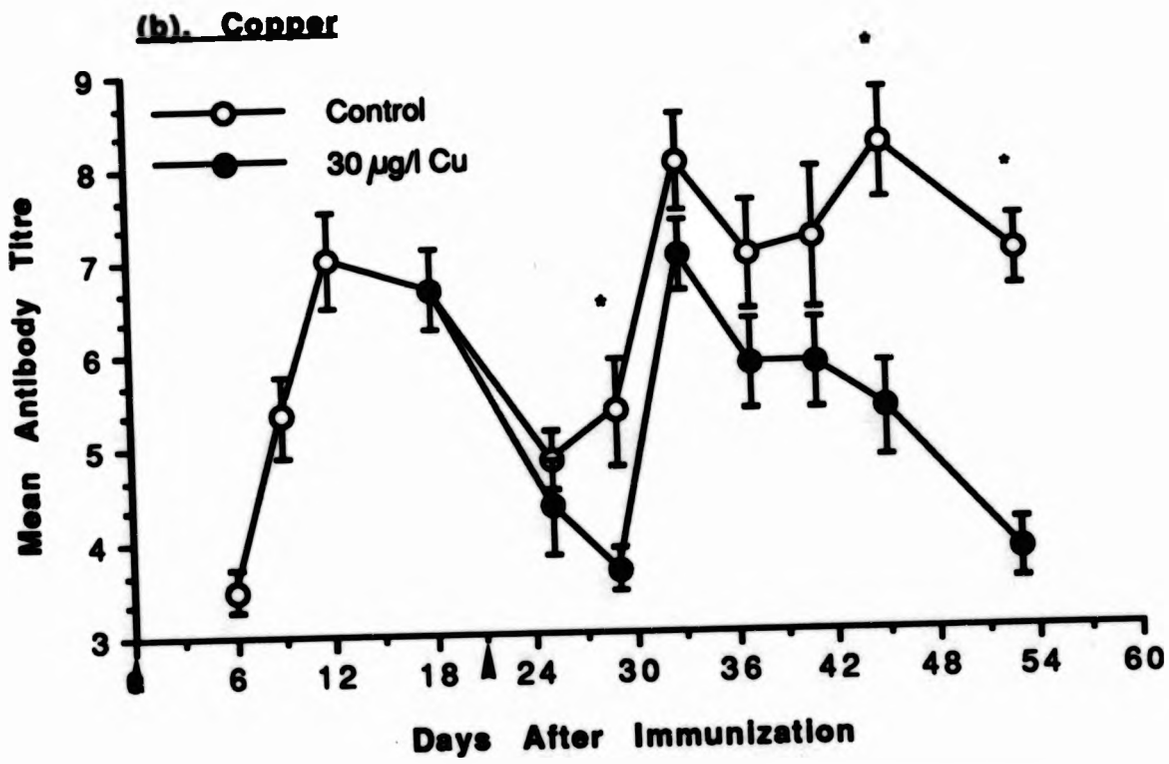


Figure 4.7 The secondary humoral anti-SRBC antibody titre (Mean ± S.E; n=6) in carp exposed to (a) cadmium and (b) copper for 35 days. Metal exposure was commenced 18 days after the primary immunization. The secondary immunization was administered 21 days after the primary. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

group. The titre then rose steeply between days 29 and 33 and reached a peak on day 37 (6.83). The mean titres in the cadmium exposed group remained lower than controls on all the sampling days following second injection. However, the titres were statistically lower ($P<0.05$) than the controls only on days 29, 33, 45 and 53.

4.3.4.2 Copper

The antibody titres following the second injection in the carp exposed to copper 18 days after the primary immunization till the end of the experimental period are presented in Figure 4.7b. As can be seen from the Figure 4.7b, the titres after the second injection in the copper exposed group were consistently lower than controls. After an initial drop to 3.67 on day 29, the titre increased rapidly to peak at 7.0 on day 33 and thereafter started to decline gradually reaching a low of 3.83 on day 53. Interestingly, the titres of the copper exposed group at all the sampling points were significantly ($P<0.01$) lower than the controls for corresponding sampling points (Figure 4.7b).

4.3.4.3 Leucocrit

The kinetics of the leucocrit response over the 53 day experimental period is illustrated in the Figure 4.8. The leucocrit in the controls increased after the primary injection, declined slightly at the time of second injection (day 21) and persisted at that level before rising to peak on day 37 (0.92%). The leucocrit increased after the second injection in the control and cadmium exposed groups. The pattern of leucocrit response in the cadmium exposed groups was very similar to that of the controls. It is clear from the Figure 4.8b that in the copper treatment, the leucocrit decreased after the second injection coinciding with metal exposure and remained lower than the control and cadmium treatments. The leucocrit ranged from 0.57 to 0.92% (Control); 0.57 to 0.91% (Cadmium); 0.40 to 0.67% (Copper). The leucocrits

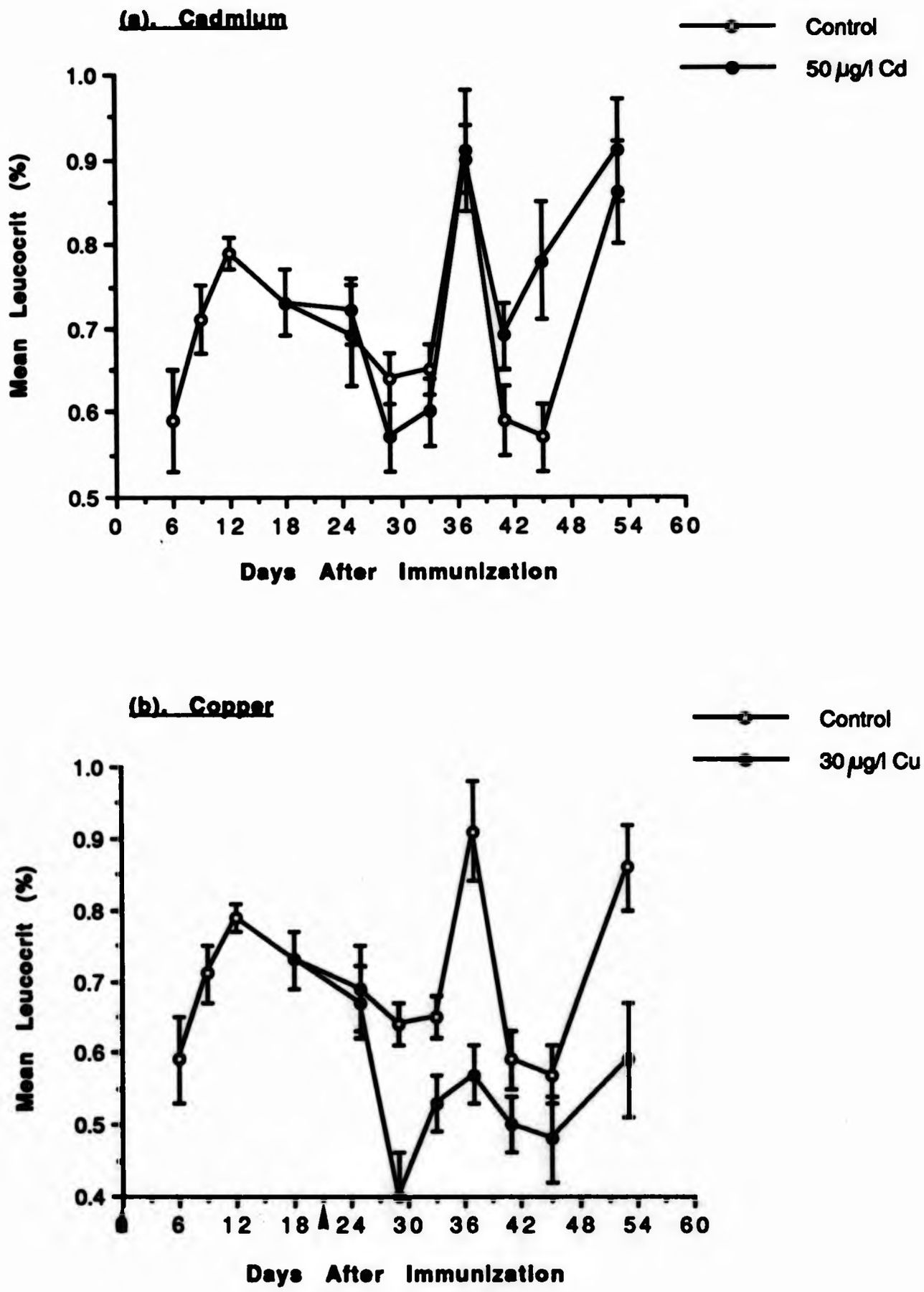


Figure 4.8 The kinetics of leucocrit (%) in carp exposed to (a) cadmium and (b) copper for 35 days. The experimental protocol same as in Figure 4.7. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

recorded in the copper treatment were significantly lower than controls and cadmium exposed group at several corresponding sampling points (Figure 4.8).

4.3.5 Experiment 5

The results presented here show the effects of concurrent immunization and metal exposure on the primary and the secondary anti-SRBC antibody response of carp. Exposure to the metals was commenced on the day of the primary injection and continued till the termination of the experiment (51 days). The second injection was given 21 days after the primary.

4.3.5.1 Cadmium

The mean antibody titre and the development of the immune response in fish exposed to the cadmium are shown in the Figure 4.9a. The control group responded to primary injection by developing a detectable level of antibody by day 6. The titre gradually increased and reached a peak of 7.7 on day 18. Relatively high titres were recorded in the controls from day 12 onwards following the primary injection. The titres in the cadmium exposed group also increased gradually from day 6 following the primary injection but did not reach any peak. The peak titre in the controls was significantly ($P<0.01$) higher than the cadmium exposed groups on days 15, 18 and 21 following the primary injection.

The titres in the controls did rise after the second injection given on day 21. After reaching a peak (10.0) on day 27, the titre remained almost at the same level till day 45 in the controls. The rise in titre in the cadmium exposed group was very gradual reaching a peak of only around 7 by day 39. The titres in the cadmium exposed group were lower than controls on all the sampling points and differed significantly during the time of peak response (days 27 to 36). It is evident from

Figure 4.9a that there was a delay in the cadmium exposed groups attaining high titres following the second injection.

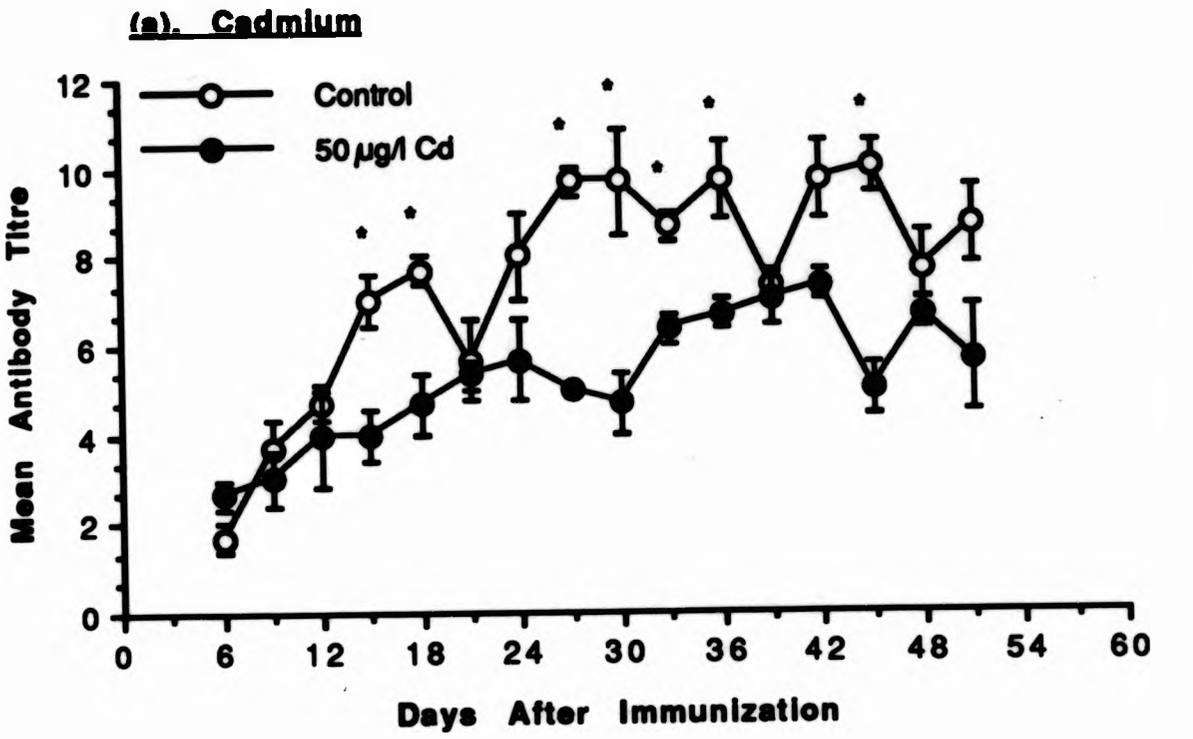
4.3.5.2 Copper

Figure 4.9b illustrates the kinetics of primary and secondary antibody response to SRBC of carp exposed to copper. After an initial increase in titre of up to 4.3 on day 15, the titre declined to a low level of only 2.3 on day 21 at the time of second injection in the copper exposed group. The titres in the copper exposed groups were significantly lower than the controls on days 15, 18 and 21 following the primary injection.

After the second injection on day 21, the titre in the copper exposed group rose sharply to a peak of around 7 on day 30 and remained around that level till day 45 and decreased later. The titre after the second injection was considerably lower than controls on most of the sampling days. The peak response titres were significantly ($P<0.01$) lower in the copper exposed groups. It is also evident from Figure 4.9b that the peak response occurred earlier in the controls than the copper exposed groups.

4.3.6 Changes in the Lymphoid Organs

All the histological observations are based on samples taken at or around the time of peak primary and secondary antibody response. The results presented here are the combined observations from samples taken from different experiments, following the primary and the secondary immunization. The most conspicuous change observed in spleen and kidney was in the melanomacrophage centres (MMC). Compared to non-immunized carp, these centres in immunized fish were numerous, large and very discrete taking on a nodular appearence. The centres stained strongly with PAS. The



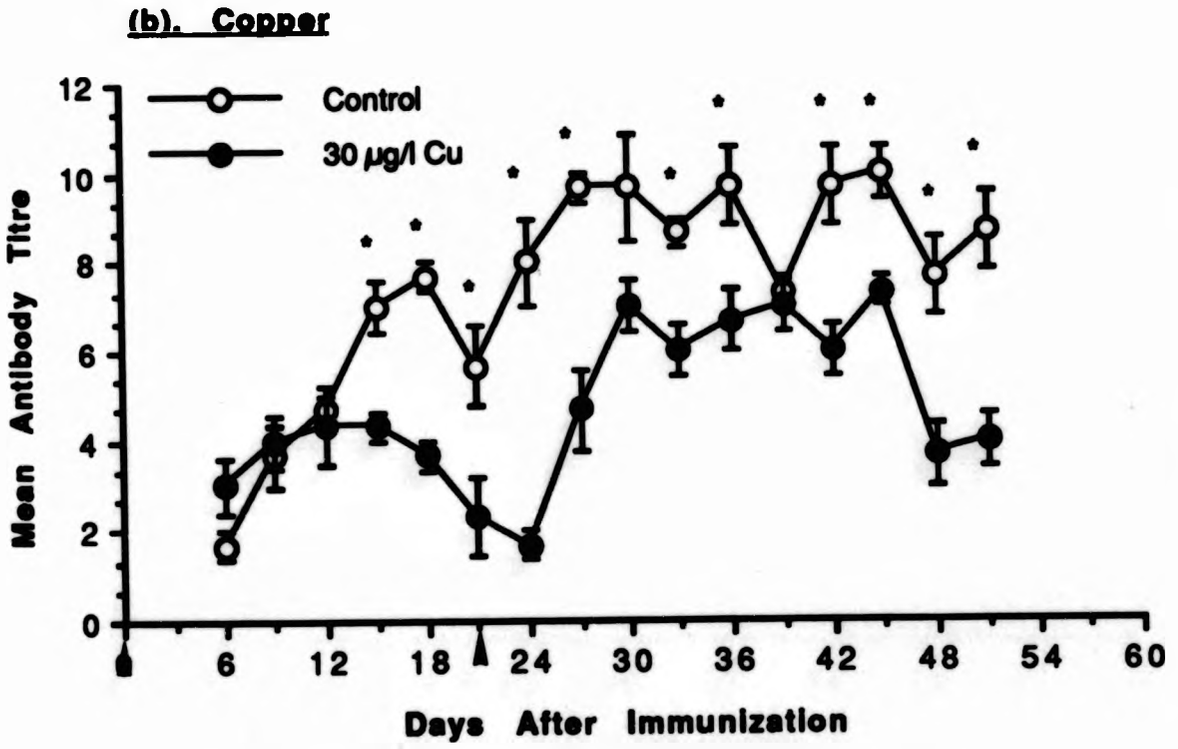


Figure 4.9 The primary and secondary humoral anti-SRBC antibody titre (Mean ± S.E; n=6) in carp exposed to (a) cadmium and (b) copper for 51 days. The primary immunization was given concurrently with the commencement of the metal exposure. The secondary immunization was carried out 21 days after the primary. Arrows denote the time of immunization. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

spleen had a higher frequency of such centres compared to the kidney. These centres in the kidney were very discrete and oval to circular in shape whereas in the spleen they were irregulary shaped (Plate 4.1 and 4.2). The overall numbers of these centres increased following the second injection. The distribution pattern of individual macrophages suggested that they were either migrating to form large aggregates by joining the existing centres, or in themselves forming new centres (Plate 4.3).

The overall distribution pattern of these centres was similar in metal exposed groups to the controls. From the light microscopical observations it appeared that there was no difference in the density of these centres per unit area of kidney or spleen as judged by eye between the different treatment groups.

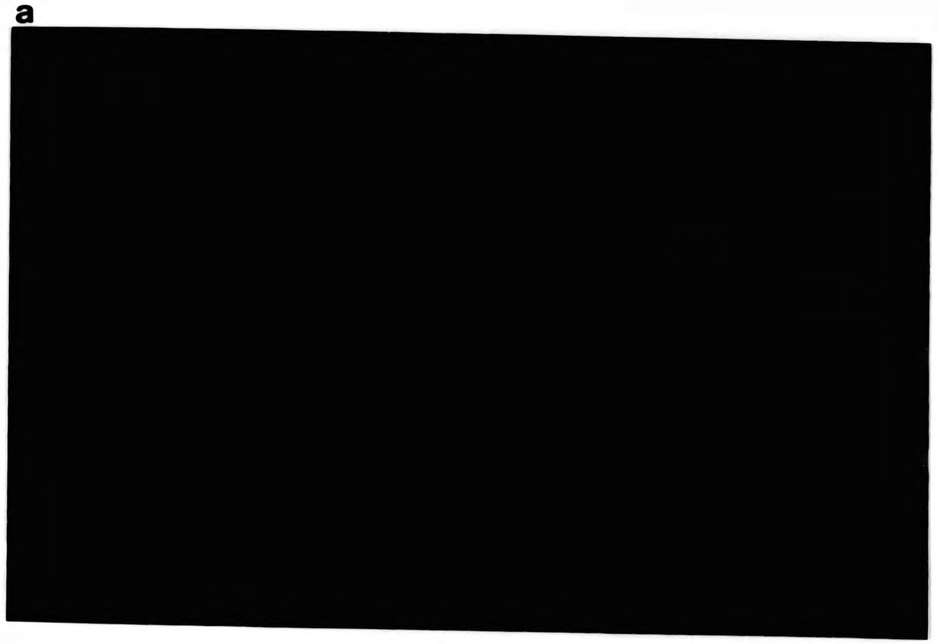
The following interesting changes were observed in the kidney MMC of metal exposed groups: (i) The number of individual macrophages scattered outside these centres appeared to be more in the cadmium and copper exposed groups (Plate 4.4). (ii) There were several patches in the kidney where macrophages were concentrated but not in well defined aggregates or centres (Plate 4.5). (iii) There were indications of fragmentation of these centres in several fish, especially in the copper exposed groups (Plate 4.6). (iv) Occasionally necrosis of individual cells both within and outside the centres was also observed in the metal exposed groups. The MMC in the spleen of metal exposed fish appeared similar to those observed in the unexposed controls (Plate 4.7).

Plate 4.1 Photomicrographs showing the melanomacrophage centres (MMC) in the kidney of control fish. The centres are round to oval in shape and nodular in appearance (arrows).

(a) 15 days following the primary immunization with SRBC. (PAS, 375X).

(b) 9 days after the secondary immunization with SRBC. (PAS, 375X).

a



b

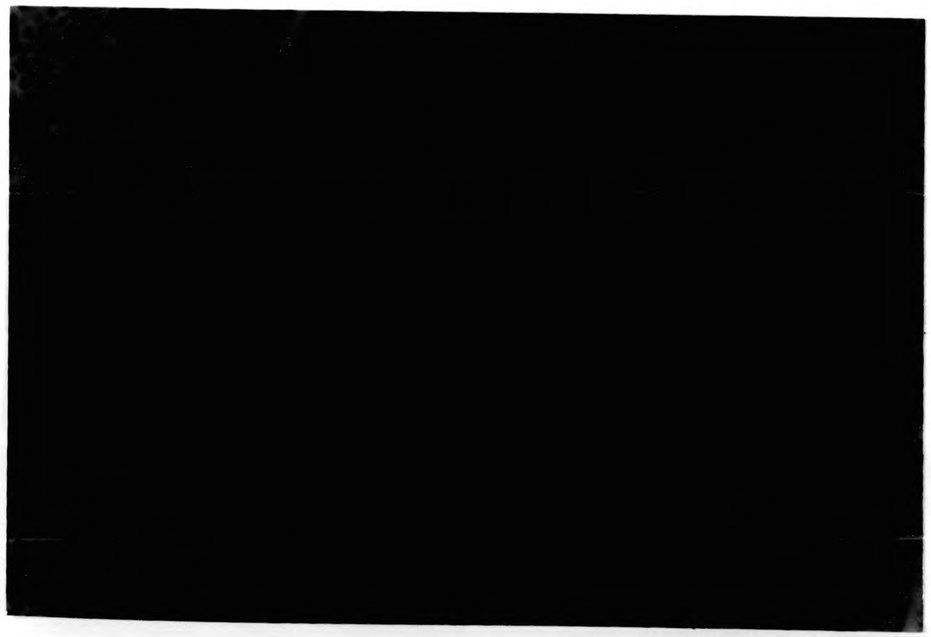


Plate 4.2 Photomicrographs showing the MMC in the spleen of control fish. Note the the irregular shape of the centres (arrow head).

(a) 15 days after the primary immunization with SRBC. (PAS, 200X).

(b) 9 days after the secondary immunization with SRBC. (PAS, 375X).

a

b

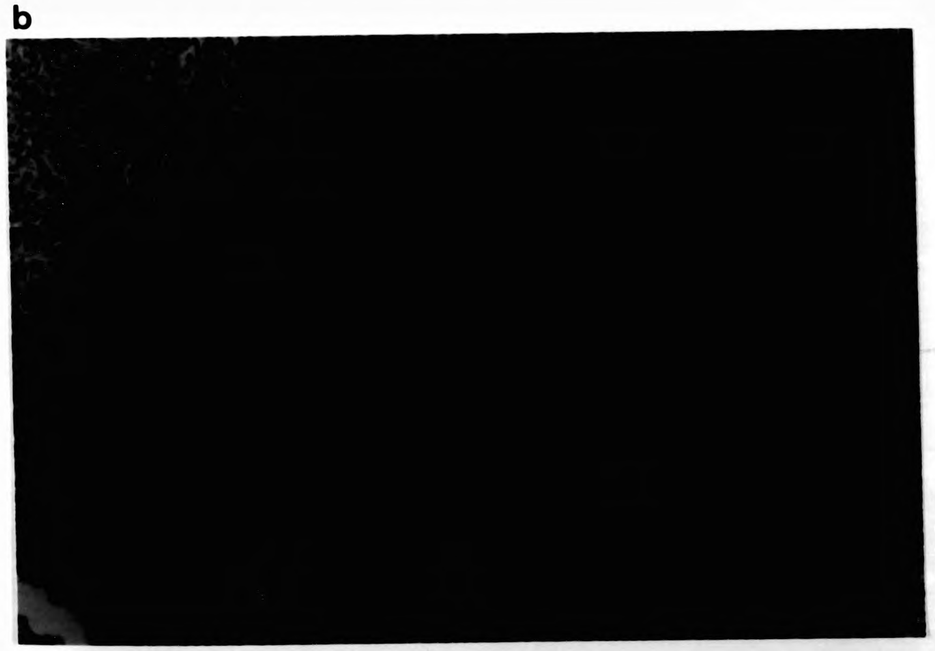
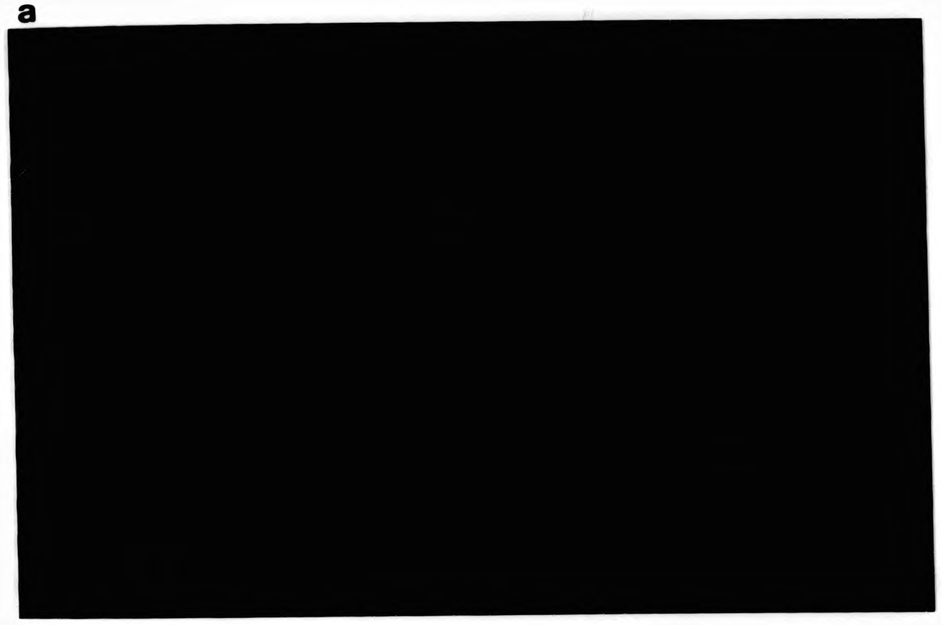


Plate 4.3 Photomicrographs showing the MMC in the control at 12 days following the secondary immunization with SRBC. Note the distribution pattern of macrophages outside the discrete centres (arrows).

(a) Kidney. (PAS, 600X)

(b) Spleen. (PAS, 600X).

a

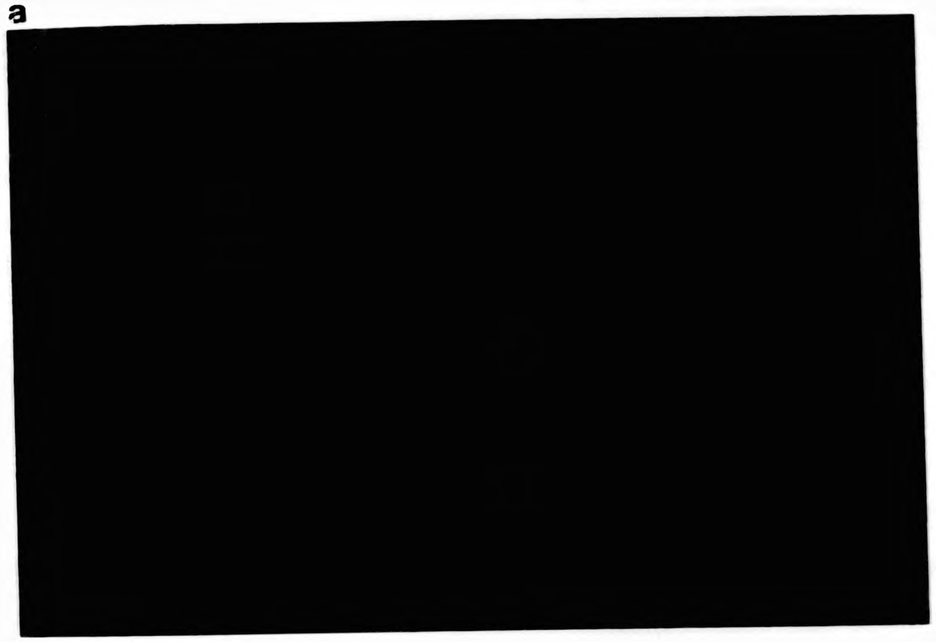


b

Plate 4.4 Photomicrographs showing the MMC in the kidney of the metal exposed fish 9 days after the secondary immunization with SRBC. Note areas of aggregates of macrophages (arrow heads) which are not in discrete centres (arrows).

(a) Cadmium (50 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS,375X).

a



(b) Copper (30 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS, 375X)

b

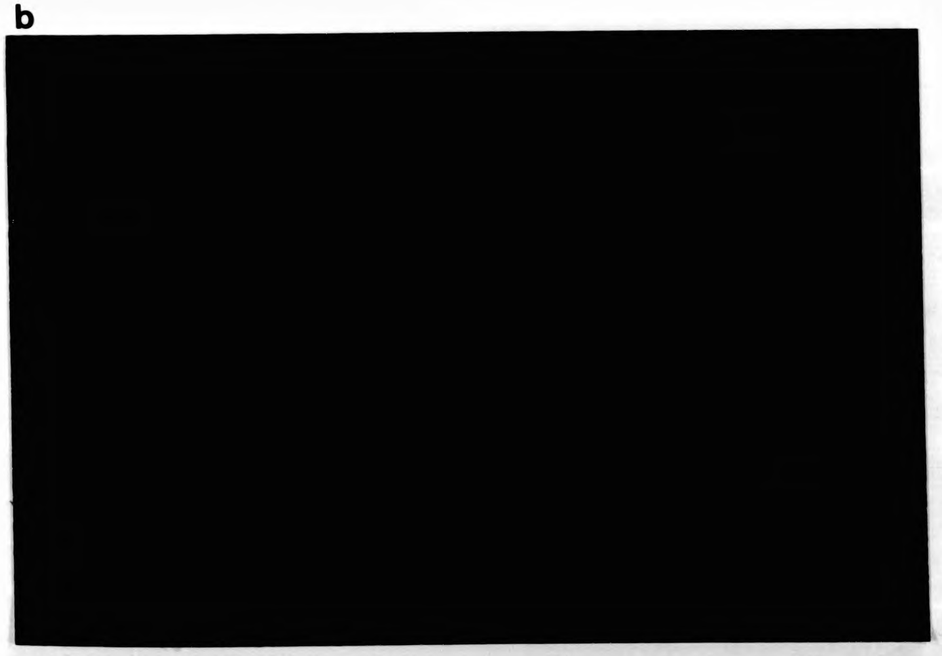
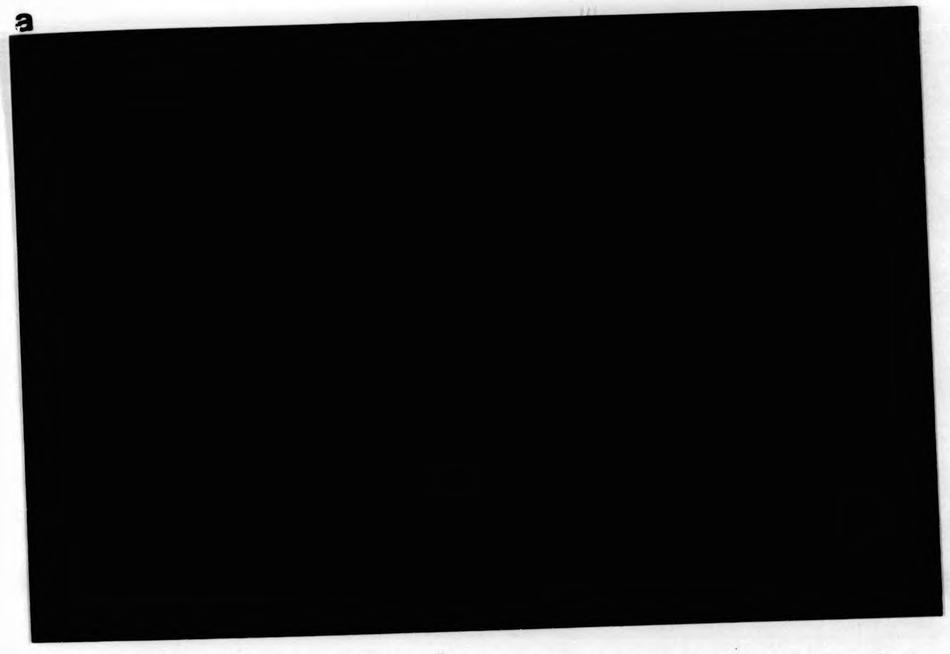


Plate 4.5 Photomicrographs showing the MMC in the kidney of the metal exposed fish 15 days following the secondary immunization with SRBC. Note higher frequency of dense aggragates (arrow heads) which are more predominant than discrete centres.

(a) Cadmium (50 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS, 375X).

(b) Copper (30 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS,375X).

a



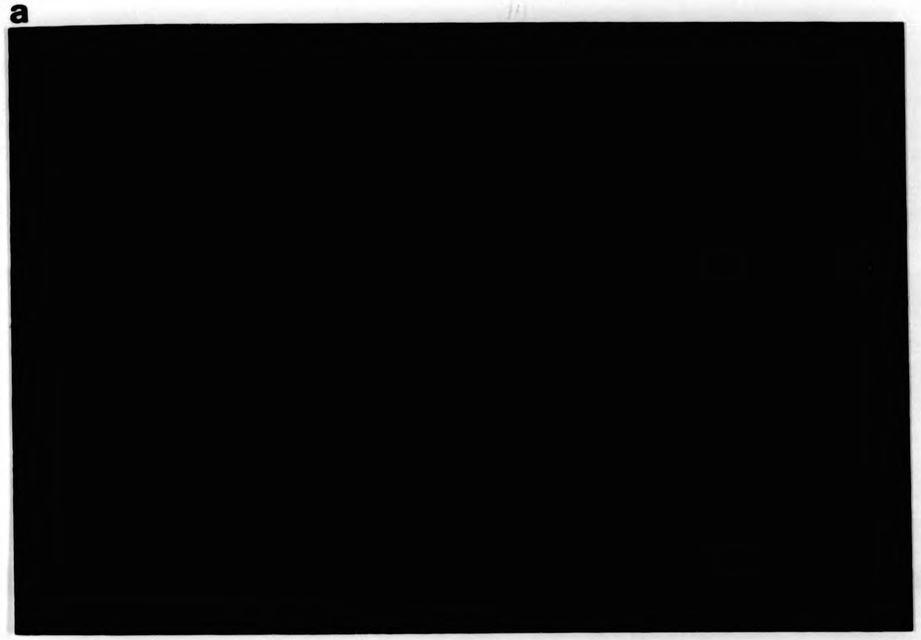
b

Plate 4.6 Photomicrographs showing the possible fragmentation (arrow head) of MMC in the kidney of the metal exposed fish.

(a) Cadmium (50 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS, 375X)

(b) Copper (30 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS, 600X).

a



b

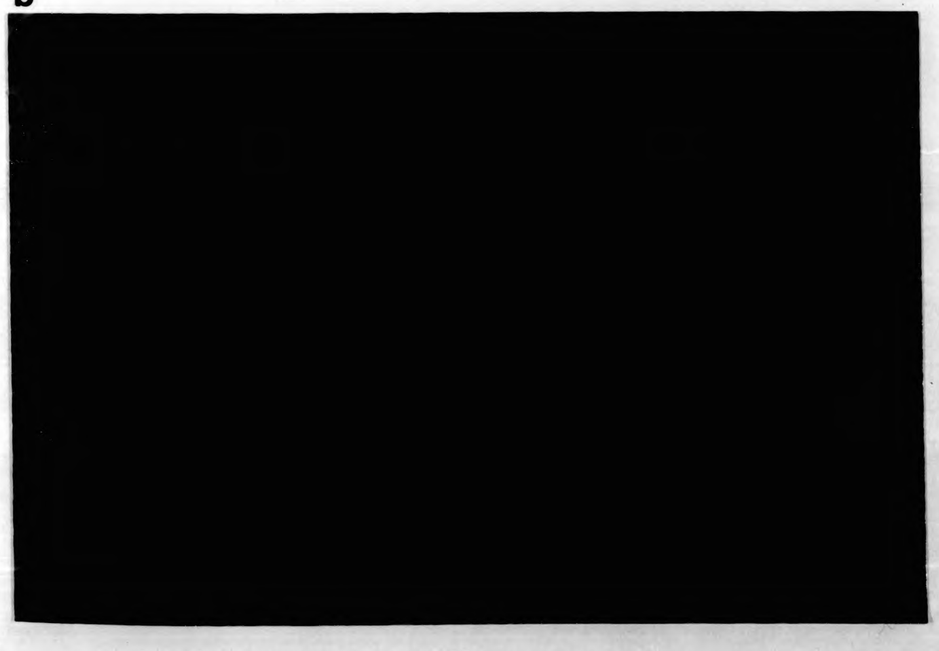


Plate 4.7 Photomicrographs showing the MMC in the spleen (arrow head) of the metal exposed fish 9 days following the secondary immunization with SRBC. Note the abundance of MMC following secondary immunization.

(a) Cadmium (50 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS, 200X).

(b) Copper (30 μgl^{-1}) exposed fish (Expt. 4.3.2), (PAS, 200X)

a

b

4.4 DISCUSSION

The following discussion is based on the effects of cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) individually on the kinetics of the primary and secondary immune response of carp, to SRBC antigens. The experiments were different from one another in that, the primary antigen was administered at different times before or after exposure to the respective metals. Secondary injection was performed 21 days after the primary.

This series of experiments demonstrated a clear suppressive effect of sublethal levels of cadmium and copper on the humoral immune response of carp to SRBC antigens, though cadmium and copper did not completely abrogate the antibody production at the concentrations tested.

The kinetics of the humoral immune response to SRBC antigens observed in controls in the present study conforms to the studies of Rijkers *et al.* (1980b). Comparing the results obtained in controls from different experiments, the peak primary response was always observed between days 15 and 18 following the primary injection. The secondary antibody response was always elevated over the first and the titre usually increased from day 6 of second injection and peaked before 12 days, remaining high until around approximately 24 to 27 days following secondary immunization. Peak PFC and RFC response normally preceeded the peak primary and secondary antibody response. The Pronephros was the key antibody producing organ compared to spleen, an observation consistent with previous finding (Rijkers *et al.*, 1980b). The lower number of PFC recorded compared to that reported by Rijkers *et al.* (1980b) could be due to the use of carp serum as the source of complement. Bream serum has been suggested to provide good assay

results (Rijkers *et al.*, 1980b).

The SRBC used in the present study are putative thymus dependent (TD) antigens in fish (Tatner, 1987). Accordingly any humoral response to these antigens would require the cooperation of "B-cell", "T-helper" cells and macrophages. Cadmium and copper both significantly reduced the number of PFC, RFC and antibody titres during the secondary response. This immunosuppression appears to be the result of interference by cadmium or copper at any one or several stages in the intricate pathway of antibody production. The lack of total suppression of immune response suggests that the initial stages of the immune response such as antigen handling and processing were not completely blocked.

Sublethal levels of heavy metals can induce perturbations in the immune response, as was shown for carp in the present study and for other fish species, with several metals (Sarot, 1973; Robohm and Nitikowski, 1974; Sarot and Perlmutter, 1976; Roales and Perlmutter, 1977; 1980; Sugatt, 1980; O'Neill, 1981a, b; Viale and Calamari, 1984; Weeks and Warinner, 1984; Weeks *et al.*, 1986; Cossarini-Dunier *et al.*, 1988; Thuvander, 1989)

The results in the present study, which follows different immunization and metal exposure regimes, has thrown light into several of the mechanisms which may be involved in the suppression of the humoral response and the ability of fish in such situations to adapt without having to suffer from total suppression. The significant effects of sublethal levels of cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) in suppressing the secondary immune response was demonstrated and are summarised below.

Exposing fish to the metals for 10 days prior to primary immunization and

continuing the metal exposure till day 9 of the secondary immunization had no affect on the kinetics of the primary antibody production, but the secondary response was reduced significantly. Both the metals significantly reduced the number of PFC and RFC followimg primary and secondary immunization. In contrast, simultaneous exposure to metals and immunization and then continuing the metal exposure throughout, had a conspicuous effect in lowering both the primary and secondary antibody response. Exposing the fish to metals 18 days after the primary immunization demonstrated the suppressive effects of the metals on the secondary response. Long-term exposure (30 days) to the metals before immunization revealed that cadmium did not result in any significant immunosuppression while copper had a significant effect in lowering both primary and secondary antibody response.

Since antibodies represent the last stage of a complex physiological pathway of the humoral immune response, it is difficult to pinpoint exactly where metals exert their detrimental effect. The fact that the overall kinetics of the immune response was unaltered, but only the magnitude of the response, especially the secondary, was significantly reduced, indicates that fish exposed to these metals were able to recognise and process the antigens and produce humoral antibody. From the consistent suppression of secondary response observed it appears that certain processes and/or cells required for the elevated secondary response were affected. The reduced number of PFC and RFC following immunization in the metal exposed groups could be responsible for the low antibody titres recorded after the second injection. The role of macrophages, helper and suppressor T-cells, B-cells and MMC and the possibility of metals interfering with any of these cells will be discussed by drawing relevant evidence from the literature.

Macrophages play a vital role in the initial process of localization, processing and

presentation of antigen to antibody producing lymphocytes. Metals have been shown to modulate the phagocytic activity of fish macrophages. Fish from polluted waters were found to have reduced phagocytic activity which returned to normal when fish were returned to clean water (Weeks and Warinner, 1984; Weeks *et al.*, 1986). Robohm and Nitkiwoski (1974) found cadmium to enhance the clearance rate of bacteria but to reduce the intracellular killing rate of phagocytes. Such a phenomenon was attributed to the interference of cadmium in the mechanism for delivering the lysosymes required for the bactericidal action. The potential of metals to exert an immediate effect on the phagocytic processes was demonstrated by Elsasser *et al.* (1986). Copper exposure immediately before the assay or after 1 or 24 hours exposure times was found to suppress the activity of phagocytes. Results with cadmium in their study showed otherwise, in that the chemiluminiscence was increased after 1 hour exposure, however, 24 hour exposure had variable results.

The ability of metals to interfere and modulate the activity of phagocytes could account for the delay and/or decrease in primary as well as secondary immune response. Total suppression of phagocytic activity of macrophages could abrogate the immune response completely to most antigens. On the other hand reduced magnitude of antigen uptake and processing by phagocytes could lead to lowered antibody titres which could be the case in the present study. The results of the present study also indirectly show that the initial process of activation of the afferent component (antigen handling, uptake and processing) of the immune response was not completely inhibited.

In homiotherms, germinal centres present in the spleen and lymph nodes have been associated with the induction of B memory cells, possibly by trapping immune-complexes via complement receptors on their surface. Fish and other poikilotherms

lack such germinal centres. It has been demonstrated that a primitive form of antigen trapping, probably as immune complexes, does occur in the spleen and pronephros of plaice (Ellis, 1980) and carp (Secombes and Manning, 1980; Secombes *et al.*, 1982a, b). In carp it is thought to be associated with proliferation and aggregation of pyroninophilic cells following immunization, to form large discrete nodules which are believed to develop into MMC (Secombes *et al.*, 1982b). These centres have been described as precursors of the germinal centre in the lymph node of higher vertebrates (Ferguson, 1976; Agius, 1985; Ellis, 1980; 1986). Damage to these centres, therefore, is likely to affect especially the secondary immune response.

Consistent changes in the number, size and frequency of MMC's observed in the present study, were very closely related to the primary and secondary immunization. These centres in spleen and pronephros were discrete and nodular. Following secondary immunization there was an increase in the frequency of macrophages and their aggregations, this observation being consistent with that of Secombes *et al.* (1982b) who found pyroninophilic cell clusters to increase after the secondary injection. The MMC and individual macrophages free in the spleen and kidney tissue were all PAS-positive, an observation also recorded by previous workers (Ferguson, 1976; Agius and Roberts, 1981). There were no obvious differences in the number of these centres between the controls and the metal exposed groups. In all the three treatments there were indications of accumulation of macrophages to form distinct nodules or centres. During their development melanomacrophages are thought to detach from the ellipsoids and migrate through the splenic parenchyma and settle in MMC (Ferguson, 1976). However, in metal exposed groups, in addition to discrete centres, there were many individual macrophages distributed in the pronephros tissue, possibly reflecting the inability of macrophages to form

aggregates. There was evidence of fragmentation of these centres in copper exposed groups. These changes would appear to have some role in suppressing the secondary response when compared to controls. A significantly lowered number haemosiderin bodies found in the spleen of blue gouramis exposed to copper and methyl mercury and immunized with *Y. ruckeri* O antigen, was attributed to the immunosuppression by Roales and Perlmutter (1980). They believed haemosiderin bodies to be the precursors of germinal centres.

Increase in MMC in fish from polluted waters (Poels *et al.*, 1980; Khan and Kicenink, 1984; Wolke *et al.*, 1985) could represent macrophages localizing necrotic cells from different tissues and aggregating at these centres. Recently Kranz and Gercken (1987) observed the splenic MMC number to increase 3 times, but their average size to be reduced significantly in plaice exposed to potassium dichromate. Decreased tendency to form large aggregates was suggested to indicate increased turnover rate of macrophages (Kranz and Gercken, 1987).

Considering the fact that MMC are believed to be responsible for "immune memory" formation or an elevated secondary response, then the observed effects of cadmium or copper on these centres could be responsible for at least some of the suppression of the secondary response observed.

The ability of fish to respond normally to immunization after long term exposure to sublethal levels of metals may suggest the possibility of physiological acclimatization. However, the results from the present study clearly show how this process could differ from metal to metal. Long term exposure to cadmium did not impair either primary or secondary response, whilst copper had a significant effect in suppressing the primary and secondary humoral response. Evidence that fish from

polluted waters were able to produce high antibody titre to a variety of pathogens (Robohm *et al.*, 1979; Robohom and Sparrow, 1981) is very interesting in this respect. Factors such as concentration of the pollutant, duration of exposure, nature of the pollutant and fish species may all have an influence on the ability of fish to respond immunologically in such situations. Individual toxicants may have a threshold level up to which the fishes can adapt and even reverse the changes after the withdrawal of the toxicant. The long term copper exposure results suggest that the suppression of primary and secondary immune response is either due to lack of a sufficient number of various cell types to initiate and execute the immune respose or to the longer time needed to acclimatize or recover from the damage caused by the copper.

In contrast, long-term (4 months) cadmium exposure with immunization administered 1 month after exposure to the metal was found to reduce the antibody to human RBC slightly in rainbow trout, whilst copper and chromium were found to have no effect (Viale and Calamari, 1984). Administration of as many as 5 immunization doses over only a 2 week period could explain why the antibody level appeared to be unaffectd in their studies, inspite of using high levels of copper (100 μgl^{-1}) in rainbow trout, a species considered to be more sensitive than carp to heavy metal toxicity.

The ability of fish to respond to the antigen administered following long-term exposure to toxicants may also depend on the nature of toxicant. Long-term (1 month) exposure of rainbow trout to endrin before immunization and continued thereafter significantly reduced the serum agglutination titre, plaque forming cells and migration inhibition factor, but not phagocytic ability according to Bennett and Wolke (1987a). The initiation of the immune response and the subsequent synthesis

and release of antibody was not affected, but only the magnitude of the response was lowered as a consequence of the reduced number of lymphocytes and antibody producing cells. This line of explanation appears to hold true for the findings of the present study, where the immune response was not suppressed totally but the magnitude was significantly reduced.

On the other hand, long-term exposure of carp to high concentration of manganese for 2.5 months (50 mgl^{-1}), immunized 7 days after the start of metal exposure was found not to lower the antibody production but significantly reduce the haematocrit (Cossarini-Dunier *et al.*, 1988). Evidence for the ability of fish to acclimatize to long-term exposure to cadmium (3.6 μgl^{-1}) and produce a normal humoral immune response also comes from the recent work of Thuvander (1989) however she found the cellular response to be significantly reduced. The results in the present study described in Chapter 3, also reveal a similar effect, that is no lowering or impairment of the humoral antibody production but a significant affect on other components of the immune response; possibly cellular immune responses and antibody or complement dependent cellular cytotoxic processes. The observations of Muller *et al.* (1977) that cadmium exposure suppresses T-cell mediated and macrophage dependent processes in mammals, rather than antibody production does to some extent support the findings in the present study.

The effect of heavy metals on the replication of immunocompetent cells in fish has not been examined in great detail. Cenini and Turner (1983) found zinc to have a suppressive effect on mitogen responsiveness of carp lymphoid cells to both B and T-cell mitogens as quantified by incorporation of 3H-thymidine into DNA. On the contrary, Ghanmi *et al.* (1989) found zinc to stimulate both T and B-like cells and enhance 3H-thymidine incorporation. However, manganese at lower concentrations

(10^{-7} to 10^{-3} M) was found to have a stimulatory effect on PHA and Con A (T cell mitogens) stimulated lymphocytes but not to LPS (B cell mitogen) stimulated cells while high concentrations (10^{-1} M) inhibited the response to T-lymphocyte mitogens but not to B-lymphocyte mitogen. Metals with the potential to interfere with calcium dependent cellular transport systems and mitochondrial protein synthesis are regarded generally to reduce the mitogen responsiveness of stimulated lymphocytes. Total suppression of mitogen responsiveness has been observed only at concentrations which are toxic to lymphocytes. The significant reduction in antibody titre recorded in the present study following secondary immunization could indirectly reflect the reduced ability of lymphocytes to proliferate *in vivo* under the influence of heavy metals. The evidence from the PFC response studies does support this view.

Lymphokines and cytokines are communicators between macrophages and T and B lymphocytes and have been reported to occur in fish (Smith and Braun-Nesje, 1982). Although interference by stressors or toxicants at this stage have not been reported in fish, Grondel and Boesten (1982) have suggested that chemicals with the potential to inhibit mitochondrial synthesis may have an influence on cellular interactions which are essential for optimal expression of the humoral response. Suppression of lymphokine-like factors has been suggested for the decrease in B cell activity following cortisol treatment (Ralph *et al.*, 1987). Such interference from cadmium and copper cannot be excluded since the secondary response in all the experiments was consistently suppressed.

The evidence available in the literature and the results of the present study indicate the importance of time and dose on immunosuppression. The time interval between immunization and exposure to the toxicant appears to have a strong influence in the ensuing immunosuppression. The greatest effect on the primary immune response

was found when there was no time interval between immunization and exposure to metals.

The conspicous drop in primary response could be a consequence of the general stress response, the components of which contribute to immunosuppression (Ellis, 1981). Corticosteroids are believed to be most effective in suppressing the immune response when administered before the antigen (Hersch, 1974). In vertebrates, stress causes multiple changes in the hormone balance and internal homeostasis which are characterised by the activation of the hypothalamus-hypophysis-adrenal axis (Pickering, 1981). This serves to return the body to the original condition of homeostasis. Such an adaptation attempt is normally accompanied by several primary and secondary effects, the components of which are immunosuppressive (Mazeaud *et al.*, 1977; Ellis, 1981). Corticosteroids, which affect both metabolic and immunologic pathways, rise dramatically in fish in response to stressful stimuli (Donaldson, 1981; Schreck, 1981). Heavy metal toxicants can indirectly act as "stressors" and induce a general stress response and modify corticosteroid levels in fish (Schreck and Lorz, 1978; Bromage and Fuchs, 1986). The duration of the primary and secondary effects of stress may depend on the nature of stressor. However, it is well known that fish are able to recover from stress and adjust the internal homeostasis (Pickering and Stewart, 1984; Pickering and Pottinger, 1987b).

Lymphocytopenia (Peters *et al.*, 1980, Pickering *et al.*, 1982; Ellsaesser and Clem, 1986; Peters *et al.*, 1988), lack of mitogen responsiveness (Grimm, 1985; Elssaesser and Clem, 1986; Ralph *et al.*, 1987), degeneration of leucocytes (Peters and Schwarzer, 1985) and reduced chemiluminescence and phagocytic activity (Stave and Roberson, 1984) are often associated with stress. The finding of few surface immunoglobulin (sIg) positive lymphocytes in fish under stress (Peters *et al.*, 1988)

might indicate reduced availabilty of lymphocytes competent enough to recognise the antigen and initiate the immune process.

The mechanism of immunosuppression might involve prevention of antigen recognition, uptake by macrophages and presentation to antibody producing cells (afferent component). But the normal primary response observed in fish exposed to cadmium or copper for 10 days prior to immunization indicated that the afferent immune system was competent to recognise and process the SRBC antigens, contrary to that seen in carp exposed to the metals and immunized simultaneously. It is interesting in this context, that Donaldson and Dye (1975) found corticosteroid concentrations in sockeye salmon exposed to copper showed a transient elevation during the first 24 hours with subsequent return to control values despite the continued presence of copper.

Cortisol administration to carp of 9 weeks of age simultaneously with the antigen allowed the antibody response to proceed, compared to the controls which failed to respond (or responded poorly) on secondary immunization (Ruglys, 1985). Such an effect was attributed to the possibility of cortisol delaying the development of suppressor cells. Such possibilites should be given consideration when evaluating the immunosuppressive effects of stressors in juvenile fish which may not have developed sufficiently to respond normally.

Antiproliferative agents are most effective in suppressing the immune response when administered between immunization and the time antibody can be detected (Hersch, 1974). The suppression of the secondary response observed in all but one experiment here could be to some extent due to the antiproliferative activity of the metals. O'Neill (1981a) found cadmium, zinc, copper and lead not to produce a

total suppression of the immune response but to bring about a greater suppression of the secondary response in fish immunized with bacteriophage MS2, a finding consistent with the present results. Reduction in the number of B cells and loss of helper and memory cell activity and suppression of clonal expansion of B-cells and/or memory cells has been suggested as the possible reasons for reduced antibody producing cells and depressed antibody titres. Reduction in the number of antibody producing cells (PFC) could have directly contributed to the reduced antibody. Whether the antiproliferative effect of the metals is directly responsible for the observed reduction in the number of antibody producing cells is not clear.

Differential effects of stressors or chemicals on T-cell sub-populations can have differing effects on the humoral immune response. This hypothesis is difficult to envisage in fish because of the uncertainty still surrounding the issue of thymus dependency of antigens in fish. A number of speculations can be made depending on the effects of toxicants on T-helper and T-suppressor cells. Suppressor T-cells, even in small numbers, can abrogate the immune response (Debre *et al.*, 1975). Reduction in the number of T-helper cells during glucocorticoid treatment (Bradley and Mishell, 1982) in conjunction with increase in percentage of T-suppressor cells (Rodgers and Mattosian-Rodjers, 1982) was proposed to be responsible for compromising both plaque forming cells and antibody production. In contrast, an elevated response is sometimes attributed to specific reduction of T-suppressor cells (Anderson *et al.*, 1982). Regardless of how immunomodulation occurs, antibody production is expected to increase or decrease depending on the specific effects of chemicals on T-cells, since they control and regulate antibody producing B-cells to many antigens.

It remains to be seen whether fish T-cell subpopulations are affected in a way

similar to that seen in mammals. Alkylating agents which effect the proliferation and mitosis of T and B cells have produced such specific effects on T cell sub poulations in fish (Anderson *et al.*, 1982). Cyclophosphamide is suggested to block suppressor T cells at certain critical stages, thereby allowing plasma cells to produce large amounts of antibody (Chen *et al.*, 1983). A large number of antibody producing cells and high titres seen in rainbow trout immunized with *Y. ruckeri* O-antigen and injected with cyclophosphamide 24 hours later, was attributed to the specific effects on T-suppressor cells (Anderson *et al.*, 1982). It is still not known whether heavy metals have such an action on specific sub-populations of fish lymphocytes. However, such an action on T-helper cells cannot be ruled out on the basis of the consistent suppression of the secondary response observed in the present study to the thymus dependent SRBC antigens.

The thymus dependency of several antigens used in fish immunology research is unclear and this makes it difficult to compare the immune response in terms of cellular requirements. Rainbow trout immunized and exposed to chromium for 2 weeks had significantly lower titres by 6 weeks after immunization (Sugatt, 1980). Blue gouramis immunized 1 week after initial exposure to copper or methylmercury, or both, had reduced titres to *P. vulgaris* and Infectious Pancreatic Necrosis Virus (IPN) (Roales and Perlmutter, 1977). These studies also emphasize the detrimental effects metals can have on the immune response of the fish to a wide variety of pathogens (antigens).

Several interesting parallels can be drawn between the present study and the studies carried out on the immunomodulatory effects of antibiotics in fish. The findings of Rijkers *et al.* (1981) is in contrast with the results recorded here. The primary response of carp to SRBC was suppressed when oxytetracycline was administered 2

weeks before immunization, but the secondary response was not altered. Interference of oxytetracycline with the interaction between phagocytic macrophages and T and B-like cells was suggested as the likely cause. It has been suggested that activated B cells would proceed with proliferation and differentiation into antibody producing plasma cells at a normal rate when oxytetracycline concentration starts decreasing, thereby, not suppressing the secondary response (Rijkers *et al.*, 1981). Such a suggestion appears to be logical in view of the present finding that long-term (30 days) cadmium exposure before immunization did not suppress the secondary anti-SRBC response. However, in contrast in all the other experiments it was the secondary response that was significantly suppressed.

Administration of oxytetracycline for a month following immunization suppressed anti-RaRBC response (van Muiswinkel *et al.*, 1980). Grondel *et al* (1987) found the primary anti-SRBC response to be most suppressed when oxytetracycline was administered 1 day before immunization. The kinetics of the PFC was not affected but in contrast the number of PFC was significantly reduced and the peak anti-SRBC antibody response was delayed by 2-4 days. Antibiotics produce dose-dependent suppression of chemiluminiscence in *S.gairdneri* pronephros leucocytes (Wishkovsky *et al.*, 1987). Incorporation of 3H-thymidine into DNA of PHA or LPS stimulated leucocytes was depressed by antibiotics in a dose-dependent manner and oxytetracycline delayed the mitogenic response, but did not reduce it (Grondel *et al.*, 1985) which might explain the suppression and delay in anti-SRBC antibody seen by Grondel *et al.* (1987). Interestingly the antibody response returned to levels comparable to the controls after 12-14 days post-immnization.

The observations of the present study regarding suppressed primary anti-SRBC response when exposure to metals and immunization were simultaneous does agree

with the findings of Grondel *et al.* (1987). It appears that most toxic agents tend to suppress the primary response significantly wherever the time gap between exposure to the toxicant and immunization is less than 1 or 2 days. As discussed earlier such an effect could be as a result of stress induced response, the components of which are known to be immunosuppressive. These findings also illustrate the ability of fish to proceed with a normal immune response in such situations provided the initial response is not completelty abolished by the toxicant. However, the magnitude of the response as seen in the present study may be lowered.

Long-term oxytetracycline treatment inhibited the primary anti-SRBC response (Grondel *et al.*, 1987), which is contrary to the observation made in the present work with cadmium but in agreement with the copper results. Such discrepencies could explain the differences in the mode of action of the different toxicants.

The consistent suppression of the secondary ("memory") anti-SRBC response, documented in the present study, appears to be due to the possible direct effects of cadmium and copper on proliferation and differentiation of B-like and T-like cells, and on MMC and indirect effects by way of interfering in the complex cooperative processes of the immune response involving the macrophages, T and B-like lymphocytes and MMC. Such a view is also supported by the fact that the secondary response was reduced even in fish exposed to the metals 18 days after the primary response. It is also evident that, inspite of exposure to sublethal levels of cadmium or copper, the immune system of *C. carpio* was competent enough to activate the afferent and efferent branches of the immune system and execute the humoral response.

The complex interaction and cooperation of immunocompetent cells which are vital

to produce an humoral immune response in fish, are in fact vulnerable to many biochemical and physiological perturbations, which can be induced by heavy metals. This is discussed further in the final Chapter (8) and comparisons are drawn between the situation in fish and the immunomodulatory effects of metals seen in mammals.

CHAPTER 5

THE EFFECTS OF CADMIUM AND COPPER ON THE SUSCEPTIBILITY, PROTECTIVE IMMUNITY AND HUMORAL ANTIBODY RESPONSE OF CARP, *C. carpio* to *Aeromonas hydrophila*.

5.1 INTRODUCTION

Studies on the influence of heavy metal pollution on the disease susceptibility and immune response in fish were reviewed by Zeeman and Brindley (1981) and Anderson *et al.* (1984). On examining these reviews it becomes evident that the majority of the knowledge available in this area is confined to studies using bacterial pathogens.

In most of the reported work, *V. anguillarum* and copper have been used as models. Considering the relative pathogenicity of different fish bacterial pathogens, it may not be surprising to find that *V. anguillarum* and *Y. ruckeri*, used in conjunction with metals or other chemical pollutants have always resulted in increased disease susceptibility (Stevens, 1977; Rodsaether *et al.*, 1977; Sugatt, 1980; Knittel, 1981; Baker *et al.*, 1983) with one or two exceptions (Cossarani-Dunier *et al.*, 1988). On the other hand, bacterial pathogens such as *F. columnaris* (MacFarlane *et al.*,1986) and *A. hydrophila* (Snarski, 1982) used for infection in metal exposed fish, did not produce any significant alterations in susceptibility to disease. From the existing literature it is also evident that the experimental protocol followed in certain cases (MacFarlane *et al.*, 1986) could have influenced the result to a great extent.

The reported effects of metal pollutants on the immune response of fish to bacterial antigens are not consistent. Metals have decreased (Roales and Perlmutter, 1977; 1980; Sugatt, 1980) and in some cases enhanced (Thuvander, 1989) the immune response, while essential metals such as manganese at high concentrations had no affect (Cossarini-Dunier *et al.*, 1988).

Most of the information available on the immunotoxic properties of metal pollutants

relates the effect of the metal to either disease susceptibility or antibody titre. Unless studies are carried out to evaluate the effect of a single toxicant on a variety of other aspects, and try to understand their interrelationship, it is difficult to define the effects of the toxicant on disease.

Aeromonas hydrophila is a gram negative bacterium regarded as being ubiquitous in fresh water where it may cause Bacterial Haemorrhagic Septicemia (BHS) in many fish species (Bullock *et al.*, 1971; Hazen *et al.*, 1978). This disease has been strongly linked to environmental stress in fish (Wedemeyer, 1974; Richards and Roberts, 1978). *A. hydrophila*, being a faculatitive, opportunistic pathogen would make an ideal model to evaluate the sublethal effects of a wide range of chemical pollutants where the expected influence of the metal is as a "stressor", as suggested in the previous Chapter.

The pathogenicity of different isolates of this bacterium has been well established and, according to Stevenson (1988) isolates with LD_{50} of 10^4 to 10^5 are regarded as virulent and, those that do not kill the fish at or above 10^7, are considerded non-virulent. The kinetics of the primary and secondary humoral immune response and memory formation in carp to heat and formalin-killed *A. hydrophila* has been demonstrated in detail (Lamers *et al.*, 1985). These features make this bacterium a good candidate for studies of the present nature.

The antigenic nature of *A. hydrophila* and the protective mechanisms of fish against this bacterium are not fully understood. It has been suggested that *A. hydrophila* has both Thymus Independent (TI) and Thymus Dependent (TD) antigenic components (Lamers *et al.*, 1985). Recently Baba *et al.* (1988a) have suggested that the protective mechanisms of immunized fish against this bacterium involve T-helper

cell and macrophage system (cellular immunity).

The two previous models (*I. multifiliis* and SRBC) used in the present investigation were different from *A. hydrophila* with respect to the cellular requirements for optimal humoral immune response. SRBC are regarded as TD antigens (Manning *et al.*, 1982) whilst the thymus dependency of "Ich" antigen is still uncertain (Houghton, 1987). Ichthyophthiriasis is a parasitic disease confined to the external epithelium while BHS is a systemic disease. The defence mechanisms of the fish against these two unrelated diseases would be expected to be different. From this point of view it was decided to use *A. hydrophila* as a model to delineate the effects of sublethal levels of cadmium and copper on the disease susceptibility, protective immunity and kinetics of the immune response in carp.

Four different approaches were taken to ascertain the effects of cadmium and copper. The first experiment was designed to investigate the effects of cadmium and copper exposure on the disease susceptibility of naive fish. In the second, previously immunized fish with established levels of antibody were exposed to cadmium and copper for 10 days to see the effects on the protective immunity. Experiments three and four were aimed at delineating the effects of cadmium and copper on the kinetics of humoral antibody response to heat-killed *A. hydrophila* whole cell bacterin.

5.2 MATERIALS AND METHODS

5.2.1 *A. hydrophila*, Maintenance and Culture

A virulent strain of *Aeromonas hydrophila* (NCMBA 1137) was used in all the immunization and challenge experiments. The strain was passaged three times intraperitoneally through carp to increase the virulence, before being used for any experiments. Isolations made from the last passage were maintained on Trypticase Soya Agar (TSA) slopes (Oxoid, U.K). Bacteria to be used for challenge infections were cultured for 24 hr on TSA at 22°C. The harvested cells were washed twice in 0.85% sterile saline by centrifuging at 6500 rpm for 5 minutes. The bacterial pellet was later resuspended in 0.85% saline to the required optical density.

The number of viable bacteria per ml was read from a standard curve of optical density against number. The standard curve was constructed as follows; From a original bacterial suspension of very high optical density (1.8), serial two-fold dilutions were made. The optical densities of each of the dilutions at 575 nm was read from a spectrophotometer. The number of viable bacteria at each of these dilutions was determined by the pour plate method. The standard curve was constructed by plotting the optical density against the number of viable bacteria. Before using the bacteria for challenge infection, the washed cells were resuspended in 0.85% saline to the required optical density (0.7-0.8 at 575nm). The number of bacteria in the suspension used for infection was always enumerated by pour plate method, in addition to reading the number from the standard curve.

5.2.2 Infection Procedure

Fish were anaesthetised with 100 ppm Benzocaine (methyl para-amino benzoate) individually and injected intraperitoneally with 0.1-0.15 ml of bacterial suspension of

known optical density. Care was taken not to damage any of the internal organs while administering the injection. For this purpose 1 ml syringe fitted with a 27 gauge needle was used.

5.2.3 Mortality Assessment

The mortalities were recorded at least twice daily. The anterior kidneys of dead fish were sampled aseptically for bacterial isolation within less than 6 hours after death, to verify the presence of *A. hydrophila* and confirm the cause of mortality. Presumptive identification was based on standard biochemical tests and carried out using API 20E strips (BioMerieux, U.K)

5.2.4 Bacterin Preparation

Bacterin for immunization was prepared from cells grown for 18 to 24 hours at 22°C on trypticase soya agar. The cells were harvested by carefully scraping the colonies from the agar slopes. Two ml of 0.85% saline was then added to wash down all the cells from the agar slopes and added to the scraped material. This bacterial suspension was centrifuged at 6500rpm for 5 minutes in a refrigerated centrifuge. The bacterial pellet was washed twice in 0.85 % sterile saline and resuspended to a final concentration of approximately 10 $mgml^{-1}$ wet weight in saline. The number of bacteria (cfu) in this suspension was determined by pour plate method. This preparation was heat killed at 100°C for 1 hour and preserved with 0.2% formalin. The whole cell bacterin preparation was tested for sterility and stored at -20°C till required for use.

5.2.5 Immunization Procedure

The bacterin pellet was washed 3 times in 0.85% saline by spinning at 6500rpm for 5 minutes to remove the formalin before being used for immunization. Fish to be

immunized were anaesthetized with 100 ppm Benzocaine and injected intramuscularly with 0.1 ml of the bacterin preparation of known optical density.

5.2.6 Collection of Blood

For measurement of serum agglutination titre, blood was collected from the caudal vein using an heparinized disposable syringe with a 27 gauge needle. Approximately 0.3 to 0.4 ml blood was withdrawn from each fish and transferred to non-heparinized Ependorf vials (1.5ml), allowed to clot at room temperature for one hour and kept overnight at 4°C. The serum was separated from the blood cells by centrifugation at 12000 rpm for 6 minutes and stored at -70°C till required for use.

5.2.7 Agglutination Assay

The serum was heat inactivated at 50°C for 30 minutes. Two-fold serial dilutions of 100 µl serum was made in phosphate buffered saline. The antigen was prepared by dilution of the immunization bacterin with 0.85% saline to a spectrophotometer reading of 0.9 O.D at 575nm. Each serum dilution and PBS control received 100 µl antigen. The microtitre plates were sealed, mixed gently and incubated at 4°C overnight. With each assay, a batch of sera collected from non-immunized carp were used to test for autoagglutination of *A. hydrophila* whole cell bacterin. The maximum dilution giving a positive agglutination was read at a magnification of X20 using a stereo microscope and the titre was recorded as the reciprocal of that dilution. A positive reaction consisted of even dispersal of the antigen over the bottom of the microtitre plate. A negative reaction was indicated by the formation of a small "button" of the antigen at the bottom of the microtitre plate. The data were reported $-\log_2$ titre + 1. Serum samples with no detectable titre were considered to have a 0 titre for the purpose of calculations and presentation of mean titres.

5.2.8 Experimental Protocol

Experiment 1 <u>The effects of metal exposure on the susceptibility of carp to infection</u>

This experiment was designed to delineate the effects of sublethal levels of cadmium or copper on the susceptibility of naive carp to *A. hydrophila* infection. Cadmium and copper experiments were run simultaneously. Three groups of 25 fish each (10.62 ± 0.99 cm; 19.35 ± 4.75 g; n=75) were exposed to 0 (control), 50 μgl^{-1} (cadmium) or 30 μgl^{-1} (copper) in a flow-through system for 10 days. At the end of the exposure period the fish were held in untreated water in the flow-through system for bacterial challenge. Fish from each treatment was divided into 5 groups of five fish each. Fish were anaesthetized individually and injected intraperitoneally with 0.1 ml of the four ten-fold dilutions (10^{-4} to 10^{-7}) of *A.hydrophila*. The number of bacteria injected per fish at 10^{-7} dilution was 1.8×10^{3}. One group was injected with 0.1 ml of sterile saline and this served as control. The fish were held in separate tanks after the challenge and were under regular observation for a week after the challenge infection. Mortalities were recorded at least twice daily. Dead fish were sampled for isolation of the bacteria from the anterior kidney and all the mortalities were confirmed to have resulted from the challenge.

The experiment was repeated and, for the purpose of presentation of results and calculation of LD_{50}, the results from these two replicate experiments were combined as they did not differ significantly from each other.

Experiment 2 <u>The effects of metal exposure on the protective immunity of previously immunized carp</u>

This experiment was aimed at evaluating the protective immunity of carp to *A. hydrophila* under the influence of cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}). A group of 60 fish (12.64 ± 1.17 cm; 20.08 ± 5.47 g; n=60) were immunized by giving a primary injection (10^8 cells/fish) on day one and a second injection (10^8 cells/fish), 15 days later (Section 5.2.5) and held in the flow-through system. Samples of sera were collected before the second injection (day 14) and before exposure to the metals (day 21) for bacterial agglutinin assay. Twenty one days after the first injection, the fish were divided into 6 groups of 10 each and allocated to 6 tanks (3 treatments in duplicate). Two groups received either cadmium or copper, while the remaining served as the control. The metal exposure duration was 10 days. At the end of the exposure duration, serum was collected from 6 fish per treatment from duplicate tanks. All the fish were challenged (Section 5.2.2) with a potentially lethal dose (10^6 bacteria/fish) of *A. hydrophila*. At this stage a batch of non-immunized fish was also challenged. Mortalities were followed over a week and isolations were made from all the moribund fish to confirm the specific cause of mortality. The results from the duplicate experiments were combined to calculate the percentage protection.

Experiment 3 <u>The effects of metal exposure on the kinetics of the humoral agglutination titre</u>

This experiment was aimed to look at the effects, exposure to metals before immunization could have on the kinetics of agglutination titre in carp to *A. hydrophila* bacterin. The fish were exposed to the metals for 10 days prior to the administration of immunization. Two groups of 12 fish each (10.56 ± 0.94 cm;

19.90 ± 3.85 g) in duplicate were exposed to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) in the flow-through toxicity system for 10 days. An additional group of 12 fish in duplicate served as controls. After 10 days, the metal dosing was discontinued and the fish from all the three treatments were immunized with *A. hydrophila* bacterin (Section 5.2.4) as described previously (Section 5.2.5). Each fish received an antigen dose of approximately 10^8 cells. On days 6, 9, 15, 18 and 21 following immunization, 3 fish per treatment were sampled and the serum agglutination titre assayed (Section 5.2.7). Fishes had to be resampled for the last sample, but a minimum time interval of 15 days was allowed in such cases.

Experiment 4 <u>The effect of concurrent exposure to metal and administration of the immunization on the primary and secondary humoral agglutination titre</u>

This experiment aimed at evaluating the primary and secondary humoral response of carp held in metal treated water to *A. hydrophila* bacterin. The fish were first immunized (10^8 cells/fish) and on the same day exposure to the metals was commenced. Three groups of 12 fish each (11.04 ± 1.20 cm; 20.47 ± 5.37 g; n=36) were first immunized (Section 5.2.4) and then allocated to 3 tanks. Two groups received either cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) and one group with no metal served as the control. A second injection (10^8 cells/fish) was given 2 weeks later. Metal exposure was continued throughout the experiment. On days 6, 9, 15, 21, 27 and 30 of immunization, 3 fish per treatment were sampled and the serum agglutinin titre measured. For the last two samples, fishes were resampled but the time interval between consecutive samplings was more than 15 days. A duplicate set of experiment was run side by side.

5.3 RESULTS

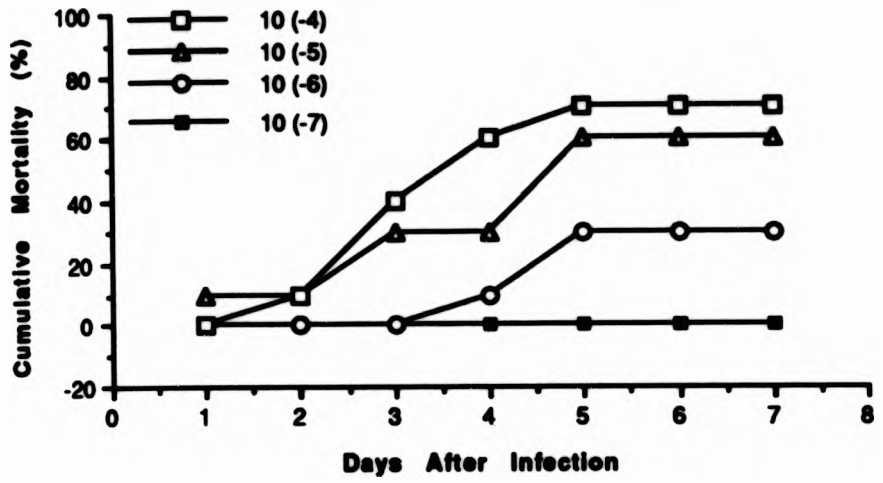
5.3.1 Experiment 1

The results presented here demonstrate the effects of sublethal levels of cadmium and copper on the susceptibility of naive carp to *A. hydrophila* infection. The daily cumulative mortality in the three treatments resulting from different challenge levels are illustrated in Figure 5.1a-c. It is evident that mortalities commenced in the cadmium and copper treatments earlier than the controls at any given challenge level. In the cadmium and copper groups, the initial mortality occurred between days one and two, especially at high challenge levels. The mortality rate observed over a week was gradual in the controls while it rose more steeply between days 1 and 5 following injection ($1.8x10^5$ and $1.8x10^6$ bacteria/fish) in metal treatments.

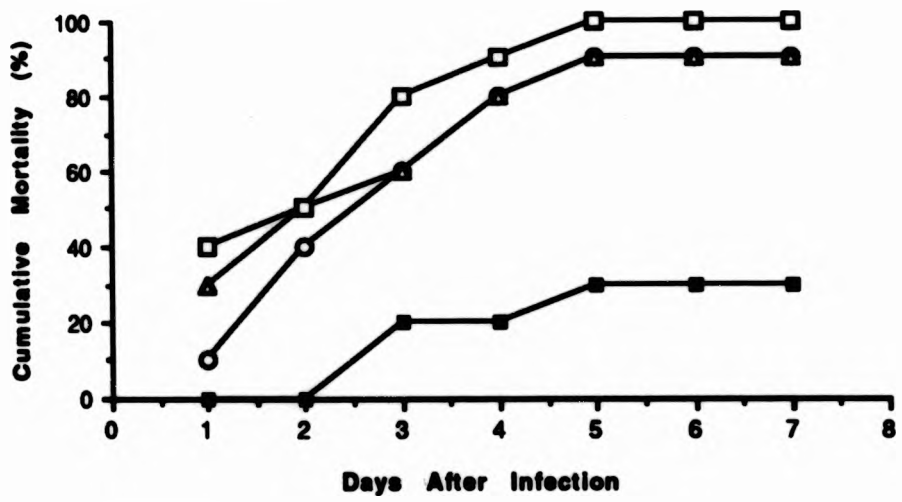
The total mortality brought about as a result of challenge infection with four graded ten-fold dilutions of *A. hydrophila* over a week is presented in Figure 5.2a. The mortality was significantly ($P<0.001$) related to the bacterial dose in all the three treatments. The combined total mortality from duplicate experiments per treatment resulting from all the four bacterial doses was 40% for control, 77.5% for cadmium and 72.5% for copper (Figure 5.2b). Statistically, the mortality caused by *A. hydrophila* in the cadmium and copper treatments was significantly higher ($P<0.05$) than the controls. Mortality was also significantly related to the presence or absence of metals.

At each challenge level, the mortality caused by *A. hydrophila* in the cadmium and copper treatments was relatively higher than the controls. The lowest challenge level tested (10^{-7}; $1.8x10^3$ bacteria/fish) did not result in any mortality in the controls, while it produced 30% mortality in the cadmium and copper exposed carp. Only

(a). Control



(b). 50 µg/l Cd



(c). 30 µg/l Cu

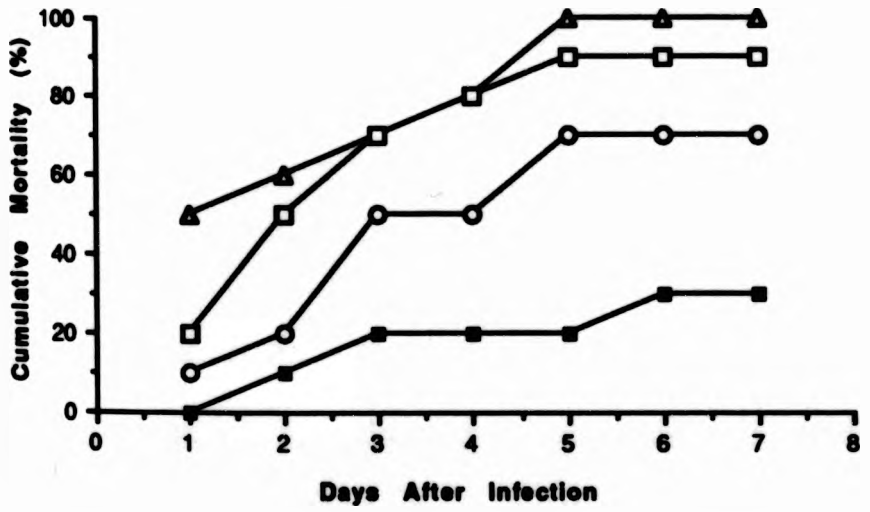


Figure 5.1 Cumulative percentage mortality of carp in three different treatments following i.p challenge with four graded ten-fold dilutions of *A. hydrophila*. The fish were exposed to the metals for 10 days before challenge infection. (a) Control (b) Cadmium and (c) Copper. The number of bacteria injected per fish at 10^{-7} dilution was 1.8×10^{3}.

challenge levels of 10^{-5} ($1.8x10^5$ bacteria/fish) and above resulted in more than 50% mortality in the controls, whereas relatively low challenge levels (10^{-6}; $1.8x10^4$ bacteria/fish) produced more than 50% mortality in the cadmium and copper groups.

The log-probit plot of bacterial number against mortality (Figure 5.3a-b) clearly shows that the number of bacteria required to produce more than 50% mortality is significantly lower in the cadmium and copper exposed groups compared to the controls.

5.3.2 Experiment 2

The effects of cadmium and copper exposure (10 days) on the protective immunity and circulating antibody titre of carp previously immunized with *A. hydrophila* bacterin is presented here. Figure 5.4a shows the cumulative percentage mortality pattern in previously immunized carp, exposed to the metals for 10 days before a potentially lethal challenge (10^6 bacteria/fish). A batch of non-immunized fish were also introduced as controls during the infection. Over a period of one week, 95% of the non-immunized fish were dead and all the mortalitites were confirmed to have resulted from *A. hydrophila* challenge. Total mortalities between duplicate tanks in treatment groups did not differ statistically. The combined total mortality at the end of one week, following challenge were as follows: Control (35%); cadmium (55%); copper (65%). As can be seen from the Figure 5.4b, the mortalities in the metal exposed groups were significantly ($P<0.05$) higher than the corresponding controls, but significantly lower than non-immunized controls. The calculated Relative Percent Survival (RPS) clearly indicated that it was significantly ($P<0.05$) higher in the controls (63%) compared to 42 and 31% in the cadmium and copper groups respectively (Figure 5.4b).

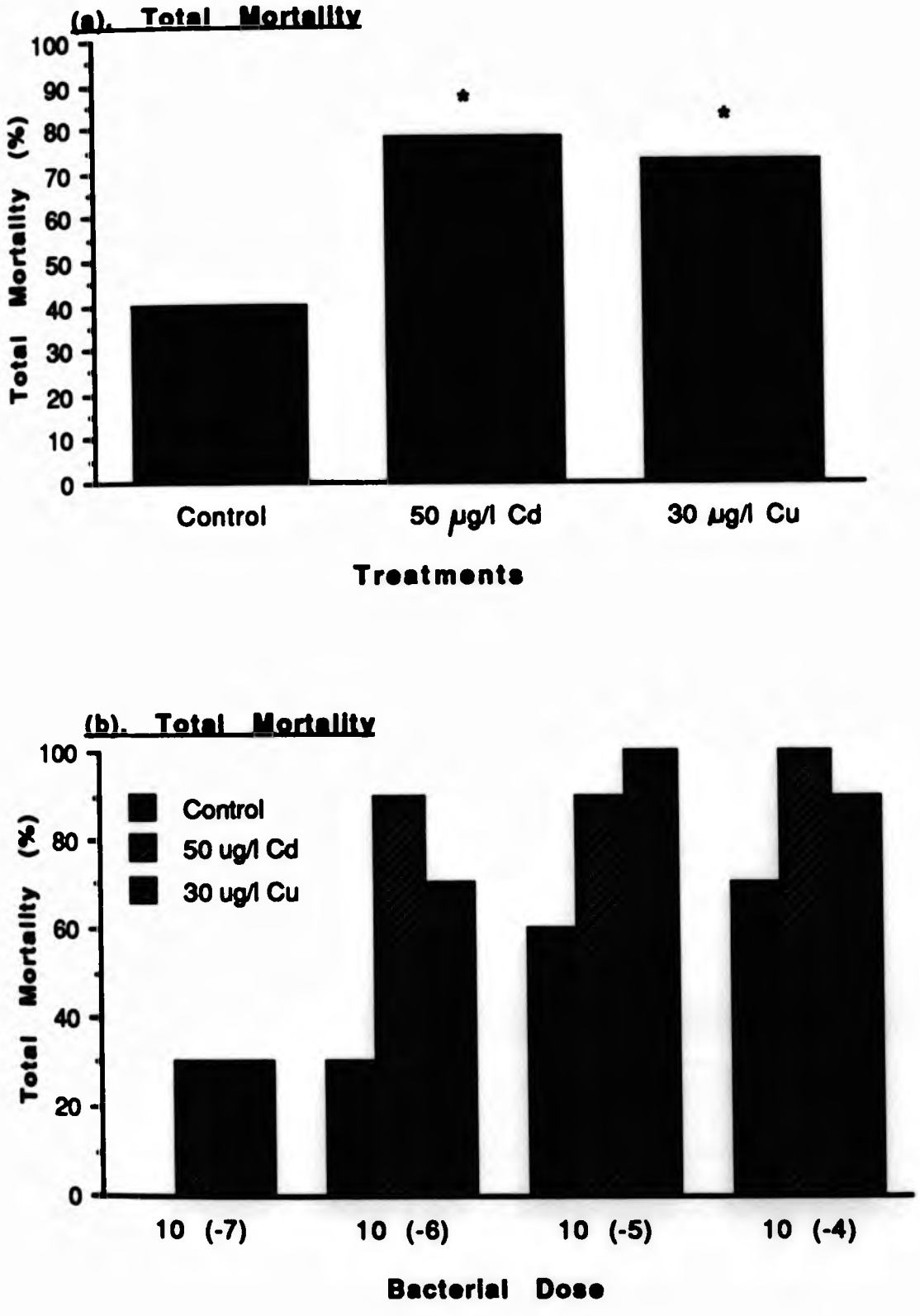
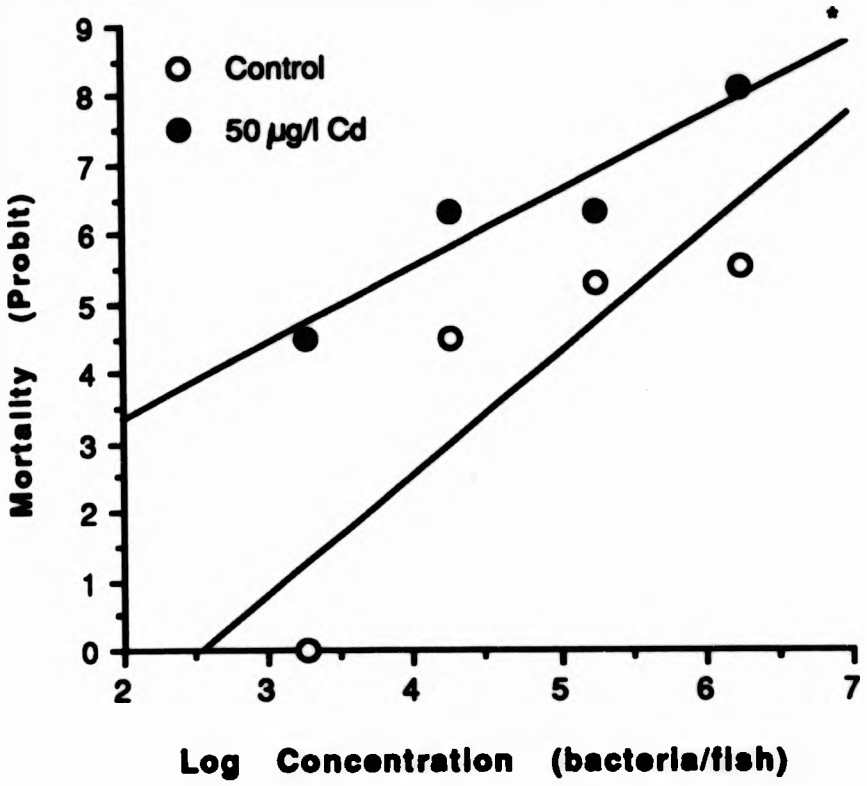


Figure 5.2 Figure showing (a) the total mortality per treatment resulting from four challenge levels combined and (b) the total mortality at each bacterial challenge level in three treatments. The experimental protocol same as in Figure 5.1. * denotes significant difference from the control ($P<0.05$).

(a). Log-Probit Plot of Mortality (Cadmium)



(b). Log-Probit Plot of Mortality (Copper)

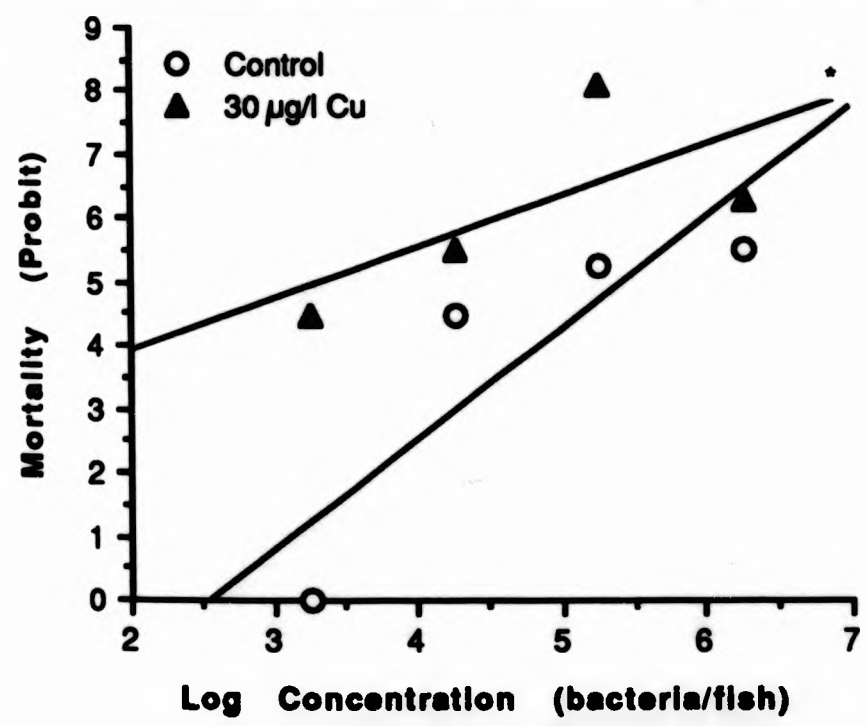


Figure 5.3 The log-probit plot of mortality against bacterial dose in carp exposed to (a) cadmium and (b) copper. The experimental protocol same as in Figure 5.1. * denotes significant difference from the control ($P<0.05$).

Serum agglutinin titre recorded in fish following immunization before and after exposure to the metals is illustrated in Figure 5.5. The titre recorded following immunization before exposure to the metals was 7.1 ± 1.2. The titre recorded a week after the second injection (on day 21) was significantly higher than the response obtained on day 14 following the first injection. The titres recorded after 10 days exposure to the metals were relatively high and did not differ between duplicate experiments. The mean titres (combined from duplicate experiments) after 10 days exposure to the metals (day 31) were as follows: control (7.75 ± 1.14); cadmium (6.58 ± 1.31); copper (5.67 ± 1.23).

Ten days exposure to cadmium or copper, had significantly ($P<0.05$) lowered the agglutinin titre. In the controls the titre on day 31 was slightly higher than that recorded on day 21 before the commencement of metal exposure. In both cadmium and copper groups, the titre after 10 days exposure was significantly lower than that recorded on day 21, before the metal dosing.

5.3.3 Experiment 3

The mean serum agglutinin titre to *A. hydrophila* in carp exposed to the metals for 10 days before immunization is presented in Figure 5.6. Low titres (0.83 to 1.33) were detected in all the three treatments 6 days after immunization. The titres increased gradually with time following immunization and peaked between days 15 and 18 in all the groups. The highest titres recorded were 5.83 (day 18) in control; 4.17 (day 18) in cadmium and 4.00 (day 15) in the copper treatment. On day 18 the peak titre in the control was significantly ($P<0.05$) higher than that observed in the cadmium and copper groups. The peak titre observed in the copper treatment on day 15, did not differ statistically from the corresponding control values. The overall titres recorded in metal exposed groups were relatively lower on all sampling points

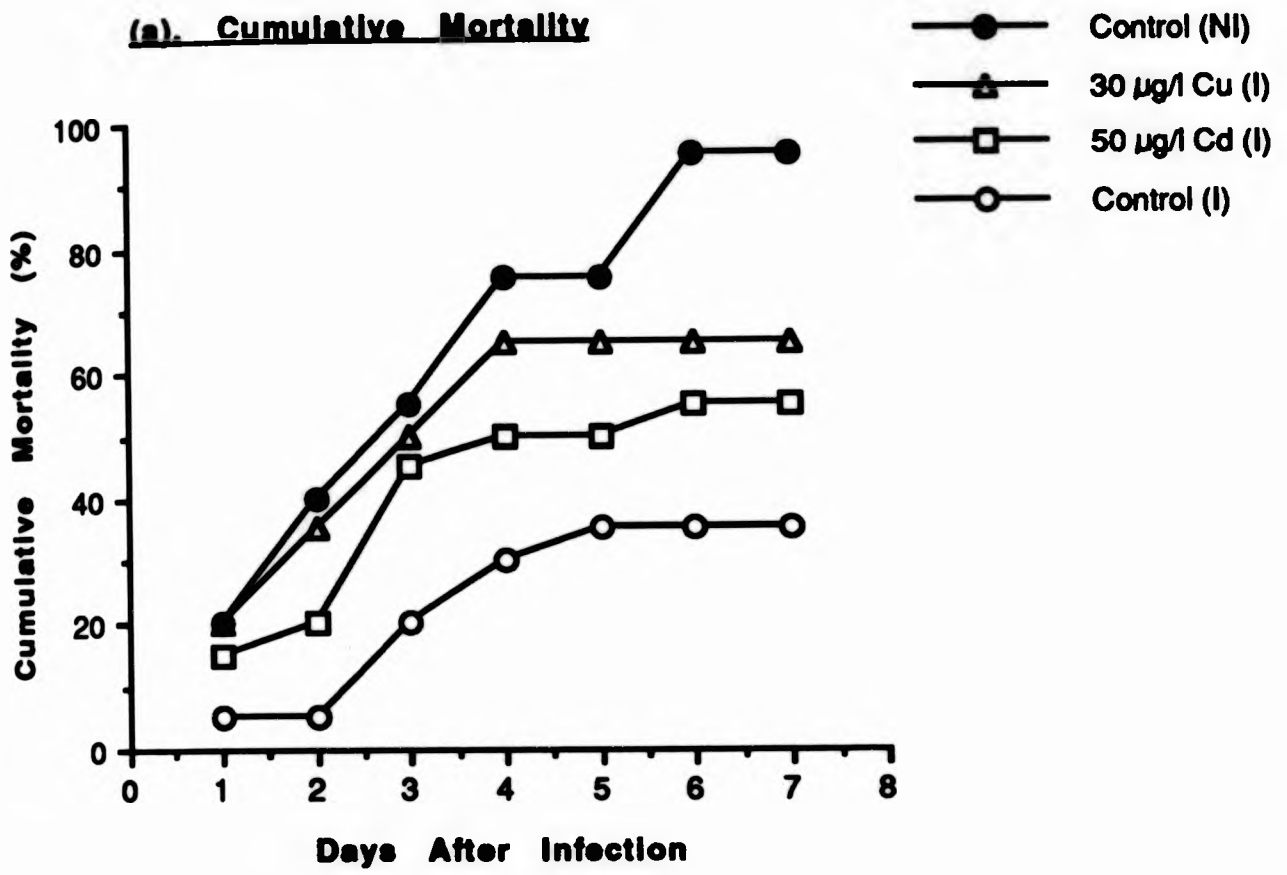


Figure 5.4 Shows the mortality pattern in previously immunized carp exposed to cadmium and copper for 10 days before i.p challenge infection with *A. hydrophila*. (a) Cumulative percent mortality and (b) Percent mortality and Relative percent survival (RPS) per treatment. * denotes significant difference from the control ($P<0.05$). I=Immunized; NI=Non-Immunized.

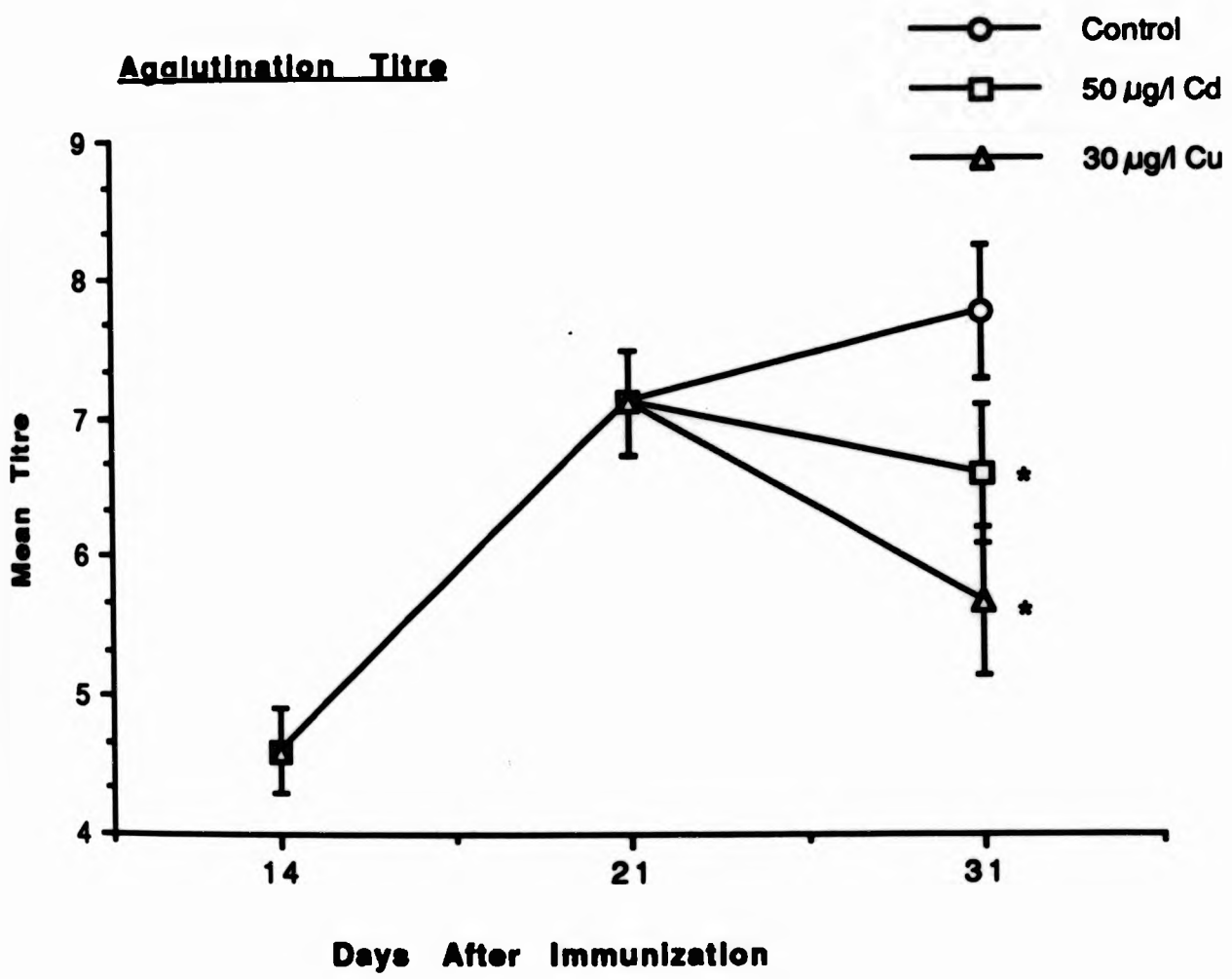


Figure 5.5 Bacterial agglutination titre (Mean ± S.E; n=10-12) in carp following immunization with *A. hydrophila* bacterin, before the commencement of metal exposure (day 21) and 10 days after the exposure to the metals (day 31). * denotes significant difference from the control (P<0.05).

than the corresponding controls.

5.3.4 Experiment 4

The results show the kinetics of the agglutinin titre in carp, given two injections of *A. hydrophila* bacterin with 15 days time interval between them with simultaneous metal exposure commencing on the day of first injection (Figure 5.7). Low agglutinin titres were detected in all the groups on day 6 of first injection. The titre recorded in the control (5.50) on the day of second injection was significantly higher than the corresponding value in cadmium (3.17) and copper (2.83) treatments. The response following second injection was significantly higher than the primary response in all the groups. The highest titre recorded after the second injection in the control was significantly higher than in the cadmium and copper treatments. The peak titres recorded were 7.50 (day 27) in controls, 6.00 (day 27) in cadmium and 5.67 (day 30) in copper.

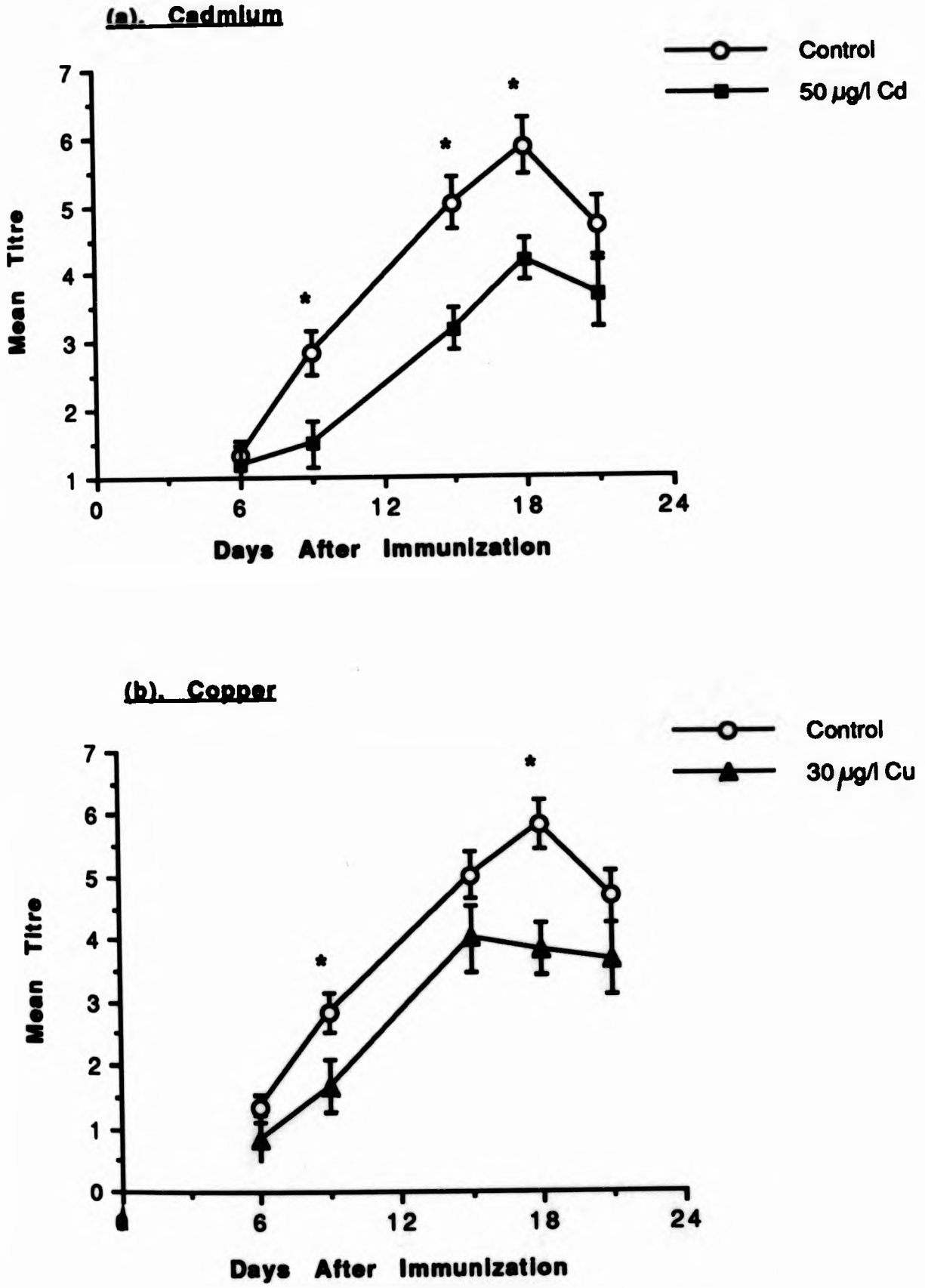
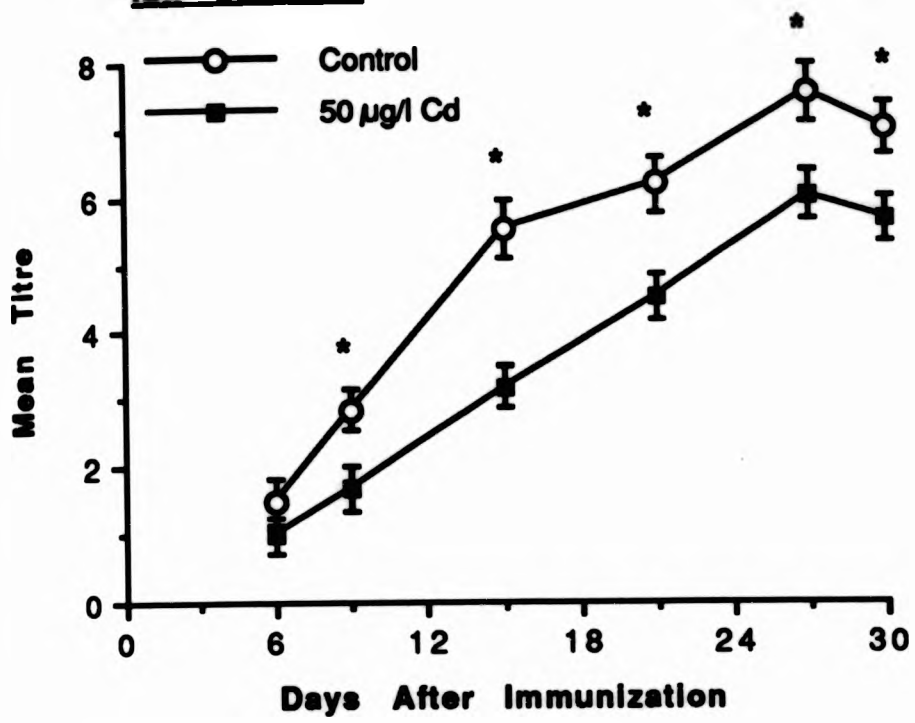


Figure 5.6 The kinetics of bacterial agglutination titre (Mean ± S.E; n=6) in carp immunized with *A. hydrophila* bacterin after 10 days exposure to (a) cadmium and (b) copper. Arrows denote the time of bacterin injection. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

(a). Cadmium



(b). Copper

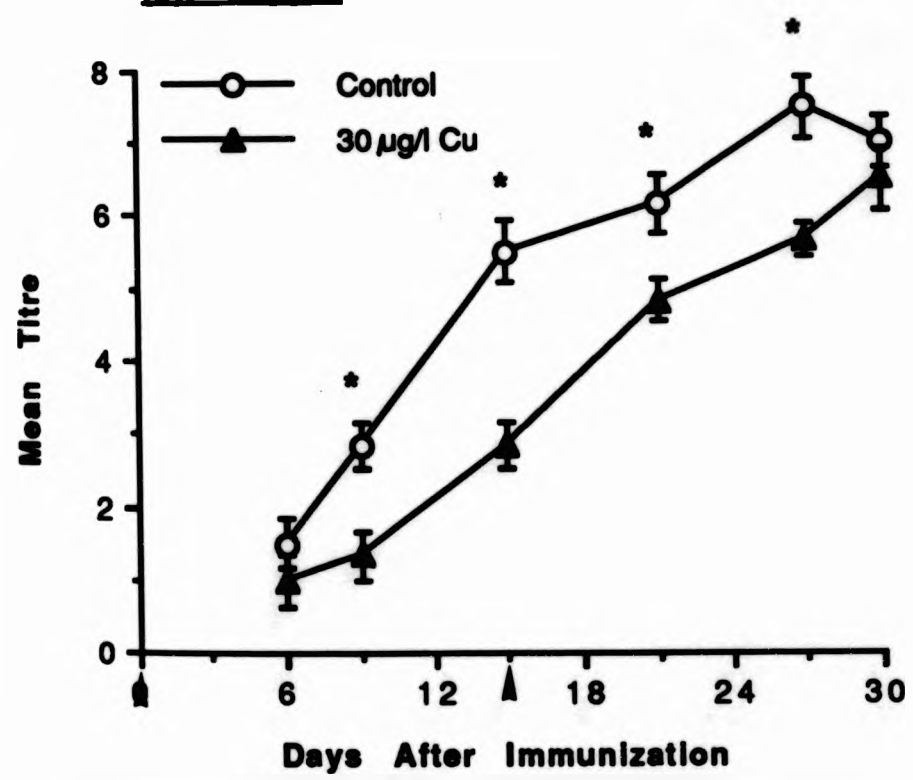


Figure 5.7 The kinetics of bacterial agglutination titre (Mean ± S.E; n=6) in carp after first and second injection with *A. hydrophila* bacterin. Exposure to the metals (a) cadmium and (b) copper was commenced on the day of first injection and continued till the end of the experiment. Arrows denote the time of bacterin injection. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

5.4 DISCUSSION

Increased susceptibility of carp to *A. hydrophila* infection after 10 days exposure to 50 μgl^{-1} cadmium or 30 μgl^{-1} copper was demonstrated in the present study. The resulting mortality was significantly related to the presence of metal and the bacterial dose. The number of bacteria required to produce more than 50% mortality was significantly reduced in fish exposed to cadmium or copper. Other laboratory studies have produced varying results with some studies showing metals predisposing the fish to infection (Hetrick, 1979; Knittel, 1981; Baker *et al.*, 1983) while others have shown metals to provide a certain degree of protection against bacterial diseases (MacFarlane *et al.*, 1986) and/or not interfering with the disease resistance mechanism of the fish (Snarski, 1982).

As noted earlier, the majority of the published work has demonstrated the effects of metal pollutants on the disease susceptibility and immune response of fish by using bacterial pathogens. Close scrutiny of the literature reveals the importance of factors such as type of the metal, type of bacteria, nature of metal exposure and infection protocol in influencing the final result.

Thus, when drawing inferences from studies of the present nature, it is essential to give wider consideration to the experimental and infection protocols followed. From a close review of the literature it is obvious that some of the protocols followed gave varying results, in particular, the method of challenge. Rainbow trout, *S. gairdneri* exposed to 7 and 10 μgl^{-1} copper for 96 hours were found to have an increased susceptibility to *Y. ruckeri* bath challenge. Interestingly, lowering the concentration of copper to 5 μgl^{-1} was observed to increase the infection

susceptibility earlier (Knittel, 1981). Similar observations were made with chinook salmon and rainbow trout exposed to copper (Baker *et al.*, 1983). The peak susceptibility in rainbow trout to a bath of *V. anguillarum* was seen after 96 hour exposure to 18-31% of their copper 96hLC$_{50}$ while it was 8-20% of 96hLC$_{50}$ for chinook salmon. The fact that higher copper levels (>50% of 96hLC$_{50}$) resulted in less mortality is very interesting. Looking at the time effect, Baker *et al.* (1983), found exposure to a higher concentration of copper caused a greater susceptibility to *V. anguillarum* after 24 to 48 hours, while it declined after 96 hour exposure time. As found by Knittel (1981) exposure to low levels of copper resulted in early susceptibility.

In experimental situations such as the above, a breach of the integrity of the primary barriers (skin and gill), alteration of mucus production and other innate mechanisms appear to be the initial stages which would allow the infection to establish and proceed. Logically, high concentrations of metals would be expected to produce more integumental damage and, therefore, higher infection and more susceptibility. The findings of both Knittel (1981) and Baker *et al.* (1983) showed otherwise. It may be that, when fish are exposed to a high concentration of metal and are removed for challenge by the bath mode of infection (as used by these authors), it is likely that metals adsorbed and/or absorbed at the surface of the fish may be directly lethal to the bacteria or indirectly may prevent primary colonization on the skin before becoming systemic.

The observations of MacFarlane *et al.* (1986) regard the ability of metals (Cu,Cd) to provide protection against infection, do give credence to the argument of the influence of the experimental and infection protocol on the result. The protocol they followed involved exposing the fish to the metals after only a two minute contact

(dip) infection with *F. columnaris*. Such an experimental regime would produce results which would appear to provide either protection or not alter the susceptibility to infection but it seems more likely that the bacteria were unable survive such treatment. However, from the studies of Knittel (1981) and Baker *et al.* (1983), it is evident that low levels of copper exposure do indeed increase the susceptibility to bacterial diseases significantly.

Injection as a method of infection has been used primarily to obtain reliable and reproducible infections in experimental fish (Ruangapan *et al.*, 1986) and this was why this method was chosen for the present study. This method of infection has been widely used in studies aimed at evaluating the effects of pollutants or stressors in altering the disease susceptibility. However, the results obtained by this method have also been shown to be inconsistent. Hetrick *et al.* (1979) found rainbow trout exposed to very low levels of copper (4-9.6 μgl^{-1}) for a week before infection to increase the susceptibility to IHPN virus. Chinook salmon, *O. kisutch* exposed to chromium (0.5 mgl^{-1}) for 2 weeks was more susceptible to *V. anguillarum* (Sugatt, 1980). These studies support the findings of the present study with regard to the ability of metal pollutants to increase the susceptibility of fish to bacterial diseases. On the other hand Snarski (1982) found 30 day exposure of *S. gairdneri* to higher copper levels of 10.9 and 21.5 μgl^{-1} did not increase the mortality in response to *A. hydrophila* infection. Taking into account the comparative sensitivity of rainbow trout and carp to copper toxicity, the contrasting results are interesting. Snarski's (1982) finding suggests the possibility of rainbow trout becoming acclimatized to low levels of copper such that it does not affect the body's innate defence mechanism since he used a duration of 30 days.

The pathogenicity, virulence and invasiveness of the pathogen selected in studies of

the present nature could influence the result to a large extent. Pathogens such as *V. anguillarum* (Stevens, 1977; Rodsaether *et al.*, 1977; Sugatt, 1980; Baker *et al.*, 1983) and *Y. ruckeri* (Knittel, 1981) have invariably resulted in increased susceptibility in metal exposed fish, whilst studies which used *A. hydrophila* (Snarski, 1982) and *F. columnaris* (MacFarlane *et al.*, 1986) observed no effect or some degree of protection by the respective metals in the study. On the other hand, *A. hydrophila* used in the present study in carp exposed to cadmium and copper for 10 days has resulted in increased disesase susceptibility.

As would be expected the toxicity, of the metal chosen would, to a certain extent, determine the susceptibility. Copper at the various low levels tested has been found to increase the susceptibility to bacterial and viral pathogens (Stevens, 1977; Rodsaether *et al.*, 1977; Hetrick *et al.*, 1979; Knittel, 1981; Baker *et al.*, 1983; Present study) with two exceptions (Snarski, 1982; Macfarlane *et al.*, 1986). There are very few studies on the ability of other metals to alter the disease susceptibility of fish. Cadmium was found to offer protection by MacFarlane *et al.* (1986) whilst chromium was seen to increase the susceptibility by Sugatt (1980). And in addition, the present study has demonstrated that cadmium exposure increases the susceptibility of carp to *A. hydrophila* infection.

Circulating macrophages would appear to offer the first line of defence especially when bacteria are introduced by injection. Increased disease susceptibility observed using this mode of infection would therefore appear to indicate the suppressive effects of metals on phagocytosis. Increased susceptibilty of naive fish to bacterial diseases of a systemic nature therefore, could be attributed to a great extent to the reduced ability and /or failure of the host's innate defence mechanisms such as phagocytosis and possibly even complement mediated lysis of gram negative bacteria

via the alternative pathway.

Phagocytosis has been recognised as one of the major lines of non-specific defence mechanism in fish (Ellis, 1981). Loss of the ability of phagocytes to clear the pathogens from the circulation and carry out the intracellular processes necessary for the killing of the bacteria could, in all probability, lead to higher infection and disease susceptibility. There is evidence that metals like cadmium and copper can interfere with phagocytosis. Elsasser *et al.* (1986) found copper to reduce the chemiluminiscence emitted by the phagocytes and such an effect was thought to be as a direct consequence of suppressive effect on the phagocytic cells. The same authors found cadmium caused a significant increase in chemiluminiscence when added 1 hour prior to or immediately before the assay, but following a 24 hour exposure the results were found to be variable, in that either no change or a decrease was observed. Primary blood clearance of MS2 bacteriophage was suppressed in copper exposed brown trout and carp (O'Neill, 1981a). A clearance time of more than 7 days was needed in brown trout compared to less than 3 days in unexposed groups, while the blood clearance was suppressed for more than 2 weeks in carp. Such a marked reduction in the ability of phagocytes and the increased time needed to clear the pathogens, could give the pathogen an upper hand and account to increased mortality.

Heavy metals such as copper and cadmium which induce a stress response leading to elevated cortisol levels in fish (Donaldson and Dye, 1975; Schreck and Lorz, 1978) can also indirectly lower the phagocytic ability in fishes operating through the suppressive effects of cortisol (Stave and Roberson, 1985). Such a possibility cannot be ruled out for the observed increased susceptibility in the present study.

Metals which suppress the phagocytic ability directly or indirectly could predispose the fish to infection, especially that of a systemic nature. On the other hand, metals which stimulate phagocytosis (Cossarani-Dunier *et al.*, 1988) or enhance clearance rate (Robhom and Nitkowski, 1974) would reduce the susceptibility. The findings on cadmium in this regard has been contradictory. Cadmium was found to induce a greater clearance rate, but reduced killing rate in the cunner, *T. adspersus* (Robhom and Nitkowski, 1974), whilst short term exposure increased the chemiluminiscence but longer term exposure reduced it (Elsasser *et al.*, 1986). Looking at the mortality figures in the present study it is clear that cadmium could not have increased the phagocytic ability of macrophages in this case.

Complement-dependent bactericidal action through the alternative pathway is considered to occur only with gram-negative bacteria and is suggested to contribute greatly to the natural resistance phenomenon (Ourth and Wilson, 1982; Ellis, 1989). There is no evidence yet to relate increased susceptibilities in naive fish to impairment of complement-dependent processes by heavy metals in fish. However, in mammals metal pollutants have been shown to interfere with this process (Hemphill *et al.*, 1971).

The present study has demonstrated the effects of cadmium and copper on protective immunity. Previously immunized carp following 10 days exposure to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) could not mount a protective response comparable to that seen in the controls. The relative percent survival of immunized fish was low in cadmium and copper exposed groups. Interestingly, in contrast to the protozoan antigen (*I. multifiliis*), this was associated with a significant drop in the humoral agglutination titre.

From the results it is obvious that the suppression of protective immunity was not total. A significant proportion of the fish survived the challenge infection with *A. hydrophila* which followed 10 days exposure to cadmium or copper. Reduced levels of antibody coupled with a reduction in the number and activity of macrophages could in theory account for the observed loss of protective ability. Reduced levels of antibody could also reduce the effectiveness of various defence mechanisms such as phagocytosis, lysis of bacteria and other complement mediated antibody dependent processes which all, in theory, could account for higher survival of bacteria. On the other hand, such explanations may appear to be simplistic because of the uncertainties surrounding the mechanisms of the immune response in fish.

Complement mediated lysis of gram negative bacteria is well established in mammals. Antigen-antibody complexes can activate the complement through the classical pathway and this serves as a major line of defence. Complement can also be activated through the alternative pathway by the endotoxins or mucopolysaccharides of the external cell wall of gram negative bacteria, and this mainly serves as a component of the host's innate defence mechanism. Antibodies and complement can act as opsonins and facilitate the process of phagocytosis in mammals as a result of the possession of mammalian macrophages of receptors for the Fc portion of the IgG and C3b portion of the complement. In mammals IgM is not an opsonin.

It is difficult to draw parallels from mammals because fishes are known to produce only one class of Immunoglobulin IgM (Ambrosius *et al.*, 1982) but the existence of immunoglobulin heterogeneity cannot be dismissed in view of the recent finding by (Sanchez *et al.*, 1989). Opsonization of particles and bacteria is controversial in fish. However, the opsonic effect of antibody has been described in fish by Griffin

(1983). IgM in fish is suggested to have a role as an opsonin in certain species, either directly via receptors for the IgM, or indirectly, via receptors for classically activated complement (Ellis, 1989). Therefore reduction in the level of circulating antibody, as observed in the present study, could have contributed to the decreased protective immunity.

There are several reports of chemical stressors, especially heavy metals, lowering the magnitude of the humoral immune response without totally suppressing it. Such findings naturally raise questions as to whether fish with reduced antibody as a result of a stress response, do indeed run the risk of being infected. Unless the specific mechanism for protective immunity and its relation to the level of antibody are known, it is not possible to speculate on such questions. The specific mechanism of the execution of the protective immunity in immunized carp to *A. hydrophila* is unclear (Baba *et al.*, 1988a, b) and comphrehensive information corelating the degree of protection with the levels of antibody titre in *A. hydrophila* immunized carp is still lacking. The study of Post (1966) provided information about protection in rainbow trout against *A. hydrophila* and attempted to correlate protection with antibody titre. In his opinion, rainbow trout with antibody titres of 1:64 or higher could be considered immune. In the present study, previously immunized carp following 10 days exposure to metals had antibody titres of 7.75 (around 1:128) in control; 6.58 (around 1:64) in cadmium and 5.67 (around 1:32) in copper treatments. The corresponding relative percent survivals (RPS) were 64% (control); 42% (cadmium) and 32% (copper). The lower antibody titre seen in metal treated groups correlated with the low RPS recorded in previously immunized carp exposed to the metals.

The present findings are in agreement with that of O'Neill (1981b) who

administered lead and cadmium intraperitoneally to *S. trutta*, 2 weeks after the 3rd successive immunization injection, and found a rapid decrease in the level of antibody. It would seem then that antibody levels can be so decreased despite there being well established levels prior to exposure to metals. Such drops in antibody levels could leave the immunized fish less protected, as was seen in the present study. It has been suggested that metals could neutralise the serum antibody activity thereby reducing the quantity of antibody in circulation. In mammals, cadmium and some other heavy metals are known to readily bind and readjust the tertiary structure of a wide range of biologically active molecules (Phipps, 1976). Such a direct action of heavy metals has been shown to impair the activity of antibody (Jones *et al.*, 1971).

As the metal exposure was commenced well after the initiation of the immune response, the interference from metals would be expected to be on the cells producing antibody or the antibody itself. The biological half life of some fish antibodies have been calculated. The half-life of lemon shark antibody was determined to be 4-5 days (Clem *et al.*, 1969), while that of rainbow trout antibody has been calculated to be around 7 days (Harrell *et al.*, 1975). Maintenance of enhanced levels of antibodies in circulation is therefore, due to a continuous process.

In addition to the above the direct effects of cadmium or copper on clonal expansion of immunocompetent "B-like" cells or "T-helper" cells would result in lowered antibody production and this was discussed in Chapter 4.

Contrary to the present finding, Thuvander (1989) recently found very low levels of cadmium (3.6 $\mu g l^{-1}$) to enhance antibody production in *S. gairdneri* to *V.*

anguillarum and not to affect the protective immunity and the mitogenic response of splenocytes to ConA and LPS. The experimental protocol followed involved cadmium exposure for a period of 12 weeks with immunization carried out after 5 weeks of exposure to the metal. Thuvander (1989) also found the blastogenesis of splenocytes to be significantly lower after 9 weeks exposure to cadmium when *V. anguillarum* whole killed cells were used as the mitogen and such an effect was attributed to the likely inhibitory effect of cadmium on T-lymphocytes. The concentration of cadmium used in the present study was atleast 15 times higher than that used by Thuvander (1989) and the difference in effects on antibody level and protective immunity are significantly different. The results of Thuvander (1989) do suggest the possibility of acclimation of fish to low levels of heavy metals such that the immune system is unaffected.

The recent work of Baba *et al.* (1988a, b) is very interesting in view of the present findings. Based on indirect evidence from a series of experiments, they suggest that the protection in carp immunized with crude *A. hydrophila*, lipopolysaccharide (LPS) to be dependent on cellular immunity regulated by a T-like cell and macrophage system. Their suggestions were based on observations of depressed Immunity in carp treated with anti-carp thymocyte serum *in vivo* and also in immunized carp whose macrophage function was impaired by dextran sulphate treatment. They were able to transfer the protective immunity passively by transferring pronephric cells (T-like cells) from previously immunized carp. They also observed carp vaccinated by the dip method with crude *A. hydrophila*, LPS to induce protection without producing any antibodies.

Considering the above argument the results obtained in the present study with regard to decreased protective ability in previously immunized carp to *A. hydrophila* and *I.*

multifiliis (Chapter 3) could have resulted from the effects of metals on other cellular processes such as antibody or complement dependent, macrophage or cytotoxic cell mediated mechanisms (cell mediated responses).

Alterations in the magnitude of the humoral immune response of carp exposed for 10 days to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) prior to immunization with *A. hydrophila* heat killed bacterin were demonstrated in the present study. The overall titre recorded in the metal exposed groups was lower than controls and the peak titres were significantly reduced. Exposure to metals for 10 days did not abrogate the immune response but only reduced the magnitude of the response. Immunization and simultaneous exposure to metals did have considerably more effect in lowering the humoral agglutinin titre, but once again the process of initiation of immune response and the production of antibody was not completely suppressed. From these results it would appear that cadmium and copper at the levels tested would not totally block the immune response. However, it will be of interest to know whether antibody levels attained under such conditions would be sufficient to offer protective immunity to the fish.

The kinetics of the humoral immune response observed in the controls in the present study agree with the findings of Lamers *et al.* (1985). However, clear-cut parallels cannot be drawn because of the short term nature of the present study where an antigen dose of 10^8 heat killed cells of *A. hydrophila* produced a peak response 18 days post-injection in carp kept at 27 ± 1°C. Lamers *et al.* (1985) found antigen doses of 10^7 to 10^9 cells produced peak primary response on day 20 in carp maintained at 22°C and a direct relationship between antigen dose and antibody response was found. They also demonstrated memory formation in carp to *A. hydrophila* bacterin. The second injection of the same dose of antigen given to carp

2 weeks after the first priming in this study, did increase the antibody titre significantly in controls. As the time interval was short, no claim to memory formation can be made in the present study.

The cellular requirements for optimal humoral response to *A. hydrophila* bacterin is still unclear because of the uncertainties surrounding the thymus dependency of this bacterin. In the opinion of Lamers *et al.*(1985), the bacterin *A. hydrophila* contains both TD and TI components, which are degraded at a different rate. The humoral immune response would require the co-operation of macrophages, T and B-like lymphocytes.

The significant increase in susceptibility observed in naive fish exposed to cadmium or copper could indirectly be attributed to a decrease in the activity and number of macrophages. Such an effect would also be expected to reduce the intial process of antigen uptake, processing and presentation to antibody producing cells.

Lamers and de Haas (1985) have recognised two stages in the *A. hydrophila* antigen localization. The first phase is non-specific and the second stage consists of antigen localization to the melanomacrophage centre (MMC) of kidney and spleen coinciding with the onset of antibody production. Lamers and Pilarczyk (1982) have made similar observations with *Y. ruckeri* O-antigen. The effects of cadmium and copper on MMC have been described and discussed in Chapter 4. Antigen localization in MMC is regarded to be an important step in the process of the immune response. Effects of metals on these centres, therefore, could have contributed to the low agglutinin titres noticed in the present study. Evidence to similar effect was put forth by Roales and Perlmutter (1980) who found complete immunosuppression associated with decrease in the amount of abnormal

haemosiderin bodies in blue gouramis immunized with *P. vulgari* bacterin and exposed simultaneously to methyl mercury and copper. They regarded haemosiderin bodies to be the precurssors of germinal centres.

Exposure of carp to very high levels of manganese (50 mgl^{-1}) for 2.5 months, with *Y. ruckeri* antigen administered 7 days after the commencemnt of metal exposure did not reduce the antibody titre (Cossarini-Dunier *et al.*, 1988). The stimulatory effect of manganese on phagocytosis observed could have accounted for the increased antigen uptake, processing and localization. Cossarini-Dunier *et al.* (1988) suggested that there was a possibility that lymphoid organs were not contaminated at the time of immunization and that this was a likely cause of the lack of immunosuppression. Such explanations appear to hold true where metal exposures are for short duration and before immunization. Since the immune system is a cell-renewal system with a high proliferation rate such arguments may not be satisfactory, especially in their studies where metal exposure was continued for 2.5 months.

Contrary to the present finding, low levels of cadmium (3.6 μgl^{-1}) were found by Thuvander (1989) not to affect the humoral antibody response in rainbow trout to *V. anguillarum* O-antigen but to enhance it. The cellular requirements for expression of the humoral immune response to *A. hydrophila* and *V. anguillarum* bacterins may differ. *V. anguillarum* O-antigen has been regarded as a TI antigen (Thuvander, 1989), while *A. hydrophila* is considered to have both TD and TI componets (Lamers *et al.*, 1985). Cadmium in mammals is known to affect T helper cells and thereby lower the secondary response to TD antigens (Koller, 1984). Effects of cadmium and copper on any one or all of the cell components (macrophages, T and B like lymphocytes) could therefore, lower the humoral response to *A. hydrophila*

bacterin.

As discussed in Chapter 4, the time of antigen administration in relation to metal exposure does seem to have a strong influence. Immunization and simulataneous exposure to the metals commencing the same day seemed to have more effect in interfering with the humoral immune response compared to immunization given 10 days after exposure to the metals. O'Neill (1981a) using two different immunization and metal exposure protocols found cadmium and copper to have immunosuppressive effects on carp and brown trout to MS2 bacteriophage. The immune response was found to be completely suppressed in brown trout and carp exposed to copper for 38 weeks, with first immunization injection given 2 weeks after metal dosing and a second after 7 or 8 weeks.

Sugatt (1980) immunized coho salmon with *V. anguillarum* bacterin and exposed them to chromium for 2 weeks. The findings that the antibody titre was unaffected at 2 or 4 weeks after immunization, but significantly reduced at 6 weeks, is obviously difficult to interpret.

Both the metals used in the present study produced similar immunosuppressive effects in carp. Considering the suppressive effects individually, copper exposure at the concentration tested, appeared to be more immunotoxic in terms of altering the suceptibility in carp to *A. hydrophila* infection and protective immunity.

A certain amount of caution needs to be exercised before drawing conclusions from studies reporting immunosuppressive effects of pollutants in general. A clear understanding of the immunomodulatory role of chemical pollutants at sublethal levels is essential. It is clearly not necessary to look for total suppression of the

immune response when defining lethality or harmfulness of environmental pollutants. This study along with others has shown that certain pollutants can lower the magnitude without total suppression of the response. The results here have clearly shown that a lowered magnitude is sufficient to suppress the protection afforded by the immune system to the extent of causing significant mortality.

CHAPTER 6

IN VIVO AND *IN VITRO* EFFECTS OF CADMIUM AND COPPER ON THE MITOGENIC RESPONSE OF CARP LYMPHOCYTES INDUCED BY CONCANAVALIN (Con A) AND LIPOPOLYSACCHARIDE (LPS).

6.1 INTRODUCTION

Mitogens induce blast transformation in normal lymphocytes. In mammals, lectins such as concanavalin A (Con A) and phytohaemagluttinin (PHA) stimulate thymus derived T cells, while lipopolysaccharide (LPS) stimulates bone marrow (Bursa) derived B cells. In contrast to the activation produced by specific antigens, these mitogens can activate a relatively high percentage of lymphocytes, probably representing a polyclonal response. Assays involving the blastogenic response of B and T lymphocytes to mitogenic stimuli *in vitro* have been widely used as tools to evaluate the immunosuppressive effects of drugs and chemicals (Mitchell, 1974). Interference with mitogenic proliferation induced by Con A suggests alterations in cell-mediated immunity, while LPS indicates humoral involvement in mammals (Koller, 1980).

Evidence for the existence of T and B equivalent lymphocytes in fish comes from studies demonstrating differential proliferative responses induced by T and B-cell mitogens (Ellis, 1989). In fish, however there appears to be no specific organ compartmentalization of lymphocyte subpopulations responding to different mitogens (Ellis, 1989). Lymphocytes from carps (Liewes *et al.*, 1982), catfishes (Clem *et al.*, 1984) and rainbow trout (Chilmonczyk, 1978; Etlinger *et al.*, 1976) have been stimulated with the majority of putative T and B-cell mitogens. In recent years, mitogen assays have been used in fish studies aimed at evaluating the immunosuppressive role of stress (Ellsaesser and Clem, 1986), temperature (Clem *et al.*,1984), cortisol (Grimm, 1985), antibiotics (Grondel and Boestein, 1982; Grondel *et al.*, 1985) and heavy metals (Cenini and Turner, 1982; Ghanami *et al.*, 1987; 1989; Thuvander, 1989).

Although several toxic metals have been shown to induce immunosuppression and increase susceptibility to disease in fish, the effects on the mitogenic response of lymphocytes have been studied only with a few metals. Most of the findings are based on the effects of metals on lymphocyte proliferation *in vitro*. The results are often contradictory and are not always correlated with the *in vivo* effects of the metal concerned on the immune response.

The available information in this area is largely confined to the *in vitro* effects of essential metals such as zinc and manganese. Zinc (10^{-7} to 10^{-3} M) was found to suppress the proliferative response of carp lymphoid cells induced by PHA (Cenini and Turner, 1982), while Ghanmi *et al.* (1987, 1989) found Zinc (10^{-7} to 10^{-3} M) to enhance 3H-thymidine incorporation by Con A, PHA and LPS stimulated carp lymphoid cells. Manganese (10^{-7} to 10^{-3} M) *in vitro* was not suppressive but mitogenic by itself and enhanced 3H-thymidine incorporation (Ghanmi *et al.*, 1989) in carp lymphoid cells.

In lymphocyte culture studies involving the addition of chemicals *in vitro*, the apparent cell responsiveness may be influenced by a number of factors. The cytotoxic properties of the chemicals, at certain concentrations, may decrease the cell viability and reduce the proliferative response, or the chemicals may directly interfere with the mitogen and the stimulation of the lymphocyte by the mitogen.

In vivo studies would normaly involve collecting lymphocytes from treated animals and stimulating with mitogens in *in vitro* cultures. Lymphocytes collected from rainbow trout which had been exposed to cadmium for 5 to 9 weeks (0.7 and 3.6 μgl^{-1}) showed a normal proliferative response to the mitogens Con A and LPS (Thuvander, 1989). In contrast, Ellsaesser and Clem (1986) found lymphocytes

obtained from stressed channel catfish which had suffered handling and transport for 18 hours could not respond to the mitogens LPS and Con A. These results highlight the need for evaluating the *in vivo* effects in addition to the *in vitro* effects.

Copper, has been shown to suppress the immune response and predispose the fish to diseases in many instances. Cadmium, on the other hand has produced contradictory results with respect disease susceptibility and immune response. This has been discussed extensively in Chapters 3, 4 and 5. There is very little information on the effects of these two metals, on the blastogenic response of lymphoid cells, either *in vitro* or *in vivo*. It is also evident from the literature that there is a need for a comprehensive study, where the effects of metals on the different aspects of disease resistance and immune response are investigated. Delineating the *in vivo* effects of metals on the blastogenic response of lymphocytes may help to understand more about the immunotoxic properties of the metal concerned.

In the present series of experiments, both cadmium and copper at low levels have increased the susceptibility of naive carp to the protozoan (*Ichthyophthirius multifiliis*) and to infection by the bacterium (*Aeromonas hydrophila*). Previously immunized carp, exposed to these metals, could not elicit a protective immune response against the protozoan or bacterial challenge. Such a loss of protective ability was not associated with any lowering of "anti-ich" humoral antibody, while humoral antibody against *A. hydrophila* was significantly lowered. Cadmium and copper exposure significantly reduced the humoral immune response of carp, especially the secondary response, to SRBC, which are putative thymus-dependent antigens.

The findings in the present study of the immunosuppressive effects are difficult to

explain but, the level of antibodies measured in circulation could largely depend on the ability of lymphocytes to proliferate, differentiate and synthesize antibody and this would seem to be a fruitful line to follow in pursuing this study.

In order to gain insight into the effects cadmium and copper might have on the DNA synthesis of different populations of lymphocytes, the following experiments were designed. The experiments were aimed at assessing the *in vitro* and *in vivo* effects of cadmium and copper on the blastogenic response of carp lymphoid cells induced by Con A and LPS, which are mammalian T and B-cell mitogens respectively.

6.2 MATERIALS AND METHODS

6.2.1 Blood Collection

Fish were individually anaesthetized with Benzocaine at 100 ppm and peripheral blood was collected from the caudal vein using heparinized 1 ml plastic syringes fitted with 25 gauge needle. Normally 1 ml blood was withdrawn from each fish. The blood was immediately transferred to sterile plastic vials containing 3 volumes of cold Eagle's Minimum Essential Medium (EMEM) supplemented with 10% foetal bovine serum and 10% pooled inactivated homologous carp serum, mixed gently and kept on ice or in a refrigerator before being used for lymphocyte separation.

6.2.2. Collection of Pronephric Cells

Fish were individually anaesthetized and bled completely before sacrificing in order to reduce erythrocyte contamination of the kidney cell preparation. The head kidney was dissected carefully under aseptic conditions and transferred to plastic vials with 5 ml of EMEM. The cells were then teased out with forceps and the resulting suspension was gently forced through a graded series of hypodermic needles from 19 down to 27 gauge. The suspension thus prepared, was allowed to stand in centrifuge tubes for 2 minutes in order to sediment larger cell clumps. The supernatant suspension was carefully aspirated into sterile plastic centrifuge tubes. The lymphocytes were separated according to the procedure described in Chapter 4 Section 4.2.6. The separated lymphocytes were washed three times in the medium, counted, and finally resuspended in a volume to yield approximately 10^5 cells/0.1ml

6.2.3 Culture Medium

Eagle's Minimum Essential medium was used in all the experiments (Wolf and Quimby, 1976). The medium was supplemented with 100 iu ml^{-1} of a mixture of

streptomycin and penicillin and 100 μg ml^{-1} kanamycin. The medium was buffered with 2.1 gl^{-1} of $NaHCo_3$ (pH=7.2). Two milliliters (200 μg ml^{-1}) of L-glutamine (Flow Laboratories, Scotland) was added to 100 ml medium. The medium was also supplemented with 10% foetal calf serum and 10% pooled heat inactivated homologous carp serum (Wolf and Quimby, 1976).

6.2.4 Metals

The metals used in these studies were cadmium and copper. The chloride salts were dissolved at 10x the final concentration desired in sterile 0.85% saline. The final concentrations used for the *in vitro* study were 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M. The metal solutions were added in a volume of 50 μl per culture well.

6.2.5 Mitogen Preparation

The mitogens Con A (Lectin from *Canavalia eusiformis*) and LPS (*E. coli*, 0127: B8) were procured from Sigma (Sigma Chemicals Company). The optimal concentrations of mitogens were determined in preliminary trials. Con A was made up fresh in EMEM for each cell culture study and was used at a concentration of 10 μg ml^{-1}. LPS was used at 50 μg ml^{-1} level. Fresh batches of mitogens were prepared each time.

6.2.6 Culture of Lymphocytes

Lymphocytes separated from peripheral blood and pronephros was washed 3 times in cold EMEM and resuspended to the required number in the medium. The viability and the number of lymphocytes per ml was estimated by the Trypan Blue exclusion method. The viability of the lymphocytes always exceeded 90%. The cells were cultured in 96 well round bottom microtitre plates (Flow Laboratories, Scotland). Each well received 100 μl of the cell suspension. The final volume of the

culture medium was 200 µl. Depending on the nature of the experiment, the other 100 µl consisted of either the medium, the mitogen in medium, metal in saline or plain saline. Normally the mitogen and the metal were added in 50 µl volumes. The particulars are described under the relevant sections. The culture plates were incubated at 22°C for 3 days in humidified air with 5% CO_2.

6.2.7 Determination of Tritiated Thymidine Incorporation

After 72 hours of incubation the cultures were pulsed with tritiated thymidine (3H-thymidine). To each well 0.5 µci of 3H-thymidine (specific activity 5 Ci mM^{-1}, Radiochemical centre, Amersham, England) in a volume of 10 µl, was added. The cultures were normally pulsed for 18 hours before harvesting.

6.2.8 Cell Harvesting and Counting Radioactivity

The cells from the culture plates were harvested using a cell harvester (Titertek Cell Harvester, Flow Laboratories, Scotland) on to filter paper discs. The filter paper discs with the cells were air dried and transferred to scintillation vials. To each vial 5 ml of liquid scintillant (Ecoscint) was added and incubated in the fridge overnight. Counting was carried out on a Packard Liquid Scintillation Counter (Packard Tri-Carb 2000 CA). On each sample, 5 minute counts were carried out, and the data was obtained as Counts Per Minute (CPM). The results were also expressed as a "Stimulation Index" which was obtained by dividing the CPM of stimulated cells by those of unstimulated cells.

6.2.9 Experimental Protocol

6.2.9.2 In Vivo Effects of Cadmium and Copper

6.2.9.2.1 Metal Exposure

The *in vivo* effects of cadmium and copper individually on carp lymphocyte

proliferation was assessed by exposing carp to the respective metals in a flow-through system. Two batches of 18 fish each (12.64 ± 1.22 cm; 25.20 ± 4.24 g) were exposed to nominal concentrations of 50 μgl^{-1} cadmium and 30 μgl^{-1} copper individually. An additional batch of 18 fish, which received no metal, served as controls.

6.2.9.2.2 Sampling

Each treatment was sampled six times, starting from day one after exposure and followed by samples at regular intervals of three days. Three fish per treatment were sampled at any single sampling point. The same fish served as both the source of peripheral lymphocytes and pronephric lymphocytes. Blood was collected first and processed for lymphocyte separation as described in Section 6.2.1, followed by collection and processing of anterior kidney cells (Section 6.2.2). From cell suspensions (peripheral or pronephric) obtained from a single fish, 9 well cultures were usually set up. This strategy remained the same for all the *in vivo* cultures. Table 6.1a gives the composition of culture wells for cells obtained from either blood or pronephros from a single fish. The composition remained the same for cells obtained from unexposed control fish and fish exposed to cadmium or copper.

In addition to 100 µl of cell suspension, the first 3 wells received only the medium (100 µl) which served as unstimulated cells. Of the remaining 6 wells, 3 received Con A (50 µl) and 3 LPS (50 µl). The final volume in each culture well was 200 µl. The difference in volume was always made up with the medium. Therefore, at each samping point from 3 fish per treatment there were 9 unstimulated and 9 each of Con A or LPS stimulated culture wells.

6.2.9.1 *In Vitro* Effects of Cadmium and Copper

For studies of the *in vitro* effect, control fish which had not been exposed to any metals were used to collect the anterior kidney cells. For both cadmium and copper, concentrations ranging from 10^{-9} M to 10^{-3} M were used. For a single experiment with a single metal, cell suspensions obtained from 3 fish (14.67 ± 0.38 cm; 40.87 ± 3.25 g) were used. Normally, 36 cell culture wells (4 rows of 9 wells each) were seeded with cells from a single fish. The first row usually served as the control (no metal). The other three rows received the metal. Therefore, from 3 fish at a time, the effects of 7 metal concentrations of any one metal were tested along with 3 separate controls.

Table 6.1b shows the composition of culture wells followed in the *in vitro* study. Each well received 100 µl of cell suspension having approximately $2x10^5$ lymphocytes. In each row (either with or without metal) the first 3 wells were unstimulated (no mitogens) the other 6 wells, 3 each, were stimulated either with Con A or LPS. In this *in vitro* exposure study, 50 µl of the media containing the mitogen and 50 µl of the saline containing the chloride salt of the metal were added to the wells. Control wells received 50 µl saline without the metal.

Where there was no significant difference between the 3 controls from 3 fish, the results were combined for the purpose of graphical and statistical analysis. In total, 4 separate experiments were conducted, each time using cell suspensions obtained from 3 fish. The procedure remained the same for both the metals studied.

Table 6.1a Composition of culture wells in *in vivo* experiments

Culture Wells	Lymphocyte Suspension	Con A	LPS	Media
Unstimulated (3 Wells)	100 µl	0 µl	0 µl	100 µl
Stimulated (3 Wells)	100 µl	50 µl	0 µl	50 µl
Stimulated (3 Wells)	100 µl	0 µl	50 µl	50 µl

Table 6.1b Composition of Culture Wells in *in vitro* Experiments

Culture Wells	Lymphocyte Suspension	Metal	Saline	Con A	LPS	Media
Control (3 wells) (Unstimulated)	100 µl	0 µl	50 µl	0 µl	0 µl	50 µl
Control (3 wells) (Stimulated)	100 µl	0 µl	50 µl	50 µl	0 µl	0 µl
Control (3 Wells) (Stimulated)	100 µl	0 µl	50 µl	0 µl	50 µl	0 µl
Metal (3 Wells) (Unstimulated)	100 µl	50 µl	0 µl	0 µl	0 µl	50 µl
Metal (3 Wells) (Stimulated)	100 µl	50 µl	0 µl	50 µl	0 µl	0 µl
Metal (3 Wells) (Stimulated)	100 µl	50 µl	0 µl	0 µl	50 µl	0 µl

* Metal concentrations tested ranged from 10^{-9} to 10^{-3} M

6.3 RESULTS

The results presented here demonstrate the effects of cadmium and copper both *in vivo* and *in vitro* on the mitogenic response of carp lymphocytes.

6.3.1 *In Vivo* Effects of Cadmium and Copper

This section shows the *in vivo* effects resulting from cadmium and copper exposure on the blastogenic response of lymphocytes. The lymphocytes collected from the pronephric kidney, and peripheral blood, of carp exposed *in vivo* to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) for varying duration were used in the *in vitro* mitogen stimulation assays. The results from both the metal treatments are presented together.

6.3.1.1 Pronephric Lymphocytes

6.3.1.1.1 Concanavalin A

The mean count per minute (CPM) and the corresponding stimulation index (SI) of Con A stimulated pronephric lymphocytes is presented in Table 6.2. The incorporation of 3H-thymidine by pronephric lymphocytes collected from carp exposed to cadmium and copper for varying duration can be seen to be significantly reduced.

The stimulation pattern as measured by 3H-thymidine incorporation by both unstimulated and Con A stimulated pronephric lymphocytes is illustrated for comparison in Figure 6.1a. Here it can be seen that the mean CPM (15275 to 21033) obtained for unstimulated lymphocytes collected from control fish from different sampling points (Days 1, 3, 6, 9, 12 and 15) remained more or less

Table 6.2 Mean CPM (SI) obtained with pronephric lymphocytes collected from carps exposed to cadmium and copper *in vivo* for varying duration and then stimulated with Con A.

Exposure Duration (Days)	Control	Cadmium 50 μgl^{-1}	Copper 30 μgl^{-1}
1	78466 ± 9295	69947 ± 6846	68422 ± 7402*
	(4.09)	(4.28)	(3.53)
3	78153 ± 6667	72847 ± 6630	55503 ± 5672***
	(3.72)	(4.38)	(3.16)
6	88303 ± 7619	57307 ± 4544***	34895 ± 5314***
	(5.78)	(3.84)	(1.99)
9	76118 ± 5427	41025 ± 8080***	29149 ± 7453***
	(4.60)	(2.66)	(1.78)
12	74584 ± 5829	32410 ± 5243***	26189 ± 3474***
	(3.97)	(2.11)	(1.76)
15	77360 ± 7006	51648 ± 5292**	47619 ± 4988**
	(4.22)	(3.28)	(3.44)

* Denotes significantly lower stimulation than the controls on corresponding sampling days.

* (P<0.05); ** (P<0.01); *** (P<0.001).

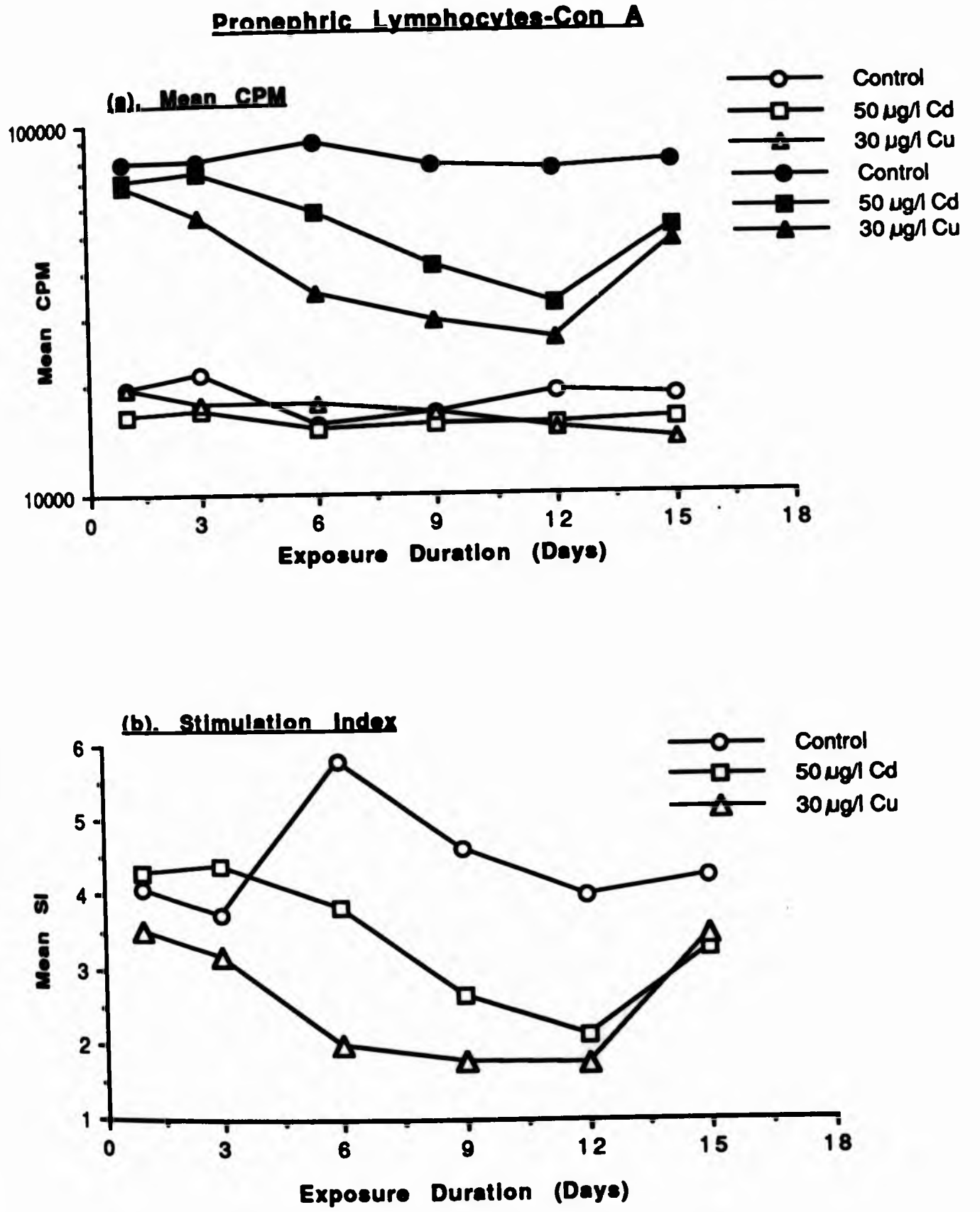


Figure 6.1 The Mean (a) CPM and (b) SI, recorded in pronephric lymphocytes collected from carp exposed to cadmium and copper for a varying duration and stimulated with the mitogen, Con A. Open symbols in (a) denote unstimulated lymphocytes whilst closed symbols denote stimulated lymphocytes.

uniform with no statistical difference between them. Cadmium exposure did not have any significant effect on the background stimulation obtained for the unstimulated cells. On the other hand copper exposure beyond 9 days reduced the background stimulation. The mean CPM obtained for cadmium and copper treated unstimulated lymphocytes were lower than controls at days 9, 12 and 15 (Fig.6.1a).

It appears from the Figure 6.1a that the mean CPM for control fish lymphocytes stimulated by Con A were statistically uniform, throughout the experimental period and ranged from 74584 to 88303. Pronephric lymphocytes collected from carp exposed to cadmium had incorporated 3H-thymidine to the same level as that seen in unexposed control fish up to day 3. However, exposure beyond 3 days (6, 9 and 12) significantly lowered 3H-thymidine incorporation and the decrease was significantly dependent on the exposure duration. Thus, duration of cadmium exposure had a significant ($P<0.001$) inhibitory effect on the proliferation of pronephric lymphocytes up to day 12. Then, after 15 days exposure, proliferation was significantly higher in lymphocytes compared to the samples of 6, 9 and 12 days, but still remained significantly lower than the corresponding control.

Copper exposure produced inhibitory effects similar to the cadmium treatment, and the level of 3H-thymidine incorporation was significantly lower than control and cadmium exposed groups on corresponding sampling points (Figure 6.1a and Table 6.2). The duration of copper exposure had a significant ($P<0.001$) effect on the level of proliferation. The inhibitory effect was more conspicuously seen between days 3 and 9, where the fall in the amount of 3H-thymidine incorporation was very sharp. As with cadmium, a recovery in proliferation was seen at day 15 where the level of 3H-thymidine incorporation was very similar to that of cadmium.

As can be seen from the Figure 6.1a, the amount of 3H-thymidine incorporation was significantly ($P<0.001$) lower in lymphocytes obtained from carp exposed to cadmium or copper compared with the corresponding controls.

Figure 6.1b shows the calculated SI for Con A stimulated pronephric lymphocytes from the three treatments. The SI obtained at different sampling points in controls was relatively uniform (3.72 to 5.78) with the exception of day 6 (5.78). The SI for cadmium and copper treatments ranged from 2.11 to 4.30 and 1.76 to 3.53, respectively. The SI obtained in both cadmium and copper treatments decreased gradually with exposure duration till day 12 before increasing. The SI obtained for cadmium (Days 6, 9, 12 and 15) and copper (3, 6, 9, 12, and 15) were significantly lower than the corresponding controls.

6.3.1.1.2 Lipopolysaccharide (LPS)

The mean CPM, with corresponding SI, of LPS stimulated pronephric lymphocytes is presented in Table 6.3. The level of 3H-thymidine incorporation by LPS stimulated control lymphocytes at different sampling points was statistically uniform ranging from 63659 to 71504 (Figure 6.2a). However, the duration of both cadmium and copper exposure had a significant effect on the amount of 3H-thymidine incorporation as can be seen from the Figure 6.2a.

Cadmium exposure for upto 3 days had no inhibitory effect, while exposure for 6 days and longer lowered the level of proliferation significantly ($P<0.001$). As can be seen from the Figure 6.2a, the inhibitory effect of cadmium was not linear with exposure duration. The maximum effect was seen at 9 days and, whilst the level continued to remain lower than the control, there was no further effect on proliferation. However, the mean CPM recorded on days 6, 9, 12 and 15 were

Table 6.3 The mean CPM (SI) obtained with pronephric lymphocytes collected from carps exposed to cadmium and copper *in vivo* for varying duration and then stimulated with LPS.

Exposure Duration (Days)	Control	Cadmium 50 μgl^{-1}	Copper 30 μgl^{-1}
1	71066 ± 10897	66683 ± 6898	63151 ± 3963
	(3.70)	(4.08)	(3.26)
3	66364 ± 5666	71099 ± 7168	59617 ± 6735*
	(3.16)	(4.27)	(3.40)
6	67404 ± 6891	57027 ± 4053***	35876 ± 3550***
	(4.13)	(3.82)	(2.04)
9	71504 ± 8107	40506 ± 5417***	34291 ± 5688***
	(4.32)	(2.62)	(2.09)
12	69885 ± 8128	42086 ± 5802***	29687 ± 7062***
	(3.72)	(2.74)	(2.00)
15	63659 ± 4548	51816 ± 6508**	47624 ± 6754**
	(3.47)	(3.29)	(3.44)

* Denotes significantly lower stimulation than the controls on corresponding sampling days.

* (P<0.05); ** (P<0.01); *** (P<0.001).

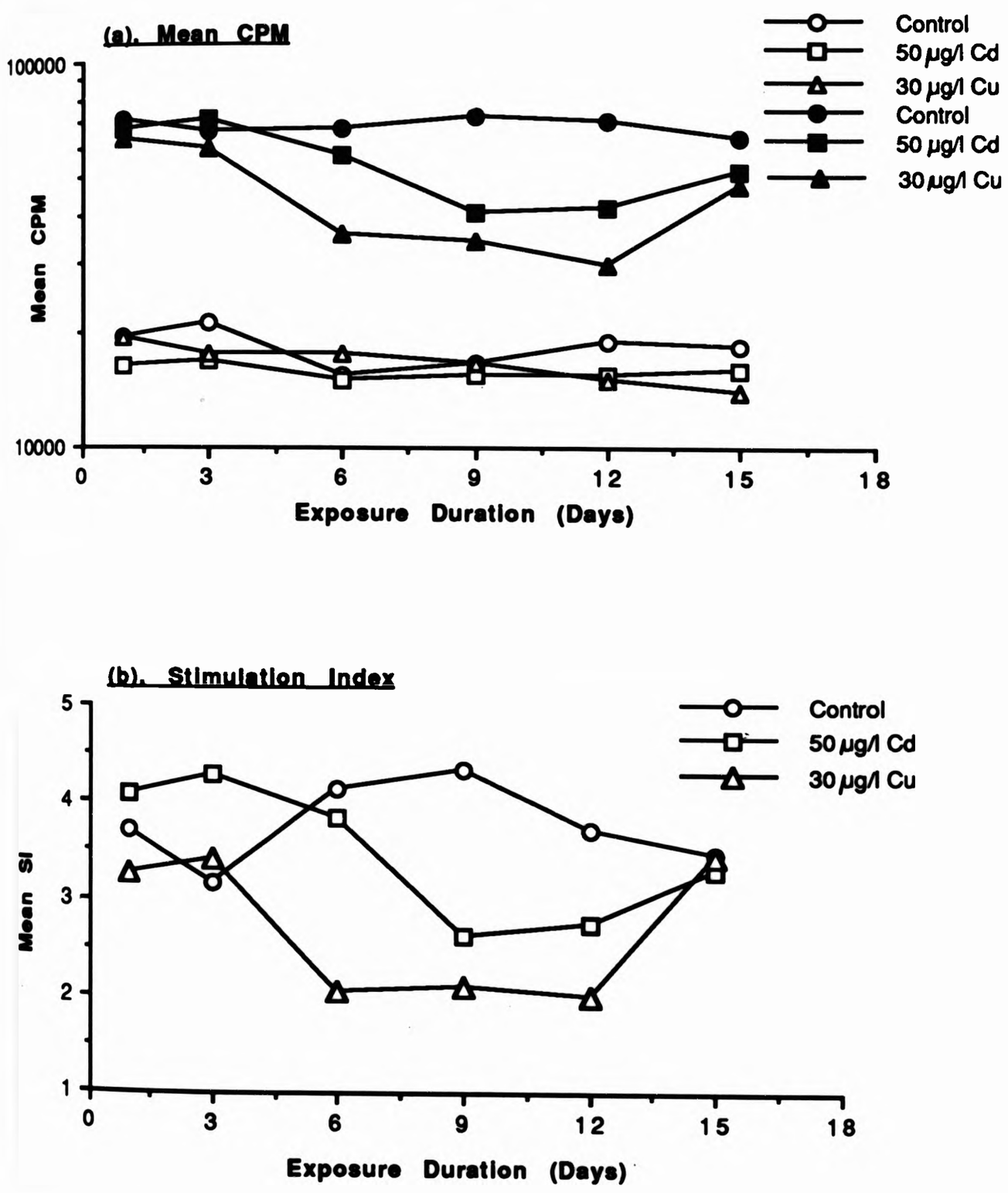


Figure 6.2 The Mean (a) CPM and (b) SI, recorded in pronephric lymphocytes collected from carp exposed to cadmium and copper for a varying duration and stimulated with the mitogen, LPS. Symbols same as in Figure 6.1

significantly (P<0.001) lower than corresponding controls Table 6.3).

The mean CPM obtained for lymphocytes collected from copper exposed treatment was lower than the control and cadmium treatments on all the sampling days. As can be seen from the Figure 6.2a, the decrease in stimulation was not linear to the exposure duration. There was an initial sharp fall in the stimulation recorded between days 3 and 6, thereafter the stimulation remained almost the same till day 12, before increasing. Copper exposure for 3 days and longer, significantly (P<0.001) lowered the amount of 3H-thymidine incorporation compared to unexposed controls.

Figure 6.2b. shows the pattern for the calculated SI for LPS stimulated lymphocytes in the three treatments. The SI found in controls ranged from 3.16 to 4.32, while it was 2.62 to 4.27 in cadmium treatment and 2.00 to 3.44 in copper treatment. The SI recorded for lymphocytes obtained after 9 and 12 days exposure to cadmium and 6, 9 and 12 days exposure to copper were significantly (P<0.001) lower than the corresponding controls. Comparing the Figures 6.2a and 6.2b, it becomes clear that, although the mean CPM on day 15 sample in cadmium and copper treatments was significantly lower than controls, the SI is statistically the same.

6.3.1.2 Peripheral Blood Lymphocytes (PBL)

6.3.1.2.1 Concanavalin A

Table 6.4 documents the mean CPM and SI obtained for PBL collected from three treatments. Figure 6.3a illustrates the stimulation pattern obtained for PBL collected from unexposed (control) carp and compares it with those exposed to cadmium and copper.

The background counts for lymphocytes from the control treatment were not statistically uniform on all the sampling points. Relatively high background counts were obtained for samples of day 9 and 15. With the exception of day 9 sample, the background counts for unstimulated lymphocytes obtained from carp exposed to cadmium were uniform. The background counts for PBL collected from the copper treatment was relatively uniform with the exception of day 15 sample.

The mean CPM for the control PBL stimulated with Con A ranged from 16379 to 22290. The response was relatively uniform on all the sampling points. As might be expected, the exposure duration had a significant effect in lowering the 3H-thymidine incorporation in both the cadmium and copper treatments (Figure 6.3a).

Cadmium exposure for 3 days and longer reduced the lymphocyte proliferation significantly, but the reduction was gradual with exposure duration. Copper exposure for 6 days and above reduced 3H-thymidine incorporation significantly. The reduction was sharp between days 6 and 9. The minimum CPM (11994) was found in samples obtained after 15 days exposure to cadmium, while the minimum (8054) was after 12 days exposure in the copper treatment. Cadmium and copper exposure for 6, 9, 12 and 15 days decreased the amount of proliferation significantly ($P<0.001$) compared with the controls on corresponding sampling days. The inhibitory effect of copper appeared to be greater than cadmium (Figure 6.3a).

Figure 6.3b represents the calculated SI for Con A stimulated PBL from the three treatments. The SI obtained for the control treatment was not very uniform on all the sampling days. It ranged from 3.13 to 7.50. On the other hand, Cadmium and copper treatments gave SI ranging from 4.06 to 6.28 and 2.12 to 6.33, respectively. The minimum SI was obtained in lymphocytes collected from cadmium and copper

Table 6.4 The mean CPM (SI) obtained with peripheral blood lymphocytes collected from carps exposed to cadmium and copper *in vivo* for varying duration and then stimulated with Con A.

Exposure Duration (Days)	Control	Cadmium 50 μgl^{-1}	Copper 30 μgl^{-1}
1	19163 ± 2686	22533 ± 4540	18903 ± 2548
	(6.63)	(5.72)	(5.53)
3	21545 ± 4489	16910 ± 2231*	19702 ± 2840
	(5.31)	(4.49)	(6.33)
6	22290 ± 5030	15646 ± 2262***	13934 ± 1705***
	(7.50)	(4.40)	(3.89)
9	16379 ± 2667	14563 ± 1772**	9354 ± 1673***
	(3.13)	(6.28)	(2.65)
12	18056 ± 2755	12728 ± 2628***	8054 ± 1955***
	(5.03)	(4.12)	(2.12)
15	17698 ± 3724	11994 ± 2589**	9440 ± 1805***
	(3.69)	(4.06)	(4.19)

* Denotes significantly lower stimulation than the controls on corresponding sampling days.

* (P<0.05); ** (P<0.01); *** (P<0.001)

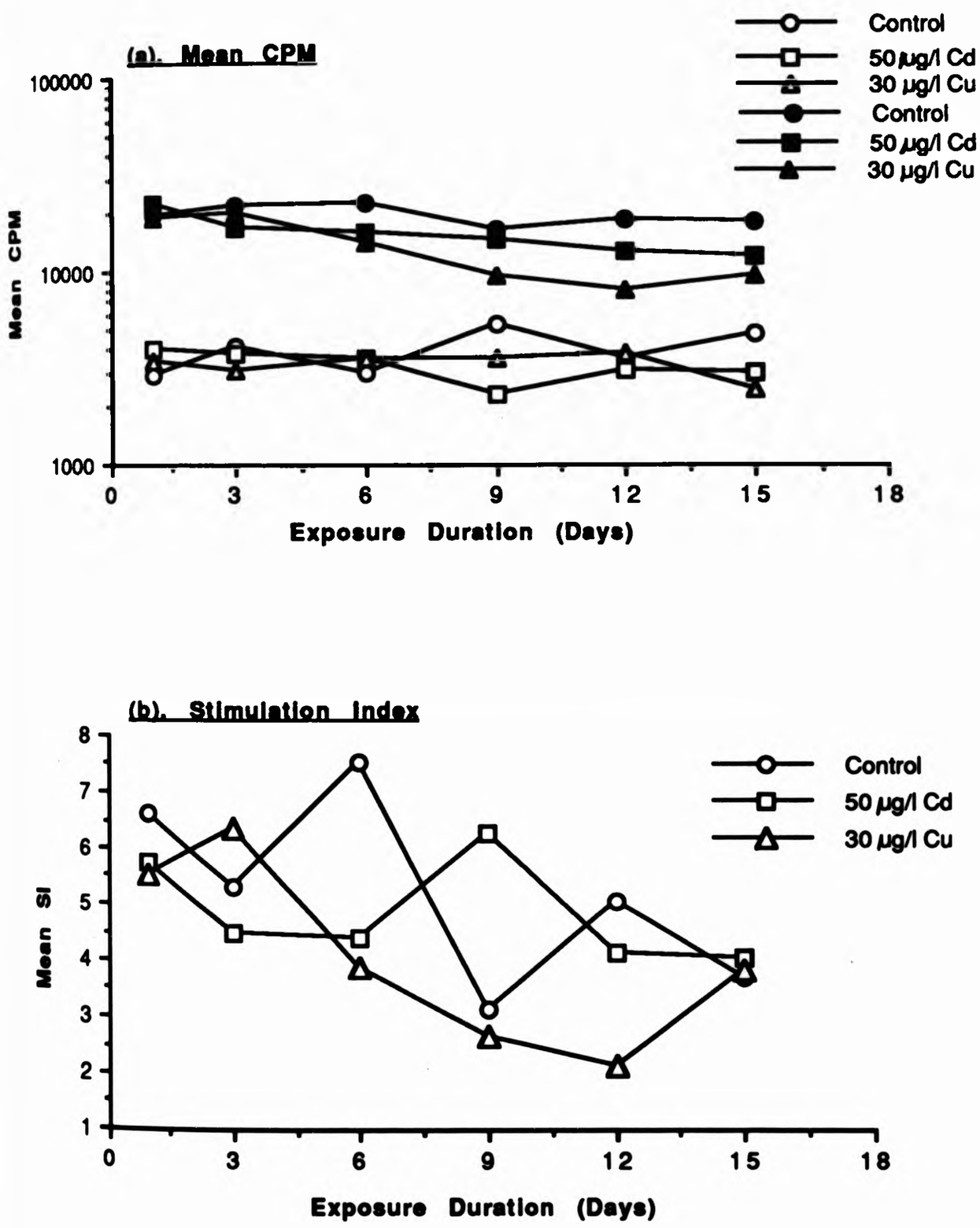


Figure 6.3 The Mean (a) CPM and (b) SI, recorded in peripheral blood lymphocytes (PBL) collected from carp exposed to cadmium and copper for a varying duration and stimulated with the mitogen, Con A. Symbols same as in Figure 6.1

treatments after 15 and 12 days exposure, respectively. The SI for lymphocytes collected following cadmium exposure was lower than corresponding controls on days 1, 6, and 12 while it was higher on other sampling points. Though the mean CPM recorded was lower on most of the sampling points, the SI did not give any clear-cut trend. In the copper treatment, an increased SI was obtained in lymphocytes collected after 3 days exposure (6.33 on day 3), but it was lower than controls on all the remaining corresponding sampling days.

6.3.1.2.2 Lipopolysaccharide (LPS)

The mean CPM and SI for PBL collected on different days from the three treatments and stimulated with LPS is documented in Table 6.5. The mean CPM of both unstimulated and LPS stimulated PBL, collected from the three treatments on different sampling days is illustrated in Figure 6.4a. The level of 3H-thymidine incorporation by PBL collected from control treatment was very uniform on all the sampling points (Figure 6.4a).

The duration of exposure to cadmium and copper had a significant ($P<0.001$) effect in lowering the proliferation. The decrease in proliferation observed in the cadmium treatment was gradual with the exposure duration. The proliferative response after 1 and 3 days exposure to copper was not statistically different from the controls but exposure beyond 3 days reduced the proliferation significantly with a sharp fall in response between day 6 and 9. The minimum CPM in cadmium (10594) and copper (9844) treatments were obtained in samples collected after 15 and 9 days exposure to the respective metals. Lymphocytes collected from carp exposed to cadmium and copper for longer than 6 days showed significantly ($P<0.05$) reduced proliferation.

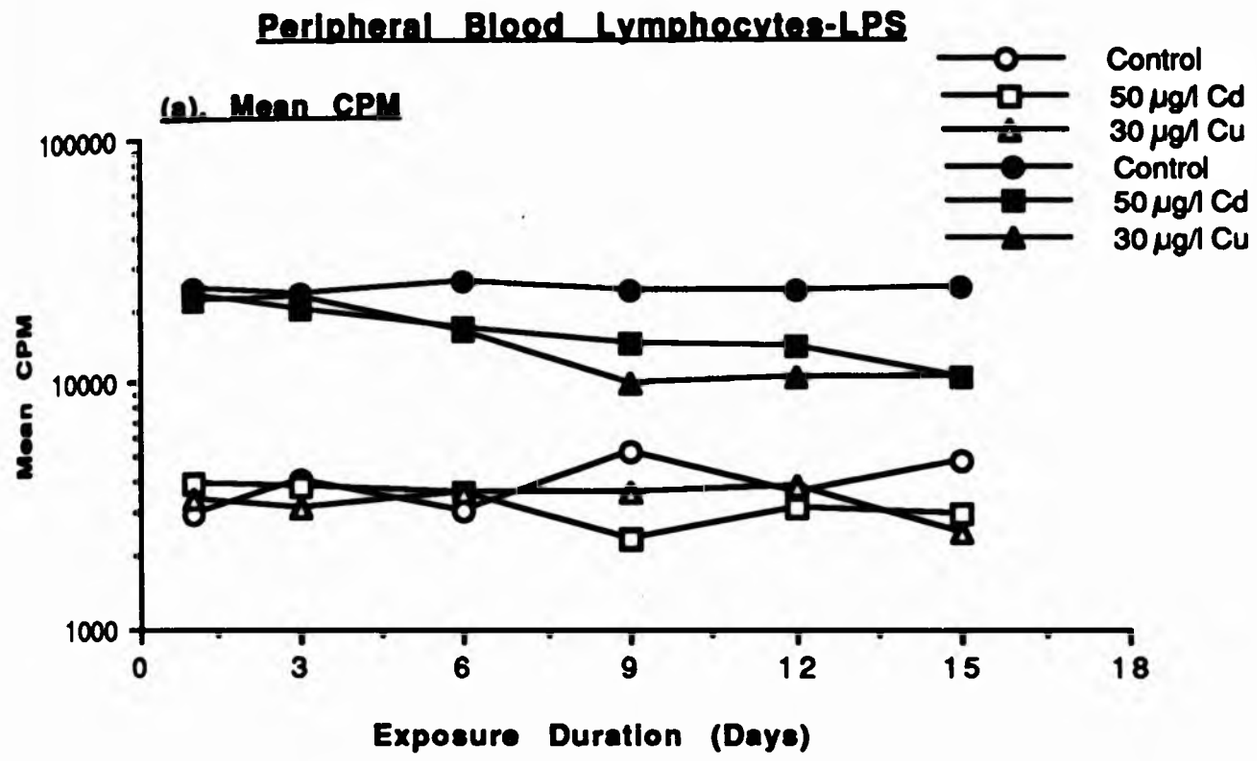
The SI calculated for PBL stimulated with LPS is shown in Figure 6.4b. The SI

Table 6.5 The mean CPM (SI) obtained with peripheral blood lymphocytes collected from carps exposed to cadmium and copper *in vivo* for varying duration and then stimulated with LPS.

Exposure Duration (Days)	Control	Cadmium 50 μgl^{-1}	Copper 30 μgl^{-1}
1	24176 ± 3078	22937 ± 4066	21821 ± 3065
	(8.37)	(5.82)	(6.39)
3	22725 ± 4431	19700 ± 4153	21994 ± 3146
	(5.60)	(5.23)	(7.07)
6	25919 ± 1701	16445 ± 2418***	16002 ± 2620***
	(8.72)	(4.63)	(4.46)
9	23832 ± 3587	14191 ± 1698***	9844 ± 2227***
	(4.55)	(6.12)	(2.79)
12	23446 ± 2109	13929 ± 2653***	10420 ± 1574***
	(6.53)	(4.51)	(2.74)
15	23976 ± 2997	10594 ± 1871***	10355 ± 962***
	(5.00)	(3.58)	(4.19)

* Denotes significantly lower stimulation than the controls on corresponding sampling days.

* (P<0.05); ** (P<0.01); *** (P<0.001)



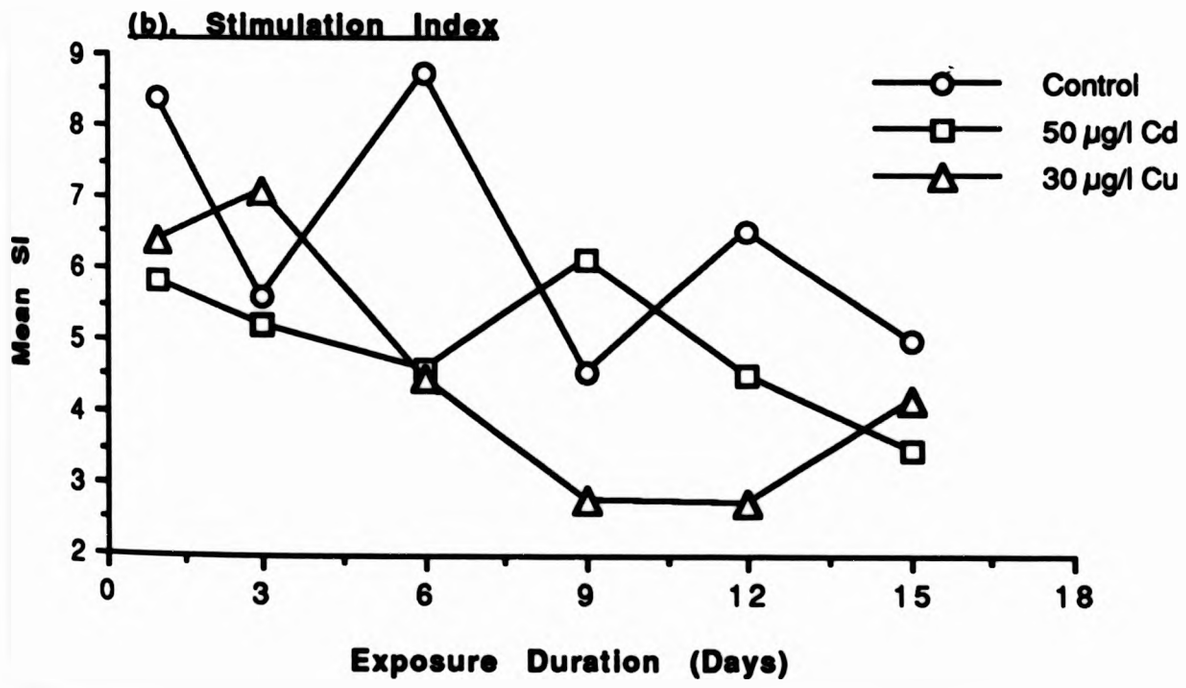


Figure 6.4 The Mean (a) CPM and (b) SI, recorded in peripheral blood lymphocytes (PBL) collected from carp exposed to cadmium and copper for a varying duration and stimulated with the mitogen, LPS. Symbols same as in Figure 6.1

found for controls was not very uniform and it ranged from 4.55 to 8.37, whilst that of cadmium treatment showed a gradual decline till day 6, then it rose to a maximum of 6.12 on day 9 before once again declining sharply. The SI was however, lower than corresponding controls on all the sampling points except for day 9 and it was statistically lower ($P<0.05$) than the controls in PBL collected after 6, 12 and 15 days exposure to cadmium. The SI ranged from 2.74 (day 12) to 7.07 (day 3) for lymphocytes collected from fish exposed to copper. The SI obtained for PBL collected from fish exposed to 6 days and longer were significantly ($P<0.001$) lower than the corresponding controls.

6.3.2 *In Vitro* Effects of Cadmium and Copper

The results presented under this section demonstrate the *in vitro* effects of cadmium and copper (10^{-9} to 10^{-3} M) on the blastogenic response of pronephric lymphocytes collected from carp, regarded as healthy, to Con A and LPS. The results of four repeat experiments are graphically presented separately while the combined result of four experiments is presented in Tables. The *in vitro* effects of cadmium and copper are dealt separately.

6.3.2.1 Cadmium

Figures 6.5a-d (Con A) and 6.6a-d (LPS) illustrate the mean CPM of four repeat experiments where pronephric lymphocytes were exposed to seven concentrations (treatment levels) of cadmium ranging from 10^{-9} to 10^{-3} M. Pronephric lymphocytes exposed to cadmium (0 and 10^{-9} to 10^{-3} M) *in vitro* showed similar levels of 3H-thymidine incorporation in all four of the repeat experiments.

The mean CPM obtained for unstimulated lymphocytes exposed to 0, 10^{-9}, 10^{-8} and 10^{-7} M cadmium did not differ statistically. On the other hand unstimulated

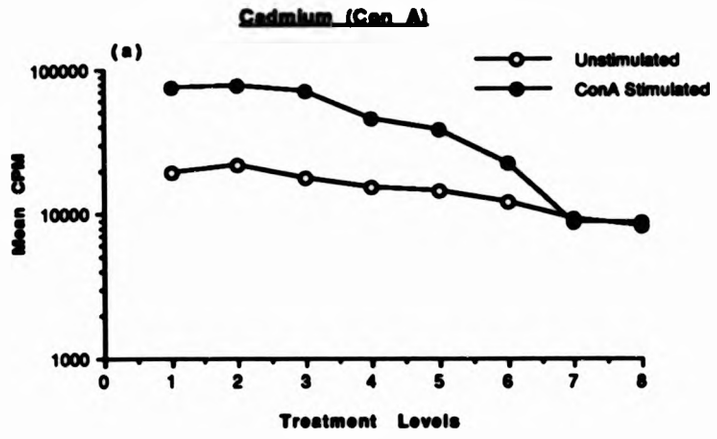


Figure 6.5 The Mean CPM obtained with carp pronephric lymphocytes exposed to varying concentrations of cadmium (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen Con A. Treatment levels 1 to 8 denote concentrations from 0 to 10^{-3} M. Treatment level 1=0; 2=10^{-9}; 3=10^{-8}; 4=10^{-7}; 5=10^{-6}; 6=10^{-5}; 7=10^{-4}; 8=10^{-3} M. Figures a-d show the results from four repeat experiments.

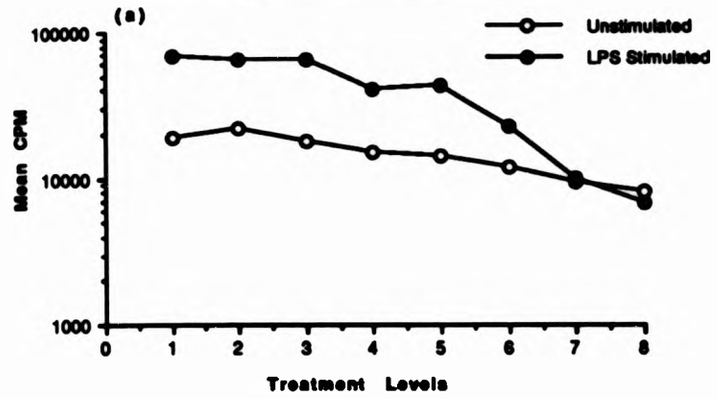


Figure 6.6 The Mean CPM obtained with carp pronephric lymphocytes exposed to varying concentrations of cadmium (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen LPS. Figures a-d show the results from four repeat experiments. Treatment levels as in Figure 6.5

lymphocytes exposed to cadmium concentrations of 10^{-6} M and above resulted in significant inhibition of background stimulation (Figures 6.5a-d, Table 6.6). The decrease in background stimulation was very gradual and was dependent on cadmium concentration.

The inhibitory effect of cadmium on Con A and LPS stimulated pronephric lymphocytes is evident from the Figures 6.5a-d and 6.6a-d respectively. The inhibitory effects of cadmium on proliferation of lymphocytes by Con A and LPS was significantly ($P<0.001$) dependent on concentration. As can be seen from the results of the four repeat experiments (Figures 6.5a-d and 6.6a-d), *in vitro* cadmium levels of 10^{-6} M and above significantly lowered the amount of 3H-thymidine incorporation by both Con A and LPS stimulated lymphocytes. The mean CPM recorded were lower than the controls and those cultures exposed to 10^{-8} and 10^{-9} M cadmium. Pronephric lymphocytes exposed to high concentrations of 10^{-4} and 10^{-3} M cadmium, failed to show any significant stimulation by either Con A or LPS. There was no statistical difference in the amount of 3H-thymidine incorporated in pronephric lymphocytes stimulated by either Con A or LPS at any given treatment level.

Figures 6.7a-d (Con A) and 6.8a-d (LPS) illustrate the SI calculated from the four repeat experiments for Con A and LPS stimulated pronephric lymphocytes exposed to different concentrations of cadmium. The SI obtained from the four experiments were not identical. However from the Figures 6.7a-d and 6.8a-d it is clear that the SI obtained decreased with increasing concentration of cadmium *in vitro* and the decrease was significantly ($P<0.05$) dependent on metal concentration. The SI obtained for lymphocytes exposed to concentrations of 10^{-8} M and lower and stimulated by either Con A or LPS did not differ significantly. On the other hand

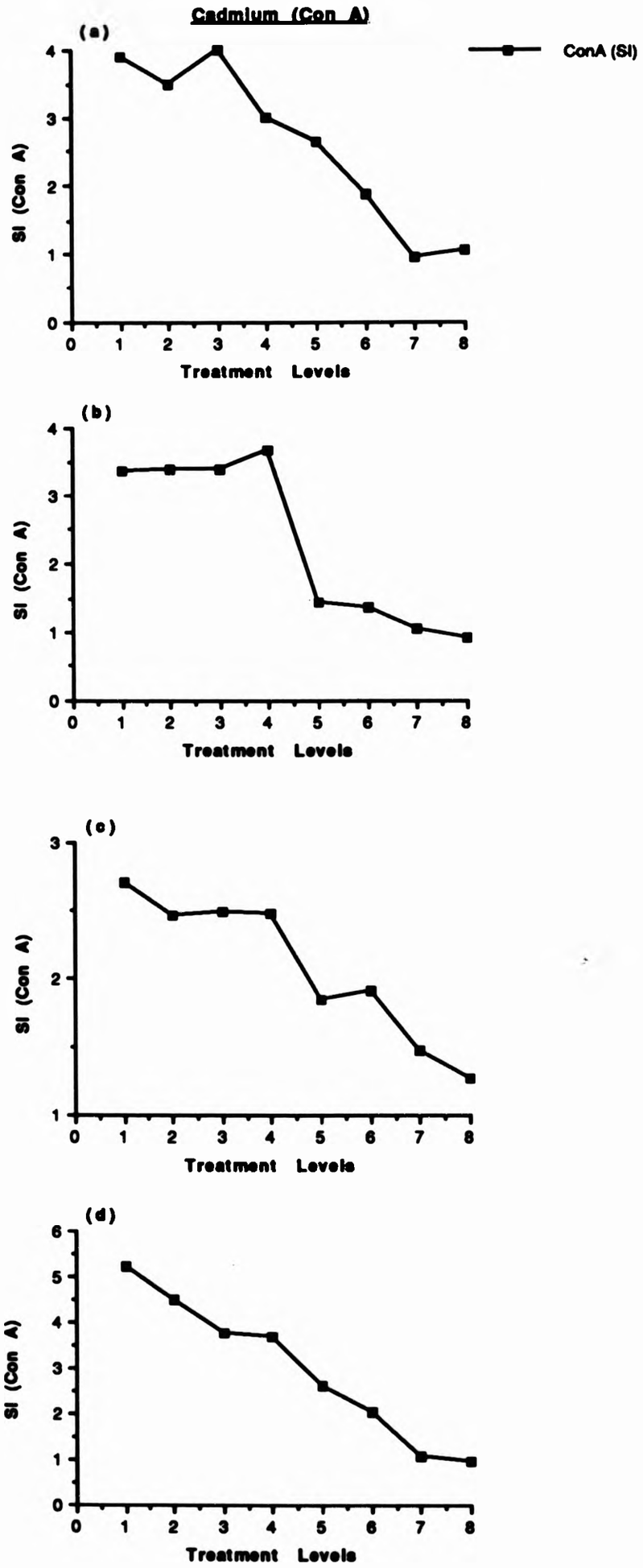


Figure 6.7 The Stimulation Index (SI) obtained with carp pronephric lymphocytes exposed to varying concentrations of cadmium (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen Con A. Figures a-d show the results from four repeat experiments. Treatment levels as in Figure 6.5

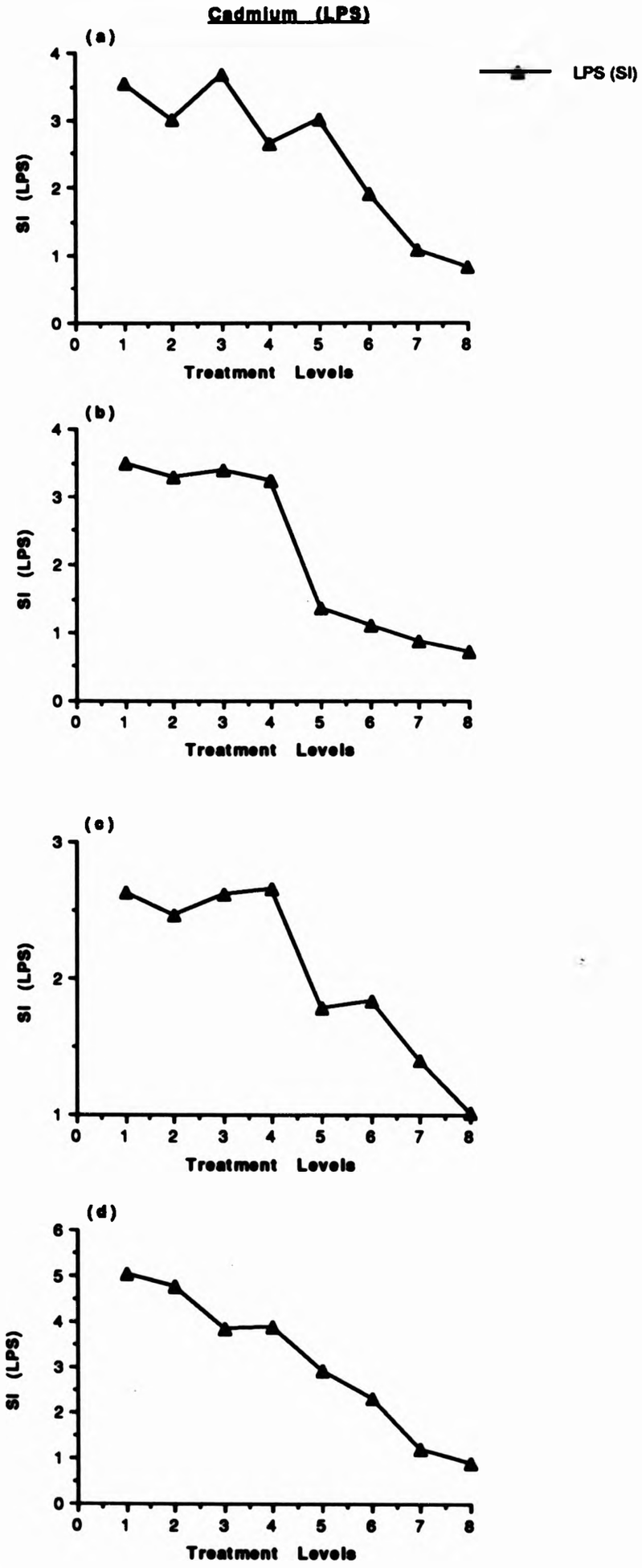


Figure 6.8 The Stimulation Index (SI) obtained with carp pronephric lymphocytes exposed to varying concentrations of cadmium (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen LPS. Figures a-d show the results of four repeat experiments. Treatment levels as in Figure 6.5

concentrations of 10^{-6} M and higher, lowered the SI significantly for both Con A and LPS stimulated lymphocytes.

The mean CPM and SI obtained by combining the results of four repeat experiments is presented in Table 6.6. The mean CPM for unstimulated lymphocytes ranged from 7767 (10^{-3} M) to 18474 (0), with the highest CPM (20325) found in lymphocytes exposed to 10^{-9} M cadmium. Mean CPM for stimulated lymphocytes ranged from 8153 (10^{-3} M) to 70016 (0=control) and 6661 (10^{-3} M) to 76864 (10^{-9} M) for Con A and LPS stimulated lymphocytes respectively. A sharp dose-dependent decline in 3H-thymidine incorporation was seen in lymphocytes exposed to cadmium concentrations of 10^{-6} and above and lymphocytes exposed to cadmium levels of 10^{-4} and 10^{-3} M, failed to show any significant proliferation.

The mean SI obtained from four experiments reveals the inhibitory effect of cadmium at concentrations of 10^{-7} M and above (Table 6.6). The mean SI found for Con A and LPS stimulated lymphocytes ranged from 1.06 (10^{-3} M) to 3.80 (0=control) and 0.86 (10^{-3} M) to 3.37 (10^{-9} M), respectively. Mean SI of more than 3 was consistently obtained only in lymphocytes which were exposed to concentrations of 10^{-7} and below. Concentrations of 10^{-6} M and above significantly reduced the SI for lymphocytes stimulated by both the mitogens.

6.3.2.2 Copper

The *in vitro* effects of copper (10^{-9} to 10^{-3} M) on Con A and LPS stimulated pronephric lymphocytes of carp from four repeat experiments are illustrated separately in Figures 6.9a-d and 6.10a-d. Background incorporation of 3H-thymidine by unstimulated lymphocytes was inhibited by copper and the inhibition was dose-dependent. Concentrations of 10^{-7} and above significantly reduced the background

Table 6.6 The mean CPM and SI of carp pronephric lymphocytes exposed to varying concentrations of cadmimum *in vitro* and stimulated with Con A and LPS.

Concentrations(M)	Unstimulated	Con A	LPS
0	18474 ± 2229	70016 ± 18747	68034 ± 18247
		(3.80 ± 1.07)	(3.68 ± 1.00)
10^{-9}	20325 ± 1443	70056 ± 14838	76864 ± 16076
		(3.46 ± 0.83)	(3.37 ± 0.98)
10^{-8}	17694 ± 1862	61021 ± 16226	60395 ± 14958
		(3.41 ± 0.66)	(3.37 ± 0.54)
10^{-7}	15938 ± 1160	50791 ± 7459	49213 ± 8229**
		(3.21 ± 0.58)	(3.10 ± 0.58)
10^{-6}	14958 ± 1309*	31731 ± 8585***	33744 ± 12096***
		(2.13 ± 0.59)*	(2.27 ± 0.82)*
10^{-5}	13863 ± 3359*	22048 ± 5175***	22116 ± 8394***
		(1.80 ± 0.29)**	(1.79 ± 0.51)**
10^{-4}	8298 ± 1162***	9217 ± 460***	9299 ± 1321***
		(1.14 ± 0.23)***	(1.14 ± 0.22)***
10^{-3}	7767 ± 764***	8153 ± 777***	6661 ± 861***
		(1.06 ± 0.15)***	(0.86 ± 0.12)***

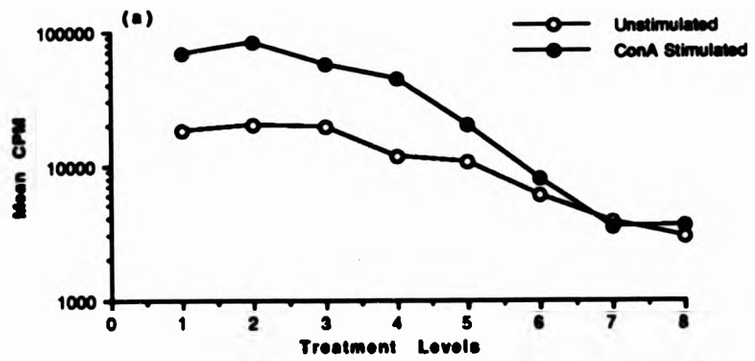
The results are combined from four repeat experiments. Each experiment had triplicate cultures (n=12).
* Denote significantly lower stimulation compared to control (0) within each category
* ($P<0.05$); ** ($P<0.01$); *** ($P<0.001$)

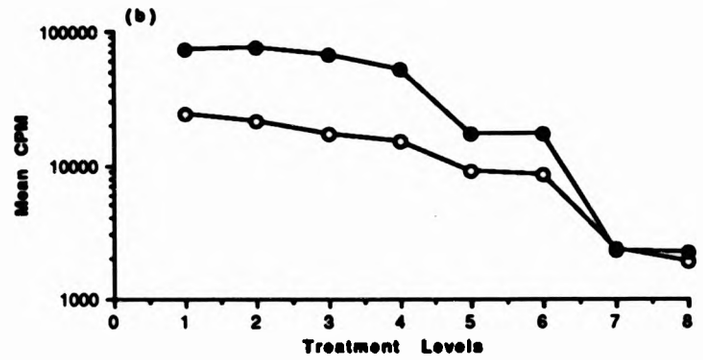
proliferation.

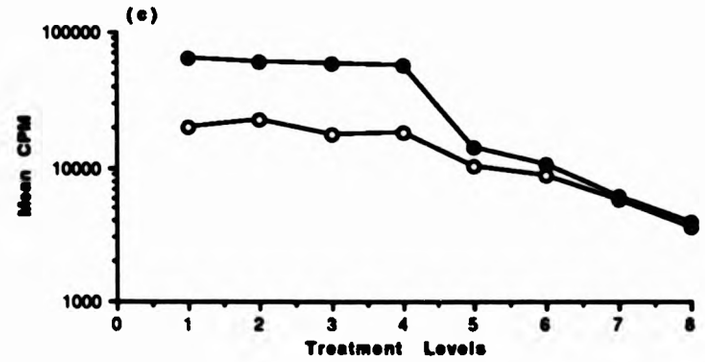
The pattern of 3H-thymidine incorporation by Con A and LPS stimulated lymphocytes observed in the four experiments was consistent and there was no statistical difference between them. In comparison to the controls, concentrations of 10^{-7} M copper and above significantly inhibited the amount of 3H-thymidine incorporation in pronephric lymphocytes stimulated by either Con A or LPS (Figures 6.9a-d and 6.10a-d). The inhibitory effect was significantly ($P<0.001$) dependent on *in vitro* copper concentration. The fall in proliferation reflected by reduction in mean CPM was sharp in cultures exposed to concentrations of 10^{-7} M and above. In all the four experiments, the proliferation was significantly inhibited in lymphocytes exposed to 10^{-4} and 10^{-3} M copper. The picture was similar in lymphocytes stimulated by both the mitogens.

The SI obtained for pronephric lymphocytes exposed to varying concentrations of copper (10^{-9} to 10^{-3} M) from four repeat experiments are shown in Figures 6.11a-d (Con A) and 6.12a-d (LPS). Between repeat experiments, there was variation in SI obtained at any given *in vitro* concentration. It is evident from the Figures 6.11a-d and 6.12a-d, that the suppression of stimulation was concentration dependent. Copper concentrations of 10^{-7} M and above consistently resulted in lower SI in all the four *in vitro* experiments. Concentrations of 10^{-6} M and above produced SI of less than 2 whilst, the higher concentrations, 10^{-4} and 10^{-3} M produced no significant stimulation.

The mean CPM and SI obtained from the combined results of the four experiments are documented in Table 6.7. The pooled CPM clearly reveals the dose-dependent inhibitory effect of copper on the background 3H-thymidine incorporation by







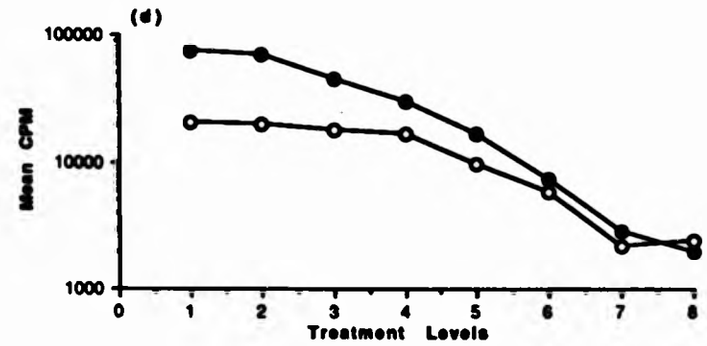
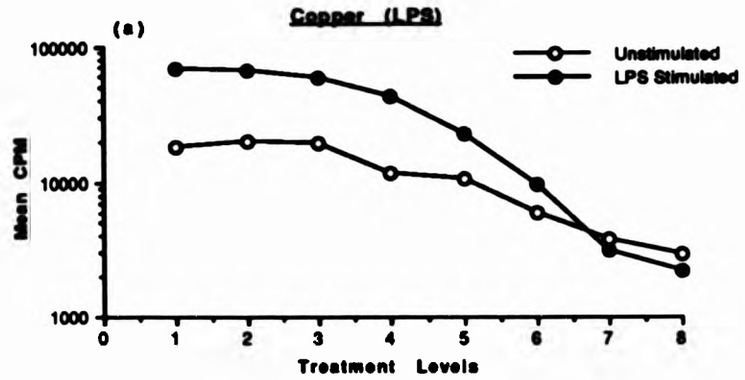
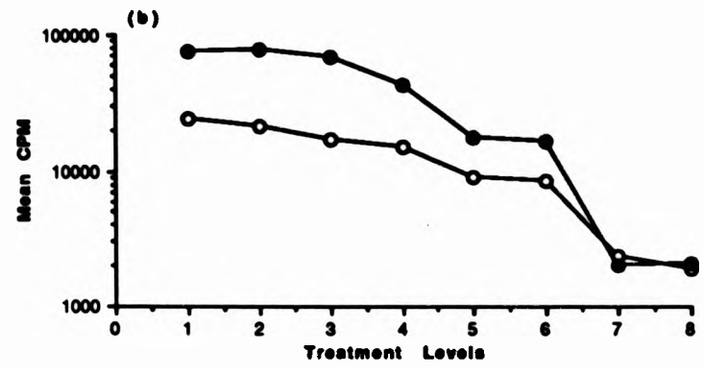
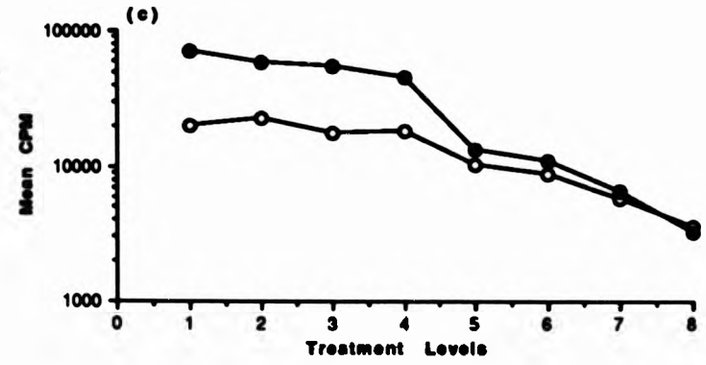


Figure 6.9 The Mean CPM obtained with carp pronephric lymphocytes exposed to varying concentrations of copper (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen Con A. Treatment levels 1 to 8 denote concentrations from 0 to 10^{-3} M. Treatment level 1=0; 2=10^{-9}; 3=10^{-8}; 4=10^{-7}; 5=10^{-6}; 6=10^{-5}; 7=10^{-4}; 8=10^{-3} M. Figures a-d show the results from four repeat experiments.







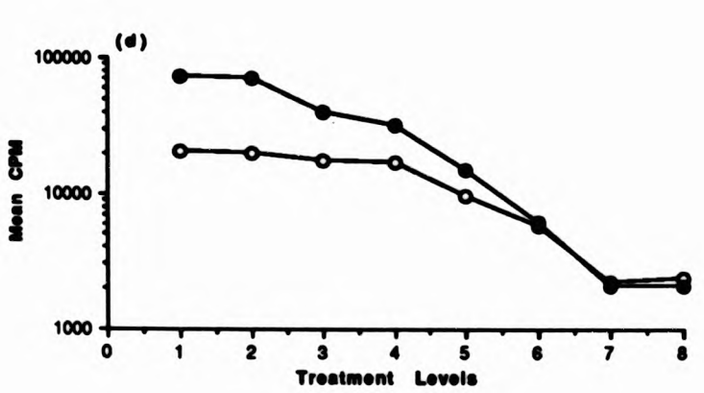


Figure 6.10 The mean CPM obtained with carp pronephric lymphocytes exposed to varying concentrations of copper (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen LPS. Figures a-d show results from four repeat experiments. Treatment levels as in Figure 6.9

unstimulated lymphocytes. The reduction in the mean CPM was most marked between 10^{-7} and 10^{-6} M copper concentration and was significantly lower than unstimulated control lymphocytes. The concentration-dependent inhibitory effect of copper on Con A and LPS stimulated lymphocytes is evident from the pooled CPM of four experiments. As with the unstimulated groups, there was a sharp fall in Con A and LPS induced stimulation between concentrations 10^{-7} and 10^{-6} M. Concentrations of 10^{-7} and above significantly reduced the amount of 3H-thymidine incorporation by Con A and LPS stimulated lymphocytes alike.

The mean SI obtained from combining four experiments confirms the significant ($P<0.001$) inhibitory effects of copper at concentrations of 10^{-6} M and above. A relatively high SI (3) was obtained only with lymphocytes exposed to *in vitro* copper concentrations of 10^{-7} M and below. At high copper concentrations there was no significant stimulation at all. As can be seen from the Table 6.7, the results obtained with both the mitogens were similar.

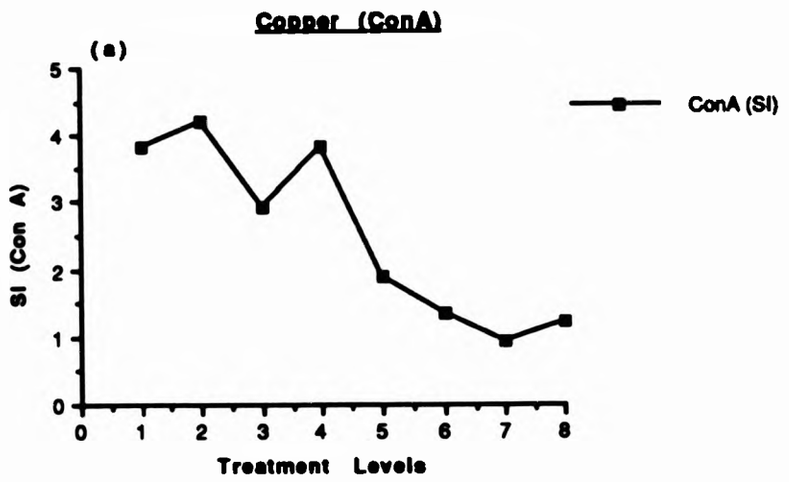


Figure 6.11 The Stimulation Index (SI) obtained with carp pronephric lymphocytes exposed to varying concentrations of copper (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen Con A. Figures a-d show the results from the four repeat experiments. Treatment levels as in Figure 6.9

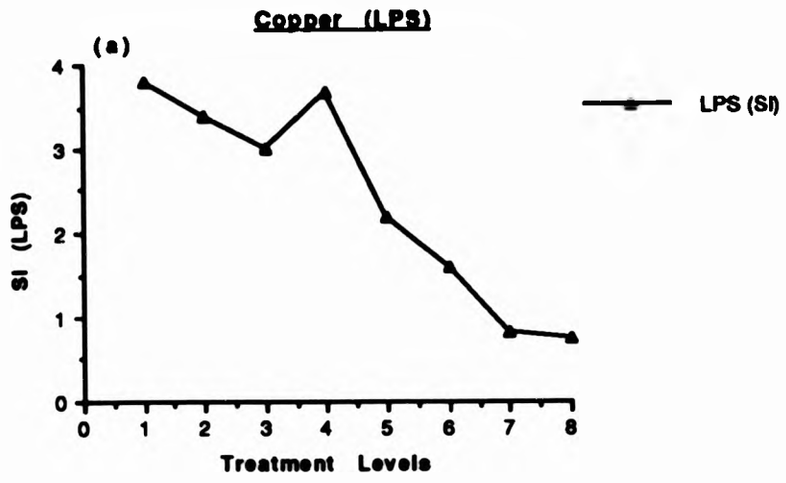


Figure 6.12 The Stimulation Index (SI) obtained with carp pronephric lymphocytes exposed to varying concentrations of copper (10^{-9} to 10^{-3} M) *in vitro* and stimulated with the mitogen LPS. Figures a-d show the results from the four repeat experiments. Treatment levels as in Figure 6.9

Table **6.7** The mean CPM and SI of carp pronephric lymphocytes exposed to varying concentrations of cadmimum *in vitro* and stimulated with Con A and LPS.

Concentrations(M)	Unstimulated	Con A	LPS
0	18474 ± 2229	70016 ± 18747	68034 ± 18247
		(3.80 ± 1.07)	(3.68 ± 1.00)
10^{9}	20325 ± 1443	70056 ± 14838	76864 ± 16076
		(3.46 ± 0.83)	(3.37 ± 0.98)
10^{-8}	17694 ± 1862	61021 ± 16226	60395 ± 14958
		(3.41 ± 0.66)	(3.37 ± 0.54)
10^{-7}	15938 ± 1160	50791 ± 7459	49213 ± 8229**
		(3.21 ± 0.58)	(3.10 ± 0.58)
10^{-6}	14958 ± 1309*	31731 ± 8585***	33744 ± 12096***
		(2.13 ± 0.59)*	(2.27 ± 0.82)*
10^{-5}	13863 ± 3359*	22048 ± 5175***	22116 ± 8394***
		(1.80 ± 0.29)**	(1.79 ± 0.51)**
10^{-4}	8298 ± 1162***	9217 ± 460***	9299 ± 1321***
		(1.14 ± 0.23)***	(1.14 ± 0.22)***
10^{-3}	7767 ± 764***	8153 ± 777***	6661 ± 861***
		(1.06 ± 0.15)***	(0.86 ± 0.12)***

The results are combined from four repeat experiments. Each experiment had triplicate cultures (n=12).

* Denote significantly lower stimulation compared to control (0) within each category

* ($P<0.05$); ** ($P<0.01$); *** ($P<0.001$)

6.4 DISCUSSION

It is widely accepted that many of the heavy metals accumulate in the lymphoid organs and are also known to influence the development and functioning of the immune system in mammals (Koller, 1980) and fish (Anderson *et al.*, 1984; Zeeman, 1986). The answer to the question as to whether metals induce any changes which prevent the blastogenic response of lymphocytes, appears to be vital in the field of immunotoxicology.

Most often, the immunosuppressive effects of chemical toxicants have been attributed to the inhibitory effects of the toxicants in question on selective populations of immunocompetent cells. In mammals, lymphocyte proliferation studies using B and T cell mitogens have enabled the immunotoxic properties of certain chemicals to be described more precisely. However, the situation in fish immunotoxicology is complicated by the uncertainty surrounding the issue of lymphocyte heterogeneity.

All the available evidence suggests the existence of B-like and T-like lymphocytes in fish (Ellis, 1989). Optimal expression of immunity to most antigens (Thymus-dependent) would require the cooperation and interaction of macrophages, T and B-lymphocytes. The observed immunosuppressive effects of cadmium and copper to SRBC (thymus-dependent) antigens in carp (Chapter 4) appeared to suggest the involvement of B and T-like lymphocytes. It was hoped to understand more about the immunosuppressive effects of cadmium and copper, by carrying out lymphocyte proliferation studies using these mammalian B and T-cell mitogens.

In studies of the present nature, where deviation of responses from what is generally

regarded as normal is the criterion, it is essential to have a proper understanding of what is a normal response. The kinetics and optimization of the normal mitogenic response of lymphocytes of some important fish species have been delineated. Factors such as incubation temperature, cell number per culture well, the type of mitogen, labelling time, and the source of lymphoid cells have all been found to influence the amount of 3H-thymidine uptake by proliferating lymphoid cells in *in vitro* mitogenic studies.

The optimum stimulation by PHA of carp lymphocytes was obtained at an incubation temperature of 33°C (Liewes *et al.*, 1982), whilst it was 32°C for blue gills (Cuchens and Clems, 1977) and 28°C for rainbow trout (Chilmonczyk, 1978). A relatively low temperature of 22°C for carp (Liewes *et al.*, 1982) and high 28°C for rainbow trout (Chilmonczyk, 1978) were found to be the optimal for LPS stimulation. In this study the incubation temperature of 22°C was adopted for both the mitogens and lymphocytes were cultured at densities of approximately 10^5 cells per 200 µl final volume. This density was found by Liewes *et al.* (1982) to be optimum for carp lymphocytes from kidney and peripheral blood.

Regarding organ compartmentalization and selective proliferation in response to different mitogens, Liewes *et al.* (1982) found carp lymphocytes from anterior kidney to respond best to PHA followed by LPS, while peripheral blood lymphocytes responded best to LPS. In contrast, thymus derived lymphocytes responded best to PHA but the response was very low to Con A and LPS. They failed to find any clear-cut compartmentalization of lymphocyte populations in carp. In the present study, there was no obvious significant difference in the stimulation pattern of pronephric lymphocytes obtained with either Con A or LPS. However, with peripheral blood lymphocytes, the highest SI was found with LPS stimulated

lymphocytes, thus supporting the findings of Liewes *et al.* (1982).

The stimulation index (SI) obtained with control lymphocytes from both anterior kidney and peripheral blood in the present study is very low compared to those reported in carp by other workers (Liewes *et al.*, 1982; Grondel *et al.*, 1985; Ghanmi *et al.*, 1989). The relatively high uptake of 3H-thymidine by unstimulated cells observed here has accounted for the low SI. It is beyond the scope of this study to determine the reasons for this high back-ground labelling. The only possible explanation is the relatively high proportion of lymphocytes in individual culture wells in this study, as lymphocytes were separated from both peripheral blood and anterior kidney preparations. Some of the earlier studies (Ghanmi *et al.*, 1989) used unseparated pronephric cell suspensions and adjusted it to contain 10^5 lymphocytes per cultutre well (200 µl). In this case the proportion of erythrocytes and other leucocytes in such culture wells could be higher.

In vivo exposure of carp to cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) had a significant effect in lowering the ability of lymphocytes to proliferate as shown by decreased uptake of 3H-thymidine by both Con A and LPS stimulated lymphocytes. The inhibitory effects could be seen after only three days exposure to the respective metals. Such an inhibitory effect was significantly related to exposure duration. The maximum inhibitory effects of cadmium and copper were seen in lymphocytes collected after 9 and 12 days of exposure to the respective metals. The presence of proliferative activity, albeit very low, as shown by the SI in the lymphocytes collected after 9 or 12 days exposure to cadmium and copper still indicated that the lymphocytes were functionally capable of activation and proliferation, in response to mitogenic stimuli. Such a significant reduction in the magnitude of the blastogenesis observed could lead to lower circulating antibodies.

This is contrary to the findings of Thuvander (1989) who found splenocytes obtained from rainbow trout exposed to very low levels of cadmium (3.6 μgl^{-1}) for 5 and 9 weeks, to have the same level of proliferation as controls (unexposed) in response to Con A and LPS. Results of this nature might indicate the ability of fish to acclimate to low levels of metals. However, in the same study, the mitogenic response of splenocytes to *V. anguillarum* antigen, used as a mitogen, was found to be significantly suppressed in cadmium exposed rainbow trout and was suggested to be due to cadmium-induced inhibition of the cellular immune response but, on the contrary, the elevated humoral antibody titre to *V. anguillarum* in cadmium exposed fish was attributed to be due to the thymus-independency of *V. anguillarum* antigen.

The suppression of the mitogenic proliferative response and potentiation of humoral antibody response observed in cadmium exposed rainbow trout to a putative T-independent antigen (Tuvander, 1989) clearly highlights the difficulties in attributing the specific effects on selective subpopulations of lymphocytes and/or branches of the immune system to the metals. The results in the present study does not reveal any possible specific effects of cadmium or copper on selective subpopulations of lymphocytes.

Acute stress such as handling and transport was shown to affect the blastogenic response of peripheral blood lymphocytes of channel catfish, *I. punctatus*, (Ellsaesser and Clem, 1986). Lymphocytes collected 18 hours after the induction of stress could no longer respond to LPS and Con A. Interestingly, they found mitogen responses to return to normal levels within three to four weeks. In addition, they observed the number of B-lymphocytes to decrease after stress with those remaining in circulation exhibiting much lower levels of surface immunoglobulin (sIg). These findings show the serious nature of stress effects on the vital components of the immune system.

In the present study, the lymphocytes collected from carp exposed to cadmium and copper were capable of responding to both B and T-cell mitogens, even though the magnitude of the response was reduced. Physiological levels of cortisol (20 to 200 ng ml^{-1}) are known to suppress lymphocyte proliferation in fish (Grimm, 1985) and this would suggest that higher cortisol levels occurring as a result of stress would function similarly *in vivo*.

The viability of lymphocytes collected after different exposure durations to cadmium and copper was always above 90%. The background incorporation of 3H-thymidine by lymphocytes collected from fish exposed to cadmium and copper was not very different from the control lymphocytes at different sampling points. It is, therefore, possible that the lymphocytes collected after exposure to cadmium and copper (days 6, 9, and 12) were in some way functionally impaired such that the putative T and B-cells were no longer capable of responding to the mitogens to the same degree that was seen in lymphocytes collected from unexposed controls.

The relatively higher proliferation rate observed with lymphocytes collected following 15 days exposure to either cadmium or copper, could possibly suggest initial stages of a recovery process. Long-term experiments would clarify whether such a recovery process operates. The possible evidence for the recovery and acclimatization with regard to lymphocyte proliferative response comes from the studies of Ellsaesser and Clem (1986) and Thuvander (1989). Fishes from polluted waters having higher antibody titres to a wide variety of pathogens could be due to such an acclimation process (Robohm and Sparrow, 1981).

Cadmium and copper exposure *in vivo* reduced the ability of lymphocytes to respond to T and B-cell mitogens *in vitro*. Taking cues from mammalian work, it could be

suggested that these metals, at low levels, appear to reduce the magnitude of cellular and humoral immune response without abrogating it totally by inhibiting the proliferation of lymphocytes.

The *in vitro* effects of cadmium and copper, revealed dose-dependent suppression of mitogenic response of pronephric lymphocytes. Cadmium at concentrations as low as 10^{-4} M, could reduce the amount of 3H-thymidine uptake significantly. The results also showed copper to be more inhibitory and concentrations of 10^{-7} M and greater produced a significant drop in the uptake 3H-thymidine. There was no indication of either metal affecting one population of lymphocytes specifically. The dose-dependent inhibitory effects seen were similar in both Con A and LPS stimulated pronephric lymphocytes. A significant drop in the uptake of the label by the unstimulated lymphocytes exposed to cadmium and copper (10^{-4} to 10^{-3} M) could indicate direct toxicity of metals at high concentrations *in vitro*.

The *in vitro* effects of other metals on lymphocyte proliferation has often produced contradictory results. Zinc was suppressive but not mitogenic to carp lymphoid cells *in vitro* at very low concentrations (10^{-7} M) and was toxic at concentrations of 10^{-3} and greater (Cenini and Turner, 1982). In contrast, it was found to stimulate 3H-thymidine incorporation by both T and B-lymphocytes of carp at concentrations ranging from 10^{-7} to 10^{-3} M, with the exception of 10^{-5} M, where it was found to be toxic (Ghanmi *et al.*, 1989). Zinc acting as a T and B-cell mitogen, especially at 10^{-3} M, but being toxic at a lower level of 10^{-5} M, is a difficult result to interpret. With Manganese, Ghanmi *et al.* (1989) found concentrations from 10^{-7} to 10^{-3} M to enhance proliferation with Con A and PHA, but not with LPS, but 10^{-1} M had an inhibitory effect on the response to Con A and PHA, while the response to LPS was unaffected.

The often contradictory results seen with *in vitro* studies which involve addition of the metal to the cell culture, highlights the problems associated with such studies. The lymphocyte responsiveness may be influenced by a number of factors, out of which the most important seems to be the cytotoxic properties of the metals at certain concentrations thereby giving decreased cell viability and reduced proliferation. Direct addition of metals to cell cultures may also cause physical problems, which could influence the result. Metals added at high concentrations may precipitate and become unavailable,thus unable to inhibit the proliferation, such an event would produce results which show that higher concentrations having an enhancing effect while lower concentrations would be toxic.

The need for correlating *in vitro* results with *in vivo* responses is strongly felt by several workers especially in mammalian immunotoxicology (Koller, 1980). In the present study, in as far as the mitogenic response was concerned, there was a very good correlation between the effects seen in *in vivo* and *in vitro* studies. *In vivo* exposure concentrations used for cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) in the present study corresponds to *in vitro* concentrations of between 10^{-7} to 10^{-6} M. In *in vitro* studies, significant inhibitory effects on lymphocyte proliferation was seen at concentrations of 10^{-6} M and greater, which was also the case with lymphocytes collected from carp exposed to cadmium and copper for 6 days and more.

The uptake of 3H-thymidine by unstimulated lymphocytes decreased with increasing *in vitro* metal concentrations, indicating cytotoxicity at concentrations of 10^{-4} M and greater. On the other hand, the viability of lymphocytes collected from fish exposed to cadmium and copper *in vivo* was always more than 90% and, the unstimulated lymphocytes incorporated 3H-thymidine to levels similar to those seen in unexposed controls at several of the sampling points. The viability of lymphocytes collected

from *in vivo* experiments and those exposed to low *in vitro* concentrations (10^{-9} to 10^{-4} M) were similar to that seen in unexposed controls, The lymphocytes exposed to the metals both *in vivo* and *in vitro* (10^{-9} to 10^{-6} M) were viable as determined by the Trypan Blue exclusion method but in some way were impaired such that their magnitude of proliferation was significantly reduced.

The mechanisms by which heavy metals interfere with DNA synthesis of lymphocytes is still unclear. Chemicals with the ability to interfere with calcium transport are suggested to inhibit lymphocyte proliferation. Several reports have indicated rapid uptake of calcium after stimulation with T-lymphocyte mitogens but not with B lymphocyte mitogens (Hart, 1978; Abboud *et al.*, 1985). Increasing the calcium concentrations in the culture medium was found to partially or completely reverse the inhibitory effects of manganese on carp lymphoid cells (Ghanmi *et al.*, 1989). It is well known that cadmium can interfere with processess requiring calcium by competitive inhibition. The chelating properties of tetracyclines for divalent cations such as calcium has been suggested to interfere with the mitogenic response of leucocytes in mammals (Diamantstein and Odenwald, 1975) and fish (Grondel *et al.*, 1985).

From *in vitro* studies in mammals it appears that cadmium tends to inhibit mitogen capabilities of T cells and potentiate the transformation of B-lymphocytes (Koller *et al.*, 1979; Gaworski and Sharma, 1978). Use of T and B cell mitogens, in the present study failed to reveal any difference in the responses of the two types of lymphocytes after *in vivo* or *in vitro* exposure to cadmium and copper. The inhibitory effects of metals seen was uniformly the same on lymphocytes stimulated by the two different mitogens.

It is, therefore, not possible from the results of the present study, to suggest that there is a differential sensitivity of subpopulations of lymphocytes (either T or B) to cadmium and copper. The present study, however, has demonstrated that exposure of lymphocytes to low levels of cadmium and copper *in vivo* and *in vitro* decreases the magnitude of their blastogenic response, significantly.

CHAPTER 7

THE EFFECTS OF CADMIUM AND COPPER ON PLASMA CORTISOL, LEUCOCRIT, HAEMATOCRIT, TOTAL ERYTHROCYTE AND LEUCOCYTE COUNT AND THE PATHOLOGY OF CARP, C. carpio.

7.1 INTRODUCTION

Environmental contaminants including heavy metals have been shown to induce a typical stress response in fish, resulting in elevation of plasma cortisol (Donaldson and Dye, 1975; Schreck *et al.*, 1978; Donaldson, 1981). It is widely believed that this response represents a part of a more general adaptive response and is related to the catabolic action of the steroid as the fish increases its energy demands, in an attempt to maintain or return to homeostasis (Schreck, 1981; Pickering and Pottinger, 1987a).

Both acute and chronic stress induce elevation of plasma cortisol levels in fish. Under acute stress the nature of the cortisol response appears to be transitory and it comes down to basal levels within 8 to 24 hours of withdrawal of stress (Pickering and Pottinger, 1987a). On the other hand, chronic stress produces prolonged elevation of plasma cortisol and it has been shown that it takes from days to several weeks for the plasma cortisol to return to basal levels (Pickering and Stewart, 1984).

Excessive or prolonged elevation of plasma cortisol has deleterious effects on the defence system of the fish (Stave and Roberson, 1985; Grimm, 1985; Ellsaesser and Clem, 1986) with a resultant increase in the susceptibility of fish to disease (Robertson *et al.*, 1969; Pickering and Dauston, 1983; Peters and Schwarzer, 1985; Pickering and Pottinger, 1985; Wechsler *et al.*, 1986; Houghton and Matthews, 1986; Woo *et al.*, 1987; Maule *et al.*, 1987; Peters *et al.*, 1988). Several of these studies demonstrating the effects of corticosteroids on disease resistance and immune response were, however, based on exogenous corticosteroid implantation.

Experimentation on the cortisol response over time, in fish stressed with environmental contaminants, have been limited to short time intervals (Donaldson and Dye, 1975; Schreck and Lorz, 1978). The question of the nature of the cortisol response in a fish which recovers from a brief encounter with an acute stress, or which acclimates to a continuous chronic pollutant stress, has not been resolved. Many environmental stresses are of a chronic nature and therefore studies examining the cortisol dynamics over a long term would enable further elucidation of the different phases of the general adaptation syndrome described by Selye (1950) and correlate it with disease resistance mechanisms.

The stress response of fish is also known to normally include a moderate to severe, delayed leucopenia; classified as a secondary effect of stress (Mazeaud *et al.*, 1977) occurring as a result of increased pituitary-interrenal activity (Peters *et al.*, 1980; 1988; Wedemeyer and McLeay, 1981; Wedemeyer *et al.*, 1983; Pickering, 1984; Ellsaesser and Clem, 1986). The leucocrit determination method (McLeay and Gordon, 1977) has been recommended as a screening test to provide information on the physiological effects of environmental stress on fish health (Wedemeyer and McLeay, 1981). The sensitivity of leucocrit to environmental stress and its relative insensitivity to blood sampling procedures supports its use as an indicator of the physiological consequences of environmental stress (Wedemeyer *et al.*, 1983).

As with cortisol there is very little information on the leucocyte response, over short and long term, to concentrations of environmental contaminants which have been shown to decrease disease resistance of fishes.

Several short and long term studies have convincingly shown the link between environmental contaminants, especially heavy metals and disease and/or immune

response in fish (Chapters 3 to 6). In the majority of these studies, much has been attributed to stress induced modulation of the immune response. However, there is very little information to support this such as the cortisol and leucocyte dynamics of the fish, at concentrations which have induced perturbations in the fish's defence system.

In the present series of short and long-term exposure experiments, both cadmium and copper at relatively low levels have been shown to produce alterations in the susceptibility to disease agents and the immune response of carp. In certain experiments with specific responses, there were indications of a recovery and/or acclimation process taking place in carp exposed to cadmium which would support the hypothesis of the involvement of the stress factor. In view of these findings, and because very little is known about cortisol dynamics of carp exposed to metals over a longer duration, this study was initiated.

The concentrations of metals used were same as those used in previous experiments (Chapters 3, 4, 5 and 6), where perturbations in the disease resistance mechanisms of carp were demonstrated. Exposure durations of 1 to 30 days were selected for this experiment so as to cover most, if not all, of the experimental regimes used in previous experiments (Chapters 3 to 6). Cortisol assessment was not undertaken in previous experiments to preclude the possible influence of other experimental factors, since in all the experiments there involved at some stage either immunization and/or challenge infections.

The primary objective of this experiment was to delineate the kinetics of plasma cortisol and leucocyte response to low levels of cadmium and copper stress. Additional parameters such as haematocrit, erythrocyte count and associated

pathology were also looked into, in an attempt to explain some of the observed effects of cadmium and copper in the previous chapters.

7.2 MATERIALS AND METHODS

This experiment was designed basically to understand the direct effects of the metals alone, on such aspects as; plasma cortisol, leucocrit, WBC counts, haematocrit, erythrocyte number and histopathology.

7.2.1 Experimental Design

A single experiment was designed to delineate all the aforesaid aspects. Three groups of 24 fish in duplicate were used in this experiment. The fish were acclimated in the flow-through system for a week before being used for the experiment. Two groups of carp (11.54 ± 1.76 cm; 19.35 ± 2.03 g; n=72) were exposed to cadmium (50 μgl^{-1}) or copper (30 μgl^{-1}) and 1 group served as the control with no metal. The duration of exposure ranged from 24 hrs to the maximum of 30 days. During the experiment the fish were fed a maintenance diet at a rate of once in 2 days.

7.2.2 Sampling Protocol

An initial sampling was made before the actual commencemnt of metal dosing into the system by randomly taking 2 fish per treatment. On days 1 (24 hrs), 2, 4, 6, 9, 15 and 30 following the metal exposure, 3 fish per treatment were sacrificed to procure samples for the different analyses. The sampling procedure followed is described below.

7.2.3 Sampling Procedure

Fish were removed from the experimental system very carefully, causing minimal disturbance, and anaesthetized using 100 ppm Benzocaine (Methyl para-amino benzoate). Blood was withdrawn from the caudal vein using heparinized 1 ml

syringes fitted with 25 gauge needle. Usually 0.7 to 0.9 ml blood was withdrawn from each fish. The blood was quickly transferred to heparinized 1.5 ml Ependorf vials. From this, samples were taken for haematocrit and leucocrit by using heparinized microcapillary tubes and their ends sealed with Critoseal. Samples were also taken for total erythrocyte and leucocyte counts using the RBC diluting tube of the Haemocytometer. The remaining blood was centrifuged at 400 g for 5 minutes and the plasma separated. Aliquots of plasma (200 µl) were transferred to Ependorf vials and stored at -70°C till until used for the plasma cortisol assay.

The fish were dissected immediately after blood withdrawal and samples from gills, spleen and kidney were taken for histopathology. Samples were fixed in 10% buffered formalin and processed and stained according to the procedure given in the Appendices 4, 5 and 6.

7.2.4 Radioimmunoassay for Plasma Cortisol

Assays for determination of plasma cortisol were carried out according to the modified method of Pickering *et al.* (1987). The materials used for the radioimmunoassay are given in the Appendix 7.

7.2.4.1 Procedure

(a). 200 µl plasma samples were pipetted into microcentrifuge tubes (1.5 ml) and 1 ml ethyl acetate added and tightly stoppered. Using a vortex mixer the samples were well mixed and then centrifuged for 5 minutes at 2500 rpm.

(b). Samples were removed from the centrifuge and a suitable volume of the supernatant was taken into marked assay tubes. Normally 200 µl aliquots of the extracted sample was taken in duplicate.

(c). A series of standards in duplicate were prepared in assay tubes as follows:

Standard	Ethyl acetate	0.5 ng ml^{-1} std.	4.0 ng ml^{-1} std.
0	200 µl	-	-
50	100 µl	100 µl	-
100	-	200 µl	-
200	150 µl	-	50 µl
400	100 µl	-	100 µl
800	-	-	200 µl

To avoid contamination, ethyl acetate was pipetted first, followed by 0.5 standard and then 4.0 standard.

(d). Sample extracts and standards were dried in a vacuum oven at less than 35°C.

(e). In the Radioactive lab, 50 µl of ^{3}H-cortisol was pipetted into a scintillation vial and 5ml scintillation fluid was added, mixed well, and the vial cap labelled as "Total count".

(f). Dried assay tubes were removed from the vacuum oven and allowed to cool to room temperature. To each tube was added 100 µl BSA-saline and 50 µl ^{3}H-cortisol which were vortex mixed.

(g). To each tube 100 µl of antibody solution was added, and again mixed well, using a vortex mixer. The assay tubes were covered and incubated in a refridgerator at 4°C for 4 hrs minimum, or overnight.

(h). After the incubation the tubes were removed and kept on crushed ice.

(i). Dextran-coated charcoal was stirred well and then 100 µl was quickly added to each assay tube and mixed well.

(j). The tubes were incubated on ice for 5 mins to allow the coated charcoal to absorb the unbound cortisol. Immediately following, the tubes were spun in a refrigerated centrifuge for 5 mins at 1200 g.

(k). 200 µl aliquots of the supernatant were transferred to marked scintillation vials.

To each vial, 5 ml of scintillation fluid was dispensed, the vials were tightly stoppered and mixed well to dissolve all the precipitate.

(l). To obtain the level of background count, 5 ml of scintillant was taken in a separate scintillation vial.

(m). Each vial (sample, standard, total and background) was counted for 10 mins in a liquid scintillation counter (Packard Tri-Carb 2000 CA). The results were recorded as dpm over a 10 min cycle per sample.

(n). The amount of cortisol present in the samples was calculated according to the method outlined below:

7.2.4.2 Calculations

1. Total counts in 50 µl ^{3}H-cortisol = T

   Standards counts = s

   Unknown counts = u

2. Since counting was performed on only 200 µl aliquots of the available 350 µl after separation;

   Corrected counts for standards = s x 350/200 = S

   Corrected counts for unknowns = u x 350/200 = U

3. % binding calculation; for standards= S/T x 100

   for unknowns= U/T x 100

4. Standard curve = % binding for each standard was plotted against the concentration in each standard (0-800 pg) in order to obtain a standard curve.

5. The concentration of cortisol in each of the unknown samples (cu) was read off the standard curve using the % binding calculated for the unknowns.

6. Cortisol concentration per ml plasma was calculated and the results presented as $ngml^{-1}$.

7.2.4.3 Verification of Assay Characteristics.

1. At regular intervals background counts were made on the entire reaction mixture less the radioactive label, to check for the contamination of the reagents.

2. Recovery checks were also made to measure the % of cortisol extracted from the plasma as described below; A range of known quantities of cortisol (0-10 ng) in ethyl acetate were dried down and 200 µl aliquots of pooled plasma added to the tubes. These were mixed well and assayed as usual. % recovery was determined by regression of measured against added cortisol. Percentage recovery was found to be between 75 and 80.

3. Variation within and between assays was assessed by regular use of pool plasma for quality control during each assay. These were always found to be less than 10%.

7.2.5 Haematocrit and Leucocrit

The methods of Snieszko (1960) for haematocrit and that of McLeay and Gordon (1977) for leucocrit was followed in the present experiment. Briefly, blood was withdrawn into the heparinized microcapillary tubes immediately after sampling and one end of the tube sealed with Critoseal. The tubes were centrifuged in a Micro-Haematocrit centrifuge (Hawksely, England) at 11500 rpm for 5 mins. Haematocrit was read from the haematocrit reader as % of Packed Red Cell Volume in relation to the total blood volume. Leucocrit was determined by measuring the height of the buffy layer (grayish-white layer separating the erythrocytes from the plasma) to the nearest 0.02 mm using a compound microscope (40 x magnification) and an occular micrometer. Precaution was taken to rotate the tube during measurement enabling the average height of the buffy layer to be approximated. The leucocrit was calculated as height of buffy layer - height of total blood x 100 and reported as % leucocrit.

7.2.5 Total Erythrocyte and Leucocyte Counts

The total erythrocyte and leucocyte counts were made with the aid of improved Neubaur ruling haemocytometer using Shaw's (1930) solution as the diluting fluid. For RBC, the cells in 4 corner and 1 central small square of the large central square were counted and their number multiplied by 10^6 and recorded as number of cells per cubic milliliter of blood. In the case of WBC, the cells in 4 large corner squares were counted and their number multipied by 500 and recorded as number of leucocytes per cubic milliliter of blood.

7.2.7 Histopathology

The samples of gill, kidney and spleen were fixed in 10% neutral buffered formalin for a minimum of 24 hours. The fixed samples were processed in an Histokine automatic tissue processer (Histokinette 2000). The processing involves passing the tissues through a series of alcohol grades, followed by absolute alcohol and chloroform, and then impregnated with molten wax. Thin sections of 3 to 5 µm were cut with Leitz-Wetzlar microtome using Richert-Jung disposable microtome blades. The sections were stained with Haematoxylin-Eosin for routine examination. Other stains such as PAS and Alcian blue were used for demonstration of specific cell changes. Staining procedures, as described in Carlton's histolab techniques (Drury and Wallington, 1980), were followed. The detailed processing and staining procedure involved are given in the Appendix 5 and 6.

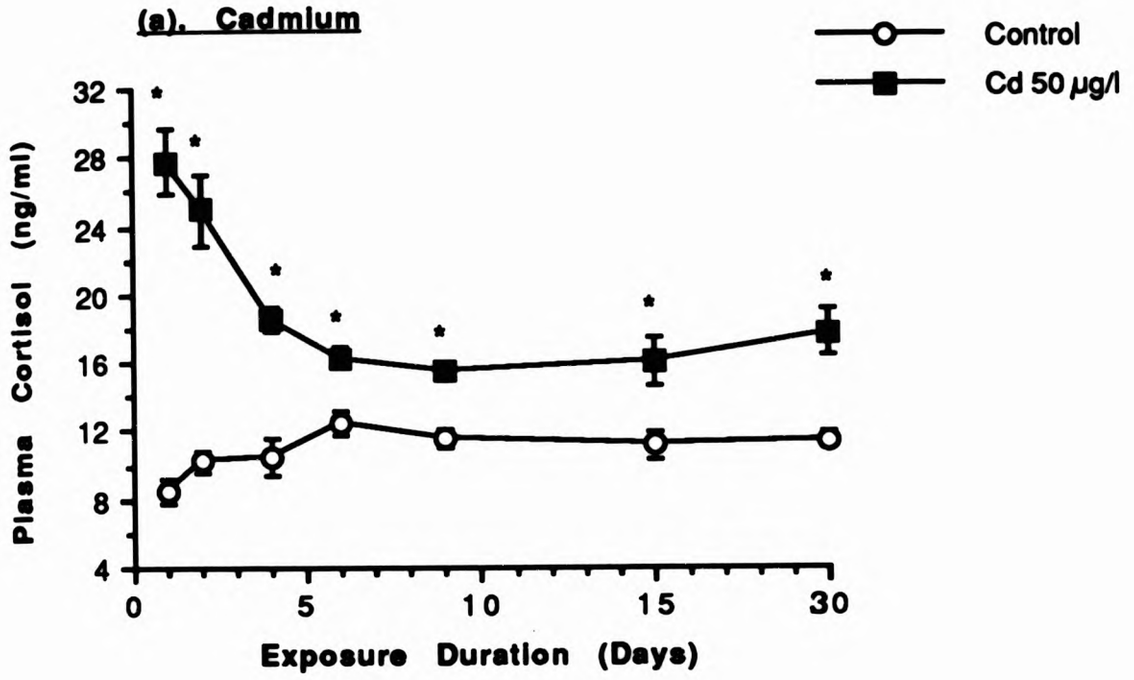
7.3 RESULTS

7.3.1 Plasma Cortisol

Cortisol concentrations were determined on seven representative samples, collected on days 1, 2, 4, 6, 9, 15 and 30 following exposure to either cadmium or copper. Figure 7.1 illustrates, the dynamics of plasma cortisol in fish exposed to cadmium, copper and compares them to unexposed controls. Cortisol concentrations on different sampling days did not differ statistically among control fish nor did they differ as a function of time. The mean cortisol levels in controls ranged from 8.5 ng ml^{-1} (day 1) to 12.47 $ngml^{-1}$ (day 6). But for a slight initial rise between days 1 and 6, cortisol levels remained almost uniform on all the sampling days.

A significant elevation in the plasma cortisol levels was recorded in cadmium exposed fish (Figure 7.1a) at the earliest sample. Exposure duration had a significant effect ($P<0.01$) on the resulting cortisol levels. The levels recorded between days 1 and 4 were significantly higher than for rest of the sampling points with the highest level (27.78 $ngml^{-1}$) recorded on day 1. With exposure duration, the cortisol levels showed a decreasing trend. Between days 6 and 30, the cortisol levels remained almost uniform, without any significant elevation or decline. As can be seen from the Figure 7.1a, the levels recorded in the cadmium exposed groups remained significantly ($P<0.05$) higher than corresponding controls on all the sampling days.

The cortisol dynamics in copper exposed groups showed several interesting changes (Figure 7.1b). The levels recorded on all the days were significantly higher ($P<0.05$) than both cadmium and control groups. Copper exposure duration had a significant effect on the cortisol dyanmics. Cortisol levels recorded showed a declining trend from peak values obtained on day 1 (56.86 $ngml^{-1}$) to a minimum on day 9 (27.31



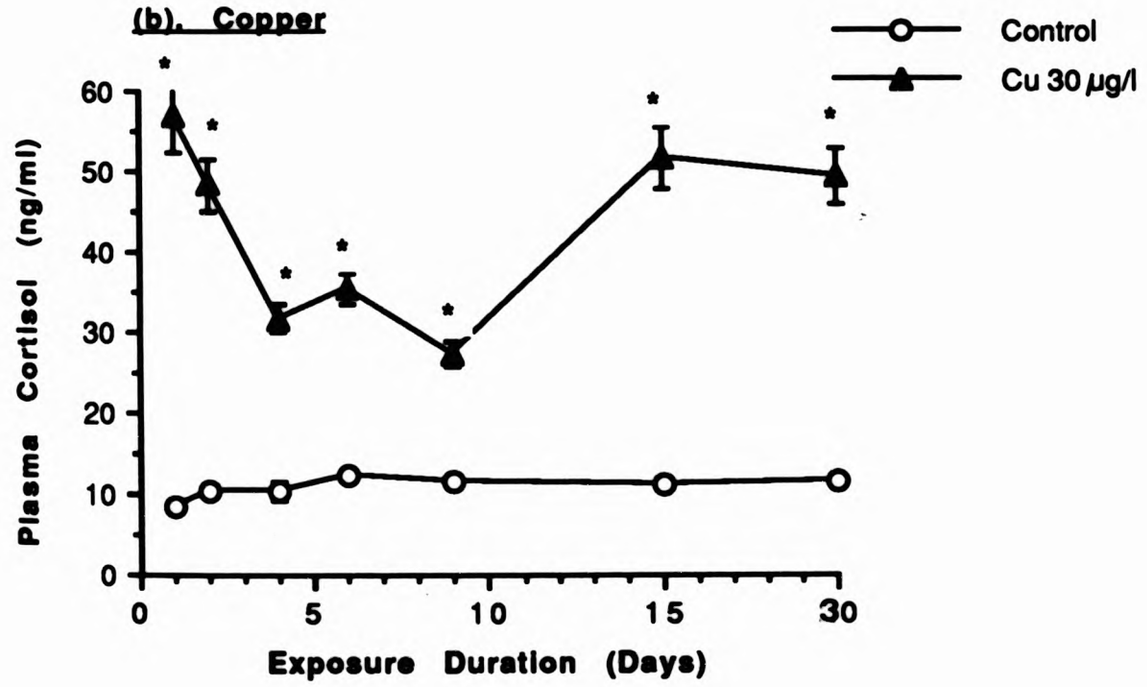


Figure 7.1 The kinetics of plasma cortisol levels ($ngml^{-1}$) in carp exposed to (a) cadmium and (b) copper for a varying duration. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

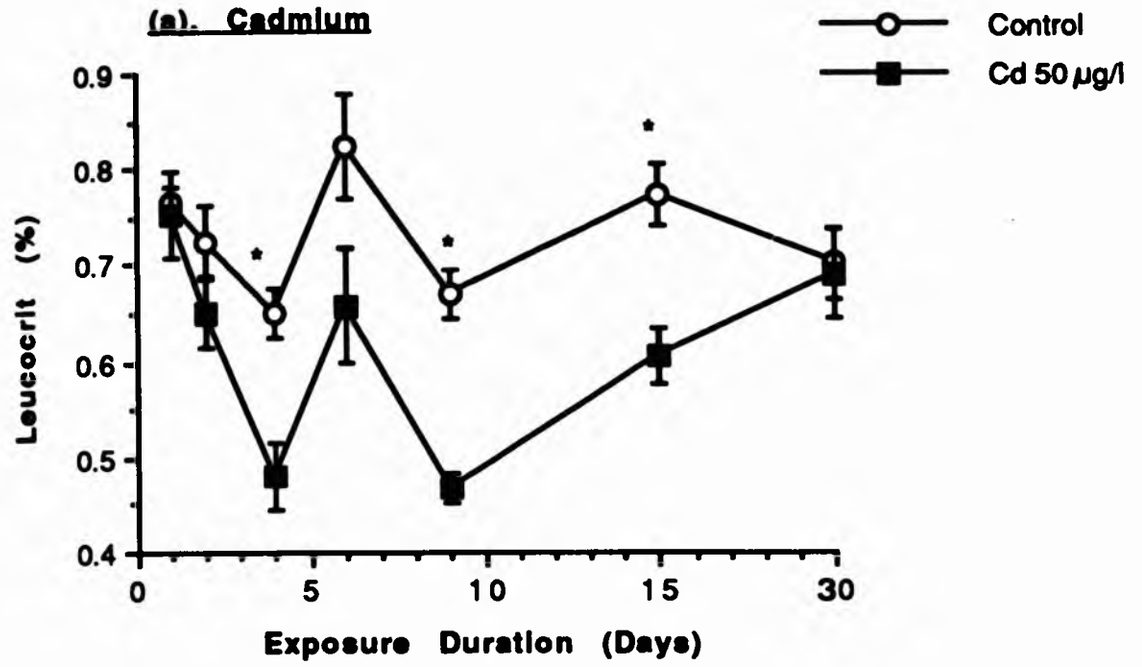
$ngml^{-1}$). However, there was a second elevation in cortisol levels beyond day 9, which was reflected by high cortisol levels on day 15 and 30. Plasma cortisol levels within the copper exposed groups were very high (48.3 to 56.86 $ngml^{-1}$) following short (days 1 and 2) and long term (days 15 and 30) exposure and were significantly higher than that recorded in samples collected between days 2 and 15, where it was at its lowest (27.31 to 35.35 $ngml^{-1}$).

7.3.2 Leucocrit

Leucocrit recorded on different sampling days in three treatments is presented in Figure 7.2. Both treatment and exposure duration had a significant effect on the resulting leucocrit response and even the interaction between treatment and exposure duration had a significant effect. The leucocrit values obtained in the controls ranged from 0.65 to 0.82%. In the cadmium and copper treatments the leucocrits recorded were lower than controls and ranged from 0.47 to 0.75 and 0.47 to 0.66% respectively. In the cadmium exposed groups the leucocrit declined sharply from day one of exposure to day four and later started to increase gradually before reaching values similar to controls on day 30 (Figure 7.2a). In the copper treatment the leucocrit values obtained showed much variation on different sampling days without any clear-cut trend (Figure 7.2b). The values obtained in the controls were significantly higher than the copper treatment on all the sampling days while it was higher than the cadmium treatment on days 4, 6, 9 and 15.

7.3.3. Total Leucocyte Count

The total number of leucocytes enumerated from the three treatments on different sampling days are shown in Figure 7.3. Treatment had a significant effect on leucocyte numbers recorded. However, exposure duration did not have any effect on the resulting leucocyte number on different sampling days. The leucocyte number in



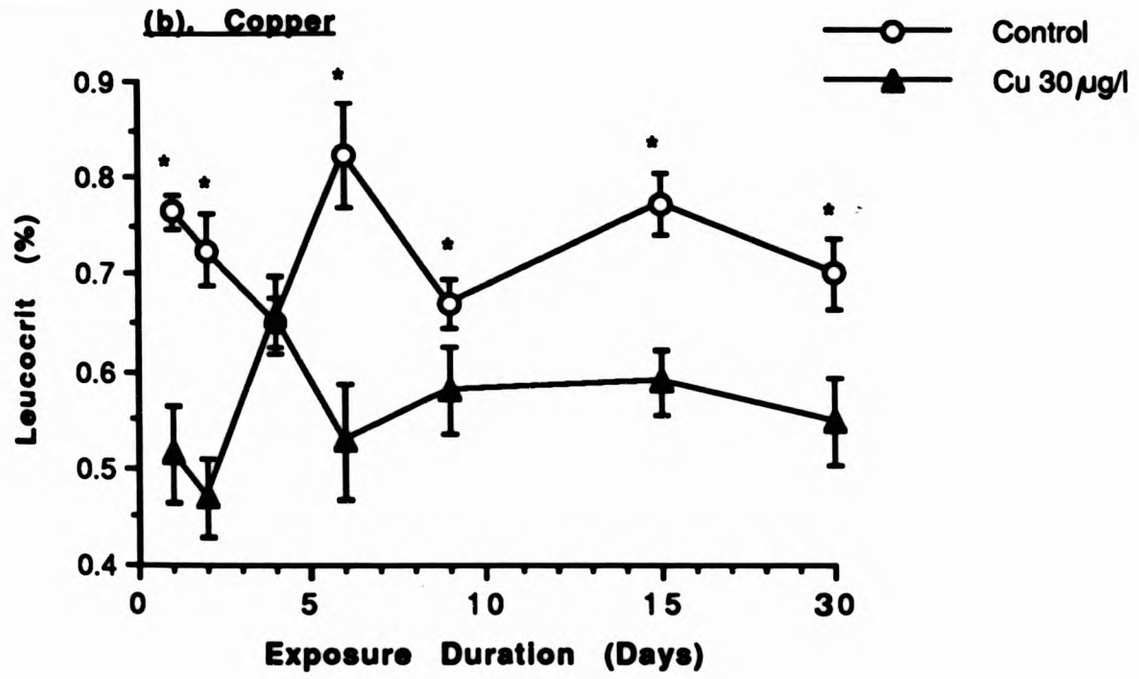


Figure 7.2 The Mean (± S.E; n=6) leucocrit (%) in carp exposed to (a) cadmium and (b) copper for a varying duration. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

the controls was consistent and it ranged from 28 to 35 (10^3) per mm^3. In the cadmium treatment there was more variation (23 to 33x10^3) in the number of total leucocytes recorded but statistically there was no difference (Figure 7.3a). In the copper treatment the leucocyte number recorded was always significantly lower than the controls (Figure 7.3b) with values ranging from 14 to 22 (10^3) per mm^3. Considering the overall response the numbers recorded per mm^3 in cadmium (26x10^3) and copper (18x10^3) were significantly lower than controls (32x10^3).

7.3.4 Haematocrit

The haematocrit recorded after different days of exposure to cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) along with unexposed controls are presented in Figure 7.4. Both the treatment and the exposure duration to the metals had significant ($P<0.05$) effect on the haematocrit and there was signifcant interaction between treatment and exposure duration. The haematocrit obtained on different days in controls was relatively uniform and ranged from 24 to 28%. The haematocrit in cadmium exposed groups was again relatively uniform on all sampling days (24 to 26%) but as can be seen from the Figure 7.4a, it was lower than the controls on most of the sampling days except on day 1 and day 30 after exposure. In the copper exposed group, the haematocrit was always significantly ($P<0.01$) higher than the corresponding control and cadmium groups (Figure 7.4b). It ranged from 25 to 33.8% reaching a maximum on day four and from thereafter started to decline gradually. In relation to the controls, copper significantly increased the haematocrit whilst in cadmium it was decreased.

7.3.5 Total Erythrocyte Count

Figure 7.5 documents the total erythrocyte count recorded in three treatments on different sampling days. Treatment had a significant effect on the erythrocyte

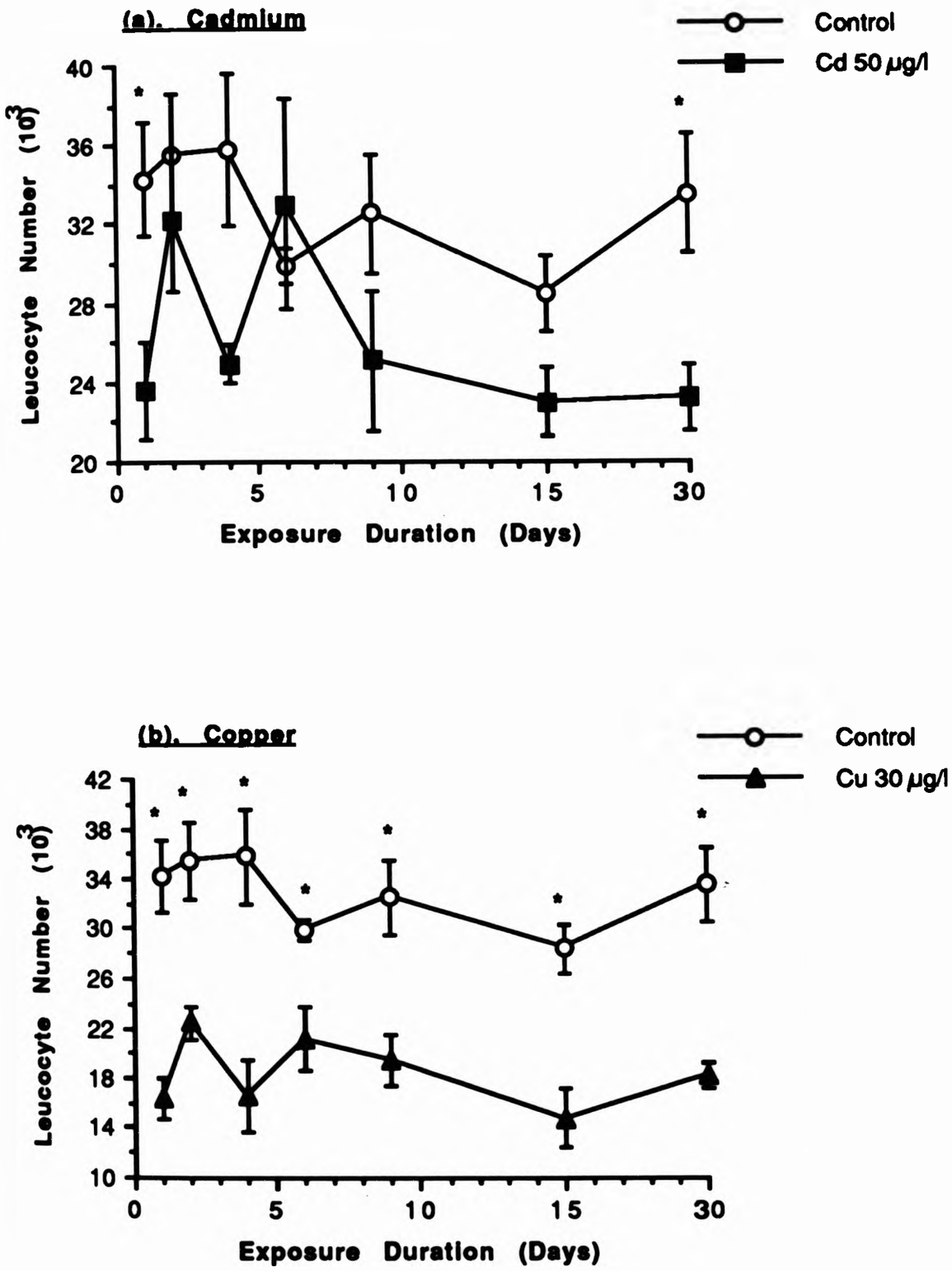


Figure 7.3 The total (Mean ± S.E; n=6) number of leucocytes (10^3) per mm^3 blood in carp exposed to (a) cadmium and (b) copper for a varying duration. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

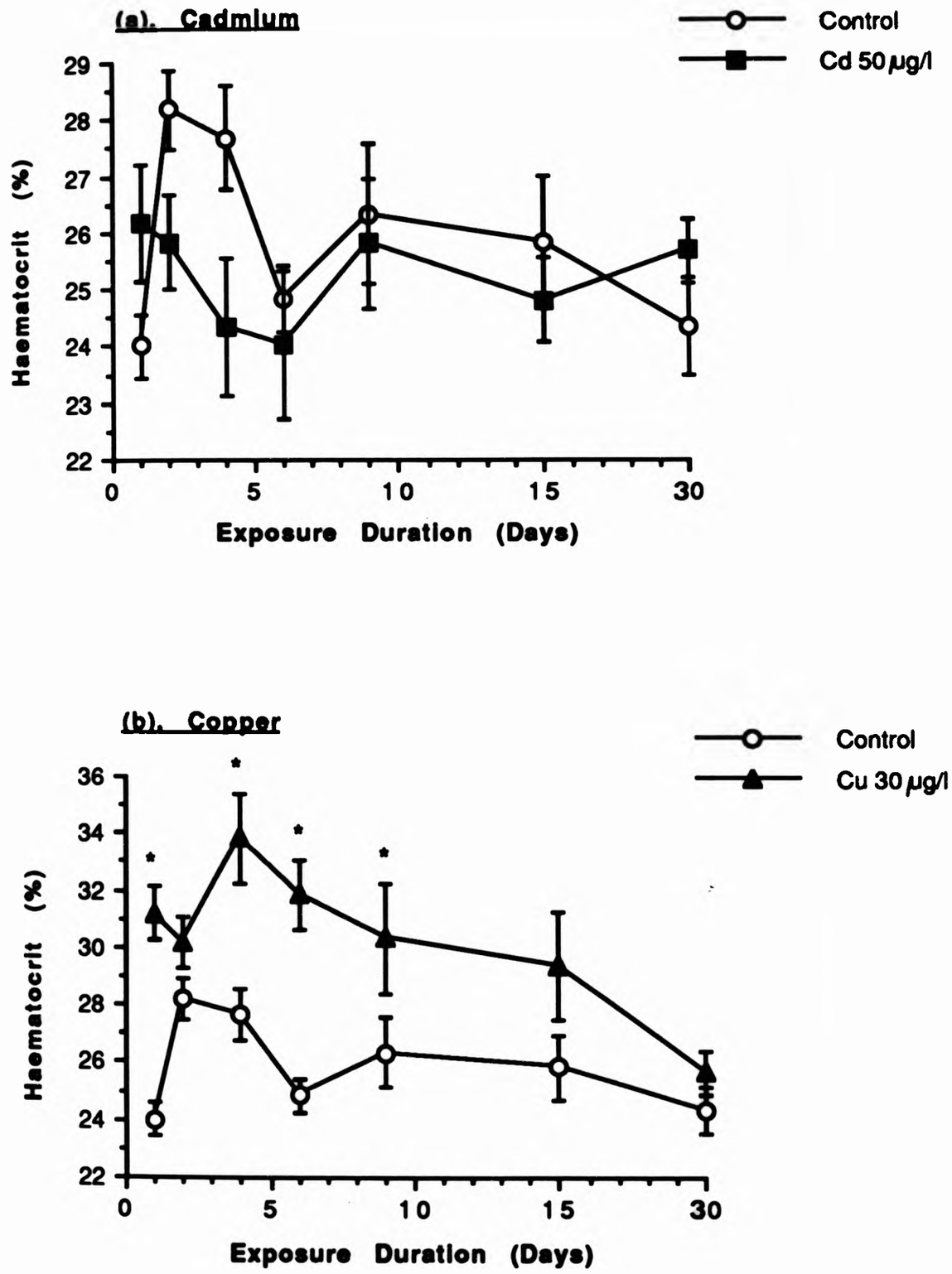


Figure 7.4 The Mean (± S.E; n=6) haematocrit (%) in carp exposed to (a) cadmium and (b) copper for a varying duration. * denotes significant difference between treatments on corresponding sampling days ($P<0.05$).

number while exposure duration did not have any statisticaly significant effect. The erythrocyte count obtained reflected closely the haematocrit record. It is evident from the Figure 7.5b, that in copper exposed groups, the total erythrocyte count was significantly ($P<0.01$) higher than both cadmium and control treatments. It ranged from 1.35 to 1.67 (10^6) per mm^3 of blood in the controls while in cadmium and copper treatments it was 1.30 to 1.64 and 1.52 to 1.84 (10^6) per mm^3 of blood, respectively.

7.3.6 Pathology

The pathological changes observed in the gills, kidney and spleen of carp exposed to cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) for varying duration are presented below. Pathological changes in relation to exposure duration were followed by light microscopy from samples taken sequentially. Of the three organs examined, the gills were the worst affected. The changes seen in kidney and spleen were not severe.

7.3.6.1 Gills

Copper treatment induced changes in gills earlier than did cadmium. Early signs of changes were noticed following 24 hours exposure to copper, whilst in the cadmium groups, it was seen only in samples taken after 48 hours exposure. Under both the metal treatments, exposure duration had a significant effect on the pathology induced. Severe changes were observed in samples taken following 6 to 9 days of exposure to cadmium or copper compared to the control gill (Plate 7.1)

The early changes included hypertrophy and necrosis of secondary lamellar epithelial cells in both cadmium and copper treatments. Severe hyperplasia, especially in the copper treated fish, hypertrophy, vacuolation and necrosis of epithelial cells in the interlamellar region and secondary lamellae were marked and consistent in the gills

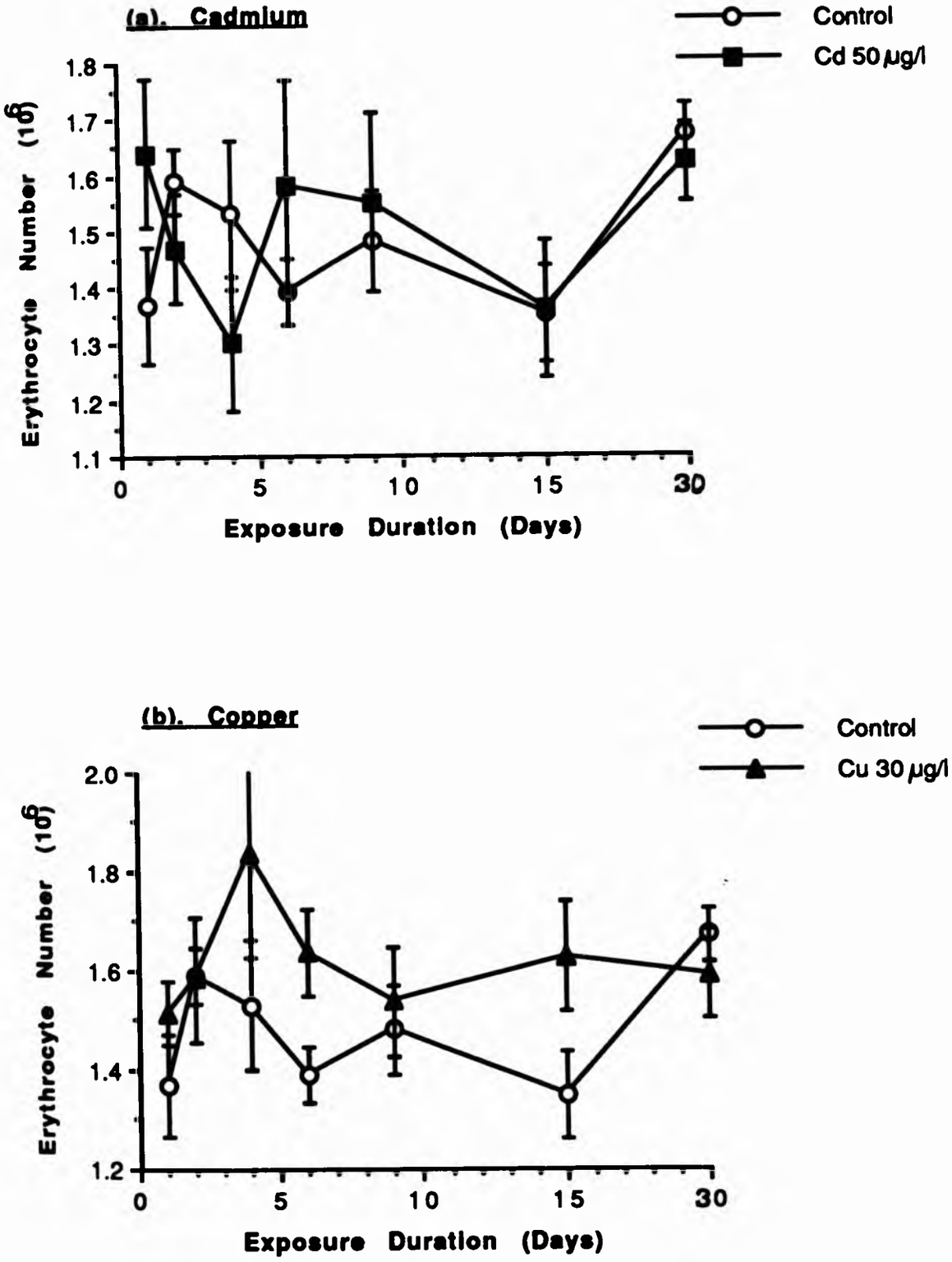


Figure 7.5 The total (Mean ± S.E; n=6) number of erythrocytes (10^6) per mm^3 blood in carp exposed to (a) cadmium and (b) copper for a varying duration. * denotes significant difference between treatments on corresponding sampling points ($P<0.05$).

of carp following 4 to 6 days of exposure to cadmium (Plate 7.2) or copper (Plate 7.3). Hyperplasia in the interlamellar region often resulted in fusion of two to four secondary lamellae and such changes were noticed only in copper exposed gills. Hypertrophy and necrosis of epithelial cells in the secondary lamellae was very common in all the treatments and such degenerating and necrotic cells were often seen to be in the process of sloughing from the epithelium (Plate 7.4). Proliferation of eosinophilic granular cells (EGC's) and their migration into necrotic areas of secondary lamellae were often observed in gills of carp exposed to cadmium or copper for more than 9 days (Plate 7.5).

The severe changes noted also included the lifting and separation of the secondary lamellar epithelium away from the pillar cells, though early signs of this separation were confined to the bases of the secondary lamellae (Plate 7.6a). Such changes became severe with exposure duration and resulted in the lifting of the entire epithelial layer of secondary lamellae, thus creating large non-tissue or lymphatic spaces (Plate 7.6b) which appeared to be invaded by leucocytes and EGC's. Gills from fish exposed to the cadmium for 30 days appeared to have relatively few cellular changes (Plate 7.7a) probably because of the severe sloughing which took place earlier (Plate 7.7b).

7.3.6.2 Kidney

The changes observed in the kidneys of carp were mild to moderate and the pathology seen was closely related to the exposure duration. Significant changes were noticed only in samples collected after 9 to 15 days exposure to the metals. Early signs of changes included indications of necrosis (pyknosis) and degeneration of cells of the kidney tubules (Plate 7.8). Structural damage to the renal glomeruli was evident in samples collected following 15 days exposure to either cadmium or

Plate 7.1 Photomicrograph showing the gill from a control fish. Note the secondary lamellar epithelium is very thin and flat (arrow head). (H&E, 600X)

Plate 7.2 Photomicrograph showing the pathological changes in the gill of cadmium (50 μgl^{-1}) exposed carp. Note hypertrophy and necrosis (arrows) of secondary lamellar epithelial cells, vacuolation and necrosis of chloride cells (arrow head).

(a) After 6 days of exposure. (H&E, 600X)

(b) After 9 days of exposure. (H&E, 1500X)

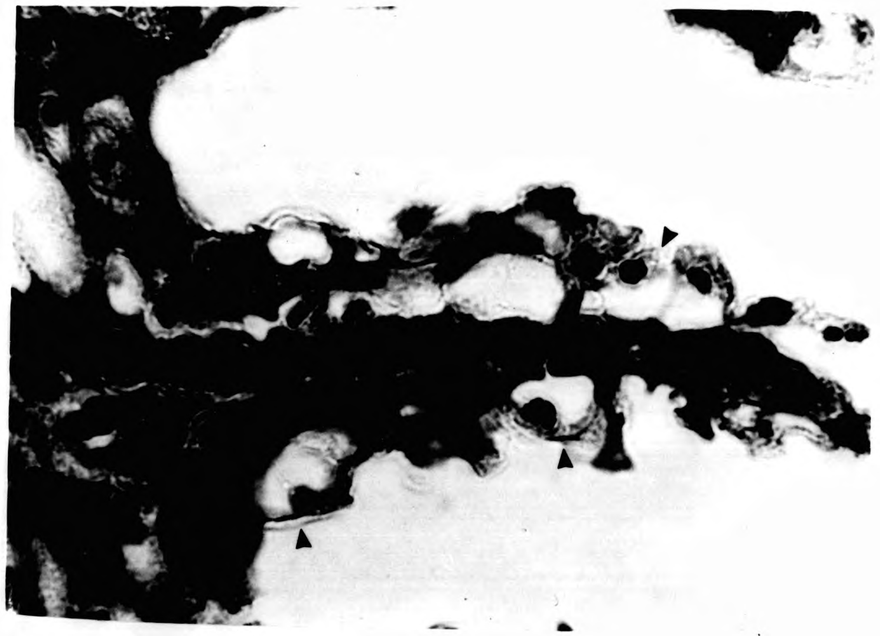
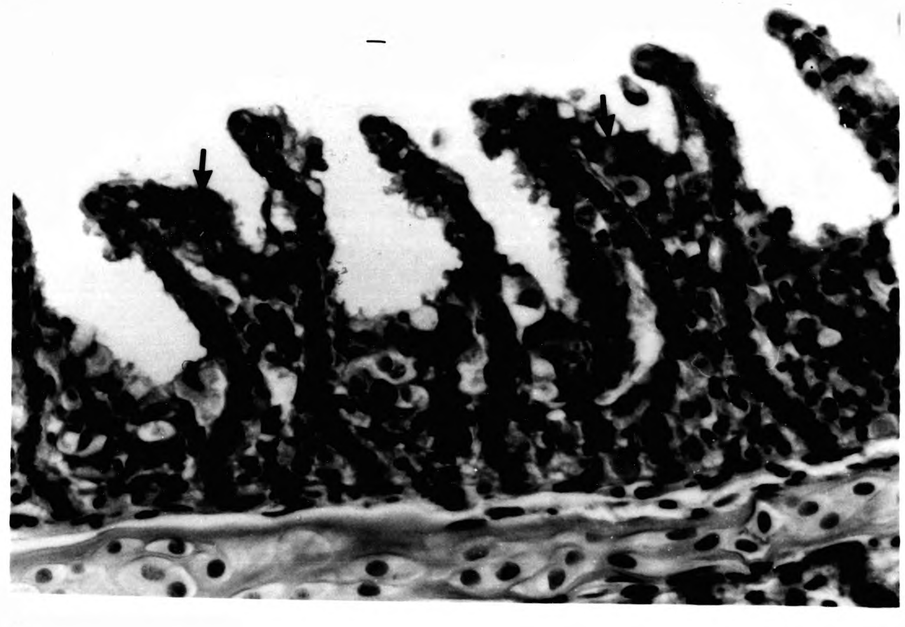


Plate 7.3 Photomicrograph showing the pathological changes in the gill of copper (30 μgl^{-1}) exposed carp. Note severe interlamellar hyperplasia (arrow) and necrosis of secondary lamellar epithelial cells (arrow head).

(a) After 6 days of exposure. (H&E, 600X)

(b) After 9 days of exposure. (H&E, 1500X)

a



b

Plate 7.4 Photomicrograph showing the sloughing (arrow head) of the secondary lamellar epithelial cells following 9 days of metal exposure.

(a). After 9 days of exposure to 50 μgl^{-1} cadmium (H&E, 600X)

(b). After 9 days of exposure to 30 μgl^{-1} copper (H&E, 1500X)

a

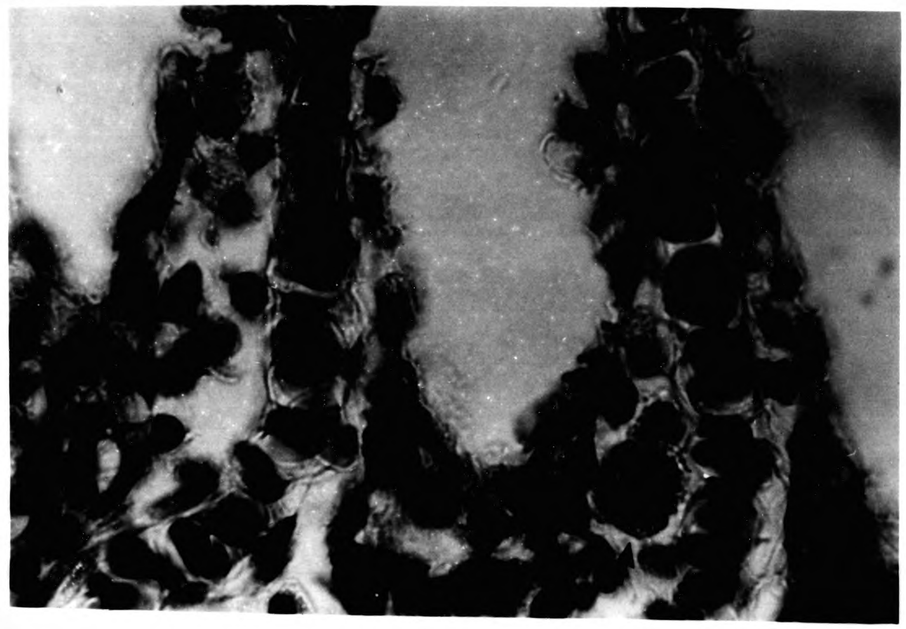
b

Plate 7.5 Photomicrograph showing the initial lifting of epithelial layers (arrow head) and the infiltration of EGC's to such spaces (arrows) in metal exposed gills.

(a) After 15 days of exposure to 50 μgl^{-1} cadmium (H&E, 1500X)

(b) After 9 days of exposure to 30 μgl^{-1} copper (H&E, 1500X)

a



b

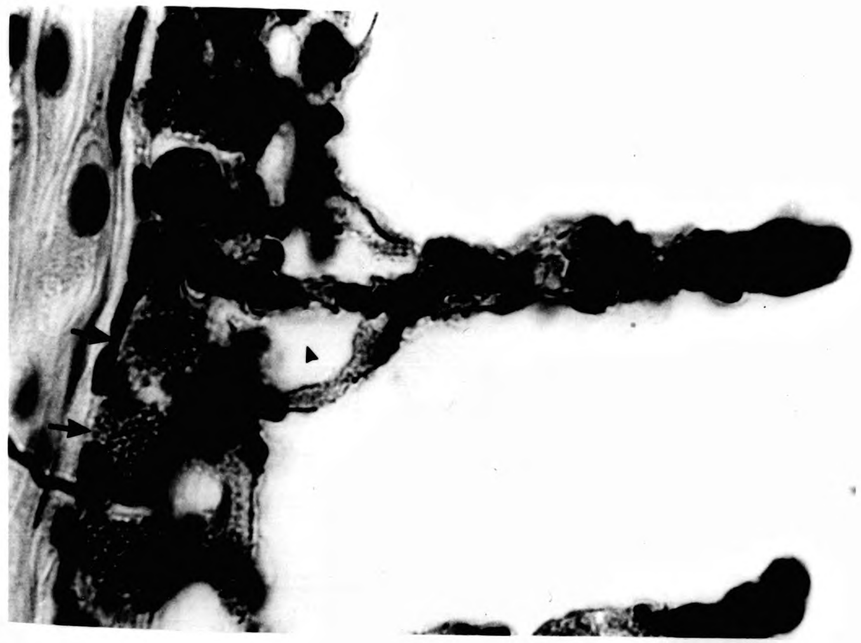
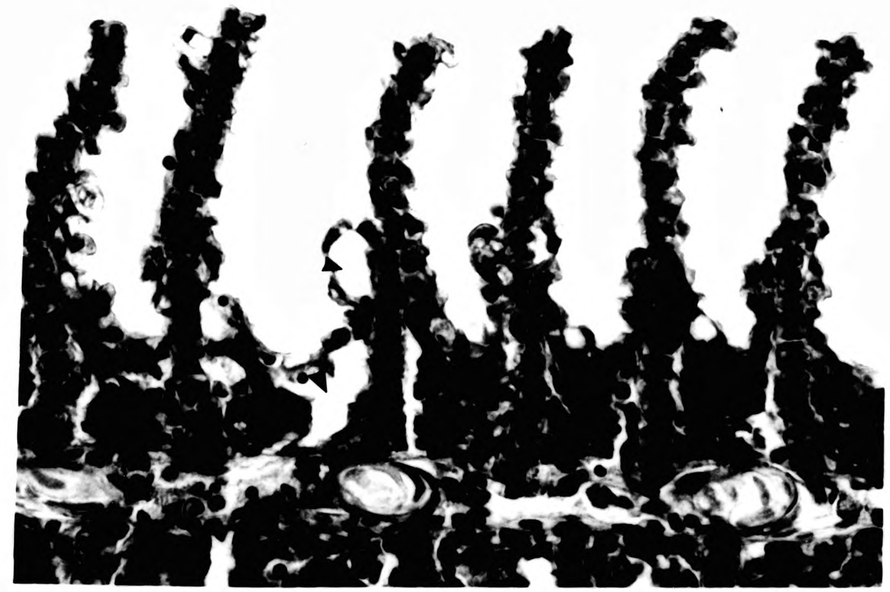


Plate 7.6 Photomicrograph showing mild to severe lifting (arrow head) of secondary epithelial layer in the metal exposed carp.

(a) After 9 days exposure to 50 μgl^{-1} cadmium (H&E, 600X)

(b) After 15 days of exposure to 30 μgl^{-1} copper (H&E, 1500X)

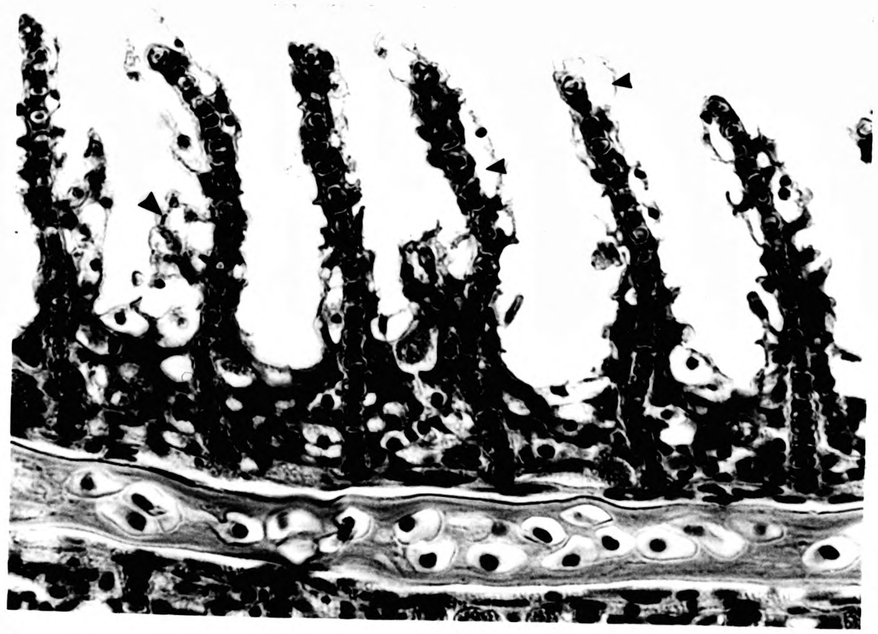
a



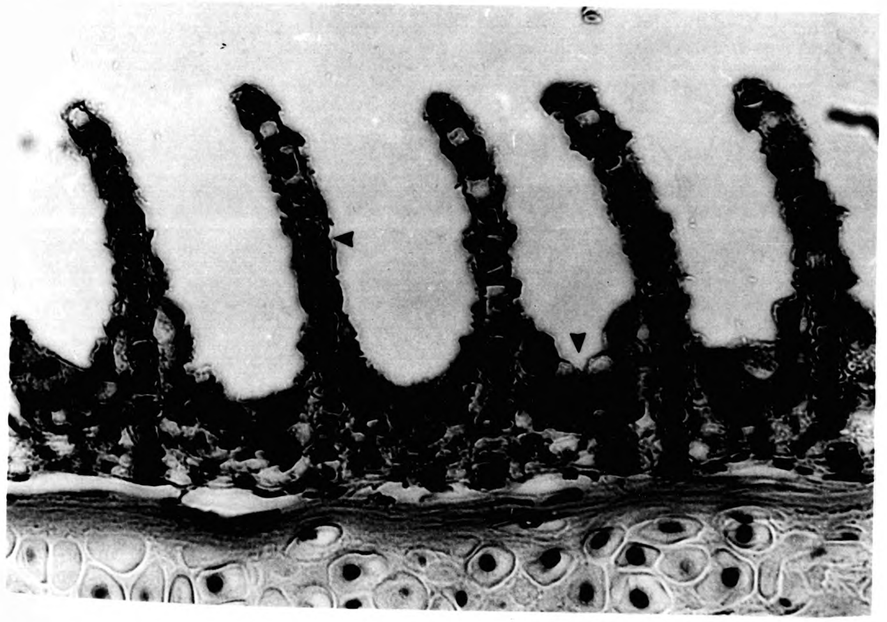
b

Plate 7.7 Photomicrograph showing (a) severe vacuolation and sloughing of secondary epithelial cells (arrow head) following 15 days exposure to 50 μgl^{-1} cadmium (b) a gill showing relatively less severe changes following 30 days exposure and possible removal of toxic material through the process of sloughing. (H&E, 600X)

a



b



copper (Plate 7.9). Accumulation of cellular debris in the lumina of kidney tubules and collecting ducts was present in some of the samples collected after 15 days exposure to cadmium or copper. In the haematopoietic tissue there was mild to moderate multifocal necrosis (Plate 7.10 and 7.11). Fragmentation of MMC in the haematopoietic tissue was often noticed in samples collected after 15 or 30 days exposure to cadmium or copper. The pigment granules and individual macrophages from the MMC were found to be dispersed amongst the haematopoietic tissue.

7.3.6.3 Spleen

The changes observed in the spleen were not as severe as in the gills and kidney and were noticed only in samples collected after 15 days exposure to the metals. The only change observed at light microscopy level was the presence of multifocal necrosis of cells in the white pulp (Plate 7.12). The MMC in the spleen of the metal exposed fish appeared to have fragmented (Plate 7.13) compared to the controls (Plate 7.14) and such changes were relatively more common in copper exposed fish.

Plate 7.8 Phtomicrograph showing initial signs of damage to kidney tubules (pyknotic nuclei = arrow heads) following 9 days of exposure to (a) 50 μgl^{-1} cadmium.(H&E, 1500X)

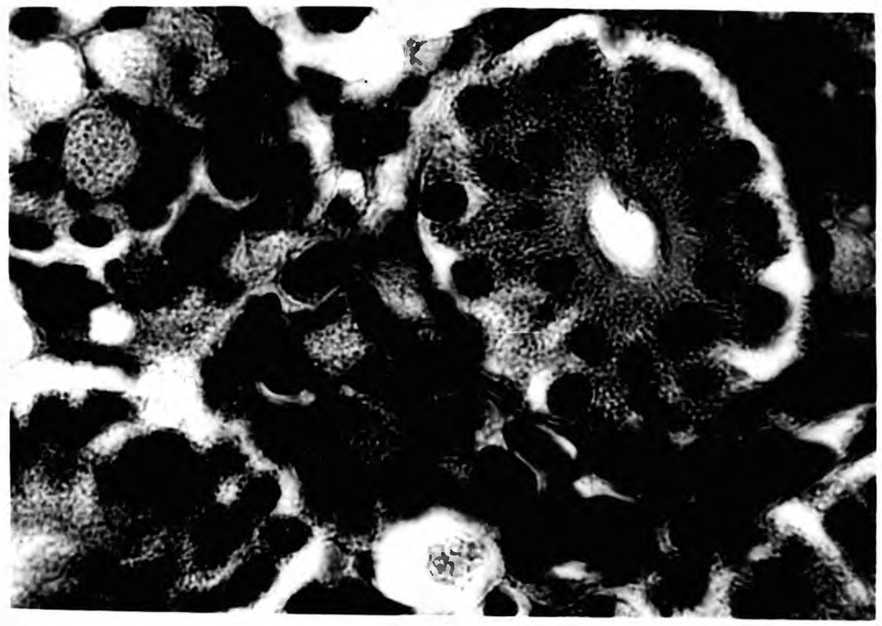


Plate 7.9 Photomicrograph showing damage to the renal glomeruli (arrow head) in the kidney of carp following 9 days exposure to 30 μgl^{-1} copper. (H&E, 600X)

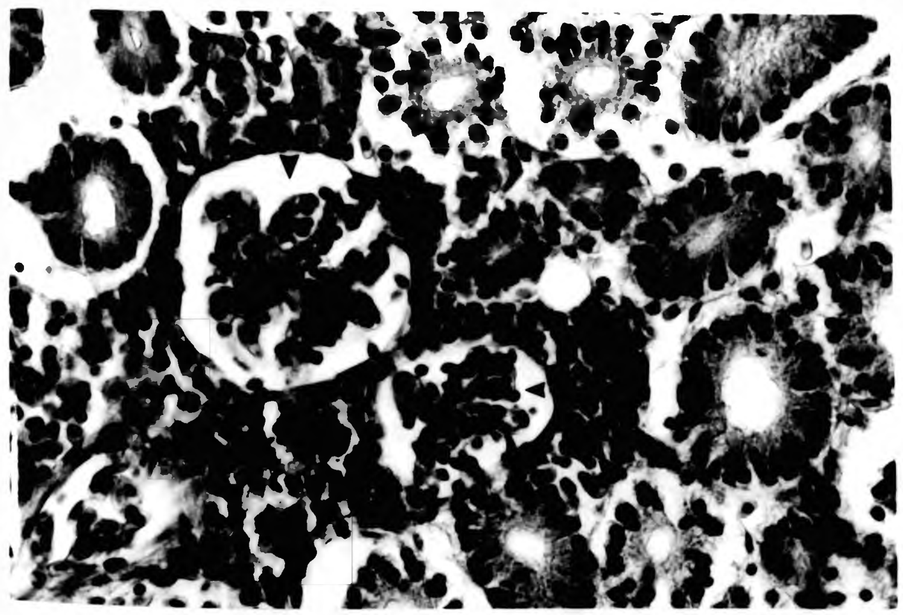
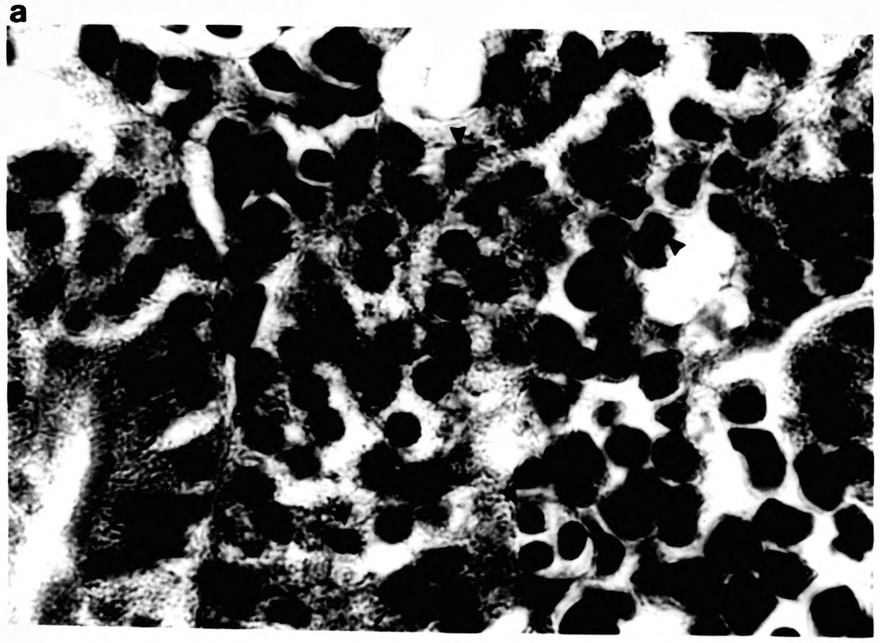


Plate 7.10 Photomicrographs showing signs of necrosis of haematopoietic cells (arrow heads) in the kidney of carp following 15 days' exposure to (a) 50 μgl^{-1} cadmium and (b) 30 μgl^{-1} copper. (H&E, 1500X).

a



b

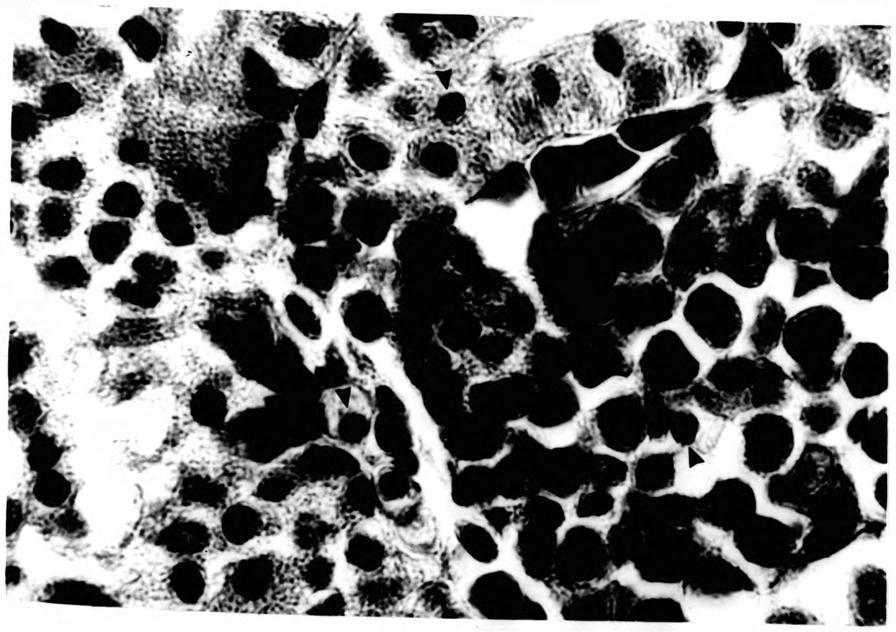


Plate 7.11 Photomicrograph showing more extensive necrosis of haematopoietic cells (arrow) and the kidney tubule (arrow head) in the kidney of carp exposed to 30 μgl^{-1} copper for 30 days. (H&E, 1500X).

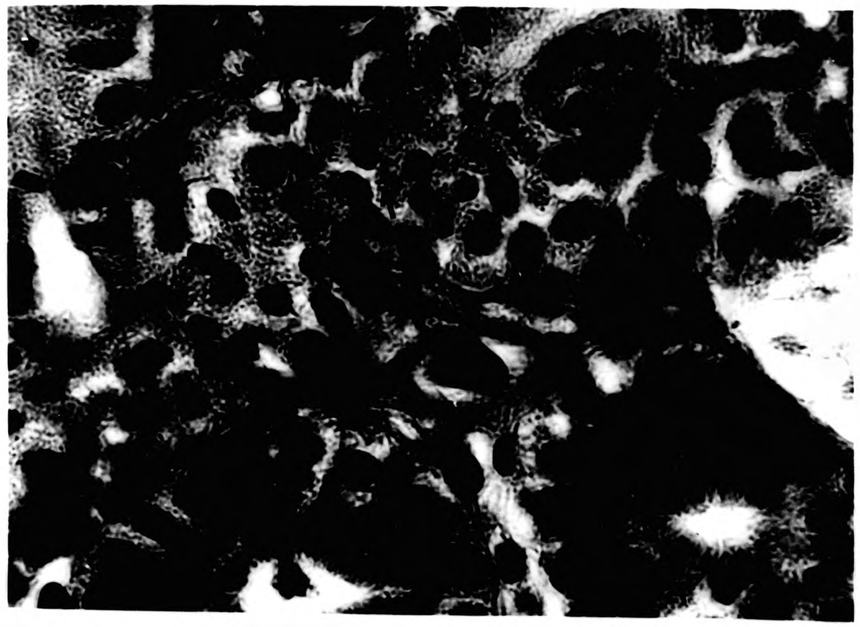


Plate 7.12 Photomicrograph showing signs of necrosis (arrow head) in the white pulp cells of the spleen in copper (30 μgl^{-1}) exposed carp. (H&E, 1500X).

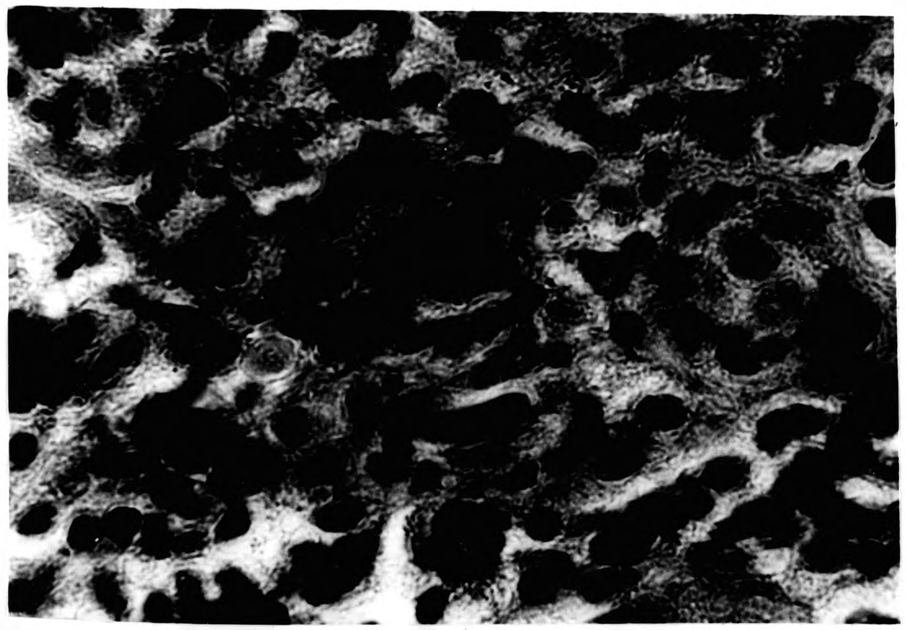
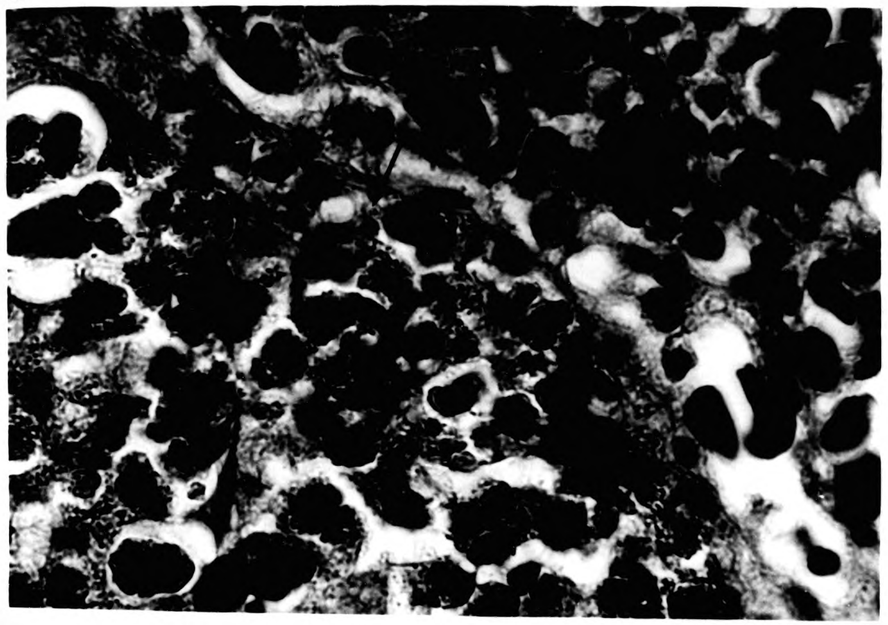


Plate 7.13 Photomicrograph showing signs of fragmentation of MMC (arrow) in the spleen of carp exposed to (a) 50 μgl^{-1} cadmium and (b) 30 μgl^{-1} copper for 30 days. (H&E, 1500X). Compare to the more discrete centres in the control carps (Plate 7.14).

a



b

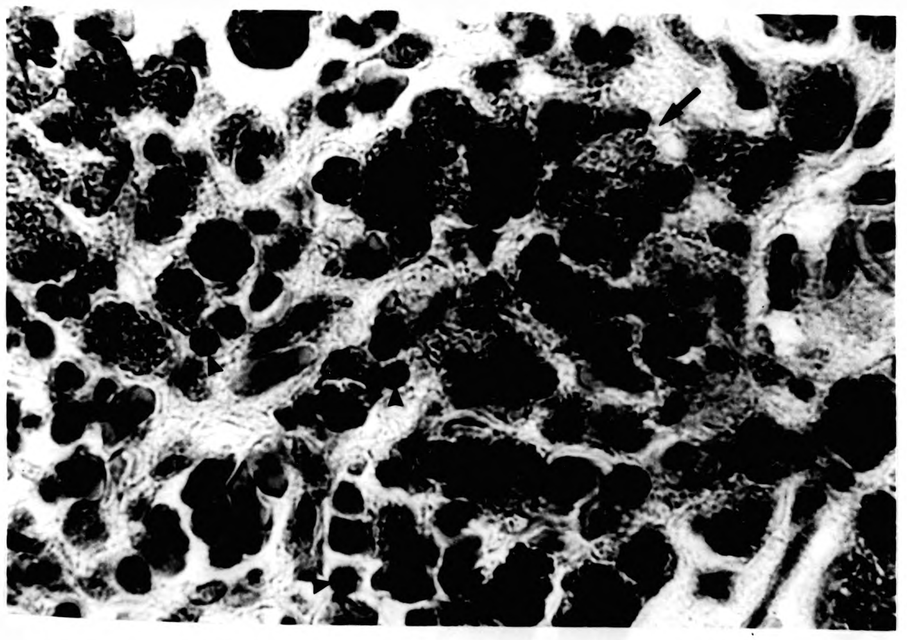
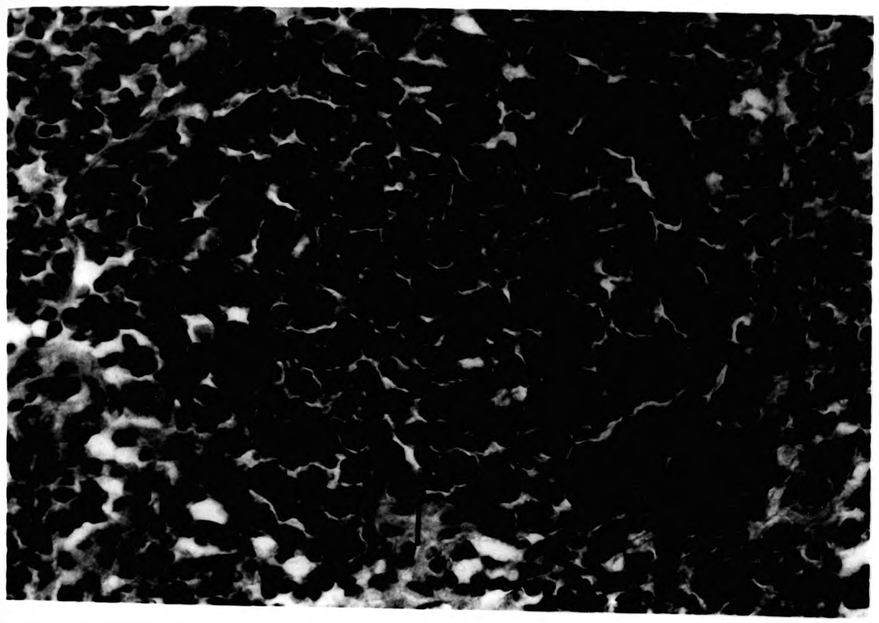
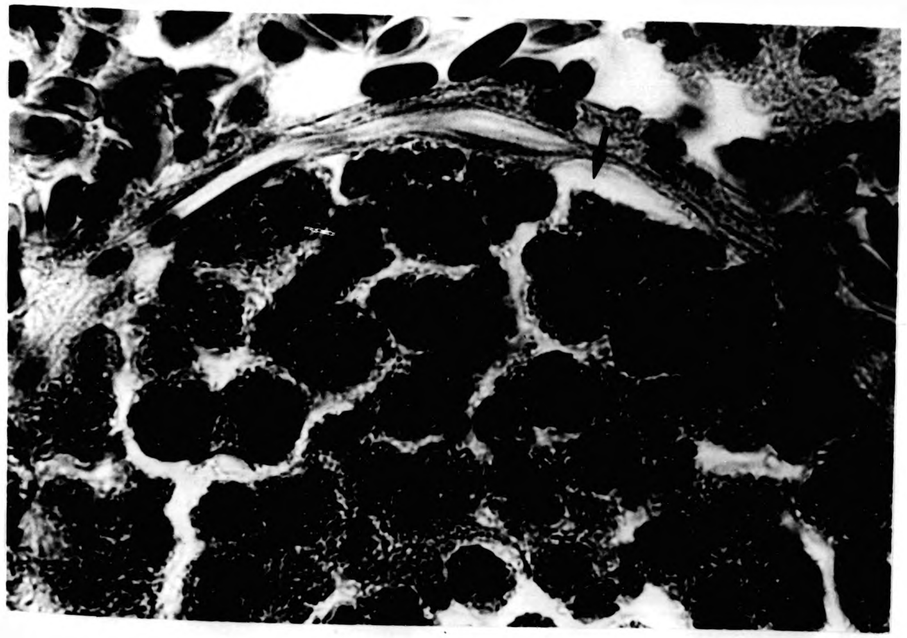


Plate 7.14 Photomicrographs showing the MMC in the spleen of control fish. Note the discrete nature of the MMC (a) H&E, 600X (b) H&E, 1500X.

a



b



7.4 DISCUSSION

Many of the environmental stresses are of a chronic rather than an acute nature and studies, therefore, aimed at examining the dynamics of cortisol over a long term are necessary to enable elucidation of the mechanisms of recovery and/or acclimation. Very few studies have examined the cortisol responses of fish to continuous chronic stimuli (Pickering and Stewart, 1984). The concentration and exposure duration used for cadmium and copper in this study have been shown in previous Chapters (3,4,5,6) to induce alterations in susceptibility to disease and the immune response of carp.

The results from these experiments clearly demonstrate the significant effects of cadmium and copper exposure on plasma cortisol concentration and its kinetics. Cadmium exposure induced a significant stress response as measured by the elevated levels of cortisol. This response, however appeared to be transitory, with plasma cortisol levels decreasing with exposure duration. Copper exposure had a similar effect on carp. Immediately following exposure there was a significant elevation of plasma cortisol which was followed by a decreasing trend till day 9, beyond which, copper exposure further increased the cortisol, to levels approaching those found immediately following exposure.

Since the first samples were not collected until 24 hours after exposure to the metals, it is difficult to speculate on the response occurring within those 24 hours following exposure. It might be expected that the levels of plasma cortisol within the first 24 hours of exposure would have been higher than that recorded in the first sample 24 hours following exposure to the metals. It has been shown by several

workers (Pickering and Pottinger, 1987a) that the maximum level of cortisol occurs between 8 and 12 hours following an acute stress.

The kinetics of the cortisol response, which was regarded as a primary stress response by Mazeaud *et al.* (1977) may to a large extent depend on the nature, severity and duration of stressor. For several acute stressors of short duration commonly encountered in aquacultural practices such as handling, confinement, transport, netting etc, the typical stress response would involve transitory elevation of plasma cortisol with the fish being able to recover and acclimatize within 24 hours as indicated by cortisol values returning to basal levels (Strange and Schreck, 1978; Strange *et al.*, 1978; Tomasso *et al.*, 1981; Pickering *et al.*, 1982; Pickering and Pottinger, 1987b). When subjected to chronic, long-term, physical and handling stress, the fish may be able to recover and adapt but this may take from a few days to several weeks (Redgate, 1974; Strange *et al.*, 1978; Pickering and Stewart, 1984).

As seen in the present study, with environmental contaminants the kinetics of cortisol response may vary and would largely depend on the concentration of the toxicant and exposure (stressing) duration. Donaldson and Dye (1975) found a slow transitory elevation of cortisol before returning to basal levels in sock-eye salmon exposed to low concentrations of copper (6.35 μgl^{-1}) for 1-24 hours. In fish exposed to higher copper concentrations (63.5 μgl^{-1}) there was a secondary elevation of cortisol at 24 hours and this was suggested to represent the inability of the fish to adapt successfully. Despite the longer exposure duration in the present study, the secondary elevation of plasma cortisol observed in copper exposed carp appears to suggest failure on the part of the fish to adapt.

In a relatively longer-term experiment, similar observations were made by Schreck

and Lorz (1978) in coho salmon, exposed to copper (15, 16, 90, 110 and 240 μgl^{-1}) for upto 7 days. At low concentrations, a dose-dependent increase in cortisol was found, followed by a return to more basal levels within 8-24 hours, while at higher concentrations the cortisol concentrations increased significantly thereafter and remained high till the end of the exposure duration. The results in the present study in copper exposed carp is therefore most similar to that obtained with high copper exposure in coho salmon (Schreck and Lorz, 1978). The present study has also shown that cortisol can remain elevated in carp exposed to low levels of copper for as long as 30 days and such prolonged elevation highlights the serious nature of environmental pollutants.

Low levels of cadmium was found not to elevate plasam cortisol levels in salmonids (Schreck and Lorz, 1978). In contrast, in the present study cadmium exposure produced transitory elevation of cortisol in carp before returning to near basal values.

It is now well known that fish need time, ranging from days to several weeks to recover and acclimatize to chronic handling and physical stress (Schreck, 1981; Pickering and Stewart, 1984). Plasma cortisol levels in brown trout subjected to continuous crowding was found to remain elevated for at least 25 days, before returning to near normal levels (Pickering and Stewart, 1984). Similarly, in carp transferred from ponds to laboratory tanks, Redgate (1974) found elevated levels of plasma cortisol persisting for a minimum of 42 days.

In both cadmium and copper exposed groups, the plasma cortisol levels remained elevated and higher than controls throughout the experimental duration (30 days) with signs of recovery only in the cadmium groups. The secondary rise of plasma

cortisol levels in copper exposed groups may indicate the failure to adapt. Therefore it appears that carp may be able to recover and adapt relatively better to cadmium than copper. The possibiltiy of fishes recovering and adapting to chronic stress conditions such that cortisol levels return to near basal levels is plausible in view of the findings with cadmium exposed carp, but the nature of the stressor is obviously very influential.

In a recent study Bennett and Wolke (1987a) demonstrated elevated levels of cortisol for upto 60 days in rainbow trout exposed to the pesticide, endrin. As in copper exposed carp in the present study, the cortisol levels did not return to basal or near control levels. Such prolonged elevation of cortisol could be the result of a more severe chronic stress and the inability of the fish to adapt.

Transient elevation of cortisol followed by its return to basal levels with subsequent persistence at that level has been equated with the three phases of Selye's (1950) General Adaptation Syndrome (GAS) i.e, alarm, resistance and recovery. All the characteristic phases of GAS could not be distinctly recognised in the present study, since the levels of cortisol did not return to pre-stress or basal levels. Carp exposed to cadmium showed signs of recovery while copper exposed ones showed signs of exhaustion.

A secondary elevation of cortisol following an acute stress has been seen during the initial stages of recovery which, according to Pickering and Pottinger (1987a) occurs 6 to 8 hours after the withdrawal of stress. They also suggested that cortisol is required during the subsequent recovery of homeostasis. In the present study, there was an indication of recovery in cadmium exposed carp, as shown by the maintenance of the near basal level of cortisol after four days of exposure but there

was no secondary rise in plasma cortisol levels. There may be some confusion therefore regarding the significance of the secondary rise and it may well be that there is a difference in the recovery process between an acute and chronic stress.

The findings on the kinetics of cortisol in carp in response to cadmium and copper exposure and its significance in relation to the immunosuppressive effects of cadmium and copper shown in previous sections (Chapters 3,4 and 5) is discussed in the final section of the thesis (Chapter 8).

Delayed lymphocytopenia (Pickering *et al.*, 1982) which occurs in response to transient elevation of plasma cortisol levels (Pickering, 1984) is one of the more conspicuous secondary effects (Mazeaud *et al.*, 1977) of acute stress. There is convincing evidence for stress induced leucopenia in fish (McLeay, 1973; McLeay and Gordon, 1977; Tomasso *et al.*, 1983; Pickering, 1984; Maule *et al.*, 1987). Leucocyte response to chronic stress in fish is not very well known, more so with environmental contaminants.

Both cadmium and copper exposure produced significant leucopenia in carp. The kinetics of the leucocyte response was not closely related to the cortisol dynamics. Of the two metals, copper produced relatively more deleterious effects on leucocrit response and it is interesting also to note that copper produced a significant elevation of plasma cortisol. Transitory leucopenia following acute stress is well documented and often this is attributed to the generalized stress response rather than to a specific cytotoxic action of the stressor concerned. Leucopenia seen in the present study was certainly not transitory in nature, therefore, the possibility of direct effects of cadmium and copper on kidney and spleen which are the haemopoietic organs of the fish can not be ruled out.

Dick and Dixon (1985) found rainbow trout exposed to acute levels (301 μgl^{-1}) of copper for 24 hours to induce leucopenia, the majority of which was found to be due to lymphocytopenia, while in contrast chronic (118 μgl^{-1}) exposure for 16 weeks did not result in any leucopenia. In contrast, in the present study very low levels of copper (30 μgl^{-1}) induced significant leucopenia in carp, a species more resistant than salmonids to metal toxicants.

Contradictory results have also been obtained with cadmium. Rao and Sharma (1982) have shown acute levels of cadmium to produce an increase in the lymphocyte population accompanied by a decrease in the thrombocyte population, whilst Garofano and Hirshfield (1982) found cadmium to produce a significant increase in total leucocyte count but with a significant decrease in lymphocyte population. Tort and Torres (1988) found 24 hour exposure to 25 mgl^{-1} cadmium to decrease leucocrit but found values returning to control levels after 96 hours exposure.

Leucopenia as measured by reduced leucocrit may not always give a true indication of lymphocytopenia. Leucocrit may remain unchanged in the face of an acute lymphocytopenia if there is a concomitant increase in circulating levels of large granulocytes (Peters *et al.*, 1980). Enumeration of a differential leucocyte count has often been suggested so as to enable the division of leucopenia into the basic components such as lymphopenia and thrombopenia.

Copper and cadmium exposure had contrasting effects on haematocrit and total erythrocyte count in carp. The haematocrits in copper exposed groups were significantly higher than controls while in the cadmium groups it was lower. The

total erythrocyte response showed a good deal of variation both within and between treatments and the treatment effect was not as distinct as that observed with haematocrit. However, copper exposed carp had higher RBC per mm^3 of blood than the control and cadmium groups on several sampling points.

The effects of cadmium on the haematocrit and RBC response of teleosts is confusing and often contradictory. Cadmium at acute or subacute levels had very little or no apparent effect on haematocrit (Calabrese *et al.*, 1975; Smith *et al.*, 1976; Garofano and Hirshfield, 1983; Houston and Keen, 1984) while others have found cadmium to decrease the haematocrit and RBC (Larsson, 1975; Johansson-Sjobeck and Larsson, 1978; Koyama and Ozaki, 1984; Gill and Pant, 1985). In the present study, cadmium certainly had a depressant effect on haematocrit which was significant in the initial one to four day exposure period, beyond which, the values returned to pre-exposure levels and persisted at that level, comparable to controls, until the end of the experiment. This observation is consistent with the recent finding of Tort and Torres (1988) where a 24 hour exposure to acute levels of cadmium (25 mgl^{-1}) were found to decrease the haematocrit, while after 96 hours the haematocrit values had returned to control levels.

Consistent with the present finding, copper has been found to increase haematocrit and RBC in several fish species (McKim *et al.*,1970; Christensen *et al.*, 1972; Mishra and Srivastava, 1979, 1980). The increase in haematocrit and haemoglobin immediately following copper exposure has been suggested to be caused by the catalyzing action of copper ions on the incorporation of body iron stores into haemoglobin; an effect which has been studied with mammals. Supporting this argument McFadden (1965) found an increase in red blood cell production, greater haemoglobin synthesis and a decrease in available liver-iron stores in brook trout

exposed to low concentrations of copper. On the contrary, Dick and Dixon (1985) have found copper to decrease erythrocyte concentration.

Metal exposure induces alterations in haematological parameters generally because of changes in blood water content. Both haemoconcentration (Mishra and Srivastava, 1979, 1980) and haemodilution (Dick and Dixon, 1985; Tort and Torres, 1988) have often been suggested as the reasons for either increase or decrease of haematocrit respectively.

Structural changes which occur in additon to changes at a chemical or cellular level may modify the function of a particular tissue or organ system. Such changes might have possible consequences on the disease susceptibility and immune response mechanisms discussed in previous Chapters (3, 4 and 5). The basic lesions seen in the present study were necrosis of either epithelial or haematopoietic cells. The severity of changes was related to exposure duration and the damage was extensive in the gill tissues which had direct contact with the metal.

The observed effects of cadmium and copper on carp gills included hyperplasia and hypertrophy of epithelial cells, necrosis of epithelial and chloride cells and sloughing off of necrotic cells from both primary and seconadry lamellar epithelium. These changes were similar to gill changes reported for other teleosts exposed to heavy metals (Gardner and Yevich, 1970; Bilinski and Jonas, 1973; Matthiessen and Brafield, 1973; Salanki *et al.*, 1982; Collvin, 1984). The severe changes noticed in the present study involved swelling, separation and lifting of the epithelial layers from the pillar cells of the secondary lamellae and the creation of large non-tissue spaces which were in some instances occupied by chloride cells and EGC's. Such changes have been observed with cadmium by other workers (Stromberg *et al.*,1983;

Oronsaye and Brafield, 1984; Karlsson-Norrgren *et al.*, 1985; Oronsaye, 1989).

Very low levels of cadmium (10 μgl^{-1}) for 6 weeks was found to result in a reduction in the height of the secondary lamellae, fusion of the tips of the secondary lamellae, rupture of pillar cells and telengeactiasis in rainbow trout and zebra fish by Karlsson-Norrgren *et al.* (1985). However, such changes were not observed in the present study, even though the cadmium concentration used was five times higher. Oronsaye and Brafield (1984) found cadmium to induce changes in the chloride cell population of sticklebacks and such a chloride cell proliferation with heavy metal toxicosis has been suggested as an adaptation mechanism to cope with influx of metals. Daoust *et al.* (1984) in a 96-hour exposure study found the most severe effect of copper (135 μgl^{-1}) on the gills of rainbow trout during the first 48 hour of exposure. In the present study the changes seen were directly related to the exposure duration and the most severe effects were seen only after 6 to 9 days exposure to 30 μgl^{-1} copper.

The pathological effects of heavy metals on fish gills and their impact on respiratory and extrarenal functions of gills has been discussed and documented by Gardner and Yevich (1970), Hughes *et al.* (1979), Majewski and Giles (1981), Stromberg *et al.* (1983), Kalsson-Norrgren *et al.* (1985) and Oronsaye (1989).

The pathological changes seen in the kidney and spleen were mild to moderate and involved multifocal necrosis of haematopoietic cells and fragmentation of MMC. Exposure duration had a direct relation with the observed pathological changes. Consistent with the present observation, Dubale and Shah (1981) found low levels of cadmium (10 to 50 μgl^{-1}) to induce changes in the excretory kidney of *Channa punctatus* and they found a direct relationship between concentration and exposure duration with the pathology. Tafanelli and Summerfelt (1975) have also shown

kidney damage to be directly related to the amount of cadmium reaching the organ. Cadmium (2 and 4 mgl^{-1}) exposure for 20 days was found not to affect the kidneys of sticklebacks while exposure to higher cadmium levels (6 mgl^{-1}) for 16 days was found to be damaging to the kidney (Oronaye, 1989). In contrast, in the present study, at nearly 100 times lower concentration (50 μgl^{-1}) cadmium has produced damaging effects on kidney and spleen of carp.

Renal tubular necrosis in carp was less severe and less extensive than necrosis in tissues with direct contact with cadmium and copper (gills). Similar observations have been made with other fresh water fishes (Oronsaye, 1989). Extensive lesions induced by heavy metals have also been reported in the excretory kidney of marine fishes (Gardner and Yevich, 1970; Newman and MacLean, 1974). Renal failure has often been implicated as the cause of death in marine fishes (Newman and Maclean, 1974) while gill damage has been thought to be the cause of death in fresh water fishes. Such differences in the toxicity of heavy metals is suggested to be related to the functional divergence in osmoregulation between fresh and marine water fishes.

In fresh water fishes, the amount of water ingestion is minimal. The amount of heavy metals reaching the kidney of fresh water fishes is believed to be the excess that had not been removed by the gills (Oronsaye, 1989). Hence kidney damage in metal exposed fish in fresh water is less extensive than in marine fish and would not be revealed at an early stage. Irrespective of the mechanism operating, the present study has clearly shown that cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) at very low levels still induce damage to both the haematopoietic and renal kidney.

Subtle effects of heavy metals on the haematopoietic cells could certainly have serious implications on the overall immunocompetence of the fish. Reduced mitogenic response of pronephric lymphocytes collected from carp exposed to

cadmium and copper (Chapter 6) further goes to show the serious nature of heavy metal toxicity.

CHAPTER 8

GENERAL DISCUSSION

8.1 SUMMARY OF THE RESULTS

The study delineated the effects of cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) on disease susceptibility, protective immunity, the kinetics of the primary and secondary humoral antibody response, the mitogenic response of lymphocytes, the cortisol response, haematological parameters and the pathological changes in common carp, *Cyprinus carpio*. Alterations in disease resistance and the immune response of carp were demonstrated using the protozoan (*I. multifiliis*), bacterial (*A. hydrophila*) and SRBC models.

Both cadmium and copper exposure for 10 days prior to a challenge infection, increased the susceptibility of naive carp to *I. multifiliis* infection significantly. Of the different external surfaces, gills were the worst affected. Previously immunized carp with established immunity and antibody titre could not mount a protective immune response to a challenge infection following 10 days exposure to cadmium and copper. The suppressive effect of cadmium was more serious. However, such a suppression of protective immunity was not associated with any lowering of the humoral "anti-ich" antibody titre. Concurrent exposure of carp to cadmium and copper with a series of controlled low level immunization exposures did not alter the kinetics and magnitude of "anti-ich" humoral antibody titre. The antibody titres of metal exposed carp remained lower than unexposed controls but were not statistically different. The unexposed controls became visibly refractory to infection following three controlled low level infections, while those exposed to cadmium and copper, became refractory only after four or five controlled challenge infections. The lethal concentration of cadmium and copper was influenced greatly by parasitization. Two stages in the life cycle of the parasite, namely, tomite penetration of the fish

epithelium and the emergence of the mature parasite from the fish epithelium appeared to lower the lethal toxic concentration of both the metals for carp significantly.

Exposure of carp for 40 days to cadmium and copper, with the primary immunization administered 10 days following the commencement of metal exposure, did not reduce the primary response but reduced the magnitude of secondary response significantly. The anti-SRBC antibody response in metal exposed carp always remained lower than unexposed controls. Experimental conditions similar to above revealed analogous results with the rosette forming and plaque forming cell responses after the primary and the secondary immunization. The degree of suppression was significant only with the secondary response. Long-term exposure (30 days) to the metals before the primary immunization produced contrasting results between the two metals. Cadmium exposure did not impair the kinetics and magnitude of either the primary or the secondary anti-SRBC antibody response. Copper exposure suppressed both the primary and secondary antibody response significantly. Exposure of carp to the metals 18 days after the administration of the primary immunization significantly suppressed the secondary response.

Primary immunization given concurrently with the commencement of exposure to the metals significantly reduced the primary response in addition to suppressing the secondary antibody response significantly. Except in carp exposed to cadmium for a long-term (30 days), both the metals reduced the magnitude of the secondary anti-SRBC antibody response significantly.

Exposure of carp to cadmium and copper for 10 days prior to experimental challenge infection with *A. hydrophila* by i/p injection significantly increased the susceptibility of carp. The bacterial dose required to bring about 50% mortality was

significantly reduced. The effect of copper in increasing the susceptibility was greater than that of cadmium. Previously immunized carp with established immunity exposed to cadmium and copper for 10 days showed significant suppression of protective immunity to subsequent challenge infection. This suppression in protective immunity was associated with a significant reduction in humoral antibody titre. Exposure to the metals for 10 days before a single immunization injection with formalin killed, whole cell bacterin, significantly reduced the magnitude of humoral anti-*A. hydrophila* antibody. Immunization administered concurrently with the commencement of metal exposure revealed significant suppression of both the primary and the secondary antibody response.

Lymphocytes collected from carp exposed to cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) showed reduced blastogenic response to the mitogens Con A and LPS. The reduction in mitogenic response was significantly related to exposure duration. Lymphocytes collected after 6 to 9 days of exposure to the metals were significantly impaired in their ability to proliferate. There was no indication of any selective suppression of either T-cell or B-cell mitogen induced proliferation. Metals showed similar effects *in vitro* on lymphocyte proliferation. Concentrations of 10^{-7} M (copper) or 10^{-6} M (cadmium) and greater suppressed the blastogenic response of lymphocytes to both the mitogens significantly. Lower concentrations did not have any effect. Both the metals at concentrations of 10^{-4} M and greater appeared to be toxic to the lymphocytes in the culture media. The concentrations of metals used in the *in vivo* exposure experiments (Cadmium= 50 μgl^{-1}; Copper= 30 μgl^{-1}) corresponds to concentrations between 10^{-7} and 10^{-6} M used in the *in vitro* studies.

Exposure to cadmium and copper produced a typical stress response, characterised by elevation of plasma cortisol. The elevation was transient in nature in cadmium

exposed carp with values returning to near basal levels and persisting at that level throughout the experimental duration (30 days). However, in copper exposed carp the response was different. From the initial maximum seen following 24 hours of exposure, the values declined gradually till day 9 before showing a secondary elevation which persisted at that level for the entire experimental duration. Both the metals induced generalized leucopenia. However, this response was not closely related to the cortisol kinetics. Cadmium exposure did not have any effect on haematocrit and total erythrocyte number, while copper exposure significantly increased the haematocrit. Both the metals produced serious gill pathology. Changes included necrosis and hypertrophy of the epithelial cells of the primary and secondary lamellae as well as the interlamellar region. Separation and lifting off of secondary lamellar epithelium resulted in large lymphatic spaces. In addition, copper exposure was characterised by severe hyperplasia in the interlamellar region. Proliferation and infiltration of EGC into the secondary lamellae was marked. The effects on the kidney and the spleen included multifocal necrosis of haematopoietic cells and fragmentation of MMC. The overall effects were not as serious as those seen in the gills.

8.2 GENERAL DISCUSSION AND CONCLUSION

Experiments carried out during the course of this investigation have demonstrated the ability of cadmium and copper at ppb levels (< 50 μgl^{-1}) to induce a response characterised by elevation of plasma cortisol and leucopenia, an increased susceptibility in naive carp to *I. multifiliis* and *A. hydrophila*, a suppression of the protective immunity, a reduction in the magnitude of the humoral antibody response, an impairment of the blastogenesis of lymphocytes, and structural and pathological changes in gills and haematopoietic organs of carp, *Cyprinus carpio*. In this final Chapter, a comprehensive account of the immunomodulatory effects of heavy metals in relation to the present findings is discussed, by drawing relevant observations from mammalian immunotoxicology.

Exposure of naive carp to cadmium or copper for 10 days increased their susceptibility to *I. multifiliis* and *A. hydrophila*. Similar findings of the ability of heavy metals to predispose fish to bacterial (Sugatt, 1980; Knittel, 1981; Baker *et al.*, 1983), viral (Hetrick, 1979) and parasitic (Ewing *et al.*, 1982) diseases are well documented. Alterations in the disease susceptibility of naive fish probably reflect the possible impairment of the non-specific, innate defence mechanisms of the host. Modulation of the non-specific defence mechanisms may involve anything from damage to the structural integrity of the primary barriers such as skin and gill epithelium, and mucus production, to reducing the competence of complement dependent processes and phagocytosis.

Structural changes produced by cadmium and copper exposure in the primary barriers, skin and gill epithelium were suggested in the present study to have

resulted in the observed increase in susceptibility of naive carp to *I. multifiliis*. It was evident that more tomites penetrated, and subsequently established successfully, in carp which were previously exposed to the metals for 10 days. It is known that tomites spend considerable amounts of energy in the process of penetrating the fish epithelium (Ewing and Kocan, 1986). Spending relatively less of their limited reserves in the process of negotiating the primary barriers which are already breached by metal exposure, may account for the higher number of tomites penetrating successfully. It is also strongly felt that the ready availability of food in the form of cell debris in carp that had been exposed to the metals, contributed to the higher survival of tomites which had penetrated successfully.

Of the several innate defence mechanisms, phagocytosis and complement induced lysis appear to be important against systemic bacterial diseases and opportunistic bacterial pathogens. Increased susceptibility to *A. hydrophila* in carp exposed to cadmium and copper compared to unexposed controls, would appear to suggest impairment in some of the major non-specific defence systems. Impaired phagocytic ability (Mushiake *et al.*, 1985; Elsasser *et al.*, 1986) and increased blood clearance time (O'Neill, 1981a) caused by metals like cadmium and copper can give systemic bacteria an upper hand. Such an effect seems likely to account for the increased susceptibility of carp to *A. hydrophila* seen in the present study.

The bactericidal action of complement in the serum, activated via the alternative pathway, is regarded as playing a major role in the natural defence mechanism, especially against gram negative bacteria (Ourth and Wilson, 1981, 1982; Sakai, 1983). Concerted action of the complement and lysozyme are regarded as being very effective in this bactericidal action against many gram negative bacteria (Ellis, 1989). There are no reports of any stress and/or chemical pollutant induced

alterations of complement mediated processes in fish.

Phagocytosis by macrophages has been established as a major line of defence against infection in fish (Ellis *et al.*, 1976). Macrophages also form an integral part of the initiation and effector phase of the humoral and cell-mediated immune system (Anderson *et al.*, 1984; Ellis, 1989). Impairment of macrophage function may not only affect phagocytic destruction of pathogens but also the presentation of antigen to antibody-producing lymphocytes (Peters *et al.*, 1988).

The phagocytic ability of macrophages collected from fish living in polluted waters was shown to be markedly reduced compared to those from pristine waters (Weeks and Warinner, 1984; Weeks *et al.*, 1986). Exogenous corticosteroids also have similar effects on macrophage activity (Stave and Roberson, 1985). The reduced ability of macrophages would automatically contribute to an increased bacterial clearance time, as seen for carp and brown trout exposed to heavy metals by O'Neill (1981a). Subtle effects like these could certainly give an upper hand to invading pathogens of a systemic nature and result in increased bacteraemia.

Heavy metals have the ability to readily bind and readjust the tertiary structure of biologically active molecules (Phipps, 1976). Metals like cadmium have a particular affinity for sulphydral and hydroxyl groups. Such a direct action has been shown to impair the activity of complement in mammals (Hemphill *et al.*, 1971).

The ability of environmental contaminants to modulate the non-specific defence mechanisms of fish deserves considerable attention. Suppressive effects of low levels of environmental contaminants on the host's innate defence mechanism will prove crucial in determining the ability of fish to combat infection and survive in natural

environments. Further work on these lines, evaluating the effects of potential environmental contaminants on non-specific defence mechanisms, especially mechanisms such as complement mediated processes, would be rewarding and, at the same time, would enable us to learn more about the function of the important non-specific defence mechanisms of fish.

Cadmium and copper at low levels have been shown in the present study to have a repressing effect on the humoral antibody response. Both these metals were shown to significantly reduce the magnitude of the secondary humoral antibody response to SRBC and *A. hydrophila*, but did not alter the kinetics and magnitude of the humoral response to "ich" antigens. Several points warrant consideration before drawing conclusions from these findings. The mode of immunization, antigen handling and localization and, most importantly, the cellular requirements for optimal expression of humoral immunity to these three different antigens, are some of the important areas which need to be considered.

SRBC are regarded as Thymus Dependent (TD) antigens and, therefore, require the participation and interaction of "T-like" and "B-like" lymphocytes and macrophages for optimal expression. In the opinion of Lamers *et al.* (1985), *A. hydrophila* has both Thymus Dependent (TD) and Thymus Independent (TI) components which are degraded at different rates. The cellular requirements for humoral "anti-ich" antibody response is uncertain (Houghton, 1987).

As cadmium and copper exposure were shown to consistently reduce the magnitude of the secondary humoral immune response to SRBC and *A. hydrophila*, it is conceivable that the metals could have interfered with and impaired the function of

T-like and B-like lymphocytes and macrophages. Lymphocytes exposed to cadmium and copper *in vitro* and those collected from carp which were exposed *in vivo* to cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) showed a reduced blastogenic response to T-cell (Con A) and B-cell (LPS) mitogens. It is important to emphasize that the proliferative response of lymphocytes was not completely suppressed, but only the magnitude of the response lowered. The duration (6 to 9 days; *in vivo*) of exposure and metal concentration (10^{-6} M and greater; *in vitro*) were significant in bringing about this effect.

The magnitude of the suppression observed in the proliferation of lymphocytes which were stimulated by Con A and LPS was similar. The general suppressive effect could have largely contributed to the lowered magnitude of the humoral immune response observed. Reduced numbers of rosette forming cells (RFC) and plaque forming cells (PFC) in cadmium and copper exposed carp support this argument.

The immunocytoadherence, rosette or antigen-binding cell (ABC) assay allows for enumeration of immunocompetent cells, while PFC assay enumerates antibody-producing lymphocytes (Rijker *et al.*,1980a; Ingram and Alexander, 1981). In mice it has been demonstrated that T-ABC are involved in helper functions and B-ABC are the precursors of antibody-producing cells. Based on the cellular immune response of rainbow trout to SRBC, Blazer *et al.* (1984) hypothesized that early ABC populations correlated with helper-T cell activity, and that the later ABC populations were the precursor B cells. The reduction in the number of RFC and PFC response shown here indicates that the effect of cadmium and copper exposure are on both antigen-binding and antibody-producing cells and this would certainly result in lowered antibody production.

In fish it is not yet clear if lymphocyte heterogeneity is as well differentiated as in higher vertebrates, but it is clear that functional differences do exist between lymphocytes in fish, and all the evidence suggests the existence of two distinct populations of lymphocytes in fish (Ellis, 1989). However, it has not been proven whether these are two distinct, embryologically different cell populations (analogous to mammalian T and B cells). From the overall results of the present study it is difficult to apportion the observed effects of cadmium and copper on any particular cell component (T-like or B-like lymphocytes or macrophages) of the immune system. From the different experiments with SRBC it is evident that both cadmium and copper suppressed the secondary humoral anti-SRBC antibody response significantly. Elevated secondary antibody response to SRBC requires "T-helper" cell involvement and, therefore, it appears that cadmium and copper could have impaired the "T-helper" cell activity in carp. Further studies are required to examine this possibility. *In vitro* antibody production studies using antigens of different cellular requirements may prove useful in this direction.

In mammals, cadmium has been shown specifically to effect the T-lymphocyte activity. In a detailed study looking at the affect of cadmium on the antibody response of mice to antigens with different cellular requirements, Blakley and Tomar (1986) have shown that the immunosuppressive effect as it relates to humoral immunity, involves T-lymphocyte function rather than macrophages or B-lymphocyte activity. The *in vivo* antibody response against DNP-Ficoll, a T-cell independent macrophage dependent response, was enhanced by cadmium; similarly the *in vivo* antibody response against *E. coli*, a T-cell and macrophagae independent response was also enhanced. In contrast, the *in vitro* antibody response to SRBC, a T-cell and macrophage dependent response, was suppressed (Blakley and Tomar, 1986).

Low levels of cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) utilized in the present study induced perturbations in humoral immunity. These perturbations, however, did not involve total immunosuppression. Both the metals altered the functional capacity of lymphocyte subpopulations (RFC, PFC and lymphocyte blastogenesis) which was manifested in the reduced magnitude of humoral antibody responses, especially the secondary response.

The immunotoxicological effects of heavy metals like cadmium, mercury and lead are well established in mammals, but there is very little information about copper. Several mammalian studies have indicated that cadmium impairs the humoral (Koller, 1973; Koller *et al.*, 1975; 1976; Blakley, 1985; Blakley and Tomar, 1986) and the cell mediated (Muller *et al.*, 1979; Fujimaki *et al.*, 1983) immune response. The impaired humoral immunity has also been associated with T-cell dependent responses (Kawamura *et al.*, 1983; Blakley, 1985; Blakley and Tomar, 1986). It has also been hypothesized that a T-helper lymphocyte deficiency may be involved (Koller *et al.*, 1975; Koller, 1984). Antibody responses to T-cell independent antigens are not affected by cadmium (Fujimaki, 1985; Blakley and Tomar, 1986).

Delayed type hypersensitivity reactions which require T-cell participation are also suppressed by cadmium (Muller *et al.*, 1979; Fujimaki *et al.*, 1983). Cadmium effects macrophage functions such as complement receptor activity (Cook *et al.*, 1984), lymphokine production and thus macrophage mobility (Kirmidijan-Schumacher *et al.*, 1981) in mammals.

From mammalian studies it is also evident that several of the altered immune responses associated with cadmium exposure involve T-cell dependent functions (Koller *et al.*, 1975; Blakley, 1985; Blakley and Tomar, 1986). Cooperation of T-

helper cells is required for elevated secondary response to SRBC antigens. The suppressed secondary response in carp which were exposed to the metals 18 days after the primary immunization with SRBC appears to suggest a possible effect of cadmium and copper on "T-helper"-like cells. T-helper cells have been suggested as the target cells for the immunosuppressive effects of cadmium, largely because of its significant suppressive effect on the secondary humoral antibody response in mammals (Koller and Kovacic, 1974; Koller *et al.*, 1975; Koller, 1984).

Several reports in mammals have indicated that there is a rapid uptake of calcium (Ca^{2+}) after stimulation of T-lymphocytes but not B-lymphocytes (Hart, 1978; Abboud *et al.*, 1985). The inhibitory effect of manganese on carp lymphocyte proliferation was found to be partially or completely reversed by increasing calcium concentration (Ghanmi *et al.*, 1989). Diamantstein and Odenwald (1974) suggested that the chelating properties of tetracyclines for divalent cations (Ca^{2+}) could interfere with the mitogenic response of leucocytes and disrupt Ca^{2+} dependent cell processes including immunomodulation. Competetitive inhibition of Ca^{2+} transport and other Ca^{2+} dependent processess by metal cations at subtle levels appears to be very important in several of the immunomodulatory effects of metals observed in mammals and fish.

Cadmium and copper exposure did not alter the kinetics and magnitude of "anti-ich" humoral antibody titre. The humoral antibody titre was monitored over 35 days at regular intervals of 7 days following each of the 5 controlled immunization exposure to tomites (day 0, 7, 14, 21 and 28). Continuous antigenic stimulation from regular infections and from the developing stages of the parasite residing within the gill and skin epithelium appears to have accounted for the relatively high tomite agglutination titre in carp.

On the other hand, the processes involved and the cells required for antigen handling, processing, presentation, and antibody production to *I. multifiliis* antigens are still largely unknown. The fact that cadmium and copper exposure did not alter the humoral antibody response to "ich" antigens, may indirectly reveal the possible differences in cellular requirements for antibody production.

A suppression of protective immunity would normally indicate a reduction in the competence of some immune effector mechanisms. Previously immunized carp exposed to cadmium and copper for 10 days could not mount a protective immune response, comparable to unexposed controls, to either *I. multifiliis* or *A. hydrophila*. With *I. multifiliis* such a suppression in protective immunity was not associated with any lowering of the humoral antibody titre, whilst there was a significant reduction in the circulating antibody level in the case of *A. hydrophila*.

All the available evidence (Graves *et al.*, 1985a, b; Houghton and Matthews, 1986; Houghton, 1987; Cross and Matthews, 1989a, b; the present study) indirectly suggests the involvement of a possible antibody and/or complement dependent cellular cytotoxic process in the execution of protective immunity in catfishes and carp to *I. multifiliis*. With *A. hydrophila*, cellular immunity regulated by T-like cells and macrophages is suggested to be pivotal in the subsequent execution of protective immunity in carp immunized with crude *A. hydrophila* LPS (Baba *et al.*, 1988b). The loss of protective immunity in the case of "ich", therefore, appears to be largely due to a suppressive effect of cadmium and copper on the cellular immune response.

Recent evidence suggests that tomites of *I. multifiliis* are able to penetrate the

epithelium of immune carp (Houghton, 1987; Cross and Matthews, 1989b). The existence of a cellular cytotoxic mechanism, therefore, seems likely in the execution of protective immunity in carp. However, the nature of this cytotoxic mechanism is unclear. The work of Graves *et al.* (1985a) suggests the involvement of non-specific cytotoxic cells in this processes. The histological results of the present study suggests the participation of eosinophilic granular cells (EGC) in the execution of protective immunity. It remains to be seen whether these cellular cytotoxic mechanisms are dependent on antibody and/or complement.

Graves *et al.* (1985a, b) have shown the mobilization and activation of NCC's from the pronephros of "ich" infected channel catfish and from *in vitro* studies they have hypothesized that contact between effector cells (NCC's) and target cells (ich tomites) immobilized by mucus antibody could initiate the cytotoxic response NCC's of fish are considered to be functionally analogous to mammalian Natural killer (NK) cells (Evans *et al.*, 1984a, b).

In mammals, antibody dependent cellular cytotoxicity (ADCC) is one of the major defence mechanisms against viruses, parasites and tumour cells (Lawman and Bell, 1984). The specificity of this mechanism is determined by the antibody, specifically bound to the antigen determinants expressed on the target cell membrane, interacting via the Fc portion of the antibody (IgG type). Binding of the Fc region of the IgG to the Fc recptors present in effector cells (macrophages, Killer-cells) is the primary interaction required for ADCC to occur.

Non-specific cytotoxic activity has been reported in several species of teleosts For example, NCC's have been found in the pronephros, spleen and peripheral blood of carp (Hinuma *et al.*, 1980), channel catfish (Evans *et al.*, 1984a; Graves *et al.*,

1984) Atlantic salmon and rainbow trout (Moody *et al.*, 1985). It is conceivable, therefore, that such cytotoxic cells may play an important part in cell mediated immunity in teleosts. In mammals cadmium has been shown to suppress immune responses which are dependent on T-cell functions (Blakley and Tomar, 1986). It remains to be determined whether such cellular immunity mediated by the cytotoxic cells in fish is affected by low levels of metal pollutants.

In mammals increased corticosteroid levels, associated with stress, have been shown to suppress the natural killer cell activity (Aarstad *et al.*, 1984; Shavit *et al.*, 1984; Mathews *et al.*, 1985). Stress associated suppression of natural cellular cytotoxicity in fish has come to light only recently (Ghoneum *et al.*, 1989). Stress induced as a consequence of social aggressiveness in tilapia was shown to suppress the natural cellular cytotoxicity in subordinates compared to dominants. Such a suppression was suggested to be due to the decreased binding capacity of the effector to target cells (Ghoneum *et al.*, 1989). There is no direct evidence to suggest such a suppression of natural cellular cytotoxicity in carp exposed to cadmium and copper.

The possible role of eosiniophilic granular cells (EGC's) in fish is very poorly understood. These cells are distributed in the connective tissue of carp and salmonids and are regarded by Ellis (1982) to be analogous to mammalian mast cells. Histamine release from mast cells in mammals is mediated by immunoglobulin IgE. When specific antigen binds to IgE on the surface of the mast cells in the presence of Ca^{2+}, degranulation and release of histamine occurs (Ellis, 1982). Teleosts are known to have only one class of immunoglobulin IgM, (Ambrosius *et al.*, 1982) although the possibility of subclasses cannot be dismissed (Sanchez *et al.*, 1989). Nevertheless, there are indications for degranulation of EGC's in fish (Ellis, 1985; Present study).

It is evident from the suppression of protective immunity by cadmium and copper that the tomites which successfully penetrated the epithelium of an immune fish were not killed by either degranulating EGC's and/or antibody or complement-dependent cellular cytotoxic processes. The histological evidence strongly indicated that the EGC's did not degranulate in carp which had lost their protective immunity, thus suggesting that the degranulation of these cells was implicated in the protection process.

Protective immune mechanisms in carp to *A. hydrophila* may depend on antibody and complement. Opsonization of *A. hydrophila* with either antibody or complement may enhance macrophage mediated processes. This is controversial in fish as they have only one class of immunoglobulin (IgM). However, opsonization has been shown to enhance the phagocytic activity of macrophages in fish (Griffin, 1983; Sakai, 1984). Thus, IgM in fish, may have a role as an opsonin in certain species, via receptors for the IgM, or indirectly via receptors for classically activated complement (Ellis, 1989). Induction of a cytotoxic reaction (cell lysis) in the case of cellular antigens by the complement system, activated by the antigen-antibody complex, is known in fishes (Ellis, 1989). The significant suppression of protective immunity shown in the case of immune carp exposed to cadmium and copper appears to suggest the effect of these two metals on antibody and complement dependent cellular immune response.

Activation of the fish complement system is known to proceed through two distinct pathways. The classical pathway is activated by antigen-antibody complexes and requires both Ca^{2+} and Mg^{2+}. The alternative pathway is activated without antigen-antibody complexes, by the lipopolysaccharides of gram negative bacteria and requires Mg^{2+} only (Matsuyama *et al.*, 1988). Existence of the classical pathway of

complement activation is well known in fish (Ellis, 1981) and was confirmed in carp by Matsuyama *et al.* (1988). It is suggested that the antibodies present in mucus may be vital in initiating cytotoxic responses via complement (Ellis, 1989).

It is very interesting to note that Ca^{2+} is required for the activation of two cytotoxic processes, namely, the degranulation of mast cells and the activation of the complement by the classical pathway. Heavy metals especially cadmium are known to affect several processes which require divalent cations like Ca^{2+} by competitive inhibition. More work on these lines may help to shed light not only on some of the unexplained subtle effects of metals on the immune system, but also on some of the antibody and/or complement dependent cellular cytotoxic processes.

Subtle structural changes produced by low levels of chemical contaminants can have serious repercussions on the disease resistance as well as on the immune response. The structural changes as a result of exposure to cadmium and copper was suggested to be the reason for the increased susceptibility of naive carp to *I. multifiliis* infection. The changes included hyperplasia and hypertrophy of epithelial cells, vacuolation of the mucus cells and degeneration and necrosis of cells in the interlamellar region. From the histological observations and from the parasite intensity figures it is strongly felt that the structural damage as a result of exposure to cadmium and copper was the main contributory factor to the increased susceptibility of naive carp to *I. multifiliis*.

It is well documented that the haematopoietic organs of teleosts are the prime target for heavy metal toxicity. The teleost pronephros is an essential component of the immune system. It has all the cellular elements for the production of lymphoid cells

(Ellis, 1989), antigen trapping (Ellis, 1980; Secombes and Manning, 1980; Lamers and Pilarczyk, 1982; Maas and Bootsma, 1982), antibody production (Rijkers *et al.*, 1980a; Anderson *et al.*, 1982), immune effector mechanisms (Graves *et al.*, 1985a, b), long-term retention of antigen or antigen-antibody complexes in the MMC (Lamers and de Haas, 1985) and possibly immune memory. The subtle structural pathological changes seen in the haematopoeitic tissue and MMC of the pronephros and spleen could have contributed to the suppressed lymphocyte proliferation and lowered the magnitude of the antibody production.

The involvement of MMC of the pronephros and spleen in the long-term retention of antigen or antigen-antibody complexes, especially particulate and whole cell antigens is well documented (Secombes and Manning, 1980; Maas and Bootsma, 1982; Lamers and Pilarczyk, 1982; Lamers and de Haas, 1985) and they are regarded as analogous to the germinal centres of higher vertebrates (Ellis, 1989). In view of the importance of MMC, the interference caused by cadmium and copper resulting in the fragmentation of MMC and the reduced ability of macrophages to form discrete nodular centres appears to be very important. Further work would be worth pursuing.

The concept of stress has evoked much attention in recent years and it has been shown that cortisol, the main glucocorticoid of teleosts (Gupta and Hanke, 1983) is released during stressful conditions (Schreck, 1982). The indirect action of the heavy metals cadmium and copper, acting as stressors and activating the hypothalamus-pituitary-interrenal axis (Schreck and Lorz, 1978; Donaldson, 1981), resulting in the elevation of plasma cortisol, has been confirmed in the present study in carp. Plasma corticosteroids which affect both metabolic and physiological pathways, rise

dramatically in fish in response to stressful stimuli (Donaldson, 1981; Schreck, 1981). Low levels of environmental contaminants including heavy metals have been previously shown to elevate plasma cortisol in fish (Donaldson and Dye, 1975; Schreck and Lorz, 1978; Bennett and Wolke, 1987a). This is a characteristic primary stress response (Mazeaud *et al.*, 1977) the components of which are thought to have immunosuppressive effects (Ellis, 1981).

Chronic stress in the form of environmental contaminants resulted in prolonged elevation of plasma cortisol, as seen here for copper in carp and elsewhere (Schreck and Lorz, 1978; Bennett and Wolke, 1987a). Chronic stress commonly encountered in aquaculture practices also often results in prolonged elevation of cortisol levels, and the fish require a relatively long time to recover and acclimatize (Redgate, 1974; Pickering and Stewart, 1984).

Elevated levels of corticosteroids have been associated with increased susceptibility of fish to bacterial (Chen *et al.*, 1983; Maule *et al.*, 1987; Peters *et al.*, 1988), viral (Wechsler *et al.*, 1986), fungal (Pickering and Duston, 1983; Pickering and Pottinger, 1985) and parasitic (Robertson *et al.*, 1969; Woo *et al.*, 1987) diseases. In the majority of these studies, exogenous corticosteroid implantation was utilized to show the link between stress induced immunosuppression and disease susceptibility. In this study however, the naturally ocurring level of cortisol was measured and related to the stressor. The increased susceptibility to disease associated with stress, possibly reveals the more covert effects of stress, on both the non-specific and specific components of the defence system.

A protracted elevation of cortisol in teleosts has been shown to suppress or reduce the magnitude of the various components of the immune system such as:

macrophage function (MacArthur and Fletcher, 1985; Stave and Roberson, 1985; Ellsaesser and Clem, 1986), lymphocyte proliferation (Grimm, 1985; Ellsaesser and Clem, 1986; Ralph *et al.*, 1987), antibody production (Anderson *et al.*, 1982), immunocytoadherence rosette response to thymus dependent antigens (Miller and Tripp, 1982), elicitation of protective immunity (Houghton and Matthews, 1986), lymphocytopenia (Pickering, 1984; Peters and Schwarzer, 1985; Ellsaesser and Clem, 1986; Maule *et al.*, 1987), and morphology of lymphoid organs (Van Muiswinkel and Van Ginkel, 1981; Chilmonczyk, 1982; Peters and Schwarzer, 1985; Ghoneum *et al.*, 1986; Saad, 1988).

In recent years several studies have demonstrated the ability of environmental contaminants to alter the susceptibility to disease and the immune response of fish (cited in Chapters 3 to 6). Several of these studies have discussed the components of the stress response in attempting to explain the immunosuppressive effects of the contaminants concerned. With the exception of Bennett and Wolke (1987a), there is no other information on the kinetics of the cortisol response to contaminants at concentrations which have induced impairment in the defence system of the fish. In the present study cadmium (50 μgl^{-1}) and copper (30 μgl^{-1}) have been shown to produce a typical stress response in carp characterised by elevation of plasma cortisol and leucocytopenia which are, respectively, the primary and secondary effects of stress according to Mazeaud *et al.* (1977). At these concentrations, both the metals have also been shown to affect other processes in the defence mechanism of carp. The involvement of the components of the stress response in bringing about some of these effects cannot be dismissed. However, it is difficult to partition and attribute the effects observed in the present study solely to either the direct action of metals or to the effects of the stress component, cortisol.

Considering the results of Chapters 3 to 6 in relation to the cortisol results several interesting inferences can be drawn. Carp exposed to cadmium for 30 days showed a transient elevation of cortisol following 24 hours of exposure, followed by a gradual decrease to significantly lower levels till day 4 after which it remained almost uniform till day 30. However, in carp exposed to copper for 30 days, the cortisol level recorded showed a declining trend from the highest value seen after 24 hours, to a minimum on day 9, after which it showed a second elevation which remained at that level till the end of the experimental duration. Transient elevation of cortisol levels before returning to basal levels within 24 hours have been known in fish subjected to low concentrations of copper (Donaldson and Dye, 1975; Schreck and Lorz, 1978). However, with high copper concentrations (Donaldson and Dye, 1975) over a long exposure period (Schreck and Lorz, 1978) a secondary elevation of plasma cortisol has been reported. Similar observations were made by Bennett and Wolke (1987a) in rainbow trout exposed to Endrin where cortisol levels remained elevated throughout the exposure period of 60 days.

The return of cortisol to basal and near basal levels as seen in the cadmium exposed carp are normally equated with the ability of the fish to recover and acclimatize, whilst secondary elevation and/or cortisol remaining high for a long time as observed with carp exposed to copper is often equated with the inability of the fish to adapt or acclimatize (Donaldson and Dye, 1975). It seems reasonable, therefore, to assume that carp can recover and adapt to low levels of cadmium relatively better than to copper. Support for such an argument comes from the long-term experiments in the present study in Chapter 4. Carp immunized following 30 days of exposure to cadmium responded with high primary and secondary anti-SRBC antibody titres and there was no indication of any suppression of the immune response. Such a response probably indicates the ability of carp to acclimatize to

low levels of cadmium. The finding of genetically selected high antibody responders in chronically polluted waters (Robohm and Sparrow, 1981) is very interesting in this respect. On the other hand, exposure to copper for 30 days before immunization significantly suppressed both the primary and secondary humoral antibody response, possibly reflecting the serious nature of long-term exposure to copper and the inability of carp to recover.

Corticosteroids are believed to be most immunosuppressive when administered before the antigen (Hersch, 1974) in the case of mammals. The significant suppression of the primary anti-SRBC response and primary anti-*A.hydrophila* response shown in carp when immunization and exposure to the metals was concurrent, provides support for such a possible action of corticosteroids. It is worth pointing out that the maximum plasma cortisol levels were recorded in carp 24 hours following exposure to both the metals. Immunization following 10 days exposure to cadmium or copper did not have any significant effect on the primary anti-SRBC respone. In this respect, the timing of the antigen administration in relation to toxicant exposure appears to have a significant effect on the ensuing immunomodulation and should be given some consideration before drawing inferences. Similar immunosuppressive effects in terms of lowering the antibody titre have been shown with exogenous corticosteroids administered to fish immediately after (Anderson *et al.*, 1982) or 24 hours before (Wechsler *et al.*, 1986) immunization. With antibiotics, Grondel *et al.* (1987) found the primary anti-SRBC response to be most suppressed when oxytetracycline treatment was commenced one day before immunization.

Carp previously immunized to *I. multifiliis* which were then exposed to cadmium or copper for 10 days could not mount a protective immune response on subsequent

challenge. This inability was not found to be associated with any lowering of the "anti-ich" antibody titre. Similar observations were made with juvenile carp administered exogenous corticosteroids 14 days after the establishment of protective immunity to *I. multifiliis* by Houghton and Matthews (1986). Chen *et al.* (1983) also found no reduction in circulating antibody to *A. salmonicida* when exogenous corticosteroid, triamcinolone acetonide, was injected 6 days after antigen administration. Thus corticosteroid induced suppression of protective immunity in fish appears to be due to impaired cellular responses and corticosteroids do not appear to lower the established antibody levels.

Stressors commonly encountered in aquaculture practices such as handling, transport, social interaction amongst individual fish and aggressiveness for relatively short periods of time are capable of remarkable immunosuppressive effects such as: suppression of natural cytotoxic cell activity in subordinate tilapia (Ghoneum *et al.*, 1988), degenerative changes in the haematopoietic organs (Peters and Schwarzer, 1985), increased susceptibility and bacteriaemia (Walters and Plumb, 1980; Peters *et al.*, 1988) and reduction in the immunocompetence of lymphoid cells (Miller and Tripp, 1982; Ellsaesser and Clem, 1986). These studies bring to light the dangers of stress respose on the non-specific and/or specific immune response, and clearly highlight the possible consequences of chronic stressors such as environmental contaminants on the immune system of fish.

The immunomodulatory effects of stress induced by low levels of environmental toxicants may be largely determined by the nature and severity of the toxicant and the duration and kinetics of the cortisol response it induces. It has been shown that cadmium and copper exposure elevate serum cortisol levels and also suppress disease resistance and the immune response. It remains to be determined whether

the immunosuppressive effects observed following cadmium and copper exposure in carp is the result of the metal, or cortisol, or a combination of the two.

In conclusion, the levels of cadmium and copper tested in this study did not bring about total immunosuppression. The ability of both the metals to reduce the magnitude of some of the components of the immune system of carp has been demonstrated. The study did not find any evidence to suggest selective suppression of different branches of the immune system. The degree of general immune suppression, both these metals were capable of producing at the concentrations tested, was significant and sufficient to increase the susceptibility to disease agents and decrease the protective immunity of carp.

In evaluating the immunosuppressive role of environmental pollutants, attention should be given to the degree and the nature of the suppression observed. At moderately low levels (as seen in the present study), contaminants may not bring about total suppression of any particular component of the immune system, but may reduce the magnitude and alter the kinetics of the response such that the fish is predisposed to infection. Only by taking a comprehensive approach, where several closely linked aspects of disease resistance and immune response are considered together, can conclusions be drawn about the immunotoxicology of potential pollutants.

The nature of the experiment, the toxicity of the contaminant and the virulence of the pathogen chosen for evaluating the effect of the toxicant should all be given careful consideration before drawing inferences. Conclusions such as that made by MacFarlane *et al.* (1986) that metals offer protection against bacterial diseases

clearly reflect the importance of the nature of the experiment.

Most toxicants would interfer with and affect a wide range of both specific and non-specific arms of the immune system. The complex nature of the immune system with its delicate interdependence makes it difficult for any study to pin-point the specific effects. From the practical point of view, the ability of fish to combat disease in contaminated waters, would appear to be an ideal tool to be used in studies aimed at evaluating the effects of chronic levels of potential aquatic contaminants. However, flexible laboratory studies permit the investigator to dissect the various segments of the immune system and use them for assessing the immunosuppressive effects of chemical toxicants.

There is, therefore, a serious need for a comprehensive approach in immunotoxicological studies. Disease resistance, specific and non-specific immunity and effector mechanisms should all be taken into consideration while delineating the effects of contaminants. Efforts should be made to correlate immune suppression with protective immunity, which will ultimately form the basis for any water quality or pollution guidelines.

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APPENDICES

Appendix 1

The nominal and measured concentration of cadmium in the experimental tanks during different experiments.

Experiment Number	Nominal Concentration (μgl^{-1})	Actual Concentrations (μgl^{-1})
3.3.1	10	9.70 ± 1.20 (n=10)
	25	22.24 ± 2.64 (n=10)
	50	45.54 ± 5.65 (n=12)
3.3.2	10	8.97 ± 2.45 (n=6)
	25	24.80 ± 4.81 (n=10)
	50	48.80 ± 5.98 (n=12)
3.3.3	10	7.80 ± 1.50 (n=6)
	25	21.50 ± 2.40 (n=8)
	50	47.75 ± 5.25 (n=10)
3.3.4	10	9.50 ± 1.90 (n=6)
	25	23.80 ± 4.65 (n=8)
	50	51.45 ± 5.35 (n=9)
4.3.1	50	53.90 ± 6.50 (n=9)
4.3.2	50	49.00 ± 4.65 (n=9)
4.3.3	50	46.85 ± 6.50 (n=10)
4.3.4	50	48.30 ± 4.80 (n=12)
4.3.5	50	51.35 ± 5.23 (n=9)
5.3.1	50	46.67 ± 4.35 (n=6)
5.3.2	50	52.70 ± 1.54 (n=9)
5.3.3	50	48.60 ± 2.65 (n=9)
5.3.4	50	50.26 ± 3.45 (n=12)
6.3.1	50	52.90 ± 3.25 (n=10)
7.3.1	50	46.75 ± 5.40 (n=12)

Appendix 2

The nominal and measured concentration of copper in experimental tanks during different experiments

Experimental Number	Nominal Concentration μgl^{-1}	Actual Concentration μgl^{-1}
3.3.1	10	8.65 ± 2.60 (n=9)
	25	21.00 ± 3.45 (n=9)
	50	45.70 ± 5.75 (n=9)
3.3.2	10	9.50 ± 3.65 (n=6)
	25	21.75 ± 4.70(n=4)
	50	46.50 ± 5.80 (n=4)
3.3.3	10	9.00 ± 2.50 (n=6)
	25	23.60 ± 3.50 (n=6)
	50	47.90 ± 3.75 (n=8)
3.3.4	10	8.50 ± 1.50 (n=3)
	25	23.85 ± 4.65 (n=6)
	50	48.90 ± 4.70 (n=9)
4.3.1	30	27.75 ± 2.90 (n=8)
4.3.2	30	28.40 ± 4.50 (n=12)
4.3.3	30	26.70 ± 2.50(n=12)
4.3.4	30	29.80 ± 4.20 (n=12)
5.3.1	30	27.50 ± 3.10 (n=9)
5.3.2	30	31.40 ± 1.50 (n=6)
5.3.3	30	28.40 ± 2.85 (n=12)
5.3.4	30	31.15 ± 1.95 (n=6)
6.3.1	30	27.80 ± 2.65 (n=8)
7.3.1	30	28.50 ± 5.30 (n=12)

Appendix 3

Physico-Chemical characteristics of the water made use in the experiments

Parameter	Mean	Range
Temperature (°C)	27.50 ± 1.05	25.50 - 28.25
Dissolved Oxygen (mgl^{-1})	8.65 ± 0.46	7.83 - 8.95
pH	6.80 ± 0.85	6.50 - 7.40
Total alkalinity (mgl^{-1} as $CaCo_3$)	43.50 ± 3.90	40 - 48
Total hardness (mgl^{-1} as $CaCo_3$)	35.70 ± 4.75	35 - 40

Appendix 4

Tissue Fixation

All tissues for histological observations were fixed in 10% neutral buffered formalin and left for atleast one week before processing.

Tissue Processing

All tissues to be processed were cassetted, labelled and autoprocessed on a Histokine tissue processor (Histokinette 2000). This involved passing tissues through different alcohol grades, followed by absolute alcohol, chloroform and then impregnation in molten wax. The detailed processing schedule is given below:

Processing Routine for Automatic Tissue Processor

50% methylated spirit	1 hour
80% methylated spirit	2 hours
100% methylated spirit	2 hours
100% methylated spirit	2 hours
100% methylated spirit	2 hours
Absolute alcohol	2 hours
Chloroform	2 hours
Chloroform	1 hour
Chloroform	1 hour
Paraffin wax	2 hours
Paraffin wax	2 hours

After processing the tissues were blocked in suitably sized moulds using molten wax and were cooled rapidly on a cold plate.

Sectioning

Tissue blocks were trimmed to bring the tissue to the surface of the block and whenever necessary surface decalcification was carried out by treating the surface of the trimmed blocks with a rapid decalcifier (RDC-Histolab). The blocks were then washed, cooled on a cold plate and 5 µm sections were cut on a Leitz-Wetzlar microtome using Richert-Jung disposable microtome blades. Thin sections were floated on a water bath maintained at 40oC and were collected on prewashed wet glass slides. The slides were then marked and dried before they were stained.

Staining

For general observations all the sections were stained with Haematoxylin and Eosin. Special stains such as periodic-acid and Schiff's (PAS) and Alcian blue (AB) were carried out as when necessary to demonstrate specific components of the tissue. Procedures as outlined in Carlton's histological techniques (Drury and Wallington, 1980) were followed for the preparation of stains and staining methods. Stained sections were mounted on synthetic mounting medium (Pertex-histolab).

Appendix 5

Haematoxylin-Eosin Staining

(1) Bring sections to water by baths in :
   Xylene 5 minutes
   Absolute alcohol 2 minutes
   Methylated spirit 1.5 minutes
(2) Haematoxylin - 10 minutes
(3) Wash in tap water - 1 minute
(4) Differentiate in 1% Acid alcohol
(5) Wash in tap water - 1minute
(6) Scott's tap water substitute - 1 minute
(7) Eosin - 3 minutes
(8) Methylated spirit - 30 seconds
(9) Absolute alcohol - 2 minutes
(10) Absolute alcohol - 1.5 minutes
(11) xylene - 5 minutes
(12) Mount in synthetic resin

Appendix 6

Periodic Acid - Schiff's (P.A.S) Staining

(1) Sections to water
(2) 1% Periodic acid - 10 minutes
(3) Wash in tap water - 5 minutes
(4) Schiff's reagent - 20 minutes
(5) Wash in tap water
(6) Haematoxylin - 5 minutes
(7) Wash in tap water
(8) Differentiate in 1% Acid aclcohol
(9) Blue in Scott's tap water substitute
(10) Wash in tap water
(11) Methylated spirit - 30 seconds
(12) Absolute alcohol - 1 minute
(13) 0.3% Tartrazine in cellosolve - 3 minutes
(14) Absolute alcohol - 1.5 minutes
(15) Xylene - 5 minutes
(16) Mount in synthetic resin

Results: P.A.S positive : Red or magenta
Nuclei : Blue

Appendix 7

Materials Used for Cortisol Radioimmunoassay

(a). BSA-Saline contains 0.9% NaCl and 0.1% bovine serum albumin.

RIA grade BSA	0.2 g
Analar grade NaCl	1.8 g

Dissolved in 200 ml distilled water and stored in fridge at 4°C.

(b). Dextran-coated charcoal contains 0.5% activated charcoal, 0.1% dextran and 0.9% NaCl.

Activated charcoal	1.0 g
Dextran	0.2 g
Analar NaCl	1.8 g

The three constituents were dissolved in 200 ml distilled water and stored in fridge at 4°C.

(c). Aristar grade Ethyl acetate.

(d). Cortisol standards (hydrocortisone) were obtained from Sigma chemicals Co in preweighed vials each containing 50 ug cortisol which was dissolved in 10 ml ethanol to give 5.0 ug/ml. From this, 100 ul was taken into a vial and made up to 10 ml in ethanol to give 50 ng/ml stock solution. From the stock two working solutions were made in ethyl acetate as follows: (1) 800 ul stock plus 9.2 ml ethyl acetate to give 4 ng/ml standard. (2) 1.0 ml from 4 ng/ml standard diluted with 7.0 ml ethyl acetate to give 0.5 ng/ml standard.

(e). Antibody (anti-cortisol-3-(0-carboxymethyl) Obtained in lyophilized form from Steranti research Ltd., was diluted to 2 ml with BSA-saline and and frozen in 100 ul aliquots. For each assay 100 ul was diluted to 5 ml with BSA-saline.

(f). ^{3}H-cortisol (1.2.6.7-^{3H} cortisol) obtained from Amersham radiochemicals was supplied as 250 uCi in 250 ul tolune-ethanol (9:1). From this 20 ul was diluted in 2 ml to give a stock of 20 uCi in 2 ml. Working solution was made up by diluting 100 ul of stock in 5 ml BSA-saline. This working solution has approximately 22 200 dpm in 50 ul.

(g). Scintillation fluid. Unisolve 1 supplied by Koch-Light was used in all the assays to measure radioactivity.