Interaction of immunostimulants and stress on innate defence mechanisms of rainbow trout, *Oncorhynchus mykiss*

By

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Table of contents

Declaration

I, Natalio Garcia Garbi, hereby declare that this thesis has been composed by myself. It is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or qualification.

Signed Date

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List of abbreviations

αMSH	alpha-melanophore stimulating hormone
2ME	2-Mercaptoethanol
ACH_{50}	50 % alternative complement pathway haemolysis
ACTH	adrenocorticotropin hormone
ADC	antibody-dependent cytotoxicity
AIR	adaptive immune response
ANOVA	analysis of variance
AO glucan	glucan supplied by Alpha-Omega Nutrition, USA
APC	antigen presenting cell
C3	third complement component
C3a	third complement fragment a
C3b	third complement fragment b
CFU	colony forming units
cHBSS	complete Hank's balanced salt solution
ConA	concavalin A
CRP	C-reactive protein
CTL	cytotoxic T lymphocyte
CV	coefficient of variation
df	dilution factor
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	disodium ethylenediaminetetraacetic acid
FcR	immunoglobulin receptor
FCS	foetal calf serum, heat inactivated
FFA	free fatty acids
fMLP	formyl-methionine-leucine-phenylalanine
GALT	gut-associated lymphoid tissue
GH	growth hormone
GI	gastrointestinal tract
GVB	gelatin veronal buffer

hIL-1α	human interleukin 1 alpha
hIL-1β	human interleukin 1 beta
HPI	hypothalamic-pituitary-interrenal axis
$hTGF\beta_1$	human transforming growth factor beta 1
hTNF-α	human tumor necrosis factor alpha
IFN-γ	gamma interferon
Ig	immunoglobulin
IgG	immunoglobulin isotype G
IgM	immunoglobulin isotype M
iHBSS	incomplete Hank's balanced salt solution (without Ca^{2+} and Mg^{2+})
IL-1	interleukin 1
IL-10	interleukin 10
IL-8	interleukin 8
IL-8R	interleukin 8 receptor
iNOS or NOS2	inducible nitric oxide synthase
ip	intraperitoneal
L-15	Leibovitz-15 culture medium
LPS	lipopolysaccharide
MAC	membrane attack complex
MAF	macrophage activating factor
MDP	muramyl dipeptide
MEM	minimum essential medium
MHC	major histocompatibility complex
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-y1)-2,5-dyphenyltetrazolium bromide
NaAz	sodium azide
NADPH	nicotinamide adenine dinucleotide phosphatase, reduced form
NBT	nitroblue tetrazolium
NCC	natural cytotoxic cells
NK	natural killer
NOS	nitric oxide synthase
OD	optical density
P/S	solution of 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin in saline

PBL	peripheral blood leukocyte
PI	phagocytic index
PMA	13-phorbol myristate acetate
PMN	polymorphonuclear leukocyte (= neutrophil)
PR	phagocytosis ratio
PRS	phenol red solution
RDA	recommended daily allowance
RNI	reactive nitrogen intermediate
ROI	reactive oxygen intermediate
RRBC	rabbit red blood cells
sIgM	surface isotype M immunoglobulin
SOD	superoxide dismutase
SRBC	sheep red blood cells
TCR	T cell receptor
TD	T cell-dependent
$TGF\beta_1$	transforming growth factor beta 1
Th	T helper cell
Th0	naïve T helper cell
Th1	T helper cell promoting a type 1 immune response
Th2	T helper cell promoting a type 2 immune response
TNF-α	tumor necrosis factor alpha
TSA	tryptone soya agar
TSB	tryptone soya broth
v/v	volume to volume
Vetregardα	Vetregard® supplied by Vetrepharm, England (manufactured in Europe)
Vetregardβ	Vetregard® supplied by Red-star Bioproducts, USA (manufactured in USA)
w/v	weight to volume
xg	times gravity

List of figures

List of tables

List of fish species

Common name used in the thesis	Scientific name
Carp (common; fancy)	*Cyprinus carpio*
Carp (ginbuna crucian)	*Carassius auratus langsdorfii*
Catfish	*Clarias gariepinus*
Catfish (channel)	*Ictalurus punctatus*
Eel (Japanese)	*Anguilla japonica*
Flatfish	*Paralichthys olivaceus*
Goldfish	*Carassius auratus*
Salmon (Atlantic)	*Salmo salar*
Salmon (coho)	*Oncorhynchus kisutch*
Sea bream (gilthead)	*Sparus aurata*
Tilapia	*Oreochromis spilurus*
Tilapia	*Oreochromis aureus*
Tilapia (Mozambique)	*Oreochromis mossambicus*
Tilapia (Nile)	*Oreochromis niloticus*
Trout (rainbow)	*Oncorhynchus mykiss*
Turbot	*Scophthalmus maximus*
Yellowtail	*Seriola quinqueradiata*

Abstract

This study investigated the use of non-specific immunostimulants to alleviate stress-mediated suppression of defence mechanisms and subsequent susceptibility to bacterial pathogens in rainbow trout (*Oncorhynchus mykiss*).

One yeast (1→3),(1→6)-β-glucan and a bacterial peptidoglycan were selected as immunostimulants from a panel of test substances on the basis of enhanced intracellular superoxide generation by kidney macrophages stimulated *in vitro*. Kidney macrophage effector activity was not affected after 1, 2, 3 or 4 weeks of in-feed treatment with 0.05 % or 5 % of glucan or peptidoglycan. However, production of bactericidal superoxide by inflammatory peritoneal macrophages did increase significantly after four weeks of oral treatment with 0.05 % peptidoglycan.

Although a single confinement of fish (93 % reduction of water volume for five minutes) caused a physiological stress response, as indicated by hyperglycaemia in plasma, kidney and inflammatory macrophage activities were only affected after six daily confinements. Phagocytosis, intracellular superoxide production and killing of *Aeromonas salmonicida in vitro* by kidney macrophages were significantly reduced. Conversely, production of extracellular superoxide, which may be associated with damage to self, was enhanced. Peritoneal macrophages displayed a similar but less marked respiratory burst response after repeated confinement.

Some of the alterations in macrophage function caused by daily confinement were prevented by feeding 0.05 % peptidoglycan four weeks before the first confinement. The increase in kidney macrophage extracellular superoxide production caused by repeated confinement was significantly alleviated by in-feed peptidoglycan. Similarly, the decrease in intracellular production by peritoneal macrophages caused by repeated confinement was prevented by in-feed treatment with peptidoglycan. Neither peptidoglycan nor repetitive confinement had any effect on complement lytic activity.

These results indicate that dietary peptidoglycan was able to reduce, by regulating macrophage function, the impact of stress on certain bactericidal defences and potential damage to self. However, there was no significant difference in the persistence of viable *A. salmonicida* in the spleen or blood of infected fish in any of the experimental treatments.

1.1 General introduction

1.1.1 Natural environment of fish

Fish are dependent on water as a medium in which to live. Vital functions, such as respiration, homeostasis, feeding, growth, reproduction, immune function and responses to stimuli, are closely associated with the characteristics of the surrounding aquatic environment (Wedemeyer, 1996). Water quality variation is common in limited water bodies such as estuarine and coastal marine areas as well as smaller fresh water lakes, channels and rivers. Temperature, salinity, dissolved oxygen and chemical run-off derived from agricultural, industrial and urban activities are amongst frequent fluctuations in water quality. Fish are highly adapted to their environment and can tolerate a wide range of variation, although adaptation has an energy cost (Pankhurst and van der Kraak, 1997). However, should tolerance limits be reached or exceeded, the animal's ability to adapt is significantly reduced with subsequent risk of reduced growth, reproduction and even survival (Wedemeyer, 1997)

1.1.2 Farming of Fish

Aquatic animals have been cultured for human consumption for centuries. Fish were initially reared under extensive conditions, where animals are kept at low stocking densities and grown in an environment similar to their natural habitat, such as large enclosed areas, without much husbandry input. Such farming conditions usually impose less strain on the physiological system than intensive farming practices (Wedemeyer, 1996).

In most countries, aquaculture procedures have become increasingly sophisticated leading to intensive aquaculture as economic pressures to improve production have grown. Total fish production can be increased by several thousand-fold through intensification (Shepherd and Bromage, 1988). If the water body is no longer able to meet the biological demands of the fish, such as increased food and oxygen consumption and

metabolic waste dispersal, artificially formulated diets and engineered holding facilities become necessary. Intensive aquaculture is a complex process where fish are grown in conditions far removed from their natural environment. Production costs are raised, and economic success is closely dependent on growth optimisation and minimisation of losses due to infectious and non-infectious agents.

In intensive salmonid aquaculture (for a review on intensive aquaculture, see Shepherd and Bromage, 1988), fish are reared through a farming cycle consisting of hatchery, on-growing and, in some cases, broodstock facilities. In the hatchery, eggs derived usually from captive broodstock are artificially fertilised and incubated in fresh water trays until they hatch. Larvae are then moved to bigger tanks or raceways and feed on nutritional reserves present in the yolk sac for several weeks depending on the species and temperature, after which, fry are weaned onto commercial feed. Once fish reach a certain size, they are moved to on-growing facilities and grown to the marketable size. On-growing of rainbow trout (*Oncorhynchus mykiss*) and other salmonids takes place in freshwater tanks, raceways or ponds as well as in cages in lakes. The on-growing phase of anadromous salmonids, such as Atlantic salmon (*Salmo salar*), is carried out in sea water, usually in marine cages. In this case, fish are transferred from the hatchery to the sea once signs of physiological change (smoltification) are observed.

Animals should be cultured in a stable environment with few disturbances to optimise growth and survival during the farming cycle. However, potentially detrimental changes in the normal day-to-day farming environment are unavoidable at times and they may be natural or artificial in origin. The former include daily or seasonal changes in water quality, physiological changes like smoltification, and interactions between fish, with microorganisms and with predators such as birds and seals. Artificial disturbances are usually associated with fish farming procedures and low water quality as a result of pollution (Wedemeyer, 1996). There are several potential disturbances in the fish environment due to farming practices and they are usually multifactorial.

Hatchery fish are often transported over long distances by road, sea or air to on-growing facilities. On-growing fish are regularly size-graded and redistributed to lessen social confrontation and to obtain similar size classes for marketing purposes. Similarly, prophylactic and therapeutic treatments such as vaccination and drug baths are also carried out during the farming cycle. For most of these procedures, fish are confined in a reduced space and often handled. Low water quality, reduced dissolved oxygen and social aggressiveness also are common consequences of farming procedures. Furthermore, commercial pressures impose high stocking densities. Farmed fish which are subjected to these experiences usually have only been domesticated for a relatively small number of generations.

Such natural or artificial adverse conditions, trigger one of the animal's most important physiological processes, the stress response, characterised by the activation of a cascade of metabolic and hormonal events well described in teleost literature (Pickering, 1981; Sumpter, 1997; Wendelaar Bonga, 1997). These include mobilisation of energy substrates and alterations in the respiratory and cardiovascular systems intended to prepare the animal to avoid, escape, acclimatise to or tolerate the adverse condition (Wendelaar Bonga, 1997). However, many adverse conditions are often chronic because farmed fish cannot escape from the culture environment. Stress-mediated physiological changes may be of little value in evading the stressor and the tolerance limit for acclimatisation may be approached or exceeded with negative consequences on the animal's performance, immune system and consequently survival (Wedemeyer, 1996).

Although inappropriate husbandry may result in high mortalities caused by non-infectious agents, it has been suggested that most losses in intensive salmonid farming have an infectious aetiology (Roberts and Shepherd, 1986). Fish microbial pathogens include viruses, bacteria, fungi and protozoan parasites (Roberts, 1989a) and stress-associated increased susceptibility to infectious diseases has been recognised as an important element of fish losses in aquaculture for several decades (Snieszko 1954;

Schreck, 1982; Wedemeyer and Goodyear, 1984; Peters *et al.*, 1988; Maule *et al.*, 1989; Wise *et al.*, 1993). In salmonid aquaculture, infections caused by ubiquitous microorganisms such as bacteria (e.g., motile aeromonads, pseudomonads and *Vibrio* spp.) and fungi (*Saprolegnia* spp.) may become a source of significant morbidity and losses in the fish stock (Inglis *et al.*, 1993; Wedemeyer, 1997). Obligate pathogens such as viruses (infectious pancreas necrosis virus, viral haemorrhagic septicaemia virus, infectious haemorrhagic necrosis virus), some bacteria (*Aeromonas salmonicida*, *Renibacterium salmoninarum*, *Yersinia ruckeri*) and several protozoan parasites are also responsible for significant disease outbreaks in salmonid aquaculture (Inglis *et al.*, 1993; Wedemeyer, 1997). In the farm aquatic environment, some microorganisms live suspended in the water, in sediments or attached to surfaces at concentrations greater than in open and cleaner waters (Enger, 1992) and, therefore, more frequent interactions between fish and opportunistic pathogens may occur. Only a small number of interactions with microorganisms result in disease, partly due to the highly adapted immune system. However, facultative as well as obligate pathogens may establish severe infections by taking advantage of immunosuppression associated with disturbances of the normal farming environment.

1.2 Host defence against infection

As in other animals, teleost defence mechanisms include external barriers to environmental microorganisms and a highly adapted immune system providing effective protection against invading microorganisms.

1.2.1 Physical barriers

The exposed surfaces of fish provide the first defence against potentially invasive microorganisms. Mucus is found covering skin, gills and the gastrointestinal (GI) tract. Besides trapping microorganisms and reducing their invasiveness, it contains important antibacterial enzymes such as lysozyme, complement and possibly antibacterial

glycoproteins (Lie *et al.*, 1989; Oohara *et al.*, 1991; Magariños *et al.*, 1995). The skin epidermis and the epithelium layering the GI tract and gills provide simple barriers to the entry and establishment of infections. Although great variation occurs in the normal structure and function of these surfaces among fish species, their anti-microbial mechanisms are fundamentally similar and include increased production of mucus and accelerated epidermal or epithelial sloughing in response to irritants like invasive microorganisms (Ferguson, 1989; Roberts, 1989b). As in higher vertebrates, a range of lactic acid producing bacteria are part of the normal microbiota of the GI tract of fish (Ringø and Gatesoupe, 1998), providing a hostile environment for invading microorganisms (Bøgwald *et al.*, 1994).

Most opportunistic pathogens are not able to establish infection on these intact surfaces, and only a small number of obligate pathogens are able to do so. However, injuries on skin and gills caused by water-borne irritants, aggressive behaviour or farming procedures become a portal of entry for opportunistic pathogens present in the aquatic environment and may allow initiation of infection (Evelyn, 1996).

1.2.2 Nonspecific Humoral factors

Once microorganisms enter the host, spread occurs mainly through the blood or direct cell-cell transmission. Although the lymphatic system is an important vehicle for certain infectious agents in higher vertebrates, very little is known about this route in fish. As in other animals, several substances which can kill bacteria or prevent them growing are found in the sera of non-immune fish. Some of these substances act as opsonins in concert with cellular defences to potentiate the microbiocidal effect. Innate humoral factors include complement, lysozyme, C-reactive proteins, interferon, transferrin, ceruloplasmin, lectins, haemolysins, proteinases, chitinases, α2-macroglobulins and others (reviewed by Ingram, 1980; Dash *et al.*, 1993; Yano, 1996). Of these, complement and lysozyme are amongst the most important and most investigated and special attention is dedicated to them in this review.

Complement

The mammalian complement is an enzymatic cascade system that plays a pleiotropic role in defence against infection and inflammation (Tizard, 1995). In protecting against invasive microorganisms, a key event is the formation of active C3b and C3a components. Depending on the stimuli triggering their synthesis, the mammalian complement system is organised in three different and independent activation systems, designated classical, alternative and lectin pathways (Muller-Eberhard, 1988; Sato *et al.*, 1994; Janeway and Travers, 1997). Once formed, C3b covalently opsonises the microorganism surface enhancing their uptake by phagocytes. Furthermore, C3b initiates a cascade reaction which leads to development of the complement membrane attack complex (MAC), a pore-like structure, on the surface of microorganisms and consequent lysis (Muller-Eberhard, 1986; Lim, 1990; Tizard, 1995). C3a and some by-products originated during the activation cascade play an important role in the inflammatory process (Marceau *et al.*, 1987; Hugli, 1990).

Functional classical and alternative pathways of complement activation resulting in target opsonisation and/or lysis are present in the sera of all major vertebrate groups (Pastoret *et al.*, 1998) including teleost fish (Sakai, 1981; Iida and Wakabayashi, 1983; Sakai, 1983; Ingram, 1987; Yano, 1988; Sakai, 1992; Press, 1998). Evidence suggesting a lectin pathway in the sera of a urochordate, the eminent predecessor of vertebrates, has been published (Ji *et al.*, 1997). However, teleost complement molecular organisation and function are poorly characterised to date. Progress is being made, however, as different proteins are being purified or their genes sequenced in several species (Lambris *et al.*, 1993; Tomlinson *et al.*, 1993; Yano, 1996; Lambris *et al.*, 1997; Nakao *et al.*, 1997; Sunyer *et al.*, 1997; Fujii and Kunisada, 1998).

Lysozyme

Lysozyme is a hydrolytic enzyme with antibacterial properties (Jollés, 1969). It is widely distributed throughout different taxa, from plants and invertebrates to all major vertebrate groups. Lysozyme digests the cell wall of bacteria by attacking β-1-4 linked N-acetylmuramamines and N-acetylglucosamines, the major components of peptidoglycan, present in the bacterial cell wall. The lytic activity of lysozyme is enhanced by previous exposure to complement MAC and this is necessary for the lysis of Gram-negative bacteria (Jollés and Jollés, 1984). Lysozyme has been shown to be bactericidal to important fish microbial pathogens (Grinde, 1989; Yousif *et al.*, 1994) and is found in most tissues and secretions of fish, especially those regularly exposed to microorganisms, such as haemopoietic organs, plasma, GI tract, gills and mucus (Grinde *et al.*, 1988; Lie *et al.*, 1989; Oohara, 1991). However, considerable variation in both activity and organ distribution exists between different species of teleost fish and even within individuals of the same species (Alexander and Ingram, 1992; Yano,1996).

1.2.3 Phagocyte microbiocidal mechanisms

While internalising invading microorganisms, phagocytes in the blood and tissues switch on potent oxygen dependent and independent microbiocidal mechanisms which, in most cases, lead to the destruction of phagocytosed material.

Phagocytosis

Engulfment of microorganisms by host cells, first discovered by Metchnikoff (1887), is a basic defence mechanism against invading pathogens. Since then, it has been demonstrated across all animal taxa (Horton and Ratcliffe, 1998).

Among mammals, the cell types displaying phagocytic activity are well characterised. They comprise the so-called professional phagocytes (neutrophils, monocytes and macrophages in blood and other tissues), non-professional phagocytes

(epithelial cells, endothelial cells and fibroblasts) and para-professional phagocytes (dendritic cells and retinal epithelial cells) (Rabinovitch, 1995). In teleost fish, phagocyte cell types are less well characterised, although it is known that macrophages, monocytes and neutrophils (PMNs) in blood, tissues and inflammatory exudates are avidly phagocytic (McKinney *et al.*, 1977; Braun-Nesje *et al.*, 1981; Thuvander *et al.*, 1987; Secombes and Fletcher, 1992; Pedrera *et al.*, 1993; Lamas and Ellis, 1994a,b; Brattgjerd *et* al., 1996; Dalmo *et al.*, 1997). Granulocytes other than PMNs are not common in the peripheral circulation of most teleosts investigated (Hine, 1992). Inflammatory basophils have been shown to be phagocytic to a lesser extent than inflammatory macrophages (Suzuki, 1986), and basophils and eosinophils in intestinal tissues of carp have been reported to be phagocytic *in vivo* (Steinhagen and Jendrysek, 1994). Far less information is available on the phagocytic ability of cells other than leukocytes, although epithelial and endothelial cells in the head kidney of rainbow trout have been shown to phagocytose *in vivo* (Dannevig *et al.*, 1994; Peters *et al.*, 1991).

Microbial internalisation is initiated by the interaction of receptors on the surface of the phagocyte with ligands present on the target cell. Different opsonin receptors have been identified in mammalian species, including those for complement fraction C3b (CD11b/CD18) and various immunoglobulins (FcRs), which promote the phagocytosis of complement or immunoglobulin opsonised microorganisms respectively (Brown, 1994a; Swanson and Baer, 1995). Certain receptors have been shown to bind directly to microbial structures, such as lipopolysaccharide-binding CD14 (Wurfel *et al.*, 1995), glucan- and LPS- binding CD11b/CD18 (Ross *et al.*, 1987; Rosen and Law, 1990; Ross and Vetvicka, 1993), carbohydrate-binding lectin receptors (Ofek, 1989) and macrophage multiligand scavenger receptors (Krieger *et al.*, 1993; Dunne *et al.*, 1994). Phagocytosis of microorganisms *in vivo* is usually mediated by the activation of different receptors (Ofek *et* al., 1995; Ernst, 1998).

There is considerable indirect evidence that phagocytosis by teleosts cells is mediated by receptors. Fish phagocytes are able to discriminate between targets (Ainsworth, 1990) and pre-treatment of macrophages with trypsin greatly reduces the uptake of opsonised particles (Matsuyama *et al.*, 1992b). Opsonisation with non-immune or immune sera as well as with C3 usually increases phagocytic activity (Honda *et* al., 1986; Sakai, 1984; Michel *et al.*, 1990; Matsuyama *et al.*, 1992b; Rose and Levine, 1992; Chen *et al.*, 1998), suggesting the existence of complement and immunoglobulin receptors on the phagocyte surface. Although FcR for IgM has been demonstrated in more primitive cartilaginous fish (Haynes *et al.*, 1988), neither FcR nor complement receptors have been identified, isolated or the relevant gene cloned from teleost fish. Very little is known about non-opsonic phagocytosis in fish. In the tilapia (*Oreochromis spilurus*), macrophage lectin-type surface receptors have been suggested to mediate phagocytosis of non-opsonised bacteria (Saggers and Gould, 1989). As in mammals, it has been shown that glucans bind specifically to a surface receptor on the teleost phagocyte (Engstad and Robertsen, 1993; Engstad and Robertsen, 1994) and, therefore, it has been suggested that a Mac-1 (or CD11b/CD18)- like receptor is present on catfish neutrophils (Ainsworth, 1994).

Recognition of foreign particles by phagocyte receptor(s) progresses, via an actin based mechanism, to internalisation of the particle within a phagosome (Kodama *et al.*, 1994b; Allen and Aderem, 1996). This process is coupled with initiation of potent intra- and extra-cellular microbiocidal mechanisms by the phagocyte. Bactericidal capacity of fish macrophages and PMNs *in vitro* is well documented (Graham *et al.*, 1988; Whyte *et al.*, 1989; Daly *et al.*, 1994; Espelid and Jørgensen, 1992; Iida *et al.*, 1993; Jørgensen *et al.*, 1993b; Lamas and Ellis, 1994b; Leung *et al.*, 1995; Hardie *et al.*, 1996; Itou *et al.*, 1997). As in mammals, microbiocidal mechanisms include (i) production of reactive oxygen- and, less conclusively, nitrogen- intermediates, (ii) release of a variety of enzymes into the phagosome (phagolysosome) and (iii) phagolysosome acidification.

Respiratory burst

The respiratory burst is a distinguishing property of all mammalian phagocytes and it is characterised by activation of plasma membrane-bound nicotinamide adenine dinucleotide phosphate (NADPH) oxidase resulting in the formation of superoxide anion ($O_2^{\cdot}$) (reviewed by Seifert and Gunter, 1991; Henderson and Chappell, 1996). NADPH oxidase is inactive in resting cells and is turned on rapidly and momentarily by particulate (zymosan, latex beads, bacteria, fungus etc.) and soluble (phorbol myristate acetate (PMA), f-Met-Leu-Phe (fMLP), etc.) stimuli. As illustrated in figure 1.1, activated NADPH oxidase catalyses the production of superoxide anion, and other reactive oxygen intermediates (ROIs) are rapidly formed from this precursor by spontaneous or enzymatic catalysis. The microbiocidal effect of ROIs is mostly mediated through protein oxidation, lipid peroxidation and deoxyribonucleic acid (DNA) damage to target cells (Gille and Sigler, 1995; Burch, 1989; Babior, 1984).

The respiratory burst is a process believed to be phylogenetically conserved, occurring across plant and animal taxa (Dikkebboom *et al.*, 1985; Nakamura *et al.*, 1985; Larson *et al.*, 1989; Bachere *et al.*, 1991; Le Gall *et al.*, 1991; Anderson *et al.*, 1992b; Oda *et al.*, 1992; Pipe, 1992; Kumazawa *et al.*, 1993; Song and Hsieh, 1994; Stabler *et al.*, 1994; Austin and Paynter, 1995; Lamb and Dixon, 1997). Teleost phagocytes in blood, haemopoietic organs and the peritoneal cavity have been reported to synthesise a variety of ROIs upon stimulation with different particulate and soluble stimulants (Scott and Klessius, 1981; Higson and Jones, 1984; Chung and Secombes, 1988; Secombes *et al.*, 1988; Zelikoff *et al.*, 1991; Anderson *et al.*, 1992a). Rainbow trout macrophages have been shown to posses a membrane bound NADPH oxidase-like enzyme (Secombes *et al.*, 1992), and the kinetics of oxygen consumption by Japanese eel (*Anguilla japonica*) PMNs during the respiratory burst was similar to the pattern observed by mammalian PMNs (Itou *et al.*, 1996).

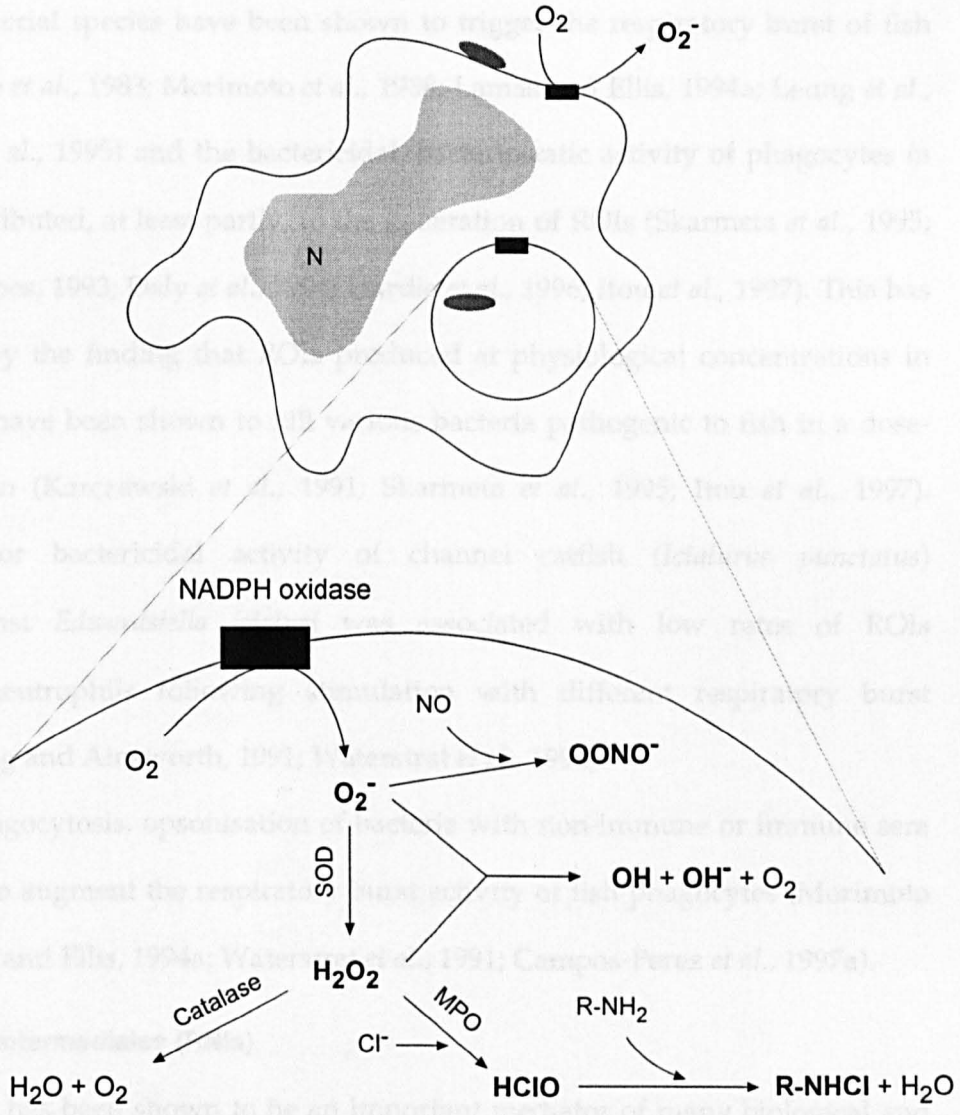

Figure 1.1 Reactive oxygen intermediates produced during the respiratory burst in MPO-positive phagocytes.

Stimulation of the plasma or phagosome membrane activates the NADPH oxidase with the subsequent release of O_2^- and other microbiocidal ROIs (in bold). In this illustration, NADPH oxidase is activated by contact with a microorganism. N, nucleus; NO, nitric oxide; $OONO^-$, peroxinitrite; SOD, superoxide dismutase; MPO, myeloperoxidase. R-NH2, free amines; R-NHCl, chloramines. Mammalian macrophages contain low levels of MPO, though they may increase its concentration by binding PMN-derived MPO via the mannose receptor (Leung and Goren, 1989). Information collated from Klebanoff and Hamon (1972); Zgliczynski and Stelmaszynska (1975); Thomas (1979)); Gabig and Babior (1981); Babior (1984); Burch (1989); Lincoln *et al.* (1995); Marcinkiewicz (1997); MacMicking *et al.* (1997).

Several bacterial species have been shown to trigger the respiratory burst of fish phagocytes (Stave *et al.*, 1983; Morimoto *et al.*, 1988; Lamas and Ellis, 1994a; Leung *et al.*, 1995; Skarmeta *et al.*, 1995) and the bactericidal/bacteriostatic activity of phagocytes *in vitro* has been attributed, at least partly, to the generation of ROIs (Skarmeta *et al.*, 1995; Sharp and Secombes, 1993; Daly *et al.*, 1994; Hardie *et al.*, 1996; Itou *et al.*, 1997). This has been supported by the finding that ROIs produced at physiological concentrations in cell-free systems have been shown to kill various bacteria pathogenic to fish in a dose-dependent fashion (Karczewski *et al.*, 1991; Skarmeta *et al.*, 1995; Itou *et al.*, 1997). Interestingly, poor bactericidal activity of channel catfish (*Ictalurus punctatus*) neutrophils against *Edwardsiella ictaluri* was associated with low rates of ROIs production by neutrophils following stimulation with different respiratory burst activators (Dexiang and Ainsworth, 1991; Waterstrat *et al.*, 1991).

As with phagocytosis, opsonisation of bacteria with non-immune or immune sera has been shown to augment the respiratory burst activity of fish phagocytes (Morimoto *et al.*, 1988; Lamas and Ellis, 1994a; Waterstrat *et al.*, 1991; Campos-Perez *et al.*, 1997a).

Reactive nitrogen intermediates (RNIs)

Nitric oxide (NO) has been shown to be an important mediator of many biological and immune functions, including inflammation and cytotoxicity to invading organisms and tumor cells (James, 1995; MacMicking *et al.*, 1997; Nathan, 1997). NO is an intermediate product generated by the activation of cytosolic nitric oxide synthase (NOS), which catalyses the oxidation of L-arginine into L-citrulline (Hibbs *et al.*, 1988; Marletta *et al.*, 1988) (figure 1.2). In mammalian species, three isoforms of NOS have been identified in most cell types (MacMicking *et al.*, 1997). Activation of the Ca^{2+}-independent inducible isoform of NOS (iNOS or NOS2), mostly present in macrophages, results in high-output cytotoxic NO production (Nathan, 1992; Xie *et al.*, 1992; Fang, 1997; MacMicking *et al.*, 1997). Full activation of NOS2 usually requires a combination of cytokine(s) and microbial products such as bacterial LPS (Nathan and Xie, 1994).

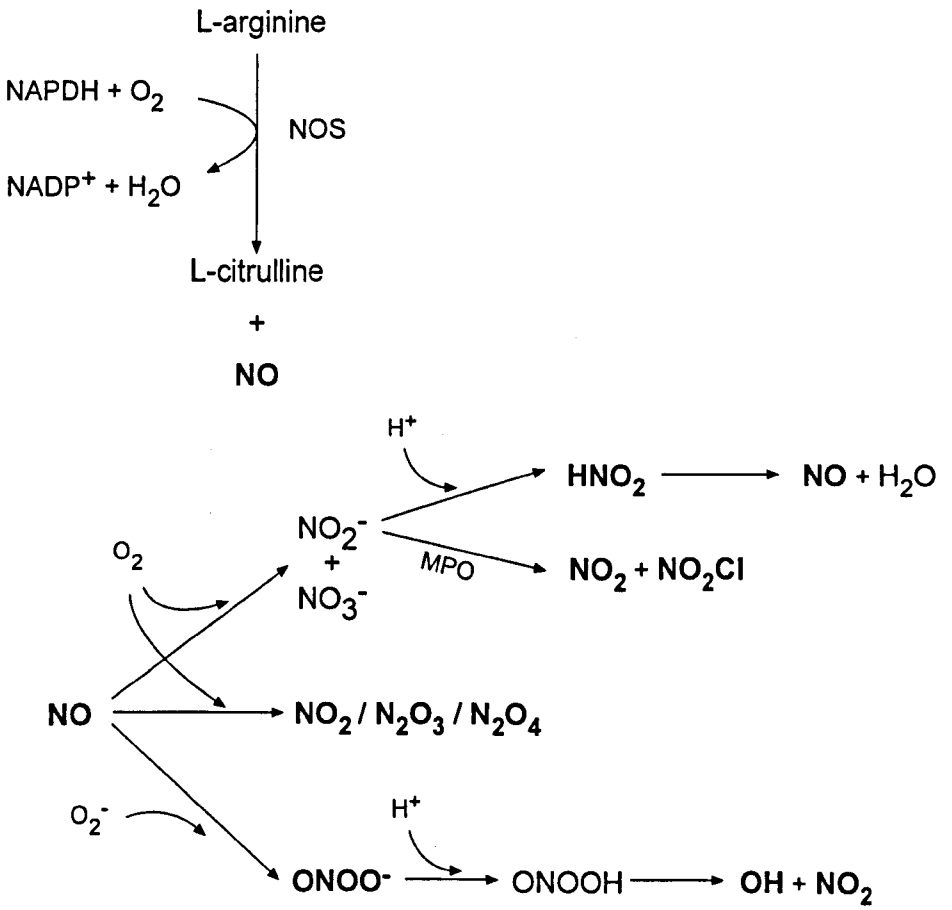

Figure 1.2 Reactive nitrogen intermediates formed during the activation of NOS2.
Nitric oxide (NO) is formed by catalytic activation of cytosolic NOS2 in stimulated phagocytes. Other RNIs are produced through subsequent oxidation and reduction. RNIs in bold are known mediators of cytotoxicity or inflammation. Information collated from Beckman *et al.* (1990); Nathan (1992); Klebanoff (1993); MacMicking *et al.* (1997); Michel and Feron (1997); Eiserich *et al.* (1998).

NO is a lipid- and water-soluble biologically active free radical gas which, in physiological *milieu*, is rapidly oxidised or reduced into other reactive nitrogen compounds collectively termed RNIs (figure 1.2). The molecular targets of RNIs in microorganisms and other cells are diverse and their effects include DNA damage, impairment of FeS-containing enzymes and interference with signal transduction (Kolb and Kolb-Bachofen, 1992; Nathan, 1992; Eiserich *et al.*, 1998).

Nitric oxide has been shown to be produced by phagocytes from a number of invertebrate and vertebrate species upon immune activation (MacMicking *et al.*, 1997). In recent years, increasing evidence has emerged indicating that teleost fish are able to synthesise nitric oxide as an inducible response. Schoor and Plumb (1994) were first to suggest iNOS activity in the head kidney of a teleost species, channel catfish, following injection of live *E. ictaluri*. Macrophages isolated from goldfish (*Carassius auratus*), catfish (*Clarias gariepinus*) and gilthead sea bream (*Sparus aurata*) have since been shown to synthesise NO *in vitro* upon stimulation with LPS and/or macrophage activating factor (MAF) (Wang *et al.*, 1995; Neumann *et al.*, 1995; Neumann and Belosevic, 1996; Neumann and Belosevic, 1997; Yin *et al.*, 1997; Mulero and Meseguer, 1998). Similarly, iNOS activity has been demonstrated in rainbow trout mixed leukocytes cultures stimulated with muramyl dipeptide (Zunic and Licek, 1997). Low concentrations of nitrite in serum have been observed in rainbow trout injected with *R. salmoninarum*, although the production pathway was not identified (Campos-Perez *et al.*, 1997b). Molecular demonstration of teleost iNOS also has been provided. iNOS messenger ribonucleic acid (mRNA) has recently been sequenced from rainbow trout and goldfish macrophages (Grabowski *et al.*, 1996; Laing *et al.*, 1996).

Very few reports have been published on the role of RNIs in the immune response of fish. Physiological concentrations of nitric oxide and/or other RNIs produced in cell-free systems are known to be toxic to several fish pathogens (Campos-Perez *et al.*, 1997b), and the bactericidal capacity of catfish activated macrophages against *Aeromonas*

hydrophila in vitro was partially arrested by blocking NO synthesis (Yin *et al.*, 1997). However, Hardie *et al.* (1996) showed that killing of the intracellular pathogen *R. salmoninarum* by rainbow trout activated macrophages was associated with a NOS independent process.

Oxygen-independent microbiocidal mechanisms

Besides ROIs and RNIs, phagocytes posses a range of enzymes and peptides displaying oxygen-independent microbiocidal capacity (Boman, 1991). Amongst them, lysosomal acid and alkaline phosphatases, lysozyme, defensins and lactoferrin are particularly important (Elsbach and Weiss, 1988; Lehrer *et al.*, 1989; Lehrer and Gantz, 1990; Lehrer *et al.*, 1993).

Very little is known about oxygen-independent killing by teleost phagocytes. Although the production of some of these substances is enhanced following activation of macrophages (Chung and Secombes, 1987; Secombes, 1988; Sveinbjørnsson and Seljelid, 1994; Dalmo and Seljelid, 1995; Bøgwald *et al.*, 1996; Dalmo *et al.*, 1996a), their role in phagocyte killing is poorly understood (Secombes, 1996).

1.2.4 Cell mediated cytotoxicity

In mammals, certain cytotoxic lymphocytes are able to target infected, tumor and non-self cells effectively. Cytotoxicity is mediated by direct recognition of target cells and extracellular release of enzymes (perforins and granzymes), which are toxic and/or induce lysis of targets (Tizard, 1995). Two different lymphocyte sub-populations are cytotoxic: natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). CTLs direct their cytotoxicity against specific targets in a major histocompatibility complex (MHC) class I restricted fashion (Rook and Balkwill, 1998). On the other hand, NK cell activation is not MHC restricted since they target infected or tumor cells which do not express MHC class I molecules but several other ligands (Ljunggren and Kärre, 1990; Moretta *et al.*, 1992; Moretta *et al.*, 1996; Lanier, 1998). In addition, NK cells can also direct cytotoxicity

against targets opsonised with IgG antibodies, a process called antibody dependent cellular cytotoxicity (ADCC) (Perussia *et al.*, 1984; Lanier, 1998).

Evidence of cell mediated cytotoxicity by teleost leukocytes other than phagocytes has been accumulating in recent years. Several reports have shown that lymphocyte-rich fractions isolated from blood, lymphoid organs and the peritoneal cavity of several teleost fish species mediate spontaneous cytotoxicity against a variety of xenogeneic and allogeneic cells, virus-infected autologous and allogeneic cells, and protozoan parasites without need of previous exposure (Evans *et al.*, 1984; Graves *et al.*, 1985; Moody *et al.*, 1985; Faisal *et al.*, 1989a; Greenlee *et al.*, 1991; Hogan *et al.*, 1996; Seeley and Weeks-Perkins, 1997; Stuge *et al.*, 1997). Teleost spontaneous cytotoxic cells appear to be the functional equivalent of mammalian NK cells and are referred to as NK-like or natural cytotoxic cells (NCC) (Evans and Jaso-Friedman, 1992). However, very little is know about the NCC mechanism of action. As in higher vertebrates, cytotoxicity has been shown to be initiated by direct contact with the target cell and possible enzyme release (Bielek, 1988; Evans and Jaso-Friedman, 1992; Carlson *et* al., 1985), which results in target apoptosis (Greenlee *et al.*, 1991). Activatory/inhibitory NCC receptors have not yet been identified, although there is some evidence indicating their presence (Evans *et al.*, 1990; Yamaguchi *et al.*, 1996).

Genetic restricted cytotoxicity in fish, the functional equivalent to mammalian CTL-mediated cytotoxicity, has been suggested in few reports (Hashimoto and Ikeda, 1987; Verlhac and Deschaux, 1987; Yoshida *et al.*, 1995; Fisher *et al.*, 1998 ; Mckinney and Schmale, 1997), and elegantly confirmed in ginbuna crucian carp (*Carassius auratus langsdorfii*) by using different isogeneic fish lines and cell lines originating from them as targets (Hasegawa *et al.*, 1998).

1.2.5 Antibody production

The adaptive immune response (AIR) is characterised by the display of diverse antigen-specific surface receptors (T cell receptor and immunoglobulin receptor), diverse class II

proteins encoded by the MHC, and cytokines regulating immune responses (Janeway and Travers, 1997). In mammalian immunology, the antibody response to many antigens is T cell-dependent (TD). In this response, complex MHC-T cell receptor (TCR) restricted interactions involving antigen presenting cells (APC), $CD4^+$ T helper (Th) cells and B cells are required for antibody production (Rook and Balkwill, 1998). Fish above agnatha (jawless fish) in the evolutionary scale have been shown to possess the basic mechanisms required for an adaptive immune response (Flajnik, 1996; Klein, 1997; Warr, 1997; Press, 1998). As a result of functional AIR, fish are able to produce antibodies against specific microbial antigens both by artificial (Ellis, 1988; Kaattari and Piganelli, 1996; Gudding *et al.*, 1997) and natural (Robohm *et al.*, 1979; Evans *et al.*, 1997) immunisation. Thus, invasive pathogens and their exotoxins for which an AIR has been produced will face, besides the innate microbiocidal mechanisms described earlier, the direct effects of the antibody molecule. The *in vitro* effects of the immunoglobulin molecule in mammalian species include antigen agglutination, precipitation and neutralisation, while the *in vivo* effects in healthy patients are immune complex formation, solubilisation by complement binding and removal (Janeway and Travers, 1997). Similar *in vitro* effects of the immunoglobulin molecule have been observed in different species of teleosts (Kaattari and Piganelli, 1996). Furthermore, antibody-opsonised microorganisms are usually more susceptible to killing by fish innate defence mechanisms (Lamas and Ellis, 1994a; Chen *et al.*, 1998).

The phenomena which characterise mammalian immunological memory (logarithmic increases in monomeric immunoglobulin (Ig) production, isotype switching to high affinity IgG, and enhanced clonal proliferation) have not been observed, at least to the same extent, in fish (reviewed by Kaattari and Piganelli, 1996). Only one Ig isotype with functional relevance, IgM, has been found in fish sera to date. However, increases in IgM production and binding affinity as well as enhanced sensitivity to antigen have all been reported, indicating that fish are able to display a differentiated response to

secondary immunisation (Arkoosh and Kaattari, 1991; Kaattari and Piganelli, 1996; Zhang *et al.*, 1997).

1.2.6 Regulation of immune response: the role of cytokines

In mammalian species, it is well known that phagocyte microbiocidal activity, lymphocyte helper function, cytotoxicity and antibody production, amongst other immune functions, are processes closely orchestrated by a range of cytokines, such as diverse interleukins, gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α). As illustrated in figure 1.3, they are secreted by phagocytes, Th cells and NK cells amongst others, and provide a link between innate and adaptive immune responses directed towards the effective clearance of invading microorganisms (Fearon and Locksley, 1996; Boehm *et al.*, 1997; Medzhitov and Janeway, 1997; Unanue, 1997b).

Although several lines of evidence indicate the existence of leukocyte-derived cytokines in fish, their diversity and regulatory role are largely unknown (Secombes *et al.*, 1996; Press, 1998). Mixed peripheral blood leukocytes from several teleost species stimulated *in vitro* with concavalin A (ConA), a potent T-cell stimulator, have been shown to secrete cytokine(s) (macrophage activating factor, MAF) which upregulate macrophage microbiocidal mechanisms (Graham and Secombes, 1990a,b; Neumann *et al.*, 1995; Mulero and Meseguer, 1998). In rainbow trout, it has been demonstrated that surface IgM negative ($sIgM^-$) cells are responsible for MAF production, suggesting a T cell origin (Graham and Secombes, 1990a). Although IFN-γ or genes encoding for it have not been identified in fish (see below), it has been suggested that rainbow trout MAF might be the equivalent to mammalian IFN-γ (Graham and Secombes, 1990b), based on similarities with that cytokine (Nathan *et al.*, 1983). Further research has strengthened this hypothesis. $sIgM^-$ lymphocytes from rainbow trout primed *in vivo* with a TD-antigen and re-stimulated *in vitro* with the same antigen produced a MAF which increased phagocyte respiratory burst (Piganelli *et al.*, 1994). Increased phagocyte nitrite production and microbiocidal activity were observed in similar experiments using mixed leukocyte cultures as secretors of MAF in catfish (Yin *et al.*, 1997).

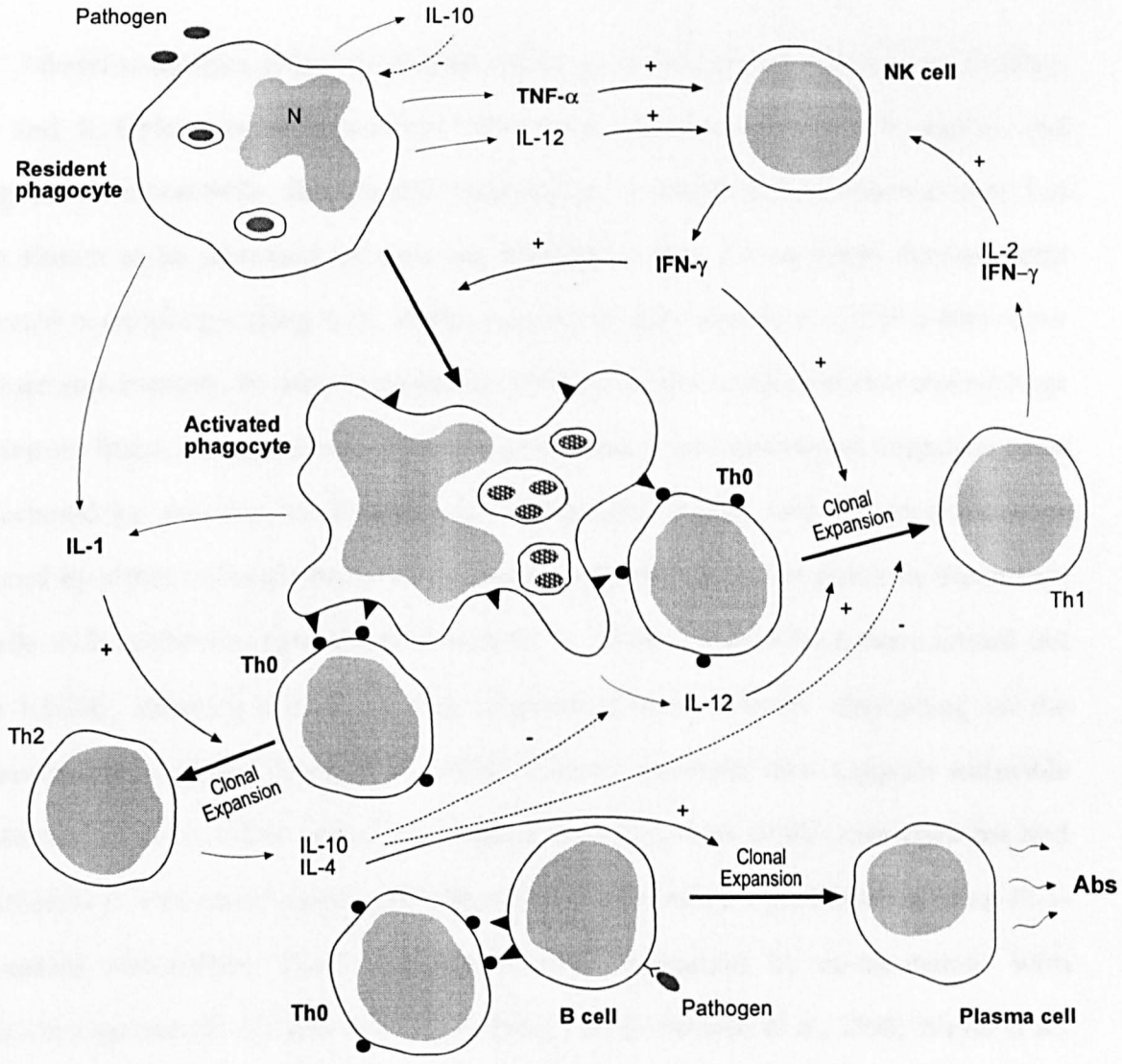

Figure 1.3 Regulation of multiple immune functions by cytokines in mice.

Resident phagocytes having ingested microorganisms are activated to a more microbiocidal state able to kill pathogens and present antigens plus MHC class II molecules (▲) more efficiently. T helper cells (Th0) recognise specific presented antigen through surface TCR (●). Depending on co-stimulatory signals between the phagocyte and Th0 and cytokines present in the surrounding *milieu*, Th0 mature into Th1 or Th2 lymphocytes. The former secrete a pool of cytokines which enhance cell mediated responses such as phagocyte and NK cell activities (type 1 response). As infection progresses, phagocytes secrete IL-10 and IL-1 which inhibit type 1 responses and stimulate the differentiation of Th0 into Th2 cells, which further inhibit type 1 response and stimulate antigen presenting B cells to differentiate into plasma cells and produce antibodies (Abs) against that particular antigen. Cells and cytokines in bold, or their functional equivalents, have been identified in teleost fish. Inhibition is represented by dotted lines and '-'; stimulation is represented by full lines and '+'. The purpose of this figure is to illustrate an example of how cytokines modulate immune function. Many other cytokines are involved in immune responses and those here mentioned display other functions not illustrated. Information collated from Cleveland *et al.* (1996); Fearon and Locksley (1996); Chambers and Allison (1997); Fresno *et al.* (1997); Medzhitov and Janeway (1997); Trinchieri (1997); Unanue (1997a); Unanue (1997b).

Likewise, indirect evidence for TNF-α-like, transforming growth factor β_1 (TGFβ_1)-like and IL-1-like factors in rainbow trout have been provided by biological and antigenic cross reactivity. Respiratory burst activity of rainbow trout macrophages has been shown to be increased by previous incubation with supernatants derived from activated macrophages (Jang *et al.*, 1995b), suggesting the presence of a TNF-α-like factor in those supernatants. In other experiments, Jang *et al.* (1995a) showed that macrophage respiratory burst, mitogen-induced lymphoproliferation and neutrophil migration were exarcebated by previous incubation with human (h) TNF-α, and all activities were reduced by either co-incubation with antibodies against hTNF-α or previous incubation of cells with antibodies against hTNF receptor 1. Similar studies have been carried out with hTGFβ_1, showing a dual effect on respiratory burst activity depending on the concentration employed (Jang *et al.*, 1994). Indirect evidence also suggests inducible production of an IL-1-like factor by common carp (*Cyprinus carpio*) macrophages and neutrophils *in vitro* since supernatants from these cells induced proliferation of an IL-1-dependent mammalian T-cell line, which was suppressed by co-incubation with antibodies against hIL-1α and hIL-1β (Verburg van Kemenade *et al.*, 1995; Weyts *et al.*, 1997b). In addition, macrophages/monocytes are required as accessory cells for the antibody response *in vitro* (Miller *et al.*, 1985) and two different monocyte-derived IL-1-like proteins been identified in channel catfish (Ellsaesser and Clem, 1994).

To date, biological and antigenic studies of cytokine-like factors in fish have not been extensively followed by DNA sequence analysis (Secombes *et al.*, 1996). The gene encoding for an IFN-γ-like product in flatfish (*Paralichthys olivaceus*) has been sequenced (Tamai *et al.*, 1993), although the low homology to the mammalian IFN-γ gene and lack of clear IFN-γ amino acid motifs suggest it is not an interferon (Secombes *et al.*, 1996). More recently, genes encoding for fish TGFβ_1-like, IL-1β-like, IL-8-like and IL8R-like products in fish species have been sequenced (Harms *et al.*, 1997; Secombes, 1997; Zou *et al.*, 1997; Fujiki *et al.*, 1998).

1.2.7 Inflammatory processes in fish

Fish, ranging from Antarctic to tropical species, are able to display an inflammatory reaction against a variety of irritants or invading microorganisms (Zelikoff *et al.*, 1991; Endo *et al.*, 1995; Silva *et al.*, 1996; Sharifpour, 1997), although the response is not as pronounced as in mammalian species (Ferguson, 1989; Roberts, 1989b). While some information is available on cells and soluble mediators involved in the inflammatory process in fish (Ellis, 1986; Suzuki and Iida, 1992; Rowley, 1996), this is very scant compared with that in higher vertebrates.

1.2.8 Evasion of killing by microorganisms, particularly bacteria

Certain microorganisms affecting mammals have successfully developed strategies to reduce or escape killing by humoral and cellular components of the host defence mechanisms (Kaufmann, 1993; Finlay and Cossart, 1997). There is no reason why such mechanisms should not exist amongst microorganisms pathogenic for fish.

Some microorganisms have been shown to attach to skin, gills or GI tract surfaces from where they are able to cause systemic infections (reviewed by Evelyn, 1996). *Flexibacter maritimus*, a bacterium which primarily attaches to the skin and gills of fish (Wakabayashi, 1993), has developed mechanisms to help attachment to mucus and reduce its bactericidal effect (Magariños *et al.*, 1995). Virulent strains of *A. salmonicida* expressing an outer protein (A) layer have been reported to resist the lytic activity of complement to a greater extent than avirulent strains (A layer negative) (Sakai, 1992).

Another important evasion strategy of some pathogens is avoidance of internalisation by phagocytes. Channel catfish neutrophils have been reported to actively internalise low virulence *A. hydrophila* and *Micrococcus luteus* cells while higher virulence *E. ictaluri* and *Edwardsiella tarda* were phagocytosed much less readily (Ainsworth and Dexiang, 1990). The capsule of *Photobacterium damsela* (previously known as *Pasteurella piscicida*) has been reported to confer increased resistance to phagocytosis by sea bream macrophages, although no correlation between capsulation

and killing by phagocytes was observed (Arijo *et al.*, 1998). Once phagocytosed, certain microorganisms have different strategies to avoid being killed by the host. Certain mammalian intracellular pathogens such as *Chlamydia* spp., *Legionella pneumophila*, *Mycobacterium* spp., and *Toxoplasma gondii* are able to inhibit phagosome maturation and/or escape into the cytoplasmic space, or else resist the microbiocidal environment in mature phagolysosomes (Sinai and Joiner, 1997). However, very little is known about the interactions between pathogens and phagosomes in fish. It has been shown that *Mycobacterium* spp. are able to resist degradation in lysosomes of rainbow trout macrophages, although opsonisation with non-immune or immune sera greatly increased the bactericidal capacity (Chen, 1996). Using gene-disrupted bacterial strains, superoxide dismutase (SOD) or catalase enzymes in *Salmonella* spp. and *Candida* spp. cells have been demonstrated to increase resistance to phagocyte killing by blocking the production of peroxinitrite and ROIs (De Groote *et al.*, 1997; Wysong *et al.*, 1998). These two enzymes have been proposed as virulence factors for the fish pathogenic bacterium *A. salmonicida* in studies involving cell-free generated oxygen and nitrogen radicals (Karczewski *et al.*, 1991; Garduño *et al.*, 1997). Furthermore, a virulent strain of *A. salmonicida* normally resistant to trout macrophage respiratory burst was more efficiently killed through incubation with a SOD inhibitor (Sharp and Secombes, 1993). Opsonised and non-opsonised strains of *E. tarda* have been associated with low respiratory burst activity of Japanese eel neutrophils (Iida and Wakabayashi, 1993), although whether this effect was due to reduced uptake by neutrophils or inhibition of the respiratory burst itself was not investigated.

Bacterial cells display an adaptive physiological change depending on the environmental conditions in which they grow and these changes may influence killing by the host immune response (Byrne and Swanson, 1998). Incubation of the fish pathogen *R. salmoninarum* with non-immune or immune sera for long periods of time (16 h) enabled the bacteria to grow considerably faster inside trout macrophages than non-

opsonised bacteria or those opsonised for shorter periods of time (3 h), suggesting that an adaptive change in bacterial physiology rather than opsonisation was the mechanism conferring increased resistance (Bandín *et al.*, 1995).

Finally, certain bacteria pathogenic to mammalian hosts have been shown to avoid effective killing mechanisms by altering the cytokine profile to which immune cells respond for effective bacterial clearance. The mechanisms by which bacteria are able to do so include alteration of cytokine synthesis, proteolytic degradation of cytokines and proteolytic cleavage of cytokine receptors on the immune cell surface (Wilson *et al.*, 1998). Although similar mechanisms may be used by fish pathogenic bacteria, more research is needed to elucidate the role of cytokines in fish immune regulation.

1.3 Stress response and modulation of immune defence mechanisms

1.3.1 Immunomodulation by aquaculture-associated stressors

Stressors related to aquaculture practices have been shown to modulate most immune functions investigated, usually causing suppression, depending on the quality and extent of the stressor and the immune parameter assayed.

Rainbow trout serum lysozyme activity has been reported to be modified by handling, transport and water quality. Short term handling induced a variable modulation of activity, while longer exposure to a stressor, such as transport or poor water quality, induced a significant decrease in lysozyme activity (Möck and Peters, 1990). A similar variable modulation of complement-mediated haemolytic and agglutinating activities as well as C3 concentration in the serum of gilthead sea bream has been observed by chasing the fish once or on a daily basis (Sunyer *et al.*, 1995; Tort *et al.*, 1996). The bactericidal capacity of rainbow trout serum was, however, enhanced after a 2 h confinement stressor, although no mechanisms involved were identified (Thompson *et al.*, 1993).

Short term stressors such as injection or noise in combination with confinement were shown to reduce macrophage phagocytosis significantly, although the effect was diminished following six daily injections (Narnaware *et al.*, 1994), suggesting a possible adaptation to the stressor. Production of microbiocidal substances by phagocytes is also influenced by stress. Short or long term confinement induced a decrease in respiratory burst activity in rainbow trout (Angelidis *et al.*, 1987) and common carp (Yin *et al.*, 1995). However, repeated confinement was not associated with significant modulation of the respiratory burst (Angelidis *et al.*, 1987). *A. salmonicida* killing by macrophages from rainbow trout confined during a 2 h period was significantly decreased, possibly as consequence of reduced macrophage respiratory burst activity (Thompson *et al.*, 1993).

Stressors associated with aquaculture practices have often been identified as possible causes for vaccine failure in the field. In mammals, proliferation of T and B cells is an important step in the production of antibodies. Peripheral lymphocytes of channel catfish exposed to handling and transport did not undergo either proliferation when incubated with mitogens or antibody responses to T-dependent and T-independent antigens (Ellsaesser and Clem, 1986). Likewise, long term crowding of rainbow trout induced a decline in numbers of antibody producing plaque forming cells isolated from the head kidney (Mazur and Iwama, 1993). Although the mechanisms involved were not investigated, serum levels of specific antibodies against *A. salmonicida* were depressed in acutely stressed rainbow trout (Thompson *et al.*, 1993). However, contradictory results are available since *A. salmonicida* antibody levels have been reported to remain stable in sera from Atlantic salmon exposed to repetitive handling (Espelid *et al.*, 1996).

Certain behavioural conduct has been shown to trigger a stress response in tilapia (*Oreochromis aureus*) and rainbow trout. In contrast to physical aquaculture-related stressors, behaviour-induced stress response is not only mediated by physical but also by psychological mechanisms (Peters *et al.*, 1991). Social interaction may result in hierarchical ranks with dominant and subordinate fish, the latter undergoing a stress

response leading to disruption of the immune homeostasis. Tilapia natural cytotoxic cell activity against YAC-1 target cells was depressed (Ghoneum *et al.*, 1988), while both phagocytosis of yeast particles and autophagocytosis of red blood cells were enhanced in subordinated rainbow trout (Peters *et al.*, 1991). As a consequence of social stress, subordinate rainbow trout possessed structural alterations in the haemopoietic organs (Peters *et al.*, 1991) and displayed increased susceptibility to *A. hydrophila* infection (Peters *et al.*, 1988). Farming practices, such as space enclosure and high stocking densities, may affect the behaviour of fish and be associated with increased social aggressiveness.

1.3.2 Neuroendocrine framework of the stress response

Cognitive stimuli, such as crowding, handling, transport and social interactions, are perceived in specific brain centres and translated by the neural and endocrine systems resulting in the secretion of a wide range of factors and hormones which mediate the stress response. Fish are equipped with the equivalent of all organs, tissues and main mediators shown to contribute to the stress response in mammals (Ottaviani and Franceschi, 1996; Wendelaar Bonga, 1997). Activation of the neuroendocrine system as part of the stress response is known to modulate the immune function through at least three different pathways: (i) release of hypothalamic, pituitary and adrenal cortex (interrenal tissue in fish) hormones during the activation of the hypothalamic-pituitary-adrenal (interrenal) axis (figure 1.4); (ii) release of catecholamines by the autonomic nervous system and adrenal medulla (figure 1.4) (renal chromaffin cells in fish), and (iii) release of neuropeptides (MacLean and Reichlin, 1981; Wendelaar Bonga, 1997).

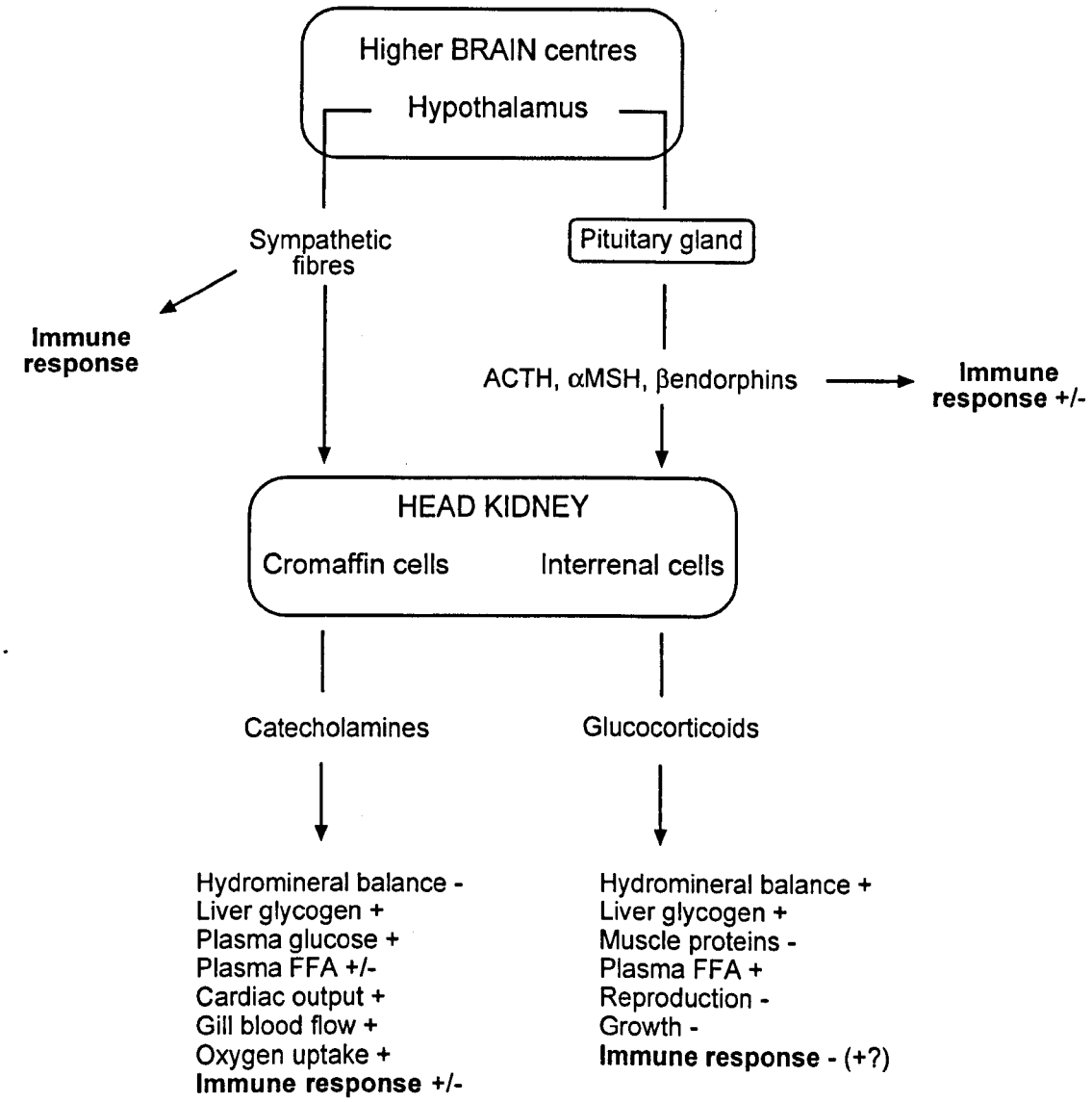

Figure 1.4 Integrated diagram of the main neuroendocrine mediators of the stress response in fish.

The consequences of activation of the hypothalamic-sympathetic-chromaffin and hypothalamic-pituitary-interrenal axes on several metabolic and immune functions are summarised. '+' indicates stimulation; '-' indicates inhibition; '?' indicates unknown. ACTH, adrenocorticotropin hormone; FFA, free fatty acids; αMSH, alpha-melanophore-stimulating hormone. Redrawn with modifications from Wendelaar Bonga (1997).

Hypothalamic-pituitary-interrenal axis (HPI)

As in higher vertebrates, activation of the HPI in fish leads to the release of many hormones and factors into the circulation which are responsible, in part, for the stress response (figure 1.4). From an immunological point of view, adrenocorticotropin hormone (ACTH) and glucocorticoids are amongst the most important mediators of the stress response. As reviewed by Brown (1994b), they have multiple effects on all aspects of the immune function (recognition, proliferation, effector and regulatory mechanisms), usually associated with suppressive actions. Other pituitary hormones, such as growth hormone (GH) and prolactin, have been shown, however, to enhance T cell proliferation and differentiation.

In fish, research on immune modulation by HPI-derived hormones has shown similarities to mammalian results. Cortisol is the main glucocorticoid in fish and its concentration in plasma has been demonstrated to peak rapidly after an acute stressor or be increased for a sustained period of time during exposure to a chronic stressor (Carragher and Sumpter, 1990; Wendelaar Bonga, 1997). Administration of physiologically high concentrations of cortisol *in vivo* has been shown to induce depression of many immune aspects, including peripheral lymphocytopenia (Pickering, 1984; Ellsaesser and Clem, 1987; Maule and Schreck, 1990a), lymphoproliferation (Ellsaesser and Clem, 1987; Carlson *et al.*, 1993a; Espelid *et al.*, 1996), plasma IgM concentration (Nagae *et al.*, 1994), complement lytic activity (Carlson *et al.*, 1993b), neutrophil and macrophage phagocytosis (Ainsworth *et al.*, 1991; Narnaware *et al.*, 1994) and the inflammatory response (Ellis, 1986; Suzuki and Iida, 1992). High affinity surface receptors for the glucocorticoids cortisol and cortisone have been demonstrated on tissue leukocytes (Maule and Schreck, 1990b), indicating a direct effect of these hormones. Suppressive effects of cortisol on lymphoproliferation and different aspects of macrophage function have also been shown *in vitro* (Tripp *et al.*, 1987; Pulsford *et al.*, 1995; Wang and Belosevic, 1995; Espelid *et al.*, 1996; Weyts *et al.*, 1997a). Therefore,

exogenous cortisol has been associated with decreased immune function, and it is generally agreed that, as in mammals, stress-related immunosuppression in fish is mainly mediated by high concentration of cortisol (Wendelaar Bonga, 1997).

However, cortisol is not the only HPI-derived hormone mediating immune modulation during the stress response and, although considerably fewer reports are available on pituitary hormones, some others are known to stimulate teleost immune function. ACTH has been shown to enhance macrophage respiratory burst in rainbow trout (Bayne and Levy, 1991a; Bayne and Levy, 1991b). GH plays an important role in the stress response of Nile tilapia (*Oreochromis niloticus*) (Auperin *et al.*, 1997) and has been shown to increase different immune functions in several fish species (Kajita *et al.*, 1992; Sakai *et al.*, 1995a; Sakai *et al.*, 1997; Muñoz *et al.*, 1998), possibly through high affinity cell surface receptors (Calduch-Giner *et al.*, 1997).

Hypothalamic-sympathetic-chromaffin axis

This axis is the teleost equivalent to the hypothalamic-sympathetic-adrenal medulla axis in mammalian species and its activation leads to the secretion of various adrenergic agents or catecholamines by renal chromaffin cells (Wendelaar Bonga, 1997) (figure 1.4). Catecholamines (adrenaline, noradrenaline and dopamine) are present in all vertebrates (Ottaviani and Franceschi, 1996) and it is well documented that in teleost fish a wide range of stressful events induce increased adrenaline and noradrenaline concentrations in plasma (Sumpter, 1997; Wendelaar Bonga, 1997).

In mammals, modulation of the immune response by adrenergic agents is complex and variable since they induce both stimulation and inhibition depending on immune parameter measured and cell type assayed (Reviewed by Brown, 1994b; Madden *et al.*, 1995). Reports on teleost immune function and catecholamines are scarce, and variable modulation has been observed. Administration of adrenaline *in vivo* or *in vitro* lowered rainbow trout macrophage phagocytic ability and this effect was neutralised by co-administration of an adrenergic blocking agent (Narnaware *et al.*, 1994; Narnaware and

Baker, 1996). However, the respiratory burst activity of rainbow trout kidney macrophages as well as lymphoproliferation were decreased or enhanced by β- or α-adrenergic analogues, respectively (Bayne and Levy, 1991b; Flory and Bayne, 1991).

Furthermore, it has been reported that cortisol and adrenergic agents interact to modulate immune function in rainbow trout. Using blocking agents, endogenous noradrenaline was shown to mediate stress-associated decrease in phagocytic activity, and exogenous cortisol administration neutralised this effect by inhibiting noradrenaline release during the stress response (Narnaware and Baker, 1996).

Neuropeptides

Several neuropeptides, such as substance P, somatostatin and endogenous opioid peptides have been shown to be released during the stress response and to regulate variably certain aspects of the mammalian immune response (Brown, 1994b; Chancellor-Freeland *et al.*, 1995).

Although some of these neuropeptides have been identified in teleost fish, their role in the stress response and immune function is unclear. As in mammals, substance P stimulates lymphoproliferation while somatostatin inhibits this response in rainbow trout (Ndoye *et al.*, 1992). Opioid peptides in fish may also contribute to the stress-mediated suppression of lymphoproliferation and NCC activity associated with stress, since the opioid antagonist naltrexone partially reversed the suppressive effects observed in socially subordinate Mozambique tilapia (*Oreochromis mossambicus*) and in lymphocytes incubated with the opioid β-endorphin *in vitro* (Faisal *et al.*, 1989b).

1.3.3 Immune framework of the stress response

The immune system has been thought traditionally to be a target of hormones and mediators released by the neuroendocrine system during the stress response. However, recent research in mammals has demonstrated that the immune system actively communicates with the brain and neuroendocrine system, and they all act in concert shaping the quality and extent of the stress response. Communication from the immune

to the neuroendocrine system is mediated, at least, at two different levels (reviewed by Brown, 1994b; Ottaviani and Franceschi, 1996; Dantzer, 1997). Firstly, cytokines secreted by different leukocytes in response to non-cognitive stimuli (such as microbial infections) are actively transported across the blood-brain barrier. In addition, certain cytokines are produced by neural and non-neural cells in the brain. Receptors for cytokines have been found throughout the brain, and have been shown to modulate the neuronal firing rate and subsequent secretion of stress hormones (corticotrophin releasing hormone, ACTH, β-endorphins, αMSH, prolactin, glucocorticoids, and catecholamines). Secondly, different cells of the immune system, mostly phagocytes, are known to secrete stress mediators like ACTH, β-endorphins, growth hormone and prolactin, in response to microbial infections as well as stress hormones. Besides modulating the immune response in an auto- and para-crine (local) fashion, these leukocyte-derived peptides modify the production of neuroendocrine-derived stress hormones by targeting the hypothalamus and pituitary gland.

Very few reports about the effect of cytokines or microbial antigens on the neuroendocrine system of fish have been published, although evidence is emerging that bi-directional communication between the immune and neuroendocrine systems is a process developed early in evolution and present in teleosts (Ottaviani and Franceschi, 1996; Balm, 1997). Some microbial infections as well as bacterial LPS are known to activate the stress response in fish (Donaldson, 1981). LPS has been demonstrated to induce increased head kidney cortisol production in Mozambique tilapia and LPS recognition by immune cells plays a central role in the response (Balm *et al.*, 1995). In another experiment, ACTH and cortisol production *in vitro* were inhibited by incubation of coho salmon (*Oncorhynchus kisutch*) pituitary gland or kidney tissue, respectively, with leukocyte-conditioned medium supposed to contain cytokines (Schreck and Bradford, 1990). Macrophage-derived supernatant, possibly containing an IL-1-like factor, has been suggested as the means of overcoming suppression of antibody producing cells in

salmon associated with cortisol treatment (Kaattari and Tripp, 1987; Tripp *et al.*, 1987). Further research with purified or engineered teleost cytokines is needed to elucidate further the communication between the immune and neuroendocrine systems during the stress response.

Finally, it is important to underline that since the stress response is an intricate process involving different systems and an ever increasing list of mediators with different and sometimes pleiotropic functions, the immunological outcome is far more complex than simple suppression (Ottaviani and Franceschi, 1996; Dantzer, 1997). However, since intensive farming is an artificial and stressful experience *per se*, additional environmental or husbandry challenges may compound the effect of existing stressors leading to immunosuppression of the fish stock and infectious outbreaks (Wedemeyer, 1997).

1.4 Prophylactic modulation of the immune response

One of the most efficient prophylactic measures against pathogens in general is adequate husbandry, where fish are grown under conditions which impose only a minimal additional strain on the host defence system. However, more virulent pathogens may be able to initiate infectious processes even in immunocompetent animals. Similarly, facultative pathogens may take advantage of lowered immune response resulting from farming practices and cause disease. Therefore, other disease controlling strategies have been sought. Antibiotic prophylaxis is used before or during animal handling, transportation of livestock, physiological alterations such as smoltification and spawning, or at times when previous clinical history of the farm has shown increased incidence in bacterial infections. Prophylactic antibiotic treatment does, however, predispose to emergence of drug resistant strains with serious implications for the bacterial disease management on farm and possible risk to human health. Its prophylactic use should therefore be restricted. Furthermore, antibiotics at levels below the minimum

inhibitory concentrations have been shown to increase virulence of certain bacteria (Morris and Brown, 1988; McKenney *et al.*, 1994).

Other more attractive preventive methods are based on artificial modulation of the immune response. The immune system is a dynamic network of cells and soluble factors able to adapt and respond to endogenous stimuli such as infections or microbial components. Appreciation of this dynamism has led to the use of artificial immunomodulation for prophylactic purposes. In general, this has been achieved by vaccines to induce a strong and effective protective response against specific pathogens. Teleost fish are amongst the first animals in evolution to develop an adaptive immune response (Klein, 1997; Warr, 1997). Although they do not display such a potent memory response as higher vertebrates, the farming life cycle is short and commercial vaccines are used against a few bacterial and viral pathogens (Gudding *et al.*, 1997). However, they are not available for some common and significant pathogens. In addition, natural or husbandry-associated immunosuppression may render vaccines less effective and the stress imposed on the fish during vaccination may lead, in some cases, to morbidity and mortality. Certain drugs and substances able to increase immune response have been suggested as a complementary strategy to vaccination against opportunistic pathogens prior to increased risk of disease outbreaks (Raa, 1996).

Precedents for this exist in human and domestic animal research. A range of vitamins and minerals administered at higher than the RDA have been shown to ameliorate immunosuppressive effects of stress in higher vertebrates (Moonsieshageer and Mowat, 1993; Nockels, 1996; Tamayo *et al.*, 1996; Wellinghausen *et al.*, 1997). Likewise, β-glucans have been applied clinically to accelerate recovery of the immune function (reviewed by Stone and Clarke, 1992). It is well established that trauma patients have increased susceptibility to infection and glucan prophylaxis in these patients increased immune competence and reduced septic morbidity (reviewed in Williams *et al.*, 1996).

Most non-specific immune mechanisms can be artificially enhanced for prophylactic and therapeutic purposes through administration of a wide range of chemically diverse compounds. These include bacterial cell wall products, (1→3)β-glucans, peptides, synthetic products, vitamins and minerals (Blazer, 1991; Secombes and Fletcher, 1992; Raa, 1996; Anderson *et al.*, 1997). Bacterial cell wall products and fungal β-glucans have aroused most interest in aquaculture and are already used as adjuvants in vaccines and additives in commercial feeds. Glucans have also been used together with antibiotics to prevent suppression of some immune parameters associated with antibiotic treatment (Thompson *et al.*, 1995).

As in mammals (reviewed by Stone and Clarke, 1992), intraperitoneal (ip) administration of soluble or particulate (1→3)-β-glucans from different origins has been shown to exert enhancement of innate and adaptive immune mechanisms of fish, leading, in some cases, to increased resistance to viral (LaPatra, 1998) and a number of bacterial pathogens (Chen and Ainsworth, 1992; Engstad *et al.*, 1992; Jørgensen *et al.*, 1993a,b; Rørstad *et al.*, 1993; Robertsen *et al.*, 1994; Thompson *et al.*, 1995; Dalmo *et al.*, 1996a; Santarém *et al.*, 1997). However, some reports have demonstrated that protection against the challenge pathogen depended on the bacterium tested and the β-glucan or immunostimulant used (Robertsen *et al.*, 1990; Nikl *et al.*, 1991; Yano *et al.*, 1991; Nikl *et al*, 1992; Matsuyama *et al.*, 1992a; Wang and Wang, 1997).

Similar results have been observed after ip administration of a wide range of products such as Freund's complete adjuvant (Kajita *et al.*, 1990), chicken egg fermented products (Sakai *et al.*, 1995b), muscle hydrolysate (Bøgwald *et al.*, 1996), algae polysaccharide (Fujiki and Yano, 1997) as well as synthetic compounds like muramyl dipeptide (Kodama *et al.*, 1993; Kodama *et al.*, 1994a) and levamisole (Kajita *et al.*, 1990).

Enhancement of defence mechanisms by ip administration of immunostimulants is, however, not a viable option in aquaculture on either economic or husbandry grounds, unless as adjuvants in conjunction with vaccination (Anderson *et al.*, 1997).

Literature on more practical delivery strategies, such as oral and bath routes, is very scarce and there is considerable scepticism in the aquaculture industry towards their prophylactic use (Raa, 1996).

Short immersion of rainbow trout in baths containing levamisole, a quaternary ammonium compound or a short-chain polypeptide enhanced innate defences and antibody response as well as survival in an *A. salmonicida* challenge (Jeney and Anderson, 1993a). Oral administration of several substances has been shown to increase fish immune activity and, in some cases, resistance to bacterial challenges. Intubation of Atlantic salmon with a single dose of soluble β-glucan induced increased superoxide production and acid phosphatase activity of macrophages (Dalmo *et al.*, 1996a). In rainbow trout, peripheral blood phagocyte activity was enhanced after in-feed particulate yeast β-glucan treatment, leading to increased resistance to *A. salmonicida* (Siwicki *et al.*, 1994) and *Flexibacter columnaris* (Jeney *et al.*, 1997). Similarly, β-glucan in combination with vitamin C has been shown to induce a significant increase in several innate and adaptive responses in rainbow trout (Verlhac *et al.*, 1998). Enhanced survival in the face of bacterial pathogens has also been reported in coho salmon treated with soluble β-glucan (Nikl *et al.*, 1992) and in rainbow trout and yellowtail (*Seriola quinqueradiata*) fed on peptidoglycan-containing diets (Itami *et al.*, 1996; Matsuo and Miyazono, 1993). In other studies, however, dietary β-glucan has been shown not to be successful in enhancing turbot (*Scophthalmus maximus*) macrophage activity and survival against *Vibrio anguillarum* (Debaulny *et al.*, 1996) or in conferring resistance to *E. tarda* in channel catfish (Ainsworth *et al.*, 1994). In-feed administration of the blue-green algae *Spirulina platensis* also failed to induce protection of channel catfish against *E. tarda*, even though peritoneal macrophage activity and antibody response to a thymus-dependent antigen were enhanced (Duncan and Klesius, 1996). These results altogether suggest that, at least in some circumstances, stimulation of fish immune activity and enhanced resistance to pathogens are achievable by bath or oral route, although further studies are

needed to optimise dosing and timing regimes and to elucidate the mechanisms involved in stimulation.

No literature was available on the prophylactic use of in-feed β-glucans or peptidoglycan to alleviate the effects of stress on the immune system of fish at the beginning of this thesis. One report has since been published (Jeney *et al.*, 1997), using yeast β-glucans in an approach complementary to the present study. In that study, in-feed glucan had a variable effect on preventing reduction of rainbow trout phagocyte activities caused by 2 h transportation. However, mortalities caused by *F. columnaris* after transportation were significantly lower in the group fed immunostimulant than in the control diet group.

1.5 Aims, rationale and structure of thesis

The aim of this study was to evaluate the potential of orally administered immunostimulants in counteracting the effects on innate defence mechanisms of husbandry-associated stress. Various opportunistic pathogens are associated with disease outbreaks at times when farming procedures induce immunosuppression in the fish stock. Conditioning the fish immune system prior to such stressful events may prepare the animals to resist challenges from pathogenic bacteria more efficiently. Several innate defence mechanisms, considered to be the first line of defence against infection, were investigated as targets of stress and immunostimulatory agents. Persistence of viable *A. salmonicida*, a pathogen causing mortalities associated with immunosuppression, in spleen and blood was quantified *in vivo* to investigate the integrated innate immune response following confinement and/or oral immunostimulation.

Confinement of fish in a reduced water volume was selected as stressor since it is a common practice during a variety of farming procedures such as transportation, vaccination, grading and chemotherapeutic bath treatments. Confinement is a

multifactorial stressor, where fish are exposed to different variables including hypoxia, physically reduced space and possibly social aggressive behaviour. A range of different substances were selected as immunostimulants based on published reports. These substances included a bacterial peptidoglycan and several β-glucans.

The thesis is presented in different experimental chapters leading to the main aim of the research:

1. Candidate substances were screened *in vitro* for their capacity to increase macrophage microbiocidal mechanisms and substances for further experiments chosen from the results (chapter 3).

2. Oral regimes with the immunostimulatory substances were investigated and designed to induce enhancement of innate defence mechanisms (chapter 4).

3. Aquaculture-associated stressful regimes leading to disruption of innate immune homeostasis were investigated (chapter 5).

4. The prophylactic potential of oral immunostimulation to revert immunomodulation by an aquaculture-associated stressful regime was investigated (chapter 6).

For simplicity, methods and protocols used in more than one experimental chapter are described in chapter 2.

Chapter 2

General materials and methods

2.1 Chemicals and reagents

All chemicals and reagents were purchased from Sigma Chemicals, England, unless otherwise indicated.

2.2 Maintenance of fish in aquarium facilities

Rainbow trout were purchased from local farms and transported by road to the freshwater aquarium facilities at the Institute of Aquaculture, University of Stirling, and maintained there under established procedures. Upon arrival, fish were quickly distributed into disinfected aerated holding tanks (370 l) in a flow-through dechlorinated water system at ambient temperature. During an acclimatisation period of 3 or more weeks, fish were fed once daily with commercial pelleted trout feed (Trouw Aquaculture, Scotland) to the manufacturer's recommended daily allowance (RDA) based on body weight and water temperature. After acclimatisation, fish were anaesthetised with benzocaine, weight-graded and distributed into disinfected flow-through dechlorinated water experimental tanks (same volume and colour as holding tanks). When required, water temperature was progressively raised, and fish were fed twice daily at the manufacturer's RDA for two or more weeks before the experiments were begun. Mortalities, feeding behaviour, water temperature and experimental procedures were recorded daily throughout the acclimatisation and experimental periods.

Fish outside the required weight range or with any signs of disease and experimental left-overs were humanely euthanised by benzocaine overdose and severing the anterior spine.

During the experiments, a higher number of fish than that required for sampling was allocated into the tanks so as not to modify considerably the stocking density after sampling and to resemble more closely the farm environment.

2.3 Peripheral blood cell counts

2.3.1 Extraction of blood

Fish were lightly anaesthetised with benzocaine and bled by caudal venepuncture using 1 or 2 ml syringes and a 25G needle (Terumo, Belgium). Approximately 0.5 ml of blood from each fish was dispensed into dried, individual Eppendorfs previously treated with 5 µl of a 0.4 M disodium ethylenediaminetetraacetic acid (EDTA) solution in distilled water (dH_2O). Extraction and handling of blood were kept constant during experiments.

Depending on the specific needs, fish were then either humanely killed by severing the anterior spine or allowed to recover in aerated water and returned to their tanks.

2.3.2 Haematocrit

Heparinised microhaematocrit tubes were filled with blood, sealed and spun in a Hawksley haematocrit centrifuge (Hawksley & son, England) for one minute. The percentage of packed cell volume (haematocrit) was calculated using a Hawksley reader (Hawksley & son, England).

2.3.3 Total peripheral blood leukocyte counts

EDTA-treated blood was immediately diluted 1:50 in 4 mM EDTA in incomplete Hank's balanced salt solution (iHBSS) and kept on ice until counts were made 2-3 h later. An aliquot of diluted blood was placed on a Neubauer haemocytometer (Hawksley, England) and cells were allowed to sediment. Peripheral blood leukocytes (PBLs) were discriminated from red blood cells since the former are rounded and refractile. The average number of PBLs per large square of the haemocytometer (each being formed of 16 smaller squares) was estimated under a phase contrast microscope at 100x magnification, and the number of PBLs ml^{-1} calculated with the following equation:

$PBLs\ ml^{-1} = PBL\ counts \times df \times 10^4$

Where *df* is the dilution factor (in this case, 50) and 10^4 is a factor to adjust for the volume between the coverslip and haemocytometer.

2.3.4 Differential peripheral blood leukocyte counts

A small drop of EDTA-treated blood was immediately smeared on an ethanol-cleaned glass slide and allowed to air dry. Blood smears were fixed and stained in Rapi-Diff (Lamb, England), a Romanowsky-based staining kit, following the manufacturer's instructions. Two hundred or more leukocytes per smear were counted using an Olympus BX50 microscope at 400x magnification and differentiated into lymphocytes, thrombocytes, neutrophils or monocytes based on morphological and staining characteristics (figure 2.1) (Rowley, 1990). No basophils or eosinophils were observed in any of the blood smears analysed. The percentage of each cell type was transformed into number of cells ml^{-1} with the total number of PBLs for each fish.

2.4 Extraction of plasma and serum from blood

Fish were anaesthetised and bled as described in section 2.3.1. For extraction of serum, 0.5-1 ml of blood was placed in sterile clean glass tubes and allowed to clot at 19 °C for approximately 3 h. Tubes were then centrifuged at 1400 xg for 5 minutes (Mistral 3000i, MSE, England) and serum extracted. If not used fresh, serum from each fish was individually aliquoted and stored at -70 °C until required. For plasma extraction, 0.5-1ml of blood was placed in dried Eppendorf tubes previously treated with 10 U ml^{-1} of heparin in Leibovitz-15 (L-15) medium, and centrifuged at 1400 xg for 5 minutes. Plasma was extracted and individual samples aliquoted and stored at -70 °C.

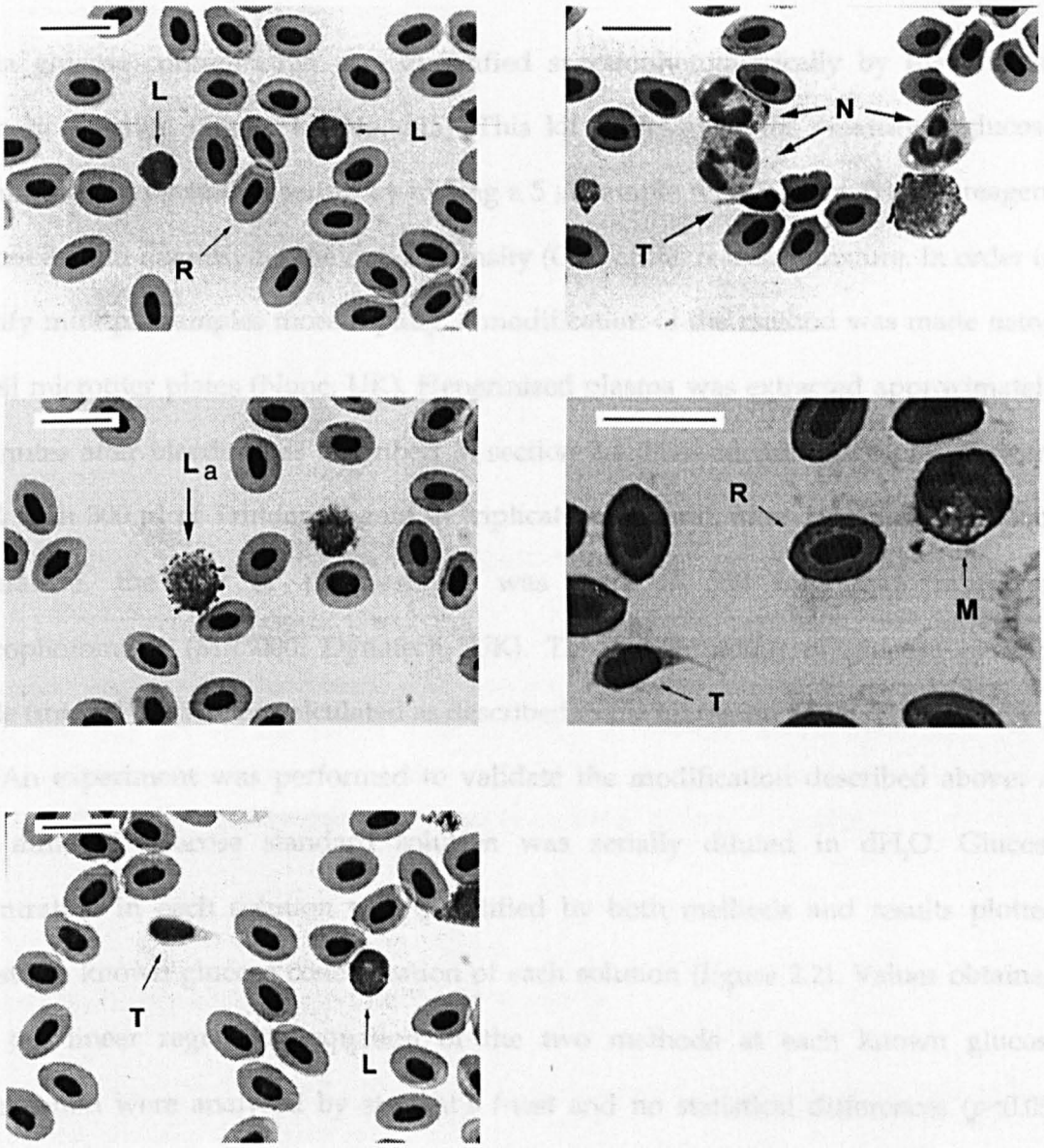

Figure 2.1 Peripheral blood cells of rainbow trout.

Smears were prepared and stained with Rapi-Diff as described in text. Photographs were taken with an Olympus BX50 microscope. R, erythrocytes; L, lymphocytes (L_a, putative activated lymphocyte); T, thrombocytes; N, neutrophils; M, monocytes. Scale bar, 10 µm.

2.5 Plasma glucose concentration

Plasma glucose concentration was quantified spectrophotometrically by the Trinder enzymatic reaction (Sigma kit No. 315). This kit is designed for measuring glucose concentration in plasma or serum by mixing a 5 µl sample with 1 ml of Trinder reagent in a cuvette and quantifying the optical density (OD) of the reaction mixture. In order to quantify multiple samples more rapidly, a modification of the method was made using 96-well microtiter plates (Nunc, UK). Heparinised plasma was extracted approximately 30 minutes after bleeding, as described in section 2.4. Five microliters of plasma were mixed with 300 µl of Trinder reagent in triplicate wells and, after 18 minutes at room temperature, the OD of the samples was read at 550 nm in a multiscan spectrophotometer (MR5000, Dynatech, UK). The concentration of glucose in each sample (mmol l^{-1}) was then calculated as described in the kit instructions.

An experiment was performed to validate the modification described above. A 16.65 mmol l^{-1} glucose standard solution was serially diluted in dH_2O. Glucose concentration in each solution was quantified by both methods and results plotted against the known glucose concentration of each solution (figure 2.2). Values obtained from the linear regression equation of the two methods at each known glucose concentration were analysed by student's *t*-test and no statistical differences ($p<0.05$) were observed.

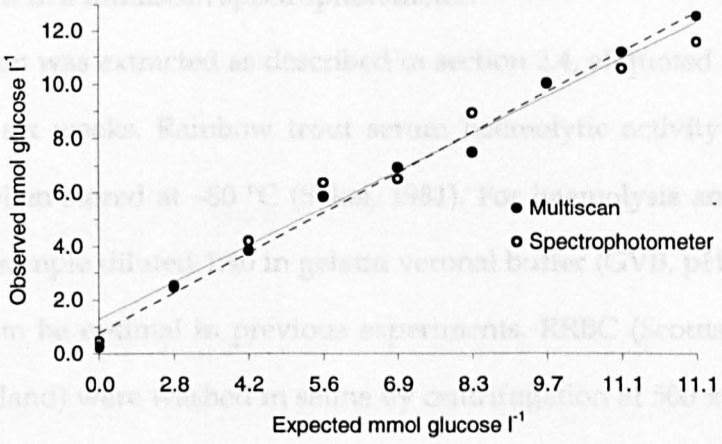

Figure 2.2 Linear regression between expected and observed glucose concentration obtained by two methods.

Glucose concentration in known dilutions was quantified by two methods as described in text. The best fits for the values obtained with the Multiscan spectrophotometer are represented by a broken line ($y = 1.491x - 0.7136$; $r^2 = 0.989$) and those obtained with a single-cuvette spectrophotometer by a full line ($y = 1.379x - 0.0983$; $r^2 = 0.973$).

2.6 Serum haemolytic complement activity

Spontaneous haemolytic activity of serum, an indicator of alternative complement lytic activity, was quantified by a method described in Yano (1992). This protocol is based in the change of absorbance at 414 nm following incubation of serum with target rabbit red blood cells (RRBC). Optical density readings were then transformed to obtain the activity of the alternative haemolytic pathway (ACH_{50}). One unit ml^{-1} of ACH_{50} is defined as the amount of serum sufficient to lyse 50 % of $4x10^7$ RRBC in a total volume of 0.7 ml (Yano, 1992). The original protocol was described for large volumes and use of a spectrophotometer. In order to allow multiple samples to be processed simultaneously, the assay was adapted for use in 96-well microtiter plates by reducing proportionally the

volume of serum and RRBC suspension. The absorbance of the samples was then determined in a multiscan spectrophotometer.

Serum was extracted as described in section 2.4, aliquoted and stored at -70 °C for less than six weeks. Rainbow trout serum haemolytic activity is stable for at least 2 months when stored at -80 °C (Sakai, 1981). For haemolysis analysis, serum was thaw and each sample diluted 1:40 in gelatin veronal buffer (GVB, pH=7.5). This dilution was observed to be optimal in previous experiments. RRBC (Scottish Antibody Production Unit, Scotland) were washed in saline by centrifugation at 500 xg for 10 minutes several times until supernatant was clear. The RRBC suspension was adjusted to a concentration of $2x10^8$ cells ml^{-1} in GVB with the aid of a haemocytometer as described in section 2.3.3. 125, 93.7, 62.5, 46.9, 31.2, 23.4, 15.6, 11.7, 7.8 and 5.8 µl of diluted serum were added to triplicate wells of a "U-bottom" 96-well plate (Immulon, Dynatech, UK) and the volume made up to 125 µl with GVB. Serum samples in the wells were mixed with 50 µl of the RRBC suspension and incubated at 19 °C with regular shaking. 0 % and 100 % lysis were obtained in triplicate wells each by mixing 50 µl of the RRBC suspension with 125 µl of GVB or 0.05 % saponin in dH2O, respectively. After 90 minutes, plates were centrifuged at 150 xg for 5 minutes and 100 µl of cell-free supernatant from each well placed in another 96-well plate. Sample absorbance at 405 nm was quantified in a multiscan spectrophotometer using 0 % lysis supernatants as blank.

Calculation of ACH_{50} value (Yano, 1992)

The degree of haemolysis at each serum dilution (Y) was calculated as follows:

$$Y = \frac{OD_A}{OD_B}$$

Where OD_A is the optical density of the sample and OD_B is the optical density of 100% haemolysis.

Values of Y in the range of 0.1 to 0.9 were disregarded and $Y/(1-Y)$ values plotted in the y axis against µl of serum in the sample (x axis) on a log-log scale. The best line to the experimental points was fitted and the ACH_{50} value obtained as follows:

$$ACH_{50}\ (units\ ml^{-1}) = \frac{(serum\ dilution)^{-1}}{k} x \frac{1}{4}^{*}$$

Where, $(serum\ dilution)^{-1}$ = 40; k = x when y = 0.5 (or $y/(1-y)$ = 1) from the linear regression equation; * is the correction factor since this protocol was performed on a 1/4 scale of the original method.

2.7 Isolation and culture of rainbow trout macrophages

2.7.1 Head kidney macrophages

Macrophages from the head kidney were isolated and cultured as originally described by Braun-Nesje *et al.* (1981) and modified by Secombes (1990). In this technique, a cell suspension enriched in macrophages is obtained by density gradient centrifugation and further purified by exploiting their ability to adhere to plastic or glass surfaces. Isolation of macrophages was conducted under sterile conditions.

Fish were anaesthetised, bled and sacrificed as described in section 2.3.1. Tail and gills were cut off to allow further bleeding. The body surface was sprayed with 70 % ethanol and fish were placed on ice. Using aseptic technique throughout, the head kidney was dissected and a single cell suspension produced by pushing the kidney sample through a 100 µm nylon mesh into 10 ml ice-cold homogenising medium (L-15 containing 10 U ml^{-1} heparin, and 100 U ml^{-1} of penicillin and 0.1 mg ml^{-1} of streptomycin solution, abbreviated P/S). The homogenised cell suspension from each fish was carefully layered onto two ice-cold Percoll density gradients (5 ml on each) previously prepared as described below. Density gradients were then centrifuged at 400 xg for 25

minutes at 4 °C and the band of cells at the interface between the two Percoll densities collected and transferred to 30 ml Universal containers. Cells from the same fish were pooled in one Universal and washed twice in 10 ml ice-cold L-15 plus P/S by centrifugation at 260 xg for 10 minutes at 4 °C. The final pellet was resuspended in ice-cold L-15 plus P/S and adjusted to $2x10^7$ viable cells ml^{-1} with the same medium. For this purpose, an aliquot of the cell suspension was diluted in trypan blue (final concentration 0.1 % w/v) and viable cells counted using a Neubauer haemocytometer as described in section 2.3.3 with a phase contrast microscope at 100x magnification. 100 µl of the cell suspension were then added to wells of a sterile flat bottom 96-well microtiter plate (Nunc, UK). Alternatively, suspensions were adjusted to $5x10^6$ cells ml^{-1} and 0.4 ml placed in wells of a sterile 8-well glass slide (Lab-Tek®, Nunc, UK).

Cells were allowed to adhere for 2 or 3 h at 19 °C (microtiter plates or glass slides respectively) and washed six times with complete Hank's balanced salt solution (cHBSS) or until a cell monolayer was observed and no cells were left suspended. cHBSS was then replaced by 100 µl of culture medium (L-15 containing 5 % heat inactivated foetal calf serum and P/S) and monolayers incubated at 19 °C until use. Figure 2.3 shows a head kidney macrophage monolayer 24 h after preparation.

Each Percoll density gradient consisted of two layers with different densities. The bottom layer (51% Percoll solution; 1.080 g l^{-1}) was made of 5.1 ml Percoll, 1 ml 10x minimum essential medium (MEM) and 3.9 ml dH_2O, whereas the top layer (34 % Percoll solution; 1.070 g l^{-1}) contained 3.4 ml Percoll, 1 ml 10x MEM medium and 5.6 ml dH_2O. Density gradients were prepared by pipetting the 51 % Percoll solution under the 34 % Percoll solution in a plastic Universal. This process was carried out carefully so that a clear-cut interface between the two solutions was seen. Gradients were formed before sacrificing the fish, kept on ice and used within 3 h of preparation.

2.7.2 Inflammatory peritoneal macrophages

Induction of an inflammatory response in the peritoneal cavity

Casein was used as irritant to induce an inflammatory response (Fernandez-Botrand and Vetvicka, 1995). Casein was solubilised at 8 % (w/v) in distilled water by raising the pH to approximately 8.5. Once casein was solubilised, the pH was adjusted to 7.5 approximately and NaCl was added to make a final concentration of 0.5 % (w/v). The casein solution was filter-sterilised (0.45 µm Ministart filter, Sartorius, England) and stored at 4 °C overnight. Fish were lightly anaesthetised with benzocaine, injected intraperitoneally with 2 ml of casein solution, allowed to recover in aerated water, and returned to their tanks.

Isolation and culture of peritoneal macrophages

High yields of peritoneal macrophages can be harvested from three days after injection of inflammatory agents (Secombes, 1990; Olivier *et al.*, 1992). Six days after injection of casein, fish were anaesthetised, bled and sacrificed as described in section 2.3.2. Tail and gills were cut off in order to reduce blood cell contamination in the peritoneal cavity during dissection. Each fish was then injected intraperitoneally with 3 ml of ice-cold iHBSS containing 10 U ml^{-1} heparin, 5 % heat inactivated foetal calf serum (FCS) and P/S. After massaging the abdomen 4-5 times, fish were transferred to a laminar flow cabinet and the skin sprayed with 70 % ethanol. Using aseptic technique throughout, a lateral incision was made and the inflammatory exudate collected by washing the peritoneal cavity thoroughly several times with 15 ml of ice-cold iHBSS containing heparin, FCS and P/S. The cell suspension from each fish was centrifuged at 500 xg for 10 minutes at 4 °C and the pellet resuspended in 5 ml of ice-cold iHBSS plus heparin, FCS and P/S. Each sample was layered onto a Percoll density gradient and macrophage-enriched suspensions obtained as described in section 2.7.1. Since inflammatory macrophages are particularly adherent, ice-cold iHBSS and 5 % FCS were used throughout the isolation procedure. Cell suspensions were finally adjusted to $5x10^6$

viable cells ml^{-1} in L-15 containing P/S without FCS as described in section 2.7.1, and 100 µl added to wells of a sterile flat bottom 96-well microtiter plate. After 2 h, wells were thoroughly washed six times with cHBSS and incubated in 100 µl of L-15 containing 5 % FCS and P/S until required. Figure 2.3 shows a peritoneal macrophage monolayer 24 h after preparation.

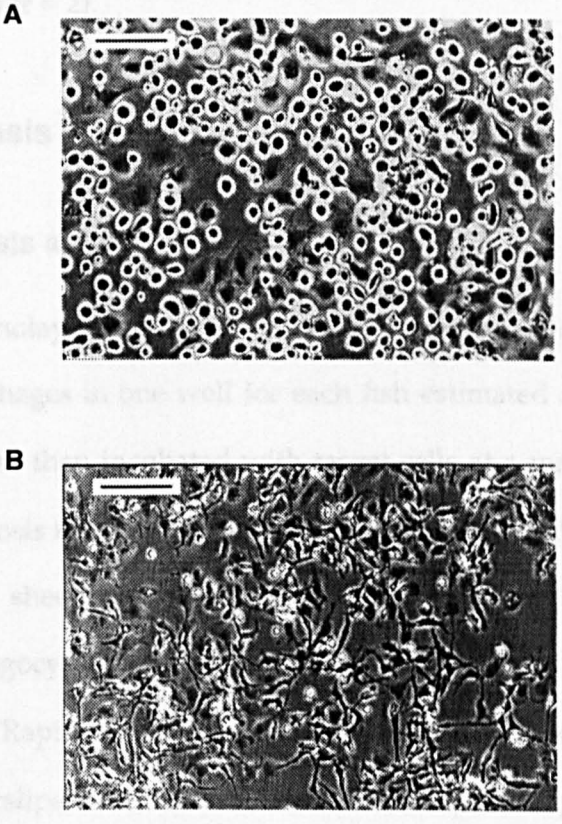

Figure 2.3 Representative head kidney and peritoneal macrophage monolayers isolated from the same individual rainbow trout.

Macrophages were isolated and cultured for 24 h as described in text. **A**, head kidney macrophages; **B**, peritoneal exudate macrophages. Note differences in cell spreading. Photographs of unstained samples were taken with an Olympus CK2 inverted microscope using phase contrast light. Scale bar, 100 µm.

2.7.3 Estimation of adhered macrophage cell numbers

Before use, macrophages adherent on the microtiter plate or slide wells were counted as described by Secombes (1990). The culture medium in some of the wells was replaced with 200 µl of ice-cold 0.2 µm-filtered lysis buffer consisting of 0.1 M citric acid, 1 % Tween 20 and 0.05 % crystal violet in dH_2O. After 5-10 minutes, nuclei in an aliquot were counted using a Neubauer haemocytometer at 200x magnification as described in section 2.3.3 (dilution factor = 2).

2.8 Phagocytosis

2.8.1 Phagocytosis assay

Macrophage monolayers in 8-well glass slides were washed twice with L-15 and adherent macrophages in one well for each fish estimated as described in section 2.7.3. Macrophages were then incubated with target cells at a macrophage:target cell ratio of 1:5 and phagocytosis allowed to proceed for 60 minutes at 19 °C. Target cells were either *A. salmonicida* or sheep red blood cells (SRBC) prepared and opsonised as described below. After phagocytosis, monolayers were washed four times with saline, and fixed and stained with Rapi-Diff (30 seconds, fixing solution; 30 seconds, acid dye; 15 seconds, basic dye). Coverslips were mounted with Pertex mounting media (Cellpath, England) and slides stored until examined. Figure 2.4 shows phagocytosis of SRBC and *A. salmonicida* by head kidney macrophages.

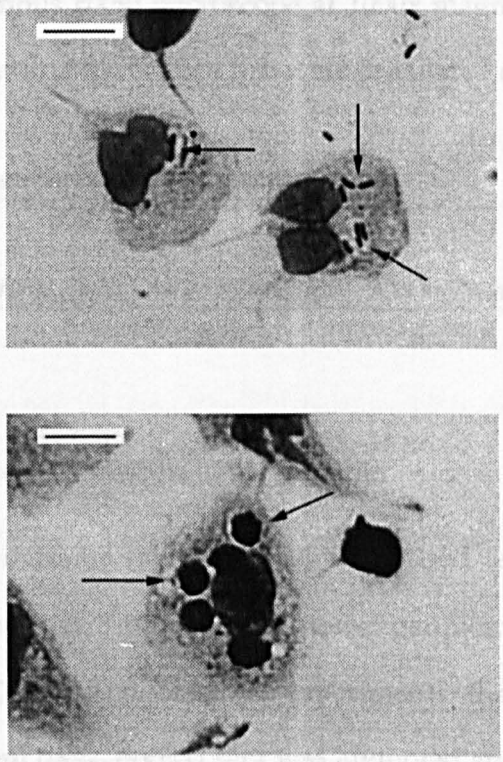

Figure 2.4 Phagocytosis of *Aeromonas salmonicida* and sheep red blood cells by head kidney macrophages.

Samples were prepared and stained with Rapi-Diff as described in text. **A**, macrophages with *A. salmonicida* cells internalised (arrows); **B**, macrophages with SRBC internalised (arrows). Scale bar, 10 µm in length.

The number of bacteria or SRBC internalised in 300 macrophages per sample was counted under an Olympus BX50 microscope at 1000x magnification. Phagocytic index (PI) and phagocytosis ratio (PR) for each fish were calculated as follows:

$$PI = \frac{Number\ of\ ingested\ target\ cells\ in\ 300\ macrophages}{300}$$

$$PR = \frac{Number\ of\ macrophages\ with\ one\ or\ more\ target\ cells\ ingested}{300} x100$$

2.8.2 Preparation and opsonisation of target cells

Pooled sera from non-immune rainbow trout were used as opsonising agent for the phagocytosis test. Sera from 3 or 4 donor, non-experimental, fish were extracted as described in section 2.4 and pooled. In experiments that required assessment of phagocytosis on different days, pooled sera was aliquoted and stored at -70 °C to ensure reproducibility of opsonisation.

Sheep red blood cells (Scottish Antibody Production Unit, Scotland) and viable cells of *A. salmonicida* were used as target particles for phagocytosis in different experiments. *Aeromonas salmonicida* B95179 was isolated from a furunculosis outbreak in Atlantic salmon (Institute of Aquaculture) and stored in cryopreservative at -70 °C until required for use. An aliquot was resuscitated and a single colony of *A. salmonicida* taken from tryptone soya agar (TSA, Oxoid, England) and incubated in tryptone soya broth (TSB, Oxoid, England) overnight at 22 °C with mild shaking. The bacterial suspension was then washed in saline with centrifugation at 1400 xg for ten minutes, and the pellet resuspended in saline to give an absorbance 1.5 at 610 nm (OD_{610}). Cells were opsonised by incubating the bacterial suspension with an equal volume of 20 % serum in saline for 30 minutes. Bacteria were then washed in saline and the OD_{610} adjusted to 1.24 (corresponding to $3.20x10^8$ colony forming units per ml). This suspension was then serially diluted in L-15 + 5 % FCS to obtain a macrophage: viable bacteria ratio of 1:5.

The number of bacterial colony forming units (CFU) per ml^{-1} was confirmed by viable counts on TSA.

SRBC (Scottish Antibody Production Unit, Scotland) were washed five times in saline with centrifugation at 500 xg for five minutes and fixed in 2.5 % paraformaldehyde in saline (w/v) for five minutes. Fixed red cells were washed five times in saline, counted as described in section 2.3.3, the density adjusted to $1x10^7$ cells ml^{-1}, and used within 10 days. Before a phagocytosis assay, macrophages adherent to one well per fish were counted as described in section 2.7.3. SRBC were opsonised with rainbow trout serum as described above, washed twice in saline and the concentration adjusted in L-15 plus 5 % FCS so that the ratio macrophage:SRBC was 1:5.

2.9 Respiratory burst activity of rainbow trout macrophages

2.9.1 Extracellular superoxide anion

Extracellular generation of superoxide anion by trout macrophages was quantified spectrophotometrically in 96-well microtiter plates by the reduction of cytochrome c using phorbol myristate acetate (PMA) to trigger the respiratory burst (Pick and Mizel, 1981, Secombes *et al.*, 1988).

Horse heart cytochrome c was solubilised in phenol red-free cHBSS at a concentration of 4 mg ml^{-1}, filter-sterilised, aliquoted and stored at -70 °C until required for use. PMA was dissolved in ethanol to a final concentration of 1 mg ml^{-1} and stored at -70 °C. Superoxide dismutase (SOD) inhibits the reduction of cytochrome c by superoxide anion and, therefore, it was used to confirm specificity of the reaction. SOD was dissolved in phenol red-free cHBBS at a concentration of 3000 U ml^{-1}, aliquoted and stored at -70 °C. Sodium azide (NaAz), a cytochrome c oxidase inhibitor, prevents oxidation of reduced cytochrome c and its use is recommended to detect the maximum response (Doyle *et al.*, 1995). A solution of 4 mM NaAz in phenol red-free cHBSS was prepared and stored at 4 °C for up to seven days before use.

Macrophage monolayers, prepared as described in section 2.7, were washed twice in sterile phenol red-free cHBSS and some wells were covered with 100 µl of a 2 mg ml^{-1} cytochrome solution containing 2 mM NaAz and 1 µg ml^{-1} PMA. As a negative control, some wells were treated with cytochrome solution containing NaAz, PMA, and 50 U SOD per well. All reactions were performed in triplicate or quadruplicate wells per fish as stated in each experimental chapter. The reaction was allowed to proceed at 19 °C and the OD of the wells read on a multiscan spectrophotometer at 550 nm at different times, usually 1, 2.5, 5, 10, 15, 30, 45, 60, 90, 120 and 240 minutes after addition of reagents.

OD results were adjusted to $2x10^5$ cells by estimating the number of adherent macrophages in duplicate wells per fish as described in section 2.7.3 using the values obtained with SOD in the reaction mixture as blanks. The nmol of O_2^- produced per $2x10^5$ cells was calculated by multiplying the OD reading adjusted to $2x10^5$ cells by a factor of 15.87 (Pick and Mizel, 1981). This factor derives from the extinction coefficient for the absorption at 550 nm of reduced minus oxidised cytochrome c in a 96-well microtiter plate.

2.9.2 Intracellular superoxide anion

Intracellular production of superoxide anion by trout macrophages was quantified spectrophotometrically in 96-well microtiter plates by the reduction of nitroblue tetrazolium (NBT) to formazan using PMA to trigger the respiratory burst (Pick *et al.*, 1981; Rook *et al.*, 1985; Chung and Secombes, 1988).

A fresh solution of NBT was prepared before the assay by dissolving 1mg ml^{-1} in cHBSS and then filter-sterilising.

Macrophage monolayers, prepared as described in section 2.7, were washed twice in sterile phenol red-free cHBSS and some wells covered with 100 µl of NBT solution containing 1 µg ml^{-1} PMA (prepared as described in section 2.9.1). Other wells were treated with NBT solution without PMA. Finally, some wells were incubated with NBT

solution plus PMA and 300 U SOD per well. All treatments were carried out in triplicate wells per fish unless otherwise stated in the experimental chapters. After 30 minutes at 19 °C, reactions were stopped and the formazan within macrophages solubilised. To do this, the supernatants were removed and cells fixed in absolute methanol for five minutes. Subsequently, wells were washed with 70% methanol to remove any trace of non-reduced NBT. Wells were air dried, and the formazan solubilised by adding 120 µl of 2 M KOH and 140 µl of dimethyl sulfoxide (DMSO) to each well. The OD of the well mixtures was then read at 610 nm in a multiscan spectrophotometer using KOH/DMSO as blank. A negative control consisting of wells without cells and treated similarly to those containing macrophages was included to confirm that all non-reduced NBT was removed before adding KOH/DMSO. The use of this negative control is recommended since non-reduced NBT turns into a blue solution with KOH/DMSO that is indistinguishable from solubilised formazan.

OD results were adjusted to $2x10^5$ cells by estimating the number of adherent macrophages in duplicate wells per fish as described in section 2.7.3.

In contrast to the cytochrome c assay, SOD is able to inhibit the PMA-triggered reduction of NBT by 40-60 % only. This is mainly due to its large molecular weight, which makes it difficult to access intracellular sites where formazan is being produced. Although NBT reduction by dehydrogenase enzymes in mitochondria may also occur, it has been shown that NBT reduction by trout macrophages stimulated with PMA truly reflects superoxide anion production (Secombes *et al.*, 1988).

2.10 Inducible nitric oxide synthase (iNOS) activity

2.10.1 Nitrite production

Activation of iNOS results in the production of large amounts of nitric oxide, a highly reactive molecule that spontaneously dismutates to nitrite, a major end product. Nitrite

is commonly quantified by the Griess reaction to give indirect measurement of iNOS activity and the assay was carried out as described by Wang *et al.*, 1995).

Head kidney macrophage monolayers were cultured in 96-well microtiter plates as described in section 2.7. Macrophages in triplicate wells were incubated with L-15 containing macrophage activating factor (MAF, obtained as described in section 2.10.2), 5 % FCS and P/S at 19 °C. MAF and bacterial LPS have been shown to synergise the production of nitrite by teleost macrophages (Neumann *et al.*, 1995; Mulero and Meseguer, 1998) and therefore 40 μg ml^{-1} of *E. coli* LPS (serotype 026:B6) were added to the MAF-containing medium. To confirm specificity of the reaction, N^G-monomethyl-L-arginine (Calbiochem, England), an inhibitor of NO production, was added to triplicate wells per fish to a final molarity of 1000 μM. After 96 h incubation, 50 μl of the culture supernatants were mixed in another 96-well microtiter plate with 100 μl of fresh Griess reagent, consisting of 1 % (w/v) sulfanilamide and 0.15 % (w/v) N-(1-naphthyl)ethyl-enediamine in 2.5 % (v/v) H_3PO_4 in dH_2O. After ten minutes at room temperature, the OD of the samples was read at 550 nm. A negative control consisting of wells without cells was included. Positive controls consisted of serial dilutions of a sodium nitrite solution ranging from 2 to 100 μM.

2.10.2 Production of macrophage activating factor (MAF)

MAF was obtained from mixed leukocyte cultures as described by Graham and Secombes (1988). Head kidneys of five rainbow trout were homogenised as described in section 2.7.1. The resulting cell suspensions were pooled and layered over a 51 % Percoll density suspension prepared as described in section 2.7.1 (no 34 % Percoll solution added in order to obtain a mixed cell suspension). After centrifugation at 400 xg for 25 minutes at 4 °C, the band of cells at the Percoll/sample interface was collected, washed and adjusted to $1x10^7$ viable cells ml^{-1} in ice-cold L-15 containing P/S as described in section 2.7.1. Cells were diluted 1:2 with pulse medium consisting of L-15 plus P/S, 10^{-4} M 2-mercaptoethanol (2ME), 10 ng ml^{-1} PMA and 20 μg ml^{-1} of concavalin A (ConA). 5 ml

of this suspension were placed in 25 cm^2 cell culture flasks (Nunc, UK). After 3 h incubation at 19 °C, plates were washed very carefully with L-15 six times. ConA induces slight adherence of lymphocytes allowing careful washing without removing these cells (Graham and Secombes, 1988). Cells were then cultured with L-15 containing 10 % FCS and P/S at 19 °C. After 72 h, MAF-containing supernatants were collected and cells/debris removed by centrifugation at 260 xg for 10 minutes and filtration through a 0.2 µm Ministart filter. Supernatants were then aliquoted and stored at -70 °C for a maximum of four months (Secombes, 1990).

To confirm the presence of MAF in the samples, head kidney macrophages from 4 fish were individually cultured in 96-well plates as described in section 2.7.1 and incubated with 1:2 serial dilutions of MAF-containing supernatants at 19 °C. After 48 h, production of extracellular superoxide anion was quantified as described in section 2.9.1 and the dilution eliciting maximum response chosen to stimulate macrophages *in vitro*.

2.11 Bacterial killing *in vitro*

Bacterial killing by head kidney macrophages *in vitro* was calculated spectrophotometrically based on mitochondrial activity as described in Stevens *et al.* (1991) with some modifications. The procedure was conducted aseptically.

Head kidney macrophage monolayers were prepared in 96-well microtiter plates and the number of adherent cells calculated as described in section 2.7. *A. salmonicida* was grown and serum-opsonised as described in section 2.8.2. The concentration of opsonised bacteria was adjusted in L-15 plus 5 % FCS to give a ratio of one macrophage to 20 CFU, which was confirmed by viable counts on TSA. Macrophages in quadruplicate wells per fish were washed with L-15 without antibiotics and the supernatants replaced with 100 µl of the bacterial suspension. Plates were centrifuged at 150 xg for 5 minutes to bring the bacterial cells into contact with the macrophages and then incubated for 5 h at 19 °C. A standard curve of bacterial concentration was prepared

using known inocula of bacteria in wells of a sterile flat bottom 96-well plate with no macrophages. The bacterial suspension was diluted in L-15 plus 5 % FCS to obtain 0, 25, 50, and 75 % reduction in bacterial cell numbers. 100 µl of each suspension were added to quadruplicate wells and the plate handled exactly in the same way as that containing macrophages and bacteria. After 5 h incubation, 50 µl of ice-cold sterile 0.8 % Tween 20 (v/v) was added to all wells to kill macrophages. Ten minutes later, all wells received 50 µl of 2 mg ml^{-1} of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) in dH_2O. Plates were incubated at 22 °C for 30 minutes to allow reduction of MTT to insoluble blue formazan by mitochondrial dehydrogenases of viable bacteria. Bacteria were then pelleted by centrifugation at 1300 xg for ten minutes and 150 µl of supernatant free of blue-coloured bacteria carefully removed. Formazan was solubilised by addition of 150 µl 0.1 N HCl in anhydrous isopropanol and thorough mixing. The OD of the reaction mixtures was quantified at 550nm on a multiscan spectrophotometer using reagents as blank. Negative controls with macrophages incubated with no bacteria were included.

The percentage of bacteria killed by macrophages was determined using the following equation:

$$\left(1-\frac{OD_{sample}-OD_{75\%}}{OD_{0\%}-OD_{75\%}}\right)\times 75$$

The OD corresponding to 0 and 75 % killing of bacteria ($OD_{0\%}$ and $OD_{75\%}$, respectively) were determined from the standard curve of bacterial concentration by linear regression analysis (in all cases, $r^2>0.96$). An example of one of the standard curves is illustrated in figure 2.5.

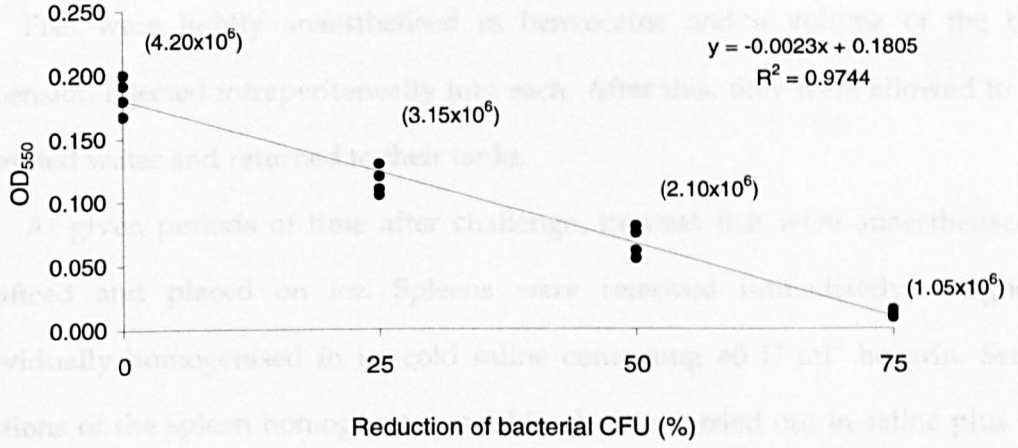

Figure 2.5 Example of one linear regression between bacterial concentration and MTT reduction.

Serial dilutions of an *A. salmonicida* suspension used to challenge macrophages *in vitro* were carried out to obtain 0, 25, 50 and 75 % reduction in bacterial cell numbers. 100 µl of each dilution were placed in quadruplicate wells and treated similarly to those containing macrophages plus bacteria. Values in parentheses indicate number of viable bacteria added per well.

2.12 Bacterial killing *in vivo*

Fish bactericidal capacity was assessed by quantifying the number of bacterial CFU present in the spleen and blood after a bacterial challenge. The spleen was chosen as target organ since it is one of the main haemopoietic and immunologically competent organs involved in antigen clearance (Zapata *et al.*, 1996) and, unlike the head kidney, is a well defined organ easily dissected.

Aeromonas salmonicida was grown as described in section 2.8.2 and washed twice in saline with centrifugation at 1400 xg for 10 minutes. The concentration of bacteria was

adjusted spectrophotometrically in saline to that required and this was confirmed by viable counts on TSA plates.

Fish were lightly anaesthetised in benzocaine and a volume of the bacterial suspension injected intraperitoneally into each. After this, they were allowed to recover in aerated water and returned to their tanks.

At given periods of time after challenge, injected fish were anaesthetised, bled, sacrificed and placed on ice. Spleens were removed immediately, weighed and individually homogenised in ice-cold saline containing 40 U ml^{-1} heparin. Serial 1:10 dilutions of the spleen homogenates and blood were carried out in saline plus heparin and viable *A. salmonicida* in the samples quantified by plating three 20 µl aliquots of each dilution on TSA. After 48 h at 19 °C, CFU were counted and CFU g^{-1} of spleen or ml^{-1} of blood calculated.

2.13 Statistical analysis

Results were analysed by Student's *t*-test, analysis of variance (ANOVA) and multiple comparison tests unless otherwise stated. Further description of statistical analysis of results is given in each experimental chapter under the section 'Materials and methods'.

Chapter 3

Modulation of macrophage microbiocidal mechanisms by β-glucans and peptidoglycan *in vitro*

3.1. Introduction

Phagocytic cells play a fundamental role in the well-being of both the immune and neuroendocrine systems. This role has been observed in many different taxa across the animal kingdom (Ottaviani *et al.*, 1993). They are important effector cells mediating microbiocidal and tumoricidal functions. Microbial killing by phagocytes is initiated by their ingestion. Phagocytosis is followed by production of microbiocidal molecules such as oxygen- and nitrogen- reactive species. In addition, phagocytes secrete an enormous range of chemical substances with different targets and functions. These products include, amongst others, cytokines, enzymes and mediators of the stress response (Langermans *et al.*, 1994; Ottaviani *et al.*, 1996). In vertebrates, the macrophage is probably the most important effector and secretor of all phagocytic cells. Some cytokines secreted by macrophages play an important role in the fine-tuning of immune processes such as T cell differentiation and the switch between cell- and humoral- mediated immunity (Trinchieri, 1997). Furthermore, the vertebrate macrophage processes and presents antigens to certain T cell subpopulations through MHC and TCR communication (Klein, 1997; Medzhitov and Janeway, 1997; Warr, 1997). Thus, the macrophage is not only an essential component of the innate immune system but also forms an essential bridge between this and the acquired component of the immune system.

A similarly wide range of factors regulates the function of macrophages. Different substances have been shown to have a modulatory activity on macrophages (Lopez-Berestein and Klostergaard, 1993; Secombes, 1994; Hauschildt and Kleine, 1995). These molecules might be synthesised by host cells themselves or produced by microorganisms inside the host animal. Exogenous modulators are, mostly, structural components of microorganisms. Endotoxin (LPS), peptidoglycans, teichoic acids and glucans are some of the molecules that fall into this category. Furthermore, secreted proteases and

exotoxins from pathogenic bacteria may also induce modulation of the macrophage activity by affecting cytokine release or recognition (Hauschildt and Kleine 1995; Henderson *et al.*, 1996; Wilson *et al.*, 1998).

Different structural components of microorganisms have been used to induce modulation of macrophage activity (Secombes, 1994; Hauschildt and Kleine, 1995). Beta glucans are the products most investigated for this purpose. Several components of the immune system are targeted by (1→3)-β-glucans. The macrophage is considered to be an important mediator of the immunostimulatory action of these molecules (Robertsen, 1994; Williams *et al.* 1996), with the response initiated through recognition by receptors on the immune cell targeted as discussed in section 1.2.3. (1→3)-β-D-glucans having β-D-glucopyranosil units attached by (1→6) linkages as single unit branches are called (1→3),(1→6)-β-D-glucans, hereafter referred to as (1→3)-β-glucans. They are widely distributed in microorganisms, mostly yeasts, and their occurrence in animals is restricted to a few invertebrates (Stone and Clarke, 1992). (1→3)-β-glucans enhance the immune system systemically, increasing tumoricidal, antibacterial, antiviral and anticoagulatory effects (reviewed by Stone and Clarke, 1992). Peptidoglycan is a major component of the cell wall of Gram-positive bacteria and it has been shown to mediate potent immunomodulatory actions on macrophages and systemically (reviewed by Schwab, 1993; Hauschildt and Kleine, 1995).

The aim of the present study was to carry out a comparative screening of rainbow trout macrophage activity enhancement by different substances. After stimulation *in vitro*, macrophage microbiocidal mechanisms were investigated to elucidate differences in the response to the different test substances. These included three (1→3),(1→6)-β-glucans, a (1→3),(1→4)-β-glucan and a peptidoglycan. The microbiocidal mechanisms investigated were production of superoxide anion and hydrogen peroxide, since they are major players of the respiratory burst. Two substances, depending on their stimulatory

capacity, were chosen for further experiments *in vivo* involving administration through the oral route.

3.2. Materials and methods

Animals

All-female rainbow trout were obtained from Trossachs Trout Farm (Scotland) and acclimatised to aquarium conditions at 5.5 ± 0.5 °C for a period of 8 weeks as described in section 2.2. Water temperature was raised gradually over 11 days and fish were kept at 13.5 ± 1 °C for further 4 weeks before the experiment commenced. The weight of the fish was 123 ± 9.5 g at the beginning of the experiment.

Immunostimulants

Different microparticulated substances were screened to assess their macrophage stimulatory capacity *in vitro*. The test substances were three (1→3),(1→6)-β-glucans isolated from the yeast *Saccharomyces cerevisae* cell wall (Macrogard®, KS Biotec Mackzymal, Norway; Vetregard®, Vetrepharm, UK; Vetregard®, Red Star Bioproducts, USA), one barley (*Hordeum vulgare*) (1→3),(1→4)-β-glucan (Alpha Omega glucan, Alpha Omega Nutrition, USA) and one peptidoglycan extracted from the cell wall of Gram positive *Lactobacillus thermophilus* (Ajinomoto PG®, Ajinomoto, Japan). For simplicity, the test substances will be referred to hereafter as Macrogard, Vetregard α, Vetregard β, AO glucan and peptidoglycan, respectively.

Stock suspensions of 1 mg ml^{-1} of the test substances were prepared in cHBSS containing P/S and then sonicated three times for 30 seconds at an amplitude of 14 microns using an internal probe sonicator (MSE, UK). The size distribution of each test substance was analysed in a Coulter® Multisizer (Coulter, UK) equipped with Coulter® Multisizer accuComp software v. 1.19. For this purpose, stock suspensions were diluted 1:100 in Isoton II® (Coulter, UK) to give a concentration level of 3-10 % before they were

analysed. Each suspension was analysed three times and the average numbers defining the size distribution for each particle recorded.

All substances were sterilised by γ-radiation by Isotron plc, (UK) before the beginning of experiments. Sterility of stock suspensions was confirmed by culturing in TSB and plating onto TSA.

Isolation and culture of head kidney macrophages

Macrophages were isolated from the head kidney of rainbow trout and monolayers cultured in 96-well plates as described in section 2.7.1. Monolayers were incubated with the test substances within 6 h of preparation.

Respiratory burst activity

Production of extracellular superoxide anion by macrophages was monitored in quadruplicate wells by the reduction of cytochrome c triggered by PMA as described in section 2.9.1. SOD was added to quadruplicate wells for each treatment to confirm specificity of the reaction.

Macrophage intracellular superoxide anion generation was quantified in quadruplicate wells by reduction of NBT in the presence or absence of PMA as described in section 2.9.2

Hydrogen peroxide production by macrophages was quantified by the peroxidase-dependent oxidation of phenol red by H_2O_2 (Pick and Keisari, 1981) in 96-well microtiter plates (Secombes, 1990). Macrophage monolayers were incubated with 100 µl of a phenol red solution (PRS) containing 0.02 % phenol red (Sigma, UK) and 0.01 % horseradish peroxidase (Sigma, UK) in phenol red-free cHBSS (final pH 7.0). The respiratory burst was triggered by the addition of 1 µg ml^{-1} PMA to the reaction mixture. After 60 minutes, the reaction was stopped by adding 10 µl of 1 N NaOH to each well and the OD_{610} was determined in a multiscan spectrophotometer (Dynatech, UK) using PRS plus NaOH only as blank. The specificity of the reaction was demonstrated by the addition of 1 mM

sodium nitroprusside (Sigma, UK), an inhibitor of SOD, to some of the wells. The reaction was conducted in quadruplicate wells for each treatment.

In all cases, duplicate wells for each treatment were used to determine the number of macrophages per well as described in section 2.7.3. Results were expressed as OD_{610} per $2x10^5$ cells for the intracellular O_2^- and H_2O_2 production tests and as nmol O_2^- per $2x10^5$ cells for the extracellular release of superoxide anion, as described in section 2.9.

Nitric oxide production

Production of nitric oxide by macrophages was determined by quantification of nitrite in culture supernatants, as described in section 2.10.1. Serial dilutions of a $NaNO_2$ solution were used as positive controls.

Macrophage stimulation

Working suspensions of each test substance were obtained by diluting stock suspensions in L-15 containing 5 % FCS and P/S. The concentration of each substance in the working suspension was adjusted to 0, 0.1, 0.5, 1, 5, 10 and 50 $\mu g\ ml^{-1}$.

Macrophage cells from 4 individual fish were used to test each substance. Monolayers were incubated with the working suspensions for 72 h at 19 °C. Monolayers were then washed three times with cHBSS prior to quantification of the intracellular respiratory burst activity and supernatants assayed for nitrite concentration as described above. The dose of each substance which elicited the maximum NBT reduction by macrophages following stimulation with PMA (see results) was chosen for further experiments. For simplicity, this dose will be referred to as optimum dose hereafter.

Macrophage monolayers from 8 individual fish were incubated with the optimum dose of each test substance for 72 h at 19 °C. Monolayers were then washed three times with cHBSS and the production of O_2^- (intra- and extra-cellular) and hydrogen peroxide quantified as described above.

Statistical analysis

The results from the experiment conducted to assess NBT reduction by macrophages incubated with different doses of the test substances were analysed by regression models and by two way ANOVA and Tukey's multiple comparisons tests. The following transformation was carried out for the regression analysis:

$y = \log([A]+1)$ where $[A]$ is the concentration of the test substance.

The software CurveExpert 1.3® was used to obtain the best regression fits. When polynomial regressions were considered, the maximum power at which the regression had statistical significance was determined by the Student *t*-test (Zar, 1984). Two variables were considered related when $p<0.05$.

Two way ANOVA was performed with one variable fixed (substance concentration) and the other randomised (fish) as described in Zar (1984). Type II two way ANOVA was chosen since all concentrations of each substance were tested on cells isolated from the same animal. Differences between treatments were considered statistically significant if $p<0.05$.

Results obtained from the NBT, cytochrome c and phenol red reduction tests when comparing the optimum dose for each substance were analysed by type II two way ANOVA and Tukey's tests as described above.

3.3. Results

Immunostimulants

The number of particles, particle mean size and the coefficient of variation (CV) of the particle size for each of the test substances were calculated from a suspension containing 10 μg ml^{-1} of the test substance (table 3.1). All yeast glucans and peptidoglycan suspensions showed a similar mean size and coefficient of variation. AO glucan suspension contained the lowest number of particles and highest CV of the particle size.

No bacterial or fungal growth was observed in TSB or TSA with any of the test substances after γ-irradiation.

Table 3.1 Number of particles, mean size (μm) and coefficient of variation of the particle size obtained from suspensions containing 10 μg ml^{-1} of each test substance.

Substance	No. of particles	Mean size ± sd	CV (%)
Peptidoglycan	$1.68x10^6$	2.87 ± 0.63	21.90
Vetregard α	$1.40x10^6$	3.99 ± 0.83	20.78
Vetregard β	$1.32x10^6$	3.82 ± 0.66	17.31
Macrogard	$3.28x10^6$	2.90 ± 0.33	11.23
AO glucan	$4.00x10^5$	3.55 ± 1.89	53.19

CV, coefficient of variation; sd, standard deviation

Respiratory burst activity

Internalisation of Vetregard α and AO glucan was demonstrated as shown in figure 3.1. All yeast glucans induced a significant dose-dependent modulation of NBT reduction by macrophages (table 3.2). The best fit (as defined by significantly higher r^2) for the test substance dose versus PMA-triggered NBT reduction activity was the quadratic or cubic polynomial regression in all yeast glucans (figures 3.2, 3.3 and 3.4). These polynomial regressions indicated that there was a dose-related increase in activity at lower concentrations which decreased progressively with higher doses. Macrogard elicited a maximum response at a lower dose than either of the two Vetregard substances (1 μg ml^{-1} *vs.* 5 μg ml^{-1} respectively, table 3.2).

The bacterial peptidoglycan also induced a significant dose-dependent modulation of intracellular O_2^- production by macrophages. However the best fit for this substance was the logistic model. This model indicated that lower doses induced an increase of activity reaching a maximum which was maintained over a wide range of concentrations (figure 3.5). The peptidoglycan concentrations which elicited the maximum response following stimulation with PMA were 0.5, 1, 5 and 10 μg ml^{-1} as shown in table 3.2.

The dose of AO glucan and macrophage NBT reduction activity were significantly related and the best fit was the polynomial regression (figure 3.6). However, there was not a significant increase of activity at any of the concentrations tested (table 3.2).

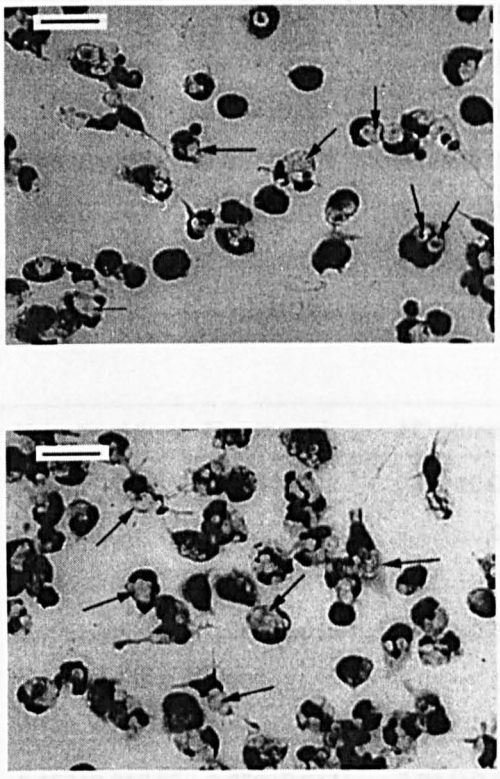

Figure 3.1 Glucan particles ingested by head kidney macrophages.

Macrophages from rainbow trout were incubated with 10 μg ml^{-1} of Vetregard α (**A**) or AO glucan (**B**). After 30 minutes slides were fixed and stained with Rapi-Diff as described elsewhere. Some internalised glucans are shown by arrows. Photographs were taken with an Olympus BX50 microscope. Bar is 20 μm in length.

Table 3.2 Effect of different doses of β-glucans and peptidoglycan on production of intracellular O_2^- by head kidney macrophages.

A

μg ml^{-1}	Vetregard α	Vetregard β	Macrogard	AO glucan	Peptidoglycan
0	0.109±0.016 ^{1}	0.109±0.016 ^{1}	0.103±0.008 ^{1}	0.112±0.025 ^{1,2}	0.112±0.025 ^{1}
0.1	0.138±0.016 ^{1,2}	0.128±0.024 ^{1,2}	0.109±0.019 ^{1}	0.098±0.024 ^{1}	0.165±0.024 ^{1,2}
0.5	0.212±0.017 ^{3,4}	0.168±0.025 ^{2,3}	0.154±0.030 ^{1}	0.111±0.020 ^{1,2}	0.227±0.035 ^{2}
1	0.230±0.041 ^{3,4}	0.188±0.035 ^{3,4}	0.237±0.077 ^{2}	0.123±0.030 ^{1,2}	0.223±0.041 ^{2}
5	0.261±0.037 ^{3}	0.252±0.284 ^{5}	0.156±0.036 ^{1}	0.132±0.028 ^{1,2}	0.227±0.042 ^{2}
10	0.214±0.037 ^{3,4}	0.234±0.016 ^{4,5}	0.122±0.031 ^{1}	0.160±0.017 ^{2}	0.224±0.042 ^{2}
50	0.190±0.325 ^{2,4}	0.195±0.013 ^{4}	0.102±0.016 ^{1}	0.135±0.030 ^{1,2}	0.188±0.046 ^{1,2}

B

μg ml^{-1}	Vetregard α	Vetregard β	Macrogard	AO glucan	Peptidoglycan
0	0.055±0.013 ^{1}	0.055±0.013 ^{1}	0.053±0.018 ^{1}	0.031±0.012 ^{1}	0.031±0.012 ^{1}
0.1	0.066±0.015 ^{1,2}	0.007±0.016 ^{1,2}	0.053±0.006 ^{1}	0.030±0.004 ^{1}	0.046±0.014 ^{1}
0.5	0.077±0.013 ^{1,2,3}	0.094±0.005 ^{1,2,3}	0.069±0.010 ^{1,2}	0.026±0.014 ^{1}	0.066±0.026 ^{1}
1	0.091±0.009 ^{2,3}	0.108±0.009 ^{2,3,4}	0.083±0.015 ^{2}	0.029±0.012 ^{1}	0.073±0.032 ^{1}
5	0.119±0.016 ^{4}	0.143±0.034 ^{4}	0.066±0.009 ^{1}	0.032±0.007 ^{1}	0.072±0.027 ^{1}
10	0.098±0.015 ^{3,4}	0.129±0.029 ^{4}	0.064±0.010 ^{1}	0.041±0.012 ^{1}	0.075±0.030 ^{1}
50	0.085±0.009 ^{2,3}	0.103±0.019 ^{2,4}	0.059±0.011 ^{1}	0.044±0.007 ^{1}	0.063±0.017 ^{1}

Results are expressed as arithmetic mean ± standard deviation. Different concentrations of each test substance were tested on cells from the same animal (repeated on four animals). Significant differences within each column are shown by different superscript numbers (p<0.05). **A**, NBT reduction activity triggered by PMA; **B**, without PMA.

Therefore, the dose for each substance inducing maximum NBT reduction activity were as follows: 5 μg ml^{-1} for the two Vetregard glucans, 1μg ml^{-1} for Macrogard and 0.5, 1, 5 and 10 μg ml^{-1} for the peptidoglycan (table 3.2). The dose of 1μg ml^{-1} peptidoglycan was chosen for further experiments. Although AO glucan did not induce any significant increase of NBT reduction, the dose of 10 μg ml^{-1} was chosen for further experiments since it elicited a non-significant increase of NBT reduction activity following stimulation with PMA.

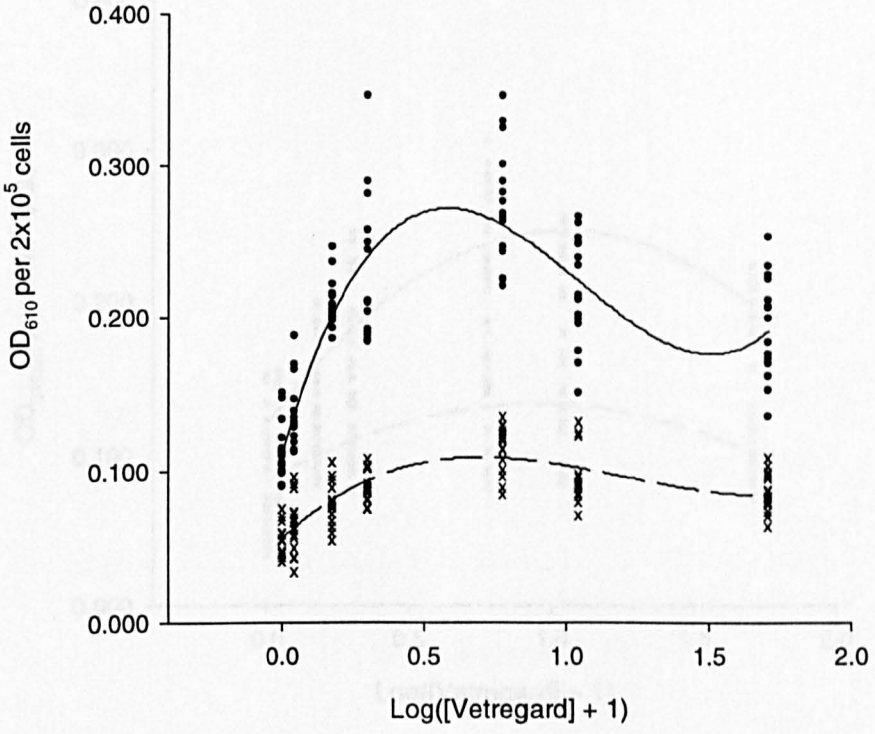

Figure 3.2 Effect of varying doses of Vetregard α on the production of intracellular superoxide anion by head kidney macrophages.

Respiratory burst was triggered by PMA (•) or without (x). The solid line indicates the regression between the concentration of the test substance and NBT reduction activity after PMA stimulation (y=0.111+0.635x-0.756x^2+0.241x^3; r^2=0.719; p<0.001). The broken line indicates the regression between the concentration of the test substance and NBT reduction activity without PMA stimulation (y = 0.055+0.179x-0.180x^2+0.049x^3; r^2=0.624; p<0.001).

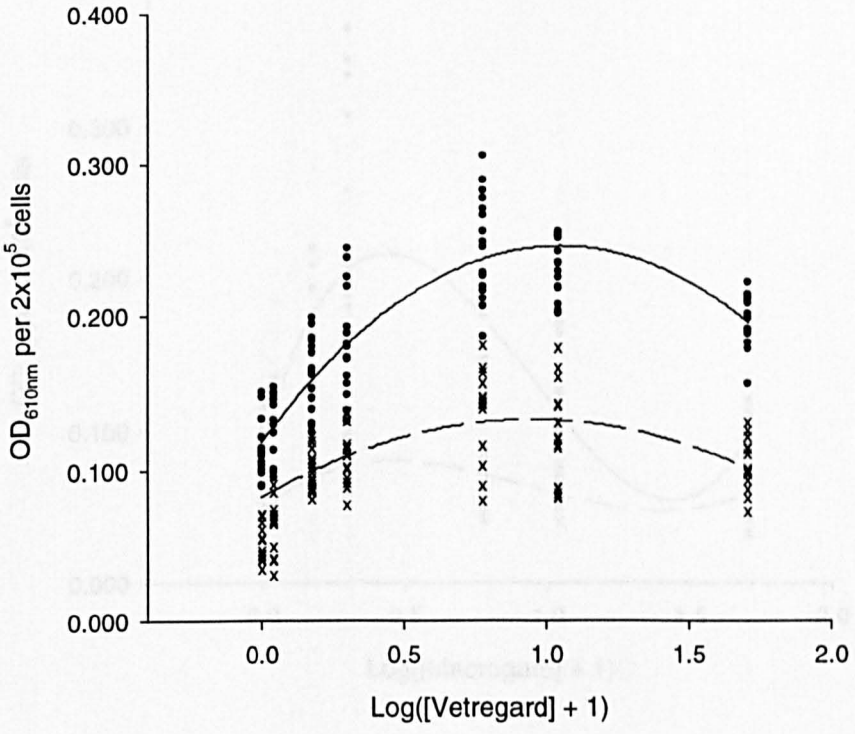

Figure 3.3 Effect of varying doses of Vetregard β on the production of intracellular superoxide anion by head kidney macrophages.

Respiratory burst was triggered by PMA (•) or without (x). The solid line indicates the regression between the concentration of the test substance and NBT reduction activity after PMA stimulation (y=0.118+0.242x-0.115x^2; r^2=0.770; p<0.001). The broken line indicates the regression between the concentration of the test substance and NBT reduction activity without PMA stimulation (y=0.083+0.105x-0.055x^2; r^2=0.331; p<0.001).

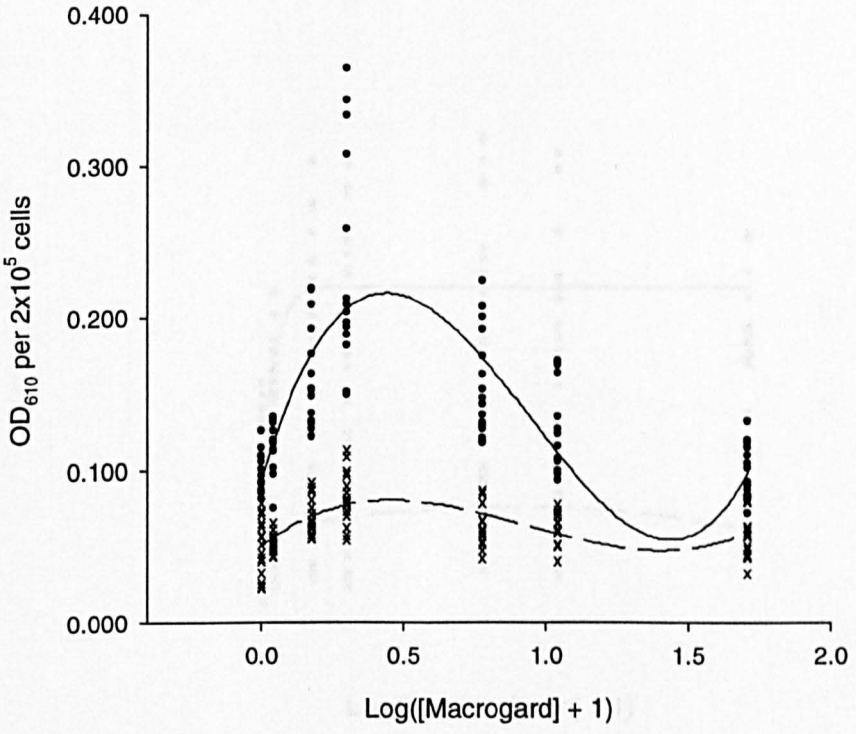

Figure 3.4 Effect of varying doses of Macrogard on the production of intracellular superoxide anion by head kidney macrophages.

Respiratory burst was triggered by PMA (•) or without (x). The solid line indicates the regression between the concentration of the test substance and NBT reduction activity after PMA stimulation (y=0.093+0.623x-0.922x^2+0.327x^3; r^2=0.548; p<0.001). The broken line indicates the regression between the concentration of the test substance and NBT reduction activity without PMA stimulation (y=0.051+0.146x-0.212x^2+0.076x^3; r^2=0.290; p<0.001).

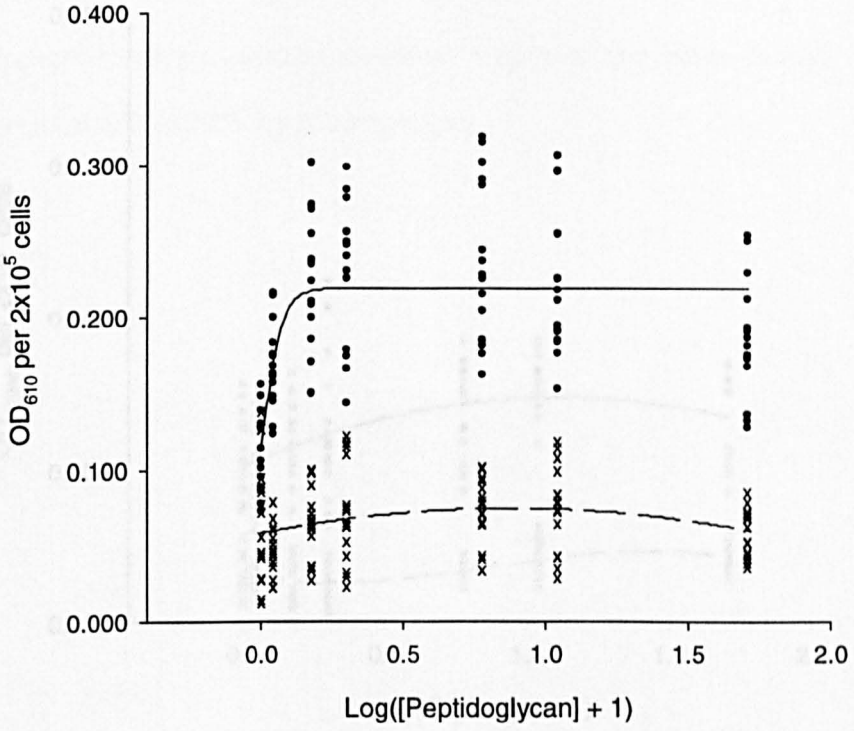

Figure 3.5 Effect of varying doses of peptidoglycan on the production of intracellular superoxide anion by head kidney macrophages.

Respiratory burst was triggered by PMA (•) or without (x). The solid line indicates the regression between the concentration of the test substance and NBT reduction activity after PMA stimulation ($y = \frac{0.219}{1 + 0.928e^{-27.4x}}$; r^2=0.450; p<0.001). The broken line indicates the regression between the concentration of the test substance and NBT reduction activity without PMA stimulation ($y = 0059 + 0.036x - 0.020x^2$; r^2=0.048; p>0.05).

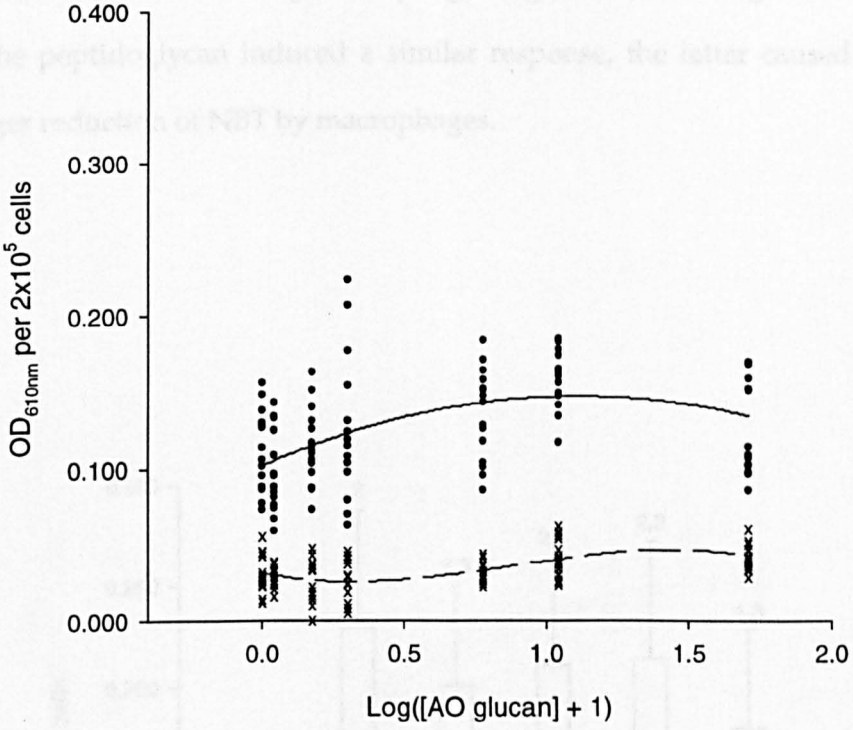

Figure 3.6 Effect of varying doses of AO glucan on the production of intracellular superoxide anion by head kidney macrophages.

Respiratory burst was triggered by PMA (•) or without (x). The solid line indicates the regression between the concentration of the test substance and NBT reduction activity after PMA stimulation (y=0.107+0.005x+0.087x^2-0.048x^3; r^2=0.239; p<0.001). The broken line indicates the regression between the concentration of the test substance and NBT reduction activity without PMA stimulation (y=0.032-0.041x+0.077x^2-0.029x^3; r^2=0.288; p<0.001).

When the optimum dose of each test substance were compared on cells from the same individuals, all substances except AO glucan were able to induce a significant increase in NBT reduction by macrophages (figure 3.7). Although the three yeast glucans and the peptidoglycan induced a similar response, the latter caused a non-significant stronger reduction of NBT by macrophages.

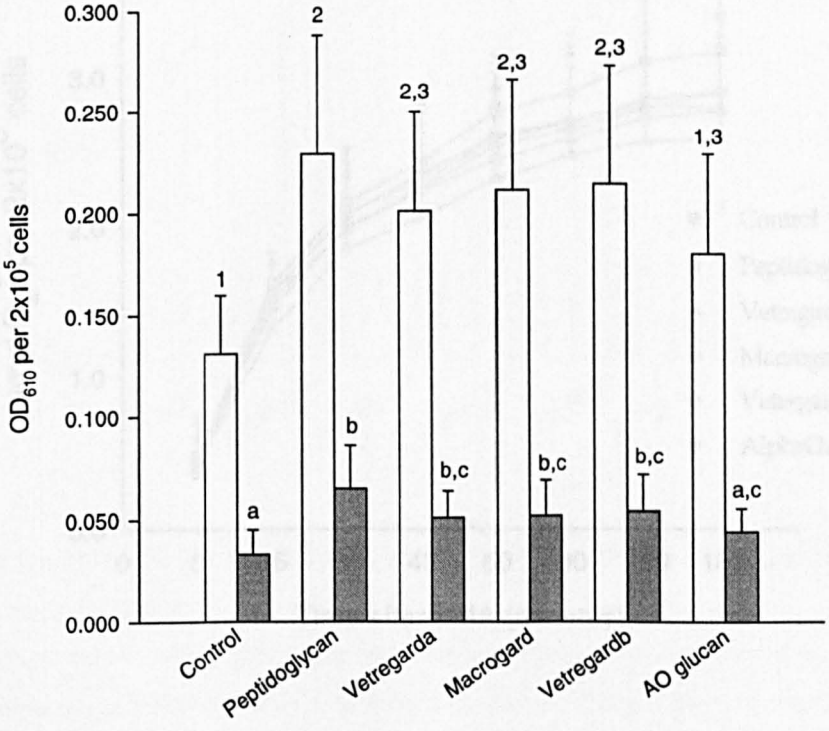

Figure 3.7 Effect of the optimum dose of each test substance on the production of intracellular superoxide anion by head kidney macrophages.
Respiratory burst was triggered by PMA (empty bars) or without (solid bars). Results are expressed as arithmetic mean ± standard deviation (n=8). Different numbers on sd bars indicate significant differences between different test substances following stimulation with PMA ($p<0.05$). Different letters on sd bars indicate significant differences between different test substances without stimulation with PMA ($p<0.05$).

However, the optimum doses of all test substances did not induce any significant difference in the production of extracellular superoxide anion as quantified by the reduction of cytochrome c (figure 3.8).

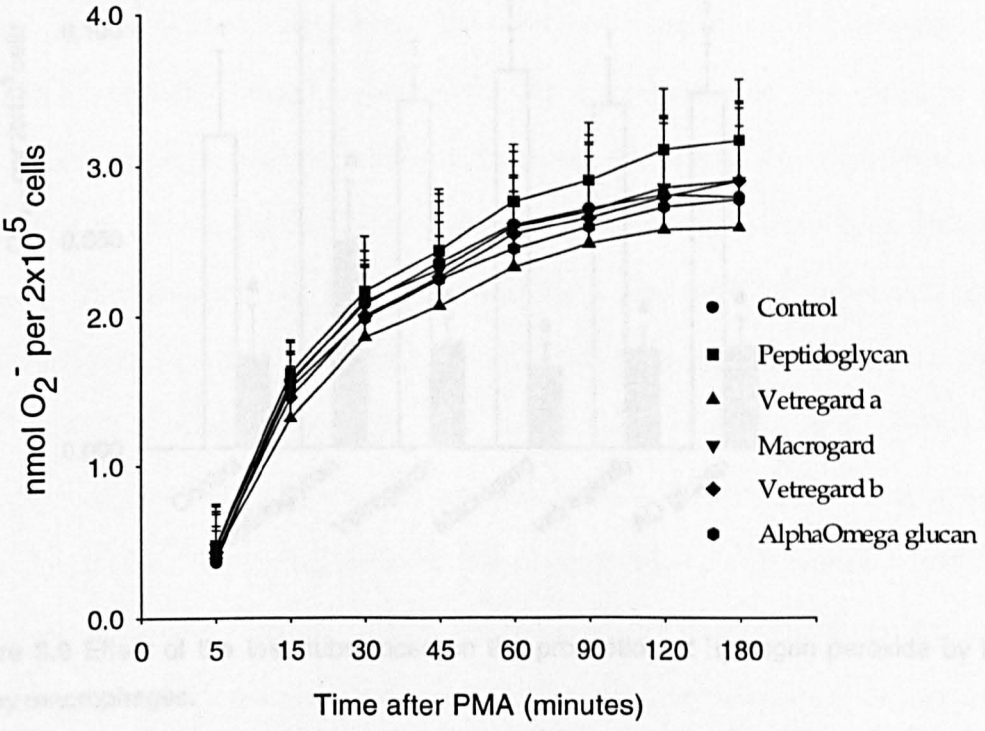

Figure 3.8 Effect of the test substances on the production of extracellular superoxide anion by head kidney macrophages.
Respiratory burst was triggered by PMA. Results are expressed as arithmetic mean ± standard deviation (n=8).

Production of hydrogen peroxide by macrophages was increased only by 1 μg ml^{-1} peptidoglycan and this increase was significant with or without stimulation with PMA (figure 3.9).

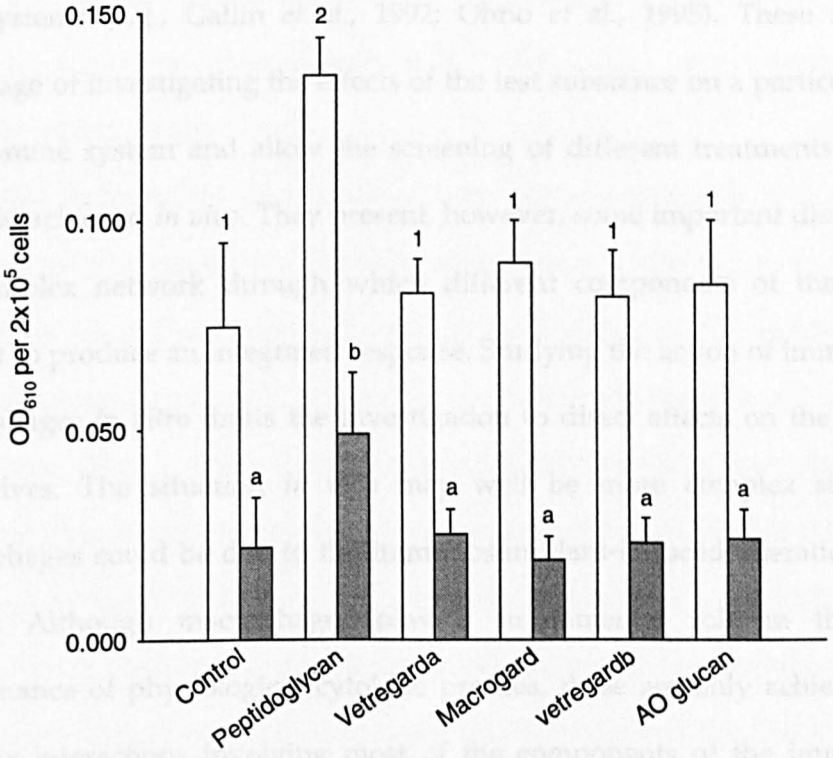

Figure 3.9 Effect of the test substances on the production of hydrogen peroxide by head kidney macrophages.
Respiratory burst was triggered by PMA (empty bars) or without (solid bars). Results are expressed as arithmetic mean ± standard deviation (n=8). Different numbers on sd bars indicate significant differences between different test substances following stimulation with PMA ($p<0.05$). Different letters on sd bars indicate significant differences between different test substances without stimulation with PMA ($p<0.05$).

Nitric oxide production

Although nitrite was measured in serial dilutions of sodium nitrite, it was not detected in the macrophage supernatants under any of the treatments applied.

3.4. Discussion

In this study, three yeast (1→3),(1→6)-β-glucans, one barley (1→2),(1→6)-β-glucan and a bacterial peptidoglycan were screened *in vitro* for their activity on macrophage respiratory burst and production of nitric oxide. Studies to compare the immunopharmacological activities of different substances are often carried out using *in vitro* systems (e.g., Gallin *et al.*, 1992; Ohno *et al.*, 1995). These systems offer the advantage of investigating the effects of the test substance on a particular component of the immune system and allow the screening of different treatments more easily than could be achieved *in vivo*. They present, however, some important disadvantages due to the complex network through which different components of the immune system interact to produce an integrated response. Studying the action of immunostimulants on macrophages *in vitro* limits the investigation to direct effects on the macrophage cells themselves. The situation *in vivo* may well be more complex since activation of macrophages could be due to the immunostimulant-induced alteration of the cytokine profile. Although macrophages play a fundamental role in the synthesis and maintenance of physiological cytokine profiles, these are only achieved as a result of complex interactions involving most of the components of the immune system. As reviewed by Bohn and Bemiller (1995), the mode of immunopotentiation by (1→3)-β-glucans in mammalian species is likely to involve not only activation of macrophages but also neutrophils, helper T cells, NK cells and promotion of T cell differentiation. Therefore, interpretation of results of immunopharmacological studies on macrophage activation *in vitro* should take account of this immune network.

It has been shown that glucans are able to induce an elevated expression of the iNOS gene in murine macrophages *in vitro* (Hashimoto *et al.*, 1997). The iNOS gene has been identified in goldfish (Laing *et al.*, 1996) and rainbow trout (Grabowski *et al.*, 1996) and published works indicate the production of inducible nitric oxide by different fish species (Schoor and Plumb, 1994; Neumann *et al.*, 1995; Mulero and Meseguer, 1998). In

the present study nitrite was not detected in the supernatants of macrophages stimulated with the glucans or peptidoglycan at different doses. The detection limit for nitrite was 3 nmol per well as determined by serial dilutions of sodium nitrite. Contradictory results exist as to whether rainbow trout macrophages are able to synthesise nitric oxide. While NO has been shown to be produced by head kidney mixed leukocyte cultures *in vitro* (Zunic and Licek, 1997), other studies have failed to demonstrate NO synthesis by macrophage cultures (Campos-Perez *et al.*, 1997b; results here presented and in chapters 4, 5 and 6 of this thesis).

PMA-independent production of intracellular superoxide anion was significantly enhanced only by yeast glucans. Single microbial components may trigger the phagocyte respiratory burst. However, full activation of the NADPH enzyme system *in vivo* requires activation of various receptors by microorganisms and host-derived stimuli (Seifert and Gunter, 1991). Soluble molecules such as PMA are strong inducers of the respiratory burst and are generally used to assay full activation of this pathway (Seifert and Gunter, 1991). All yeast glucans and the bacterial peptidoglycan tested in the present study induced a significant modulation of intracellular superoxide anion production by head kidney macrophages after PMA stimulation. Peptidoglycan induced, however, a significant increase of intracellular O_2^- production that was maintained over a significantly wider range of concentrations. This was reflected both in the regression analysis and the Tukey's multiple comparison test. Previous reports also indicate that (1→3),(1→6)-β-glucans induce maximum production of O_2^- by fish macrophages over a narrow range of concentrations (Jørgensen, 1994; Tahir and Secombes, 1996). All yeast β-glucans induced a similar pattern of modulation of macrophage activity, however, Macrogard was able to induce a maximum production of intracellular superoxide anion at a lower dose. Linear (1→3)-β-glucans possessing (1→6)-linked β-glucosyl side branches ((1→3),(1→6)-β-glucans) are generally considered to be the most effective β-glucan immunostimulants (Bohn and Bemiller, 1995). In this study, barley (1→2),(1→6)-

β-glucan did not induce any significant increase in the production of intracellular superoxide anion, unlike results with the substances containing (1→3),(1→6)-linked moiety. It should be noted that regression analyses are only valid for the range of concentrations applied. If higher concentrations of the test substances had been used, the model lines for the PMA+ response in figures 3.2, 3.3 and 3.4 might have shown a decrease of activity at the highest concentrations.

Extracellular superoxide anion production is important in killing microorganisms as well as in immunomodulation (Koner, 1997) but due to its high reactivity it may cause damage to self if not properly regulated (Gille and Sigler, 1995). In all cases, it was demonstrated that the test substance dose which induced maximum production of intracellular superoxide anion did not significantly modulate the production of extracellular superoxide anion. Enhancement of respiratory burst activity has been shown to be mediated by synthesis *de novo* of at least some cytosolic and membrane components of the NADPH oxidase (Tennenberg *et al.*, 1993). NADPH oxidase components synthesised *de novo* after incubation with immunostimulants might be preferentially translocated to the immunostimulant-containing phagosome membrane rather than to the plasma membrane. This would result in increased O_2^- generation predominantly in the intracellular compartment. In this study, the substances tested were quickly phagocytosed by most macrophages and induced the maximum NBT reduction activity at a similar number of particles per well (1 macrophage:1-4 particles approximately). Although low doses of particulate β-glucans have been shown to enhance production of extracellular O_2^- by teleost fish, higher doses such as used in this study did not induce any significant change (Tahir and Secombes, 1996).

The production of hydrogen peroxide by macrophages is a result of dismutation of superoxide anion following initiation of the respiratory burst. Hydrogen peroxide synthesis is an important moiety in the killing of pathogens by phagocytes and, in myeloperoxidase (MPO) - positive phagocytes, it is catalysed by MPO to yield highly

reactive HClO (Gabig and Babior, 1981). High doses (25-100 μg ml^{-1}) of particulate yeast β-glucans have been shown to induce a rapid increase in hydrogen peroxide production by murine macrophages (Okazaki *et al.*, 1996). In this study, only peptidoglycan was able to induce a significant increase in the production of H_2O_2, although macrophages were incubated with lower doses over longer periods of time than in the murine experiment.

Finally, incubation of macrophages with latex particles of different sizes may provide information on the effect of the particle size of the immunostimulants used. The use of latex beads in experiments to investigate the effect of particulate test substances on macrophages is therefore recommended.

Thus, a different modulation of the respiratory burst was observed amongst the different groups of test substances. Yeast β-glucans and bacterial peptidoglycan were able to induce an increased production of intracellular superoxide anion. This increase was greater and sustained over a wider range of concentrations in the case of the peptidoglycan. The wide dose tolerance eliciting maximum production of intracellular O_2^- by peptidoglycan may have important practical consequences in the field. A key factor in the success of pharmacological treatment is the accuracy in the dose delivered to individual animals. In aquaculture, treatment of animals is usually carried out orally in combination with the feed. It is well known that feed uptake, and consequently the treatment associated with it, is very variable amongst a population of farmed fish. This variability may result from differences in the physical, social, immune, etc. status of the animal. The use of the peptidoglycan *in vivo* may, therefore, have certain advantages over the other substances tested. Peptidoglycan and Vetregard α were chosen for further experiments *in vivo*.

Chapter 4

Modulation of rainbow trout innate defence mechanisms by β-glucan and peptidoglycan *in vivo*.

4.1 Introduction

Since prophylactic use of antibiotics is associated with undesired emergence of resistant bacterial strains, other methods to reduce the impact of infectious diseases become more important. A wide range of chemically diverse compounds has been shown to increase fish immune performance and resistance to infectious challenges (reviewed in Raa, 1996). These include bacterial cell wall products, (1→3)-β-glucans, peptides and synthetic compounds. Current knowledge of the effects of immunostimulants on fish defence mechanisms and resistance to bacterial infections is summarised in section1.4.

The aim of this study was to investigate the use of bacterial peptidoglycan in feed to enhance innate defence mechanisms of rainbow trout and to compare efficacy with similar application of yeast (1→3),(1→6)-β-glucan. Peptidoglycan and Vetregard α (yeast β-glucan) were selected as potential immunostimulants since they induced the highest stimulation of macrophages *in vitro* (chapter 3). Initial experiments were conducted to confirm their stimulatory capacity *in vivo*. For this purpose, the test substances were administered intraperitoneally and peritoneal macrophage activity assayed. Further experiments involved the assessment of immunostimulation by in-feed administration of the test substances. Different treatment regimes were applied and serum haemolytic activity as well as function of resident and inflammatory macrophages investigated. In addition, *A. salmonicida* killing *in vivo* was assessed in animals orally treated with peptidoglycan and in controls.

4.2. Materials and methods

Animals and bacterial strain

All-female rainbow trout were obtained from Almond Bank Trout Farm (Scotland) and acclimatised to flow-through aquarium conditions at 6 ± 2 °C for a minimum of 4 weeks as described in section 2.2 before experiments began. Animals from this stock were used for all experiments described in this chapter.

Aeromonas salmonicida isolate B95179 (isolate details are given in section 2.8.2) was used in phagocytosis and bacterial killing experiments in this chapter.

Intraperitoneal administration of immunostimulants

After acclimatisation, groups of 12 fish weighing 133.2 ± 6.6 g were allocated to two similar 370 l aerated tanks with a flow-through water supply. Fish were fed twice daily with commercial trout pellets to the manufacturer's RDA (Trouw Select No. 40; 1.84% of body weight, bw). Water temperature was raised gradually over a period of 14 days and kept constant at 14.5 ± 0.5 °C during the experiment. Six fish in each tank were then anaesthetised with benzocaine and injected intraperitoneally with 2 ml of a filter-sterilised 8 % casein solution in saline containing either 0.25 mg ml^{-1} peptidoglycan or β-glucan. The remaining 6 fish in each tank were handled similarly and injected with 2 ml of a sterile 8 % casein solution only.

Fish were sacrificed 6 days after injection and peritoneal macrophages harvested and assayed for intra- and extra-cellular respiratory burst activity and nitric oxide production.

In-feed administration of immunostimulants

Commercial trout pellets were thoroughly mixed with β-glucan or peptidoglycan at the desired concentration and surface-coated with 2 % (v/w) fresh cod liver oil. Control feed was prepared by mixing pellets with 2 % cod liver oil only. Experimental feed was kept in the fridge and used within 30 days of preparation to avoid rancidity developing.

Groups of 35 fish, individual average weight 140.6 ± 14.7 g, were distributed into three similar flow-through 370 l aerated tanks. Water temperature was gradually raised over a period of 13 days and kept constant at 14 ± 0.5 °C throughout the experiment. Fish were fed twice daily to the manufacturer's RDA (Ewos Select No. 40; 1.84 % bw). One group of fish was fed with control feed while the other two groups of fish were fed on a diet containing β-glucan at 0.05 % and 5 % of feed weight respectively. Six fish per treatment were sacrificed after 1, 2, 3 and 4 weeks on the experimental or control diets and sampled for haematological parameters (serum haemolytic activity, haematocrit, PBL counts) and head kidney macrophage activity (phagocytosis, intra- and extra-cellular respiratory burst, nitric oxide production and killing of *A. salmonicida*).

The experiment described above was repeated using peptidoglycan instead of β-glucan as immunostimulant. The average fish weight was 172.4 ± 19.2 g and water temperature was kept constant at 14 ± 0.5 °C during the experiment.

Since peptidoglycan showed some advantages over yeast glucans in enhancing macrophage activity *in vitro*, a final experiment was carried out with this compound. Groups of 35 fish individually weighing 241.9 ± 11.7 g were put in four 370 l aerated tanks with flow-through water supply. Water temperature was raised progressively over a period of 14 days and then kept constant at 13.5 ± 0.5°C. Fish in two tanks were fed twice daily to the RDA (Trouw Select No. 50; 1.62 % bw) on control feed while those from the other two tanks were fed on peptidoglycan-containing feed (0.05% of feed weight). Four weeks after the beginning of treatment, six fish from one control tank and six fish from one treatment tank were sacrificed and sampled for serum haemolytic activity and head kidney macrophage activity (phagocytosis, respiratory burst, nitric oxide production and *A. salmonicida* killing). The remaining fish in those two tanks were injected intraperitoneally with *A. salmonicida* to monitor bacterial clearance from the spleen. Fish in the other two tanks were used to elicit an inflammatory peritoneal exudate. For this purpose, 10 fish from each of the two tanks were injected

intraperitoneally with 2 ml of a filter-sterilised 8 % casein solution in saline four weeks after the beginning of treatment. Six fish from each tank were sacrificed six days after casein injection to assess peritoneal macrophage respiratory burst. After *A. salmonicida* or casein injection, fish in all groups were fed on control diet.

Haematology

Haematocrit and PBL counts were determined in EDTA-treated blood from individual fish as described in section 2.3

Serum from individual fish was isolated, stored and assayed for lytic activity against rabbit red blood cells as detailed in section 2.6.

Isolation and culture of macrophages

Head kidney and peritoneal macrophages were enriched in suspension and monolayers prepared on 96-well microtiter plates and 8-well glass slides as described in section 2.7.1. Macrophages were then cultured at 19 °C in L-15 containing 5 % FCS and P/S. No antibiotics were added to monolayers prepared to study killing of *A. salmonicida.* Monolayers were used within 5 h of preparation after three washes with cHBSS.

Phagocytosis

A. salmonicida was grown overnight in TSB at 22 °C with continuous shaking. Bacterial cells were opsonised with fresh pooled sera from five trout donors and adjusted to a concentration of $3.2x10^8$ CFU ml^{-1} as described in section 2.8.2. The number of adherent macrophages per well was calculated as described in section 2.7.3. Head kidney macrophage monolayers were incubated at 19 °C with 0.4 ml of a diluted *A. salmonicida* suspension to give a ratio of 1 macrophage to 5 bacterial cells. After 60 minutes, monolayers were washed three times with cHBSS and stained with Rapi-Diff. Phagocytic index and phagocytosis ratio were estimated, as described in section 2.8.1, by examining 300 macrophages per fish under an Olympus optical microscope at 1000x magnification.

Respiratory burst

The production of extracellular superoxide anion by macrophages was quantified in triplicate wells by the PMA-triggered reduction of cytochrome c as described in section 2.9.1. Superoxide dismutase was added to triplicate wells per fish to confirm specificity of the reaction.

Macrophage intracellular respiratory burst activity was measured in triplicate wells by the reduction of NBT in the presence or absence of PMA for 30 minutes as described in section 2.9.2

In both assays, duplicate wells were used to estimate the number of macrophages per well and results were adjusted to OD_{610} or nmol O_2^- produced per $2x10^5$ cells for the NBT and cytochrome c assays respectively.

Nitric oxide

Inducible nitric oxide synthase activity was measured as described in section 2.10. Macrophage monolayers were stimulated with MAF and 40 μg ml^{-1} of *E. coli* LPS (serotype 026:B6, Sigma, UK) in L-15 containing 5 % FCS and P/S for 96 h at 19 °C. Supernatants were then assayed for nitrite by the Griess reaction. Serial dilutions of sodium nitrite were used as positive controls.

A. salmonicida killing *in vitro*

A. salmonicida was grown overnight in TSB at 22 °C with continuous shaking. Bacteria were washed three times in PBS, opsonised with the same pooled sera as that used in the phagocytosis assay, and adjusted to $3.2x10^8$ CFU ml^{-1} as described in section 2.8.2. Head kidney macrophage monolayers were incubated with serum opsonised *A. salmonicida* at a ratio of 1 macrophage to 20 bacterial cells for 5 h at 19 °C. Viable bacteria in the mixture wells were then quantified by the reduction of MTT and the percentage of bacteria killed obtained as described in section 2.11.

A. salmonicida clearance *in vivo*

A. salmonicida was grown overnight in TSB at 22 °C with continuous shaking. The bacterial suspension was washed twice in saline and adjusted spectrophotometrically to a concentration of $3.2x10^8$ CFU ml^{-1} (OD_{610}= 1.24 in TSB). A suspension containing $1x10^5$ CFU ml^{-1} in saline was prepared as challenge dose and the concentration confirmed by viable counts. Each fish received an intraperitoneal dose of 200 µl of $1.1x10^5$ *A. salmonicida* CFU ml^{-1}. Six fish per treatment were sacrificed and spleens and blood sampled for viable bacteria 6, 12, 24, 48, 72 and 96 h after challenge as described in section 2.12.

Statistical analysis

Comparisons between the results of intraperitoneally administered immunostimulant samples and controls were carried out by the Student *t*-test.

Results from the experiments on oral administration were analysed by repetitive measurements one-way ANOVA and Newman-Keuls test when three doses were compared or by *t*-test when only two doses were investigated. After confirming by *t*-test that no differences were found within the same treatment group throughout the different sampling times, samples from the same treatment were pooled together and compared by one-way ANOVA and Newman-Keuls test.

Normality and homogeneity of variance was confirmed before any parametric test was applied. When normality failed, the logarithmic transformation was applied. All percentage values were made normal by the square root arcsin transformation. One way ANOVA on ranks was used when transformed data were still not normal. Statistical tests were carried out with SigmaStat® (Jandel Scientific) and, in all cases, $p<0.05$ was the accepted significant level. Regression analyses were used to confirm linearity between number of viable *A. salmonicida* and MTT reduction in the bacterial killing *in vitro* assay.

The relative risk of *A. salmonicida* being recovered from the spleen was investigated using the software EPI-INFO 6.04 (Centre for Disease Control and Prevention, USA, and World Health Organisation, Switzerland).

4.3 Results

Intraperitoneal administration of immunostimulants

Production of intra- and extra-cellular superoxide anion by peritoneal macrophages was significantly enhanced following administration of either β-glucan or peptidoglycan (figures 4.1 and 4.2). No significant differences were found between the respiratory burst activity of macrophages from fish treated with β-glucan or peptidoglycan.

Nitrite, a major product of nitric oxide spontaneous turnover, was not detected in any culture supernatants of peritoneal macrophages incubated with or without MAF plus LPS. The lowest detection of nitrite using several dilutions of $NaNO_2$ was 3.5 nmol per well.

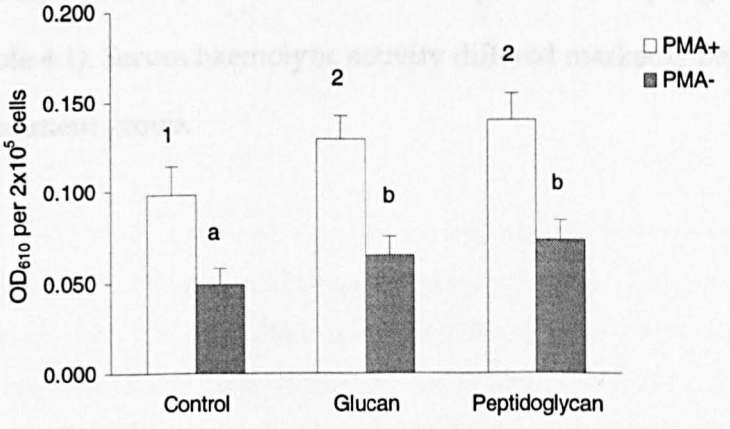

Figure 4.1 Reduction of NBT by peritoneal macrophages.
Results are expressed as arithmetic mean ± sd (n=6). Different numbers or letters on sd bars indicate significant modulation of activity stimulated with or without PMA respectively.

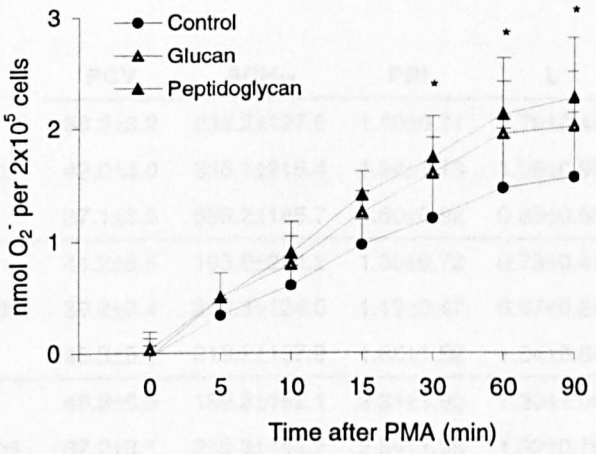

Figure 4.2 Production of extracellular O_2^- by peritoneal macrophages.
Results are expressed as arithmetic mean ± sd (n=6). * indicates significant difference between the two treatments and control.

In-feed administration of immunostimulants

In-feed administration of β-glucan or peptidoglycan did not have a significant effect on serum haemolytic activity or PBL counts at any of the sampling times compared with controls (table 4.1). Serum haemolytic activity differed markedly between animals within the same treatment group.

Table 4.1 Haematological parameters of in-feed β-glucan and peptidoglycan treated fish.

A		PCV	ACH_{50}	PBL	L	T	N	M
1 week	G 0	38.2±3.2	239.2±127.5	1.40±0.71	0.79±0.40	4.03±2.24	1.66±0.75	2.09±1.27
	G 0.05	42.0±3.0	335.1±215.4	1.94±1.73	1.09±0.98	5.66±5.24	2.15±1.54	2.68±1.99
	G 5	37.1±3.5	356.2±185.7	1.50±0.92	0.85±0.52	4.29±2.91	1.73±0.72	2.09±1.07
2 weeks	G 0	41.2±5.5	195.9±254.2	1.30±0.72	0.73±0.40	3.71±2.16	1.57±0.92	2.00±1.50
	G 0.05	39.2±3.4	250.3±124.0	1.19±0.47	0.67±0.26	3.39±1.53	1.44±0.55	1.79±1.02
	G 5	45.3±5.6	219.2±167.9	1.85±1.52	1.04±0.84	5.21±4.29	2.32±2.16	3.02±3.32
3 weeks	G 0	48.3±6.5	189.2±152.1	2.31±1.90	1.30±1.06	6.65±5.47	2.75±2.42	3.60±3.60
	G 0.05	37.2±3.1	259.3±145.7	2.34±1.36	1.32±0.76	6.70±4.07	2.82±1.67	3.57±2.70
	G 5	42.6±4.8	384.7±287.9	2.34±0.42	1.24±0.26	7.48±1.62	2.78±0.30	3.97±0.91
4weeks	G 0	42.1±3.4	322.8±154.7					
	G 0.05	40.2±2.1	350.7±124.6					
	G 5	50.4±2.6	225.8±154.6					

B		PCV	ACH_{50}	PBL	L	T	N	M
1 week	Pg 0	51.2±5.1	225.6±154.9	3.06±0.86	2.35±0.64	5.81±2.44	1.43±0.84	1.92±2.01
	Pg 0.05	55.4±6.2	199.4±235.1	3.30±1.25	2.55±0.98	6.27±2.82	1.43±0.71	1.90±1.93
	Pg 5	49.1±5.3	189.6±154.3	3.33±0.94	2.55±0.68	6.24±2.29	2.08±1.94	2.60±3.70
2 weeks	Pg 0	49.2±4.5	335.9±124.3	3.29±1.10	2.55±0.88	5.96±2.09	1.89±1.85	2.76±3.45
	Pg 0.05	47.3±3.2	189.4±174.8	2.61±1.42	2.07±1.17	4.39±2.03	1.52±2.01	2.89±3.53
	Pg 5	52.7±4.6	215.6±195.3	2.91±1.05	2.28±0.92	5.11±0.98	1.67±1.91	2.92±3.56
3 weeks	Pg 0	45.3±2.6	185.9±266.3	2.57±1.67	2.05±1.36	4.53±2.87	0.56±0.54	1.68±2.01
	Pg 0.05	46.8±2.3	335.4±223.7	3.33±2.37	2.65±1.96	5.48±3.42	1.87±2.66	3.98±4.73
	Pg 5	51.3±4.1	175.9±198.6	2.16±0.31	1.69±0.22	3.85±0.82	1.22±1.19	1.78±2.21
4 weeks	Pg 0	52.4±4.1	228.5±147.9					
	Pg 0.05	51.9±4.8	175.9±199.8					
	Pg 5	55.4±5.7	325.9±129.7					

Samples were taken for haematological assessment 1, 2, 3 and 4 weeks after the onset of treatments. Results are expressed as mean value ± sd. n= 6. **A**, β-glucan treatment; **B**, peptidoglycan treatment. Treatments were as follows: G 0, 0% glucan; G 0.05, 0.05% glucan; G 5, 5% glucan. Pg 0, 0% peptidoglycan; Pg 0.05, 0.05% peptidoglycan; Pg 5, 5% peptidoglycan. ACH_{50}, serum alternative complement units per ml; PCV, haematocrit (%); L, lymphocytes ml^{-1} ($x10^7$); T, thrombocytes ml^{-1} ($x10^6$); N, neutrophils ml^{-1} ($x10^6$); M, monocytes ml^{-1} ($x10^5$).

Head kidney macrophages were able to phagocytose *A. salmonicida* (table 4.2) and generate intra- and extra-cellular superoxide anion in response to PMA (table 4.3). The metabolic activity of *A. salmonicida* was slightly reduced as indicated by decreased MTT reduction after 5 h incubation with renal macrophages (table 4.4). However, no significant differences were observed in head kidney macrophage activity from β-glucan or peptidoglycan treated fish compared with control animals (table 4.2 to 4.4, respectively). Likewise, no significant differences were found even when pooled haematological and cellular results for each treatment were compared with their respective pooled controls. Nitrite was not detected in the supernatants of head kidney macrophages stimulated with MAF and LPS under any conditions tested. The lower detection limit was 3.5 nmol nitrite per well.

Table 4.2 Phagocytosis of *A. salmonicida* by macrophages from fish orally treated with β–glucan or peptidoglycan.

A

		PR	PI
1 week	G 0	35.4 ± 11.2	0.56 ± 0.22
	G 0.05	42.6 ± 16.7	0.72 ± 0.36
	G 5	28.9 ± 14.5	0.55 ± 0.27
2 week	G 0	27.9 ± 9.8	0.48 ± 0.18
	G 0.05	35.4 ± 9.7	0.56 ± 0.21
	G 5	29.7 ± 10.2	0.39 ± 0.18
3 week	G 0	33.4 ± 7.8	0.55 ± 0.18
	G 0.05	35.7 ± 4.7	0.49 ± 0.21
	G 5	29.7 ± 5.4	0.53 ± 0.19
4 week	G 0	42.8 ± 0.4	0.39 ± 0.21
	G 0.05	39.7 ± 11.8	0.29 ± 0.09
	G 5	32.7 ± 15.7	0.27 ± 0.11

B

	PR	PI
Pg 0	37.9 ± 9.8	0.37 ± 0.08
Pg 0.05	32.1 ± 8.7	0.29 ± 0.11
Pg 5	27.4 ± 10.4	0.31 ± 0.15
Pg 0	25.4 ± 7.9	0.21 ± 0.09
Pg 0.05	35.9 ± 10.5	0.28 ± 0.08
Pg 5	29.7 ± 4.6	0.32 ± 0.09
Pg 0	35.7 ± 4.6	0.38 ± 0.11
Pg 0.05	29.7 ± 9.7	0.35 ± 0.09
Pg 5	22.9 ± 10.7	0.42 ± 0.15
Pg 0	25.6 ± 12.4	0.28 ± 0.08
Pg 0.05	37.9 ± 5.6	0.25 ± 0.12
Pg 5	29.4 ± 9.8	0.29 ± 0.11

A, β-glucan treatment; **B**, peptidoglycan treatment. Results are expressed as mean values ± sd (n=6). Treatment abbreviations as for table 4.1. PR, phagocytosis ratio; PI, phagocytic index.

Table 4.3 Respiratory burst activity by head kidney macrophages from fish treated with in-feed β-glucan or peptidoglycan.

A

		PMA+	PMA-
1 week	G 0	0.144 ± 0.028	0.053 ± 0.007
	G 0.05	0.157 ± 0.053	0.062 ± 0.025
	G 5	0.179 ± 0.049	0.060 ± 0.030
2 week	G 0	0.170 ± 0.041	0.076 ± 0.022
	G 0.05	0.162 ± 0.046	0.055 ± 0.028
	G 5	0.174 ± 0.046	0.039 ± 0.032
3 week	G 0	0.179 ± 0.045	0.044 ± 0.018
	G 0.05	0.187 ± 0.022	0.060 ± 0.022
	G 5	0.177 ± 0.071	0.091 ± 0.019
4 week	G 0	0.153 ± 0.061	0.055 ± 0.018
	G 0.05	0.145 ± 0.035	0.062 ± 0.026
	G 5	0.159 ± 0.052	0.053 ± 0.032

B

	PMA+	PMA-
Pg 0	0.114 ± 0.019	0.073 ± 0.010
Pg 0.05	0.137 ± 0.032	0.072 ± 0.029
Pg 5	0.135 ± 0.017	0.085 ± 0.021
Pg 0	0.155 ± 0.057	0.075 ± 0.019
Pg 0.05	0.153 ±0.035	0.043 ± 0.027
Pg 5	0.195 ± 0.072	0.055 ± 0.011
Pg 0	0.157 ± 0.022	0.075 ± 0.043
Pg 0.05	0.123 ± 0.036	0.090 ± 0.026
Pg 5	0.198 ± 0.048	0.099 ± 0.059
Pg 0	0.156 ± 0.026	0.042 ± 0.017
Pg 0.05	0.178 ± 0.024	0.049 ± 0.036
Pg 5	0.135 ± 0.040	0.081 ± 0.020

C

G 0	3.3 ± 0.4
G 0.05	3.4 ± 1.0
G 5	3.7 ± 0.4
G 0	3.8 ± 0.6
G 0.05	4.1 ± 0.7
G 5	3.6 ± 0.6
G 0	3.5 ± 0.7
G 0.05	3.5 ± 0.7
G 5	3.9 ± 1.4
G 0	3.2 ± 0.8
G 0.05	3.7 ± 0.7
G 5	3.3 ± 0.9

D

Pg 0	3.3 ± 0.9
Pg 0.05	3.5 ± 0.7
Pg 5	3.5 ± 0.1
Pg 0	3.6 ± 0.9
Pg 0.05	3.5 ± 0.1
Pg 5	3.8 ± 1.0
Pg 0	3.6 ± 0.4
Pg 0.05	3.8 ± 0.3
Pg 5	3.6 ± 0.5
Pg 0	3.5 ± 0.7
Pg 0.05	3.7 ± 0.8
Pg 5	3.7 ± 1.4

A and **B**, reduction of NBT after 30 min of reaction; **C** and **D**, nmol of extracellular O_2^- produced 60 min after addition of PMA. Results are expressed as mean values ± sd (n= 6). Treatment abbreviations as for table 4.1.

Table 4.4 Killing of *A. salmonicida* by head kidney macrophages from fish treated with in-feed β-glucan or peptidoglycan.

	A		B	
1 week	G 0	2.9 ± 1.9	Pg 0	3.7 ± 1.4
	G 0.05	3.8 ± 0.8	Pg 0.05	4.3 ± 1.2
	G 5	2.5 ± 1.6	Pg 5	3.9 ± 1.1
2 week	G 0	3.9 ± 2.0	Pg 0	4.5 ± 1.9
	G 0.05	4.5 ± 1.3	Pg 0.05	3.8 ± 1.1
	G 5	3.8 ± 2.1	Pg 5	4.2 ± 0.9
3 week	G 0	5.1 ± 2.5	Pg 0	4.9 ± 2.1
	G 0.05	4.9 ± 1.6	Pg 0.05	3.6 ± 1.9
	G 5	4.2 ± 1.5	Pg 5	4.0 ± 1.1
4 week	G 0	3.8 ± 1.7	Pg 0	5.2 ± 1.5
	G 0.05	4.5 ± 1.7	Pg 0.05	4.9 ± 1.8
	G 5	5.5 ± 2.1	Pg 5	5.4 ± 2.4

A, Glucan treatment; **B**, Peptidoglycan treatment. Results are expressed as mean % of bacteria killed (as indicated by decreased MTT reduction) ± sd after 5 h incubation at a ratio of 1effector:20 target cells (n=6). Treatment abbreviations as for table 4.1

In the last experiment, fish were fed on a 0.05% peptidoglycan-containing diet for four weeks and head kidney and peritoneal macrophage activity as well as persistence of *A. salmonicida* in spleen and blood were investigated. The activity of head kidney macrophages was similar to the previous experiment (data not shown). However, the treatment induced a significant increase in PMA-triggered extracellular production of superoxide by casein-elicited peritoneal macrophages (figure 4.3).

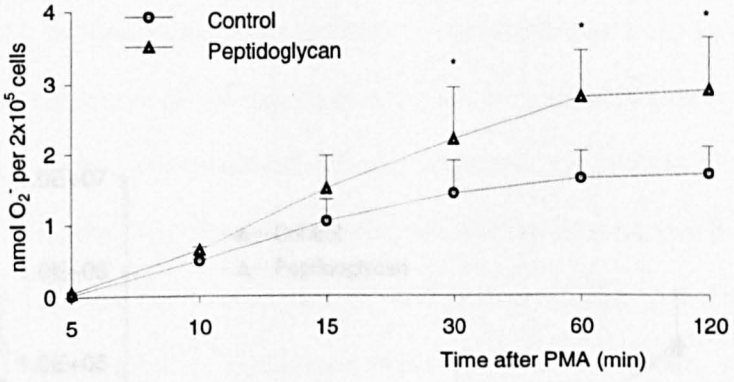

Figure 4.3 Production of extracellular O_2^- by peritoneal macrophages.
Results are expressed as arithmetic mean ± sd (n=6). * indicates significant difference between treatment and control groups.

The number of viable *A. salmonicida* in the spleen of challenged fish increased with time (figure 4.4). There were, however, very large individual variations in the number of bacterial CFU g^{-1} of spleen within the two treatment groups (average CV= 252.6%) and no significant differences were found between control and peptidoglycan treatments. There were no differences between the two treatments in the relative risk to viable *A. salmonicida* cells persistence in the spleen of infected fish either (results shown in appendix 1). No viable bacteria were recovered from the blood of infected fish at any sampling time.

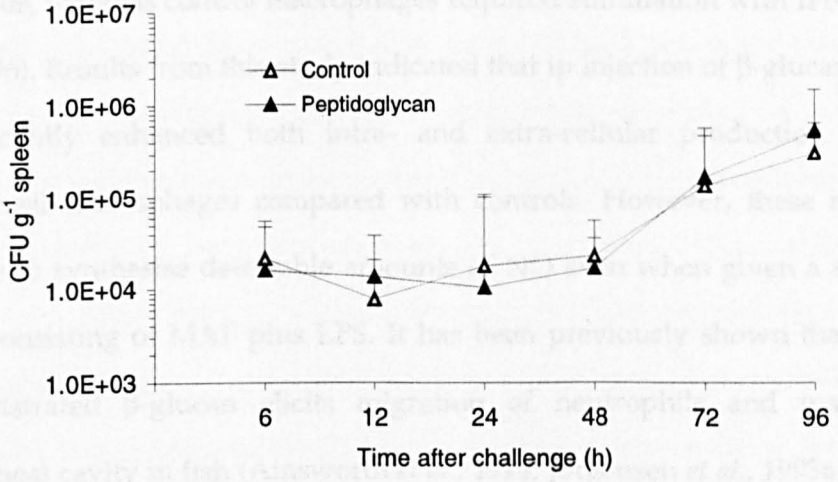

Figure 4.4 Persistence of *A. salmonicida* CFU in spleens of control and in-feed peptidoglycan treated fish.

Results are expressed as mean values ± sd (n=6). Each fish received an ip dose of 2.2×10^4 CFU.

4.4. Discussion

Chapter 3 described the immunostimulatory activities of (1→3)-β-glucans and peptidoglycan *in vitro*. In the present study, enhancement of innate immune mechanisms *in vivo*, both by intraperitoneal and in-feed administration, was investigated.

Enhancement of peritoneal macrophage activity following ip administration of β-glucans has been demonstrated by several authors. Derivatives of β-glucan administered intraperitoneally into mice have been reported to increase superoxide anion and nitric oxide formation as well as acid phosphatase activity of peritoneal macrophages (Yoshida *et al.*, 1996). Furthermore, mice peritoneal macrophages were able to produce nitric oxide after administration of soluble or particulate β-glucans *in vivo* without need for a second stimulus, whereas control macrophages required stimulation with IFNγ *in vitro* (Ohno *et al.*, 1996). Results from this study indicated that ip injection of β-glucan or peptidoglycan significantly enhanced both intra- and extra-cellular production of superoxide in peritoneal macrophages compared with controls. However, these macrophages were unable to synthesise detectable amounts of NO even when given a second stimulus *in vitro* consisting of MAF plus LPS. It has been previously shown that intraperitoneally administrated β-glucan elicits migration of neutrophils and macrophages to the peritoneal cavity in fish (Ainsworth *et al.*, 1994; Jørgensen *et al.*, 1993a), but these studies did not include measurement of functional activity of peritoneal macrophages. The results given here present data on levels of activity in stimulated and control peritoneal macrophages.

Rainbow trout haematological parameters such as haematocrit and PBL counts were not affected by in-feed administration of either β-glucan or peptidoglycan after 1, 2, 3 or 4 weeks of treatment and these results are similar to those previously reported by Siwicki *et al.* (1994). No significant differences in haemolytic activity were observed between the sera of control and treated animals. Due to the high variation in haemolytic

activity observed within animals in the same group, larger numbers of fish would be needed to investigate this further. Similarly, head kidney macrophage activity was not modulated by administration of different doses of β-glucan or peptidoglycan in the feed for periods of up to 4 weeks. Kidney macrophages from control animals were able to produce superoxide anion and to phagocytose and kill *A. salmonicida* to the same extent as macrophages from treated fish, although the degree of killing was very limited in all groups. Strain pathogenicity of *A. salmonicida* and *A. hydrophila* has been shown to be an important factor in determining the outcome of interaction between macrophages and bacteria (Sharp and Secombes, 1993; Leung *et al.*, 1995). No detailed information on the pathogenicity of the challenge strain used in the present study is available, however, in the experiments on bacterial killing *in vivo*, numbers of viable *A. salmonicida* in the spleen of challenged fish increased with time.

An interesting finding was that casein-elicited peritoneal macrophages showed significantly increased superoxide production following 4 weeks of in-feed peptidoglycan treatment. Duncan and Klesius (1996) observed increased phagocytosis and chemotaxis of peritoneal macrophages in channel catfish orally treated with the immunostimulant *Spirulina platensis*. Unfortunately, activity of kidney macrophages was not assessed in that study and, therefore, comparison of this aspect with the results described here is not possible. In mice, several studies have also demonstrated enhanced local immune activity after oral administration of immunostimulants. For instance, intestinal lymphocytes have been shown to be primed for proliferation by orally administered MDP (Zunic, 1996) and peritoneal and alveolar macrophages were activated by oral β-glucan treatment (Suzuki *et al.*, 1990; Sakurai *et al.*, 1992).

Thus, results from the present study show that although peptidoglycan and β-glucan exerted immunostimulatory activity *in vivo* after ip injection, oral administration was unable to elicit a systemic immune response as indicated by enhanced serum complement and head kidney resident macrophage activities. Haemopoietic organs such

as spleen and head kidney have been shown to be the main centres for the clearance of blood-borne antigens in rainbow trout (Alexander *et al.*, 1983; Marsden *et al.*, 1996; Zapata *et al.*, 1996). Peptidoglycan was, however, effective in enhancing the activity of inflammatory macrophages, which play a fundamental role in combating persistent infection foci in different organs. The fate of orally administered immunostimulants and their mode of action may be of importance in explaining this variable modulation. Laminarin, a soluble β-glucan, has been demonstrated to be absorbed in the gut and readily cleared from the circulation by reticuloendothelial cells of haemopoietic organs of fish (Dalmo *et al.*, 1994; Sveinbjørnsson *et al.*, 1995; Dalmo *et al.*, 1996a,b). There are no similar studies on peptidoglycan in fish. The fate of peptidoglycan in mice is very different to that of soluble glucan in fish. Peptidoglycan is poorly absorbed in the gut and quickly excreted in the urine (Parant *et al.*, 1979; Tomasic *et al*, 1980; Ladesic *et al.*, 1993). The mechanisms of action of these two substances may be, therefore, different. While peptidoglycan may not be found in haemopoietic organs after administration, orally administered soluble glucan in fish interacts directly with kidney macrophages (Dalmo *et al.*, 1996a,b) through ligation with specific surface receptors (Engstad and Robertsen, 1994). Nevertheless, no reports are available on the fate of orally administered particulate β-glucan in fish or other animals. A few studies have observed potentiation of head kidney macrophage activity after oral administration of particulate β-glucan (see section 4.1), although no such activation was found by Debaulny *et al.* (1996) in turbot, nor in the present study in rainbow trout. A possible explanation for these contradictory results is that head kidney of teleost fish is a highly haemopoietic organ (Zapata *et al.*, 1996), thus, actively generating and exporting leukocytes depending on the requirements to maintain an adequate immune function and surveillance.

Beta-glucans have been shown to enhance mice splenic haemopoiesis *in vivo* (Patchen and MacVittie, 1985; Patchen and MacVittie, 1986) and to increase the proportion of precursor cells differentiating into macrophages and granulocytes

(Burgaleta and Golde, 1977). In addition, β-glucan administration into mice induces mobilisation of bone marrow cells into the peripheral circulation (Patchen and MacVittie, 1986). Thus, increased haemopoiesis and leukocyte trafficking are induced by β-glucans in murine models. Although similar studies are not available in fish literature, sera from fish treated with MDP and Freund's complete adjuvant, of which MDP is the main immunoactive molecule, possessed enhanced macrophage colony formation activity *in vitro* (Kodama *et al.*, 1994a), suggesting that haemopoiesis in fish also could be enhanced by immunostimulants.

Enhanced haemopoiesis and/or leukocyte trafficking may induce changes in macrophage sub-population structure of fish head kidney as cells at certain stages of maturation leave the organ to become resident in other tissues. Therefore, the functional activity of the macrophage population in the head kidney as a whole may not be modulated by in-feed administration of small doses of immunostimulant. However, further studies on leukocyte trafficking and haemopoiesis in fish are required to investigate this hypothesis.

Finally, clearance of pathogenic or opportunistic bacteria from different organs and blood has been used successfully to monitor susceptibility to bacterial infection after different interferences with the immune system of fish (Marsden et al, 1996; Sakai et al, 1989) or mammalian species (MacMiking et al, 1997). In this study, higher numbers of viable *A. salmonicida* were found in spleen with time after ip injection into rainbow trout, although no significant differences were observed in persistence of viable *A. salmonicida* cells in spleen between treatments. Other studies have also reported stimulation of fish immune components by in-feed administration of glucans which have not led to increased survival in bacterial challenges (Ainsworth *et al.*, 1994; Debaulny *et al.*, 1996).

In summary, peptidoglycan and β-glucan induced a similar increase of peritoneal macrophage activity when administered intraperitoneally. Oral treatment did not, however, modify kidney macrophage effector or serum haemolytic activities. On the

other hand, the respiratory burst response of inflammatory peritoneal macrophages was significantly augmented after oral treatment with peptidoglycan. This increase in activity was not reflected in the clearance of viable *A. salmonicida* from spleen *in vivo*. Therefore, a regime whereby stimulation of macrophage activity is achieved following in-feed peptidoglycan was identified. Further experiments will involve investigation of immunomodulation mediated by an aquaculture-associated stressor and the prophylactic use of peptidoglycan to compensate possible suppressive effects.

Chapter 5

Modulation of rainbow trout innate defence mechanisms by confinement.

5.1 Introduction

Successful intensive farming of aquatic animals is based on minimising disease incidence and optimising growth and reproduction. All these aspects depend on husbandry of animal stocks under low stress conditions. However, certain stressful events are inherent to intensive farming. High stocking density, low water quality, handling, transportation of live stock and elevated microbial load in water are considered to be amongst the most important stressors faced by farmed aquatic animals (Wedemeyer, 1996).

Increased susceptibility to infectious disease following stressful events is thought to be, in part, a consequence of immunomodulation mediated in most cases by the immune and neuroendocrine mediators of the stress response (summarised in section 1.3). Serum complement, lysozyme, phagocyte activity, cell-mediated natural cytotoxicity and specific antibody production are amongst important defence mechanisms of fish against invading microorganisms (Iwama and Nakanishi, 1996). Aquaculture-associated stressful events have been shown to induce a variable modulation of all these immune parameters depending on the quality and extent of the stressor and the immune parameter investigated (summarised in section 1.3.1).

The aim of this study was to identify a stressful regime inherent in the practice of aquaculture that induced depression of innate immune activity and reduced resistance to bacterial challenge. Confinement was selected as stressor and, for this purpose, rainbow trout were subjected to single or repeated daily confinement and several innate immune parameters investigated. The ability of confined fish to respond to an LPS challenge *in vivo* also was assessed. Finally, persistence of viable *A. salmonicida* cells in the spleen and blood of control and repeatedly confined animals was quantified after ip injection of *A. salmonicida*.

5.2 Materials and Methods

Animals and bacterial strain

All-female rainbow trout were obtained from Almond Bank Trout Farm and acclimatised to aquarium conditions at 6 ± 2 °C for at least three weeks as described in section 2.2. Fish from this stock were used for all experiments described in this chapter. Fish were fed on expanded (floating) pellet feed to monitor feeding behaviour.

A. salmonicida B95179 (isolate details given in section 2.8.2) was used to assess bacterial killing *in vitro* and *in vivo*.

Experimental design

Immunomodulation by confinement

After acclimatisation, groups of 62 fish individually weighing 45.2 ± 3.2 g were allocated to four similar 370 l aerated tanks receiving a flow-through water input. Water temperature was raised progressively over a period of ten days and kept constant at 12.5 ± 0.5 °C throughout the experiment. Fish from two tanks were subjected to severe confinement by reducing the water depth to 7 cm for five minutes before returning it to the original depth of 104 cm. The complete procedure lasted 25 minutes and water flow was kept constant during this operation. Fish from the other two tanks were left undisturbed. Animals were fed twice daily with a commercial trout pellet to the manufacturer's RDA (Ewos Select No. 40; 1.70 % bw). On the day confinement was applied, animals were fed only once, 6h after the water level was lowered.

Blood samples for measurement of plasma glucose concentration were taken 4 h after the onset of confinement from three fish from each tank, totalling six control and six confined fish.

Four individuals from each tank, totalling eight control and eight confined animals, were sampled for assessment of haematological and immunological parameters at several sampling points. Sampling took place 24 h before confinement and 24 h, 2, 4, 8

and 16 days after confinement. Parameters assayed were haematocrit, PBL counts, serum haemolytic activity, head kidney macrophage phagocytosis, respiratory burst (unstimulated and MAF plus LPS stimulated) and production of nitric oxide.

Immunomodulation by LPS challenge in confined animals

Groups of 25 fish with an average weight of 52.1 ± 2.6 g were placed in two similar 370 l flow-through aerated tanks. Water temperature was raised gradually over a period of 12 days and kept constant at 14.0 ± 0.5 °C during the experiment. Fish in one tank were subjected to a single severe confinement experience as described above while those in the other tank were left undisturbed.

Twelve hours after the onset of confinement, blood samples for measurement of glucose concentration were taken from five fish from each tank and were then sacrificed. At this time, ten control and ten confined fish were lightly anaesthetised with 2.5 % benzocaine, then injected intraperitoneally with 100 µl of 10 mg ml^{-1} of *E. coli* LPS (serotype 026:B6, Sigma, UK) in saline, marked and returned to their respective tanks. Five control and five confined individuals were sampled 2.5 days after injection of LPS to assess macrophage activity (superoxide and nitric oxide production).

Immunomodulation by repeated confinement

Groups of 28 fish weighing 110 ± 6.5 g were distributed in eight similar 370 l aerated flow-through tanks. Water temperature was elevated gradually over a period of ten days and kept constant at 12.3 ± 0.5 °C throughout the experiment. Four tanks were used to assess the effects of repeated confinement on haematological and immunological parameters (two control and two confinement tanks) while killing of *A. salmonicida in vivo* after ip challenge was monitored in the other four tanks (two control and two confinement tanks). Fish from confinement tanks were subjected to a severe confinement as described above, while control fish were left undisturbed. This procedure was repeated on six consecutive days before fish were sampled to assess their haematological and immunological condition, or challenged with *A. salmonicida*. Trout were fed twice

daily to the manufacturer's RDA (Ewos Select No. 40; 1.62 % bw) during the experiment. On the days that confinement was applied, they were fed only once 5-7 h after the onset of confinement.

Samples for measurement of plasma glucose concentration were taken from three fish from each tank, totalling six control and six confined animals. Blood was withdrawn 4 h post confinement on the first and last day of confinement to examine changes in plasma glucose concentration.

Sampling for haematological and immunological condition of the fish took place 30 minutes after the last confinement and it consisted of four individuals from each tank, totalling eight control and eight confined fish. Parameters assayed were haematocrit, PBL counts, serum haemolytic activity, head kidney macrophage phagocytosis, respiratory burst (unstimulated and MAF plus LPS stimulated), production of nitric oxide and killing of *A. salmonicida in vitro*.

All fish in the tanks used to study bacterial killing *in vivo* were injected ip with *A. salmonicida* and samples were taken at different times, up to 72 h after injection, for quantification of viable *A. salmonicida* in spleen and blood. Confinement was applied as described above until the last sampling. Therefore, these fish were subjected to repeated confinement for nine consecutive days.

Haematology

Plasma glucose concentrations were measured as described in section 2.5.

Haematocrit and PBL counts were quantified in EDTA-treated blood as described in section 2.3. Serum was extracted from blood and the spontaneous haemolytic activity against rabbit red blood cells determined in accordance with section 2.6.

Isolation and culture of macrophages

Macrophages were isolated from the head kidney and monolayers cultured in 96-well microtiter plates or in 8-well glass slides at 19 °C for 12 h as described in section 2.7.

Monolayers were washed three times with cHBSS before use. Macrophages monolayers assessed for killing of *A. salmonicida* were incubated without P/S.

Phagocytosis

Opsonised sheep red blood cells were prepared and adjusted to the desired concentration as described in section 2.8.2.

The number of adherent macrophages from each fish was estimated in spare wells as described in section 2.7.3. Macrophage monolayers in 8-well slides were incubated with 0.4 ml of opsonised SRBC suspension to a ratio of 1 macrophage to 5 SRBC at 19 °C. After 60 minutes, monolayers were washed three times and stained with Rapi-Diff. Phagocytic ratio and phagocytic index were obtained as described in section 2.8.1.

Respiratory burst

The production of extracellular superoxide anion by macrophages was quantified in triplicate wells by the reduction of cytochrome c triggered by PMA as described in section 2.9.1. Superoxide dismutase was added in triplicate wells per fish to confirm specificity of the reaction.

Macrophage intracellular respiratory burst activity was measured in triplicate wells by the reduction of NBT in the presence or absence of PMA as described in section 2.9.2.

Duplicate wells were used to count the number of macrophages per well and results adjusted to OD_{610} per $2x10^5$ cells or nmol O_2^- produced per $2x10^5$ cells for the NBT and cytochrome c assays respectively.

Some of the monolayers were incubated with culture medium containing MAF plus 40 $\mu g\ ml^{-1}$ *E. coli* LPS (serotype 026:B6) at 19 °C for 72 h and intra- and extra-cellular superoxide production quantified as described above.

Nitric oxide

The supernatants of macrophages incubated with MAF plus LPS were assayed for nitrite, a major end product of nitric oxide, by the Griess reaction as described in section 2.10. Serial dilutions of sodium nitrite were used as positive controls.

A. salmonicida killing *in vitro*

A. salmonicida was grown in TSB at 22 °C for 12 h with continuous shaking. The bacterial suspension was washed three times, opsonised with trout serum and adjusted to the desired concentration in L-15 plus 5% FCS as described in section 2.8.2. Macrophage monolayers were exposed to a ratio of 1 macrophage to 20 bacterial cells at 19 °C. After 5 h, percentage of bacteria killed was quantified as described in section 2.11.

A. salmonicida clearance *in vivo*

A. salmonicida was grown in TSB at 22 °C overnight and adjusted spectrophotometrically to a concentration of $3.2x10^8$ CFU ml^{-1} (OD_{610}= 1.24 in TSB). This bacterial suspension was diluted in saline to obtain a suspension of $1.0x10^5$ CFU ml^{-1}, as challenge dose, and the concentration confirmed by viable plate counts..

Fish were lightly anaesthetised with 2.5 % benzocaine and 200 µl of $1.5x10^5$ CFU ml^{-1} were injected intraperitoneally into each fish. After 6, 12, 24, 48 and 72 h, samples consisting of spleens and blood from four fish per tank, totalling eight control and eight confined fish, were taken and processed to quantify viable *A. salmonicida* cells as described in section 2.12.

Statistical analysis

Comparison between mean values was conducted by the Student *t*-test unless otherwise stated. After confirming that mean values from duplicate tanks of the same treatment were not significantly different, they were pooled into one group for comparison between control and confinement treatments. Statistical differences within control and confinement groups during the time-course study following confinement were

investigated by the repetitive measurements one-way analysis of variance and Student-Newman-Keuls multiple comparison test. Normality and homogeneity of variance were confirmed before any parametric test was applied. When normality failed, the logarithmic transformation was performed. All percentage values were normalised by square root arcsine transformation. When transformed data were still not normal, ANOVA on ranks was applied. Statistical tests were performed with SigmaStat™ 1.0 (Jandel Scientific) and in all cases $p<0.05$ was the accepted significance level. Regression was also used to analyse bactericidal capacity of macrophages *in vitro*.

Differences in the relative risk of viable *A. salmonicida* persistence in the spleen of infected fish were investigated using the software EPI-INFO 6.04.

5.3 Results

Feeding behaviour and mortalities

In all experiments, fish accepted the RDA and no morbidity or mortalities were observed throughout the experiments. Trout injected ip with LPS accepted the RDA 6h after injection and continued to do so for 15 days, when all fish were sacrificed.

Haematology

Single confinement induced a significant increase in plasma glucose concentration as compared with control animals in all experiments. However, glucose concentration in the plasma of fish subjected to repeated confinement for six consecutive days was not significantly different from control fish (Table 5.1)

Table 5.1 Plasma glucose concentration in confinement experiments

	Control	Stress
Single confinement	4.40 ± 0.67	8.49 ± 1.43 *
Single confinement + LPS	4.42 ± 0.72	6.05 ± 1.14 *
Repetitive confinement (1st day)	4.71 ± 1.36	9.05 ± 2.59 *
Repetitive confinement (last day)	5.15 ± 1.20	6.76 ± 3.22

Data are expressed as mean nmol glucose l^{-1} ± sd. * indicates significant difference respect to control ($p<0.05$). n=6 (n=5 in single confinement + LPS experiment).

A significant increase in neutrophil numbers in fish confined for six consecutive days was observed. No other haematological parameter was significantly altered by confinement (table 5.2).

Table 5.2 Haematological parameters in control and confined fish

A	ACH_{50}	PCV	PBL	L	T	N	M
Day -1							
Control	1.13±0.12	41.6±6.62	1.66±0.45	9.31±.3.20	5.41±1.32	2.10±0.71	1.85±1.51
Confined	2.25±1.15	44.2±6.06	1.39±0.40	7.42±2.61	4.71±0.11	1.61±0.64	1.88±1.46
Day +1							
Control	3.25±0.72	43.7±6.95	1.46±0.43	7.82±2.61	5.10±1.60	1.45±0.57	2.03±1.40
Confined	3.12±0.55	46.4±6.36	1.37±0.43	6.30±1.21	5.81±1.89	1.52±0.47	2.80±0.87
Day +2							
Control	0.66±0.11	51.1±7.00	1.45±0.39	7.62±2.69	5.12±1.80	1.64±0.77	1.66±1.58
Confined	0.68±0.07	48.0±7.60	1.60±0.52	8.71±3.20	5.30±1.99	1.72±0.86	1.70±1.58
Day +4							
Control	5.35±0.57	42.0±8.11	1.48±0.46	8.60±3.01	4.31±2.20	1.65±0.98	1.94±1.57
Confined	5.91±1.06	44.8±7.79	1.57±0.61	8.60±3.03	5.13±3.10	1.80±1.15	1.71±1.87
Day +8							
Control	9.92±3.41	45.6±7.82	1.69±0.57	8.92±2.80	6.09±3.82	1.72±0.69	2.14±1.61
Confined	9.02±1.34	43.6±6.18	1.53±0.46	8.11±3.20	5.00±1.91	2.01±1.09	2.19±1.68
Day +16							
Control	3.23±1.53	44.8±6.36	1.56±0.52	8.51±2.84	5.61±2.50	1.33±0.26	1.32±1.23
Confined	4.56±2.13	44.8±6.18	1.73±0.67	9.32±4.30	6.51±2.40	1.28±0.57	1.92±1.67

B	ACH_{50}	PCV	PBL	L	T	N	M
Control	5.65±2.37	33.3±2.75	2.57±0.99	19.4±0.61	4.76±2.10	1.33±0.99	4.40±2.20
Confined	4.64±1.24	35.6±5.41	4.37±1.50	26.5±0.46	4.03±1.74	4.15±1.96*	6.08±3.61

A, after single confinement; confinement took place at day 0. **B**, after repeated confinement. Results are expressed as mean ± sd (n= 8). ACH_{50}, units of alternative complement haemolysis ml^{-1} ($x10^2$); PCV, haematocrit; PBL, leukocytes ml^{-1} ($x10^7$); L, lymphocytes ml^{-1} ($x10^6$); T, thrombocytes ml^{-1} ($x10^6$); N, neutrophils ml^{-1} ($x10^6$); M, monocytes ml^{-1} ($x10^5$). No significant differences ($p<0.05$) were found between control and confined animals at any sampling time, except for neutrophil counts in repeatedly confined animals (*).

Head kidney macrophage activity

Single confinement did not significantly modulate macrophage phagocytic activity (table 5.3), even when results obtained at different times after confinement were pooled and compared.

Table 5.3 Phagocytosis of *A. salmonicida* by head kidney macrophages from control and confined fish

	Phagocytic index		Phagocytosis ratio	
	Control	Confined	Control	Confined
Day -1	0.804 ± 0.225	0.633 ± 0.193	51.28 ± 15.29	45.34 ± 16.79
Day +1	0.711 ± 0.146	0.594 ± 0.169	46.39 ± 13.66	39.06 ± 11.36
Day +2	0.828 ± 0.187	0.646 ± 0.175	42.40 ± 12.77	34.75 ± 15.97
Day +4	0.810 ± 0.177	0.634 ± 0.182	40.71 ± 12.17	32.66 ± 15.01
Day +8	0.786 ± 0.207	0.788 ± 0.210	48.87 ±14.53	49.20 ± 9.02
Day +16	0.741 ± 0.315	0.716 ± 0.280	41.87 ± 11.87	45.74 ± 13.47

Confinement took place at day 0. Results are expressed as mean ± sd (n= 8). No significant changes were observed between control and confined animals at any sampling time.

Phagocytosis of SRBC by macrophages was significantly lower after repeated confinement as compared with control fish (figure 5.1).

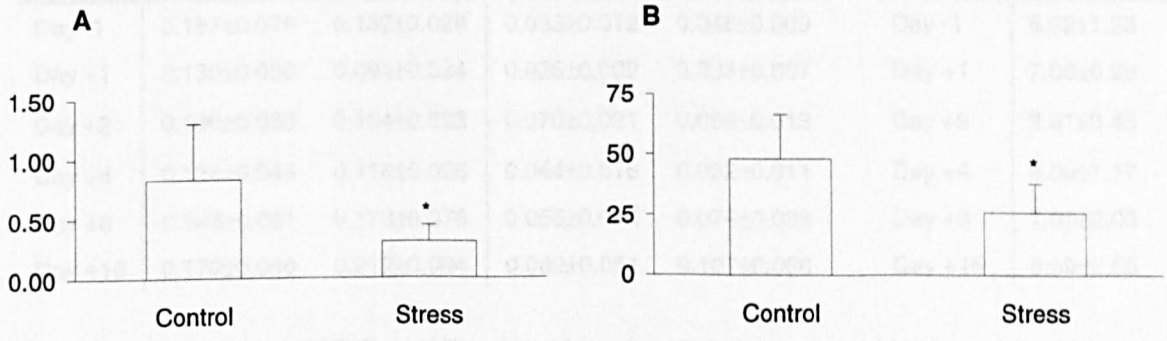

Figure 5.1 Phagocytosis of SRBC by head kidney macrophages from control and repeatedly confined animals

A, phagocytosis index; **B**, phagocytosis ratio (%). Results are expressed as mean value ± sd (n=8). * indicates significant difference ($p<0.05$).

Production of intra- or extra-cellular superoxide anion triggered by PMA was not significantly modulated by single confinement. Furthermore, no significant differences were observed when the activity of macrophages stimulated with MAF plus LPS *in vitro* was compared between single confined and control fish. Even when results from samples taken at 24 h, 4, 8 and 16 days were pooled, no significant differences were observed between confined and control animals. There was, however, a non-significant increase in extracellular superoxide production and an initial decrease of PMA-triggered NBT reduction after confinement (table 5.4).

Table 5.4 Respiratory burst by head kidney macrophages from control and confined fish

	PMA +		PMA -	
A	Control	Confined	Control	Confined
Day -1	0.067±0.035	0.060±0.013	0.017±0.006	0.020±0.006
Day +1	0.045±0.024	0.029±0.008	0.014±0.005	0.016±0.005
Day +2	0.062±0.035	0.052±0.023	0.011±0.007	0.017±0.009
Day +4	0.044±0.029	0.053±0.023	0.019±0.012	0.022±0.010
Day +8	0.047±0.027	0.057±0.025	0.011±0.007	0.016±0.006
Day +16	0.057±0.032	0.074±0.033	0.016±0.010	0.024±0.013

B	Control	Confined
Day -1	2.62±0.49	2.41±0.24
Day +1	2.76±0.51	3.17±1.75
Day +2	1.14±0.48	1.81±0.57
Day +4	1.79±0.52	2.40±1.04
Day +8	2.73±0.79	3.03±1.32
Day +16	2.00±0.84	2.17±0.68

	PMA +		PMA -	
C	Control	Confined	Control	Confined
Day -1	0.167±0.079	0.132±0.029	0.033±0.012	0.045±0.009
Day +1	0.130±0.068	0.084±0.024	0.025±0.009	0.033±0.007
Day +2	0.136±0.030	0.104±0.023	0.070±0.021	0.058±0.013
Day +4	0.128±0.045	0.116±0.026	0.044±0.016	0.032±0.011
Day +8	0.143±0.061	0.173±0.076	0.055±0.034	0.074±0.036
Day +16	0.170±0.096	0.212±0.094	0.082±0.051	0.107±0.056

D	Control	Confined
Day -1	6.92±1.28	6.65±0.67
Day +1	7.08±0.98	8.14±4.17
Day +2	2.81±0.48	2.68±0.35
Day +4	4.06±1.17	5.39±2.32
Day +8	7.03±2.03	7.27±3.17
Day +16	6.09±2.55	5.14±2.09

A and **C**, reduction of NBT; **B** and **D**, production of extracellular superoxide by macrophages from control and confined trout. **A** and **B**, unstimulated macrophages; **C** and **D**, macrophages stimulated with MAF + LPS. Confinement took place at day 0. Results are expressed as mean values ± sd (n= 8). Data on extracellular superoxide is 60 minutes after addition of PMA. No significant differences were found between control and confined fish.

Similarly, LPS challenge after confinement did not induce any significant difference in the respiratory burst activity between experimental and control animals (table 5.5). A trend in decreased PMA-triggered intracellular O_2^- production and increased extracellular O_2^- generation was, however, observed in macrophages from single confined animals.

Table 5.5 Respiratory burst by head kidney macrophages from control and confined fish challenged with LPS

A

PMA +		PMA -	
Control	Confined	Control	Confined
0.188 ± 0.106	0.160 ± 0.070	0.028 ± 0.018	0.041 ± 0.022

B

Control	Confined
3.35 ± 0.81	4.22 ± 0.76

A, reduction of NBT; **B**, production of extracellular superoxide, by macrophages from control and confined trout challenged with LPS *in vivo*. Results are expressed as mean ± sd (n= 5). Data on extracellular superoxide is 60 minutes after addition of PMA. No significant differences were found between control and confined fish.

In contrast, this pattern of respiratory burst modulation was observed to be statistically significant in macrophages from repeatedly confined animals (figure 5.2). Macrophages isolated from repeatedly confined fish had a significantly increased extracellular superoxide and decreased intracellular superoxide productions. The same pattern was observed when macrophages were stimulated *in vitro* with MAF plus LPS before assaying for respiratory burst.

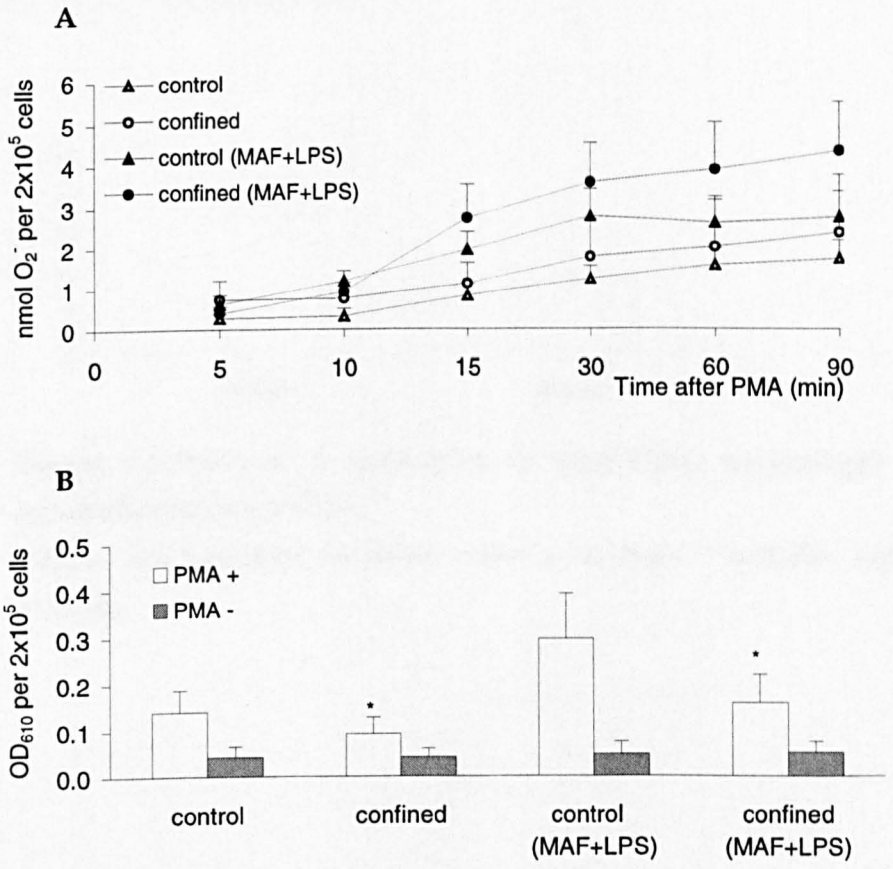

Figure 5.2 Respiratory burst of head kidney macrophages from control and repeatedly confined animals.

A, production of extracellular superoxide (n=8). Results are expressed as mean values ± sd. Significant differences between control and confined animals were observed from 15 minutes after addition of PMA ($p<0.05$). **B**, reduction of NBT (n=8). Results are expressed as mean values ± sd. In graph B, * indicates significant differences between control and confined groups ($p<0.05$). In all cases, MAF+LPS induced a significantly higher amount of superoxide.

Nitrite was not detected in any of the supernatants assayed. The lowest concentration of nitrite detected in the serial dilutions of $NaNO_2$ was 2.5 nmol/well.

Incubation of macrophages from repeatedly confined animals with serum-opsonised *A. salmonicida* for 5 h resulted in killing of bacterial cells. Macrophages from confined animals showed a significantly decreased capacity to kill *A. salmonicida* compared with control macrophages (Figure 5.3). Conversion of MTT to formazan by suspensions containing 25 %, 50 % and 90 % of the bacterial challenge dose was directly related to CFU ml^{-1} (r^2= 0.979; p<0.05).

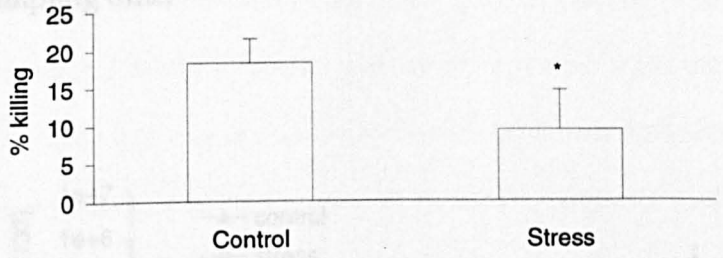

Figure 5.3 Killing of *A. salmonicida* by head kidney macrophages from control and repeatedly confined animals.
Results are expressed as mean values ± sd (n=8). * indicates significant differences (p<0.05).

A. salmonicida clearance *in vivo*

Viable *A. salmonicida* were recovered from the spleen from 6 h after ip injection in both control and confined groups. Bacteria were entrapped and/or multiplied quickly in this organ in both groups as shown in figure 5.4. There was a high variation in *A. salmonicida* CFU g^{-1} spleen within groups at all sampling times (coefficient of variation ranged from 84.5% to 156%) and the increase in CFU in spleens from confined fish was found not to be statistically significant. Confinement did not induce increased relative risk of viable *A. salmonicida* persistence in the spleen compared to control fish either (results shown in appendix 2). No viable bacteria were recovered from the blood of infected fish at any sampling time.

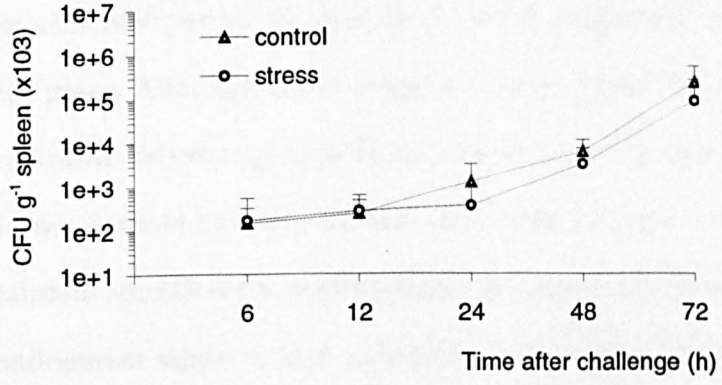

Figure 5.4 Clearance of *A. salmonicida* by spleens of control and repeatedly confined animals.

Results are expressed as mean values ± sd (n=8). Fish were intraperitoneally challenged with $2x10^4$ CFU.

5.4. Discussion

Elevated plasma glucose concentration in response to aquaculture-associated stressors has been extensively reported (example, Iwama *et al.*, 1995). This elevation has been shown to be mediated by glycolysis and/or glycogenolysis due, at least in part, to catecholamine and possibly cortisol release during the stress response (Pankhurst and Sumpter, 1997). In this study, plasma glucose concentration was significantly increased by single confinement in all experiments. However, a non-significant elevated glucose concentration was observed in plasma of animals subjected to repeated daily confinement. This trend in plasma glucose concentration has also been reported in sea bream following a 16-day period of repeated acute stressor, although plasma cortisol concentration was significantly increased in fish subjected to the stressor throughout the experimental period (Sunyer et al, 1995), suggesting that no physiological adaptation took place. Although confinement induced a stress response in the present study, it was not known whether physiological adaptation to repeated confinement occurred since no plasma cortisol or other plasma indicators of stress were measured. The inability to maintain significantly higher plasma glucose concentrations during extended periods of confinement might be due, at least in part, to the animal switching from glycogenolysis to gluconeogenesis when hepatic glycogen reserves are depleted (Vijayan *et al.*, 1997; Wendelaar Bonga, 1997). However, the plasma glucose of carp remained significantly higher in animals subjected to a crowding stress for 30 days (Yin *et al.*, 1995).

It has been reported that acute physical stressors induce several cellular and immunological changes in peripheral blood of fish. Amongst these, neutrophilia and lymphocytopenia have been most extensively reported (example, Ellsaesser and Clem, 1986). Reduced serum complement haemolytic and bactericidal activity also have been demonstrated following confinement (Yin *et al.*, 1995) and handling (Sunyer *et al.*, 1995).

In this study, however, only neutrophilia, amongst all haematological parameters tested, was observed to be significant following repeated daily confinement.

Results obtained in the present experiments showed that modulation of head kidney macrophage antimicrobial mechanisms depended on the frequency of confinement. Nitrite, the main product of nitric oxide spontaneous turnover, was not detected in any of the macrophage supernatants under any conditions tested. These results are similar to previous observations on nitric oxide production (chapters 3 and 4), where different strategies of macrophage stimulation *in vitro* or *in vivo* did not yield detectable nitrite production. In contrast to mouse macrophages, human macrophages require a previous infectious, inflammatory or autoimmune disease process *in vivo* to switch on NO production *in vitro* (reviewed by MacMicking *et al.*, 1997), and this may also be the case of rainbow trout macrophages.

Phagocytosis of foreign particles by fish macrophages has been reported to be both increased (Peters *et al.*, 1991) and suppressed (Narnaware *et al.*, 1994) by aquaculture-associated stressors. In this study, significant modulation of phagocytic activity depended on the frequency of confinement. SRBC engulfment by head kidney macrophages was not significantly modulated by single confinement of fish at any sampling time, not even when results from all control and all confined animals were pooled into two groups and compared. A non-significant depression in both phagocytic index and phagocytosis rate was observed, however, following a single confinement. This depression was statistically significant when fish were subjected to repeated confinement for six consecutive days.

Likewise, head kidney macrophage respiratory burst activity was significantly modulated by repetitive confinement but not by single confinement at any sampling time. However, in the single confinement experiments, the production of PMA-triggered extra- and intra-cellular superoxide anion was significantly decreased two days after confinement. This effect was not due to confinement since it was observed in both

control and experimental animals and differences between the two groups were not found to be significant. Although the cause was not determined, Jørgensen *et al.* (1993b) also observed significant modulation of extracellular production of superoxide within the control group in a time-course study. Since, in our study, significant differences within groups occurred at one sampling time only, technical error on that sample may be the most likely cause. Significant differences between control and confined animals were not observed even when results from samplings at 1, 4, 8 and 16 days after confinement were pooled in one control and one confined group and compared. However, a tendency of increase in extracellular superoxide and decrease in intracellular superoxide production triggered by PMA was observed. This trend, as in results from phagocytosis, was found to be statistically significant when fish were exposed to daily repeated confinement.

Results from studies on stimulation of macrophages with MAF plus LPS *in vitro* showed that macrophages from single confined animals were able to produce superoxide anion to the same extent as control macrophages. This response was also observed in macrophages from confined animals and subsequently challenged with LPS *in vivo*. LPS challenge *in vivo* has been shown to exert multiple immunological (Ingram and Alexander, 1980) and physiological (Balm *et al.*, 1995) effects on teleost fish. However, unlike mammalian species, where even low doses of LPS are associated with fatal septic shock (Easmon, 1990), challenge of brown trout with high doses of bacterial LPS did not induce mortality or loss of appetite (Ingram and Alexander, 1980; Balm *et al.*, 1995). In this study, although direct comparisons are not possible, trout injected with LPS showed a significant increase in respiratory burst activity compared with non-injected animals from the same fish stock. Therefore, present results on immunostimulation, survival and feeding behaviour after high LPS dose injection are similar to those reported in the literature (Ingram and Alexander, 1980; Balm *et al.*, 1995). These results altogether underline the ability of rainbow trout to mount an adequate

respiratory burst process following a single stressor not only under resting conditions but also after a considerable challenge with LPS *in vivo*, or stimulation *in vitro*.

As in the studies on modulation of macrophage activity by confinement, the percentage of *A. salmonicida* killed *in vitro* was affected by the extent of confinement. Phagocytosis and respiratory burst were not significantly modulated by single confinement and neither was *A. salmonicida* killing. However, repetitive confinement induced significant reduction of killing capacity by macrophages *in vitro*, and this was correlated with decreased phagocytic and intracellular respiratory burst activities observed in those cells.

Increased production of extracellular superoxide and other reactive oxygen intermediates *in vivo* have been associated with a range of physiopathological and tissue injuries (reviewed by Gille and Sigler, 1995) as well as alteration of immune competence (Koner *et al.*, 1997) in mammalian species. Therefore, assuming similarities between mammalian species and rainbow trout, increased extracellular superoxide anion production caused by repeated confinement in this study, may play an important role in the pathological and immunological consequences associated with stress.

In this study, viable *A. salmonicida* were intraperitoneally injected into fish and CFU were recovered from the spleen from 6h after injection (first sampling time). As in experiments described in chapter 4, numbers of *A. salmonicida* CFU g^{-1} spleen increased with time, indicating growth of *A. salmonicida* in spleen and/or effective clearance from the circulatory system. Increased CFU numbers in spleens from repeatedly confined animals were observed from 24 h after infection, suggesting an impaired killing/clearance capacity as compared with control animals. However, these differences were not statistically significant. Similarly, the relative risk of viable bacterial persistence in spleen was not modified by repetitive confinement. The variation in recovered numbers of CFU from spleen amongst individuals from the same experimental group was very high at all sampling times (mean coefficient of variation was 125.41 %) and

viable bacteria were not recovered from all animals. Therefore, significant differences would be very difficult to demonstrate. This high variation in the bacterial clearance from spleens might be also part of the reason for the high variation found in challenge trials where mortalities are quantified (Nordmo, 1995).

In conclusion, results from this study indicate that rainbow trout were able to mount an adaptive stress response following confinement since plasma glucose concentration was elevated. However, no loss of appetite or morbidity was observed throughout the experiment. The immune response differed depending on the extent of confinement. Innate immune mechanisms assayed were only significantly altered after repetitive confinement, indicating that the adaptive response following single confinement did not adversely affect the immune parameters tested, while that to repeated confinement did have a detrimental effect. Certain stressful events may render animals more susceptible to opportunistic infections not only through immunosuppression but also by self-damage induced by increased production of extracellular reactive oxygen intermediates.

Finally, having identified a confinement strategy inducing immunomodulation, and an immunostimulation regime through the use of peptidoglycan in-feed (see chapter 4), further experiments focused on the prophylactic use of peptidoglycan to ameliorate the impact of confinement on rainbow trout innate immune condition (see chapter 6).

Chapter 6

Modulation of rainbow trout innate defence mechanisms by confinement and peptidoglycan

6.1 Introduction

Reducing the impact of infectious diseases is central to successful aquaculture. As in farming of other animals, strategies of livestock husbandry and disease treatment have been designed for this purpose. Vaccines have been developed against some fish pathogens. However, they are not available for many opportunistic bacteria present in the water such as motile aeromonads, pseudomonads and some *Vibrio* spp. Opportunistic pathogens can cause substantial losses in fish populations when animals become immunocompromised due to environmental stress or at times of physiological change or alterations in farming practice (Wedemeyer and Goodyear, 1984; Wedemeyer, 1996). In such situations, attempts are usually made to improve holding conditions and, in addition, oral antibiotic treatment may be applied. However, antibiotic prophylaxis is strongly contra-indicated in aquaculture to help control emergence of drug resistant strains and, moreover, the range of antibiotics licensed for therapeutic use is limited.

Strategies based on genetic selection are being investigated to select stocks more able to resist stress-associated bacterial diseases. Selection of salmonids for high or low stress response has not been very successful in improving survival in face of a range of pathogens. In fact, some pathogens induced higher mortalities in low stress responders (Fevolden *et al.*, 1992; Fevolden *et al.*, 1994). There is still scope for improvement using genetic selection, but other strategies based on stimulation of host immune response have been proposed as a means to counteract stress-mediated immunosuppression in aquaculture.

Although different substances including microbial components, vitamins and minerals have been shown to enhance fish immune response (reviewed in Blazer, 1991; Secombes and Fletcher, 1992; Raa, 1996), their potential to compensate for or prevent immunosuppression due to stress has been less extensively studied. High levels of dietary vitamin C have been shown to enhance immune function in salmonids (Verlhac

and Gaboudan, 1994) and it has been suggested that feeding vitamin C to fish larvae increased survival to bacterial challenges after stress (Merchie *et al.*, 1997). However, conflicting evidence has also been produced since suppression of rainbow trout kidney macrophage activity (Thompson *et al.*, 1993) and diminished resistance of channel catfish to *E. ictaluri* (Li *et al.*, 1998) associated with confinement were independent of the vitamin C status of the fish. Prophylactic use of oral β-glucan has been successful in improving recovery of peripheral phagocyte function after transportation of rainbow trout, which then showed increased survival against a spontaneous, uncontrolled, infection with *F. columnaris* (Jeney *et al.*, 1997).

Chapters 4 and 5 identified dietary peptidoglycan and confinement as factors that induced modulation of macrophage function. The present study was carried out to investigate whether the alteration of macrophage function associated with confinement could be limited or prevented through prophylactic peptidoglycan administration. The ability of fish to coordinate innate defence system to kill challenge bacteria also was investigated by injecting *A. salmonicida* into treated fish and measuring bacterial clearance from the spleen.

6.2 Materials and methods

Animals and bacterial strain

All-female rainbow trout were purchased from Trossachs Trout Farm and acclimatised to aquarium conditions at 11.5 ± 0.5 °C for a period of 6 weeks as described in section 2.2 before experiments begun.

Aeromonas salmonicida strain FCS (details given in Inglis *et al.*, 1991) was used in all experiments in this study. Preliminary studies on viable bacterial persistence in the spleen and blood of fish involved the use of *A. salmonicida* B95179, *Vibrio anguillarum* NCIMB 6 and *A. hydrophila* NCIMB 1134 as challenge organisms.

Experimental design

After acclimatisation, groups of 50 fish individually weighing 175 ± 9.3 g were placed in six similar 370 l fresh water flow-through tanks with aeration. Water temperature was kept constant at 11.5 ± 0.5 °C throughout the experiment.

In order to investigate the potential prophylactic use of peptidoglycan in feed prior to stressful situations, four different treatments were applied: (1) fish in one tank were subjected to 'control treatment', which involved feeding on the control diet with no disturbance of the animals; (2) fish in another tank were fed on the peptidoglycan diet and left undisturbed ('peptidoglycan' treatment); (3) fish in another two tanks were fed on the control diet and subjected to repeated confinement ('confinement' treatment); (4) finally, fish in the remaining two tanks were fed on the diet containing peptidoglycan and subjected to repeated confinement ('peptidoglycan plus confinement' treatment).

Diets were prepared and stored as described in section 4.2. Floating pellets were chosen to allow feeding to be more easily monitored. The experimental diet contained 0.05 % peptidoglycan (w/w). Fish were fed daily to the manufacturer's RDA (Ewos Select No. 40; 1.44 % bw) on experimental or control diets. On the days that confinement was applied fish were fed only once daily 5-7 h after confinement. As it can be seen in figure 6.1, four weeks after the beginning of feeding treatments, trout were sampled to quantify PBLs and assess head kidney and peritoneal macrophage activity. In order to elicit migration of macrophages to the peritoneal cavity, 2 ml of filter-sterilised 8 % casein solution in saline were injected intraperitoneally into 12 fish per tank six days before sampling. Injected fish were then marked and returned to their tanks. After this, animals in the 'confinement' and 'peptidoglycan plus confinement' treatment tanks were subjected to a confinement stressor, which was repeated daily for six days, as described in section 5.2.

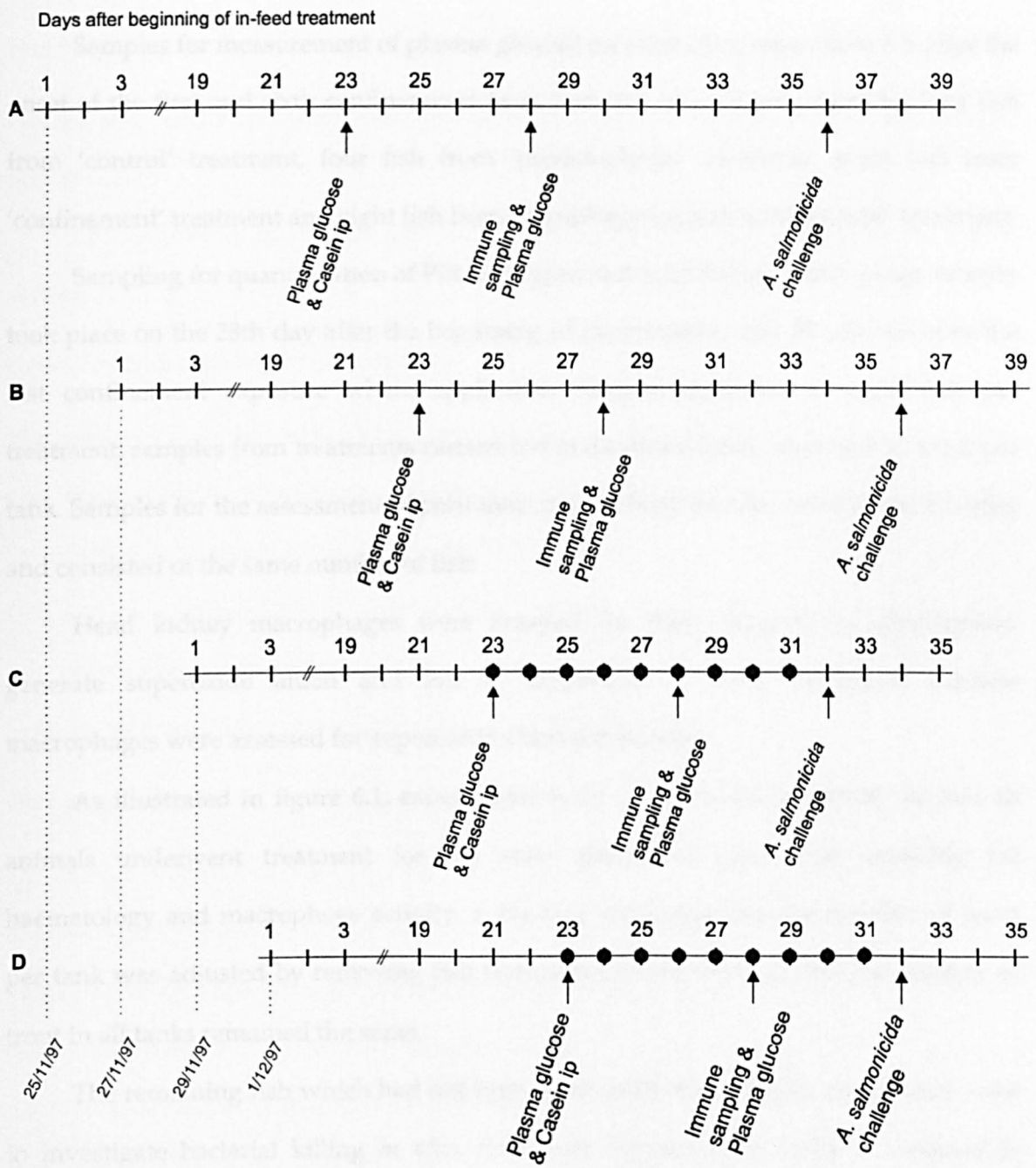

Figure 6.1 Experimental design to investigate potential prophylactic use of peptidoglycan in feed prior to stressful events.

A, 'control' treatment; **B**, 'peptidoglycan' treatment; **C**, 'confinement' treatment; **D**, 'peptidoglycan plus confinement' treatment. ● indicates days when confinement was applied.

Samples for measurement of plasma glucose concentration were taken 5 h after the onset of the first and sixth confinements from four fish in each tank, totalling four fish from 'control' treatment, four fish from 'peptidoglycan' treatment, eight fish from 'confinement' treatment and eight fish from 'peptidoglycan plus confinement' treatment.

Sampling for quantification of PBL cell types and head kidney macrophage activity took place on the 28th day after the beginning of experiments, and 30 minutes after the last confinement exposure where applicable. Samples consisted of eight fish per treatment; samples from treatments carried out in duplicate tanks consisted of 4 fish per tank. Samples for the assessment of peritoneal macrophage function were taken 4 h after and consisted of the same number of fish.

Head kidney macrophages were assayed for their capacity to phagocytose, generate superoxide anion and kill *A. salmonicida in vitro*. Peritoneal exudate macrophages were assessed for superoxide anion production.

As illustrated in figure 6.1, experiments were carried out sequentially so that all animals underwent treatment for the same period of time until sampling for haematology and macrophage activity. Following each sampling, the number of trout per tank was adjusted by removing fish from some of the tanks so that the number of trout in all tanks remained the same.

The remaining fish which had not been previously injected with casein were used to investigate bacterial killing *in vivo*. Fish were injected with viable *A. salmonicida* intraperitoneally and six fish per treatment were sampled 5, 12, 24 and 48 h after injection; fish were bled, sacrificed and the spleens collected. Samples from treatments carried out in duplicate tanks consisted of 3 fish per tank. Fish were subjected to their respective treatment until *A. salmonicida* challenge, after which they were fed on commercial trout pellets at the RDA until the end of the experiment.

Haematology

Haematocrit and PBL counts were determined in EDTA-treated blood as described in section 2.3.

Quantification of plasma glucose concentration was performed as detailed in section 2.5.

Isolation and culture of macrophages

Macrophages from the head kidney and peritoneal cavity were enriched in suspension and monolayers on 96-well microtiter plates or 8-well glass slides prepared as described in section 2.7. Monolayers were then cultured at 19 °C in L-15 containing 5 % FCS and P/S and used within 12-18 h of preparation. P/S was not added in the culture medium for macrophage monolayers prepared to assess *A. salmonicida* killing *in vitro*. Macrophage monolayers were washed 3 times with cHBSS before use.

Phagocytosis

Opsonised sheep red blood cells were prepared and adjusted to the desired concentration as described in section 2.8.2. Freshly opsonised SRBC were used each time. The number of adherent macrophages from each fish was estimated in spare wells as described in section 2.7.3. Monolayers in 8-well glass slides were then incubated with 0.4 ml of diluted SRBC suspension in L-15 plus 5 % FCS to obtain an average ratio of 1 macrophage to 5 SRBC. After 60 minutes at 19 °C, phagocytic index and phagocytosis ratio were calculated as described in section 2.8.1.

Respiratory burst

PMA-triggered reduction of cytochrome c was assessed to quantify generation of extracellular superoxide anion in 96-well microtiter plates as described in section 2.9.1. Superoxide dismutase was added to some of the wells to confirm specificity of the reaction.

Intracellular superoxide anion production was measured in 96-well microtiter plates as the reduction of NBT in the presence or absence of PMA as described in section 2.9.2.

All reactions were carried out in triplicate wells. Duplicate wells per fish and assay were used to count the number of macrophages per well and results adjusted to OD_{610} per $2x10^5$ cells or nmol O_2^- produced per $2x10^5$ cells for the NBT and cytochrome c assays, respectively.

A. salmonicida killing *in vitro*

A. salmonicida was grown in TSB overnight at 22 °C with mild continuous shaking. Bacteria were then washed three times in PBS and opsonised as described in section 2.8.2 with the same serum stock as that used for opsonising SRBC. Bacteria then were washed three times in PBS and the concentration adjusted spectrophotometrically to $1x10^9$ CFU ml^{-1} (OD_{610}= 1.24). The bacterial suspension was diluted in L-15 containing 5% FCS to give a ratio of 1 macrophage to 20 *A. salmonicida* CFU, later confirmed by viable plate counts. Head kidney macrophage monolayers were then incubated with the bacterial suspension at 19 °C. Five hours later, viable bacteria in the wells were quantified by the reduction of MTT and the percentage of bacteria killed calculated as described in section 2.11.

Detection of specific antibodies to *A. salmonicida* in serum

The titre of antibodies specific to *A. salmonicida* in sera of rainbow trout was assessed by enzyme-linked immunosorbent assay (ELISA). Sera from six donor fish were extracted as described in section 2.4 and store frozen at -20 °C until analysed. Donor fish had not been previously exposed to *A. salmonicida* experimental infection or vaccination. *A. salmonicida* was grown in TSB and adjusted to a concentration of $1x10^9$ CFU ml^{-1} in saline as described above. 96-well microtiter plates were coated with 50 µl of 0.05 % (w/v) poly-L-lysine in 0.05 M carbonate-bicarbonate buffer (pH 9.6). After 60 minutes, plates

were washed twice with low salt wash buffer (0.020 M Trizma base, 0.38 M NaCl, 0.05 % (v/v) Tween 20 and 0.01 % (w/v) Merthiolate in distilled water; pH 7.3). Supernatants were replaced with 100 µl of $1x10^8$ *A. salmonicida* CFU ml^{-1} in saline and plates incubated overnight in a humid chamber at 4 °C. Bacterial cells were then fixed by adding 50 µl per well of 0.05% (v/v) glutaraldehyde in PBS (0.02 M $NaH_2PO_4.2H_2O$, 0.02 M $Na_2HPO_4.2H_2O$, 0.15 M NaCl in distilled water; pH 7.2). After 20 minutes incubation at room temperature, plates were washed three times with low salt wash buffer and non-specific binding sites blocked with H_2O_2 and bovine serum albumin (BSA). For this purpose, each well was treated with 100 µl of 10 % (v/v) H_2O_2 in PBS for 1 h and washed three times with low salt wash buffer. Supernatants were then replaced with 250 µl of 1 % (w/v) BSA and plates incubated for 2 h at room temperature before washing three times with low salt wash buffer. Wells were incubated with 100 µl of serially diluted fish serum in PBS for 2 h at room temperature and washed 5 times with high salt wash buffer (0.02 M trizma base, 0.50 M NaCl, 0.1 % (v/v) Tween 20 and 0.01 % (w/v) Merthiolate in distilled water; pH 7.7), incubating for five minutes on last wash. Supernatants were then replaced with 100 µl of first antibody (mouse IgG antibody specific to Atlantic salmon IgM; diluted 1/1000 in 1 % (w/v) BSA in PBS) for 60 minutes at room temperature. Plates were washed five times with high salt wash buffer and supernatants replaced with 100 µl of second antibody (rabbit antibody specific to mouse IgG labelled with horseradish peroxidase; diluted 1/1000 in 1 % (w/v) BSA in PBS). After 60 minutes incubation at room temperature, plates were washed five times with high salt wash buffer, incubating five minutes on last wash. Supernatants were replaced with 100 µl of chromogen solution (prepared by mixing 150 µl of 42 mM 3'3'5'5'-tetramethylbenidine dihydrochloride in acetic acid diluted 1/2 with distilled H_2O and 15 ml of 0.1 M citric acid, 0.1 M sodium acetate, 0.03 % (v/v) H_2O_2 in distilled H_2O; pH 5.4). After 10 minutes at room temperature, 50 µl of 2 M H_2SO_4 were added to each well to stop the reaction and the optical density of the reaction mixtures was read at 450 nm in a multiscan

spectrophotometer. ELISA was performed in triplicate wells for each test serum dilution. Negative controls consisted of wells with no bacteria, wells with bacteria and no first antibody, and wells with bacteria and no second antibody. All readings were blanked against wells treated with chromogen and stop solutions only.

A. salmonicida clearance *in vivo*

A. salmonicida was grown as described above and the bacterial suspension adjusted to give a concentration of $1x10^9$ CFU ml^{-1} in TSB (OD_{610}= 1.24). This was diluted in saline to obtain a challenge suspension and the concentration confirmed by viable counts. Each fish received an ip dose consisting of 400 µl of $2.3x10^5$ *A. salmonicida* CFU ml^{-1}. Four fish per tank were sacrificed and spleens and blood sampled for viable bacteria 5, 12, 24, 48 and 72 h after injection as described in section 2.12.

Statistical analysis

Differences in individual mean values between different treatments were investigated by one way ANOVA and the Student-Newman-Keuls multiple comparison test. After confirming by the Student *t*-test that individual mean values from duplicate tanks of the same treatment were not significantly different, they were pooled into one group for analysis. Normality and homogeneity of variance were confirmed before any parametric tests were applied. Non-normal data underwent logarithmic transformation except percentage data which was transformed by the square root arcsin. When transformed data were still non-normal, one way ANOVA on ranks was applied. Statistical tests were performed with SigmaStat™ 1.0 and in all cases $p<0.05$ was the accepted level of significance. Regression analysis were also used to confirm linearity between *A. salmonicida* CFU and reduction of MTT. Differences in the relative risk of persistence of viable *A. salmonicida* in the spleen and blood were investigated using the software EPI-INFO 6.04.

6.3 Results

Feeding behaviour and mortalities

Trout in all tanks fed actively throughout the experiment and no pellets were observed floating on the water surface immediately after feeding. During dissection of fish, feed was observed in the guts of all sampled fish. No morbidity or mortality was observed during the experiment.

Haematology

Confinement of fish caused plasma hyperglycaemia compared with control treatment. However, differences were only statistically significant after the first confinement. Peptidoglycan in feed did not affect plasma glucose concentration (figure 6.2).

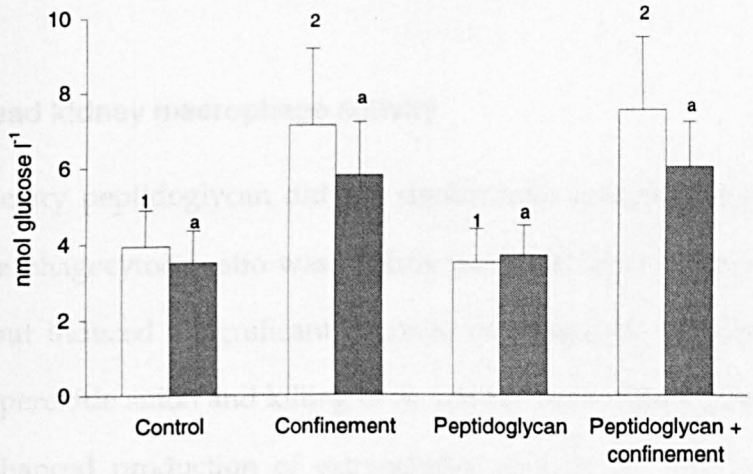

Figure 6.2 Plasma glucose concentration.
Treatments were as for figure 6.1. Results are expressed as mean values + sd (n=6). Empty bars show plasma glucose concentration 5 h after first confinement or at the equivalent time for control and peptidoglycan treatments; solid bars, 5 h after sixth confinement or equivalent time. Different numbers or letters on bars indicate significant differences between treatments ($p<0.05$)

None of the treatments induced a significant effect on total or differential PBL counts. However, neutrophilia was observed in trout subjected to confinement or peptidoglycan plus confinement treatments and, to a lesser extent, in fish fed peptidoglycan (table 6.1).

Table 6.1 Total and differential PBL counts.

Treatment	PBL	L	T	N	M
Control	32.2 ± 11.1	24.3 ± 8.1	5.95 ± 2.92	1.66 ± 0.99	0.55 ± 0.22
Peptidoglycan	38.9 ± 17.1	29.2 ±15.1	7.05 ±3.84	2.52 ±1.70	0.25 ±0.07
Confinement	41.7 ± 5.4	32.5 ± 3.8	4.48 ± 1.93	4.61 ± 2.20	0.33 ± 0.19
Peptidoglycan + confinement	40.3 ± 6.5	28.1 ± 2.0	6.97 ± 2.76	5.06 ± 5.70	0.29 ± 0.23

Treatments were as for figure 6.1. Data are expressed as mean values ($x10^6$) ml^{-1} ± sd (n=8). No significant differences were found between any treatment. PBL, total peripheral blood leukocyte counts; L, lymphocytes; T, thrombocytes; N, neutrophils; M, monocytes.

Head kidney macrophage activity

Dietary peptidoglycan did not significantly modulate macrophage function, although the phagocytosis ratio was slightly increased after treatment. Confinement of rainbow trout induced a significant decrease in phagocytic activity, intracellular generation of superoxide anion and killing of *A. salmonicida in vitro* by kidney macrophages as well as enhanced production of extracellular superoxide. Prophylactic use of peptidoglycan treatment had a variable influence in compensating for the effects of repeated confinement on macrophage activity. Peptidoglycan did not significantly compensate for decreased phagocytic activity, intracellular production of superoxide anion and killing of *A. salmonicida in vitro,* although all these parameters were increased compared with confined fish held without peptidoglycan supplement (figures 6.3, 6.4A and 6.5 respectively). The significant enhancement of extracellular superoxide anion production

by kidney macrophages from confined animals was significantly neutralised by feeding fish on the diet containing peptidoglycan, although, as it can be seen in figure 6.4B, it was still enhanced compared with macrophages from control animals.

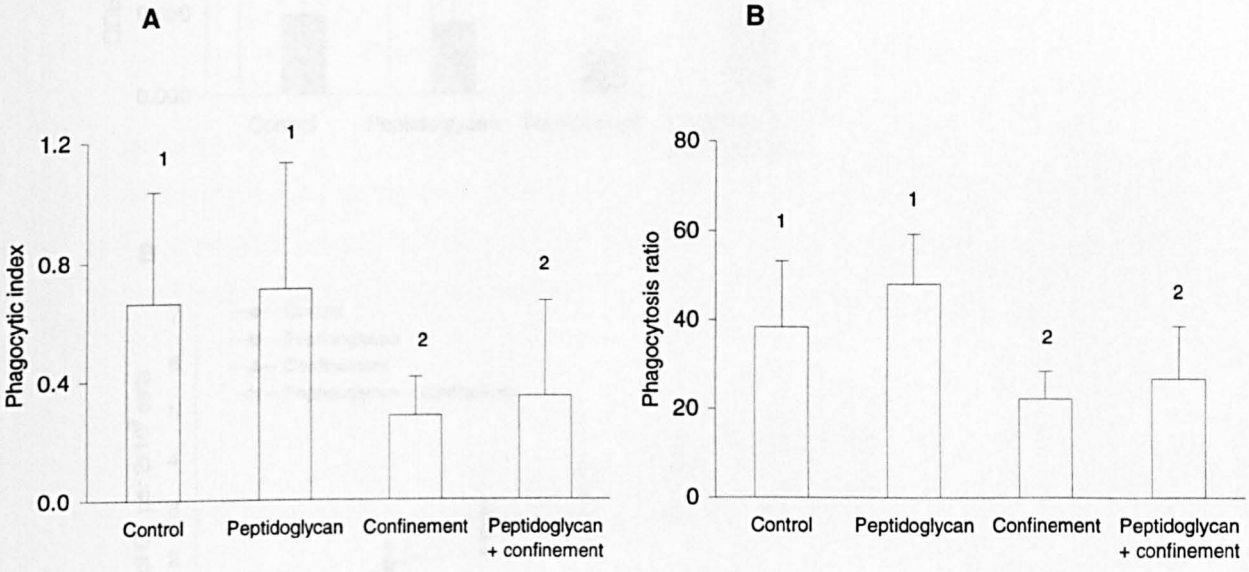

Figure 6.3 Phagocytosis of SRBC by head kidney macrophages.
Treatments were as for figure 6.1. **A**, phagocytic index; **B**, phagocytosis ratio (%). Results are expressed as mean values ± sd (n=8). Different numbers on sd bars indicate significant differences between treatments ($p<0.05$).

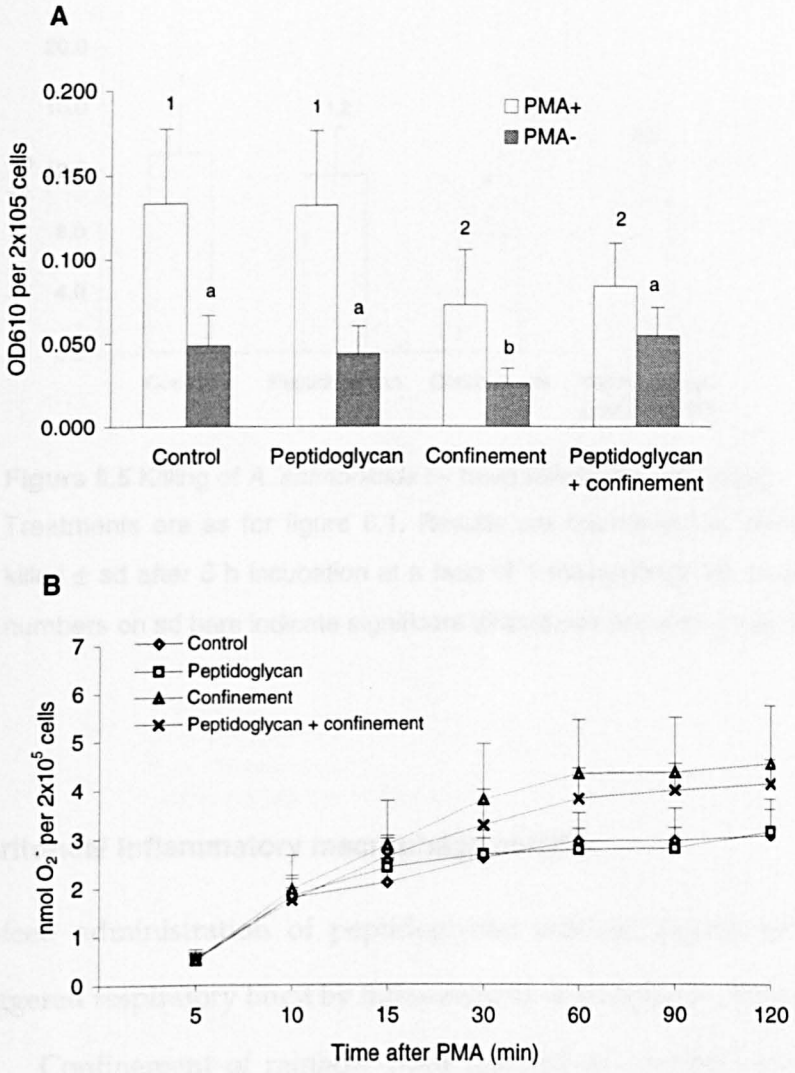

Figure 6.4 Respiratory burst activity of head kidney macrophages.

Treatments are as for figure 6.1. **A**, reduction of NBT; results are expressed as mean values ± sd (n=8). Different numeric or alphabetic characters on sd bars indicate significant differences between treatments ($p<0.05$). **B**, production of extracellular superoxide anion; results are expressed as mean values ± sd (n=8); significant differences were found from 60 minutes after addition of PMA between confinement treatment and control, peptidoglycan and peptidoglycan plus confinement treatments only ($p<0.05$).

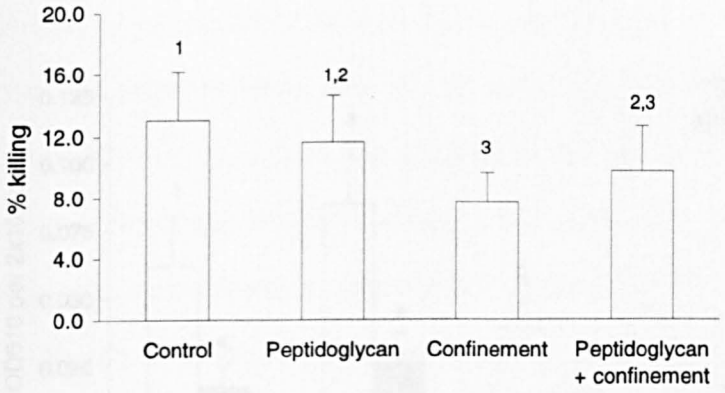

Figure 6.5 Killing of *A. salmonicida* by head kidney macrophages.
Treatments are as for figure 6.1. Results are expressed as percentage of *A. salmonicida* killed ± sd after 5 h incubation at a ratio of 1 macrophage:20 bacterial cells (n=8). Different numbers on sd bars indicate significant differences between treatments ($p<0.05$).

Peritoneal inflammatory macrophage activity

In-feed administration of peptidoglycan induced significant enhancement of PMA-triggered respiratory burst by inflammatory macrophages (figure 6.6).

Confinement of rainbow trout resulted in variable modulation of inflammatory macrophage respiratory burst. NBT reduction was significantly depressed while production of extracellular superoxide anion was significantly increased (figure 6.6).

Although the use of peptidoglycan in feed did not have any regulatory effect on the confinement-mediated increase in extracellular production of superoxide anion, it compensated the suppressive effect of confinement on NBT reduction by macrophages (figure 6.6).

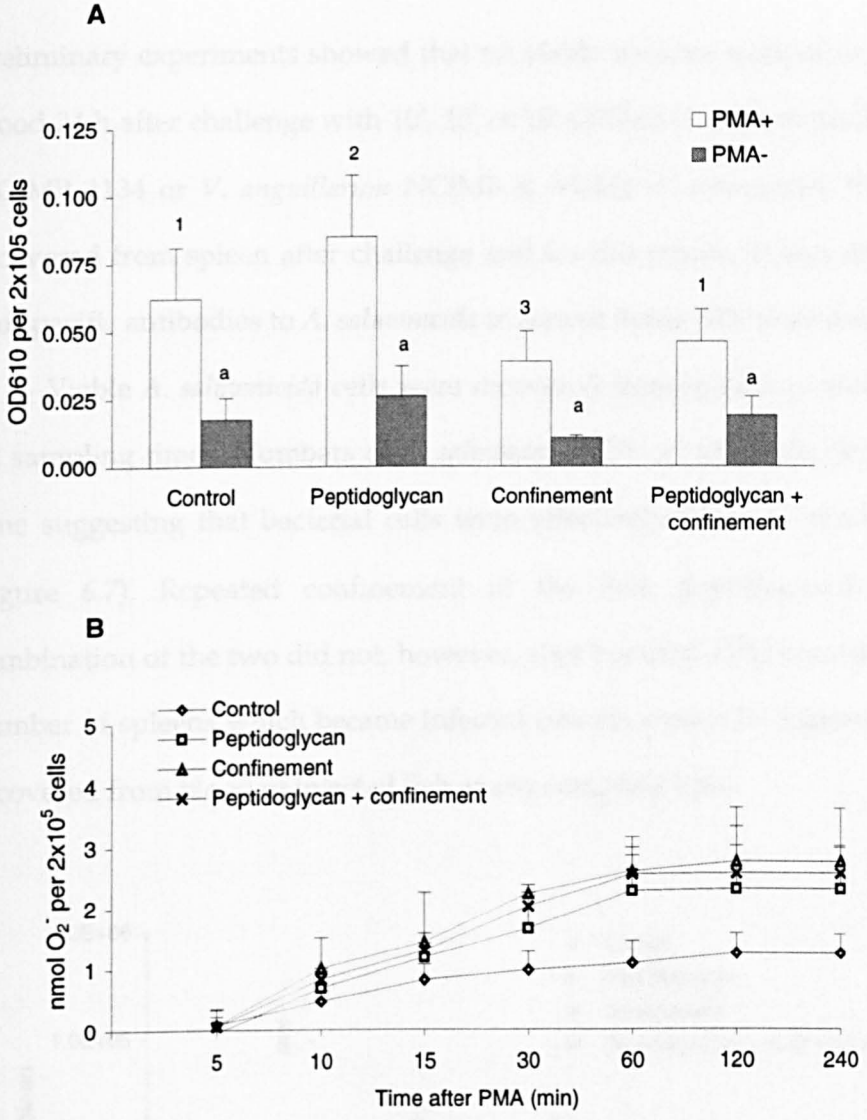

Figure 6.6 Respiratory burst activity of inflammatory peritoneal macrophages.

Treatments are as for figure 6.1. **A**, reduction of NBT; results are expressed as mean values ± sd (n=8). Different numeric or alphabetic characters on sd bars indicate significant differences between treatments ($p<0.05$). **B**, production of extracellular superoxide anion; results are expressed as mean values ± sd (n=8); significant differences were found from 30 minutes after addition of PMA between control treatment and peptidoglycan, confinement and interaction treatments only ($p<0.05$).

Bactericidal activity *in vivo*

Preliminary experiments showed that no viable bacteria were recovered from spleen or blood 24 h after challenge with 10^4, 10^5 or 10^6 CFU of *A. salmonicida* B95179, *A. hydrophila* NCIMB 1134 or *V. anguillarum* NCIMB 6. Viable *A. salmonicida* FCS strain cells were recovered from spleen after challenge and for this reason, it was chosen for this study. No specific antibodies to *A. salmonicida* in sera of donor fish were detected by ELISA.

Viable *A. salmonicida* cells were recovered from spleens of most inoculated fish at all sampling times. Numbers of *A. salmonicida* CFU g^{-1} of spleen decreased rapidly with time suggesting that bacterial cells were effectively killed or rendered non-culturable (figure 6.7). Repeated confinement of the fish, peptidoglycan administration or combination of the two did not, however, alter bacterial CFU counts from spleens or the number of spleens which became infected (results shown in appendix 3). No CFU were recovered from blood of injected fish at any sampling time.

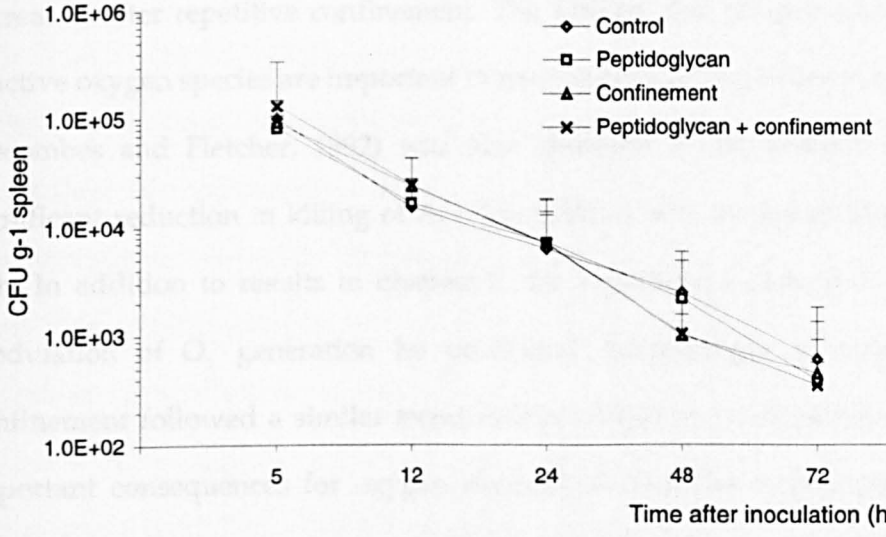

Figure 6.7 *A. salmonicida* CFU in the spleen of challenged fish.
Results are expressed as mean values ± sd (n=6). Each fish received a dose of 9.2×10^4 CFU. No significant differences were observed between any treatment.

6.4 Discussion

The results here reported on immunomodulation by oral treatment with peptidoglycan for a 4-week period are similar to those reported in chapter 4. Total and differential PBL counts and head kidney macrophage activity were not significantly affected by oral peptidoglycan. However, inflammatory peritoneal macrophage activity, as indicated by extracellular superoxide anion generation, was significantly enhanced. Furthermore, results from this experiment showed that intracellular production of O_2^- by peritoneal macrophages was also augmented in response to dietary peptidoglycan.

Likewise, results obtained on the modulation of plasma glucose concentration and innate immune activity by daily repeated confinement of rainbow trout were consistent with those of chapter 5. Confinement induced a stress response as indicated by plasma hyperglycaemia compared with control treatment. Head kidney macrophage activity was variably modulated by repetitive confinement. While phagocytosis of SRBC and production of intracellular O_2^- were depressed, generation of extracellular O_2^- was increased after repetitive confinement. The finding that phagocytosis and intracellular reactive oxygen species are important in macrophage killing of invading microorganisms (Secombes and Fletcher, 1992) was also observed in the present work through the significant reduction in killing of *A. salmonicida in vitro* by macrophages from confined fish. In addition to results in chapter 5, the experiments described here showed that modulation of O_2^- generation by peritoneal macrophages in response to repeated confinement followed a similar trend to that of kidney macrophages. These may have important consequences for oxygen dependent microbial killing and damage to self. Increased superoxide production is associated with phagocyte-mediated tissue damage in mammalian species (Baggiolini and Wymman, 1990). On the other hand, an increased neutrophil count was observed in the blood of confined animals, although, in contrast with that reported in chapter 5, it was not statistically significant. This may be explained

by the fact that the variation in neutrophil numbers within control and within confined animals in this experiment was greater than the observed in chapter 5.

Since hyperglycaemia was recorded and no mortalities or loss of appetite seen, an adaptive stress response appears to have been elicited during the six days that fish were subjected to repetitive confinement. This response, however, resulted in impaired activity of macrophages from different sources, namely those resident in the head kidney and the inflammatory cells in the peritoneal cavity.

The serum hyperglycaemic response to confinement was independent of peptidoglycan treatment, indicating that dietary peptidoglycan did not prevent the stress response elicited by confinement. This observation was consistent with similar studies on the use of glucan (Jeney *et al.*, 1997) and vitamin C (Thompson *et al.*, 1993; Li *et al.*, 1998) as feed additives in rainbow trout, Atlantic salmon and channel catfish. Compensatory effects of in-feed peptidoglycan on confinement-mediated immunomodulation were therefore more probably achieved at a tertiary level (immunological) rather than at a secondary (physiological) level of the stress response.

Dietary peptidoglycan limited the effects on macrophage function associated with stress, but the magnitude of the effect recorded depended on both the activity measured and source of macrophages. The confinement-mediated suppression of phagocytosis, PMA-triggered production of intracellular superoxide anion and bacterial killing by head kidney macrophages was not significantly modulated by in-feed peptidoglycan treatment. Nevertheless, in peptidoglycan plus confinement treated fish, all these activities were slightly increased compared with results from fish subjected to confinement only. The enhanced production of extracellular superoxide anion by head kidney macrophages in response to confinement was significantly reduced by feeding fish on the peptidoglycan diet prior to confinement of animals. However, as it can be seen in figure 6.4B, extracellular $O_2^{\cdot}$ generation was still considerably higher than that in control fish. Since in-feed peptidoglycan alone did not affect head kidney macrophage

activity, this finding may suggest that peptidoglycan administration caused limitation of confinement-mediated over-production of extracellular O_2^- through regulatory mechanisms. However, these mechanisms were not able to elicit a similar effect on the other activities of renal macrophages.

The prophylactic activity of peptidoglycan on stress-induced changes in the respiratory burst of inflammatory peritoneal macrophages was different to that of resident head kidney macrophages. Dietary peptidoglycan was able to significantly restore the reduced generation of intracellular O_2^- caused by confinement, although the resultant level was still lower than in control fish. However, in contrast to head kidney macrophages, the over-production of extracellular superoxide caused by confinement was not modified when fish were fed on the peptidoglycan-containing diet.

Viability of *A. salmonicida* cells in the spleen of challenged fish was independent of the treatment applied. Since confinement caused significant depression of important macrophage bactericidal functions, it seems likely that the activity at which macrophages are able to kill *A. salmonicida* was not affected by confinement. Alternatively, other immune parameters that were not assayed may be important in *A. salmonicida* clearance in the spleen. These results underline the difficulties encountered when comparing individual specific immune activities with susceptibility of fish to laboratory-induced infections (Anderson *et al.*, 1997).

Very few published reports are available on the use of feed additives to compensate immunomodulation caused by stressful events in fish. Although peptidoglycan has been used as feed additive to enhance immune performance of fish (Matsuo and Miyazono, 1993; Itami *et al.*, 1996), there are no available reports on its prophylactic use prior to controlled stressful conditions to the author's knowledge. Some work is, however, available on use of dietary β-glucan. It has been reported that peripheral blood leukocytes from rainbow trout fed on different doses of fungal β-glucan showed increased phagocytosis as well as intra- and extra-cellular superoxide generation

compared with cells from control fish (Jeney *et al.*, 1997). Dietary glucan in that study did not restore the immunosuppression of those parameters observed 2 h after transportation of the fish. However, macrophage function from treated fish recovered to a greater extent than that of control fish one week after the stressor.

Results from the experiments here described showed that dietary peptidoglycan was able to compensate for the impairment of some macrophage functions caused by a common aquaculture-related stressor. This prophylactic effect was observed to occur to a greater extent than previous reports on dietary β-glucan and vitamin C (Thompson *et al.*, 1993). Inflammatory macrophages play a fundamental role in combating foci of infection in a variety of organs (Secombes, 1996), and dietary peptidoglycan was able to restore diminished intracellular production of microbiocidal O_2^- by these cells. However, peptidoglycan was not able to do so in head kidney macrophages. This may have important consequences for successful prophylactic use of the immunostimulant since haemopoietic organs such as spleen and head kidney have been shown to be the main centres for the clearance of blood-borne antigens in rainbow trout (Alexander *et al.*, 1983; Marsden *et al.*, 1996).

Therefore, these results taken together with those observed by Jeney *et al.* (1997) should be viewed as encouraging, although further laboratory and field experiments are required to accurately establish the role of dietary immunostimulants in the management of infections in stressed fish stocks.

Chapter 7

General discussion

7.1 General discussion

7.1.1 Haematological and immune parameters investigated

Preliminary work involved the screening of different immune assays for application in this study. Some of the assays investigated showed large individual variation between replicate fish and it was decided not to use them for that reason. These included serum lysozyme activity (as described by Ellis, 1990 and Siwicki and Anderson, 1993), intracellular production of superoxide anion in whole blood samples (as described by Siwicki and Anderson, 1993) and luminol-enhanced chemiluminescence response of head kidney macrophages to PMA.

Chemiluminescence is a very sensitive assay of respiratory burst activity by phagocytes (Dahlgren *et al.*, 1991) but available equipment, a liquid scintillation counter (Packard, England), was able to analyse only one sample at a time. The use of a microplate luminometer might have reduced within-group variation since all samples are read at once and this should be pursued when access becomes available.

Lysozyme is of considerable importance in defence against certain bacteria in vertebrate species. Application of specific antibodies to quantify titres in serum and organs would provide complementary data to the highly variable results obtained with classical functional assays.

C-reactive protein (CRP) is an important component of the innate defences against infection (Roberts, 1989b). Its concentration in serum rises during inflammation (Fletcher and Baldo, 1974), infection (Ramos and Smith, 1978) and immunisation (Kodama *et al.*, 1989). However, CRP has received little attention and further work is needed to investigate its role as indicator of immune competence.

Plasma glucose concentration

There was a low variation in glucose concentration within groups in all experiments. As commonly stated in the literature, increased plasma glucose concentration is a reliable

indicator of stress in rainbow trout subjected to a single confinement. However, after fish were confronted by the stressor on a daily basis, no significant differences between confined and control groups were observed. Quantification of other indicators of stress such as plasma cortisol, catecholamine(s) and lactate concentration would be beneficial in further investigations of physiological adaptation to the stressor.

Peripheral leukocyte counts

Total and differential peripheral blood leukocytes can be readily quantified, and neutrophilia as well as lymphocytopenia have been reported in the blood of fish subjected to a variety of stressors. In the present study, there was a large variation in peripheral leukocyte counts, particularly those of neutrophils, and their values were not significantly altered by any experimental procedure. Only in one out of the two experiments involving repetitive confinement, was a significant neutrophilia observed in the blood of confined animals.

As suggested by Borregaard (1996), quantification of cell types in the blood is often of little relevance to immune status and functional aspects should be investigated. Although more laborious, cellular function studies on head kidney and inflammatory macrophages were conducted in the present study.

Serum haemolytic activity

Serum complement-mediated lysis of xenogeneic red blood cells has been reported to be affected by stress in sea bream (Tort *et al.*, 1996) or ip glucan treatment in yellowtail (Matsuyama *et al.*, 1992a). The results presented here, however, showed no significant differences between experimental and control fish, possibly due to large variation in ACH_{50} values within groups. However, since the microassay for haemolytic activity reported here allows simultaneous processing of numerous samples, ACH_{50} quantification in rainbow trout should be carried out with a still higher number of individuals (in this thesis n=6 or 8).

Macrophage activity

Three different aspects of macrophage effector function were evaluated: ingestion of target cells, production of microbiocidal substances and actual killing of bacteria. Since alteration of one activity was not necessarily correlated with the others, it is suggested that a range of macrophage assays should be carried out to evaluate the response of macrophages to experimental conditions. This was particularly necessary in the case of superoxide anion production. In most occasions, extra- and intra-cellular superoxide were affected in opposite directions by the experimental conditions.

Quantification of phagocytosis by optical microscopy is subjected to some degree of inaccuracy since relatively few macrophages are examined (300 per sample) and some target cells may be counted as internalised when they are only attached or associated with the phagocyte membrane. Nevertheless, phagocytosis is a key event in macrophage killing and the variation in activity was not large within the same experimental groups. Efforts to quantify phagocytosis automatically based on fluorimetry and flow cytometry were made, but the technique finally used in this study was preferable overall due to poor access to equipment. Automation of measuring phagocytic activity should remain a priority in further studies.

Quantification of bacterial killing by macrophages *in vitro* by the method described here was found to be reproducible with no large within-group variation. However, interpretation of results was sometimes difficult due to the low percentage of bacteria usually killed. Opsonisation of bacterial cells with specific antibodies may usefully increase the percentage killed and should be explored.

7.1.2 Immunostimulation by glucans and peptidoglycan

All yeast (1→3),(1→6)-β-glucans and the bacterial peptidoglycan induced a dose dependent effect on macrophage intracellular superoxide anion generation when applied *in vitro* and significant differences were observed between the two types of substances (chapter 3). Peptidoglycan induced a maximum response over a wider range

of concentrations and this suggested that it might be more suited for in-feed administration due to differential feeding rates by different animals. Furthermore, production of hydrogen peroxide by macrophages was enhanced only by peptidoglycan.

Pharmacological studies *in vitro* are often carried out in order to screen different test substances and doses. They are also used to obtain data on the effects of the test substance on the specific immune target (examples, Jeney and Anderson, 1993b; Jørgensen, 1994). Results in this study showed that the stimulation of macrophage activity by *in vitro* treatment with glucan and peptidoglycan did not occur when the substances were administered in the feed. Resident macrophages isolated from the head kidney of fish fed on different doses of peptidoglycan and yeast glucan over various periods of time did not show any significant change in activity compared with fish fed on control diet (chapters 4 and 6). Neither was there any difference detected in the haematological parameters tested. Likewise, recovery of viable *A. salmonicida* cells from the spleen of challenged fish did not differ between peptidoglycan treated and untreated fish (chapters 4 and 6). Inflammatory peritoneal macrophages did, however, display an enhanced respiratory burst associated with the application of immunostimulants orally or intraperitoneally. A similar peritoneal macrophage response to the immunostimulant *Spirulina platensis* administered in-feed has been reported in channel catfish (Duncan and Klessius, 1996).

No reports have been published on the fate of dietary particulate (1→3),(1→6)-β-glucan or peptidoglycan. Furthermore, MDP (the immunologically active component of peptidoglycan) has been shown to be rapidly excreted into the urine of mice and not trapped in lymphoid organs (Ladesic *et al.*, 1993). Since head kidney macrophage activity was not enhanced by dietary stimulants, this would suggest that the tests substances did not accumulate in the head kidney and that the stimulatory activity of peptidoglycan on peritoneal cells may have been mediated by gut mucosal immune cells. Teleosts fish posses a diffuse but functionally distinct gut mucosal immune system (Zapata *et al.*,

1996; Press, 1998). GALT cells may respond to dietary peptidoglycan by secreting cytokines which could affect immune functions in other body compartments, for example the peritoneal cavity. Mice GALT lymphocytes have been shown to be activated following in-feed glucan administration (Zunic *et al.*, 1996) and peritoneal as well as alveolar macrophages showed an increased activity after oral glucan treatment of mice (Suzuki *et al.*, 1990; Sakurai *et al.*, 1992). In this case, screening test substances *in vitro* to study their direct effect on macrophage function may have limited value since they may not have access to sites where macrophages are found. Instead, assessment of the effect on macrophages of supernatants derived from GALT cells isolated from animals given immunostimulant in the feed or stimulated *in vitro* may yield more comprehensive data.

7.1.3 Immune response to stress

Rainbow trout produced an adaptive stress response to confinement indicated by plasma hyperglycaemia but with no loss of appetite and no morbidity or mortalities throughout the experiment. Modulation of the immune parameters investigated was dependent on repetition of confinement. Single confinement did not modify any of the immune functions measured. Furthermore, macrophage activity was not significantly modified by single confinement even when confined fish, or macrophages isolated from them, were challenged with LPS injection or stimulated with MAF plus LPS, respectively. Therefore, single confinement did not affect the ability of macrophages to increase their function in response.

The immune response to six daily confinements was different however and results from the two experiments assessing immune activity after repetitive confinement were similar (chapters 5 and 6). Although increased plasma glucose concentration was not significantly different from control animals, all head kidney macrophage functions were significantly affected. Phagocytosis, production of intracellular superoxide anion and, possibly as a result, killing of *A. salmonicida* by head kidney macrophages were all depressed while generation of extracellular superoxide was increased by repetitive

confinement. Superoxide anion production by inflammatory peritoneal macrophages showed a similar response. As discussed by Ottaviani and Franceshi (1996) and Dantzer (1997), these findings indicate that the stress response does not necessarily imply immunosuppression. Unregulated activation of some functions such as superoxide anion production may result in damage to self (Gille and Sigler, 1995) and may contribute to disease associated with the stress response and opportunistic pathogens. Some studies in fish have also shown variable modulation of different immune functions after stressful events (example Thompson *et al.*, 1993), although no differential induction of extra- and intra-cellular superoxide anion generation during stress have been reported.

7.1.4 Immune response to dietary peptidoglycan and confinement

Peptidoglycan administered orally was able to prevent some of the effects of repeated confinement on macrophage activity. Peptidoglycan significantly reduced some of the extracellular superoxide generation by head kidney macrophages from these confined fish. However, extracellular superoxide formation in the group fed peptidoglycan and exposed to repeated confinement was still considerably (but not significantly) higher than that of cells isolated from non-confined trout and there would still have been the potential for damage to self in these animals. The immunostimulant did not, however, lead to modulation of the confinement-associated suppressive effects on head kidney macrophage phagocytosis, namely production of intracellular superoxide and killing of A. *salmonicida in* vitro. It is, therefore, doubtful if peptidoglycan in-feed had any significant effect on restoring the bactericidal capacity of kidney macrophages from repeatedly confined fish.

The response of inflammatory macrophages to dietary peptidoglycan was different to that of kidney macrophages since the stimulant reversed the suppressive effects of repeated confinement on intracellular superoxide production. Extracellular superoxide production remained significantly higher in inflammatory macrophages from confined animals fed on the immunostimulant.

7.1.5 Clearance of *Aeromonas salmonicida in vivo*

The competence of an integrated immune response after in-feed immunostimulant treatment and/or repeated confinement was evaluated through the persistence of viable *A. salmonicida* in the spleen and blood of fish up to 96 h after ip injection. Challenge experiments where mortalities of infected fish are recorded over a defined period of time have certain advantages in that more natural routes of infection may be studied. However, results are expressed as mortality or survival indexes and do not provide information on morbidity amongst survivors. More accurate and precise information on the development of infection may be obtained by quantifying viable bacteria present in target organs, tissues or blood. As natural *A. salmonicida* infections develop, there is septicaemia and bacterial colonisation of visceral organs, muscle and gills (Munro and Hastings, 1993). The spleen was chosen as target organ to quantify viable *A. salmonicida* CFU because of its haemopoietic nature and the ease with which it can be dissected.

Even though *A. salmonicida* infection is associated with stressful conditions (Munro and Hastings, 1993) and the treatments applied in this study affected the activity of important bactericidal macrophage functions, neither confinement nor in-feed peptidoglycan resulted in higher or lower bacterial recovery from the spleen.

In the experiments described in chapters 4 and 5, numbers of viable *A. salmonicida* in the spleen increased with time after challenge, whereas in chapter 6, numbers dramatically decreased after challenge. No serum antibodies specific for *A. salmonicida* were found in the sera of non-challenged fish. Furthermore, no viable cells of injected *A. salmonicida* strain B95179, *A. hydrophila* NCIMB 1134 or *V. anguillarum* NCIMB 6 were recovered from spleens or blood of the stock fish used for the experiments in chapter 6. These results suggest that the fish used in chapter 6 were less susceptible to *A. salmonicida* persistence. Whether this was due to genotypic or phenotypic differences between the fish stocks remains unresolved.

In all the experiments, there was large variation in the recovery of *A. salmonicida* from the spleen (within groups), with no viable bacteria recovered from some of the fish. In spite of this variation, the mean CFU recovered per gram of spleen was very similar between the different treatments. The large variation observed may explain some difficulties in standardising and reproducing infectious challenges where only mortalities are recorded (Nordmo, 1995).

It is important to note that the spleen surface was not sterilised before counting *A. salmonicida* CFU in the organ and, therefore, viable cells present on the spleen surface may have been counted. An improvement in this technique would be to sterilise the spleen surface with 70% ethanol after dissection and this is recommended for future work.

7.1.6 Comparative microbiocidal activity of kidney and peritoneal macrophages

Macrophages present in haemopoietic organs, such as kidney, play an important role in the clearance of blood-borne antigens (Zapata *et al.*, 1996), while those migrating towards inflammatory stimuli are an important part of the defence against infectious agents in localised foci and, possibly, in the first stages of infection being established. Inflammatory macrophages can be obtained by injection of different inflammatory agents into the peritoneal cavity and the magnitude of their activity depends on the substance injected (Rowley, 1990; Secombes, 1990).

Casein-elicited peritoneal macrophages consistently produced less superoxide anion than resident head kidney macrophages, although, taking all results together, they were more responsive to confinement and immunostimulation. Furthermore, in response to an *A. salmonicida* ip challenge, peritoneal macrophages showed a significantly higher superoxide production than renal macrophages, indicating that bacterial presence triggers a greater microbiocidal activity in inflammatory macrophages (results not included).

Inducible NOS has been identified in rainbow trout head kidney cells (Grabowski *et al.*, 1996) and nitric oxide production by kidney mixed leukocyte cultures has been detected (Zunic and Licek, 1997). In this study, kidney macrophages did not produce detectable amounts of nitric oxide following any of the treatments applied (immunostimulants *in vitro* and *in vivo* and confinement). The findings of Zunic and Licek (1997) suggest that trout macrophages may need cellular interactions to switch on production of NO. Further work would be pursued to confirm the results reported by Zunic and Licek (1997) and identify the cells producing NO. Also, investigation of the cellular/soluble requirements as well as immune status *in vivo* necessary to trigger NO synthesis are necessary to understand better the microbiocidal mechanisms of rainbow trout phagocytes.

7.2 Summary of main conclusions

The main conclusions of this thesis are as follows:

i. Bacterial peptidoglycan *in vitro* induced a maximum kidney macrophage respiratory burst response over a wider range of concentrations than the yeast glucans. Although this observation was not found in the in-feed treatment experiments in this study, the use of peptidoglycan *in vivo* may have important advantages since feed uptake, and therefore that of associated treatment, varies considerably amongst individual fish in a farmed population.

ii. Dietary peptidoglycan elicited a significant activation of inflammatory macrophages but did not affect the activity of resident macrophages in the head kidney. Bacterial killing by inflammatory phagocytes may therefore be enhanced in natural infections through oral treatment with peptidoglycan. However, since the activity of kidney macrophages was not affected, killing of pathogens once established in haemopoietic

organs may not necessarily be enhanced by peptidoglycan, unless they are then recognised as an inflammatory focus. Therefore peptidoglycan may be beneficial in the prophylaxis of infectious diseases or treatment early in the infection.

iii. The stress response is not necessarily followed by changes in macrophage and complement activities. Single confinement produced a physiological stress response which was not reflected in disturbance of macrophage function even when confronted to an immunological challenge. However, repeatedly confined animals showed disturbed macrophage function, both in the head kidney and peritoneal cavity, with possible implications for pathogen killing and damage to self.

iv. Dietary peptidoglycan limited some of the effects of confinement on macrophage function and this was more evident in cells isolated from the peritoneal cavity. Taken together, these results and those reported by Jeney *et al.* (1997) suggest that dietary immunostimulants may prove beneficial in increasing resistance of fish to bacterial pathogens after husbandry-related stressful events. Further work should be carried out to develop this hypothesis (see section 7.3).

v. Even though confinement and dietary peptidoglycan treatments did induce changes in certain macrophage activities, the integrated innate immune response, as reflected by persistence of *A. salmonicida* in spleen and blood, was not affected by experimental conditions. There may have been several explanations for this observation. The macrophage activity observed following treatments may not have altered the effect on *A. salmonicida* viability. In addition, lytic complement activity, which was not affected by any treatment, or other parameters which were not measured may have a significant role in the control of *A. salmonicida* persistence.

7.3 Recommended future work

Before oral immunostimulation can be confidently recommended for use in aquaculture, field studies on the effect of dosing and timing regimes should be undertaken. These studies could also be paired with assessment of immune parameters which are readily quantifiable on-farm, such as whole blood respiratory burst activity (as described by Siwicki and Anderson, 1993), on a large number of fish.

There are no reports on the absorbance of particulate glucans and peptidoglycan from the digestive tract and their subsequent fate in fish. Such information is important to understand the mechanism of immunostimulation, since it may be mediated directly by the given substance and/or by immunostimulant-responsive GALT. Cytokine research is in its early stages in fish (Secombes, 1996) and little is known about teleost gut mucosal immunity (Press, 1998). Further knowledge of these aspects of immunity would be advantageous not only in elucidating the mechanisms behind oral immunostimulation, but also in developing oral vaccines against important fish pathogens.

Development of a bacterial challenge more responsive to experimental treatments would offer advantages when interpreting data regarding the immune parameters investigated. In this study, several bacterial species pathogenic to rainbow trout were used for this purpose, but only *A. salmonicida* was recovered from injected fish 24 h after challenge. This highlights the complex nature of the interaction between animal and pathogen in the development of infection. However, infectious challenges are a fundamental tool for the investigation of prophylactic treatments. Much more effort is needed to develop a series of reproducible challenges by different routes, including bath and cohabitation, and able to give different levels of infection, so that the degree of protection or susceptibility given by the experimental treatment can be assessed.

Studies involving assessment of cellular innate defence mechanisms in fish and other animals are usually carried out by isolating a particular cellular population and

assaying for their activity at a given period of time after treatment (e.g., immunostimulants). This implies that the treatment, if successful, induces activation of the parameter studied. However, it might well be that the treatment, rather than activation, induces priming of defence mechanisms and, therefore, no increase in activity would be observed. An alternative study may be the application of an immune challenge, such as an infection or LPS injection, after the experimental treatment has been completed and before the cells of interest are isolated. In this case, the immune challenge may activate primed immune cells for higher activity, and the performance of such cells when confronting a challenge would be investigated. This may also apply to the study of the immune response to stress, where the basal activity of immune cells may not necessarily be affected during the stress response, but their capacity to respond to an immune challenge could.

It is well known that there are major differences in the susceptibility of different fish species to individual bacterial pathogens and also to the damaging effects of stress. Tilapia, carp and, to a lesser extent, rainbow trout and catfish are recognised as very resistant in this respect. Work now needs to be targeted on the more vulnerable species such as Atlantic salmon. However, their sensitivity to environmental changes makes them difficult to work with and is likely to result in even greater within-group variation than has been observed in this study. The present work provides a basis on which build. A method similar to that developed here should be applied to evaluate the use of immunostimulants in protecting such fish from the effects of husbandry related stressors.

Appendices

Appendix 1: *Aeromonas salmonicida* CFU per gram of spleen of fish treated with peptidoglycan in feed and with control diet at different times after ip challenge.

PG 0.05%

Fish	6 h	12 h	24 h	48 h	72 h	96 h
1	58114	-	29556	-	-	210472
2	-	-	-	44100	1005598	-
3	175	-	11512	-	5900	626000
4	-	18143	23479	32109	5912	-
5	67234	64875	-	-	-	-
6	-	-	-	26321	-	2416474

Control

Fish	6 h	12 h	24 h	48 h	72 h	96 h
1	-	-	-	54000	-	750148
2	-	32198	23387	-	-	-
3	76524	-	45198	76117	-	-
4	-	-	-	-	820298	419834
5	54812	15775	41043	10187	3100	34887
6	-	-	-	-	-	649698

- indicates no viable bacteria recovered from the sample.

Appendix 2: *Aeromonas salmonicida* CFU in spleen of fish repeatedly confined for nine consecutive days and in controls at different times after ip challenge.

Stress

Fish	6 h	12 h	24 h	48 h	72 h
1	-	651	-	-	100235
2	1052	-	563	10564	502162
3	-	895	435	4521	-
4	-	-	-	3521	-
5	-	-	-	-	25648
6	132	320	1520	-	-
7	150	-	350	-	65498
8	-	312	-	3698	-

Control

Fish	6 h	12 h	24 h	48 h	72 h
1	351	-	5216	-	639874
2	-	465	2654	-	-
3	-	523	-	14562	125469
4	426	-	-	8564	569213
5	-	146	-	5319	459867
6	123	-	1260	10256	3264
7	-	351	-	-	-
8	236	456	995	9561	-

- indicates no viable bacteria recovered from the sample.

Appendix 3: *Aeromonas salmonicida* CFU in spleen of fish treated with peptidoglycan and repeatedly confined, treated with peptidoglycan only, repetitively confined only and in controls at different times after ip challenge.

PG 0.05 % plus confinement

Fish	5 h	12 h	24 h	48 h	72 h
1	15673	222436	320513	7143	2143
2	568902	704545	7576	16837	325
3	394737	214189	11486	5804	-
4	6336957	198333	5556	3017	3017
5	895455	108333	99074	14493	-
6	735714	163793	-	14024	-
7	1163043	192708	74479	14375	256
8	325397	145513	40385	6098	-

Confinement

Fish	5 h	12 h	24 h	48 h	72 h
1	804795	103788	7576	-	25014
2	1644026	197023	63012	-	-
3	1418787	70192	28846	22561	-
4	170495	379375	12500	2431	-
5	1215165	625410	117500	8500	3123
6	1250000	189423	50962	13514	-
7	155405	271667	183333	4730	-
8	702381	60714	91667	27500	7512

PG 0.05 %

Fish	5 h	12 h	24 h	48 h	72 h
1	640816	113889	-	-	4464
2	594286	119231	237500	31625	10938
3	973529	105882	30263	-	-
4	1068519	332000	18548	54687	-

Control

Fish	5 h	12 h	24 h	48 h	72 h
1	664572	252679	38393	-	-
2	1514317	74074	173148	4868	-
3	1287879	250000	-	n.d.	24038
4	526952	35463	37037	n.d.	-

- indicates no viable bacteria recovered from the sample. n.d. indicates not determined.

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