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Modulation of the health status of ornamental fish by stress and dietary immuno-stimulants

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University of Plymouth

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Modulation of the health status of ornamental fish by stress and dietary immuno-stimulants

by

Stuart Maudling

A thesis submitted to the University of Plymouth
in partial fulfilment for the degree of

DOCTOR OF PHILOSOPHY

(October 2006)

Department of Biological Sciences
Faculty of Science
in partnership with the Waltham Centre for Pet Nutrition

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Modulation of the health status of ornamental fish by stress and dietary immuno-stimulants

Stuart Maudling

ABSTRACT

Three trials were conducted to investigate the effects of dietary immuno-stimulant supplementation on various physiological, haematological and immunological parameters of ornamental fish following stress and vaccination.

The first series of experiments investigated the mucosal stress response of mirror carp, (Cyprinus carpio), using urinalysis strips and established stress indicators within peripheral blood. A netting stressor, which combined aerial exposure and confinement, of 6 minutes duration was applied and the response of fish was measured 1.5, 3, 5, 24 and 168 hours post-stress. Mucosal haemoglobin and protein were established as consistent indicators of stress and strong relationships could be drawn between them and plasma glucose concentration and peripheral monocytes and lymphocytes counts, firmly establishing them as secondary stress indicators. Visual assessment of the strips was found to overestimate mucosal parameters compared to results obtained using instrument based analysis, but the underlying changes were similar. Further experiments conducted on goldfish, (Carassius auratus), and tilapia, (Oreochromis niloticus x. O. mossambicus), showed that the mucosal response to stress was conserved between fish species.

The second trial investigated the potential of dietary immuno-stimulants to modify the immune status of fish and their response to stress. Fish were fed either a control, beta-glucan (0.2 % w/w), nucleotide (0.2 % w/w) or combined supplement (0.2 % beta-glucan + 0.2 % nucleotide) diet for 3 weeks at 1 % BW.d^{-1} and then to the same stressors as used in Trial 1. Significant stress-induced changes in mucosal protein (p = 0.006), plasma glucose (p = <0.004) and protein concentration (p = <0.003), peripheral lymphocytes (p = 0.001) and monocytes (p = <0.001) and blood NBT reduction (p = <0.001) were observed. Fish fed diets containing immuno-stimulants showed a quicker recovery from the stress-induced hyperglycaemic response than those fed the control diet. Fish that received the beta-glucan diets showed lower blood NBT reduction immediately post-stress than all other diets.

The final trial investigated the effects of the control, beta-glucan and combined-supplement diet on the response of fish to vaccination. Fish were fed the diets for 2 weeks prior to and 1 week following IP vaccination against Aeromonas salmonicida at 1.0 % BW.d^{-1}. The immune response was measured every 2 weeks for 12 weeks following vaccination. Vaccination induced a significant stress response as seen by a large hyperglycaemic response (p = <0.05). Significant reductions in peripheral monocyte count were seen following vaccination (p = <0.04). All groups showed elevated NBT reduction at 4 weeks post-vaccination. Serum lysozyme activity showed evidence of long term immuno-suppression following vaccination. No clear effect of the immuno-stimulating diets could be found on any of the physiological or immune parameters tested.

The trials conducted found that ornamental fish have a well developed mucosal stress response and this can be used to make inferences about the stress state of fish. The effects of beta-glucans and nucleotides on either the response to stress and immune status were subtle. The nucleotide supplement appeared most effective at reducing the effects of stress, whilst beta-glucans may exert more influence on the immune parameters tested. However, no evidence of synergy between the two types of immuno-stimulant was found in fish fed both immuno-stimulants together.
All experimental work involving animals was carried out under the Home Office project licence #30/2135 and personal licence #30/6797
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Author’s Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other University award.

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A programme of advanced study was undertaken which included courses in fish immunology and nutrition.

A number of scientific conferences were attended at which some of the work was presented.

List of conference contributions


Signed ........................................
Date ........................................
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Dedication

I would like to dedicate this thesis to my mother, Brenda and brother, Robert for all the support and encouragement offered during the completion of the thesis, and in loving memory of my father, Derek.
Abbreviation List

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<tr>
<td>ACH&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Alternative Complement Activity Units</td>
</tr>
<tr>
<td>ACP</td>
<td>Alternative Complement Pathway</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic Hormone</td>
</tr>
<tr>
<td>AIS</td>
<td>Adaptive Immune System</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Monophosphate</td>
</tr>
<tr>
<td>AmPS</td>
<td>Antimicrobial peptides</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic Nervous System</td>
</tr>
<tr>
<td>B</td>
<td>β-glucan supplement diet</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BW.d&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>Body weight per day</td>
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<td>Classical Complement Pathway</td>
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<td>Immunoglobulin D</td>
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<td>IgE</td>
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<td>IgG</td>
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<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>IIS</td>
<td>Innate Immune System</td>
</tr>
<tr>
<td>IL-1</td>
<td>Interleukin 1</td>
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<tr>
<td>IL-2</td>
<td>Interleukin 2</td>
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<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IPN virus</td>
<td>Infection Pancreatic Necrosis virus</td>
</tr>
<tr>
<td>IPT</td>
<td>Immunologically Permissible Temperature</td>
</tr>
<tr>
<td>IQ</td>
<td>Inter-quartile</td>
</tr>
<tr>
<td>ISA virus</td>
<td>Infectious Salmonid Anaemia virus</td>
</tr>
<tr>
<td>KT</td>
<td>Kendall’s Tau</td>
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<tr>
<td>LCP</td>
<td>Lectin Complement Pathway</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>M</td>
<td>Machine</td>
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<tr>
<td>Mab</td>
<td>Monoclonal Antibody</td>
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<tr>
<td>MAC</td>
<td>Membrane Attack Complex</td>
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<tr>
<td>MBL</td>
<td>Mannan Binding Lectin</td>
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<tr>
<td>MDP</td>
<td>Muramyl Dipeptide</td>
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<td>MS222</td>
<td>Tricane methyl sulphonate</td>
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<td>N</td>
<td>Nucleotide supplement diet</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>NBT</td>
<td>Nitro-blue Tetrazolium</td>
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<tr>
<td>NCC</td>
<td>Naturally Cytotoxic Cell</td>
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<tr>
<td>NSP</td>
<td>Non-Starch Polysaccharide</td>
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<tr>
<td>O</td>
<td>Oral</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>RM</td>
<td>Residual mucus</td>
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<tr>
<td>ROS</td>
<td>Reactive Oxygen Species</td>
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<tr>
<td>SD</td>
<td>Standard Deviation</td>
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<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SG</td>
<td>Specific Gravity</td>
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<tr>
<td>S Ig-</td>
<td>Specific Immunoglobulin negative</td>
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<tr>
<td>S Ig+</td>
<td>Specific Immunoglobulin positive</td>
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<tr>
<td>SM</td>
<td>Salivette® mucus</td>
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<tr>
<td>SNS</td>
<td>Sympathetic Nervous System</td>
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<tr>
<td>SRBCs</td>
<td>Sheep Red Blood Cells</td>
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<tr>
<td>(TMP)</td>
<td>Thyamine Monophosphate</td>
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<tr>
<td>TNF-α</td>
<td>Tissue Necrotic Factor -α</td>
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<tr>
<td>TSA</td>
<td>Tryptic Soya Agar</td>
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<td>UMP</td>
<td>Uracil Monophosphate</td>
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<td>v/v</td>
<td>Volume per Volume</td>
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Chapter 1. A review of the current literature on the effects of stress, β-glucans and nucleotides on the immune system of fish and other vertebrates.
1.1 Ornamental fish and the challenges of the captive environment

An ornamental fish is broadly defined as "any fish that is kept for any purpose other than providing a source of food" (Davenport, 2001). This definition of ornamental fish is flexible and shows considerable variation between countries; some popular ornamental species in the United Kingdom are food fish in other countries. However, for the purposes of this thesis, the above term serves adequately and the two primary experimental species are typical ornamental fish.

Research by the Ornamental Aquatic Trade Association (OATA) (2004) found that ornamental fish rate are the third most popular class of household pet in the UK, behind cats and dogs, with around 14% of the population owning fish. It is estimated that this represents some 144 million ornamental fish and Davenport (2001) calculated that in 1999, the UK ornamental fish trade was worth around £12 million.

The majority of ornamental fish in the UK originate from temperate freshwater, tropical freshwater or tropical marine habitats, with temperate freshwater fish comprising 99% of the total ornamental fish imported annually by the UK. Of these approximately half are pond fish such as the goldfish (*Carassius auratus*) and Koi carp, (*Cyprinus carpio*) (Davenport, 2001) making these two species of great importance to the ornamental trade both in the UK and in the countries involved in their breeding and supply.

All fish experience physiological stress in some form; however fish held in captivity often experience a higher degree of stress than wild fish due to the nature of the captive environment and an inability to escape from stress. The environments experienced by ornamental fish are particularly conducive to physiological stress; small water volumes are prone to large fluctuations in temperature and water quality. Numerous biotic stressors are present for ornamental fish including overcrowding, social
hierarchies, predation, an unsuitable environment and pathogenic organisms. Other stressors are unique to captive environments, such as netting, handling, transportation, anaesthetisation and certain disease treatments, which are the result of techniques essential to the maintenance of the captive environment in which the fish live.

Although short term exposure to stress is generally harmless, prolonged exposure to stress can cause numerous detrimental effects (Pruett, 2001, Moynihan, 2003). One of the most important to ornamental fish is the risk of immuno-suppression from long term exposure to stress. This is particularly detrimental to coldwater species of fish, due to the highly temperature dependent nature of the Adaptive Immune System (AIS).

Because of these factors it is imperative to consider ways of reducing stress and enhancing immune function in ornamental fish to prevent disease outbreaks, which aside from causing mortalities, can be detrimental to the aesthetic value of the fish.

### 1.2 The vertebrate immune system

The immune system is designed to recognise antigens present on a wide range of pathogenic organisms, from viruses to metazoan parasites. A response is generated by the presence of these antigens, at the cellular and biochemical level. The Adaptive Immune System (AIS) generates specific antibody and the response is tailored to a particular pathogen, whilst the Innate Immune System (IIS) utilises a broad spectrum of defences to combat a range of pathogens. There is considerable overlap and synergy between the two wings of the immune system, despite functioning in distinct ways to destroy pathogens (Dalmo et al., 1997, Press and Evensen, 1999, Sakai, 1999). Generally, the IIS is more active against opportunistic pathogens, with the AIS tailored towards more specialised, obligate pathogens (Robertson et al., 1994).
The immune system of vertebrates shows widespread similarities in form and function from fish to mammals. Cellular defences, such as macrophages, have similar functions as do many of the extra-cellular humoral immune factors. Recent studies have highlighted the similarities in the chemical control of the immune system throughout the vertebrates (Press, 1998). However, despite these similarities the immune system of teleost fish is fundamentally simpler due to its ancestral nature (Dalmo et al., 1997, Sakai, 1999).

1.2.1 The Adaptive Immune System (AIS)

All vertebrates possess some form of AIS that responds to specific antigens through the production of specific immunoglobulin (Ig) and which possesses a degree of immune memory. The mammalian AIS shows a high level of specialisation, with five Ig classes performing distinct roles within an immune response. The duration of protection from these specific antibodies often lasts years. In comparison, teleost AIS is poorly developed with only a single class of Ig and protection is short-lived (Smith et al., 2000). The ancestral nature of the teleost immune system becomes more apparent at the organ level. The immune organs of fish are more diffuse than those of higher vertebrates; in addition, fish lack both bone marrow and lymph nodes, which are characteristic features of the immune systems of higher vertebrates. In fish, primary lymphoid tissues are the thymus, kidney (anterior and body regions), spleen and gut associated lymphoid tissues (GALT) (Press, 1998). The function of these organs has been retained by mammals, with the exception of the kidney.

Evidence for a distinct lymphatic system in fish is contradictory; Vogel and Claviez (1981) and Steffensen and Lomholt (1992) suggest that what was previously considered a lymphatic system may in fact be an erythrocyte deficient secondary circulatory system; a feature consistent with the lack of lymph nodes in fish.
Cells of the adaptive immune system

The AIS utilises two cell types to generate a response to specific antigen, the B and T lymphocytes. B-lymphocytes produce and express specific Ig on their cell surface membrane (CSM); these are designated slg+ cells. T-lymphocytes come in a variety of forms but all lack surface Ig, earning them the designation of slg- cells.

T-lymphocytes respond to antigen presented by macrophages and once stimulated, they induce B-lymphocytes to produce specific antibody against the presented antigen. In general, B-lymphocytes are dependent on presented antigen and cytokines produced by T-lymphocytes to initiate antibody production, however there are some T-lymphocyte independent antigens which can directly initiate a B-lymphocyte response without the assistance of T lymphocytes (Mond et al., 1995).

Mammals possess two classes of helper T lymphocytes, which are identified by their cytokine profile; T\(_{H1}\) cells secrete cytokines associated with the promotion of cellular immunity and T\(_{H2}\) cells produce cytokines associated with antibody production. The cytokines produced by each subclass of T lymphocyte inhibits the activity and production of the other class, allowing the immune system to tailor its response to the particular pathogen present without wasting resources on proliferation of ineffective immune defences.

Both B and T lymphocytes have been isolated from fish. B lymphocytes have been conclusively identified by the presence of surface antibody (slg+). Surface antibody negative cells (slg-) have also been isolated and these have been classified as T lymphocytes, and appear functionally identical to the T lymphocytes of higher vertebrates, as indicated by a dependency of cell mediated immunity on these cells (Press, 1998).
Immunoglobulin (Ig)

Although mammals possess five classes of Ig (IgA, IgD, IgE, IgG and IgM), fish rely primarily on a single class, IgM, although other classes may be present. IgM represents the ancestral Ig molecule and teleost IgM is similar to, but distinct from, mammalian IgM. In fish its structure is tetrameric, comprising only four Ig molecules, as opposed to the pentameric arrangement found in mammals.

In common with all immune parameters, serum Ig titres show considerable intra and inter-specific variation. Values reported for unchallenged fish range from between 0.2 and 0.8 mg.ml\(^{-1}\) \((\text{Oreochromis niloticus})\) \((\text{Dominguez et al., 2004})\), 22 - 42 mg.ml\(^{-1}\) \((\text{Rutilus rutilus})\) \((\text{Aaltonen et al., 1994})\), 0.1 – 5.3 mg.ml\(^{-1}\) \((\text{Clupea pallasii})\) \((\text{Davies et al., 1999})\) and ~ 19 mg.ml\(^{-1}\) \((\text{Ictalurus punctatus})\) \((\text{Hrubec and Smith, 1999})\). A study by Shelby et al., (2002) highlights the variability in antibody titre; Atlantic menhaden \((\text{Brevoortia tyrannus})\) show mean Ig concentrations of 7.4 mg.ml\(^{-1}\), but values range from 0.3 to 23.5 mg.ml\(^{-1}\), highlighting intra-specific variation of this parameter.

Research conducted by Shariff et al., (2001) illustrated the effects of pathogen challenge on antibody titres; unchallenged Javanese carp \((\text{Barbonymus gonionotus})\) showed Ig concentrations of around 16 mg.ml\(^{-1}\), following pathogen challenge, this rose to around 21-24 mg.ml\(^{-1}\). Similar increases have been found in salmon \((\text{S. salar})\) by Melingen and Wergeland (2000), where vaccinated fish showed approximately double the concentration of unchallenged fish.

Ig production is influenced by a range of environmental factors including size, age, pH and temperature, with temperature being particularly important in determining the antibody response of a fish \((\text{Shelby et al., 2002, Dominguez et al., 2004})\). Whilst Ig production increases proportionally with temperature \((\text{Dominguez et al., 2004})\), at low temperatures production of specific Ig by the AIS ceases. Bly and Clem (1992) and Tatner (1996) define the threshold temperature at which this occurs is termed the Immunologically Permissible Temperature (IPT). For the majority of temperate
ornamental fish, including goldfish (*C. auratus*) and carp (*C. carpio*), the IPT is often at or only slightly above the mean winter temperature. Fluctuations in temperature over the winter can leave fish without the ability to produce specific Ig for extended periods. Even when temperatures exceed the IPT, the AIS can be slow to respond to pathogens or vaccines, with specific Ig production often taking four to six weeks or more (Ellis, 2001). In contrast, many pathogens are capable of reaching lethal numbers in a few days at the same water temperatures, leaving even healthy fish at severe risk from pathogens (Ellis, 2001).

### 1.2.2 The Innate Immune System (IIS)

The IIS differs from the adaptive immune system in several key areas. The immune response generated occurs in response to target molecules, including lipopolysaccharide (LPS) and β-glucans, which are present on a range of pathogenic organisms, so is not tailored to a specific pathogen. Rather, a suite of immune components are activated that are general in action, allowing the subsequent response to be rapid, with responses taking at most a few days. Activation of the IIS shows a greater thermal independence than the AIS, an important factor for poikilothermic organisms; as shown by the greater reliance on the IIS by fish in temperate regions compared to those in the tropics (Avtalion, 1999, Ellis, 2001). Several studies have shown the importance of the IIS to embryonic and larval fish, prior to the development of the AIS, which can take several months (Ellis, 2001, Magnadottir, 2006, Zapata *et al.*, 2006).

The purpose of the IIS is to immediately tackle pathogens resulting in their destruction or limiting their spread, allowing the AIS to respond and eliminate the pathogen fully, if necessary.
Cellular defences

The IIS of fish relies on a lower diversity of cellular defences than that of mammals, reflecting its ancestral status (Press, 1998), although the cell types present are largely consistent with their mammalian analogues.

Granulocytes

Dalmo et al., (1997) report that many fish species, including carp (*Cyprinus carpio*), reportedly possess three classes of granulocyte: neutrophils, eosinophils and basophils, although others, such as channel catfish (*Ictalurus punctatus*), possess only neutrophils (Ellsaesser and Clem, 1986).

Neutrophils are the most clearly defined of the three classes and are present in the majority of teleosts (Ferguson, 1976, Hine, 1992). Neutrophils are present in the circulation in low numbers; the majority remain attached to the vascular walls in a dormant state, only becoming activated in the presence of cytokines or specific activation molecules such as β-glucans or other bacterial structural molecules (Styrt, 1989, Castro et al., 1999). Upon activation neutrophils detach and enter the circulation, migrating to the source of infection where they exit the circulation and enter the tissues (Roberts, 1989, Suzuki and Iida, 1992).

Activated neutrophils can undergo phagocytosis and respiratory burst in a similar fashion to activated macrophages. The Reactive Oxygen Species (ROS) produced by activated neutrophils are primarily used to destroy phagocytosed pathogens but are commonly released into the extra-cellular environment (Dahlgren and Karlsson, 1999, Hampton and Winterbourn, 1999). This makes the neutrophil a potent immune cell, so they only remain activated for short periods, before undergoing apoptosis to limit damage from oxidative stress on host tissues.
Macrophages

Macrophages, although classified as cells of the IIS, fulfil additional immune roles as part of the AIS. Macrophages are found throughout the body, with the majority being located in the head kidney and other immune tissues, including the intestinal mucosa and GALT of the hindgut where they conduct immune surveillance and control the subsequent immune responses (Rombout et al., 1986a, Temkin and McMillan, 1986, Rombout and Van den Berg, 1989, Rombout et al., 1989b). Wandering phagocytes have also been isolated from fish mucus (Ourth, 1980, St-Louis Cormier et al., 1984, Wood et al., 1988). Macrophages are generally uncommon in or absent from the circulation; instead macrophage precursor cells, monocytes are present. Ellis (1986) found that monocyte activation and migration into tissues from the circulation occurs in response to cytokines in a similar fashion to that seen in neutrophils.

Activation of macrophages occurs when receptors on the CSM are stimulated by factors produced by pathogens or other macrophages; factors include cytokines, lipopolysaccharide (LPS), peptides, proteins, mannose and β-glucans (Seljelid and Eskeland, 1993, Press, 1998). Once activated, macrophages undergo respiratory burst and produce lysosomal enzymes, chemokines and cytokines (Karnovsky and Lazdins, 1978, Karnovsky et al., 1982, Adams and Hamilton, 1984, Gorden et al., 1992). These cytokines and chemokines help modulate the immune response of the IIS.

Macrophage phagocytosis was studied by Seljelid and Eskeland (1993) and they observed that the range of phagocytosed particles is 1-10 μm with the completion of phagocytosis requiring attachment of the particle to the phagocyte, ingestion through the formation of a phagosome and breakdown within the phagosome. Following ingestion, products of respiratory burst activity are employed to break the particle down. These include the production of superoxide (O₂⁻), hydrogen peroxide (H₂O₂) and singlet oxygen (O¹) which react to create highly active hydroxyl radicals; although nitrogen based radicals are also produced (Karnovsky, 1962, Hibbs, 1992). In addition to radicals,
macrophages employ various enzymes, including lysozyme, to destroy pathogens. The oxygen independent mechanisms employed by macrophages have received comparatively little study, although it is known that several enzymes are involved, of which lysozyme is thought to be one. Although macrophages typically digest pathogens internally, the contents of the phagosome can be released into the extracellular environment (Grinde, 1989, Jaeschke, 1995). Macrophages have a role to play in the response of the AIS to pathogens through the processing and expression of ingested antigen on their CSM, allowing detection by T-lymphocytes (Balm, 1997). This antigen presentation forms an important part of the development of a specific response to a pathogen.

Non-specific cytotoxic cells (NCCs)

These are agranular leukocytes that do not require prior activation by cytokines to initiate their cytotoxic activity. There is evidence to suggest that NCCs are involved in the defence against viral and parasitic infection; NCCs are active against protozoan parasites including white-spot (*Ichthyophthirius multifilis*). The effect of a number of parameters on NCCs was studied by Kiron et al., (1993) and diet, especially the lipid component, was found to have a strong effect in addition to the usual parameters of stress, age and temperature.

Humoral defences

This section of the IIS comprises immune molecules which are produced by various immune cells and secreted into the blood or other bodily fluids such as mucus. The humoral defences of the teleost IIS generally show greater activity than those found in mammals, highlighting the reliance on the IIS in general and humoral defences in particular.
The complement system

The complement system comprises an enzyme cascade chain that acts to destroy invading organisms through the production of membrane attack complex (MAC). The complement system of teleosts comprises at least 35 plasma proteins and is initiated by three pathways; the alternative (ACP), classical (CCP) and lectin (LCP) pathways (Boshra et al., 2006).

The CCP and LCP are fundamentally very similar; however the CCP is antibody dependent, whereas the lectin pathway is antibody independent. This is the main difference between the CCP and LCP and the ACP; the initiator molecule of the ACP, protein C3, is produced spontaneously in the blood, whilst the other two pathways require activation by molecular complexes to proceed. These are immunoglobulin in the case of the CCP and mannan-binding lectin (MBL) and ficolins, which are present on the CSM of bacteria, in the case of the LCP (Holmskov et al., 1994, Boshra et al., 2006).

Boshra et al., (2006) report that the ACP represents an ancient immune defence and is highly active at low temperatures, making it of great importance to teleost fish. This is highlighted by ACP titres that far exceed those found of the mammalian ACP and teleost complement is able to lyse a far wider range of target cells (Yano et al., 1985, Lobb and Hayman, 1989, Boshra et al., 2006). This enhanced activity has been attributed by Boshra et al., (2006) to the presence of a wide range of isotypes of several key complement components.

Complement proteins are associated with a variety of immune processes and can bind to the CSM of intact foreign cells and the bound protein is recognised by all immune cells capable of phagocytosis (neutrophils, macrophages and monocytes) which then phagocytose the cell. Complement proteins are also involved in inflammatory processes, solubilisation of immune complexes, uptake and presentation of antigen by macrophages and can increase the ability of B-lymphocytes to respond to antigens (Boshra et al., 2006).
The direct lysing activity of the ACP is directed mainly against a-virulent Gram-negative strains of bacteria (Iida and Wakabayashi, 1983, Sakai, 1983, Iida and Wakabayashi, 1993). Studies have found that Gram-positive bacteria and virulent Gram-negative strains can suppress the ACP through the expression of molecules, for example sialic acid, on the cell membrane (Munn et al., 1982, Ourth and Bachinski, 1987, Jenkins et al., 1991). Though the ACP shows limited activity against such bacteria, lysozyme can attack the cells walls of Gram-positive bacteria, making them susceptible to attack by the MAC. A study conducted by Miles et al. (2001) suggests that complement may be inhibitory to fungal cell growth and the ACP has been found to be active against a variety of metazoan parasites, including gyrodactylids and ciliates (Bakke et al., 1998, Buchmann, 1998, Sigh et al., 2004). Studies on the epidermal mucus of fish have found that complement is active in the mucosal surfaces, in addition to its presence in the serum (Sakai, 1992, Shepard, 1994).

**Lysozyme**

This enzyme is common throughout the vertebrate taxa and is active against a wide range of micro-organisms. Lysozyme cleaves the β-1,4 linkages between N-acetylmuramic acid and N-acetylgalactosamine in the peptidoglycan cell walls of Gram-positive bacteria. By disrupting these membrane components, lysozyme causes the cell to lyse, killing it. Lysozyme is also active against fungal cell walls (Fletcher and Grant, 1968, Fletcher and White, 1973b) and some strains of Gram-negative bacteria (Grinde, 1989, Lie et al., 1989). The enzyme is unable to act on the outer cell wall of these latter strains, but it can act on the inner peptidoglycan layer if the outer layer has been disrupted by a MAC (Hjemeland et al., 1983). As described above, the ACP and MAC show limited ability to act on Gram-negative strains, so this activity is generally limited to non-virulent strains which do not express LPS or sialic acid on their cell envelopes.
Lysozyme is produced and secreted by leukocytes and is present in numerous body fluids, including blood and epidermal and intestinal mucus (Fletcher and White, 1973b, Fange et al., 1976, Lindsay, 1986). Takahashi et al., (1986) and Grinde et al., (1988a) have isolated two distinct types isomers of lysozyme from carp (C. carpio) and rainbow trout (Oncorhynchus mykiss), although some differences are apparent between the isotypes present in each species. A lysozyme-like molecule with a different iso-electric point from other isomers has been found in rainbow trout (O. mykiss) mucus by Smith et al., (2000), so further isomers of lysozyme may exist. The combination of complement and lysozyme in the mucus provides an effective defence against a variety of bacterial pathogens.

Research conducted by Grinde et al., (1988b) found that lysozyme activities show considerable variation between species, ranging from 8 - 20 U.ml\(^{-1}\) in tusk (Brosme brosme) and ling (Molva molva) to 2 - 6000 U.ml\(^{-1}\) in species such as turbot (Scophthalmus maximus), halibut (Hippoglossus hippoglossus) and rainbow trout (Oncorhynchus mykiss). Extreme inter and intra-specific variation is a common feature of many immune parameters and activity is greatly affected by a variety of environmental factors.

**Other humoral factors**

In addition to complement and lysozyme, numerous other enzymes and immunologically active molecules are employed by the IIS.

Protease enzymes are present in the mucosal secretions of a variety of fish species with the majority exhibiting trypsin-like activity (Braun et al., 1990, Kristjansson, 1991, Aranishi and Mano, 2000). Proteolytic enzymes may protect against Gram-negative bacteria such as *Vibrio anguillarum* (Hjemeland et al., 1983, Ellis, 2001) and fulfil other roles in immunity. Investigations into the action of proteases in the mucus of fish conducted by Concha et al., (2003) found that certain non-immune molecules, such as
apolipoproteins and high-density lipoproteins, yield low molecular weight, anti-bacterial peptides following enzymatic modification. These cleaved fragments, along with others from different sources, are collectively known as antimicrobial peptides (AmPs). Boman (1995) defines AmPs as being less than 10 kDa in mass, with stoichiometric, not enzymatic, functions. These AmPs assume a key role where fish lack specific Ig against a pathogen; molecules fitting this description have been isolated from plaice (Pleuronectes platessa) finless sole (Pardachirus marmoratus), winter flounder (Pseudopleuronectes americanus) and rainbow trout (O. mykiss) (Oren and Shai, 1996, Cole et al., 1997, Smith et al., 2000). Action of AmPs is through disruption of the CSM of pathogens through the formation of an ion-channel, termed the barrel-stave model (Reddy et al., 2004), or by interaction with the hydrophilic region of membrane components, which is termed the carpet model (Reddy et al., 2004).

Mucus often contains larger peptides and proteins in the range of 13-65 kDa which also have antibacterial properties; examples have been isolated from epidermal mucus of carp (C. carpio), rainbow trout (O. mykiss) and Queen parrotfish (Scaurus vetula) (Lemaitre et al., 1996, Viedeler et al., 1999, Smith et al., 2000). Again, action of these peptides is through the ion channel formation or destabilisation of the CSM (Lemaitre et al., 1996, Fernandes et al., 2002). AmPs and larger peptides and proteins in mucus are effective at lysing numerous strains of Gram-positive and Gram-negative strains although some of the smaller compounds are specific to one type of bacteria only (Lemaitre et al., 1996, Viedeler et al., 1999, Smith et al., 2000).

The innate immune system of fish and other vertebrates utilises a wide range of other humoral factors in addition to those discussed above. Lectins, for example mannan-binding lectin, bind to specific carbohydrates on the CSM of target cells. The presence of bound lectins targets cells for phagocytosis and lectins may help initiate complement pathways (Aranson, 1996, Magnadottir, 2006). There appears to be a high level of conservation in the specificity and structure of lectins present in fish and
mammals, although fish do exhibit lectins with no known mammalian homologues (Magnadottir, 2006). Acute-phase proteins, such as C-reactive protein and serum amyloid protein, are primarily associated with the acute phase response in vertebrates, and increase in concentration following tissue injury or infection. In addition to this role, these proteins show lectin-like properties and bind carbohydrate, aiding phagocytosis and activating complement (Steel and Whitehead, 1994, Bayne and Gerwick, 2001, Magnadottir, 2006). The exact acute-phase proteins present varies between fish species: cod (*Gadus morhua*) and channel catfish (*Ictalurus punctatus*) only express C-reactive protein, whilst salmon (*Salmo salar*) and halibut (*Hippoglossus hippoglossus*) express serum amyloid protein (White *et al.*, 1981, Lund and Olafson, 1998). Other fish species, including rainbow trout (*Oncorhynchus mykiss*) express both proteins, a situation common with higher vertebrates (White *et al.*, 1981, Lund and Olafson, 1998). Both the concentration and structural diversity of acute-phase proteins, particularly C-reactive protein, are greater in fish than in higher vertebrates (Lund and Olafson, 1999, Magnadottir, 2006).

Another important class of immune protein present in vertebrates are metal-ion sequestering proteins. The proliferation of various bacteria is limited by the presence of certain ions, especially free iron in the blood and tissues. By hinging free ions from the blood, the innate immune system prevents their utilisation by bacteria limiting their spread (Dalmo *et al.*, 1997).

In common with other aspects of the innate immune system, there is considerable conservation of humoral factors between fish and higher vertebrates. However, teleosts generally show a wider structural diversity in humoral immune components and components are generally show either higher activity or are present at greater concentrations in teleosts than in higher vertebrates, reflecting the greater reliance on the innate immune system in lower vertebrates.
For more detailed information regarding this humoral components, the reader is directed to the reviews of (Dalmo et al., 1997) and (Magnadottir, 2006).

**Cytokines and chemokines**

These are small protein-based molecules produced by various leukocytes, which function as intracellular signals and regulate cellular interactions, communication and behaviour. Cytokines have a key role in the control of immune responses and in the interaction between the immune and endocrine systems.

Interleukins 1 and 2 (IL-1 and IL-2) are secreted by slg- cells, macrophages and granulocytic cells. IL-1 has a broad spectrum of effects on numerous immune cells and tissues and is associated with the stimulation of both the AIS and IIS, inducing lymphocyte proliferation, granulocyte activation and mobilisation (Ellsaesser and Clem, 1994, Verburg-van Kemenade et al., 1995). IL-2 is more specific in function, only causing proliferation of lymphocytes (Caspi and Avtalion, 1984, Grondel and Harmsen, 1984). Interferon (IFN) is a glycoprotein that inhibits viral replication and is produced by leucocytes in numerous fish species (Ingram, 1980, Alexander and Ingram, 1992, Secombes, 1994b, Secombes, 1994a, Ellis, 2001) and production is up-regulated by the presence of viral DNA (Dorson et al., 1994, Robertsen et al., 1997, Nygaard et al., 2000). Graham and Secombes (1990) found that IFN enhances the respiratory burst and antibacterial activity of macrophages.

### 1.2.3 Mucus and mucosal immunity

The integument of fish and the protective mucosal coating act as the first defence against pathogens acting as physical barriers to infection (Dalmo et al., 1997). Pathogens in the mucus layers are prevented from reaching the tissues by entrapment and removal of pathogens via sloughing of mucus. Secondary to this are the actions of the various
components of the immune systems contained within the mucus matrix (Ellis, 2001).

Of all the mucosal surfaces of fish, the gut has the most developed mucosal immune defences, yet even without immune components, its numerous digestive enzymes make it a hostile environment (Hofer and Schiemer, 1990, Kristjansson and Nielson, 1992). Despite this vertebrates have evolved potent immune defences in the gut. Although lacking the gut-associated lymphoid organs present in mammals; the intestinal mucosa of carp (C. carpio) contains B-lymphocytes, T-lymphocytes and Ig presenting macrophages similar to those present in mammals (Rombout et al., 1993, Sveinbjornsson et al., 1996).

The hindgut of carp (C. carpio) is analogous to the mammalian caecum and is adapted to absorb and transport antigen to mucosal immune cells (Georgopoulou and Vernier, 1986, Rombout et al., 1989a), which process and present antigen, making the hind gut an important region for the initiation of mucosal immune responses (Rombout et al., 1986a, Rombout and Van den Berg, 1989, Koumans-van Diepen et al., 1994).

The mucosal immune system of the skin surfaces is less developed, although evidence suggests that antigen processing and presentation occurs in the epidermis in a similar fashion to that in the gut (Lobb, 1987, Iger and Wendelaar Bonga, 1994). Epidermal mucus fulfils a variety of roles in addition to immune defence, including maintenance of osmotic homeostasis (Handy, 1989) and is equipped with numerous components of the both IIS (Fange, 1984, Shepard, 1994, Yang and Albright, 1994, Nilsen, 1995, Ellis, 2001) and specific Ig, bestowing a range of antibacterial and antifungal properties (Wildgoose, 2001). The blood and mucosal immune responses of fish appear to show a degree of separation. Following vaccination both blood and the mucosa express antigen specific Ig, though expression of Ig is highly dependent on the route of administration. Mucosal immunity responds strongly to bath and oral antigen administration with heightened levels of Ig in the mucus of fish, despite low serum Ig titres (Fletcher and White, 1973a, Rombout et al., 1986a, Lobb, 1987, Rombout et al., 1989b). Conversely, vaccination via injection induced elevated serum Ig titres but
elicited only a weak response in the epidermal mucosa.

1.3 Neuro-endocrine-immune interaction

The interaction between stress and disease onset and development has received scientific study for over 100 years. Biondi and Zannino (1997) suggest that research into neuro-endocrine-immune interaction started with the observation by Pasteur in 1878 that resistance to anthrax in chickens could be modified by the application of a cold water stressor. Since this initial observation, considerable study has been made and the concept of physiological stress has been identified and the definition refined by a number of authors (Selye, 1950, Selye, 1953, Brett, 1958).

Unstressed and healthy fish exist in equilibrium with their environment and the pathogens within it and the balance of this equilibrium can be significantly altered following exposure to stress. Such alteration of this equilibrium has many consequences; immune defences may be weakened or pathogen virulence may be altered, thus fish are affected by both environmental and pathogenic factors, amplifying the effects of stress (Wedemeyer, 1996). Evidence of these complex interactions has received considerable study in mice. Previte and Berry (1962) noted that stress prior to infection with a virulent strain of bacteria did not significantly alter susceptibility, yet greatly increased the susceptibility of an organism to mildly virulent stains.

Similarly, when fish experience stress their susceptibility to a range of opportunistic pathogens, including fungal, (Saprolegnia sp.) and bacterial pathogens, (Aeromonas hydrophila, Flexibacter columnaris and strains of Pseudomonas sp. and Vibrio sp.) increases. These organisms are common in the aquatic environment are not able to infect healthy fish, only affecting immuno-compromised fish. Biondi and Zannino (1997) consider that host immuno-competence is the most crucial factor in defining the spread of disease.
1.3.1 Mechanisms of neuro-endocrine-immune interaction

The complex interactions between the immune system and the endocrine systems is thought to be due, at least in part, to a similar evolutionary history (Maier et al., 1994).

The IIS, when undergoing a localised inflammatory response or more widespread acute phase response, requires significant amounts of energy to sustain that response and so mobilises energy reserves from the liver and muscles to yield free glucose. The bulk of this mobilised energy is diverted to granulocyte activation, leukocyte migration and wound healing (Maier et al., 1994, Maier and Watkins, 1998). In addition, various behavioural modifications may be invoked to support these processes, including a reduction in organism mobility, aggression and increased sheltering behaviour (Maier et al., 1994).

The vertebrate stress response also requires mobilisation of tissue energy reserves to produce free glucose, which is required to support the fight-flight response of the organism. However, the energy demands of the stress response are almost diametrically opposed to those of the inflammatory response (Maier et al., 1994). The greatest metabolic activity of a stressed organism occurs in the brain and skeletal muscles, to support the demands of these tissues energy is diverted from non-essential organs and the immune system. Similarly, the behavioural modifications invoked by stress are geared towards defence and escape.

The metabolic demands of the inflammatory response and the stress response are mutually exclusive and mechanisms have evolved to promote one system over the other. This is facilitated by the reliance of both inflammatory and stress responses on the same suite of molecules and receptors for regulation and signalling, allowing the two systems to interact effectively (Maier et al., 1994, Turnbull and Rivier, 1999, Baigent, 2001).

The glucocorticoids play an essential role in the regulation of both systems. It is thought that the inflammatory response evolved prior to the stress response and the earliest metabolic role for glucocorticoids was the provision of energy for and the
ultimate regulation of the inflammatory response. Following injury or infection, pro-
inflammatory cytokines (including IL-1 and TNF-α) are produced, primarily by 
macrophages, which act on the brain and trigger the release of glucocorticoids (Maier et 
al., 1994, Balm, 1997).

When released into the blood from the anterior kidney, glucocorticoid hormones 
mobilise glycogen and protein stores to provide the free glucose required by the 
inflammatory response, but they also have a regulatory role. In addition to producing 
pro-inflammatory cytokines, numerous classes of leukocyte produce intermediate stress 
hormones, including cortisol releasing hormone (CRH) and adrenocorticotropic 
hormone (ACTH) (Weigent et al., 1988, Lyons and Blalock, 1997, Baigent, 2001). As 
noted by Engelsma et al., (2002) and Turnbull and Rivier (1999) despite a low 
contribution to the total plasma concentration of these hormones, these hormones have 
significant localised effects and are fundamental in the regulation of inflammatory 
processes. Such production of intermediate stress hormones has been observed in 
goldfish (C. auratus) (Ottaviani et al., 1995, Ottaviani et al., 1998) and channel catfish 

The presence of glucocorticoids alters both the production of the pro-
inflammatory cytokines and the affinity of their receptors, regulating them at both source 
and target. The gradual accumulation of cytokines initiates the inflammatory response 
and resultant increase in glucocorticoid hormones provides energy and eventually down-
regulate the inflammatory response through negative feedback.

The stress response comprises a series of physiological adjustments made by an 
organism in response to cues from the nervous and endocrine systems. Two major 
pathways are initiated by stress. The catecholamine response is initiated and regulated by 
the autonomic nervous system (ANS) (Eskandari et al., 2003), whilst the endocrine 
system of fish is activated along the hypothalamus-pituitary-interrenal (HPI) axis with the 
resultant release of corticosteroid hormones from the anterior kidney. Both stress
pathways exert a strong influence on, and in turn, are influenced by the both the IIS and AIS. The initiation of a stress responses results in a massive and rapid increase in the concentration of circulating stress hormones. At such high levels, glucocorticoid hormone concentrations rapidly attain levels where the pro-inflammatory cytokines are not produced, thus suppressing the inflammatory processes and allowing the stress response to divert energy to the core organs (Maier et al., 1994). The stress response takes precedent over the inflammatory response, allowing the organism to escape the stressful situation at hand; when the stressor has been lifted and the concentration of glucocorticoids has decreased the inflammatory response can re-establish itself.

Although stress has been largely thought to be a suppressive force, evidence of stress-induced immuno-stimulation has been found in a range of vertebrates. The duration of the stressor appears to be the key factor responsible for determining the response of the immune system to stress.

Catecholamines are released following stress and increase in the blood even more rapidly than the glucocorticoid hormones. Fish leukocytes possess both α- and β-adrenergic receptors (Narnaware et al., 1994, Finkenbine et al., 2002) which bind to catecholamines and regulate the immune system following stress. Generally the response of immune cells to catecholamines released following acute stress is stimulatory, however following prolonged catecholamine release, stimulatory effects are lost and the response becomes suppressive (Narnaware et al., 1994, Engler et al., 2004).

Receptors for glucocorticoid hormones are present on the CSM of leukocytes in many species of fish, including salmon (Salmo salar) and carp (C. carpio) (Maule and Schreck, 1990, Weyts et al., 1998). Two classes of receptor are present, both of which bind to cortisol but with greatly different affinities, a feature which allows a differential response to stress to be elicited in leukocytes following stress (Greenwood et al., 2003). The response to glucocorticoids is again dependent on the duration of the stressor, with short-duration acute stress generally having a stimulatory effect and chronic stress a
suppressive effect. In addition to catecholamines and corticosteroids, other hormones products of the brain and intermediates in the HPI axis also affect the immune systems, although to a lesser extent than the key stress molecules.

1.3.2 Stimulatory effects of stress on the immune system

The release of catecholamines and corticosteroids by the ANS and HPI axis has both stimulatory and suppressive effects on the immune system. Acute stressors, of short duration, result in a rapid and transient increase in catecholamines and corticosteroids within the blood, generally have a stimulatory effect on vertebrate immune systems (Pruett, 2001, Moynihan, 2003).

The evolutionary basis behind stress induced stimulation of immunity is clear and has been succinctly explained by Viswanathan, Daugherty, et al., (2005):

"Since stressful natural encounters often result in wounding and infection, it is unlikely that eons of evolution would select for a system exquisitely designed to escape the jaws and claws of a lion to only have it eaten inside out by bacteria."

It is thought that the stress response, utilising the cytokines and hormones already employed by the immune system has evolved into a mechanism that primes and activates the IIS in advance of actual injury or trauma (Maier and Watkins, 1998). This represents an important adaptation as production of the cytokines required to generate inflammatory responses (IL-1 & TNF-α) takes several hours, during which time infection can take hold. By activating the immune system in advance, this lag period can be overcome reducing the response time to infection (Maier and Watkins, 1998).

Stimulation of the immune system following stress can be found throughout the vertebrates, from fish to mammals (Pruett, 2001, Dhabhar, 2002, Moynihan, 2003, Engler
et al., 2004, Dhabhar and Viswanathan, 2005) and typically manifests itself as a mobilisation of leukocytes, particularly neutrophils, monocytes and NCCs, to enable them to move towards potential sites of infection and combat disease effectively should injury occur.

Mobilisation of granulocytic cell classes into the circulation, such as monocytes and neutrophils and under certain conditions, lymphocytes, is triggered by catecholamines (Benschop et al., 1996, Carlson et al., 1997, Engler et al., 2004). In the case of neutrophils, glucocorticoid hormones may also trigger mobilisation (McEwen et al., 1997), indicating that more complex mechanisms may be responsible. Stress-induced neutrophilia appears to be a combination of both direct and indirect mechanisms. In addition to responding to hormonal cues, granulocytic cells detach from the vascular epithelium in response to increased heart rate and blood velocity (Boxer et al., 1980, Moazzam et al., 1997, Engler et al., 2004). Further recruitment of granulocytes to the circulation occurs from immune organs innervated by adrenergic nerve bundles (Engler et al., 2004). This interaction of direct and indirect effects on one single class of immune cells illustrates the sophisticated nature of the control exerted by the nervous and endocrine systems on the immune system at time of stress.

Studies on various mammalian models have demonstrated that stress can result in significant immune enhancement. For example, forced physical exercise increased resistance to certain bacterial pathogens Francisella tularensis and Streptococcus pneumoniae in rats, mice and guinea pigs (Nicholls and Spaeth, 1922, Oppenheimer and Spaeth, 1922, Friman et al., 1982, Ilback et al., 1984). In fish, stress was found to enhance macrophage respiratory burst activity and increase lysozyme activity (Fevolden et al., 1994, Kurogi and Lida, 1999), along with a reduction in neutrophil apoptosis, prolonging their effective life in the circulation (Weyts et al., 1998). Some stimulatory effects of stress on the immune system are summarised in Table 1.1.
<table>
<thead>
<tr>
<th>Effect</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased circulating NK cell numbers</td>
<td>Rats [1]</td>
<td>[1](Engler et al., 2004)</td>
</tr>
<tr>
<td>Increased circulating monocyte numbers</td>
<td>Rats [1]</td>
<td>[1](Engler et al., 2004)</td>
</tr>
<tr>
<td>Increased circulating phagocyte numbers</td>
<td>Limanda limanda [2]</td>
<td>[2](Pulsford et al., 1994)</td>
</tr>
<tr>
<td>Increased circulating neutrophil numbers</td>
<td>Ictalurus punctatus [3]</td>
<td>[3](Ellsaesser and Clem, 1986)</td>
</tr>
<tr>
<td>(neutrophilia)</td>
<td>Oreochromis aureus [4]</td>
<td>[4](Ascencio'Valle et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>O. mykiss [5]</td>
<td>[5](Hlavv and Bulkley, 1980)</td>
</tr>
<tr>
<td></td>
<td>Rats [1]</td>
<td>[1](Engler et al., 2004)</td>
</tr>
<tr>
<td>Reduced apoptosis of neutrophils</td>
<td>Cyprinus carpio [7]</td>
<td>[7](Weyts et al., 1998c)</td>
</tr>
<tr>
<td>Increased neutrophil ROS production</td>
<td>Oreochromis niloticus [8]</td>
<td>[8](Kurogi and Lida, 1999)</td>
</tr>
<tr>
<td>Increased lysozyme production</td>
<td>Oncorhynchus mykiss [9]</td>
<td>[9](Fevolden et al., 1994)</td>
</tr>
</tbody>
</table>

NK = natural killer. ROS = Reactive Oxygen Species.
Table 1.2. Suppressive effects of stress on aspects of the immune system of teleosts.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced circulating lymphocytes (Lymphopenia)</td>
<td>Oncorhynchus kisutch[^1]</td>
<td>[1](McLeay, 1973)</td>
</tr>
<tr>
<td></td>
<td>Salmo trutta[^2]</td>
<td>[2](Pickering, 1984)</td>
</tr>
<tr>
<td></td>
<td>Ictalurus punctatus[^3]</td>
<td>[3](Ellsaesser and Clem, 1986)</td>
</tr>
<tr>
<td></td>
<td>Salmo salar[^4]</td>
<td>[4](Epselid et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>L. punctatus[^3]</td>
<td>[5](Ellsaesser and Clem, 1986)</td>
</tr>
<tr>
<td></td>
<td>O. kisutch[^5]</td>
<td>[6](Tripp et al., 1987)</td>
</tr>
<tr>
<td></td>
<td>Limanda limanda[^6]</td>
<td>[7](Weyts et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>Cyprinus carpio[^7]</td>
<td></td>
</tr>
<tr>
<td>Reduced lymphocyte proliferation</td>
<td>Carassius auratus[^11]</td>
<td>[8](Anderson et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Oncorhynchus mykiss[^12]</td>
<td>[9](Ruglys, 1985)</td>
</tr>
<tr>
<td></td>
<td>C. auratus[^11]</td>
<td>[10](Tripp et al., 1987)</td>
</tr>
<tr>
<td>Reduced phagocytic activity</td>
<td>C. carpio[^7]</td>
<td>[11](Wang and Belosevic, 1995)</td>
</tr>
<tr>
<td></td>
<td>Oreochromis mossambicus[^13]</td>
<td>[12](Naraware et al., 1994)</td>
</tr>
<tr>
<td></td>
<td>O. mykiss[^8]</td>
<td>[13](Bury et al., 1998)</td>
</tr>
<tr>
<td></td>
<td>C. carpio[^9]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>O. kisutch[^5]</td>
<td>[14](Yin et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>Oncorhynchus tsawyscha[^10]</td>
<td>[15](Ortuno et al., 2001)</td>
</tr>
<tr>
<td>Reduced macrophage respiratory burst</td>
<td>C. carpio[^14]</td>
<td>[16](Montero et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Sparus aurata[^15,16]</td>
<td></td>
</tr>
</tbody>
</table>
1.3.3 Suppressive effects of stress on the immune system

Following repeated or prolonged exposure to a stressor, or combination of stressors, a chronic stress state may develop where circulating catecholamines and corticosteroids remain elevated for extended periods. Under these conditions, the stress hormones cease to have stimulatory effects on the immune system and instead become suppressive (Pruett, 2001, Moynihan, 2003). A summary of the suppressive effects of stress on the immune system are presented in Table 1.2. Even short-term acute stressors can be strongly immuno-suppressive but these effects are usually seen on aspects of the AIS, not the IIS (Maier and Watkins, 1998). This may in part stem from suppression arising from aspects of the IIS, for example the respiratory burst products of macrophages, in particular nitric oxide, inhibit T and B lymphocyte proliferation (Albina and Henry, 1991). Aspects of the IIS, many of which are stimulated by short duration acute stressors are also suppressed by chronic stress (Maier et al., 1994, Maier and Watkins, 1998).

The glucocorticoid hormones are strongly immuno-suppressive under chronic stress conditions, which is consistent with their role as regulatory molecules for the inflammatory response (Maier et al., 1994). In addition, catecholamines and intermediate stress hormones (ACTH and CRF) may also be suppressive under certain conditions (Harris and Bird, 2000). For example, macrophage phagocytic activity and respiratory burst is suppressed by both catecholamines and cortisol, although cortisol has the most pronounced effect (Narnaware et al., 1994, Wang and Belosevic, 1995, Narnaware and Baker, 1996).

A reduction in circulating lymphocyte number (lymphopenia) is common following stress and appears to be a result of interactions between catecholamines and corticosteroids, in much the same way as neutrophilia (Benschop et al., 1996, Carlson et al., 1997, Engler et al., 2004). Catecholamines induce lymphocytes to undergo apoptosis and redistribution of cells from the circulation to immune organs, particularly the spleen, occurs in response to both catecholamine and glucocorticoid signals (Maier and Watkins,
Thus both cell survival and migration may be modified following stress (Engler et al., 2004).

In addition the widespread changes to cellular immune defences, humoral immune components also show stress-induced immuno-suppression. The complement system in particular has been found to be highly susceptible to stress-induced immuno-suppression (Boshra et al., 2006).

In numerous trials, the doses of cortisol reported to trigger immuno-suppression in vitro and to a lesser extent, in vivo greatly exceed those experienced by organisms following stress (Narnaware et al., 1994, Eskandari et al., 2003). Eskandari et al., (2003) found that when doses closer to the physiological values for a species are used, cortisol ceased to be completely immuno-suppressive and numerous enhancing and regulatory functions become apparent. Thus caution must be exercised when interpreting the effects of cortisol on the immune system in vitro, as conclusions may be drawn regarding suppressive effects from concentrations never experienced by organisms in vivo.

Physical immune barriers may also experience stress-induced alterations in effectiveness (Biondi and Zannino, 1997). Increases in circulating stress hormones resulting from numerous stressors have been shown to inhibit mucus production, examples being rapid temperature shock, acidic conditions, water contaminated with fertiliser and exposure to heavy metals (Iger et al., 1988, Rajan and Barnajee, 1991, Iger and Wendelaar Bonga, 1994, Quiniou et al., 1998). In all of these studies, the initial response to a stressor was an increase in mucus production, a mechanism thought to prevent ionic imbalance and reduce the risk of injury through abrasion (Handy, 1989, Shepard, 1994). Subsequently, a few hours post-stress, significant reductions in goblet cell number resulted in a thinning of the mucus coat, resulting in "dry skin syndrome" leaving fish immuno-compromised in all cases (Iger et al., 1988, Rajan and Barnajee, 1991, Iger and Wendelaar Bonga, 1994, Quiniou et al., 1998).

1.4 Disease management strategies

1.4.1 Chemo-therapeutic agents

The deleterious effects of inadequate nutrition and disease, which include effects of viral, protozoan, metazoan and bacterial pathogens and parasites, are the two largest external biotic factors influencing the growth of vertebrates (Rintamaki-Kinnunen et al., 2005).

Disease is a problem that has the potential to affect all ornamental fish at some point during their lives. Fortunately, the majority of disease experienced by ornamental fish is non-infectious and can be attributed to genetics, inadequate nutrition and poor water quality (Shao, 2001). Disease arising from these sources can be combated effectively through simple husbandry techniques, through meeting the nutritional requirements and through the prevention of extensive in-breeding, a particular problem for ornamental fish.

Infectious diseases often require treatment with some form a therapeutic agent. Various drug treatments are employed to treat disease in ornamental fish, but considerable limitations exist on those available to aquarists in some countries. In the UK, antibiotics are only available on prescription from a veterinarian, greatly restricting their use for disease management of ornamental fish. In general, the costs of veterinary medicine in the UK ensure that the majority of fish do not receive specific treatment, unless they have an exceptionally high monetary or sentimental value. In the US, the Food and Drug Administration (FDA) does not approve any antibiotics for use with
ornamental fish, however, Yanong (2003) found that this does not prevent their use on ornamental fish.

There are numerous general treatments available for use on ornamental fish, formalin, malachite green and salt being examples. Such chemicals, despite being effective, can have undesirable side effects and may have carcinogenic or toxic properties which can affect both the fish and the aquarist. Such drugs are often controlled by local licensing laws; for example malachite green is prohibited for use on fish destined for consumption (Rintamaki-Kinnunen et al., 2005) although no restrictions, as yet, apply for usage in ornamental fish.

In addition to deleterious effects these agents may have on the user, many commonly employed drug treatments have considerable deleterious side effects on fish and the environment. Although highly effective, antibiotics can affect biological filtration systems, limiting their ability to effectively purify water in addition to their highly restricted nature and expense. Yanong (2003) found that excessively high doses can affect liver and kidney function and may induce organ failure.

Malachite green can lower the oxygen capacity of the water in addition to damaging susceptible plant and fish species; copper at medicinal concentrations is unsafe for use with some plants or invertebrates; formalin forms paraformaldehyde which is toxic, so correct dose rates and durations are essential.

All the common chemical treatments used for disease control in fish have the potential to induce a measure of stress on the fish which can in turn weaken the immune response of the fish further, compounding the effects of disease (Donaldson, 1981).

Despite the costs and detrimental effects of disease treatment, there is a requirement to control pathogens. Disease has numerous detrimental effects on the physiology and aesthetics of a fish, and the high value attached to ornamental fish ensure that considerable care to protect investment is made. The limitations and expense of conventional drug treatments and a general desire of the public to employ more natural
disease control strategies have led to increased research into the promotion of the fish's own immune defences through the application of immuno-stimulant compounds.

1.4.2 Immuno-stimulants

An immuno-stimulant has the potential to enhance a fish's immune defences via activation aspects of the immune system, primarily the monocyte-macrophage system of the IIS (Bricknell and Bowden, 2003). The stimulation of host defence mechanisms presents no specific factors for pathogenic organism to develop resistance against, allowing them to be used widely as prophylactic measures (Sakai, 1999); the development of resistant strains is a major argument against the widespread use of antibiotics (Aoki, 1992).

Types of immuno-stimulant

Numerous compounds with immuno-stimulating properties have been identified including chemicals, drugs, vitamins and minerals. Additionally, as reported by Sohn et al., (2000) and Dhabhar and Viswanathan (2005) under specific conditions stressors may also be immuno-stimulatory in nature.

The majority of immuno-stimulant compounds are derived from natural sources, although some synthetic compounds show immuno-stimulant properties, including the anti-helminthic drug Levamisole (Kajita et al., 1990) and the synthetic peptide FK-565 (Kitao and Yoshida, 1986, Kitao et al., 1987). These synthetic compounds have been shown to increase the phagocytic activity of neutrophils, elevate leukocyte and phagocyte numbers and enhance lysozyme activity in mammals (Symoens and Roesenthall, 1977). Similar results have been observed in fish following administration of Levamisole (Sakai, 1999) with enhanced neutrophil phagocytosis, enhanced lysozyme activity and leukocyte numbers (Siwicki, 1987, Siwicki, 1989). Levamisole administration lead to improved
resistance to challenge by bacterial pathogens such as *Aeromonas salmonicida* and *Vibrio anguilarum* (Olivier *et al.*, 1985, Kajita *et al.*, 1990).

The majority of organically derived immuno-stimulants are derived from pathogenic organisms. Intact bacteria have been used extensively as immuno-stimulants; the major component of Freund's complete adjuvant (FCA) is intact *Mycobacterium butyricum*. In addition, bacteria are a particularly rich source of immuno-stimulatory compounds, including lipopolysaccharide (LPS), muramyl dipeptide (MDP) and others (Sakai, 1999, Sohn *et al.*, 2000). These purified extracts from bacterial cell walls are highly effective immuno-stimulants.

Yeast and fungal cells are a similarly rich source of immuno-stimulant compounds. Chitin and chitosan can be purified from various fungal sources and are immuno-stimulatory in vertebrates. Chitin has been found to enhance macrophage activity in fish and increases bacterial resistance in both fish (Sakai *et al.*, 1992, Kawakami *et al.*, 1998) and mammals (Nishimura *et al.*, 1985). Chitosan (de-N-acetylated chitin) promotes increased respiratory burst activity, bacterial killing activity and elevates immunoglobulin concentrations in fish (Anderson *et al.*, 1995), with the sum of these immune enhancements granting enhanced resistance to various bacterial pathogens (Siwicki *et al.*, 1994, Anderson *et al.*, 1995).

Yeast cells are the source of a number of commercially viable immuno-stimulants. These include chitin and chitosan and other immuno-stimulatory molecules, such as the β-glucans and nucleotides that form the basis of this investigation. The mode of action and effects on immunity of β-glucans and nucleotides are covered in more detail below.

**Limitations of Immuno-stimulants**

As promoters and enhancers of the immune system, immuno-stimulants have been found to have greatest effect when administered prophylactically. Administration following
disease outbreaks reduces or negates any stimulatory effects (Sohn et al., 2000). This may be due to the immuno-stimulants increasing the burden on the immune system by eliciting a response to the immuno-stimulant compound in addition to that already generated by the presence of the pathogen. However, under certain conditions, the administration of immuno-stimulants alongside antibiotics can be effective at limiting pathogen spread in both humans and fish (Dietrich et al., 1981, de Felippe et al., 1993, Thompson et al., 1993).

Immuno-stimulants are ineffective at preparing the immune system against certain pathogens. The typical mode of action is through activation of macrophages which are effective against most bacteria, however certain pathogens, for example Renibacterium salmoninarum, Pseudomonas piscicida and Edwardsiella ictaluri can survive phagocytosis and survive within the phagosome (Nelson et al., 1989, Baldwin and Newton, 1996, Gutenberger et al., 1997, Sakai, 1999), rendering immuno-stimulation ineffective against these bacteria.

The majority of immuno-stimulant compounds show a non-linear dose/response relationship, with greatest stimulatory effects found at lower dose rates. Numerous studies have found that higher doses typically fail to enhance immune defences or may even induce immuno-suppression (Kitao et al., 1987, Kajita et al., 1990, Robertsen et al., 1994, Sohn et al., 2000). This may be due to desensitisation of the immune system to the immuno-stimulant and tolerance of that compound may develop, preventing a response to the cue when present on pathogens (Castro et al., 1999).

**Administration of immuno-stimulants**

Administration of immuno-stimulants can be achieved via several routes although the results obtained may differ depending on the route of administration. Presentation of immuno-stimulants can be made via the following routes: injection, orally or bath immersion.
Injection into the body cavity typically generates a strong response as the compound remains unaltered by stomach acid or digestive enzymes. Immuno-stimulants administered in this way generate a strong immune reaction in the blood; however there may only be a limited mucosal response. This may be due to a degree of separation between blood and mucosal immune systems (Fletcher and White, 1973a, Rombout et al., 1986b, Lobb, 1987, Rombout et al., 1989b). Injection is time-consuming, labour intensive and not suitable on fish below a threshold size. In addition, there is a small risk of associated mortality through the handling of the fish, which is unacceptable for high value ornamental fish (Sakai, 1999).

Therefore, for the ornamental market, the most practical routes of immuno-stimulant administration are bath immersion or orally via the feed. Research by Ainsworth et al., (1994) has shown that both routes stimulate the intestinal and epidermal mucosa, priming the immune system at the source of infection.

Immersion is generally non-stressful, requiring minimal handling; however, immune responses to the immuno-stimulant are typically reduced in both intensity and duration (Baba et al., 1993, Nikl et al., 1993, Anderson et al., 1995) when compared to oral supplementation or injection.

Oral administration appears an effective route of administration for immuno-stimulant compounds, which are small enough to be absorbed across the gut wall intact. For larger molecules, for example β-glucans, modification by the gastro-intestinal tract may reduce the effectiveness of the immuno-stimulant compound (Veerabagu et al., 1996, Rust, 2002). Supplementation via the oral route directly stimulates the immune defences of the intestinal mucosa, which may create a reservoir of activated neutrophils and macrophages in the mucosal tissues, allowing pathogens to be effectively overwhelmed before triggering a disease outbreak (Ainsworth et al., 1994).

The results of a study conducted by Bricknell and Bowden (2003) showed that continuous feeding of immuno-stimulants led to one of two outcomes; the rarer situation
is that the immune system attains and remains at heightened level of activity, which is maintained by the continued feeding of the compound. Contrastingly, studies conducted by Matsuo and Miyazano (1993) found that rainbow trout (O. mykiss) administered peptidoglycan showed a reduction in immune status, a finding confirmed by Yoshida et al., (1995) who studied catfish (Clarias gariepinus) fed β-(1,3) glucan with β-(1,6) linked side chains (Macrogard®) or mannan oligosaccharide (Vetregard®). This desensitisation of the immune system and tolerance to immuno-stimulant compound developed after several weeks of continual feeding. In extreme cases, immuno-suppression may stem from continued administration of an immuno-stimulant, with the immune system being weakened below its un-stimulated state (Bricknell and Bowden, 2003). In these situations, the optimal feeding strategy appears to be administration of immuno-stimulants in cycles of 4–6 weeks duration causing the immune system of the fish oscillates between its resting and stimulated state. When the immuno-stimulant is removed from the feed, the stimulatory effect is lost and the immune system reverts to its basal level of activity. Cyclical feeding also allows for greater flexibility in immuno-stimulant administration, allowing supplemented feed to be offered prophylactically in anticipation of stressful events (Bricknell and Bowden, 2003).

### 1.5 β-glucans and Nucleotides

#### 1.5.1 β-glucan structure

This important group of immuno-stimulants take the form of long chain carbohydrate polymers (Sakai, 1999). These glucan molecules consist of a main chain of 1-3-linked β-glucopyranosyl units along which random β-glucopyranosyl units are attached by 1-6 linkages (Robertson et al., 1994, Bohn and BeMiller, 1995) (Figure 1.1). This class of glucan has potent immuno-stimulant properties, although the actual immuno-stimulatory
properties are dependent on the position and configuration of the \( \beta-(1,6) \) side branches (Bohn and BeMiller, 1995).

\( \beta-(1,3:1,6) \) glucans are ubiquitous structural molecules found in bacterial, yeast and fungal cell walls, with yeasts being a popular source of commercially available \( \beta \)-glucans. The status of \( \beta \)-glucans as ancient structural molecules in a plethora of pathogenic organisms has led the vertebrate immune system to evolve numerous recognition devices for these molecules (Robertsen et al., 1994). Ainsworth et al. (1994) found that macrophages have a specific glucan receptor on their CSM; upon binding to this receptor macrophage become activated and increase antigen presentation, cytokine production and initiate respiratory burst activity, thus enhancing resistance to bacterial infection (Robertsen et al., 1990, Raa et al., 1992).

### 1.5.2 Effects of \( \beta \)-glucans on the vertebrate immune system

All \( \beta-(1,3:1,6) \) glucans, irrespective of their origins, have been shown to enhance antitumour, antibacterial, and antiviral activity of the immune system (Bohn and BeMiller, 1995, Bricknell and Bowden, 2003).

The stimulatory effects of \( \beta \)-glucans are typically limited to those immune cells that possess specific receptors for \( \beta \)-glucan molecules which are macrophages, granulocytes and NCCs in fish (Engstad and Robertsen, 1993, Engstad, 1994, Engstad and Robertsen, 1994). Through activation of these cells \( \beta \)-glucans enhance several aspects of the IIS, which are summarised in Table 1.3. Immune enhancement following administration of \( \beta \)-glucans has been found to enhance resistance to bacterial challenge in a range of fish species from salmonids to commercially important ornamental species (Table 1.3).

Although \( \beta \)-glucans directly stimulate cellular and humoral components of the IIS, they can have both primary and secondary stimulatory effects on the AIS. Immunoglobulin can be produced by the AIS in response to \( \beta \)-glucans, but is typically of
low specificity, due to the difficulties of raising immunoglobulin against large polysaccharides (Tzianabos, 2000). The duration of the specific immune response generated against β-glucans is also weaker than that generated by protein antigens (Tzianabos, 2000). β-glucan supplementation may increase the efficacy of vaccines, probably through enhancement of antigen presentation via macrophages in both farmed salmonid fish (Rorstad et al., 1993; Aakre et al., 1994; Baulny et al., 1996) and commercially important ornamental fish species (Russo et al., 2006). Further activation of the AIS by β-glucans occurs through the production of cytokines (Sohn et al., 2000); yeast glucans have been shown to enhance IL-1 production in salmonids which induces proliferation and differentiation of T and B lymphocytes (Robertsen et al., 1994).

In addition to their direct immuno-stimulatory benefits, β-glucans possess secondary beneficial effects including the enhancement of growth in a variety of vertebrate species including fish (Onarheim, 1992; Cook et al., 2003) and swine (Schoenherr et al., 1994; Dritz et al., 1995; Boddez, 1998). It is thought that this may, in part, be due to a reduced bacterial loading on the organism (Sohn et al., 2000). The potential of β-glucans to act as stress modulators has received only limited study, with most of the research focussing on the reversal of stress-induced immuno-suppression.
Figure 1.1. Generic structure of a β (1-3)-glucan molecule showing β (1-6) linked β (1-3) side branches.
(Source: www.β13dglucan.org)

Skjermo et al., (2002) reported enhanced stress tolerance and enhanced survival post-stress in juvenile marine fish. Similar findings were reported by Liu et al., (2004) and Su et al., (1995) using shrimp post-larvae, suggesting that β-glucan supplementation may have secondary beneficial effects on the ability of organisms to respond to adverse conditions, particularly with reference to recovery times. β-glucans have been shown to significantly reduce blood cortisol concentrations following stress, although blood glucose levels were unaffected, in a clear demonstration of their stress mediating effects (Cain et al., 2003).

β-glucans show the most widespread immuno-stimulatory effects when injected or when used as purified compounds in vitro (Table 1.3). The effects of oral
supplementation with β-glucans has received less study, but in trials which have used this route of administration, the effects of supplementation appear limited to enhanced bacterial resistance, macrophage respiratory burst and phagocytosis.

In common with other immuno-stimulants, high doses of β-glucans can be detrimental to the efficiency of the immune system. In vitro studies by Castro et al., (1999) confirmed the dose dependent nature of β-glucan stimulation. Low doses of 10 – 25 μg.ml\(^{-1}\) were sufficient to elevate macrophage respiratory burst activity. Respiratory burst activity increased proportionally with dose up to concentrations of 100 – 250 μg.ml\(^{-1}\). However, further increases of dose up to 500 μg.ml\(^{-1}\) failed to induce any further increases and were observed to decrease activity in some cases. Similar results were found in rainbow trout (O. mykiss) by Jorgensen and Robertsen (1995). It was concluded that the higher doses of β-glucans induce exhaustion of macrophages and neutrophils through sustained respiratory burst activity.
<table>
<thead>
<tr>
<th>Effect</th>
<th>Species</th>
<th>Pathogen</th>
<th>Route</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased bacterial resistance</td>
<td>Salmo salar</td>
<td>Vibrio anguilfarum</td>
<td>IP &amp; O</td>
<td>[1] (Robertsen et al., 1990)</td>
</tr>
<tr>
<td></td>
<td>Ictalurus punctatus</td>
<td>Yersinia ruckerii</td>
<td>IP</td>
<td>[3] (Chen and Ainsworth, 1992)</td>
</tr>
<tr>
<td></td>
<td>Epalzeorhynchos bicolor</td>
<td>Yersinia ruckerii</td>
<td>IP</td>
<td>[4] (Raa et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Morone chrysops X M.</td>
<td>Streptococcus iniae</td>
<td>O</td>
<td>[12] (Li and Gatlin, 2003)</td>
</tr>
<tr>
<td></td>
<td>saxatilis</td>
<td></td>
<td></td>
<td>[17] (Li and Gatlin, 2004)</td>
</tr>
<tr>
<td></td>
<td>Xiphophorus helleri</td>
<td>A. iniae</td>
<td>O</td>
<td>[18] (Tuemau et al., 2000)</td>
</tr>
<tr>
<td></td>
<td>Barbus cochinus</td>
<td></td>
<td>O</td>
<td>[19] (Kumari and Sahoo, 2006a)</td>
</tr>
<tr>
<td></td>
<td>Gymnocorymbus ternetzi</td>
<td></td>
<td>O</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Clarias batrachus</td>
<td></td>
<td>O</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Oncorhynchus mykiss</td>
<td></td>
<td>IP</td>
<td>[5] (Jorgensen et al., 1993)</td>
</tr>
<tr>
<td>Increased lysozyme activity</td>
<td>O. mykiss</td>
<td></td>
<td>IP</td>
<td>[6] (Thompson et al., 1995)</td>
</tr>
<tr>
<td></td>
<td>C. batrachus</td>
<td></td>
<td>IP</td>
<td>[7] (Jorgensen and Robertson, 1995)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IP &amp; O</td>
<td>[8] (Jorgensen and Robertson, 1995)</td>
</tr>
<tr>
<td></td>
<td>O. mykiss</td>
<td></td>
<td>IP</td>
<td>[9] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Clarias gariepinus</td>
<td></td>
<td>IP</td>
<td>[10] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Pseutca maxima</td>
<td></td>
<td>IP</td>
<td>[12] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Sparus auratus</td>
<td></td>
<td>IP</td>
<td>[13] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>M. chrysops X M.</td>
<td></td>
<td>IP</td>
<td>[14] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>saxatilis</td>
<td></td>
<td>IP</td>
<td>[15] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>Cyprinus carpio</td>
<td></td>
<td>IP</td>
<td>[16] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>C. batrachus</td>
<td></td>
<td>IP</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Scophthalmus maximus</td>
<td></td>
<td>IP</td>
<td>[17] (Engstad et al., 1992)</td>
</tr>
<tr>
<td></td>
<td>S. salar</td>
<td></td>
<td>IP</td>
<td></td>
</tr>
<tr>
<td>Increased respiratory burst</td>
<td>I. punctatus</td>
<td></td>
<td>IP &amp; O</td>
<td>[18] (Yoshida et al., 1995)</td>
</tr>
<tr>
<td>activity</td>
<td></td>
<td></td>
<td>IP</td>
<td></td>
</tr>
<tr>
<td>Increased phagocytic activity</td>
<td>Cyprinus carpio</td>
<td></td>
<td>IP</td>
<td>[19] (Yoshida et al., 1995)</td>
</tr>
<tr>
<td>Increased granulocyte number</td>
<td></td>
<td></td>
<td>IP</td>
<td></td>
</tr>
</tbody>
</table>

IP = intra peritoneal; O = oral.
1.5.3 Exogenous nucleotide structure

Nucleotides are low molecular weight structural components of both DNA and RNA and are utilized by several metabolic pathways and processes within the vertebrate body (Low et al., 2003). Nucleotides are essential to cells undergoing active cell division for the synthesis of new genetic material.

Structurally, nucleotides are derived from either purine or pyrimidine sources; pyrimidines consist of a single, nitrogen-containing hydrocarbon ring whilst purines consist of two ring structures. A complete nucleotide molecule comprises: a purine or pyrimidine base linked to a phosphate group and ribose sugar. Structures of common nucleotides are shown in Figure 1.2.

Nucleotides are derived from three sources: *de novo* synthesis within the body, from salvage pathways and diet sources (Low et al., 2003).

*De novo* synthesis of nucleotides from precursors is an energetically costly process, as is their salvage from sources within the body. Thus supplementation of nucleotide compounds in the diet is an effective way of overcoming the energy costs associated with cell proliferation (Low et al., 2003). All dividing cells require nucleotides to synthesize new genetic material and supplementation of preformed nucleotides reduces the metabolic costs of cell division, enhancing growth and tissue repair. Certain cells and tissues are particularly limited by the availability of nucleotides, notably the gut and immune tissues (Li and Gatlin, 2006). This may be due to either a total inability to synthesize nucleotides in the case of lymphocytes, macrophages and erythrocytes, or by demand exceeding production as is common in the gut of numerous organisms (Carver, 1994, Carver and Walker, 1995). Due to these limiting factors, these tissues benefit greatly from nucleotide supplementation. The specific effects of nucleotides on immunity will be discussed in detail below.
Figure 1.2. Structures of commonly occurring nucleotides.
A: Uridine 5' monophosphate (UMP), B: Guanosine 5' monophosphate (GMP), C: Cytidine 5' monophosphate (CMP), D: Adenosine 5' monophosphate (AMP).
(Source: www.sigmaaldrich.com)
1.5.4 Effects of nucleotides on the vertebrate immune system

Nucleotides are present in virtually every food source, yet vertebrates do not appear to specifically require an external source of nucleotides; mice fed diets devoid of nucleotides grow and survive as normal, although immune responses may be impaired (Jyonouchi et al., 1994, Jyonouchi and Sun, 1996, Jyonouchi et al., 1996, Calder, 2000, Jyonouchi et al., 2000, Grimble and Westwood, 2001). Ordinary immune function was found to be restored following re-feeding with nucleotides (Kulkani et al., 1989).

The mode of action of nucleotides on the immune system is thought to be through the sparing of energy during cell division through the provision of preformed molecules (Li and Gatlin, 2006). This removes the requirement of dividing cells to synthesise or salvage nucleotides to produce new genetic material in daughter cells. The immune system, particularly the cells of the IIS, is particularly nucleotide-limited in this way, as are the tissues of the gastrointestinal tract, making nucleotide supplementation of great potential benefit to these tissues.

Nucleotide supplementation was found to enhance resistance to challenge by a range pathogenic and parasitic organisms in salmonids (Carver, 1994, Carver and Walker, 1995, Burrells et al., 2001a). Various immune parameters, including phagocytosis, lysozyme and complement activity were enhanced in nucleotide supplemented carp, in addition to a reduction in the number of pathogenic bacteria in the blood (Sakai et al., 2001).

The effects of nucleotide supplemented diets on the immune system of fish are summarised in Table 1.4. Unlike β-glucans, the commonest route of administration for nucleotides is via the feed due to the small size of nucleotides, however Russo et al., (2006) express concern that even these molecules may undergo modification in the gastro-intestinal tract which may lower their efficiency as immuno-stimulants. Indeed, nucleotide injection has been found to elicit a stronger immune response than oral supplementation in mammals (Veerabagu et al., 1996).
Table 1.4. Effects of dietary supplementation with exogenous nucleotides on pathogen resistance and the teleost immune system.

<table>
<thead>
<tr>
<th>Effect</th>
<th>Species</th>
<th>Pathogen</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased bacterial resistance</td>
<td><em>Cyprinus carpio</em>[^1^]</td>
<td><em>Aeromonas hydrophila</em></td>
<td>[^1^](Sakai et al., 2001)</td>
</tr>
<tr>
<td></td>
<td><em>Oncorhynchus mykiss</em>[^2^]</td>
<td><em>Vibrio anguillarum</em></td>
<td>[^2^](Burrells et al., 2001a)</td>
</tr>
<tr>
<td></td>
<td><em>Oncorhynchus kisutch</em>[^2^]</td>
<td><em>Piscirickettsia salmonis</em></td>
<td>[^2^](Burrells et al., 2001a)</td>
</tr>
<tr>
<td></td>
<td><em>Morone chrysops X,</em></td>
<td><em>Streptococcus iniae</em></td>
<td>[^7](Li and Gatlin, 2004)</td>
</tr>
<tr>
<td></td>
<td><em>Morone saxatilis</em>[^6^,^7^]</td>
<td></td>
<td>[^8](Russo et al., 2006)</td>
</tr>
<tr>
<td></td>
<td><em>Epalzeorhynchos bicolor</em>[^8^]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased viral resistance</td>
<td><em>O. mykiss</em>[^2^]</td>
<td><em>Infectious Salmonid Anaemia</em> (ISA)</td>
<td>[^2^](Burrells et al., 2001a)</td>
</tr>
<tr>
<td></td>
<td><em>O. mykiss</em>[^5^]</td>
<td>(IPN) virus</td>
<td>[^5](Leonardi et al., 2003)</td>
</tr>
<tr>
<td>Increased parasite resistance</td>
<td><em>Salmo salar</em>[^2^,^3^]</td>
<td><em>L. salmonis</em></td>
<td>[^2^](Burrells et al., 2001a)</td>
</tr>
<tr>
<td>Increased lysozyme activity</td>
<td><em>C. carpio</em>[^1^]</td>
<td></td>
<td>[^1](Sakai et al., 2001)</td>
</tr>
<tr>
<td>Increased respiratory burst activity</td>
<td><em>C. carpio</em>[^1^]</td>
<td></td>
<td>[^6](Li et al., 2004)</td>
</tr>
<tr>
<td></td>
<td><em>M. chrysops X,</em>[^6^,^7^]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><em>M. saxatilis</em>[^6^,^7^]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Increased phagocytic activity</td>
<td><em>C. carpio</em>[^1^]</td>
<td></td>
<td>[^1](Sakai et al., 2001)</td>
</tr>
<tr>
<td>Increase specific antibody titre</td>
<td><em>S. salar</em>[^4^]</td>
<td></td>
<td>[^4](Burrells et al., 2001b)</td>
</tr>
<tr>
<td>Increased lymphocyte number</td>
<td><em>O. mykiss</em>[^5^]</td>
<td></td>
<td>[^5](Leonardi et al., 2003)</td>
</tr>
<tr>
<td>Increased complement activity</td>
<td><em>C. carpio</em>[^1^]</td>
<td></td>
<td>[^1](Sakai et al., 2001)</td>
</tr>
</tbody>
</table>
Nucleotides possess health promoting properties in addition to their direct immune enhancing abilities. Gut development, lipid metabolism and liver regeneration (Sanchez-Pozo et al., 1998, Gil, 2002b) and the development of a fully functioning GALT system (Gil, 2002a) have all been found to be enhanced following nucleotide supplementation.

In fish, nucleotide supplements have been shown to have positively effect stress resistance and growth performance in a variety of species. Growth enhancements have been widely reported in salmon (S. salar), carp (C. carpio) and tilapia (Oreochromis sp.) (Ramadan and Atef, 1991, Adamek et al., 1996, Burrells et al., 2001b, Sakai et al., 2001). Burrells et al., (2001b) found that Atlantic salmon (S. salar) show enhanced survival during saltwater transfer, a process known to induce significant stress, whilst Leonardi et al., (2003) found reduced plasma cortisol levels in rainbow trout (O. mykiss) following nucleotide supplementation, confirming the potential of nucleotides to modulate the stress response of fish.

Enhanced gut morphology, including improved villi height and gut muscle thickness were observed in nucleotide supplemented salmonids, promoting more efficient food handling and absorption (Burrells et al., 2001b). This improved feed uptake, coupled with the ability of certain nucleotides to act as feeding stimulants reported by Miyasaki and Harada (2003) may account for the enhanced growth commonly seen in nucleotide supplemented fish.

In common with other immuno-stimulants, detrimental effects can arise following high levels of supplementation; these include reductions in growth rates and lowered energy and nitrogen retention in juvenile European bass (Dicentrarchus labrax), although no direct immuno-suppressive effects were reported (Peres and Oliva-Teles, 2003).
1.6 Conclusion

The current chemotherapeutic agents available for the treatment of disease in ornamental fish have severe limitations and often have detrimental side effects either on the fish or its environment. Immuno-stimulants, such as β-glucans and nucleotides, can be administered easily through the feed and have no negative effects on environmental parameters. They promote health through activation and enhancement of the fish’s own immune defences and have secondary beneficial effects on growth and stress resistance. The administration of β-glucans and nucleotides as prophylactic measures prior to the onset of disease and as mechanisms of overcoming stress-induced immuno-suppression has great potential and could be regarded as an essential means of controlling disease in ornamental fish.

Due to the strong linkage between stress and immune function it is impossible to consider one without reference to the other. However, due to the ornamental nature of the fish species used in the current research and the policy of the Waltham Centre for Pet Nutrition, it was necessary to use only mild, non-invasive techniques for both the assessment of both stress and immune status. This explicitly limits sampling to the epidermal mucosa and peripheral blood. To this end, one of the aims of this research is to develop and validate a non-invasive stress monitoring method which allows inferences on stress status to be made from sampling the epidermal mucus. Once developed, this method will be used in conjunction with established assays of stress and immune status to assess the relative effects of acute and chronic stress on the immune status of ornamental fish. The potential of β-glucans and nucleotides supplied in the diet to modify the immune status of both unstressed and stressed fish will be investigated to assess the potential of these compounds to improve the general health status of ornamental fish. Finally, the specific and innate immune response to vaccination will be assessed. Due to the non-invasive nature of the research, a challenge with a live pathogen was
unacceptable so an attenuated vaccine was chosen to simulate infection. The effect of dietary immuno-stimulation at the time of vaccination will be assessed to determine the specific immuno-modulating effects of these compounds.

These research directions will contribute greatly to the knowledge of the general health status of a range of ornamental fish and their responses to stress, immuno-stimulation and the presence of a pathogen.
Chapter 2. General Research Protocols
2.1 Fish and Husbandry

Unless otherwise stated, goldfish (Carassius auratus), mirror carp (Cyprinus carpio) and tilapia (Oreochromis niloticus X. O. mossambicus) were obtained from commercial suppliers and transferred to the University of Plymouth packaged in filtered water in clear polythene bags with pure oxygen. Fish were allowed to recover from transport in darkness for 24 hours, after which light levels were slowly increased. Food was offered during this period, gradually increasing in response to the appetite of the fish. Approximately three to five days after arrival, fish were transferred to the experimental tanks. Following this transfer, fish were fed on a commercially available flake or pellet diet, Aquarian® [Masterfoods Complimentary Petcare, Birstall, Leeds, UK (MF)] until they were consistently feeding at 2 – 3 % of their body weight per day (BW.d-1)

2.1.1 Experimental Systems

Experiments were conducted in one of two systems, as detailed below:

System A (Plate 2.1) comprised 20 tanks arranged in a recirculation system in the experimental aquarium of the University of Plymouth. Tank capacity was 170 litres of water with a flow input of approximately 10 L.min⁻¹ through the tank.

System D (Plate 2.2) comprised 10 tanks arranged in a recirculation system in the experimental aquarium of the University of Plymouth. Tank capacity was 75 litres of water with a flow input of approximately 8 L.min⁻¹ through the tank.

Unless otherwise stated, water temperature was maintained automatically at 23 ± 1 °C and pH at 7 ± 0.5, with a photoperiod set to 12 hours light: 12 hours dark using artificial light from fluorescent tubes.
Plate 2.1. Re-circulating tank systems located in the experimental aquarium at the University of Plymouth as used in the course of this research.

a) System A

b) System D
Other water quality parameters were monitored and maintained within the tolerance limits for the carp (*C. carpio*) and goldfish (*C. auratus*) reported by Rottmann and Shireman (1990). Specific parameters were maintained as follows: unionised ammonia (NH$_3$) <0.01 ppm, nitrite (NO$_2$) <0.01 ppm, nitrate (NO$_3$) < 20 mg.l$^{-1}$ and dissolved oxygen (DO$_2$) 5-7 mg.l$^{-1}$.

2.1.2 Diets

Four diets were formulated according to specific nutrient requirements of the species used (NRC (National Research Council), 1999) and their composition is presented in Table 2.1. Dietary analysis was carried out and supplied by MF.

Control diet

Aquarian$^\circledR$ (MF) food in either flake or pellet form was used during acclimation prior to and as the control diet during all trials. The nutrient composition of this diet is identical to that available commercially, but for experimental proposes this diet was supplied and used in an un-pigmented form to minimise variation in nutritional content between flake and pellet version of the diet.

β-glucan diet

Un-pigmented Aquarian$^\circledR$ flake or pellet supplemented with 0.2% w/w Macrogard$^\circledR$, [Biotec Ltd, Tromso, Norway] at the formulation stage by MF. The β-glucans in this supplement were derived from the yeast *Saccharomyces cerevisiae* which provides both β-1,3 and β-1,6 linked structures as shown in Table 2.2.
Table 2.1. Nutritional analysis of the experimental diets  
(Source: Master Foods Complementary Petcare, Leeds, UK).

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Unit</th>
<th>Control</th>
<th>β-glucan</th>
<th>Nucleotide</th>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture</td>
<td>%</td>
<td>5.00</td>
<td>4.50</td>
<td>5.30</td>
<td>4.00</td>
</tr>
<tr>
<td>Protein</td>
<td>%</td>
<td>35.50</td>
<td>35.75</td>
<td>37.00</td>
<td>36.75</td>
</tr>
<tr>
<td>Lipid</td>
<td>%</td>
<td>11.75</td>
<td>11.70</td>
<td>11.40</td>
<td>11.55</td>
</tr>
<tr>
<td>Ash</td>
<td>%</td>
<td>8.40</td>
<td>8.55</td>
<td>8.65</td>
<td>8.70</td>
</tr>
<tr>
<td>Fibre</td>
<td>%</td>
<td>4.70</td>
<td>4.40</td>
<td>5.80</td>
<td>5.00</td>
</tr>
<tr>
<td>Gross Energy</td>
<td>kJ/100 g⁻¹</td>
<td>2008.66</td>
<td>2015.29</td>
<td>2000.66</td>
<td>2024.42</td>
</tr>
<tr>
<td>ME (est.)</td>
<td>kJ/100 g⁻¹</td>
<td>1693.53</td>
<td>1698.33</td>
<td>1677.02</td>
<td>1701.05</td>
</tr>
<tr>
<td>P / ME</td>
<td>mg.kJ⁻¹</td>
<td>20.96</td>
<td>21.05</td>
<td>22.07</td>
<td>21.60</td>
</tr>
</tbody>
</table>

ME = metabolisable energy; P / ME = protein / metabolisable energy.

Table 2.2. Composition of Macrogard®  
(Source: Biotec Ltd., Tromso, Norway)

<table>
<thead>
<tr>
<th>Structure</th>
<th>Amount (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-1,3 linkages</td>
<td>83</td>
</tr>
<tr>
<td>β-1,6 linkages</td>
<td>5</td>
</tr>
<tr>
<td>β-1,6 branching points</td>
<td>6</td>
</tr>
<tr>
<td>Non-reducing terminal glucose molecules</td>
<td>5</td>
</tr>
</tbody>
</table>

Nucleotide diet

Un-pigmented Aquarian® flake or pellet supplemented with 0.2% w/w of Optimun® [Chemoforma Ltd, Augst, Switzerland] at the formulation stage by the MF. Optimun® contains the following nucleotide bases: cytidine mono-phosphate (CMP), uridine mono-phosphate (UMP), adenosine monophosphate (AMP) and guanidine mono-phosphate (GMP) in addition to intact RNA.

Combined-supplement diet

Un-pigmented Aquarian® supplemented with 0.2% w/w Macrogard® and 0.2% w/w Optimun® during the formulation process by MF.
Nucleotide and β-glucan analysis

The amount of β-(1,3:1,6) glucan in the four diets was determined at the University of Plymouth using a commercially available test kit [Megazyme, Bray, Ireland]. The β-glucan content (% w/w) of the diets was as follows: control, 3.8 %; nucleotide, 0.4 %; β-glucan, 7.8 % and combined (beta-glucan + nucleotide) diet, 8.4 %.

The nucleotide content of the four diets was determined by Chemoforma using HPLC. The total nucleotide content of the diets was determined as follows: control, 0.3 %; β-glucan 0.3 %; nucleotide, 0.6 % and combined (beta-glucan + nucleotide) diet 0.6 %.

2.2 Stressors

Following capture in hand-nets, all the fish within a tank were confined in a single net and subjected to a stressor modified from the method described by Schwalme & Mackay (1985). A combination of aerial exposure and confined immersion was applied to the fish in cycles of 30 seconds of aerial exposure followed immediately by 30 seconds of confined immersion. The number of cycles varied depending on the level of stress required and details of the duration of the stressor are given in the relevant chapters. Once the stressor had been applied, all fish were released back into the tank.

2.3 Anaesthetisation and sample collection

All fish were removed from their respective tanks and transferred into an anaesthetic bath containing Tricane Methane Sulphonate (MS222) [Pharmaq, Fordingbridge, UK] at a concentration of 100 mg.L⁻¹ (Ross, 2001). Anaesthesia was monitored by loss of equilibrium in the fish which indicated it was suitably sedated for subsequent sampling.
Following anaesthetisation, blood was drawn from the caudal vein using a 1 ml syringe fitted with a 25 gauge needle. Following sampling, fish were allowed to recover in a well aerated water bath and were immediately returned to a holding tank when judged fully recovered from anaesthetisation. Anaesthetisation was not applied to fish which were only being sampled for mucus using Multistix® (section 2.4.1).

Prior to the drawing of blood the syringe was flushed with a 5000 U.ml$^{-1}$ of sodium-heparin [Sigma-Aldrich, Poole, UK], where plasma was being prepared. Blood was centrifuged at 4000 x g for 5 minutes and the resulting plasma was taken off and stored at -80 °C until used for assay purposes.

Serum was prepared using an un-heparinised syringe; collected blood was allowed to clot at room temperature for 3 – 4 hours before centrifugation at 9000 x g for 5 minutes. The resulting serum was removed and re-centrifuged at 9000 x g for a further 1 minute before storage at -80 °C until used for assay purposes.

2.4 Analytical techniques

2.4.1 Mucosal parameters

Mucosal stress indicators were analysed using Multistix® 10 SG [Bayer Plc., Newbury, UK] which have reagent sites for the measurement of ten physiologically important molecules. The reagent sites are as follows: glucose, acetoacetate, bilirubin, specific gravity (SG), erythrocytes, pH, protein, urobilinogen, nitrite and leukocytes. The target molecules and specificity of the reagent sites are presented in Table 2.3. Fish were removed from the water and a Multistix® 10 SG strip was quickly swabbed along the flank of the fish, avoiding the head and vent regions, ensuring that each reagent site was coated with mucus (Plate 2.2). The strip was then left for the reagent sites to develop colour and was read at the times specified by the manufacturer against the supplied colour chart.
Table 2.3. Reagent site specificity of Multistix® 10 SG.
(Source: Bayer Plc. Newbury UK)

<table>
<thead>
<tr>
<th>Site</th>
<th>Sensitivity</th>
<th>Target</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>4 – 7 mmol.L(^{-1})</td>
<td>Glucose</td>
<td></td>
</tr>
<tr>
<td>Bilirubin</td>
<td>7 – 14 µmol.L(^{-1})</td>
<td>Bilirubin</td>
<td></td>
</tr>
<tr>
<td>Acetoacetate</td>
<td>0.5 – 1.0 mmol.L(^{-1})</td>
<td>Acetoacetate</td>
<td></td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>150 – 620 µg.L(^{-1})</td>
<td>Haemoglobin</td>
<td>Equally sensitive to Myoglobin.</td>
</tr>
<tr>
<td>pH</td>
<td>0.5 pH unit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>0.15 – 0.30 g.L(^{-1})</td>
<td>Albumin</td>
<td></td>
</tr>
<tr>
<td>Nitrite</td>
<td>13 – 22 µmol.L(^{-1})</td>
<td>Nitrite ion (NO(_2)(^-))</td>
<td></td>
</tr>
<tr>
<td>Leukocytes</td>
<td>5 – 15 cells.µL(^{-1})</td>
<td>Leukocyte esterase</td>
<td></td>
</tr>
</tbody>
</table>

In order to perform statistical analysis, the following conversions were made for haemoglobin, bilirubin and nitrite from the manufacturers scale to numerical data:

Haemoglobin: negative = 0, trace = 1, ++ = 3, +++ = 4;

Bilirubin: negative = 0, ++ = 2, +++ = 3;

Nitrite: negative = 0, positive = 1

Colour change was either read visually by the experimenter, or read automatically using a Clinitek® strip reader [Bayer Plc., Newbury, UK] to ensure consistency between readings, the exact method is detailed in the relevant chapters.
Plate 2.2. Sampling of epidermal mucus using Multistix® 10 SG.

a) The strip was applied to the flank of the fish above the lateral line

b) The strip was drawn along the flank of the fish towards the caudal region. This process was repeated two to three times
c) Once the reagent sites were coated with mucus, the strip was left to develop colour according to the manufacturer's specifications.
2.4.2 Haematological Parameters

Plasma glucose was analysed on an individual fish basis, unless otherwise stated, using a commercially available test kit [Sigma-Aldrich], which utilises the glucose oxidase method. The enzymatic reaction was terminated using 12 N H₂SO₄ and the resultant colour change was read at 450 nm using a JASCO V-350 spectrophotometer. Calibration was carried out using a 1.0 mg.ml⁻¹ glucose standard solution [Sigma-Aldrich]. Differential leukocyte counts were conducted on peripheral blood. Blood smears were prepared immediately following blood removal. Blood smears were air-dried, fixed in methanol for 5 minutes and stained with Giemsa stain [BDH, Poole, UK], rinsed with water, air-dried and examined under a light microscope. A minimum of 200 cells per slide were counted to generate a statistically significant sample. Neutrophils, monocytes, thrombocytes and lymphocytes were identified following the classifications of (Hibiya, 1982, Yasutake and Wales, 1983) and the total number of leukocyte class was expressed as a percentage of the total leukocyte population.

2.5 Statistical analysis

Unless otherwise stated all data are presented as median (inter-quartile range). The group of fish contained in each tank was treated as the unit of replication, not individual fish themselves. Data were analysed using the non-parametric Kruskal Wallis test with post hoc multiple comparisons testing using Minitab 14. Non parametric testing was necessary due to both the non continuous nature of the data obtained by Multistix® and the consistent lack of normality in the data sets. Differences between medians are considered significant when the associated probability (p) ≤ 0.05. Significant differences between medians within the same treatment are indicated by superscripts pairs (i.e. a/b, c/d etc.), where applicable, differences between treatments are indicated by asterisks.
Relationships were calculated using Kendall’s Tau (Minitab 14) with an associated p value of ≤ 0.05 being required to consider a relationship significant.
Chapter 3. Development of a non-invasive stress monitoring technique. A: Validation of urinalysis strips and a comparison between visual and instrument assessment methods
3.4.1 Introduction

The neuro-endocrine and immune systems of vertebrates are inextricably linked (see section 1.3), a situation that is perhaps even more pronounced in fish. This ensures that accurate measurement and assessment of immune status must take into account the stress loading acting on the fish. Physiological stress can have both immuno-enhancing and immuno-suppressive effects, with the nature and duration of the stressor being the determining factor (Dhabhar and McEwen, 1997).

The metabolic and physiological adjustments associated with an organism’s stress responses are complex and numerous authors have adapted the original definition of stress, and its subsequent modifications, proposed by Selye (1936, 1950, 1953). However, the underlying themes remain constant; that of a stimulus (termed the stressor), a consequential brain reaction following perception of the stressor which initiates physiological systems associated with the fight-flight reaction (Dhabhar and McEwen, 1997, Dhabhar, 2002). It is this last component that comprises the stress response of an organism. A more complete definition of stress and stressor, which is used for the purposes of this thesis is that outlined by Plytycz and Seljelid (2002) which builds on the original definitions of stress and integrates them with more recent work (Selye, 1936, Selye, 1978, Tsigos and Chrousos, 1995, Chrousos, 1998) to state a stress response as:

"... a state of threatened homeostasis or disharmony and is counteracted by a complex repertoire of physiologic and behavioural adaptive responses that establish homeostasis."

As stated in section 1.3, the response to stress, especially that of the immune system, is largely dependent on the duration of stress and to this end, stressors have being labelled acute for short duration and chronic for prolonged exposures (Adams, 1990). An
alternative concept described by Plytycz and Seljelid (2002) defines stressors by the physiological response generated; those that induce stimulatory responses beneficial to survival, well-being or reproduction can be termed eustress, whilst those inducing detrimental effects are termed distress. These definitions become useful when determining the effects of stress on the immune system.

Stressors themselves have numerous classifications and can be broadly divided into three groups; physical, psychological and physiological. However, as discussed by Dhabhar (2002), a stressor rarely falls purely into one category and may induce stress in more than one way, making it difficult to isolate the causal elements of stress. Wounding for example, can be defined as a physical stressor, yet may involve some psychological and physiological components (Dhabhar, 2002).

The physiological changes experienced by fish following stress comprise two key pathways: the autonomic nervous response and the neuro-endocrine response. The autonomic response is controlled primarily by the Sympathetic Nervous System (SNS) which acts on the chromaffin tissues to release catecholamines. The neuro-endocrine response involves activation of the HPI axis with the resultant release of glucocorticoid hormones from the inter-renal cells.

The use of blood indicators in the diagnosis of stress has a long history and the dynamics of several parameters have received considerable study in a wide variety of fish species and in response to a range of stressors.

Cortisol, as the primary glucocorticoid in fish, is often used to assess the degree of stress. Due to the relative ease of detection for cortisol in blood and tissues, the dynamics of numerous fish species are known, including those of cyprinids, such as Cyprinus carpio, to several stressful situations (Davis and Parker, 1986, Pottinger, 1998, Tanck et al., 2000, Ruane et al., 2001, Ruane et al., 2002a, Ruane et al., 2002b). The rapid release of cortisol and its role as a primary stress hormone make it less applicable when
considering the physiological adaptations invoked by the application of a stressor. In such conditions, the secondary stress indicators, such as blood glucose concentration and peripheral leukocyte number can be more indicative of the duration and degree of stress experienced by the fish. Other indicators of stress in blood used to quantify stress in cyprinids include; lactate levels, haematocrit and leukocrit (Ruane et al., 2001, Ruane et al., 2002a, Ruane et al., 2002b). Standard values for common stress indicators in fish blood are presented in Table 3.A.1. However, it should be noted that it is virtually impossible to obtain a stress-free sample due to the inherently stressful nature of blood sampling.
<table>
<thead>
<tr>
<th>Species</th>
<th>Cortisol (ug.dL⁻¹)</th>
<th>Glucose (mg.dL⁻¹)</th>
<th>Haematocrit (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 – 30 [8]</td>
<td></td>
</tr>
<tr>
<td></td>
<td>~ 0.4 [10]</td>
<td>~ 40 [9]</td>
<td></td>
<td>[8] (Koss and Houston, 1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[13] (Ruane et al., 2002a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[14] (Houston and de Wilde, 1968)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[15] (Fourie and Hattingh, 1976)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[16] (Van Vuren and Hattingh, 1978)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[17] (Nolan et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>1.0 – 1.3 [20]</td>
<td></td>
<td></td>
<td>[20] (Iwama et al., 1997)</td>
</tr>
</tbody>
</table>
Secondary stress indicators often show highly variable or inconsistent responses to stress, limiting their use as diagnostic tools. In particular haematocrit and blood lactate levels are commonly employed stress indicators despite a lack of consistent responses following exposure to stress (Houston, 1997).

Due to the need for the majority of stress quantification techniques to utilise blood samples, there remains the inherent danger of sample-induced stress either preventing an accurate diagnosis or having the effect of increasing the loading on already stressed fish. Catecholamines and glucocorticoids can show significant elevations within minutes following stress, (Davis and Parker, 1986) making it imperative that sampling is conducted quickly to preserve the quality of data obtained. However, blood sampling can be difficult to achieve with small fish, particularly where subsequent recovery is required, a situation common with high value fish species, such as Koi carp (*C. carpio*).

In addition to the constraints of sample collection, there is often a considerable delay between sample acquisition and the generation of data for these techniques, coupled with a reliance on skilled personnel and laboratory facilities which limits their application, particularly in the ornamental fish trade. There is currently a niche for non-invasive methods that may allow the acquisition of rapid results for use as stress diagnosis tools. These would allow significant improvements in the assessment of disease and health in the aquarium and ornamental fish industry.

One potential non-invasive means of determining the level of stress experienced by fish is through the measurement of cortisol in tank water. This method has been successfully developed for salmonids and cyprinids (Scott and Sorensen, 1994, Scott et al., 2001, Ruane and Komen, 2003). However, a dependency on laboratory equipment remains and the considerable associated lag time and inability to accurately assess the level stress experienced by individual fish within a group limit the applicability of this method to the ornamental fish industry.
The integument of fish and its protective coating of mucus provide a suitable surface for the assessment of stress. As described in section 1.2.3, goblet cell numbers and mucus production are altered by stress (Wood and Yasutake, 1957, Ojha and Munshi, 1974, Pickering and Macey, 1977, Marshall, 1979, Zuchelkowski et al., 1981, Laurent, 1984). In addition, stress induces compositional changes in the mucus matrix, which are reflected in alterations in pH and viscosity (Fletcher and Grant, 1968, Lebedeva and Golovkina, 1987).

The inclusion of molecules within the mucus matrix is similarly affected by stress, in particular protein and haemoglobin concentration show strong stress-linked dynamics (Smith and Ramos, 1976, Isaacson and Morrisson, 1980, Peters et al., 1980, Lebedeva et al., 1999, Lebedeva, 1999). Ketone bodies, especially acetoacetate, appear to be linked to the nutritional history of the fish; high mucosal levels are indicative of nutritionally deficient or fasting fish (Ramos and Smith, 1978, Perrier et al., 1986, Heming and Paleczny, 1987, Lebedeva, 1999). Values for the normal unstressed values of these parameters with fish mucus (assessed using urinalysis strips) are presented in Table 3.A.2.

Changes in mucosal parameters have been detected and quantified using commercially available urinalysis strips in the mucus of several fish species (Smith and Ramos, 1976, Isaacson and Morrisson, 1980, Lebedeva, 1999, Lebedeva et al., 2000, Lebedeva et al., 2001). The authors of these trials concluded that clear stress-induced changes in both mucus and the molecules included within its matrix occur.
Table 3.A.2. Biochemical profile of the epidermal mucus of unstressed fish.

<table>
<thead>
<tr>
<th>Species</th>
<th>Haemoglobin (Arb. Units)</th>
<th>Acetoacetate (mmol.L(^{-1}))</th>
<th>Protein g.L(^{-1})</th>
<th>pH</th>
<th>Specific Gravity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypophthalmichthys molitrix (^{[1,3]})</td>
<td>1</td>
<td>1.3 – 2.1</td>
<td>1.1 – 2.0</td>
<td>5.8 – 6.9</td>
<td>--</td>
<td>(Lebedeva, 1999)</td>
</tr>
<tr>
<td></td>
<td>0-1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>(Lebedeva et al., 1999)</td>
</tr>
<tr>
<td>Cyprinus carpio (^{[1,3]})</td>
<td>1</td>
<td>0.4 – 1.4</td>
<td>0.65 – 1.2</td>
<td>6.6 – 6.7</td>
<td>--</td>
<td>(Lebedeva, 1999)</td>
</tr>
<tr>
<td></td>
<td>0-1</td>
<td>~ 8</td>
<td>~ 0.35</td>
<td>--</td>
<td>--</td>
<td>(Lebedeva et al., 1999)</td>
</tr>
<tr>
<td>Ctenopharyngodon idella (^{[1]})</td>
<td>1</td>
<td>0.85</td>
<td>0.93</td>
<td>7.1</td>
<td>1.013</td>
<td>(Lebedeva, 1999)</td>
</tr>
<tr>
<td>Misgurnus fossilis (^{[1]})</td>
<td>1</td>
<td>0.6</td>
<td>0.3</td>
<td>6.8</td>
<td>1.020</td>
<td>(Lebedova, 1999)</td>
</tr>
<tr>
<td>Oncorhynchus mykiss (^{[1,2,1]})</td>
<td>0-1</td>
<td>0.5 – 0.15</td>
<td>0.33 – 1.3</td>
<td>5.7 – 7.0</td>
<td>--</td>
<td>(Lebedeva, 1999)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.1</td>
<td>0.44</td>
<td>5.9</td>
<td>1.010</td>
<td>(Heming and Paleczny, 1987)</td>
</tr>
<tr>
<td>Clarias gariepinus (^{[1]})</td>
<td>1-2</td>
<td>0.85</td>
<td>0.72</td>
<td>8.00</td>
<td>--</td>
<td>(Lebedeva, 1999)</td>
</tr>
<tr>
<td>Acipenser gueldenstaedti (^{[1]})</td>
<td>0-2</td>
<td>0 – 0.5</td>
<td>0.5 – 1.0</td>
<td>5.5 – 7.9</td>
<td>--</td>
<td>(Lebedeva, 1999)</td>
</tr>
<tr>
<td>Oreochromis sp. (^{[3]})</td>
<td>0-1</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>(Lebedeva et al., 1999)</td>
</tr>
</tbody>
</table>
However, despite the identification and publication of stress-induced changes in a variety of mucosal parameters, no comparisons appear to have been drawn with the dynamics of known and validated stress indicators within the blood to support the use of mucosal stress indicators.

The current trial aimed to assess the validity of the detection of mucosal parameters using a commercially available urinalysis strip, Multistix® 10 SG [Bayer Plc.] in a model ornamental species, the mirror carp (Cyprinus carpio). This species was selected on merit of its status as the genetic source of two important ornamental strains; Koi carp and Ghost Koi (Michaels, 1988, Balon, 1995).

In addition to quantifying the mucosal stress response in this species, comparisons were made between mucosal parameters and established and validated stress indicators in the blood to establish any link between the blood and mucosal stress response. Finally comparisons were made between results obtained from visual assessment of the strips and those obtained using the dedicated Clinitek-Status® strip reader [Bayer Plc.].

Specifically, the hypotheses tested were as follows:

H₀₁ – mucosal acetoacetate, haemoglobin, protein and leukocyte concentration as determined by Multistix® will show significant relationships with plasma glucose and protein concentrations.

H₀₂ – mucosal acetoacetate, haemoglobin, protein and leukocyte concentration as determined by Multistix® will positively correlate with peripheral neutrophil, monocyte and thrombocyte counts and negatively correlate with peripheral lymphocyte counts.
$H_0.3$ – results obtained for mucosal stress indicators following visual assessment and instrument assessment of Multistix® will not differ significantly.
3.A.2 Materials and Methods

Two hundred and ninety six (296) mirror carp (*Cyprinus carpio*) were obtained from S&L Fisheries (Kemberton, UK) and divided between eight tanks in system D in the experimental aquarium at the University of Plymouth as described in section 2.1. Two replicate groups of fish were analysed together in four repeats of the experiment and the mean weights of the fish for each run are presented in Table 3.A.3. Fish were fed to satiation twice daily on standard Aquarian® pellet (section 2.2).

3.A.2.1 Stressors

All fish received a six minutes (six cycles) netting stress administered as described in section 2.2. The sampling strategy is outlined in Table 3.A.4. Five fish per group were sampled for mucosal stress indicators at each sample point. Three fish per group were sampled for haematological stress indicators at each sample interval. Fish were removed from the trial following sampling, ensuring no re-sampling of fish occurred.

3.A.2.2 Analytical techniques

Mucosal parameters

Mucosal stress indicators were analysed using Multistix® 10 SG [Bayer Plc.] as described in section 2.4.1.

Haematological parameters

Blood was collected as described in section 2.3.
Table 3.A.3. Fish weights of mirror carp (Cyprinus carpio) used in the investigation
Values are means (± 1 S.D.)

<table>
<thead>
<tr>
<th>Run</th>
<th>Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>32.9 (± 1.3)</td>
</tr>
<tr>
<td>2</td>
<td>36.3 (± 1.1)</td>
</tr>
<tr>
<td>3</td>
<td>42.0 (± 2.5)</td>
</tr>
<tr>
<td>4</td>
<td>39.6 (± 1.6)</td>
</tr>
</tbody>
</table>

Table 3.A.4. Experimental Protocol (Validation trial)

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Sampling times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Mucosal Stress indicators</td>
<td>Pre-stress 1.5, 3, 5, 24 &amp; 168 hours post-stress</td>
</tr>
<tr>
<td>2. Plasma protein</td>
<td>Pre-stress 1.5, 3, 5, 24 &amp; 168 hours post-stress</td>
</tr>
<tr>
<td>3. Plasma glucose</td>
<td>Pre-stress 1.5, 3, 5, 24 &amp; 168 hours post-stress</td>
</tr>
<tr>
<td>4. Differential leukocyte counts</td>
<td>Pre-stress 1.5, 3, 5, 24 &amp; 168 hours post-stress</td>
</tr>
</tbody>
</table>

Total protein

Plasma protein concentration was assayed using a commercially available test kit [Bio-Rad, Hemel Hempstead, UK] which uses a modification of the Bradford method (Bradford, 1976).

Glucose

Plasma glucose concentration was assayed as described in section 2.4.2.

Differential leukocyte counts

Blood smears were prepared and analysed as described in section 2.4.2.
3.A.2.3 Statistical analysis

Unless otherwise stated, data is presented as median (interquartile-range). Data was analysed using the non-parametric Kruskal-Wallis test with post hoc multiple comparison analysis (Minitab 14). Relationships were tested using Kendall’s Tau (Minitab 14), with an associated p value of ≤ 0.05 being required for a relationship to be considered significant.
3.A.3 Results

3.A.3.1 Mucosal parameters

Upon contact with fish mucus, no colour change was observed in the reagent sites for glucose, bilirubin, urobilinogen or nitrite, indicating their absence from epidermal mucus. Colour change was observed in the reagent sites for acetoacetate, specific gravity, haemoglobin, pH and protein following contact with fish mucus.

The results obtained from the reagent site for specific gravity were inconsistent and very easily contaminated by tank water, making the data spurious, therefore the data was not considered robust enough for statistical analysis and is omitted here. Similarly the reagent site for pH was limited in its precision, only giving results to the nearest pH unit, which ensured that the data set was again spurious so has been omitted. Care was taken to ensure that there were no false positive results; the reagent sites for haemoglobin, acetoacetate, protein and leukocytes did not show any colour change following contact with the researcher’s hands or from tank water.

The observed mucosal acetoacetate concentration was significantly altered following stress when the strips were visually assessed (Table 3.A.5); with a significant reduction at 3 hours post-stress compared to pre-stress values. Contrastingly, when analysed using the instrument, no acetoacetate was observed in the mucus, concentrations are shown in (Table 3.A.5).

Mucosal haemoglobin increased following stress, with significant observed elevations from 1.5 – 5 hours after stress (Figure 3.A.1).
Table 3.A.5. Mucosal acetoacetate (mmol.L\(^{-1}\)) of mirror carp (Cyprinus carpio) following netting stress. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found.

<table>
<thead>
<tr>
<th>Run</th>
<th>Time elapsed after stress (hr(^{-1}))</th>
<th>Pre</th>
<th>1.5</th>
<th>3</th>
<th>5</th>
<th>24</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual</td>
<td></td>
<td>0.3^b</td>
<td>(0-0.5)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median 1-3</td>
<td></td>
<td>0^a</td>
<td>(0-0)</td>
<td>(0-0)</td>
<td>(0-0.5)</td>
<td>(0-0.1)</td>
<td>(0-0.1)</td>
</tr>
</tbody>
</table>

Where strips were visually analysed, elevated levels were apparent at 3 hours post stress (compared to pre 24 and 168 hours post stress) and at 1.5 and 5 hours post stress (compared to 168 hours post-stress). Elevation in mucosal haemoglobin as determined following instrument reading of the strips occurred at 3 and 5 hours post stress in all runs (compared to pre and 168 hour post-stress values). Differences in the recovery pattern of the fish were apparent, with fish that had been tested and the strips visually assessed showing recovery to pre-stress values at 24 hours; those strips that had undergone machine assessment indicated recovery took longer to occur, at 168 hours (Figure 3.A.1). Mucosal haemoglobin concentrations reported by the strips that had been assessed using the Clinitek machine were lower than those assessed visually. The response of the three instrument analysed runs was highly consistent, especially in the first 5 hours post stress (Figure 3.A.1).

Mucosal protein concentrations were significantly elevated from 1.5 – 5 hours compared to the 24 and 168 hour recovery samples regardless of the method used to read the strips (Figure 3.A.2). For the machine analysed strips, the increases in mucosal protein were significant compared to the pre-stress values for each of the runs.
Figure 3.A.1. Mucosal haemoglobin (arbitrary units) of mirror carp (*Cyprinus carpio*) following netting stress. Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by run code (V, IR₁, IR₂ or IR₃) and superscript letter pairs (i.e. a/b, c/d etc.).

Figure 3.A.2. Mucosal protein (g.L⁻¹) of mirror carp (*Cyprinus carpio*) following netting stress. Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by run code (V, IR₁, IR₂ or IR₃) and superscript letter pairs (i.e. a/b, c/d etc.).
Table 3.A.6. Mucosal leukocytes (cells,\(\mu l^{-1}\)) of mirror carp (\textit{Cyprinus carpio}) following netting stress. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found.

<table>
<thead>
<tr>
<th>Run</th>
<th>Pre</th>
<th>1.5</th>
<th>3</th>
<th>5</th>
<th>24</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visual</td>
<td>15\textsuperscript{a} (15-70)</td>
<td>125\textsuperscript{b} (70-125)</td>
<td>125\textsuperscript{bd} (125-1250)</td>
<td>125\textsuperscript{bf} (112-125)</td>
<td>70\textsuperscript{c} (15-125)</td>
<td>42.5\textsuperscript{ee} (15-125)</td>
</tr>
<tr>
<td>Clinitek</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
<tr>
<td>Median 1-3</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
<td>0 (0-0)</td>
</tr>
</tbody>
</table>

As was the case for mucosal haemoglobin, protein concentrations reported by the machine method were generally lower than those obtained by visual analysis of the strips (Figure 3.A.2).

Clear differences in the reported number of mucosal leukocytes were observed. When visually assessed, reported mucosal leukocytes concentrations were significantly elevated above pre-stress values from 1.5 – 5 hours post-stress (Table 3.A.6). Values at 3 and 5 hours post stress were also higher than those at 24 and 168 hours post stress. However, when the instrument was used to analyse the strips, no leukocytes were detected and no significant alterations in mucosal leukocytes were found in these fish (Table 3.A.6).

Having established that mucosal data from the visually assessed strips were spurious this data was removed from further analysis. The corresponding data for blood stress indicators sampled during this part of the experiment were also not analysed.
Figure 3.A.3. Percentage peripheral leukocyte counts of mirror carp (Cyprinus carpio) following netting stress. A: lymphocytes B: monocytes

Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by run code (1, 2 or 3) and superscript letter pairs (i.e. a/b, c/d etc.).
Figure 3.A.4. Percentage peripheral leukocyte counts of mirror carp (*Cyprinus carpio*) following netting stress.

A: neutrophils  
B: thrombocytes  

Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by run code (1, 2 or 3) and superscript letter pairs (i.e. a/b, c/d etc.).
3.A.3.2 Haematological Parameters

All the classes of leukocyte present showed significant alterations following stress (Figures 3.A.3 & 3.A.4). Percentage neutrophils were elevated above pre-stress values from 1.5 – 5 hours post stress, in the first run, with the duration of elevation decreasing with run number (Figure 3.A.4 A). Monocytes showed similar elevations with values 1.5 – 5 hours being significantly greater than pre-stress values. These elevations were consistent between runs (Figure 3.A.3 B). Significant elevations in thrombocyte number were apparent from 1.5 – 24 hours post-stress (Figure 3.A.4 B), with the most sustained elevation being shown by fish sampled during the second run of the experiment. Reductions in peripheral lymphocyte numbers were observed over a similar time period (1.5 – 24 hours) (Figure 3.A.3), with values for both leukocyte classes returning to pre-stress levels by 168 hours post-stress (Figures 3.A.3 A & 3.A.4 B). The most sustained reductions in lymphocyte number were observed in fish sampled during the first run of the experiment, and occurred between 1.5 – 24 hours post-stress (Figure 3.A.3 A), whilst those sampled during the second and third runs showed less sustained reductions in lymphocyte number, at 3 and hours post-stress.

Of the blood stress indictors measured, plasma protein showed no changes following stress and plasma protein concentration ranged from 2.7 – 2.9 g.dl⁻¹.

Plasma glucose concentrations were consistently elevated above pre and 168 hour post-stress values from 1.5 – 5 hours post-stress (Figure 3.A.5). The glucose response to stress was very consistent across the three runs of the experiment.
Figure 3.A.5. Plasma glucose concentration (mg.dl$^{-1}$) of mirror carp (*Cyprinus carpio*) following netting stress.

Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by run code (1, 2 or 3) and superscript letter pairs (i.e. a/b, c/d etc.).

### 3.A.3.3 Relationships

Relationships between blood and mucosal parameters were sought in order to establish any linkage between the blood and mucosal stress response. Relationships between variables dependent on each other, for example peripheral leukocyte counts, were ignored. Only the instrument analysed data was used for the purposes of establishing relationships, the visually assessed strips being considered too spurious for this purpose. In order to preserve equal sample sizes, the corresponding haematological data from this run was not included.
Figure 3.A.6. Relationship analysis (Kendall’s Tau) of glucose and mucosal stress indicators of mirror carp (*Cyprinus carpio*) following netting stress.

A: plasma glucose vs. mucosal haemoglobin, KT = 0.86, p = < 0.001
B: plasma glucose vs. mucosal protein, KT = 0.70, p = 0.002

Each value represents the run median (n = 10) of both sampled parameters at a single time point.
Figure 3.A.7. Relationship analysis (Kendall's Tau) of glucose and peripheral leukocytes of mirror carp (Cyprinus carpio) following netting stress.

A: plasma glucose vs. peripheral monocytes, KT = 0.88, p < 0.001
B: plasma glucose vs. peripheral lymphocytes, KT = -0.63, p < 0.001

Each value represents the run median (n = 10) of both sampled parameters at a single time point.
Figure 3.A.8: Relationship (Kendall’s Tau) analysis of mucosal haemoglobin and protein and other stress indicators of mirror carp (Cyprinus carpio) following netting stress.

A: mucosal haemoglobin vs. mucosal protein, KT = 0.83, p = 0.004
B: mucosal haemoglobin vs. peripheral monocytes, KT = 0.86, p = < 0.001
C: mucosal protein vs. peripheral monocytes, KT = 0.69, p = 0.01

Each value represents the run median (n = 10) of both sampled parameters at a single time point.
As a well established and validated stress indicator glucose was used as the primary reference point for assessing the validity of the mucosal stress indicators. Both mucosal haemoglobin and protein showed significant relationships with plasma glucose, with Kendall’s Tau (KT) values of 0.86, \( p = < 0.001 \) and 0.70, \( p = 0.002 \) respectively (Figure 3.A.6, A & B). Plasma glucose showed significant relationships with peripheral monocytes and lymphocytes (KT = 0.88, \( p = < 0.001 \) & -0.63, \( p = < 0.001 \) respectively) (Figure 3.A.7, A & B).

Further relationships could be established between both mucosal haemoglobin and protein and peripheral monocytes (KT = 0.86, \( p = < 0.001 \) and 0.69, \( p = 0.01 \) respectively) (Figure 3.A.8, B & C). Additionally, a strong relationship could also be drawn between mucosal haemoglobin and protein concentration (KT = 0.83, \( p = 0.004 \)) (Figure 3.A.8, A).
3.A.4 Discussion

The netting stressor used was sufficient to induce a significant stress response in both the blood and mucus of the C. carpio tested. The values for the key mucosal stress indicators, haemoglobin and protein, were consistent with those reported in the literature.

The indicators of stress in the blood, namely glucose and peripheral leukocyte counts, showed dynamics consistent with those reported in the literature for both un-stressed and stressed C. carpio (Pottinger, 1998, Svobodova et al., 1999, Ruane et al., 2001, Ruane et al., 2002a, Ruane et al., 2002b). Peripheral leukocyte percentages followed the widely documented stress induced dynamics reported for fish, with significant neutrophilia, elevated monocyte numbers with a corresponding lymphopenia (Pickering, 1984, Pickering and Pottinger, 1987, Ainsworth et al., 1991).

Plasma protein concentration was unaffected by stress, which is consistent with results of previous trials using the rainbow trout (Oncorhynchus mykiss) (Vijayan and Leatherland, 1989). Increases in blood protein content have been reported following stress in winter founder (Pleuronectes americanus) and have attributed to a reduction in blood volume and the resulting haemoconcentration (Fletcher, 1975). Although not affected by stress in the current trial, more severe forms of netting or other stressors may induce alterations in blood volume and thus affect the protein content.

The urinalysis strips used in the current trial, were able to detect changes in several mucosal parameters. Although the sites for pH and specific gravity reacted with epidermal mucus they were discarded as useful biological indicators. The reagent site for pH showed limited precision and specific gravity was very susceptible to contamination by water from the tank during sampling, reducing confidence in the results obtained.

Mucosal haemoglobin has been found to show a strong linkage to the degree of stress experienced by a fish; prior to stress it is present in the mucus low amounts but rapidly increases in concentration in proportion to the degree of stress applied (Smith and
Ramos, 1976, Isaacson and Morrission, 1980, Lebedeva et al., 1999, Lebedeva, 1999). The mechanism of haemoglobin transfer to the epidermal mucus is still uncertain but appears to be linked increased blood pressure, elevated erythrocyte fragility and superficial damage to the skin. Wechsler (1984) reported the presence of erythrocytes in the mucus of fish stressed by either handling or anoxia; there were none in the mucus of unstressed fish. Unfortunately when sampling the mucus it is impossible to entirely eliminate possible sample induced damage to the dermal tissues, but using the Multistix®, this is kept to the minimum and the results obtained are thought to reflect the underlying physiology of the fish rather than a response to sampling.

Mucosal protein concentrations are reported to increase following stress (Lebedeva et al., 1999) which may in part be due to increased mucosal haemoglobin concentration. The strong relationship between mucosal haemoglobin and protein found in this trial supports this hypothesis. In the current trial, both haemoglobin and protein showed significant increases following stress, confirming previously reported patterns.

When visually analysed, values from the strips were consistently higher than those generated by the instrument. This discrepancy was especially apparent on the reagent sites for acetoacetate and leukocytes, which showed a significant patterns following stress when visually assessed. When assessed using the instrument, virtually no acetoacetate or leukocytes were detected, and no trends were apparent. The disparity between results obtained from the two methods was reduced in the sites for haemoglobin and protein, but there was still a tendency for the visually assessed strips to return a higher value. Despite this, the underlying trend for these parameters remained the same regardless of the assessment method.

It is apparent that caution must be expressed when visually assessing Multistix® due to the tendency to return artificially high results. This increases the risk of false-positive results, which in turn lead to unsound conclusions being drawn. The use of the instrument seems to greatly reduce this risk through removal of operator error, through
individual colour perception variations. This is most apparent on the leukocyte and acetoacetate reagent sites, where the colour difference between a negative, trace and positive result are very slight. Where the colour changes are more distinct, for example on the haemoglobin and protein sites, the error is less. Thus it follows that the use of the instrument is essential to increase confidence in the results obtained when using Multistix® in this fashion.

Significant relationships were found between plasma glucose, mucosal haemoglobin and protein, and peripheral monocytes and lymphocytes. These parameters showed positive relationships with glucose, with the exception of peripheral lymphocytes, which showed a negative relationship. These relationships indicate a strong link between the blood and mucosal stress responses and allow inferences to be made about the status of the blood response from the mucus and vice versa. It can therefore be accepted that mucosal haemoglobin and protein are parameters that closely follow the dynamics of known secondary stress indicators in the blood. This observation permits inferences of the stress status of fish to be drawn from sampling the mucus, removing the need to rely on blood sampling and subsequent analysis. This non-invasive method, being rapid, cheap and not reliant on extensive laboratory equipment or skills is widely applicable to the field of fish welfare and husbandry, particularly the ornamental trade, where conventional indicators of stress are not applicable.

In conclusion, the data from the current trial indicate that urinalysis strips, such as the Multistix® 10 SG used in the current trial have potential applications as rapid non-invasive stress diagnostic tools in fish. Of the parameters tested by the strips, mucosal haemoglobin and protein showed strong stress-linked patterns, allowing partial acceptance of $H_0$.1. The other reagent sites that reacted with mucus (pH, specific gravity, acetoacetate and leukocytes) are limited in application by limited precise, risk of contamination or difficulties in interpretation. Relationships between mucosal haemoglobin and protein and peripheral monocytes and lymphocytes were clearly
defined, allowing partial acceptance of $H_0.2$. The clear differences observed between visual and machine assessment of the strips ensures that $H_0.3$ is firmly rejected. Thus, it is concluded that the mucosal stress response, as quantified by mucosal haemoglobin and protein has the potential to act as a secondary stress indicators and their ease of sampling make them suitable parameters for non-invasive stress monitoring.
Chapter 3. Development of a non-invasive stress monitoring technique. B: Utilisation of urinalysis strips for the detection of stress in commercially important ornamental fish
3.B.1 Introduction

The epidermal mucus secretions of fish perform a multitude of roles, from physical and immune protection, to aiding swimming and maintenance of osmoregulatory homeostasis (Shepard, 1994). Numerous molecules and some cell types are included within the mucus matrix, with the presence and subsequent concentration being highly dependent on a variety of physiological and environmental conditions, including the development of stress. Mucosal haemoglobin, protein, pH and specific gravity have all been documented as being influenced by stress (Smith and Ramos, 1976, Isaacson and Morrisson, 1980, Lebedeva et al., 1999, Lebedeva, 1999).

In the previous section, the mucosal and blood stress response of C. carpio was investigated to establish potential linkage between the two systems and a comparison between visual and machine assessment of the strips was conducted. The clearest response to stress was observed for mucosal haemoglobin and protein, which confirmed previously published results (Smith and Ramos, 1976, Isaacson and Morrisson, 1980, Lebedeva and Golovkina, 1987, Lebedeva et al., 1999, Lebedeva, 1999). It was also apparent from the previous trial that visual assessment of the strips carries with it an attendant risk of over-estimating the presence of a particular compound. This was particularly apparent on the sites for acetoacetate and mucosal leukocytes which were greatly over estimated following visual assessment.

Although stress-induced changes in mucosal pH and specific gravity have been reported (Lebedeva and Golovkina, 1987, Lebedeva et al., 1999, Lebedeva, 1999), results obtained in previous trials (section 3.A.3.1) highlighted limitations with both sites, reflecting their ease of contamination by tank water and a general lack of precision, making it difficult to draw physiologically relevant conclusion from these particular reagent sites. As such, the only sites considered to have strong biological significance and sufficient reliability are those for mucosal haemoglobin and protein.
The mirror carp (*Cyprinus carpio*), was used in the previous trial and acted as a good model for a range of ornamental fish. *C. carpio* is the genetic source of a number of highly important ornamental fish species including all varieties of the highly prized Koi carp (Balon, 1995) and also of Ghost Koi, a robust mirror carp / Koi hybrid strain popular in the UK (Michaels, 1988). This makes them an ideal model species for ornamental fish species in the UK.

The aim of the current trial was to build on the establishment of the mucosal sampling as a rapid means for the detection of stress in ornamental fish by applying the method to other key ornamental fish species.

The first of these species is the goldfish (*Carassius auratus*), which for many people is the ubiquitous ornamental fish species. This species has been domesticated over the course of the last 2000 years (Hecker, 1993) and is possibly the most common companion animal in the UK (Ornamental Aquatic Trade Association, 2004). The other species tested is the hybrid red tilapia (*Oreochromis niloticus X. Oreochromis mossambicus*) which is a representative of the large and diverse cichlid family. Whilst the species tested is primarily farmed for food across the globe, it can be used as model species for the large numbers of ornamental cichlids present within the ornamental fish trade.

The phylogenetic distance between these two species is considerable so assessment of their response to stress will allow inferences to be made that are applicable to a wide range of fish, of which many will have a high commercial value. This will allow an assessment of the validity and applicability of the urinalysis testing method for the detection of stress in the wider ornamental industry.

Despite the tendency for visually assessed strips to over-estimate the concentration of mucosal parameters, this method was used in the current trial to best represent the perceived use of the strips in the ornamental industry. Whilst the dedicated strip reading machine may reduce the over-estimation of stress, the majority of users will
only require an indication of stress, which visual assessment supplies. The current trial also investigated the relative effects of both acute and chronic stressors on the mucosal stress response, to further establish the application potential of urinalysis as practical tools for stress diagnosis in fish.

The specific hypotheses tested in this investigation are:

- **H$_0$1** – mucosal haemoglobin and protein, as detected by Multistix$^\text{®}$ 10 SG, will be altered by the application of an acute stressor

- **H$_0$2** – mucosal haemoglobin and protein, as detected by Multistix$^\text{®}$ 10 SG, will be altered by the application of a chronic stressor

- **H$_0$3** – Stress induced changes in mucosal haemoglobin and protein will be consistent between goldfish (*C. auratus*) and tilapia (*O. niloticus* X *O. mossambicus*) and with those observed previously for carp (*C. carpio*).
3.B.2 Materials and Methods

3.B.2.1 The mucosal stress response of goldfish (*Carassius auratus*)

**Trial A**

One hundred and twenty (120) goldfish (*C. auratus*) were obtained from Prestopets Ltd [North Weald, UK] and transferred to the experimental aquarium at the University of Plymouth. The fish had a mean (± 1 S.D.) initial body weight of 5.48 g (± 0.02) and were distributed between twelve tanks of system A, as described in section 2.1.1. Each tank of fish was randomly allocated one of the experimental diets (control, β-glucan, nucleotide and combined supplement) described in section 2.1.2, with each diet being administered to fish in triplicate. For this trial, diets were administered in the form of flakes and feeding was commenced at an initial rate of 5 % BW.d⁻¹, decreasing to 4 % and finally 3% by the end of the trial. Feed intake was reduced as fish size increased to account for decreases in feed efficiency in larger fish as a measure to prevent overfeeding (Shimeno *et al.*, 1977, Wedemeyer, 1996).

**Stressors**

A mild netting stressor was applied to all fish, consisting of a combination of netting and aerial exposure as described in section 2.2. Two cycles of stress were applied to the fish giving total stress duration of 2 minutes. Five fish were sampled from each tank at each sample point. Following sampling fish were removed from the trial and not re-sampled. The experimental protocol is outlined in Table 3.B.1.
Table 3.B.1. Experimental Protocol (Trial A)

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Sampling times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Growth rate Wks 1 – 4 (weekly)</td>
</tr>
<tr>
<td>2</td>
<td>Mucosal Stress indicators Pre-stress, 5, 60, 120 &amp; 180 minutes post-stress</td>
</tr>
</tbody>
</table>

(1) No measurements of weight were made in the final two weeks of the trial to minimise exposure to stress. During this two-week period, increases in feeding rate were calculated from previous growth data.

Analytical Techniques

Mucosal stress indicators were assessed using Multistix® 10 SG as described in section 2.4.1.

Trial B

One hundred and twenty five (125) goldfish (C. auratus), were obtained from Prestopets Ltd [North Weald, UK] and transferred to the University of Plymouth. The mean (± 1 S.D.) initial body weight of these fish was 32.5 g (± 2.2) and they were divided equally between five tanks of system A as described in section 2.1.1. Fish were fed to satiation twice daily on a 50:50 % ratio of Aquarian® flake and Trouw Excel 16 pelleted trout diet [Trouw UK Ltd., Northwich, UK]. Fish were maintained under these conditions for several weeks prior to the start of the trial.

Stressors

Two levels of acute stress were applied during this trial. The low stress exposed fish, comprising two replicate groups, received a four minute (four cycles) netting stress as described in section 2.2. The high stress exposed fish, again comprising two replicate groups, received a six minute (six cycles) netting stress applied in the same manner as the low stress group. The control fish, comprising a single group, received no netting stress. The sampling strategy for this experiment is outlined in Table 3.B.2.
Table 3.B.2. Experimental protocol (Trial B)

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Sampling Times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal stress indicators</td>
<td>Pre-stress, 1, 2, 3, 6, 24, 48, 72, 96, &amp; 168 hours post-stress</td>
</tr>
</tbody>
</table>

Five fish were randomly sampled from each group at each time point and fish were subsequently returned to the experimental tanks following sampling.

**Analytical Techniques**

Mucosal stress indicators were assessed using Multistix® 10 SG as described in section 2.4.1.

**Trial C**

Two hundred and forty (240) goldfish (*C. auratus*), with a mean (± 1 S.D.) initial weight of 10.7 g (± 0.2) were obtained from Prestopets Ltd. and were divided equally between twelve tanks within of System A as described in section 2.1.1. Following allocation to experimental tanks, fish were not weighed in order to minimise stress; subsequent adjustments to feed intake, to account for growth, were predicted using Specific Growth Rate (SGR) data obtained for (*C. auratus*) maintained under similar conditions. Predicted growth increments were added to existing tank weights estimate growth and feeding intake. Following acclimation, each group of fish was allocated one of the four experimental diets (control, β-glucan, nucleotide and combined supplement) in flake form as described in section 2.1.2, with each diet being tested in triplicate. Food was administered at a rate of 2.0 % BW.d⁻¹.
Table 3.B.3. Experimental protocol (Trial C)

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Sampling Times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Mucosal stress indicators</td>
<td>1.5, 3, 5 &amp; 24 hours post-stress</td>
</tr>
<tr>
<td>2 Plasma cortisol</td>
<td>1.5, 3, 5 &amp; 24 hours post-stress</td>
</tr>
<tr>
<td>3 Plasma glucose</td>
<td>1.5, 3, 5 &amp; 24 hours post-stress</td>
</tr>
</tbody>
</table>

**Stressors**

All fish received a four minute (four cycles) netting stress administered as described in section 2.2. The sampling strategy is outline below in Table 3.B.3. Three fish per tank were sampled at each time point. Following sampling fish were removed from the trial and not re-sampled.

**Analytical techniques**

Mucosal stress indicators were assessed using Multistix® 10 SG as described in section 2.4.1. Peripheral blood was obtained from three fish per tank as described in Section 2.3 and the plasma analysed for glucose as described in section 2.4.2. Plasma cortisol concentration was determined on a pooled sample of plasma from three fish per tank. Analysis was carried out by Triphasic CTL [Plymouth, UK] using a commercially available ELISA kit [DSL, Oxon, UK]. The assay used has a sensitivity of 2.76 nmol.L⁻¹ / 0.1 μg.L⁻¹.

**3.B.2.2 The mucosal stress response of Tilapia (Oreochromis niloticus X. Oreochromis mossambicus)**

One hundred and forty (140) hybrid red tilapia (O. niloticus X. O. mossambicus) were obtained from the University of Wales, Swansea and transferred into an eight tank recirculation system within the experimental aquarium at the University of Plymouth (Plate
3.B.1). Water parameters were as follows; temperature $28 \pm 1 ^\circ C$, pH $6.3 - 7.1$, NH$_3$ <0.01 ppm, NO$_2$<0.01 ppm, NO$_3$ <20 mg.l$^{-1}$ and DO$_2$ 5-7 mg.l$^{-1}$. The photoperiod was maintained at twelve hours light/ twelve hours dark using fluorescent lighting.

Fish were acclimated for three weeks prior to the start of the trial and were fed to satiation twice daily on Skretting Standard Expanded 40 [Trouw UK Ltd.]. Mean fish weight at the start of the trial was 38.6 (± 3.7) g.
Plate 3.B.1. Eight tank re-circulation aquarium system located in the experimental aquarium of the University of Plymouth as used during Tilapia trial.
Stressors

Both chronic and acute stressors were applied during the course of this experiment. Chronic stress was applied by varying stocking density; twenty five fish per tank which gave an approximate density of 10.3 kg.m\(^{-3}\) and ten fish per tank, which equated to approximately 2.6 kg m\(^{-3}\). Each stocking density was replicated four times. For the duration of the trial all fish were fed to satiation twice daily on Skretting Standard Expanded 40 pellet diet.

Acute stress was applied to all fish using the netting stress described in section 2.2, with a total duration of six minutes (six cycles). Mucosal stress indicators were recorded for five fish per tank at all sampling times. Blood samples were taken from three fish per tank at each sample interval. The sampling strategy is outlined in Table 3.B.4.

Mucosal parameters

Mucus from five fish per tank was sampled at each time point. Mucosal stress indicators were assessed using Multistix\textsuperscript{®} 10 SG as described in Section 2.4.1.

<table>
<thead>
<tr>
<th>Table 3.B.4: Experimental protocol (Tilapia trial)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Parameters Measured</strong></td>
</tr>
<tr>
<td>1 Mucosal stress indicators</td>
</tr>
<tr>
<td>2 Haematocrit</td>
</tr>
<tr>
<td>3 Differential leukocyte counts</td>
</tr>
<tr>
<td>4 Total erythrocyte counts</td>
</tr>
</tbody>
</table>
Haematological parameters

Blood was collected as described in section 2.3 and was used to determine haematocrit, total erythrocyte count and to prepare slides for differential leukocyte counts.

Blood for haematocrit determination was drawn into heparinised micro-centrifuge tubes which were plugged with putty and spun at 3600 x g for 3 minutes. Two tubes per fish were prepared and the packed cell volumes were measured using a Hawksley reader and are presented as a percentage of the total blood volume.

To perform total erythrocyte counts, 5 μl of heparinised blood was diluted in 195 μl of Rees-Ecker solution (Wedemeyer and Yasutake, 1977), to give a 1:200 dilution. An Improved Neubauer haemocytometer was used to count the number of erythrocytes under a light microscope (Houston, 1990).

3.B.2.3 Statistical analysis

Unless otherwise stated, data is presented as median (inter-quartile range). Data were analysed using the non-parametric Kruskal-Wallis test with post hoc multiple comparison analysis (Minitab 14).
3.B.3 Results

3.B.3.1 Goldfish (C. auratus)

Trial A

Haemoglobin was present in the mucus of all fish sampled prior to and following stress. No significant differences in mucosal haemoglobin concentration between fish sampled prior to and following stress. During recovery from stress significant changes in mucosal haemoglobin concentration were apparent in fish fed the β-glucan diet (Table 3.B.5); haemoglobin of the β-glucan fed fish was elevated at 1 and 2 hours post stress compared to the 3 hours post-stress sample.

Mucosal protein concentrations were largely unaffected by the application of stress, with only fish from the control group showing significant change. Mucosal protein concentration of these fish was significantly lower at 3 hours post stress than either pre-stress or 1 hour post-stress (Table 3.B.6). Fish that had received the immuno-stimulating diets showed no significant changes in mucosal protein concentration at any time during the trial.

Trial B

Unstressed fish in Trial B showed no significant alteration in mucosal haemoglobin concentration during the course of the trial (Table 3.B.5). Both groups of fish that received stress showed clear stress-induced elevation in mucosal haemoglobin. Fish that received the lower level of stress showed significant elevations in mucosal haemoglobin above pre-stress values at 1 hour post-stress above pre-stress values. Mucosal haemoglobin values of these fish were also significantly elevated at 1 and 2 hours post-stress relative to the 168 hours post-stress sample. Elevations in mucosal haemoglobin concentration of fish receiving the higher level of stress occurred later, with mucosal
haemoglobin concentrations at 2 and 3 hours post-stress being significantly elevated compared to pre-stress and 168 hours post-stress samples (Table 3.B.5).

Stress-induced reductions in mucosal protein content were apparent over the longer time scale used in Trial B. Fish that received the high level of stress showed higher levels of protein prior to stress and after 24 hours recovery than at 72 - 168 hours post stress, additionally, protein concentration was greater at 1 - 3 hours post stress than at 24, 72 - 168 hour (Table 3.B.6). Fish that received the lower level of stress showed similar, but less sustained, long term reductions, with values recorded prior to stress and at 1 - 3 hours post stress being significantly higher than 48, 72 - 168 hours. Despite having not experienced a direct stressor; the control group of fish showed a significant reduction in mucosal protein at 168 hours post stress compared to values taken pre-stress and at 1, 2, 6 and 24 hours post-stress.

**Trial C**

The mucosal haemoglobin concentration of fish sampled in Trial C showed no stress-induced changes following stress (Table 3.B.5). Similarly, the protein content of epidermal mucus of all groups of fish sampled was unaffected by stress (Table 3.B.6).

Cortisol values for fish sampled as part of Trial C were consistently high and were unaffected by stress (Table 3.B.7). Considerable variation in cortisol values returned between groups of fish was apparent, which contributed to a lack of statistical significance.

Plasma glucose was significantly altered by stress in all groups of fish, with the exception of those fed the β-glucan diet. Fish fed the control, nucleotide and combined supplement diet, showed increases in plasma glucose post-stress, with peak values being observed at 1.5 hours post stress in all groups (Table 3.B.7). Fish fed the β-glucan diet showed no change in plasma glucose concentration between pre and post-stress values, but significant changes were seen during recovery from stress. In these fish, plasma
glucose concentration at 1.5 hours post-stress was significantly higher than at 3 – 24 hours post-stress (Table 3.B.7).
Table 3.B.5. Mucosal haemoglobin (arbitrary units) of goldfish (*Carassius auratus*), previously fed immuno-stimulating diets and subjected to a mild netting stressor.

Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® flake food. No significant differences between treatments were found.

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<th>Nucleotide</th>
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*-- = no sample taken at this time point*
Table 3.B.6. Mucosal protein concentration (g.L⁻¹) of goldfish (Carassius auratus) previously fed immuno-stimulating diets and subjected to a mild netting stressor.

Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® flake food. No significant differences between treatments were found.

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<th>Nucleotide</th>
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-- = no sample taken at this time point
Table 3.B.7. Plasma cortisol (μg.dl⁻¹) and glucose (mg.dl⁻¹) concentration of goldfish, (Carassius auratus), previously fed immunostimulating diets and subjected to a mild netting stressor.

Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.).

Control = Aquarian® flake food. No significant differences between treatments were found.

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<th>Glucose</th>
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3.B.3.2 Tilapia (O. niloticus X O. mossambicus)

Mucosal Parameters

Mucosal haemoglobin concentration was unaffected by the application of stress, whilst protein concentration showed significant stress-induced change (Table 3.B.8). During recovery from stress, the concentration of haemoglobin in the mucus of fish previously held at the higher stocking density was significantly higher at 3 hours post-stress compared to the 5 and 24 hours post-stress. Fish from the lower stocking density tanks showed no change in mucosal haemoglobin concentration at any point during the trial (Table 3.B.8).

Significant stress-induced changes in mucosal protein content occurred in both groups of fish at similar times with elevated mucosal protein concentrations at 1.5 and 3 hours post-stress, when compared to pre-stress values. These time points were also significantly elevated compared to the 24 hour post-stress sample. Additionally, fish from the higher stocking density showed higher mucosal protein concentration at 5 hours post-stress compared to the 24 hour post-stress sample.

Haematological parameters

The application of netting stress did not induce any changes in peripheral neutrophil, thrombocyte or lymphocyte numbers in fish previously held at the lower stocking density, although neutrophils numbers were elevated at 24 hours post-stress compared to the 168 hour post-stress sample in these fish (Table 3.B.9). Fish held at the higher stocking density showed significant elevations in thrombocytes at 24 and 168 hours post-stress and a corresponding significant reduction in lymphocyte numbers at the same time points was observed (Table 3.B.9).

Total erythrocyte counts and haematocrit of fish held at the higher stocking density were unaffected by stress (Table 3.B.10). Fish held at the lower stocking density
showed significantly elevated erythrocyte numbers at 24 and 168 hours after stress, and whilst no difference between pre and post-stress haematocrit values was observed, haematocrit values at 24 hours post-stress were significantly higher than those recorded at 168 hours post-stress (Table 3.B.10).
### Table 3.B.8. Mucosal parameters of red tilapia (*Oreochromis niloticus X Oreochromis mossambicus*) held at two stocking densities for six weeks and subjected to a mild netting stressor.

Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. No significant differences between treatments were found.

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<th>Time elapsed after stress (hr⁻¹)</th>
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</tr>
<tr>
<td>Pre-stress</td>
<td>4 (3-4)</td>
<td>3 (3-4)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3.5 (3-4)</td>
</tr>
<tr>
<td>1.5</td>
<td>3.5 (3-4)</td>
<td>1b (3-4)</td>
</tr>
<tr>
<td>3</td>
<td>3.5 (3-4)</td>
<td>1b (3-4)</td>
</tr>
<tr>
<td>5</td>
<td>3 (3-4)</td>
<td>1c (3-4)</td>
</tr>
<tr>
<td>24</td>
<td>3 (3-4)</td>
<td>0.3c (2-4)</td>
</tr>
</tbody>
</table>

### Table 3.B.9. Percentage peripheral leukocyte counts of red tilapia (*Oreochromis niloticus X Oreochromis mossambicus*) held at two stocking densities for six weeks and subjected to a mild netting stressor.

Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found.

<table>
<thead>
<tr>
<th>Time elapsed after stress (hr⁻¹)</th>
<th>Neutrophils</th>
<th>Thrombocytes</th>
<th>Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Pre-stress</td>
<td>0.0 (0-0.5)</td>
<td>14.0a (6.2-24.8)</td>
<td>20.3 (8.3-38.5)</td>
</tr>
<tr>
<td>24</td>
<td>0.2 (0-1.8)</td>
<td>26.7a (21.3-38.1)</td>
<td>35.2 (24.3-40.8)</td>
</tr>
<tr>
<td>168</td>
<td>0.0 (0-0.48)</td>
<td>33.0a (24.3-37.3)</td>
<td>32.2 (26.4-37.7)</td>
</tr>
</tbody>
</table>

### Table 3.B.10. Erythrocyte counts and haematocrit of red tilapia (*Oreochromis niloticus X Oreochromis mossambicus*) held at two stocking densities for six weeks and subjected to a mild netting stressor.

Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found.

<table>
<thead>
<tr>
<th>Time elapsed after stress (hr⁻¹)</th>
<th>Erythrocytes (cells × 10⁶ µL⁻¹)</th>
<th>Haematocrit (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>High</td>
<td>Low</td>
</tr>
<tr>
<td>Pre-stress</td>
<td>2.1 (1.6-2.9)</td>
<td>2.0a (1.7-2.3)</td>
</tr>
<tr>
<td>24</td>
<td>3.5 (2.5-3.7)</td>
<td>2.7b (2.5-3.2)</td>
</tr>
<tr>
<td>168</td>
<td>3.2 (2.6-3.9)</td>
<td>3.3b (2.9-4.3)</td>
</tr>
</tbody>
</table>
3.B.4. Discussion

Mucosal haemoglobin concentration of stressed *C. auratus* and tilapia (*O. niloticus X O. mossambicus*) was found to increase above either pre-stress levels or that of levels after extended recovery (24 or 168 hours), which is consistent with previously reported patterns (Smith and Ramos, 1976, Isaacson and Morrisson, 1980, Lebedeva et al., 1999, Lebedeva, 1999). However the relationship between stress and mucosal haemoglobin change was less consistent than seen in section 3.A. Dynamics of mucosal haemoglobin change closest to those seen previously were observed in fish sampled during Trial B. A consistent feature of the remaining trials using both *C. auratus* and *O. niloticus X O. mossambicus* was high pre-stress values, possible as a result of sample-induced stress, which appear to have prevented the detection of a clear difference between pre and post-stress states. The patterns observed during recovery from stress closely followed those seen previously, indicating that the underlying physiological response was conserved. Values for haemoglobin and protein content of *O. niloticus X O. mossambicus* epidermal mucus were very similar to those observed for *C. auratus* both prior to and following stress, despite the large phylogenetic differences between the two species. Mucosal haemoglobin values were also largely consistent with those observed in *C. carpio* in the previous trial.

Mucosal protein concentration was affected differently by stress in the two species sampled, however the values were largely consistent between the two species and also with the *C. carpio* sampled during the previous trial. In *O. niloticus X O. mossambicus*, protein concentration increased immediately post stress, returning to pre-stress values by 24 hours post-stress. In the case of goldfish (*C. auratus*), a reduction in mucosal protein content was observed following stress, which continued for up to a week. The protein content of the epidermal mucus is thought to receive contributions from numerous sources from within the dermal tissues and blood, including
haemoglobin, (Shepard, 1994, Lebedeva, 1999) so the mechanisms responsible for these changes are unclear and can not be resolved using the current method.

The overall similarity of the response of epidermal mucus to stress of the two species sampled in the current trial and that of *C. carpio* sampled in the previous trial, indicating that the suitability of Multistix® as a tool to detect changes in mucosal parameters is not restricted to a single species or even a group of closely related species.

The other stress indicators used in Trial C to support the use of Multistix® as diagnostic tools provided mixed results. Cortisol concentrations were not significantly altered by stress but were consistently at the upper range of values reported for cyprinids (Pottinger, 1998, Svobodova *et al.*, 1999, Ruane *et al.*, 2001, Ruane *et al.*, 2002a, Ruane *et al.*, 2002b), suggesting that significant sample-induced stress affected circulating cortisol concentrations. This is a common problem with the use of cortisol as a stress indicator; its rapid release in response to the stress of handling can prevent the detection of subtle effects if sampling is not conducted within the latency period of the species. Research on the stress response of cyprinids conducted by Davis and Parker (1986) found that a detectable increase in circulating cortisol can occur within 4 - 6 minutes of a stressful event.

Despite the lack of a cortisol response, a significant hyperglycaemic response was observed following stress; a response well documented for a variety of fish species, including cyprinids (Fletcher, 1975, Schwalme and Mackay, 1985, Vijayan and Leatherland, 1989, Pottinger, 1998, Svobodova *et al.*, 1999, Grutter and Pankhurst, 2000). This indicates that a significant stress response was generated following the application of the netting stressor employed, confirming that the mucosal patterns are indeed likely to be stress-induced. This hyperglycaemic response of *C. auratus* was comparable with that seen in *C. carpio* in section 3.A, but also reflects the shorter duration of the stress, increasing from pre-stress values of 26 - 44 mg.dl⁻¹ to peak values of 46 - 56 mg.dl⁻¹ at 1.5 hours after stress. However, unlike in the previous trial, there
were no significant relationships between the mucosal and blood parameters tested. There was a lack of a significant stress-induced increase in plasma glucose concentration in those *C. auratus* fed the β-glucan diet. As was the case for mucosal haemoglobin, the lack of response was through to be due an unusually high pre-stress value of 43.5 mg.dl$^{-1}$. Again, this may be due to sample-induced stress and may have prevented the detection of stress-induced change. The response of glucose following stress suggests that a standard hyperglycaemic response had occurred, with values recovering along the same timescales as those seen in fish fed other diets.

For the tilapia trial, peripheral leukocyte and erythrocyte counts were employed as additional indicators of stress. Following stress, lymphopenia, a common response of fish lymphocytes to stress (Pickering and Pottinger, 1987) was observed. Such reductions in the number of circulating lymphocytes are the result of a variety of factors including redistribution from blood into the tissues, and increased apoptosis in response to the presence of the stress hormones (Pruett, 2001, Dhabhar, 2002, Moynihan, 2003, Engler *et al.*, 2004). Significant stress-induced neutrophilia was not observed in either group of fish, although neutrophils did significantly decrease during recovery from stress. Changes in these leukocyte classes were only observed in fish from the low stocking density tanks, whilst fish sampled from the high stocking-density tanks showed stress-induced increases in thrombocyte numbers. The presence of stress-induced lymphocyte reductions and thrombocyte increases, albeit in different treatments, are features of the stress response seen in *C. carpio*. This may indicate some difference in the physiological tolerance of or response to stress between the two species sampled.

Measurements of haematocrit and total erythrocyte counts produced different results between *O. niloticus* X *O. mossambicus* held at the two different stocking densities; fish from the higher stocking showed no significant changes in either measure of erythrocyte number following stress, whilst those from the lower stocking density did. The differences in stress response may be due to suppression of aggressive behaviour at
the higher stocking density, which resulted in a lower level of chronic stress. Many
tilapias (*Oreochromis* sp.) are naturally aggressive and territorial fish and at low stocking
densities will create and defend territories, resulting in highly stressful interactions
occurring (Toguyeni *et al.*, 1997, Wedemeyer, 1997). Conversely, at higher stocking
densities, similar territories are unable to be formed due to the sheer number of fish so
aggression is reduced.

In fish from the lower stocking density tanks, both haematocrit and erythrocyte
count increased following stress. Increases in haematocrit are typically caused by
erthrocyte swelling or haemoconcentration due to water loss (Houston and DeWilde,
1972, Houston, 1997), but in this case the corresponding increase in erythrocyte number
suggests that cells were recruited into the circulation. In particular this occurs from the
spleen of fish, which is innervated by adrenergic nerve fibres and when stimulated
The cellular data indicate that the *O. niloticus X O. mossambicus* held at the lower
stocking density underwent a more pronounced reaction to the acute stressor applied.

The Multistix® employed in the current trial to assess the mucosal stress response
were successful in detecting changes in both haemoglobin and protein allowing the
acceptance of Hₐ₁. Conversely, no strong evidence in support of Hₐ₂ was detected:
significant effects of chronic stress on the mucosal stress response were not observed,
despite a differential blood response occurring in the tilapia trial. Similarly there was
only limited evidence to suggest that the degree of stress increased the magnitude of the
mucosal stress response, although all the stressors applied were comparatively mild in
nature and more severe stressors may result in more severe mucosal stress responses.
However at this level of stress exposure the response appears to be largely consistent.
Finally the response of the two key mucosal parameters, haemoglobin and protein, were
consistent between the cyprinid, *C. auratus* and the cichlid *O. niloticus X O.
mossambicus* sampled, indicating that the underlying physiology responsible for the
mucosal stress response is conserved. The similarity of the responses to each other and to the ones observed for *C. carpio* allow H₃ to be accepted, although the linkage between stress and mucosal parameters seen previously was noticeably weaker in these trials. Therefore, it is concluded that Multistix® have potential to act as diagnostic tools for the detection of stress in the epidermal mucus secretions of ornamental fish.
Chapter 4. Modulation of the immune status of mirror carp (Cyprinus carpio): effects of dietary immuno-stimulants and acute stress
4.1 Introduction

The immune system of fish constantly encounters and neutralises pathogenic organisms. As described previously, fish possess both an adaptive immune system (AIS) (section 1.2.1) and innate immune system (IIS) (section 1.2.2), but the latter is of greater importance to temperate fish species such as goldfish (C. auratus) and Koi carp (C. carpio) that regularly experience temperatures below their respective immunologically permissible temperature (IPT) (Bly and Clem, 1992, Tatner, 1996). At these temperatures fish are totally reliant on the cellular and humoral defences of the IIS; even at temperatures above the IPT, as production of specific immunoglobulin (Ig) is usually slow.

The strong linkage between the hormones of the hypothalamus-pituitary-interrenal (HPI) axis and the immune system (section 1.3) gives stress the potential to modify the ability of an organism to neutralise pathogenic organisms, thus altering its disease resistance. The effects of stress varies with duration; acute stress may activate the immune system whilst chronic stress may induce immuno-suppression (Pruett, 2001, Moynihan, 2003), although the definitions of stressor types are flexible. Fish that have experienced stress generally show a reduction in disease resistance, particularly to opportunistic bacterial pathogens such as Aeromonas hydrophila, Flexibacter columnaris and the fungus Saprolegnia sp. which are usually incapable of affecting unstressed fish (Biondi and Zannino, 1997).

The oral administration of immuno-stimulants in general, and β-glucans and nucleotides in particular, have been found to enhance various aspects of the IIS, including phagocytic activity (Chen and Ainsworth, 1992, Duncan and Klesius, 1996, Sakai et al., 2001), complement activity (Sakai et al., 2001), macrophage respiratory burst (Castro et al., 1999, Cook et al., 2001, Cook et al., 2003, Li and Gatlin, 2004, Li et al., 2004) and lysozyme activity (Sakai et al., 2001, Kumari and Sahoo, 2006b). In addition, both β-
glucans and nucleotides may possess additional beneficial effects on growth and stress resistance (Burrells et al., 2001b, Skjeremo et al., 2002, Leonardi et al., 2003).

Despite similar stimulatory effects, the mode of action of β-glucans and nucleotides is different. β-glucans are recognised by specific receptors on the CSM of a variety of immune cells, triggering activation of their immune function (Engstad and Robertsen, 1993, Engstad, 1994, Engstad and Robertsen, 1994). This manifests itself through increased respiratory burst activity and phagocytosis, although other immune parameters are also enhanced (see Table 1.3). Nucleotide supplementation provides immune cells normally deficient in nucleotides with preformed molecules, reducing the energy costs of cell division, facilitating cell proliferation and repair. Enhancements to both cellular and humoral components of the IIS are also reported following nucleotide supplementation (see Table 1.4)

The differences in mode of action of these two classes of immuno-stimulant offer the potential for greater enhancement if provided together. Such a combination of β-glucans and nucleotides should complement each other through the activation of components of the IIS and by allowing greater cell production and repair.

In addition to direct immuno-stimulatory effects, dietary nucleotides have been reported to confer greater stress resistance, with enhanced survival and reduced blood cortisol levels, following stressful situations (Burrells et al., 2001b, Leonardi et al., 2003). β-glucans may also have stress mediating effects, but less study has focussed on this aspect of their action, although enhanced growth and body condition is a widely reported consequence of β-glucan supplementation, equipping fish to deal with stress more effectively (Onarheim, 1992, Cook et al., 2001).

This investigation assessed the effects of a period of dietary supplementation with β-(1,3;1,6) glucans and exogenous nucleotides, both derived from yeast, on immune parameters and stress response of mirror carp (Cyprinus carpio). Dietary immuno-
stimulant administration commenced immediately prior to the application of an acute stressor and the subsequent immune response was monitored.

The specific hypotheses tested were as follows:

\( H_01 \) – dietary supplementation with either \( \beta \)-glucans or nucleotides at 0.2% weight / weight (w/w) of feed will positively enhance serum haemolytic complement and serum lysozyme activity and increase respiratory burst activity, quantified by NBT reduction, of whole blood, both prior to and following stress.

\( H_02 \) – any stress-induced immuno-suppression will be less in fish fed immuno-stimulants, and recovery from a suppressed state will be quicker.

\( H_03 \) – dietary supplementation with either \( \beta \)-glucans or nucleotides at 0.2% w/w of feed will enhance stress resistance of the fish, reducing changes in plasma glucose, peripheral leukocytes and mucosal parameters.

\( H_04 \) – those fish supplemented with a combination of \( \beta \)-glucans and nucleotides will show the greatest enhancements to both immune parameters and stress resistance.
4.2 Materials and methods

Four hundred and twenty (420) mirror carp (Cyprinus carpio) with a mean (± SD) initial body weight of 26.67 g (± 0.2) were obtained from Hampshire Carp Hatcheries (Twyford, UK). The Fish were separated and maintained in twelve groups within System A in the experimental Aquarium of the University of Plymouth as described in section 2.1.1. Water temperature was maintained at 18 ± 1 °C. Groups of fish were randomly allocated one of the experimental diets, in pellet form, described in section 2.1.2, and were administered to triplicate tanks. Each diet was administered at a rate of 1.0 % BW.d⁻¹ for three weeks prior to stress; adjustments to feed intake to account for growth, were calculated using Thermal Growth Coefficient calculations based on growth data obtained during acclimations (Cho and Bureau, 1998).

4.2.1 Stressors

A mild netting stressor was applied to all fish as described in section 2.2. The stressor duration was six minutes (administered in six cycles).

4.2.2 Sampling Strategy

Blood and mucus parameters were sampled prior to stress and following stress as outlined in Table 4.1. Five fish per tank were sampled at each time point. All five were sampled for mucosal parameters; heparinised blood and plasma were prepared from two of these fish and serum prepared from the remaining three fish. Following sampling fish were removed from the experiment and not re-sampled.
### Table 4.1. Experimental protocol

<table>
<thead>
<tr>
<th>Parameters measured</th>
<th>Sampling times</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosal parameters</td>
<td>1.5, 4, 6.5, 24 &amp; 168 hours</td>
</tr>
<tr>
<td>(5 fish per tank)</td>
<td>post-stress</td>
</tr>
<tr>
<td></td>
<td>Pre-stress</td>
</tr>
<tr>
<td>NBT reduction (whole blood)</td>
<td>1.5, 4, 6.5, 24 &amp; 168 hours</td>
</tr>
<tr>
<td>(2 fish per tank)</td>
<td>post-stress</td>
</tr>
<tr>
<td></td>
<td>Pre-stress</td>
</tr>
<tr>
<td>Differential leukocyte counts</td>
<td>1.5, 4, 6.5, 24 &amp; 168 hours</td>
</tr>
<tr>
<td>(2 fish per tank)</td>
<td>post-stress</td>
</tr>
<tr>
<td></td>
<td>Pre-stress</td>
</tr>
<tr>
<td>Plasma protein</td>
<td>1.5, 4, 6.5, 24 &amp; 168 hours</td>
</tr>
<tr>
<td>(2 fish per tank)</td>
<td>post-stress</td>
</tr>
<tr>
<td></td>
<td>Pre-stress</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>1.5, 4, 6.5, 24 &amp; 168 hours</td>
</tr>
<tr>
<td>(2 fish per tank)</td>
<td>post-stress</td>
</tr>
<tr>
<td></td>
<td>Pre-stress</td>
</tr>
<tr>
<td>Serum lysozyme</td>
<td>1.5, 4, 6.5, 24 &amp; 168 hours</td>
</tr>
<tr>
<td>(3 fish per tank)</td>
<td>post-stress</td>
</tr>
<tr>
<td></td>
<td>Pre-stress</td>
</tr>
<tr>
<td>Serum spontaneous haemolytic activity</td>
<td>1.5, 4, 6.5, 24 &amp; 168 hours</td>
</tr>
<tr>
<td>(3 fish per tank)</td>
<td>post-stress</td>
</tr>
</tbody>
</table>

### 4.2.3 Mucosal Parameters

Mucosal stress indicators were assessed using Multistix® 10 SG as described in section 2.4.1. Assessment of the colour change of the reagent pads was carried out using a Clinitest Status strip reader [Bayer Plc.] as described in section 2.4.1.
4.2.4 Haematological parameters

Fish were anaesthetised and blood collected as previously described in section 2.3. Serum and plasma were prepared as described in section 2.3.

**Differential leukocyte counts**

Blood smears were prepared and differential leukocyte counts were conducted as described in section 2.4.2.

**Glucose**

Plasma glucose was assayed using the GOD-POD method [Sigma-Aldrich] as described in section 2.4.2.

**Total protein**

Plasma protein concentration was determined using the micro scale version of the Bio-rad modification of the Bradford method (Bradford, 1976) (section 3.A.2.2).

4.2.5 Immunological parameters

**Nitroblue tetrazolium (NBT) reduction ability of whole blood**

The NBT reduction ability of whole blood was assayed using the method of Ispir and Dorucu (2005). Briefly, 200 µl of heparinised blood was mixed with 200 µl of 0.2 % NBT solution in phosphate buffered saline (PBS) and incubated at room temperature for 30 minutes. 10 µl of blood / NBT solution was added to 1000 µl of N,N, dimethyl formamide in a glass tube and centrifuged at 900 x g for 5 minutes. The optical density (OD) of the resulting supernatant was read at 620 nm using a glass cuvette in a JASCO spectrophotometer.
Serum spontaneous haemolytic activity

Serum spontaneous haemolytic activity was assayed using the method of Ortuno, Esteban *et al.*, (2001). Sheep red blood cells (SRBCS) [TCS Biosciences, Bodolph Claydon, UK] were used as targets. SRBCS were washed 3 times at 1000 x g for 10 minutes in complement fixation test (CFT) buffer [Oxoid, Basingstoke, UK]. Following the final wash, cells were made up to an approximate 5 % solution in CFT buffer. One part SRBCS was added to 14 parts 0.1 % anhydrous Na₂CO₃ (v/v) and absorbance was measured against a CFT buffer blank at 540 nm. A 5 % suspension (10⁹ SRBC.ml⁻¹) has an absorbance value of 0.7 at 540 nm (Ingram, 1990). The approximate solution was adjusted to an accurate 5 % solution using the following formula (Mayer, 1961).

\[
V_f = V_i \times \frac{\text{absorbance of haemolysate}}{0.700}
\]

Where \( V_i \) is the initial volume of the approximate 5 % suspension and \( V_f \) is the final volume to which \( V_i \) has to be adjusted to give an accurate 5 % SRBC suspension. For assay purposes the 5 % solution was diluted to 3 % using CFT buffer.

Test serum in 500 µl aliquots was diluted in CFT buffer and added to 500 µl of SRBCS to give final serum concentrations of 10, 5, 2.5, 1.25, 0.625, 0.313, 0.1565 and 0.078 %. Serum and SRBCS were incubated for 1 hour at 25 °C and the samples were centrifuged at 1000 x g for 7 minutes to remove un-lysed SRBCS. The relative haemoglobin concentration of the supernatants was assessed at 540 nm using a spectrophotometer. 100 % lysis was achieved using 500 µl distilled water, minimum lysis was achieved using 500 µl CFT.

The degree of haemolysis (\( y \)) of the sample relative to the 100 % control was calculated and the lysis curve for each sample obtained by plotting \( \frac{y}{(1-y)} \) against the
volume of serum added (ml) on a log-log graph. From this graph, the number of ACH$_{50}$ units per ml of serum was calculated following the method of Yano (1992) The amount of serum giving 50% haemolysis (K) is calculated as the: $x = K$ when $y = 0.5$). From this the ACH$_{50}$ units per ml serum were calculated as follows.

$$ACH_{50} \text{ U.ml}^{-1} = \frac{1}{K} \times 10^*$$

*Initial serum dilution factor

**Serum lysozyme activity**

Serum lysozyme activity was assayed using the turbidometric method of Ellis (1990). 50 µl of serum was added to 950 µl of 0.2 mg.ml$^{-1}$ of *Micrococcus lysodeikticus* in 0.05 M Na$_2$HPO$_4$ (pH 6.2). 1 unit of lysozyme activity being defined as creating a decrease in absorbance of 0.001 absorbance units per min.

**4.2.6 Statistical analysis**

Unless otherwise stated, data is presented as median (inter-quartile range). Analysis was conducted using the non-parametric Kruskal-Wallis test with post hoc multiple comparison analysis (Minitab 14). Relationships were tested using Kendall's Tau (Minitab 14), with an associated p value of $ \leq 0.05$ being required for a relationship to be considered significant.
4.3 Results

4.3.1 Mucosal parameters

Epidermal mucus reacted with the reagent pads for acetoacetate, specific gravity, haemoglobin, pH, protein and leukocytes. Data from the specific gravity and pH reagent sites were not analysed due to the problems outlined previously (section 3.A.3.1). No significant amounts of either acetoacetate or leukocytes were detected in the epidermal mucus of the fish tested. The presence of protein and haemoglobin was consistently detected by the Multistix® are presented in Tables 4.2 and 4.3.

No significant differences in mucosal haemoglobin were apparent between fish fed the different diets at any point during the trial (Table 4.2). Additionally, no significant effect of stress was apparent on mucosal haemoglobin concentrations. During recovery from stress, fish fed the combined diet showed greater mucosal haemoglobin at 4 hours post-stress than at 1.5 and 168 hours post-stress. Despite the medians of these time points being identical, the variability of the data obtained at 4 hours post-stress was lower than those at the other time points. Fish fed the other diets showed no change in mucosal haemoglobin concentration at any point during the trial.

As was the case for mucosal haemoglobin, mucosal protein was unaffected by the diet fed to the fish (Table 4.3). During recovery from stress, changes in mucosal protein concentration were apparent in all groups of fish except the control group. These changes manifested themselves as a significant reduction in mucosal protein at 24 hours post-stress compared to 1.5 hours post-stress, with additional reductions at 6.5 hours post stress in fish fed the β-glucan and nucleotide diets (Table 4.3). Fish fed the nucleotide diet showed the only significant difference between pre and post-stress mucosal protein concentration with pre-stress values being significantly higher than those at 24 hours post-stress.
Table 4.2. Mucosal haemoglobin (arbitrary units) of mirror carp (Cyprinus carpio) following netting stress. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® pellet food.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Time after stress hr⁻¹</th>
<th>Pre</th>
<th>1.5</th>
<th>4</th>
<th>6.5</th>
<th>24</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2-4)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(3-3)</td>
<td></td>
</tr>
<tr>
<td>β-glucan</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
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<td></td>
</tr>
<tr>
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<td>(2-3)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(3-3)</td>
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<tr>
<td>Nucleotide</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2-4)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(2-3)</td>
<td>(2-2)</td>
<td>(3-3)</td>
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<tr>
<td>Combined</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>(2-4)</td>
<td>(2-3)</td>
<td>(3-3)</td>
<td>(2-3)</td>
<td>(3-3)</td>
<td>(3-3)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.3. Mucosal protein (g.L⁻¹) of mirror carp (Cyprinus carpio) following netting stress. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Time after stress hr⁻¹</th>
<th>Pre</th>
<th>1.5</th>
<th>4</th>
<th>6.5</th>
<th>24</th>
<th>168</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>0.3</td>
<td>0.3</td>
<td>1</td>
<td></td>
</tr>
<tr>
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4.3.2 Haematological parameters

Peripheral neutrophil numbers were unaffected by both diet and the application of stress in all groups of fish (Table 4.4). Change during recovery from stress was only seen in fish fed the control diet, which showed elevated neutrophil numbers at 4 hours post-stress compared to 1.5 and 168 hours post-stress (Table 4.4).
All fish, irrespective of diet, showed lower percentage peripheral thrombocytes at 168 hours post-stress than at earlier points in the trial. However, post-stress thrombocyte numbers were not significantly different from pre-stress numbers (Figure 4.1). The greatest reductions in thrombocytes at this time point were observed in fish fed the nucleotide diet, where the 168 hour sample was significantly lower than those taken at 1.5 – 24 hours post-stress, with the least reduction found in fish fed the combined supplement diet. The only significant difference that could be attributed to diet was found at 1.5 hours post-stress, where fish fed the β-glucan diet showed higher peripheral thrombocyte at 1.5 hours after stress than fish fed the combined diet (Figure 4.1).

As was the case for thrombocytes, post-stress lymphocyte numbers were not significantly different from pre-stress numbers, with the exception of fish fed the combined supplement diet (Figure 4.2). These fish showed significantly lower lymphocytes at 6.5 hours post-stress than prior to the application of stress.

Significant reductions in peripheral lymphocytes were observed following stress, with all groups of fish showing lower lymphocytes at 4 and 6.5 hour post-stress than the 168 hour post-stress sample. Fish fed the nucleotide diet showed the most sustained reductions in lymphocyte number, from 1.5 – 24 hours (Figure 4.2). Fish fed the control diet showed significant reductions only at 4 and 6.5 hours post-stress with the β-glucan and combined supplement fed-fish being intermediate between the nucleotide and the control groups of fish. At 168 hours post-stress, fish fed the nucleotide showed significantly higher peripheral lymphocyte numbers than those fed the β-glucan diet (Figure 4.2).

No differences in peripheral monocyte numbers were apparent between treatments at any point in the trial. Peripheral monocytes were elevated, irrespective of diet, at 4 and 6.5 hours post-stress compared to pre-stress counts (Figure 4.3).
Table 4.4. Percentage peripheral neutrophil counts of mirror carp (*Cyprinus carpio*) following netting stress.

Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Pre</th>
<th>1.5</th>
<th>4</th>
<th>6.5</th>
<th>24</th>
<th>168</th>
</tr>
</thead>
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<tr>
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<td>1.5b</td>
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<td>0.4</td>
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<td>(0.4-0.9)</td>
<td>(0.4-0.5)</td>
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<td>(0-0.4)</td>
</tr>
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<td>Combined</td>
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<td>0.6</td>
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<tr>
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<td>(0-2.0)</td>
<td>(0-2.7)</td>
<td>(0-0.1)</td>
</tr>
</tbody>
</table>
Figure 4.1. Percentage peripheral thrombocyte counts of mirror carp (*Cyprinus carpio*) following netting stress. Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by diet code (C (control); β (β-glucan); N (nucleotide); Co (Combined)) and superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® pellet food.

Figure 4.2. Percentage peripheral lymphocyte counts of mirror carp (*Cyprinus carpio*) following netting stress. Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by diet code (C (control); β (β-glucan); N (nucleotide); Co (Combined)) and superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® pellet food.
Figure 4.3. Percentage peripheral monocyte counts of mirror carp (Cyprinus carpio) following netting stress. Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by diet code (C (control); β (β-glucan); N (nucleotide); Co (Combined)) and superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® pellet food.

Figure 4.4. Plasma glucose (mg.dL⁻¹) of mirror carp (Cyprinus carpio) following netting stress. Values are medians; vertical bars represent inter-quartile range. Significant differences between time points are indicated by diet code (C (control); β (β-glucan); N (nucleotide); Co (Combined)) and superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® pellet food.
Significant hyperglycaemia was observed in all groups of fish at 1.5 hours post-stress compared to pre-stress values; with secondary elevations in plasma glucose at 168 hours post-stress (Figure 4.4). At 4 hours post-stress, fish fed the control diet produced a significantly greater hyperglycaemic response than all other groups of fish. Fish fed the nucleotide diet showed significantly higher plasma glucose than the β-glucan fed fish at 6.5 hours post-stress and fish fed the combined supplement diet at 24 hours post-stress (Figure 4.4). All groups of fish fed dietary immuno-stimulants showed a quicker recovery from the hyperglycaemic spike seen at 1.5 hours post stress. These fish showed a return to pre-stress plasma glucose concentrations by the 4 hour post-stress sample, whilst those fed the control diet took until 24 hours post-stress to return to their pre-stress glucose concentration (Figure 4.4).

All fish, except those fed the β-glucan diet which showed no change, experienced a reduction in plasma protein concentrations following stress relative to their pre-stress state (Table 4.5). In the all groups, these reductions in plasma protein were seen at 4 and 168 hours post-stress. All fish fed the immuno-stimulant containing diets showed significant change shortly post-stress, with values at 4 hours being significantly lower than both the 1.5 and 6.5 hour samples (β-glucan and combined diets) or 1.5 and 24 hour samples (nucleotide diet) (Table 4.5). During recovery from stress, (4 – 168 hours), fish fed either the control or β-glucan diet displayed significantly higher plasma protein concentrations than those fed the nucleotide or combined diet (Table 4.5).

### 4.3.3 Immunological parameters

Significant differences in the NBT reduction ability of blood were apparent at various points during the trial (Table 4.6). Prior to stress, fish fed the control and β-glucan diets showed greater reduction than those fed the nucleotide diet. At 1.5 hours post-stress the β-glucan fed fish showed significantly lower NBT reduction than all other
groups of fish. At 6.5 hours the nucleotide fed fish showed significantly greater reduction than those fed the β-glucan diet. At 24 hours post-stress fish fed the control diet showed greater reduction than those fed the nucleotide and combined diets. Finally, at 168 hours post-stress, fish fed the control or β-glucan diets showed greater reduction than those fed the nucleotide diet.

The only fish to show significant change in blood NBT reduction following stress were those fed the nucleotide diet. Here, NBT reduction increased immediately post-stress compared to pre-stress values (Table 4.6). All groups of fish showed changes in their NBT reduction ability during recovery from stress, with the exception of those fish fed the combined supplement diet, which showed no significant change in NBT reduction throughout the trial (Table 4.6). In addition to the post-stress increase in NBT reduction, fish fed the nucleotide diet showed significantly higher NBT reduction at 1.5 and 6.5 hours post-stress compared to the 4, 24 and 168 hour post-stress samples. The control group of fish showed greater NBT reduction at 1.5 hours than at 6.5 hours post-stress, whilst NBT reduction in blood of β-glucan fed fish was lower at 1.5, 6.5 and 24 hours compared to the 168 hour sample.

There were no significant differences in serum haemolytic complement activity of any individual group of fish following stress (Table 4.7). At 4 hours post-stress, fish fed the combined supplement diet showed significantly greater haemolytic activity than those fed the β-glucan diet. At 6.5 hours post-stress, β-glucan supplemented fish showed significantly greater haemolytic activity than those fed the nucleotide diet.
Table 4.5. Plasma protein (g.dl⁻¹) of mirror carp (Cyprinus carpio) following netting stress. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

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<tr>
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</table>

Table 4.6. Nitroblue tetrazolium reduction by blood of mirror carp (Cyprinus carpio) following netting stress. Values are median (OD₆₂₀ nm) per 10μl blood. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). Differences between treatments are indicated by asterisks. Control = Aquarian® pellet food.

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Table 4.7. Spontaneous haemolytic activity (ACH$_{50}$ U.ml$^{-1}$) of mirror carp (Cyprinus carpio) serum following netting stress. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). Differences between treatments are indicated by asterisks. Control = Aquarian® pellet food.

<table>
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<td>(39.8-183.7)</td>
<td>(11.6-41.5)</td>
<td>(41.2-179.1)</td>
<td>(24.8-136.1)</td>
</tr>
<tr>
<td>Combined</td>
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<td>28.6</td>
<td>98.8*</td>
<td>89.1</td>
<td>117.8</td>
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<td>(-)</td>
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<td>(89.7-154.5)</td>
<td>(22.0-164.6)</td>
<td>(33.9-251.0)</td>
<td>(28.0-136.2)</td>
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(-) = no IQ range calculated due to small sample size

Table 4.8. Lysozyme activity (U.ml$^{-1}$ serum) of mirror carp (Cyprinus carpio) serum following netting stress. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

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<th>6.5</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>8.3</td>
<td>6.4</td>
<td>4.5</td>
<td>11.6$^b$</td>
<td>9.6</td>
<td>1.8$^a$</td>
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<tr>
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<td></td>
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<td>(4.6-12.2)</td>
<td>(--)</td>
<td>(7.1-17.3)</td>
<td>(--)</td>
<td>(1.3-3.6)</td>
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<td>16.4</td>
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<td>2.2</td>
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<td></td>
<td></td>
<td>(--)</td>
<td>(--)</td>
<td>(--)</td>
<td>(0.4-20.9)</td>
<td>(0.9-11.1)</td>
<td>(--)</td>
</tr>
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<td>(--)</td>
<td>(2.2-12.9)</td>
<td>(--)</td>
</tr>
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</table>

(-) = no IQ range calculated due to small sample size
The diet fed to the fish prior to stress had no significant effect on serum lysozyme activity and the application of stress did not elicit any significant change from pre-stress activity in any group of fish (Table 4.8). Fish fed the control diet showed elevated lysozyme activity at 6.5 hours post-stress compared to the 168 hours post-stress sample (Table 4.8). Statistical analysis of lysozyme activity was hampered by small sample sizes.

4.3.4 Relationships

Relationships were sought between known stress indicators and the immune and mucosal parameters tested. Three significant relationships could be drawn, all between haemolytic complement activity and plasma glucose (Figure 4.1). Fish fed the β-glucan diet showed a positive relationship between the two parameters (KT = 0.73, p = 0.04), whilst those fed the nucleotide and combined supplement diet showed negative relationships (KT = -0.73, p = 0.04 and -0.87, p = 0.02 respectively).
Figure 4.5. Relationship (Kendall’s Tau) analysis of plasma glucose and spontaneous haemolytic complement of mirror carp (Cyprinus carpio) serum following netting stress.

A: β-glucan diet, KT = 0.73, p = 0.04
B: Nucleotide diet, KT = -0.73, p = 0.04
C: Combined supplement diet, KT = -0.87, p = 0.02

Each value represents the median treatment value (n = 3) of both sampled parameters at a single time point.
4.4 Discussion

Changes in several parameters associated with the epidermal mucus of fish were detected during the course of this trial although, the same problems encountered in previous trials (section 3.A & 3.B) meant that only mucosal haemoglobin and protein were analysed fully in fish in the current trial. However, clear differences in the response of the epidermal mucus to stress were apparent between the current trial and those conducted previously.

Stress-induced increases in mucosal haemoglobin of fish, which were a consistent feature of previous trials, were not seen in the current trial. Only one group of fish, those fed the combined supplement diet, showed any significant change in mucosal haemoglobin concentration during recovery from stress.

All fish fed the immuno-stimulant containing diets showed a reduction in mucosal protein concentration during recovery from stress; a pattern of results consistent with previous trials. As for mucosal haemoglobin, the significant stress-induced changes seen in previous trials were absent in the current trial. Mucosal protein concentration has been found to be less closely linked to blood stress indicators and the lack of a strong haemoglobin response suggests that the mucosal stress response was modified in the fish used in the current trial.

Despite this modification of the mucosal stress response, the haematological parameters tested indicated that a significant stress response was induced by the netting stressor applied and this response was consistent with that observed in fish in previous trials using the same stressor.

Significant hyperglycaemia was apparent in the blood of fish in this trial as observed in previous trials. Pre-stress values of 21–24 mg.dl$^{-1}$ increased to 56–70 mg.dl$^{-1}$ by 1.5 hours after stress. This compares to data obtained under similar conditions for similar sized *C. auratus* and *C. carpio* (25–43 mg.dl$^{-1}$ increasing to 46–56 mg.dl$^{-1}$
& 28 mg.dl\(^{-1}\) increasing to 90 mg.dl\(^{-1}\) (sections 3.A and 3.B respectively)). These data are also comparable with values reported for *C. carpio* (Price *et al.*, 1997, Pottinger, 1998, Ruane *et al.*, 2001, Ruane *et al.*, 2002a, Ruane *et al.*, 2002b). These data indicate that in the case of the current trial, the fish's mucosal stress response, especially the appearance of haemoglobin, has been modified from that seen in previous trials (section 3.A & 3.B).

The fish's plasma protein concentration was significantly altered both by stress and during recovery from stress, a result which differs from that observed in previous trials (section 3.B). The pre-stress values obtained (2.7 – 3.0 g.dl\(^{-1}\)) were comparable with those reported for healthy, unstressed wild *C. carpio* (~3 g.dl\(^{-1}\) (serum)) (Price *et al.*, 1997), Koi carp (*C. carpio*) (~4.7 g.dl\(^{-1}\)) (Yin *et al.*, 1995) and for rohu (*Labeo rohita*) (1.2 – 3.5 g.dl\(^{-1}\)) (Choudhury *et al.*, 2005, Misra *et al.*, 2006).

Alterations in serum protein concentration have been reported in immuno-stimulated fish; with elevations in serum protein concentration following supplementation with β-glucans (Misra *et al.*, 2006) and nucleotides (Siwicki *et al.*, 1994, Choudhury *et al.*, 2005). However the data did not support these observations and the alterations observed appear to be stress-linked.

The release of stress hormones has been reported to have numerous effects on the physiology of the fish, resulting in increased metabolic activity (Mazeaud *et al.*, 1977). To facilitate this heightened metabolic activity, blood flow to the gills increases, with a resulting increase in gill surface. Although this increased surface area allows greater oxygen uptake, there are associated negative effects including increased fluid transfer resulting from heightened gill permeability. Fletcher (1975) found that in marine fish, such as winter flounder (*Pleuronectes americanus*), increased gill permeability causes greater loss due to osmosis, concentrating the blood and increasing blood protein concentration. The opposite is true in freshwater fish, such as *C. carpio*, where the relative osmotic strengths of the tissues and water cause a net water influx (Adedire and
Oduleye, 1984). This influx may result in haemodilution, resulting a corresponding decrease in blood protein levels; Yin et al., (1995) observed this haemodilution in Koi carp (C. carpio) following crowding stress. In the current trial, high levels of plasma glucose present in the blood of the fish sampled at the same times as low protein concentration suggest that haemodilution was the causative agent for decreases in protein concentration.

Injection with β-glucans has been found to significantly enhance the numbers of peripheral neutrophils and monocytes (Jeney and Anderson, 1993, Jorgensen et al., 1993, Siwicki et al., 1994, Selvaraj et al., 2005); no evidence of this was found in the current trial. However, the route of immuno-stimulant administration is important and whilst injection with β-glucan can enhance leukocyte number, presentation by either bath or oral routes was found not to elicit the same level of response (Selvaraj et al., 2005).

Following the application of the netting stressors, peripheral blood leukocyte numbers underwent patterns similar to those observed previously following similar stressors. Two classic leukocyte patterns were observed, namely neutrophilia, and lymphopenia, which are common features of the teleost stress response (Pickering and Pottinger, 1987, Ainsworth et al., 1991). Neutrophilia was limited to the fish fed the control diet and occurred during recovery from stress, although lymphopenia was observed in the peripheral blood of all groups of fish following stress. The only stress induced change in lymphocyte number was seen in the combined supplement fed fish. The duration of the post-stress lymphopenia was greatest in the fish fed the nucleotide diet and may represent a more rapid mobilisation of these immune cells into the tissues in the presence of nucleotides than in their absence. Significant increases in monocytes were observed on a similar timescales to that of the neutrophilia, indicating a mobilisation of key immune cells following stress, a widely reported response of the vertebrate immune system (Hlavek and Bulkley, 1980, Elsasser and Clem, 1986, Pulsford et al., 1994, Weyts et al., 1998, Ascencio'Valle et al., 2004, Engler et al., 2004).
Monocyte mobilisations in response to stress were not significantly altered by the diet fed to the fish.

There is some evidence that the diet fed to the fish influenced their subsequent response to fish; in particular the duration of the post-stress hyperglycaemic response was greatest in the control group of fish (1.5 – 6.5 hours), whilst fish fed the immuno-stimulants diets had shown recovery to pre-stress values by 4 hours post-stress. This suggests that the addition of dietary immuno-stimulants down-regulated the response to stress of this parameter and enhanced the recovery of these groups of fish. Contrastingly, the effect of stress on peripheral lymphocytes was greatest in fish fed the nucleotide-containing diets and the least in fish fed the control diet. Whilst a reduction in the magnitude of the hyperglycaemic response is consistent with the reported stress-alleviating properties of immuno-stimulants (Burrells et al., 2001b, Leonardi et al., 2003), the greater lymphocyte migration out of the circulation is less clearly attributable to stress. However it is the opinion of several researchers that the mobilisation of lymphocytes from peripheral blood represents an enhancement of the immune response following stress, so the greater mobilisation of these cells in nucleotide supplemented fish may be an adaptive response (Benschop et al., 1996, Carlson et al., 1997, Engler et al., 2004). However, losses of lymphocytes through apoptosis can not be ruled out.

The greatest-stress induced changes in plasma protein concentration were seen in fish fed the nucleotide-containing diet, which may in part be due to increases in gill permeability. As this is an adaptive mechanism to support stress-induced metabolic shifts (Fletcher, 1975, Adedire and Oduleye, 1984, Yin et al., 1995), these changes may be indicative of a greater ability to adapt to the burden of stress. This is conducive with the reported energy sparing nature of nucleotides (Li and Gatlin, 2006). Fish fed the β-glucan diet showed a response to stress intermediate to the extremes, again this may be due to the stress-reducing effects of β-glucans.
The range of serum lysozyme activity recorded in the current was 1.78 – 16.4 U.ml\(^{-1}\) which is comparable to values reported for several species of Indian major carp, which typically show serum lysozyme activities in the range of 2 – 11 U.ml\(^{-1}\) (Sahoo et al., 2005) and for C. carpio; 12 – 18 U.ml\(^{-1}\) (Jian strain) (Jian and Wu, 2004), but lower than the 42 – 55 U.ml\(^{-1}\) reported for un-stimulated C. carpio from cold water environments (Price et al., 1997, Sakai et al., 2001). In C. carpio supplemented with either nucleotides or β-glucans, serum lysozyme activity has been observed to significantly increase, although with β-glucans this has again been largely in response to injection (Engstad et al., 1992, Jorgensen et al., 1993) Oral supplementation with nucleotides induced significant increases in lysozyme activity of C. carpio up to ~140 U.ml\(^{-1}\) compared to un-stimulated fish (Sakai et al., 2001). These data suggest that the immuno-stimulant diets fed to the fish had no stimulatory effects on lysozyme production, a result observed in hybrid striped bass (Morone chrysops X. M. saxatilis) supplanted orally with β-glucans and nucleotides from intact yeast cells (Li and Gatlin, 2003, Li and Gatlin, 2004, Li et al., 2004).

Serum spontaneous haemolytic activity of fish in the current trial ranged 20 – 158 ACH\(_{50}\) U.ml\(^{-1}\) which fall in the range of values quoted for C. carpio and other species. Indian major carp species typically exhibit values in the range of 29 – 90 ACH\(_{50}\) U.ml\(^{-1}\) (Dash et al., 2000, Sahoo et al., 2005) with common carp (C. carpio) showing values of 53 - 68 ACH\(_{50}\) U.ml\(^{-1}\) (Matsuyama et al., 1988, Yano, 1992, Sakai et al., 2001), although values of up to ~560 ACH\(_{50}\) U.ml\(^{-1}\) have been reported for un-stimulated C. carpio (Selvaraj et al., 2005).

The NBT reduction ability of whole blood were affected by both diet and stress, however, there was not a consistent pattern for these differences. The level of NBT reduction ability was similar to that reported for healthy un-stimulated fish Chen et al., (2001) suggesting that the immuno-stimulating diets did not act to increase the NBT activity of blood. Indeed in a separate trial, blood OD\(_{620nm}\) values for un-stimulated C.
carpio were ~ 0.28 (Malina et al., 2002), suggesting that NBT reduction by whole blood of the fish in the current trial may have actually been suppressed, possibly due to the different temperature. However differences in the duration of incubation period of blood and NBT solution between the current trial and that conducted by Malina et al., (2002) may account in part for the differences. This method of assessing the oxidative production of blood is difficult to interpret as it only provides relative assessment of oxidative potential.

Of the humoral immune parameters tested, serum lysozyme and spontaneous haemolytic activity showed some effects of both diet and stress, but no consistent patterns were apparent. In the case of lysozyme, this may in part have been due to a lack of replication due to small sample volumes, which prevented extensive replication, factors that may have compounded the inherently large variation shown by lysozyme activity (Grinde et al., 1988b, Anderson and Siwicki, 1995). Significant change in serum lysozyme activity was apparent in fish fed the control diet only, and then only during recovery from stress; fish that received diets containing immuno-stimulants showed no change. This is contradictory to trials using both β-glucan and nucleotide supplements in which enhancement of lysozyme activity was found (Engstad et al., 1992, Thompson et al., 1995, Sakai et al., 2001).

The β-glucan content of the diet appears to have influenced spontaneous haemolytic activity; where significantly higher activity was seen in groups of fish that had been fed β-glucans. Intra-peritoneal injection of β-glucan has been found to stimulate spontaneous haemolytic activity (Engstad et al., 1992, Jeney and Anderson, 1993, Jorgensen et al., 1993), which may have contributed to the results seen. Although fish fed the combined diet showed elevated spontaneous haemolytic activity and nucleotides have been found to enhance this immune parameter (Sakai et al., 2001) the lack of an enhancement in fish receiving the nucleotide supplement only suggests that the causative agent was β-glucan and not nucleotides. However, orally supplemented
immuno-stimulants are less effective at enhancing spontaneous haemolytic activity those administered by injection (Baulny et al., 1996, Verlhac et al., 1998, Cook et al., 2003, Selvaraj et al., 2005, Kumari and Sahoo, 2006b).

Some differences in the ability of blood to reduce NBT were seen. Prior to stress fish fed the nucleotide diet showed significantly lower reduction than the other groups of fish. Following stress the control and nucleotide-supplemented fish showed significant elevation in NBT reduction at 1.5 hours post-stress, whilst those fed the β-glucan and combined diet showed no change. This indicates that nucleotides enhance the ability of cells to undergo respiratory burst following stress, whilst β-glucans limit respiratory burst activity. This is conducive with research by Castro et al., (1999) who found that β-glucan stimulation inhibited respiratory burst through prolonged macrophage activation.

Of the relationships that were sought, the only ones that proved significant were between glucose and spontaneous haemolytic activity. The marked difference between responses appear to be attributable to the nucleotide content of the diet; where nucleotides had been fed to fish negative relationships were present, where β-glucan was the sole immuno-stimulant in the diet, a positive relationship was found. The control group showed no relationship between plasma glucose concentration and spontaneous haemolytic complement activity. These results suggest that in fish that received the β-glucan supplement an immuno-stimulatory effect on spontaneous haemolytic activity occurred following stress, whilst the inclusion of nucleotides had the opposite, suppressive effect. The inclusion of nucleotides themselves may not have been entirely responsible for this decrease as stress-induced suppression of spontaneous haemolytic activity has been reported in a variety of fish species (Yin et al., 1995, Montero et al., 1999, Ortuno et al., 2001). However, given that lack of response shown by the control group of fish, it would appear that the condition was greater in those fish that had received nucleotides.
In conclusion, the current trial suggests that oral presentation diets supplemented with 0.2 % w/w β-glucans, 0.2 % w/w nucleotides individually or together significantly may subtly modify the stress response or immune status of the fish used in the current trial. No significant enhancements of immune parameters were observed prior to stress, rejecting $H_{01}$.

In particular the response to the netting stressor, as characterised by the glucose and cellular immune response, appeared reduced in fish that had received immuno-stimulants, regardless of their type. The nucleotide diet may be more effective at limiting the effects of stress, possibly due to the energy sparing potential of the nucleotide supplement, allowing acceptance of $H_{03}$.

The effects of stress on other immunological parameters appear heightened in some cases and reduced in others. Respiratory burst activity appears to have been negatively affected by the presence of β-glucan, whilst spontaneous haemolytic activity appeared enhanced by β-glucan. Finally, there appears some evidence that β-glucan may have alleviated some aspects of stress-induced immuno-suppression, allowing partial acceptance of $H_{02}$. The effects of nucleotides on the immune parameters tested was minimal and there was no evidence that the combination of nucleotides and β-glucans in the diet generated a significantly better response than when administered singly, rejecting $H_{04}$. 
Chapter 5. The effects of dietary immuno-stimulation on the innate and adaptive immune response of mirror carp (Cyprinus carpio) following vaccination with Aeromonas salmonicida
5.1 Introduction

Ornamental fish are at risk of infection from a range of opportunistic and obligate bacterial pathogens. Due to the limited virulence of opportunistic bacteria, only immunocompromised fish, typically those experiencing stress, are likely to become infected. In contrast, obligate pathogens are specialised organisms that must infect a host organism to complete their life cycle. These pathogens express a range of virulence factors which are extracellular products or structural components of the cell wall, which whilst they can initiate strong immune responses in fish, have toxic properties or are otherwise capable of inhibiting host immune defences and reducing immuno-competence. Examples include the carbohydrate-lipid complex, lipopolysaccharide (LPS) expressed on the outer membrane of Gram-negative bacteria and sialic acid, which is immunosuppressive on its own but can work in tandem with LPS (Munn et al., 1982, Ourth and Bachinski, 1987, Jenkins et al., 1991). These pathogens can cause widespread damage to captive fish populations and are often fatal. One such pathogen is Aeromonas salmonicida which can cause large lesions within the surface tissues of the fish. If the fish survives infection, these lesions may cause scarring, reducing their aesthetic appeal. Other fish pathogens of note include Streptococcus iniae which has the potential to cause massive losses to farmed food and ornamental fish in both marine and freshwater (Baya et al., 1990, Zlotkin et al., 1998, Colorni et al., 2002).

Although vaccines are available against Aeromonas salmonicida and are commonly employed as disease prevention methods in aquaculture, they are of limited applicability to ornamental fish as discussed in section 1.4.1. Similarly, antibiotics have limited application in the ornamental fish industry due to cost and licensing regulations as described in section 1.4.1. Immuno-stimulants either administered singly or in combination with a vaccine, if the conditions allow, have been reported to be effective at
increasing survival in a number of fish species following both bacterial and viral pathogen challenge.

Injected or orally supplemented β-glucans have been found to increase resistance to bacterial challenge in a range of fish species, from important aquaculture species such as salmon (S. salar) (Robertsen et al., 1990, Raa et al., 1992), channel catfish (Ictalurus punctatus) (Chen and Ainsworth, 1992) and hybrid striped bass (Morone chrysops X. M. saxatilis) (Li and Gatlin, 2003, 2004) to important ornamental fish species including the red-tailed black shark (Epalzeorhynchos bicolor) (Russo et al., 2006), rosy barbs (Puntius conchonius) and black tetras (Gymnocorymbus ternetzi) (Tuernau et al., 2000). Similarly, oral supplementation with nucleotides has been shown to increase both bacterial and viral resistance in both commercially farmed food fish (Burrells et al., 2001b, Sakai et al., 2001, Li and Gatlin, 2004, Li et al., 2004) and ornamental fish species (Russo et al., 2006).

In addition to generally boosting the immune response and priming the fish to combat pathogen challenge, β-glucans and nucleotides have been reported to increase the efficacy of vaccination and have been implicated as potential adjuvants. In this respect the immuno-stimulant augments the specific immune response of the fish to the vaccine, potentially enhancing the duration of immunological memory (Rorstad et al., 1993). The adjuvant properties of β-glucans have been shown in salmonids (Nikl et al., 1993), turbot (Scophthalmus maximus) (Toranzo et al., 1995) and ornamental fish species (Russo et al., 2006). Nucleotides have been found to enhance the post-vaccination antibody titre and survival in tilapia (Oreochromis sp.) (Ramadan et al., 1994), salmon (S. salar) (Burrells et al., 2001b) and ornamental fish (Russo et al., 2006).

This aim of this trial was to investigate the effect of dietary supplementation with immuno-stimulants prior to and following infection with an obligate bacterial pathogen A. salmonicida. A commercially available vaccine was used to simulate the presence of a pathogen without inducing a disease state. The responses of both the IIS and AIS were
assessed to investigate the effects of dietary immuno-stimulants on the post-infection response of fish.

The specific hypotheses tested were as follows:

\[ H_01 \] – increased vaccine efficacy, as quantified by specific antibody titre, will be seen in fish receiving diets containing 0.2 % (w/w) β-glucan or a combination of 0.2 % (w/w) β-glucan and 0.2 % (w/w) nucleotide.

\[ H_02 \] – serum lysozyme, serum spontaneous haemolytic activity and respiratory burst activity of blood will be enhanced in fish receiving diets containing 0.2 % (w/w) β-glucan or a combination of 0.2 % (w/w) β-glucan and 0.2 % (w/w) nucleotide following vaccination.

\[ H_03 \] – the response of fish to the stress of vaccination, as indicated by glucose and peripheral leukocyte counts, will be reduced in fish receiving diets containing 0.2 % (w/w) β-glucan or a combination of 0.2 % (w/w) β-glucan and 0.2 % (w/w) nucleotide.
5.2 Materials and Methods

5.2.1 Fish and husbandry

One hundred and eighty (180) mirror carp (Cyprinus carpio) with a mean (± S.D.) initial body weight of 26.67 ± 0.2 g were obtained from Hampshire Carp Hatcheries [Twyford, UK]. Fish were maintained in nine tanks within system D in the experimental Aquarium of the University of Plymouth as described in section 2.1.1. Water temperature was maintained at 23 ± 1 °C. Fish were randomly allocated one of the following experimental diets (control, β-glucan or combined supplements), in pellet form, as described in section 2.1.2. Diets were tested in triplicate and administered at a rate of 1.0 % BW.d⁻¹. Fish were fed the experimental diets for two weeks prior to vaccination and for one week following vaccination, for total immuno-stimulation duration of 3 weeks. After this period, all fish were fed the control diet (section 2.1.2) for the duration of the trial. Adjustments to feed intake to account from growth were calculated using Thermal Growth Coefficient calculations based on growth data obtained during the acclimation period (Cho and Bureau, 1998).

5.2.2 Vaccination

Fish were vaccinated via the intra-peritoneal route with 0.1 ml of Aquavac® Furovac 5 [Schering-Plough Aquaculture, Saffron Waldon, UK], which contains intact, formalin-killed Aeromonas salmonicida in a non-mineral oil adjuvant.

5.2.3 Sampling Strategy

Haematological and immunological parameters were measured prior to the commencement of immuno-stimulant supplementation, two weeks prior to vaccination.
Table 5.1. Experimental protocol

<table>
<thead>
<tr>
<th>No.</th>
<th>Parameters measured</th>
<th>Sampling times</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Specific Ig ELISA</td>
<td>Pre supplementation</td>
</tr>
<tr>
<td></td>
<td>(3 fish per tank)</td>
<td>2, 4, 6, 8, 10 &amp; 12 weeks</td>
</tr>
<tr>
<td>2</td>
<td>NBT reduction (whole blood)</td>
<td>post-vaccination</td>
</tr>
<tr>
<td></td>
<td>(2 fish per tank)</td>
<td>2, 4, 6, 8, 10 &amp; 12 weeks</td>
</tr>
<tr>
<td>3</td>
<td>Differential leukocyte counts</td>
<td>Pre supplementation</td>
</tr>
<tr>
<td></td>
<td>(2 fish per tank)</td>
<td>2, 4, 6, 8, 10 &amp; 12 weeks</td>
</tr>
<tr>
<td>4</td>
<td>Plasma protein</td>
<td>post-vaccination</td>
</tr>
<tr>
<td></td>
<td>(2 fish per tank)</td>
<td>Pre supplementation</td>
</tr>
<tr>
<td>5</td>
<td>Plasma glucose</td>
<td>2, 4, 6, 8, 10 &amp; 12 weeks</td>
</tr>
<tr>
<td></td>
<td>(2 fish per tank)</td>
<td>post-vaccination</td>
</tr>
<tr>
<td>6</td>
<td>Serum lysozyme (pooled sample, 1 per tank)</td>
<td>Pre supplementation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2, 4, 6, 8, 10 &amp; 12 weeks</td>
</tr>
<tr>
<td>7</td>
<td>Serum spontaneous haemolytic activity</td>
<td>post-vaccination</td>
</tr>
<tr>
<td></td>
<td>(3 fish per tank)</td>
<td></td>
</tr>
</tbody>
</table>

Following vaccination, haematological and immunological parameters were sampled as outlined in Table 5.1. Following sampling fish were returned to their respective tanks and may have been re-sampled during the experiment.

5.2.4 Haematological parameters

Blood was collected and serum and plasma prepared as described in section 2.3. Serum was prepared from three of the five fish sampled at each time point and plasma prepared from the remaining two fish.

Differential leukocyte counts

Blood smears were prepared and differential leukocyte counts were conducted as described in section 2.4.2.
Glucose

Plasma glucose was assayed using the GOD method [Sigma-Aldrich] as described in section 2.4.2.

Total protein

Plasma protein concentration was determined using the micro scale version of the Bio-rad modification of the Bradford method (Bradford, 1976) (section 3.A.2.2).

5.2.5 Immunological parameters

NBT reduction of whole blood

The NBT reduction ability of whole blood was assayed as described in section 4.2.5.

Serum spontaneous haemolytic activity

Spontaneous serum haemolytic activity was assessed as described in section 4.2.5.

Serum lysozyme activity

Serum lysozyme was assayed using the turbidometric method as described in section 4.2.5.

Specific Antibody Enzyme Linked Immunosorbant Assay (ELISA)

Specific Ig raised by vaccinated fish against *A. salmonicida* was measured using indirect ELISA [Aquatic Diagnostics, Stirling, UK].
Antigen culture

A virulent strain of *Aeromonas salmonicida* previously isolated from wild, infected Atlantic salmon (*S. salar*) in the Tamar river (maintained as part of the University of Plymouth Culture Collection) was grown on Tryptone Soya Agar (TSA) [Oxoid, UK] + 1.5 % NaCl for 24 hours at 25 °C. Cell counts were carried out to determine the concentration of bacterial cells. 1 ml of culture was diluted with PBS in a ten-fold dilution series to $10^8$. 0.1 ml of each dilution ($10^6 - 10^8$) were spread plated onto duplicate TSA + 1.5 % NaCl plates and incubated at 25 °C for 24 – 48 hours. The colonies on the most countable plate (30 – 300 colonies) were enumerated and the cell count of the original culture was calculated in Colony Forming Units (CFU) ml$^{-1}$. The initial concentration was $1 \times 10^9$ CFU ml$^{-1}$.

One colony was used to inoculate 50 ml of TSA + 1.5 % NaCl which was then incubated in a shaking water bath at 25 °C for a further 24 hours. The resulting bacteria were washed twice in sterile PBS at 3600 x g for 15 minutes and were re-suspended in sterile PBS and diluted to $1 \times 10^8$ CFU ml$^{-1}$ before use in the ELISA procedure.

ELISA procedure

A 96 well ELISA plate was coated with 0.05 % (w/v) poly-L-lysine in coating buffer (pH 9.6) (Appendix A), 50 μl well$^{-1}$ for 60 min and washed twice with low salt wash buffer (pH 7.3) (Appendix A). 100 μl$^{-1}$ *Aeromonas salmonicida* ($1 \times 10^8$ bacteria ml$^{-1}$ in PBS) was added to each well and incubated overnight at 4 °C. $50 \mu l$ well$^{-1}$ of 0.05 % (v/v) gluteraldehyde in PBS (Appendix A) was added to the antigen and incubated for a further 20 min at 22 °C. The plate was then washed three times with low salt wash buffer. Non-specific binding sites were blocked using 1 % bovine serum albumin (BSA) in PBS; 250 μl well$^{-1}$ was added and incubated at 22 °C for 2 hours. The plate was then washed three times with low salt wash buffer. Doubling dilutions of fish serum in PBS were prepared starting with a 1:2 dilution, with pre-vaccination serum being used as a control and PBS...
as a negative control. 100 μl well⁻¹ of test serum and control dilutions were added and incubated overnight at 4 °C. The plate was then washed five times with high salt wash buffer (pH 7.7) (Appendix A), incubating for 5 minutes on the last wash. One vial (200 μg) of anti-carp monoclonal antibody (Mab) [Aquatic Diagnostics, Stirling UK], was reconstituted in 1 ml of PBS and added to 32 ml antibody buffer (1 % BSA solution) (Appendix A) and 100 μl of Mab solution was added to each well and the plate incubated at 22 °C for 60 minutes. The plate was then washed five times with high salt wash buffer, incubating for 5 minutes on the last wash. 100 μl well⁻¹ conjugate (Goat anti-mouse IgG-HRP diluted 1/1000 in 1 % BSA solution [Sigma-Aldrich]) (Appendix A) was added and incubated for 60 minutes at 22 °C. The plate was then washed five times with high salt wash buffer, incubating for 5 minutes on the last wash. 100 μl well⁻¹ of chromagen (42 mM 3’3’5’5’ tetramethylbenizidine dihydrochloride in 1:2 acetic acid: distilled water) in substrate buffer (pH 5.4) (Appendix A) was added and the plate incubated for 10 minutes at 22 °C. The reaction was stopped with 50 μl well⁻¹ of 2M H₂SO₄. The plate was read at 450 nm in an ELISA reader, using wells filled with chromagen and stop solution as a blank.

5.2.6 Statistical analysis

Unless otherwise stated, data is presented as median (inter-quartile range). Data was analysed using the non-parametric Kruskal-Wallis test with post hoc multiple comparison analysis (Minitab 14). Relationships were tested using Kendall’s Tau (Minitab 14), with an associated p value of ≤ 0.05 being required for a relationship to be considered significant.
5.3 Results

5.3.1 Haematological parameters

No clear effect of treatment on plasma glucose was apparent (Figure 5.1 A). All treatments showed significantly elevated plasma glucose concentration at 2 weeks post-vaccination and again at 12 weeks post-vaccination, compared to the pre-supplementation/vaccination sample and all samples from 4 – 10 weeks post-vaccination (Figure 5.1 A).

Fish fed the control diet showed the highest plasma protein concentration from week 4 to 10 post-vaccination, being significantly elevated relative to the β-glucan fed fish from weeks 4 – 6 and against the combined supplement fed-fish at weeks 8 – 10 (Figure 5.1 B). The only fish to show significant differences between pre and post-vaccination protein concentrations were those fed the combined supplement diet, where protein concentrations at 8 weeks post-vaccination were significantly lower than concentrations prior to supplementation/vaccination and those at 6 weeks post-vaccination (Figure 5.1 B). In all groups of fish, peak plasma protein concentration was observed at 4 weeks post-vaccination (Figure 5.1 B). Fish from the control group showed higher plasma protein at this sample point than at 2, 8 and 12 weeks post-vaccination. Fish given the β-glucan diet showed significantly lower plasma protein at week 6 compared to week 4, whilst those fed the combined supplement diet showed reduced protein at week 8 compared to 4 weeks post-vaccination.
Figure 5.1. Blood metabolites of mirror carp (Cyprinus carpio) fed immuno-stimulants and vaccinated against Aeromonas salmonicida.

A: plasma glucose (mg.dl⁻¹)
B: plasma protein (g.dl⁻¹)

Values are medians; vertical bars indicate inter-quartile range. Significant differences are indicated by run code (C (control); β (β-glucan); Co (Combined) and superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® pellet food.
There were no differences in percentage peripheral leukocyte count, for any of the leukocyte classes assessed, that could be attributed to the diet fed to the groups of fish.

Following vaccination significant elevations in peripheral neutrophil count were observed in the control group of fish, with the other groups showing no change (Figure 5.2). Following vaccination, fish fed the control and combined supplement diet showed significantly elevated neutrophil numbers at 2 weeks post-vaccination compared to 12 weeks post-vaccination (Figure 5.2). In the control group of fish, peripheral neutrophil counts at 2 weeks post-vaccination were also significantly higher than those at 8 weeks post-vaccination. Fish fed the β-glucan diet showed no change in peripheral neutrophil numbers during the course of the trial.
Table 5.2. Percentage peripheral thrombocyte counts of mirror carp (*Cyprinus carpio*) fed immuno-stimulants and vaccinated against *Aeromonas salmonicida*. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Pre supp.</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
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<td>17.6</td>
<td>21.0</td>
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<td>(7.4-15.7)</td>
<td>(7.5-20.1)</td>
<td>(6.1-28.9)</td>
<td>(13.1-23.8)</td>
<td>(13.8-21.3)</td>
<td>(16.5-27.0)</td>
</tr>
<tr>
<td>β-glucan</td>
<td>18.1</td>
<td>10.7</td>
<td>13.3</td>
<td>16.5</td>
<td>8.9</td>
<td>14.8</td>
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<td></td>
<td>(7.8-32.1)</td>
<td>(7.5-13.8)</td>
<td>(7.7-24.0)</td>
<td>(9.0-24.8)</td>
<td>(5.0-16.3)</td>
<td>(5.9-17.9)</td>
<td>(8.5-22.2)</td>
</tr>
<tr>
<td>Combined</td>
<td>15.6</td>
<td>10.9</td>
<td>11.3</td>
<td>13.5</td>
<td>11.7</td>
<td>12.11</td>
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<td></td>
<td>(6.7-17.9)</td>
<td>(9.2-12.7)</td>
<td>(9.9-23.7)</td>
<td>(9.7-20.0)</td>
<td>(4.6-25.4)</td>
<td>(9.9-22.3)</td>
<td>(9.2-21.3)</td>
</tr>
</tbody>
</table>

Table 5.3. Percentage peripheral lymphocyte counts of mirror carp (*Cyprinus carpio*) fed immuno-stimulants and vaccinated against *Aeromonas salmonicida*. Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Pre supp.</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
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<tbody>
<tr>
<td>Control</td>
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<td>86.9</td>
<td>88.9</td>
<td>79.6</td>
<td>84.6</td>
<td>82.2</td>
<td>78.2</td>
</tr>
<tr>
<td></td>
<td>(81.7-91.4)</td>
<td>(81.7-91.4)</td>
<td>(79.5-92.2)</td>
<td>(69.5-92.6)</td>
<td>(75.2-86.2)</td>
<td>(78.2-85.5)</td>
<td>(72.3-83.1)</td>
</tr>
<tr>
<td>β-glucan</td>
<td>80.1</td>
<td>87.5</td>
<td>86.3</td>
<td>82.9</td>
<td>90.5</td>
<td>84.6</td>
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<td>(84.4-90.2)</td>
<td>(75.8-92.0)</td>
<td>(74.7-90.5)</td>
<td>(83.5-94.1)</td>
<td>(81.3-93.5)</td>
<td>(77.1-90.5)</td>
</tr>
<tr>
<td>Combined</td>
<td>83.6</td>
<td>87.7</td>
<td>87.9</td>
<td>86.1</td>
<td>87.5</td>
<td>87.7</td>
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<td>(86.1-88.8)</td>
<td>(76.1-90.1)</td>
<td>(79.3-89.5)</td>
<td>(73.8-95.0)</td>
<td>(77.4-89.8)</td>
<td>(78.4-90.8)</td>
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</tbody>
</table>

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Figure 5.3. Percentage peripheral monocyte counts of mirror carp (*Cyprinus carpio*) fed immuno-stimulants and vaccinated against *Aeromonas salmonicida*. Values are medians; vertical bars indicate inter-quartile range. Significant differences are indicated by run code (C (control); β (β-glucan); Co (Combined) and superscript letter pairs (i.e. a/b, c/d etc.). Control = Aquarian® pellet food.

Peripheral thrombocyte counts were unaffected by supplementation and vaccination. No significant differences between diets were apparent at any point during the trial (Table 5.2). Similarly, peripheral lymphocyte counts were also unaffected by supplementation and vaccination (Table 5.3).

Peripheral monocyte counts of fish fed the control diet were unaffected by vaccination (Figure 5.3). Fish fed the β-glucan and combined diets showed significantly lower numbers of peripheral monocytes at 4 and 10 weeks post vaccination respectively compared to the pre-supplementation/vaccination and 2 week post-vaccination samples (Figure 5.3).
Figure 5.4. Nitroblue Tetrazolium reduction (OD$_{620}$ nm) by whole blood of mirror carp (Cyprinus carpio) fed immuno-stimulants and vaccinated against Aeromonas salmonicida.

Values are medians; vertical bars indicate inter-quartile range. Significant differences are indicated by run code (C (control); β (β-glucan); Co (Combined) and superscript letter pairs (i.e. a/b, c/d etc.). No samples were taken prior to vaccination (week 0). Control = Aquarian® pellet food.

5.3.2 Immunological parameters

Due to a problem with the assay system, which resulted in a precipitate forming during the final incubation step, no results were obtained from the specific Ig ELISA assay. No samples were analysed for NBT reduction prior to supplementation/vaccination. Fish fed the control diet showed significantly elevated NBT reduction ability at week 4 post-vaccination compared to weeks 2, 8 – 12 (Figure 5.4). Additionally, fish from the control group also showed significantly greater NBT reduction at week 6 than weeks 8 -12 post vaccination. Similar elevations were present for β-glucan fed fish, with NBT reduction at week 4 post-vaccination significantly elevated above values for weeks 2, 6, 10 and 12 post vaccination. Fish maintained on the
combined supplement diet showed significantly elevated NBT reduction at week 4 compared to weeks 6 and 12 post vaccination. The NBT reduction of whole blood was significantly lower a 6 weeks post-vaccination than at week 8 and 10 post-vaccination. Control and β-glucan fed fish showed significantly greater NBT reduction than combined-supplement fed fish at the start of the trial (weeks 4 and 6), this was reversed by the end of the trial with β-glucan and combined supplement fed fish showing elevated activity at the end of the trial (weeks 8 and 10). No significant differences were apparent between diets at weeks 2 and 12 post vaccination.

All lysozyme activities from samples taken prior to the onset of dietary supplementation were analysed as a single treatment and comparisons of post-vaccination changes were made against this single value. No significant differences were seen between treatments at any point during the trial. Fish fed the control diet showed significantly lower lysozyme activity at 2 and 12 weeks post vaccination than the pre-supplementation/vaccination sample and at 4 weeks after vaccination (Table 5.4). Similarly the fish fed the β-glucan and combined supplement diets showed significantly lower lysozyme activity at 2, 6 and 12 weeks after vaccination than the pre-supplementation/vaccination sample.

As was the case for serum lysozyme, all spontaneous haemolytic activity samples taken pre-supplementation/vaccination were analysed as a single treatment and comparisons of post-vaccination changes were made against this single value. No significant effects of either vaccination or diet fed to the fish were apparent on serum spontaneous haemolytic activity of fish sampled during this trial (Table 5.5). A high degree of variability in haemolytic activity was apparent following vaccination, especially in fish fed the control diet and particularly at 2 weeks post-vaccination (Table 5.5).
Table 5.4. Serum lysozyme activity (U.ml⁻¹) of mirror carp (Cyprinus carpio), fed immuno-stimulants and vaccinated against Aeromonas salmonicida.
Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

<table>
<thead>
<tr>
<th>Pre supp.</th>
<th>Diet</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
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<td>0.7ᵃ</td>
<td>4.9</td>
<td>4.7</td>
<td>0.5ᵃ</td>
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</tr>
<tr>
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<td>(2.3-68.7)</td>
<td>(0-2.2)</td>
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<td>(0-1.2)</td>
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</tr>
<tr>
<td>13.1ᵇ</td>
<td>β-glucan</td>
<td>2.2ᵃ</td>
<td>6.0</td>
<td>1.8ᵃ</td>
<td>6.2</td>
<td>4.4</td>
<td>0.7ᵃ</td>
</tr>
<tr>
<td>(9.8-16.9)</td>
<td></td>
<td>(0-3.6)</td>
<td>(2.2-11.6)</td>
<td>(0.7-3.3)</td>
<td>(4.6-10.9)</td>
<td>(4.4-8.0)</td>
<td>(--)</td>
</tr>
<tr>
<td>Combined</td>
<td>1.1ᵃ</td>
<td>3.6</td>
<td>0.9ᵃ</td>
<td>5.3</td>
<td>5.6</td>
<td>1.3ᵃ</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(0.1-3.5)</td>
<td>(2.2-8.9)</td>
<td>(0-2.2)</td>
<td>(3.1-7.1)</td>
<td>(4.3-7.2)</td>
<td>(0.4-2.2)</td>
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</tr>
</tbody>
</table>

(--) no inter-quartile range calculated due to small sample size.

Table 5.5. Serum spontaneous haemolytic activity (ACH₅₀ U.ml⁻¹) of mirror carp (Cyprinus carpio) fed immuno-stimulants and vaccinated against Aeromonas salmonicida.
Values are medians (inter-quartile range). Significant differences between values are indicated by superscript letter pairs (i.e. a/b, c/d etc.). No significant differences between treatments were found. Control = Aquarian® pellet food.

<table>
<thead>
<tr>
<th>Pre supp.</th>
<th>Diet</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
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<td>73.7</td>
<td>413.0</td>
<td>162.0</td>
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<tr>
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<td>(248-1130)</td>
<td>(10-400)</td>
<td>(16-262)</td>
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<tr>
<td>29.4ᵇ</td>
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<td>16.1</td>
<td>89.4</td>
<td>232.2</td>
<td>56.5</td>
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<td>(--)</td>
<td>(4-164)</td>
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<tr>
<td>Combined</td>
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<td>306.4</td>
<td>19.4</td>
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<td>(197-321)</td>
<td>(5-148)</td>
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</tbody>
</table>

(--) indicates that no inter-quartile range could be calculated due to small sample sizes.
Figure 5.5. Relationship analysis (Kendall’s Tau) of haematological and immunological parameters of carp (*Cyprinus carpio*), vaccinated against *A. salmonicida* and fed the control diet.

Plasma glucose (mg.dl⁻¹) vs. serum lysozyme (U.ml⁻¹), KT = -0.62, p = 0.05

Each value represents the median treatment value (n = 3) of both sampled parameters at a single time point. No NBT samples were taken prior to vaccination.

Relationships were sought between the various immune and haematological parameters measured. No significant relationships were found in fish that had been fed the β-glucan diet.

For fish fed the control diet, plasma glucose showed a weakly significant negative relationship (KT = -0.62, p = 0.05) with serum lysozyme activity (Figure 5.5). For fish fed the combined supplement diet, the post-vaccination ability of blood to reduce NBT showed significant positive relationships with serum haemolytic activity (KT = 0.73, p = 0.04) and serum lysozyme activity (KT = 0.73, p = 0.04) (Figure 5.6 A & B).
Figure 5.6. Relationship analysis (Kendall’s Tau) of haematological and immune parameters of carp (*Cyprinus carpio*) vaccinated against *A. salmonicida* and fed the combined supplement diet.

A: blood NBT reduction (OD₆₂₀nm) vs. serum haemolytic activity (ACH₅₀ U.ml⁻¹), KT = 0.73, p = 0.04

B: blood NBT reduction (OD₆₂₀nm) vs. serum lysozyme activity (U.ml⁻¹), KT = 0.73, p = 0.04

Each value represents the median tank value (n = 3) of both sampled parameters at a single time point. No NBT samples were taken prior to vaccination.
5.4 Discussion

Following vaccination various stress and immunological parameters were measured to monitor the physiological and immunological response of mirror carp (*Cyprinus carpio*) to the presence of the attenuated pathogen. Despite being fed immuno-stimulant containing diets for two weeks prior to and for a week following vaccination, there was little variation in the immune parameters measured between the different groups of fish.

The vaccination process appears to have induced significant stress on all fish regardless of group. Throughout the trial, plasma glucose concentrations were elevated above the values seen in previous trials. For example, pre-vaccination glucose levels were in the range of 33 – 48 mg.dl\(^{-1}\) which is slightly higher than pre-stress values observed in previous trials (21 – 24 and 25 – 43 mg.dl\(^{-1}\)), but still in the range indicative of unstressed *C. carpio* (Price *et al.*, 1997, Pottinger, 1998, Ruane *et al.*, 2002a, Ruane *et al.*, 2002b, Ruane and Komen, 2003). All fish expressed a significant hyperglycaemic response at 2 weeks post-vaccination, with glucose concentrations ranging from 80 – 101 mg.dl\(^{-1}\), which is consistent with the highest post stress values seen for *C. carpio* in section 3.B (~90 mg.dl\(^{-1}\)). These data indicate that vaccination is a highly stressful event and that the metabolic changes initiated persist for a considerable time (two weeks in this case). Recovery to pre-vaccination levels occurred by the 4 week sample. A second hyperglycaemic response was observed at 12 weeks post-vaccination where glucose values were in the range of 78 – 85 mg.dl\(^{-1}\), again exceeding the peak stress values seen in previous trials.

Vaccination is widely recognised as a stressful event for fish, involving anaesthetisation and considerable handling time (Van Muiswinkel and Wiegertjes, 1997). The presence of cortisol following such a stressor can greatly impact on both the innate and specific response. Anderson *et al.*, (1982) studied the effects of cortisol on the response of rainbow trout (*Oncorhynchus mykiss*) to vaccination with *Yersinia ruckerii*.
and found that high cortisol levels induced significant immuno-suppression. The suppressive effects of cortisol on the innate immune system are widespread and are detailed in section 1.3.3. The stress experienced at the time of vaccination may have had a considerable effect on the other physiological and immunological parameters measured, masking any effects of the immuno-stimulant diets fed and impairing the immune response.

In addition to the acute stress experienced by the fish immediately following vaccination, the data suggest that a chronic stress condition may have developed during the course of the trial. This is a common result of repeated sampling and disturbance resulting from insufficient recovery time between stressors. The metabolic changes induced to compensate for the presence of the initial stressor do not return to pre-stress levels, causing subsequent stressors to act in a cumulative fashion. Several studies have reported this process occurring in stressed salmonids and gilthead sea bream (Sparus aurata) (Barton et al., 1986, Javis, 1990, Pages et al., 1995). These studies also found that habituation to a repetitive stressors occurred, where the subsequent stress response was downgraded but there was a new higher baseline level of cortisol and other stress indicators. Pages et al., (1995) found that daily application of a stressor could induce habituation within three weeks, whilst Javis (1990) found that longer intervals between stress events delayed the onset of habituation to around ten weeks of the first stressor application. The twelve week post-vaccination period is long enough for the disturbance and repeated sampling to have induced chronic stress and subsequent habituation, which may be responsible for the elevated plasma glucose concentrations seen twelve weeks after vaccination.

As with plasma glucose, pre-vaccination plasma protein concentrations, and indeed those across the entire trial (1.6 – 3.3 g.dl⁻¹) were consistent with those seen in C. carpio in previous trials (2.7 – 3.0 g.dl⁻¹) and those reported in the literature (Yin et al., 1995, Price et al., 1997, Choudhury et al., 2005, Misra et al., 2006).
Fish fed the immuno-stimulating diets showed significant reductions in plasma protein following vaccination, whilst those fed the control diet showed significant increases. Similar decreases in plasma protein have been observed in a variety of fish species following vaccination, including cod (*Gadus morhua*) (Magnadottir et al., 2001), sea bass (*Dicentrarchus labrax*) (Coeudacier et al., 1997), rainbow trout (*O. mykiss*) (Olensen and Vestergard Jorgensen, 1986) and brown trout (*Salmo trutta*) (Ingram and Alexander, 1977). The reasons for these decreases have largely been attributed to seasonal variations or sexual maturation, neither of which was thought to have happened during the course of the current trial. It is considered unlikely that the lower plasma protein concentration of fish fed the immuno-stimulant containing diets was due to water imbalance due as is thought to have occurred previously (section 4) due to the timescales involved. From the current trial, the decrease in plasma protein seen is unexplained.

There was no significant effect of either vaccination or the diet fed to the fish on peripheral thrombocytes and lymphocytes. Peripheral thrombocytes ranged from 10 – 21 % and lymphocytes from 78 – 91 % of the total peripheral blood leukocyte population which was consistent with the ranges observed for *C. carpio* in previous trials (section 4).

In contrast, the percentage of circulating granulocytic cells did alter during the course of the trial and appeared to have been influenced by diet. Peripheral neutrophils counts of fish fed the control diet were significantly elevations at 2 weeks post-vaccination than prior to vaccination, increasing from ~ 0 % to ~ 0.6 % of the leukocyte population. These elevations are consistent with the elevations in neutrophils seen post-stress and may be attributable to the stress induced mobilisation of immune cells in response to various cues (Hlavec and Bulkley, 1980, Ellsaesser and Clem, 1986, Pulsford et al., 1994, Weyts et al., 1998, Ascencio'Valle et al., 2004, Engler et al., 2004). However, in the fish that had been fed the either of the immuno-stimulant containing diets, no significant changes in the number of circulating neutrophils post vaccination were apparent, although significant change was observed in the period following
vaccination. Injection of β-glucans typically enhances the number of these cells in the blood (Jeney and Anderson, 1993, Jorgensen et al., 1993, Siwicki et al., 1994, Selvaraj et al., 2005) although oral administration has been found to be ineffective at generating a comparable response (Selvaraj et al., 2005).

Another explanation for the increased numbers of circulating neutrophils is the presence of the vaccine. In a study on vaccinated carp, (C. carpio) Kodama et al., (2002) reported rapid increases in the number of circulating granulocytic cells following vaccination or pathogen injection. These increases typically occurred within 24 – 48 hours of injection, with values returning to pre-vaccination levels after 72 – 96 hours (Kodama et al., 2002). The subsequent decrease in circulating granulocytes is due to migration out of the circulation and into the tissues, usually towards the site of infection. In a study on sea bass, (Dicentrarchus labrax) Afonso et al., (2005) observed this initial increase and decrease in circulating neutrophils was followed by similar increases and decreases in circulating monocytes, which occurred over a period of up to 7 days. It is considered unlikely that these processes were responsible for the increased number of granulocytic cells seen in the current trial, due to the time scales involved. The first post-vaccination sample was taken at 2 weeks post-vaccination, so it is expected that this process would have already occurred with the bulk of the granulocytic cells present in the peritoneal cavity at the site of the infection (Afonso et al., 2005). This may have been the case in fish that had received the β-glucan diet; activation of the cells in the presence of β-glucan would allow more rapid migration into the tissues, preventing stress-induced changes from occurring. In the control and combined-supplement fed fish both circulating neutrophils and monocytes were still significantly elevated at 2 weeks post-vaccination suggesting that other factors, for example the stress of vaccination, may have been responsible. Following the establishment of plasma glucose as a good predictor of granulocyte number in section 3.B, and the high plasma glucose concentration seen at 2
weeks post-vaccination, it seems highly probable that the response of these two groups of fish is due to the stress of vaccination.

However, despite the secondary hyperglycaemia at 12 weeks post-vaccination, there was no concurrent neutrophilia in control fish, indicating either acclimation to stress or a movement out of the blood into the tissues in response to the presence of the vaccine, again this is considered unlikely due to the time scales involved. The notable lack of any significant change in neutrophil number in fish fed the β-glucan diet throughout the trial is interesting as β-glucans have been reported to increase the presence of granulocytic cells in the blood of salmonids and C. carpio (Jeney and Anderson, 1993, Jorgensen et al., 1993, Siwicki et al., 1994, Selvaraj et al., 2005). However, due to the long intervals between samples, any change could have been missed.

The respiratory burst activity of whole blood, as quantified by NBT reduction, was greatest at 4 weeks post-vaccination in all groups of fish regardless of the diet fed to them, with the greatest enhancements seen by fish fed β-glucan as a sole supplement. The least elevation in NBT reduction was seen in the control group of fish. The presence of a specific receptor on the CSM of neutrophils and macrophages allows these cells to react to the presence of β-glucans rapidly and maintains them in an activated state (Engstad and Robertsen, 1993, Engstad, 1994, Engstad and Robertsen, 1994). This may have been responsible for the greater NBT reduction seen in β-glucan supplemented fish; the presence of more activated neutrophils allowed a greater response to the presence of the vaccine resulting in heightened respiratory burst activity.

Of the humoral immune parameters measured, lysozyme activity was greatest in the serum of un-vaccinated fish and decreased following vaccination, a pattern consistent with the reported effects of stress on lysozyme activity. Lysozyme has been shown to be greatly affected by stress and the reductions in activity may have been attributable to the effects of the stress of vaccination and then the subsequent development of a chronic condition. Following short term stressors such as brief handling, lysozyme may be
increased, as reported by Fevolden et al., (1994) and Mock and Peters (1990). However after longer duration stressors such as transportation or smoltification, serum lysozyme activity can exhibit considerable suppression compared to un-stressed samples (Mock and Peters, 1990, Schrock et al., 2001). The reductions seen in the current trial, from 13.1 U.ml⁻¹ prior to vaccination, to 0.5 – 8.5 U.ml⁻¹ following vaccination reflect this tendency for suppression. These pre-vaccination values are largely consistent with values for C. carpio obtained in previous trials, but are generally lower than those reported in the literature; 2 – 18 U.mL⁻¹ for cultured Indian major carps and both cultured and 42 -55 U.ml⁻¹ for wild common carp (C. carpio) (Price et al., 1997, Jian and Wu, 2004, Sahoo et al., 2005). Further evidence for immuno-suppression was obtained from the negative relationship between plasma glucose and serum lysozyme activity in the control group of fish. This relationship was not present in fish receiving the immuno-stimulants, so their presence may have help alleviate some of the immunosuppressive effects of stress.

Post-vaccination spontaneous haemolytic activity showed considerable variation, yet the median pre-vaccination value of 29.4 ACH₅₀ U.ml⁻¹ is consistent with those seen in previous trials and with the range of values (29 – 90 ACH₅₀ U.ml⁻¹) reported for C. carpio and Indian carps (Matsuyama et al., 1988, Yano, 1992, Dash et al., 2000, Sakai et al., 2001, Sahoo et al., 2005). Following vaccination huge amounts of variation were apparent with values ranging from 16 – 413 ACH₅₀ U.ml⁻¹, with an exceptionally high value at 2 weeks post-stress (1227 ACH₅₀ U.ml⁻¹) seen in fish fed the control diet. Despite these large increases compared to pre-stress values, these data were not significantly different. This is partly due to the inherent high, natural variation in complement activity, although most values for C. carpio are in the range of 29 – 90 ACH₅₀ U.ml⁻¹ (see above), values of up to 560 ACH₅₀ U.ml⁻¹ have been reported in healthy, un-vaccinated C. carpio (Selvaraj et al., 2005). This variation has been attributed to large degree of intrinsic genetic variation associated with this immune characteristic (Wiegertjes et al., 1993). Magnadottir et al., (2001) conclude that this
variation is a considerable problem when studying complement activity as it prevents statistical confidence and masks the more subtle effects of the environment.

When relationships were sought between immunological and haematological parameters mixed results were found. Fish fed the β-glucan diet showed no significant interaction between parameters, whilst those fed the control and combined diet did. The weakly significant negative relationship between plasma glucose concentration and serum lysozyme activity in the control group of fish is thought to be indicative of stress-induced immuno-suppression and has been observed in previous trials conducted on C. carpio in the current investigation. The NBT reduction potential of whole blood was found to be linked to both serum lysozyme and spontaneous haemolytic activity in the fish that received the combined supplement diet. Unlike the relationship with plasma glucose concentration seen in the control fish, these are harder to attribute to a source event and may represent coincidental relationships.

In conclusion, vaccination against Aeromonas salmonicida via IP injection was found to induce significant stress on all groups of fish tested, yet no conclusive evidence suggests that the presence of immuno-stimulants helped reduce the response to the stressor. An increase in respiratory burst activity was seen in all groups of fish at 4 weeks post-vaccination and the magnitude of this response was greatest in those fish receiving the immuno-stimulant containing diets. Spontaneous haemolytic activity was unaffected by the presence of the vaccine and the immuno-stimulant diets and serum lysozyme activity reduced during the course of the trial. Lysozyme is thought to have been detrimentally affected by stress, a condition that the immuno-stimulant containing diets may have reduced slightly. Therefore, it is possible to partially accept H₂. However, the data suggest the majority of changes observed may have resulted from the stress of the vaccination process rather than the presence of the immuno-stimulants or the vaccine. There was no evidence that the immuno-stimulant containing diets had any stress-modulating effects, rejecting H₃. Due to the failure of the ELISA system it was
impossible to draw any conclusions on the effects of the immuno-stimulants on specific antibody titre, so $H_0$ remains untested.
Chapter 6. General Discussion
The presence of a mucosal stress response was confirmed in the three species of fish used in the current trial. In particular, clearly defined post-stress changes in mucosal haemoglobin and protein were observed in these fish confirming the work of earlier authors (Smith and Ramos, 1976, Isaacson and Morrisson, 1980, Lebedeva et al., 1999, Lebedeva, 1999, Lebedeva et al., 2001). Of the species tested, goldfish (*Carassius auratus*) and mirror carp (*Cyprinus carpio*) are commercially important in their own right (Davenport, 2001) and the tilapia (*Oreochromis niloticus X. O. mossambicus*) is a model species for the numerous species of cichlids which are commonly encountered in the ornamental fish trade as well as in established aquaculture.

When the mucosal stress response of these species was compared, the responses seen were very consistent with each other despite large phylogenetic differences between the species indicating that urinalysis strips may be able to detect stress in a range of fish species. The epidermal mucus of numerous fish species has been sampled using urinalysis strips, with the majority of species tested being cyprinids (Isaacson and Morrisson, 1980, Lebedeva, 1999), although salmonids, catfish (*Clarias gariepinus*), sturgeon (*Acipenser gueldenstaedtii*) and perichiform fish have also been sampled (Isaacson and Morrisson, 1980, Heming and Paleczny, 1987, Lebedeva, 1999). All fish species showed heighten levels of mucosal haemoglobin following stress, but time course studies charting the response of fish to clearly defined stressors have not been carried out.

A detailed time course study of both secondary stress indicators in both epidermal mucus and blood was conducted and during this, comparisons between visual and instrument-based assessment of strips were made. From the data obtained it became apparent that visual reading of the strips consistently lead to an over-estimation of the value of mucosal parameters when compared to those values reported by the Clinitek machine. This over-estimation was particularly apparent on the reagent sites for both acetoacetate and leukocytes, to the extent that significant stress-induced patterns could be seen when the strips were visually assessed that were absent when strips were read using
the strip-reading machine. Whilst overestimation was apparent for the sites for haemoglobin and protein, it was less pronounced and the same patterns could be discerned using both assessment methods, allowing the results to be viewed and interpreted with greater confidence. The application of an instrument based assessment method may have resulted in a greater perception of the mucosal response; in a study on wild fish, Isaacson and Morrisson (1980) reported that a maximum response to haemoglobin was obtained within 5 minutes of stress. This high degree of sensitivity was cited by these authors as a major limitation of using the strips to detect stress. However, during the course of the current research, the increases in mucosal haemoglobin have been more gradual, which may in part be due to differences in the stressors used or to the use of the dedicated instrument for strip analysis. It is the finding of the current research that the strips are much more versatile in the detection of stress than previously thought. Despite the tendency for visually assessment of strips to overestimate the values of mucosal haemoglobin and protein, the underlying patterns of stress-induced change remained consistent, allowing confidence in visual assessment where the dedicated strip-reading machine is not available. Visual assessment was used in three species of ornamental fish and the results obtained were consistent across the species, supporting the findings of other authors (Isaacson and Morrisson, 1980, Heming and Paleczny, 1987, Lebedeva, 1999).

Despite the wide recognition of stress-induced increases in mucosal haemoglobin and other parameters in these fish species, to the author’s knowledge little or no attempt has been made to quantify mucosal parameters through reference to blood stress indicators. Comparisons were drawn between mucosal stress indicators, assessed using the machine and blood stress indicators, which to the author’s knowledge has not been demonstrated in fish. Mucosal haemoglobin and protein concentration showed significant relationships with plasma glucose concentration which had been chosen as the key reference stress parameter in the blood. The hyperglycaemic response of fish to
stress is a highly conserved metabolic adjustment and is well characterised in *C. carpio*, making it highly suited as a reference parameter (Pottinger, 1998, Tanck *et al.*, 2000, Ruane *et al.*, 2002a, Ruane *et al.*, 2002b, Ruane and Komen, 2003). The link between the blood and the mucosal stress response in this way clearly demonstrates that mucosal haemoglobin and protein are secondary stress indicators within epidermal mucus and operate on the same timescales as other, more established, stress indicators in the blood. Additionally, changes in the blood leukocyte populations, which have also been well documented as secondary stress indicators in the blood of fish and other vertebrates (Pickering, 1984, Pickering and Pottinger, 1987, Ainsworth *et al.*, 1991, Dhabhar and McEwen, 1997, Engler *et al.*, 2004) were also found to be significantly linked to both mucosal stress indicators. Again, it is thought that this is the first demonstration of a clear link between mucosal and secondary blood stress indicators in fish and lends weight to the use of epidermal mucus as a diagnostic surface for the non-invasive detection of stress.

Although the mucosal stress response was validated against blood indicators of stress, no specific assays were carried out to detect mucosal stress indicators. For example the haemoglobin concentration could have been confirmed by conducting assays using the Drabkins method (Drabkins and Austin, 1932). These methods were not conducted due to several complicating factors.

The main species used goldfish (*C. auratus*) and carp (*C. carpio*), were found to only produce limited amounts of epidermal mucus, which made the collection of epidermal mucus problematic. It was necessary to scrape the mucus off the flank of the fish, using a method modified from that of Handy (1989). Although applicable to larger fish, this process has the potential to induce significant stress on the fish and inflict damage to the epidermal tissues of the fish. This significant sample-induced stress could have been avoided through the sampling strategy, but the potential for skin damage could greatly not have been avoided. In addition to the detrimental effect this would have had
on the fish, the potential effect on the results could not be ignored. Skin damage would have released both haemoglobin and protein into the collected mucus, which would have been additive to that released by the stress response. This would have induced significant error and reduced confidence in the results obtained. The overly high results obtained would have little bearing on the actual stress status of the fish sampled.

To combat the potential for stress-induced skin damage, a less abrasive mucus collection was devised. This used a commercially available medical-grade cotton wadding [Salivette®, Sarstedt Ltd, Beaumont Leys, UK], which was rubbed along the flank of the fish. The mucus was then extracted through high-speed centrifugation. The protein profiles of mucus collected using this method were compared with those of mucus remaining in the collection tray used during sampling, which minimised the risk of skin damage through scraping, using SDS-PAGE separation. The analysis of the resulting Salivette extract found that large amounts of protein were being retained upon the wadding, reducing confidence in this method as a suitable alternative mucus collection method (for a fuller description see Appendix B).

Further compounding the specific analysis of mucus, were the difficulties in the standardisation of the resulting analysis. The mucosal secretions of fish have a highly variable water content, varying from a thick gel to a liquid with a density only slightly greater than water (Handy, 1989, Silberberg, 1989, Shepard, 1994). This makes it difficult to make direct comparisons based on untreated mucus due to its variable nature. To overcome this mucus was lyophilised and analysed on a dry-weight basis samples, however it was noted that total re-solubilisation of lyophilised mucus was almost impossible. This lowered confidence in the subsequent results as a considerable amount of mucus remained out of solution. Aggregation during lyophilisation is reported by Gindzienski et al., (2003) to be a non-reversible process, and the use of fresh mucus is recommended where possible. Due to the size of the fish used in the current
investigations, this was not feasible and thus has proven impossible to conduct specific analytical techniques on either haemoglobin or protein within epidermal mucus.

Whilst the relationship between secondary stress indicators in the blood and epidermal mucus was established during the current research, caution must be expressed in its wider application. Other parameters may affect the response of either system to stress, for example a decrease in temperature in one of the trials may have been the causative factor in the lack of a mucosal stress response for those fish. Additionally fish in higher stress environments, for example in glass aquaria, may have an elevated baseline level of stress, resulting in higher levels of mucosal stress indicators (Alexander, L. personal communication). Further investigation into the mucosal stress response needs to be conducted, particularly in response to other stressors that ornamental fish might encounter, such as temperature shock and transportation, to establish the validity of urinalysis strips as a general stress diagnosis tool. Following the results of the current investigation where temperature was established as a potential modifying factor for the stress response, the effects of this parameter should be further elucidated. Ornamental fish, especially pond fish, may experience considerable fluctuations in temperature across the year and the effects of these changes on the physiology of the fish and its response to stress may be profound.

Throughout the course of the current research, evidence modulation of stress and immune status was sought by the provision of β-glucans and nucleotides in the diets. Although the effects of both β-glucans and nucleotides on stress induced immuno-suppression are well documented, their effects on the primary and secondary stress responses of fish, as quantified by blood cortisol and glucose concentrations respectively, are less well investigated. Dietary nucleotides have been found to reduce plasma cortisol and glucose levels in fish (Burrells et al., 2001b, Leonardi et al., 2003), with β-glucans also reducing
cortisol, but not glucose concentrations post-stress in fish (Cain et al., 2003). In humans, both dietary nucleotides and β-glucans have been found to lower the immunosuppressive effects of different types of exercise stress (McNaughton et al., 2006, Nieman and Bishop, 2006), yet direct evidence for cortisol reduction has only been reported following nucleotide supplementation (McNaughton et al., 2006). Evidence for stress modulation by both nucleotides and β-glucans was found following acute stress, where both classes of immuno-stimulant reduced the duration of the hyperglycaemic response relative to the control group of fish following the application of netting stress. This shortening of the hyperglycaemic stress response was observed in and consistent between all groups of fish supplemented with immuno-stimulants.

The addition of nucleotides appeared to aid lymphocyte migration from the blood into the tissues following stress, which may be evidence of greater adaptation by the cells of the specific immune system to stress (Dhabhar and McEwen, 1997). Similarly the increased perturbations in plasma protein concentration seen in fish which had received a nucleotide supplemented diet may be indicative of a greater potential adaptation to stress through increased gill blood flow. Whilst β-glucans in the diet shortened the duration hyperglycaemic response, no evidence of further modifications of the stress response was found. The stress mediating effects of nucleotides have been established in fish and may in part be due to their energy sparing properties or to enhancements to the structure of gut (Burrells et al., 2001a, Burrells et al., 2001b, Li and Gatlin, 2006) which may have prepared the fish more effectively for stress. The energy sparing nature of nucleotides has also been explored using equine models and it was found that nucleotides could directly enter the Kreb’s cycle, contributing to carbohydrate metabolism (Art and Lekeux, 1993, Art et al., 1994) Due to the restrictions on tissue sampling imposed on the current research, the structure of the gut was not investigated. The potential for β-glucans to act as stress-mediators has received little study in fish, but preliminary studies suggest that they can mediate the effect of stress with respect to survival (Su et al., 1995,
Skjermo et al., 2002, Liu et al., 2004) and modifications in stress hormone level in both fish (Cain et al., 2003). Thus, to the author's knowledge, the current research represents the first comparative study of the stress mediating effects of β-glucans and nucleotides in fish. The results of the current research indicate that both dietary nucleotides and β-glucans have potential as stress mediators and further research into the relative merits of these two classes of compound and their effect on the response of fish to various stressors is clearly warranted.

Limited evidence was found in support of immune modulation resulting from immuno-stimulant supplementation in the diet. Of the two classes of immuno-stimulant administered during the current research, β-glucans showed the greatest potential for immune modification; however the effects observed were subtle. The immune response of β-glucan supplemented fish was modified compared to control and nucleotide-fed fish following both stress and vaccination, with β-glucans partially overcoming stress-induced immuno-suppression seen in fish fed other diets. Despite these positive effects, there was some evidence of detrimental effects of β-glucan administration, particularly related to respiratory burst potential, as quantified by the NBT reduction potential of whole blood. Although β-glucans are highly effective at stimulating granulocytic cells and inducing respiratory burst, Castro et al., (1999) found that prolonged supplementation can induce cellular exhaustion through prolonged activation. Immediately following stress, fish fed the β-glucan diet showed significantly lower NBT reduction in the blood compared to all other diets. This situation arose from (non-significant) increases in blood NBT reduction following stress in all except the β-glucan fed fish. This may indicate an inability to increase respiratory burst activity in response stress in β-glucan supplemented fish, but the reasons are uncertain; no evidence of heightened NBT reduction in prior to stress was found in either group of fish that received β-glucan prior to stress. This makes it unlikely that the immune cells were already undergoing maximal respiratory burst activity and so
were unable to respond as all diets showed similar levels of NBT reduction. In the light of research by numerous authors stating that acute stress is an immuno-stimulatory force (Pruett, 2001, Dhabhar, 2002, Plytycz and Seljelid, 2002, Moynihan, 2003, Dhabhar and Viswanathan, 2005), it would appear that β-glucans were detrimental in this instance. No clear evidence of either immuno-stimulation or immuno-suppression by nucleotides was found during the current research, leading to the conclusion that nucleotides had no significant effects on the immune parameters tested during the current research.

The failure of the ELISA system designed to detect the specific antibodies raised against the vaccine prevented any inference into the antibody status of the fish. As such, the potential of immuno-stimulants to act as adjuvants and increase antibody protection could not be elucidated from this trial.

For a comprehensive test of the ability of immuno-stimulants to promote good health, a live pathogen challenge is the most effective means of doing so. Whilst in vitro studies and testing of immune parameters in unchallenged fish are informative, unless these manifest themselves into enhanced survival following infection the effectiveness of the immuno-stimulant remains largely un-quantified under the conditions most applicable to the fish. The limitations of immune parameter testing compared to pathogen challenge were raised by Fevolden et al., (1994) and Binuramesh et al., (2005) who found that heightened levels of immune parameters, especially lysozyme, do not necessarily translate to heightened survival following challenge. Under certain conditions, high levels of immune parameters may be indicative of a weak immune system already stretched to its limits, resulting in poor post-challenge performance.

Under the current research conditions, it was not possible to conduct a live pathogen challenge due to ethical concerns raised by the sponsor. However, under different circumstances a live pathogen challenge would perhaps represent a more effective means of assessing the potential of both β-glucans and nucleotides to heighten the immune status of the fish. It would also be beneficial to assess the effects of
stressors, such as those used in the current research on the post-challenge performance of both control and immuno-stimulated fish. The effect of stress on the immune system was apparent throughout the current research, highlighting the importance of stress monitoring in health management. In particular, evidence of immuno-suppression was seen following both acute stress and vaccination. This effects of stress were particularly apparent on the humoral parameters of the IIS tested in the peripheral blood are particularly susceptible to stress. Of these, lysozyme has been repeatedly observed to be strongly suppressed by stress in a variety of fish species (Mock and Peters, 1990, Demers and Bayne, 1997, Binuramesh et al., 2005). For these reasons a combined stress and challenge study would be highly informative for the field of ornamental fish health management.

Throughout the current research, it was apparent that the addition of immuno-stimulants elicited only subtle effects on both stress and immune status. It is thought that this may be partially attributable to the route of administration. When injected, β-glucans and nucleotides have been shown to generate strong immune responses; the effects of the same compounds administered via the oral route are often considerably less. In a trial that compared the response of C. carpio to either IP injection or oral supplementation of β-glucan, it was found that injection conferred 100 % survival following bacterial challenge and enhanced a variety of immune parameters, whilst oral supplementation did not significantly alter survival or immune parameters from those of control fish fed diets devoid of β-glucans (Selvaraj et al., 2005).

The authors of this study concluded that the failure of orally supplied β-glucans to elicit an immune response was due to an inability of the gut to absorb particles of glucan greater than 1 μm in size.

At alternate explanation for the generally lower response of fish to orally supplemented β-glucans compared to that elicited by injection, may be modification
within the gastro-intestinal tract. β-glucans are classed as non-starch polysaccharides (NSP) and fish and other vertebrates exhibit limited potential for their breakdown in the gastro-intestinal tract due to a lack of required enzymes (Trowell et al., 1976). Krogdhal et al., (2005) report that carnivorous fish lack the enzymes required to degrade NSP, but work conducted by Rust (2002) found that omnivorous and herbivorous fish possess the ability to digest NSP, although enzyme activity is usually low. It is unclear whether the required enzymes are products of the intestinal microbial population; in several herbivorous fish species the ability to digest NSP was found to be lessened or absent following antibiotic treatment (Das and Tripathi, 1991). C. carpio and other cyprinids may utilize these bacteria to digest cellulose and further breakdown products (Das and Tripathi, 1991, Kihara and Sakahata, 2002). Studies on the digestion of β-glucan by pigs conducted by Johansen et al., (1993) found that extensive de-polymerisation of β-glucans occurred in the upper gastrointestinal tract as a result of enzymes originating from the gut microbial fauna. These studies highlight the importance of gut organisms in the nutrition of vertebrates and the contribution they make to the digestion of NSP.

In the majority of trials where β-glucans have been supplied in the diet, the test species are carnivorous, such as salmon (S. salar), trout (O. mykiss) and striped bass (M. saxatilis X M. chrysops), which are thought to be unable to digest NSP. Modification of β-glucans in the gut of these fish is unlikely, so if they can indeed cross the gut wall, they will do so intact. The majority of other species tested, including C. carpio, are omnivores with a varying carnivorous component to their diet, giving the potential for greater modification of β-glucans in the gut, which may lower the effectiveness of these compounds.

The nucleotide nutrition of fish is also poorly understood and much of the current knowledge is extrapolated from mammalian models (Li and Gatlin, 2006, Russo et al., 2006). In mammals, free nucleotides undergo a series of modifications through removal of the phosphate group to leave nucleosides, which are further digested to free purine and
pyrimidine bases (Li and Gatlin, 2006). The extent of nucleotide breakdown varies considerably in mammals; mice primarily absorb nucleosides across the gut whilst humans absorb a range of nucleotide breakdown products (Sonoda and Tatibana, 1978). In a comparative study conducted on mice, Veerabagu et al., (1996) found that injected nucleotides elicited a stronger immune response in mice than those administered orally. This difference in response was attributed to degradation of nucleotides in the gastrointestinal tract which lowered their immuno-stimulating properties. It is thought that no comparable studies have been conducted in fish to assess the relative merits of injection and oral supplementation as routes of administration for nucleotides.

Even before the immuno-stimulant supplement enters the gastrointestinal tract of the fish, there is potential for considerable modification. The formulation and production of diets can involve high pressures and temperatures which can cause considerable nutrient losses from the diet. This is compounded by difficulties in the detection of immuno-stimulants in the finished diet. This especially applies to the diets containing $\beta$-glucans, where values for $\beta$-glucan content ranged from 0.4 – 8 % w/w. There are reservations as to the validity of the $\beta$-glucan analysis; original analysis of Macrogard® by Biotec Ltd [Tromso, Norway] found differing results which did not reflect the $\beta$-glucan inclusion at formulation. Subsequent analysis of the experimental diets using a commercially available assay kit, found levels of $\beta$-glucan that reflected the amount of $\beta$-glucan supplement added to the diets at formulation. However, the accuracy of these results are questionable: such assay kits show a limited ability to distinguish between (1,3: 1,6) $\beta$-glucan and other $\beta$-glucans such as cellulose, cellulose derivatives, or (1,3: 1,4) $\beta$-glucans from cereal sources, due to the necessary inclusion of an acid hydrolysis in the method (Engstad, R. personal communication) The experimental diets contain between 2.6 and 3.0 % of insoluble dietary fibre, the majority of which is though to comprise of cellulose; following acid hydrolysis this would be reported as (1,3:1,6) $\beta$-
glucan. Thus caution must be expressed with regard to the detection of β-glucan in diets which contain cellulose as a carbohydrate source.

These results of nucleotide analysis by HPLC are held in more confidence than those for the β-glucan diets due to a more specific assay for nucleotides which was less prone to interference. Analysis using this method resulted in levels close to those added to the diets at the formulation stage; yet still do not totally reflect what was added to the diets during production. Despite the more specific method, during analysis an unknown peak was discovered comprising between 0.1 and 0.3 % of the total nucleotide content of the diet. It is thought that this peak represents guanine (Hoffmann, K. personal communication) but highlights the difficulties of assaying these feed additives even with a more specific method. The uncertainty in the actual inclusions of the immuno-stimulant compounds used in the current trial highlight the problems of immuno-stimulation via the oral route and the complexity of characterising the nature of active functional components in the diet.

From the data obtained in the current trial and that obtained by other researchers it is clear that whatever the mechanisms involved (modification or loss during formulation, modification in the gastro-intestinal tract or an inability of the immuno-stimulant to cross the gut wall) oral administration of immuno-stimulants is less effective at generating a systemic immune response than injection, confirming the previously available scientific literature.

The restrictions on sampling imposed during the current trial prevented investigation of the response of the gastro-intestinal tract to the presence of immuno-stimulants. The importance of the gastro-intestinal tract as an immune organ should not be underestimated and studies investigating the effectiveness of vaccination by different routes have found that oral and bath immersion generates a strong, but localised immune response in the gut of fish, without necessarily inducing a strong systemic response
Similarly, nucleotides contribute to heightened cell turnover and replacement in the gut tissues of fish (Burrells et al., 2001a, Burrells et al., 2001b, Li and Gatlin, 2006), again indicating that the response to oral supplementation may be more localised. Due to the non-invasive nature of the assays of immuno-competence used in the current trial, the response and condition of this important immunologically active surface remained un-sampled. Thus the effect of β-glucans and nucleotides on the gut remain unclear, a situation that warrants further study, especially given the knowledge that oral supplementation stimulates the gastrointestinal tract. Similarly, comparative trials assessing the relative abilities to degrade β-glucans and nucleotides in fish that feed at different trophic levels is required to determine if they are more effective in one or other class of fish.

Conclusion

In conclusion, the results of the current programme of research suggest that the oral supplementation with 0.2 % w/w (1,3:1,6) β-glucan or 0.2 % w/w nucleotides either singly or in combination has no significant effect on the immune status of fish compared to those fish fed an immuno-stimulant free diet.

The current investigation highlights the highly stressful nature of simple husbandry-related techniques such as netting, events that are a common occurrence in the lives of ornamental fish. The prophylactic administration of immuno-stimulants, helped modulate the response of fish to stressors and β-glucans were, in some instances, able to combat stress-induced immuno-suppression. However, at no point in the current research was there strong evidence that the combination of β-glucans and nucleotides worked significantly better than the immuno-stimulants administered singly. Therefore, prophylactic administration of immuno-stimulants has a role to play in stress
management, yet it still remains imperative to identify and prevent or at least reduce the severity of stressful events experienced by ornamental fish to safeguard their health and well being. This can only be achieved through regular monitoring of the environmental conditions that the fish are kept in and also through regular monitoring of the stress profile of fish. The epidermal mucosal surfaces have the potential to give at least an indication of the stress status of the fish which can provide extra information for aquarists on the general well being and health profile of their fish.
Appendix A. Regants for enzyme linked immunosorbent assay (ELISA)
1: COATING BUFFER (carbonate bicarbonate solution)

\[
\begin{align*}
\text{Na}_2\text{CO}_3 & \quad 1.59 \text{ g} \\
\text{NaHCO}_3 & \quad 2.93 \text{ g} 
\end{align*}
\]

Dissolved in one litre of distilled water. Adjusted to pH 9.6. Prepared fresh for each coating.

2: LOW SALT WASH BUFFER

\[
\begin{align*}
\text{Trisma base} & \quad 24.2 \text{ g} \\
\text{NaCl} & \quad 222.2 \text{ g} \\
\text{Merthiolate} & \quad 1 \text{ g} \\
\text{Tween} 20 & \quad 5 \text{ ml} 
\end{align*}
\]

Dissolved in one litre of distilled water. Adjusted to pH 7.3 with concentrated HCl.

3: HIGH SALT WASH BUFFER

\[
\begin{align*}
\text{Trisma base} & \quad 24.2 \text{ g} \\
\text{NaCl} & \quad 292.2 \text{ g} \\
\text{Merthiolate} & \quad 1 \text{ g} \\
\text{Tween} 20 & \quad 10 \text{ ml} 
\end{align*}
\]

Dissolved in one litre of distilled water. Adjusted to pH 7.7 with concentrated HCl.

4: PHOSPHATE BUFFERED SALINE (PBS)

Oxoid [Basingstoke, UK] phosphate buffered saline tablets were dissolved in distilled water (1 tablet per 100 ml)
5: ANTIBODY BUFFER (1% BSA solution)
   Bovine Serum Albumin (BSA) 1 g
   Dissolved in 100 ml of PBS

6: CONJUGATE IN CONJUGATE BUFFER
   Goat anti-mouse IgG-HRP diluted $1/1000$ in conjugate buffer

   CONJUGATE BUFFER
   BSA 1 g
   Dissolved in 100 ml low salt wash buffer

7: CHROMAGEN IN SUBSTRATE BUFFER
   CHROMAGEN
   42 mM 3'3'5'5'-Tetramethylbenizidine dihydrochloride (TMB) in 1:2 acetic acid:
   distilled water. 150 ml of this solution was added to 15 ml substrate buffer.

   SUBSTRATE BUFFER
   Citric Acid 21.0 g
   Sodium acetate 8.2 g
   Dissolved in one litre of distilled water. Adjusted to pH 5.4 with 1 M NaOH. 5 µl of
   H$_2$O$_2$ was added to 15 ml substrate buffer.
Appendix B. Comparison of epidermal mucus collection methods
B (i) Mucus collection

Fish were anaesthetised in 2 litres of water containing MS222 (Tricane methane sulphonate) added at a concentration of 100 mg.L\(^{-1}\) (Ross, 2001). Mucus was collected by swabbing the flank of the fish, avoiding the head, vent and caudal regions, using a Salivette\(^{\circledR}\) [Starstedt Ltd., Leicester, UK] trimmed to fit an Eppendorf tube. Once coated with mucus, the Salivette\(^{\circledR}\) was centrifuged at 5,000 x g for 60 seconds. The Salivette was then re-orientated and further centrifuged at 7,000 x g for 15 seconds. Mucus collected by this method was designated “Salivette” mucus (SM). During sampling in this way, residual mucus and water was retained in the sampling vessel (foil tray). This was collected separately to the S mucus and was designated “Residual” mucus (RM). Mucus was collected, using both methods, from thirty goldfish (Carassius auratus). After sampling, fish were allowed to recover in a well-aerated tank.

B (ii) Mucus preparation

Following collection, mucus was frozen at -80 °C prior to lyophilisation and subsequently stored in a dessicator until analysis. 0.5, 1 and 5 mg of lyophilised mucus was added to 100 µl of sample buffer (4 % SDS, 12 % glycerol, 50 mM tris, 2 % mercaptoethanol, 0.01 % bromophenol blue, pH 8.6). One replicated of each sample received no sonication, the other was sonicated in a water bath at 25 °C for 10 minutes to break up the sample and allow for easier solubilisation. Samples were boiled for 15 minutes, with vortexing being carried out every 5 minutes. Samples were finally spun at 5,000 x g to remove any remaining insoluble material and the supernatant only loaded on the gel.
B (iii)  **Sodium Dodecyl Sulphate-Polyacrylamide Gel**

**Electrophoresis (PAGE)**

Protein separation was performed using tris-tricine SDS-PAGE as described by Shagger & Von Jagow (1987). A 4 % stacking gel was layered over a 10 % main gel and was run at 140 volts for 4-5 hours. Protein bands were visualised using Coomassie Blue staining. Molecular markers used were as follows: Myosin (208 kDa), Phosphorylase B (105 kDa), Glutamic Dehydrogenase (53 kDa), Carbonic Anhydrase (34 kDa), Myoglobin (blue) (23 kDa), Myoglobin (red) (17 kDa), Lysozyme (13 kDa), Aprotinin (6 kDa) and Insulin (B chain) (4 kDa) [Multimark standard, Invitrogen, Paisley, UK]

The use of a Salivette to collect epidermal mucus of fish resulted in a dramatic reduction in the protein classes visualised on the gel at all sample concentrations (Plate B1). A concentration of 1 mg.100μl⁻¹ sample buffer (Lanes 3&4) generated the sharpest bands without overloading the gel (Lane 6) (Plate B1). The 208 kDa marker did not enter the separating gel within the running time imposed; proteins separated ranged from around 105 kDa down to 4 - 6 kDa in size (Plate B1).

Clear bands were resolved at approximately 6 kDa, 12 kDa, 14 kDa, 23 kDa, 38 kDa, 42 kDa, 51 kDa & 61 kDa. These bands were consistent across the different sample concentrations used (Plate B1).
Plate B1. Tricene-PAGE gel of epidermal mucus fractions from *Carassius auratus*.

The gel was run to identify proteins in epidermal mucus using two collection methods. All samples were boiled in 10% SDS for 10 minutes prior to loading. The gel was visualised using Coomassie Blue stain. M = molecular weight markers in kDa; 0.5 SM = 0.5 mg mucus collected using a Salivette; 0.5 RM = 0.5 mg mucus collected without Salivette (residual mucus); 1.0 SM = 1.0 mg “Salivette” mucus; 1.0 RM = 1.0 mg “Residual” mucus; 5.0 SM = 5.0 mg “Salivette” mucus; 5.0 RM = 5.0 mg “residual” mucus.
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