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THE EFFECT OF DIETARY INCLUSION OF CATEGORY THREE ANIMAL BY PRODUCT MEALS ON RAINBOW TROUT (O.MYKISS, WALBAUM) MINERALISED TISSUE AND IMMUNE FUNCTION

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The effect of dietary inclusion of category 3 animal by-product meals on rainbow trout (*O. mykiss* Walbaum) mineralised tissues and immune function.

By

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ABSTRACT

The effect of dietary inclusion of category 3 animal by-product meals on rainbow trout (*O.mykiss* Walbaum) mineralised tissues and immune function.

M.A.G.Owen

Aquaculture is growing rapidly worldwide and is projected to become the major source of fish used for human consumption. A major factor that limits aquaculture reaching its full potential is an adequate supply of the raw materials necessary for formulated fish feeds. The dependence of modern aquaculture on fishmeal obtained from wild fisheries is not environmentally sustainable and replacements for fishmeal must be found. Some animal by-products are viable replacements for fishmeal, and can provide sufficent nutrition for high growth rates, but little is known about the potential of animal by-products to adversely affect fish health. The objectives of these experiments were to determine if animal by-products used in fish feeds impair immune response or alter bone physiology in cultured juvenile rainbow trout. Four animal by-product containing diets (poultry meat meal (PMM)/ PMM plus feathermeal / PMM plus bloodmeal) and two reference diets (fishmeal or soya) were evaluated to determine their effect on innate immune response, the ability of fish to cope with normal husbandry stressors, and bone physiology. PMM was then selected due to its favourable amino acid profile and high digestibility and assessed to determine if the high levels of fishmeal replacement that may be required in the future, impact the health of rainbow trout. Due to the lack of reliable indicators of bone quality and quantity in salmonids the effects of exercise and phosphorus deficiency in rainbow trout were also examined. Relative to the fishmeal control diet,

fish fed diets with PMM [(PMM) 50% crude protein, by substitution], PMM plus two percent blood meal, or PMM plus five percent feather meal, did not have an impaired innate immunity (lysozyme, alternative complement, phagocytosis, intracellular respiratory burst, differential counts of peripheral blood leukocytes) or changes in bone physiology as assessed by dynamic bone histomorphometry. Higher levels of PMM (0-70% digestible protein, by substitution) caused a reduction in apparent net mineral retention of phosphorus and calcium ($P<0.001$), a lower vertebral bone mineral content ($P<0.001$) and reduced vertebral mechanical properties (compressive extension ($P=0.04$), Young's Modulus ($P=0.03$)), but fish growth was not affected. Exercise influenced bone modelling, with exercised animals having a reduced bone area and trabecular thickness ($P=0.01$), increased autocentrum width ($P=0.04$), and higher bone mineral content ($P= 0.02$); however, bone mechanical properties were unaffected. Induction of genes (receptor activator nuclear factor kappa beta and osteoprotogenerin), involved in the resorption of mineralised tissue, was not observed in fish fed phosphorus deficient diets although scales were evidenced to be an important source of labile minerals. Overall our results indicate that low level replacement of fish meal by poultry meat meal, and blends of poultry meat meal with blood or feathermeal do not affect fish innate immune response, bone physiology, or growth however the greatly elevated levels of poultry meat meal that may be required in future salmonid aquafeeds could increase the risk of spinal malformations. Thus the category 3 animal by products tested are valuable fishmeal replacements for aquaculture based on the endpoints measured in this study.

All experimental work involving animals was carried out under the Home Office project licence #30/2135 and personal licence #30/3190

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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.

The study was jointly funded by the University of Plymouth and Prosper de Mulder as part of a HEIF 3 studentship.

A programme of advanced study was undertaken which included courses in fish immunology (Fish Immunology Workshop, Wageningen, Holland), nutrition, biological statistics and advanced molecular techniques.

Additionally specific skills training included sessions on: Preparing for the viva, interview techniques (applying for jobs, applying for funding), and applying for research funding.

Presentations and Conferences Attended:

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10th International Symposium on the culture of the tench, Turin, Italy Sept 29-Oct 4th 2008.Light colour affects the stress response and behaviour of juvenile tench (*Tinca tinca* L.). Oral Presentation.

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Nur tote Fische schwimmen mit dem Strom

Abbreviations

ALP	Alkaline phosphatase
APS	Animal protein source
ANMU	Apparent net mineral utilisation
ANPU	Apparent net protein utilisation
BFC	Bakers formol calcium
Bm	Body mass
BM	Blood meal
Con A	Concanavalin A (a lectin from *Canavalia ensiformis*)
DBH	Dynamic bone histomorphometry
EFM	Enzyme treated feathermeal
EU	European Union
FBS	Foetal bovine serum
FCR	Feed conversion ratio
FE	Feathermeal
FM	Fishmeal
HSI	Hemato-somatic index
IMS	Industrial methylated spirits
L	Litres
L-15	Leibowitz 15 media
$L\text{-}15^{PR}$	Liebowitz 15 media without phenol red
LPS	Bacterial lipopolysaccharide
MBM	Meat and bone meal
Md.Pm	Mineralising perimeter
MS222	Tricane methane sulphonate
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NFE	Nitrogen free extract

NSD	No significant differences
OPG	Osteoprotegerin
P/S	Penicillin/ Streptomycin
PAP	Processed animal protein
PBS	Phosphate buffered saline
PBL	Peripheral blood leukocytes
PMA	Phorbol 12-myristate 13-acetate
PMM	Poultry meat meal
PrP	'Normal' prion protein
PrP^{sc}	Protease resistant prion protein
RANK	Receptor activator of nuclear factor- κβ
RANKL	Receptor activator of nuclear factor- κβ ligand
RBT	Rainbow trout
ROS	Reactive oxygen species
SDH	Spray dried haemoglobin
SEM	Scanning electron microscope
SGR	Specific growth rate (% BM day^{-1})
SHFE	Steam hydrolysed feathermeal
Tb.N	Trabecular number
Tb.Th	Trabecular thickness
Tb.Sp	Trabecular separation

Chapter 1 : General Introduction

1.1 Introduction

World population is expected to grow from the present 6.8 billion people to approximately 9 billion by 2050 (Garcia and Rosenberg 2010). The growing need for nutritious and healthy food will increase the demand for fisheries products from marine sources, whose productivity is already highly stressed by excessive fishing pressure, growing organic pollution, toxic contamination, coastal degradation and climate change. Thus the global rise in aquaculture must come from the more efficient use of declining resources (Garcia and Rosenberg 2010). Aquaculture continues to be the fastest growing animal food-producing sector and to outpace population growth, with per capita supply from aquaculture increasing from 0.7 kg in 1970 to 7.8 kg in 2006, an average annual growth rate of 6.9 percent. It is set to overtake capture fisheries as a source of food fish. From a production of less than 1 million tonnes per year in the early 1950s, production in 2006 was reported to be 51.7 million tonnes with a value of US$78.8 billion, representing an annual growth rate of nearly 7 percent (FAO 2009). This rapidly expanding aquaculture industry requires increasing amounts of formulated feed, traditionally based upon high inclusion levels of fishmeal (30-60% dry matter) however the FAO also report that the maximum wild capture fisheries potential from the world's oceans has probably been reached. Therefore this increase in demand for fishmeal and oil, compared to supply, has led to escalating prices placing an even higher demand and global market for fishmeal and its derivatives. This reliance on a singular ingredient presents considerable risk associated with the supply, price, and quality fluctuations therefore as a strategy to reduce this risk, the identification, development and use of alternatives to fishmeal and oil in aquaculture remain a high priority (Glencross, Booth and Allan 2007). Additionally as a result of the volumes of fishmeal and oil

used in aquaculture, especially for high value carnivorous species, aquaculture of these species is still seen as a net consumer rather than producer, and this practice has raised concerns about the long-term sustainability of these industries, consequently the very future of the growing aquaculture industry depends on the progressive reduction of wild fish inputs into fish feed (Naylor, Goldburg, Primavera, Kautsky, Beveridge, Clay, Folke, Lubchenco, Mooney and Troell 2000).

In order to address this problem a wide range of potential alternative to fishmeal and oils have been evaluated over the last decade; and these can be broadly classified into those derived from plant or terrestrial animal origin: Plant derived sources include soybean meals, canola meals, and lupin meals. Major animal derived protein sources have included rendered meat meal, blood meals, and poultry meals.

Animal by-product proteins have been proven to be viable replacements for a significant portion of the fishmeal in aquafeeds (Bureau, Harris and Cho 1999; Allan, Parkinson, Booth, Stone, Rowland, Frances and Warner-Smith 2000; Bureau, Harris, Bevan, Simmons, Azevedo and Cho 2000; Sugiura, Babbitt, Dong and Hardy 2000; Yanik, Dabrowski and Bai 2003; Tidwell, Coyle, Bright and Yasharian 2005; Wang, Guo, Bureau and Cui 2006; Rahnema and Borton 2007). However the traditional assessment has been by the use of growth indices and signs of gross pathology. These novel sources must now be validated for use in terms of sub-clinical effects upon health. Health is an ill-defined word when used in the context of animals with the commonly accepted definition – the absence of disease –recognised to be inadequate. For an intensively cultured animal to be healthy it must be able to cope with husbandry stressors, effectively eliminate intra-and extra

cellular pathogens and maintain normal homeostatic function (Sealey and Gatlin 1999; Lall 2000; Gatlin 2002). The future profitability of aquaculture operations depends on the ability of nutritionists to formulate diets that provide all essential nutrients, in the correct proportions, to maintain the increased growth rates and stringent health requirements of modern intensive fish farming operations. At present the greatest influence on the profitability of an aquaculture facility is the incidence, and severity, of disease and deformity. Disease reduces profitability through a reduction in the numbers of fish reaching harvest size; while obvious deformities can downgrade harvested salmon (*Salmo salar*) from 'superior' to lower grades, significantly impacting profitability. In addition to the downgrading of deformed fish there are increased processing costs due to the irregular shape of the animal resulting in extra fillet trimming and a concomitant reduction in yield (Branson and Turnbull 2007).

Diet is known to have an effect on both immune response and skeletal function in higher vertebrates and there is now an increasing trend in aquatic animal science to link health and nutrition (Blazer and Wolke 1984). However at present there is a paucity of information on the nutritional modulation of disease resistance and deformity in fish outside of specific additives, (probiotics and immunostimulants), or over fortification with key elements e.g. Vitamin C. Thus a brief description of the teleost immune system and skeletal tissue/ mineral homeostasis is required followed by the known effects of selected nutrients regardless of dietary source. Once the physiological systems and known effects of selected nutrients/ ingredients have been detailed, investigations that specifically examine the effect of animal protein sources will be discussed.

As a consequence of the demand for aquaculture products the evolution of fish farming has moved towards higher yields and faster growth for maximum profitability. In order to support these trends the culture of high value species such as salmon (*Salmo salar)* and trout (*Oncorhynchus mykiss*) are dependent upon the use of formulated feeds. The nutritional requirements of teleost fish do not vary greatly between species but differences do occur between both freshwater and marine, and temperate and tropical species. A typical salmonid diet currently contains 35-50% protein and 10-30% lipid, with both of these nutrients coming from a variety of ingredients.

1.2 The teleost skeletal system

The skeleton of fish is a complex and metabolically active tissue that undergoes continuous remodelling throughout the organism's life (Huysseune 2001). The skeletal system consists of bones and cartilage and serves multiple functions, the most important being to support the structural integrity of the body for normal posture, development and locomotion (Bone and Moore 2008). The skeleton provides a site for muscle attachment protects vital organs and serves as a reservoir of ions. Morphologically fish bones consist of the dermal head bones, internal skeleton, and scales. However in contrast to mammals, fish haematopoiesis occurs in the spleen and anterior kidney rather than within the bone marrow. The bones and scales of fish consist of inorganic calcium hydroxyapatite salts embedded within a matrix of organic collagen fibres with the organic matrix mostly comprised of collagen and hydroxyapatite while cartilage consists of cells in an extracellular matrix, which may or may not be mineralised depending on the cartilage type (Stiassny 2001). Similar to higher vertebrates a large number of forms of cartilage and several other tissues with distinct histological characteristics

between bone and cartilage have been identified in fish and play an important role in skeletal development (Yasutake 1983).

Similar to other vertebrates, skeletal elements represent a reservoir of calcium and phosphorus and other ions that are in a continual exchange with electrolytes found in the blood and extracellular fluids. They are therefore vital in maintaining homeostasis by exerting a buffering effect on changes in plasma electrolyte levels. These functions require continuous remodelling; both bone resorption and deposition, and also the presence of specialised cell types. The three types of cell are osteoblasts that form bone; osteocytes which are osteoblasts that have become entrapped within the mineral matrix; and osteoclasts, multinucleated bone resorption cells. Fish bone can be either cellular (containing osetocytes), or acellular. Cellular bones are restricted to a small group of fish; Salmonidae (including *O.mykiss*), Cyprinidae, and Clupeidae while acellular bones are found in all other teleosts. In mammals osteocytes are postulated to participate in bone mineral exchange and assist in the regulation of bone remodelling by detecting strain changes (Burger, Kleinnulend, Vanderplas and Nijweide 1994). Acellular bones are formed by osteoblasts moving away from the site of mineralisation as bone deposition occurs and were thought to be incapable of remodelling (Moss and Freilich 1963). However more recent studies have indicated that acellular bones are capable of some remodelling with vertebral cells expressing high levels of the osteoclast specific marker tartrate resistant acid phosphatase (TRAP) in the Japanese medaka (*Oryzias latipe*) in the absence of characteristic multinucleated osteoclasts (Nemoto, Higuchi, Baba, Kudo and Takano 2007) .

The organisation of the bone, especially the orientation of the collagen and the order placement of the bone cells within the matrix, is one of the criteria used to

classify bone. The two general categories commonly used are lamellar and non-lamellar bone. Non-lamellar bone (=fibro-lamellar; woven bone) is characterised by the disorderly, irregular arrangement of collagen within the matrix and is typical of fast growing bone. Lamellar bone is characterised by orderly, regular arrangement of collagenous fibres within the matrix, usually accompanied by the regular orientation of bone cells and is typical of slow growing bone. In the salmonid vertebrae lamellar bone is found in a continuous layer around the notochord with a layer of interconnecting trabecular processes formed over the top in cancellous bone (Nordvik, Kryvi, Totland and Grotmol 2005).

Growth in the thickness of bone is achieved by the addition of new bone on top existing outer bone surface. The growth occurs through the activity of osteoblasts within the periosteum, a connective sheath that covers the outer bone surface. As new bone is being deposited by osteoblast activity on the external surface, osteoclasts within the bone dissolve the bony tissue adjacent to the endosteal surface. Thus bone deposition and bone resorption normally go on concurrently, so that the bone is constantly being remodelled. Bone remodelling serves two purposes: (1) it keeps the skeleton 'engineered' for maximum effectiveness in its mechanical uses and (2) it helps to maintain the plasma Ca^{2+} level (Stiassny 2001).

Teleost vertebrae exhibit variation in both development and morphology, however they have been described as being derived from four major elements; the chordacentrum, the autocentrum, the cartilages of the neural and haemal arches and the arcocentrum (Arratia, Schultze and Casciotta 2001). The chordacentra are the first structures in the vertebral body to develop, and they appear as metameric mineralised zones within the notochordal sheath. In Atlantic salmon the notochord seems to generate the segmental pattern of the vertebral column through the

formation of the chordacentra, which may thus play a key role in the further development of the vertebral bodies by patterning the sclerotome (Grotmol, Kryvi, Nordvik and Totland 2003; Fleming, Keynes and Tannahill 2004; Grotmol, Nordvik, Kryvi and Totland 2005). The chordacentrum acts as a foundation for the initial layer of perinotochordal bone, the autocentrum which is formed by sclerotomal osteoblasts through direct ossification. The arcocentrum originates from direct ossification on the surfaces of the cartilage of the neural and haemal arches and may, dependent upon the species, fuse with the autocentrum. A schematic representation of a salmonid vertebra is shown in figure 1-2.

In addition to the skeleton fish posses scales that have been shown to be an important site of Ca and phosphorus metabolism and deposition. The calcium exchange rate of fish scales is three times that of bones (Berg 1968) with Garrod and Newell (1958) reporting that the scales of *Tilapia esculenta* accounted for between 19 and 24% of the dry mass thus making a significant contribution towards the mineral content of fish.

Elasmoid scales of teleosts are anchored in separate scale pockets by sparse bundles of collagen fibres attached to the calcified layer (Whitear 1986). Four fields are distinguished at the scale surface: the anterior field, two lateral fields and the posterior field. The anterior field and the lateral fields are deeply inserted within the dermis. The posterior field is covered by the epidermis with the calcified layer facing the epidermis (Zylberberg, Bonaventure, Cohen-Solal, Hartmann and Bereiterhahn 1992). The posterior field is not overlapped by other scales (Whitear 1986).

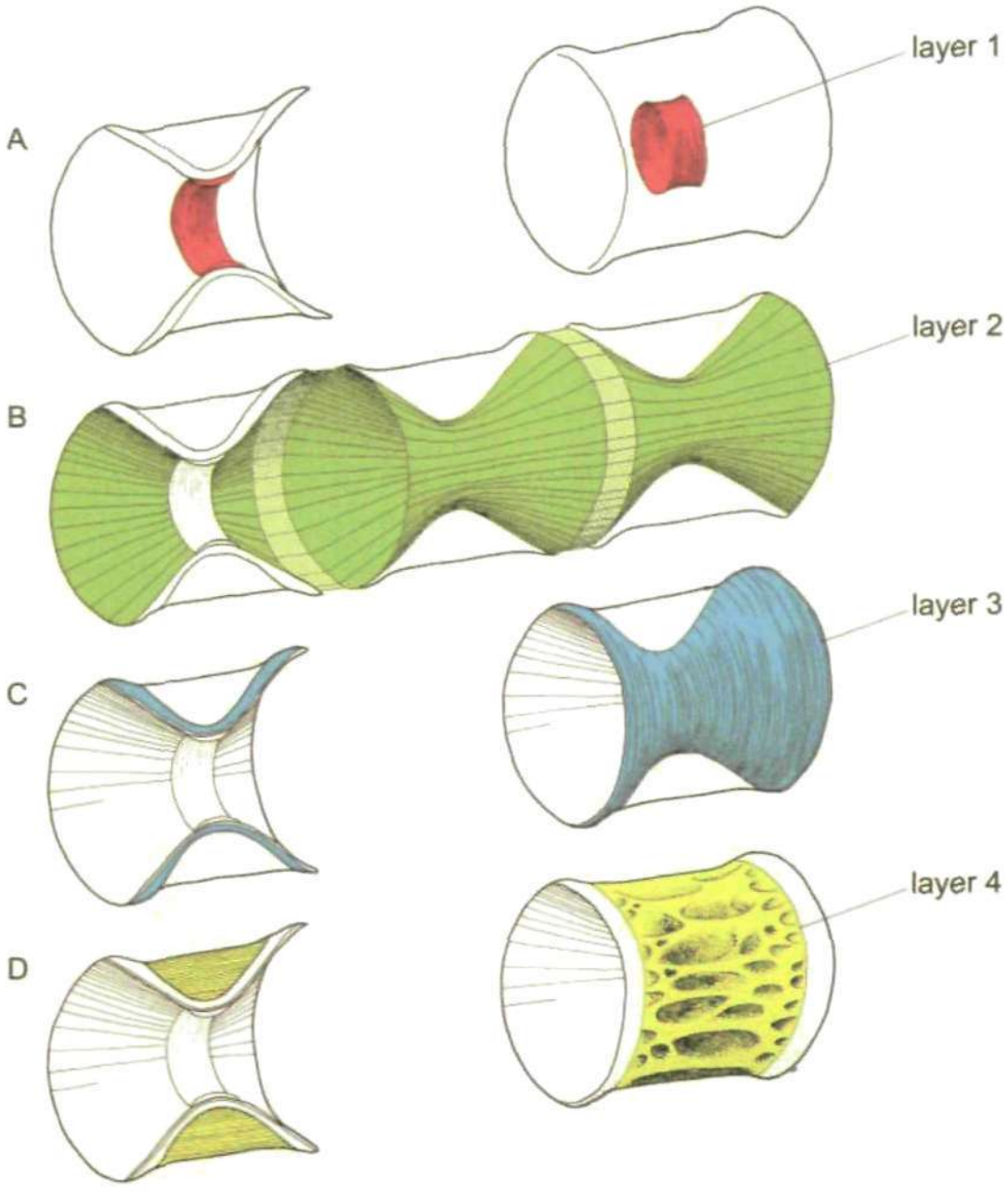

Figure 1-1: Schematic illustrations of the layers within the vertebral body of the Atlantic Salmon. The layers are displayed both in medial section and within the whole vertebra. The main direction of the collagen fibres within the matrix of each layer is indicated by lines. (A) Layer 1 (chordacentrum), which forms through the mineralisation of the notochord sheath. (B) Layer 2 is a continuous, thin layer that encases the entire notochord. (C) Layer 3 makes up the bulk of the laminar bone in the amphicoel. (D) Layer 4 consists of cancellous bone mainly with an interconnected mesh of longitudinal and transverse trabeculae. (Nordvik *et al.* 2005)

The elasmoid scales of teleost fish are composed of hydroxyapatite ($Ca_{10}(PO4)_6(OH)_4$) and extracellular matrix, mainly type I collagen fibers, which together form a highly ordered three-dimensional structure. Each scale consists of two distinct regions: an external (osseous) layer and an internal fibrillary plate (Onozato and Watabe 1979; Zylberberg and Nicolas 1982). In the upper external layer, collagen fibres are randomly arranged and embedded in a proteoglycan matrix. Within the fibrillary lower layer, in contrast, the collagen fibres are co-aligned and organized into lamellae that are superimposed to produce an orthogonal and/or a double-twisted plywood pattern (Olson and Watabe 1980; Zylberberg and Bereiter-Hahn 1991; Nicolas, Gaill and Zylberberg 1997). The collagen fibres are produced within the fibrillary layer by scleroblasts located at the base of the scales (Onozato and Watabe 1979; Zylberberg *et al.* 1992). The fibres are organized through the cooperative involvement of microtubules and actin microfilaments that are subjected to consecutive alterations during the formation of plies of the basal plate (Byers, Fujiwara and Porter 1980; Zylberberg and Nicolas 1982; Zylberberg and Bereiter-Hahn 1991; Zylberberg *et al.* 1992). In general, the spatial organization of collagen fibres is of key importance for the mechanical properties of different connective tissues (Weiner, Traub and Wagner 1999). Mineralization of fish scales occurs continuously throughout the life of the organism. The external layer is initially mineralised with matrix vesicles, and then the internal layer is developed (Onozato and Watabe 1979; Zylberberg and Nicolas 1982). Needle like or flaky crystals of apatite in random orientation are observed in the outer layer (Onozato and Watabe 1979; Olson and Watabe 1980). Calcification of the internal layer, in contrast, occurs in the absence of matrix vesicles (Schönbörner, Boivin and Baud 1979), and the orientation of the crystallographic

c-axis of apatite crystals parallel to collagen fibres in bony fish (*Leuciscus cephalus*) has been recently determined using small-angle X-ray diffractometry (XRD) (Bigi, Burghammer, Falconi, Koch, Panzavolta and Riekel 2001).

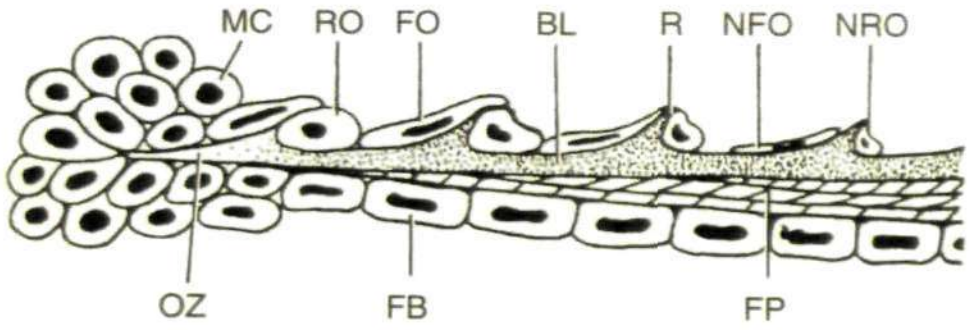

Figure 1-2: Schematic diagram showing the structure of the anterior margin of a teleostean scale and the scale forming cells. BL bony layer, FB fibroblast, FO flattened osteoblast, FP fillibrary plate, MC marginal cell, NFO necrotic flattened osteoblast, NRO necrotic round osteoblast, OZ osteoid zone, R ridge, RO round osteoblast. (Kobayashi, Yamada, Maekawa and Ouchi 1972).

The main constituents of fish mineralised tissues include calcium, phosphate and carbonate with small amounts of magnesium, sodium, strontium, lead, citrate, fluoride, hydroxide, and sulphate.

1.2.1 Deformity

Deformity can be defined as a major difference in the shape of a body part or organ compared to the average shape for that part. Thus deformities can occur in both mineralised and non-mineralised tissues. In aquaculture common deformities are a complex mixture of different bone disorders including vertebral and spinal malformations such as kyphosis (humpback, hunchback), lordosis (saddleback, swayback), scoliosis (lateral curvature with rotation of the vertebrae), and platyspondyly (short-tail, compressed vertebrae). Some of these disorders may also include fusion of vertebrae. Other deformities include "neck-bend" or "stargazer" and in the head region involve compressed snout (pugheadness), bent jaw (crossbite), jaw (harelip or front and downwards protuberance of jaw; reduction of

lower jaw), short operculum, and other defects (reduced or asymmetric fins, etc.). Often these deformities may be a combination of several deformities, however, neck, vertebral, and spinal disorders are most prevalent (Lall and Lewis-Mcrea 2007). Clinical data and the biochemical basis for pathogenesis of bone malformation during the prevalence of this condition are poorly defined. One of the major bottlenecks is the lack of information on bone formation during various stages of development and reliable biochemical markers and bone histology data to characterize specific changes associated with multiple nutrient deficiencies and water quality.

Phosphorus is an essential element in the diet of vertebrate animals and is required for the formation of hydroxyapatite in hard tissues. P is also recognized to play a major role in a large number of metabolic processes (Sugiura *et al* (2004). The association between phosphorus deficiency and the incidence of deformity has long been understood, with sigmoid deformities of the distal ends of pleural ribs and dorsal ventral spines diagnostic for P deficiency (Beaverfjord *et al* 1998, Robert *et al* 2001). Clinically P deficiency can result in reduced growth, reduced mechanical properties of mineral tissues, reduced scale and bone mineral content and an impaired immune response (Sugiura *et al* 2004). P deficiency and the resultant syndromes are further discussed in Chapter 2.

1.2.2 Mineral homeostasis.

The biochemical mechanisms involved in skeletal tissue metabolism of fish can differ substantially from mammals; in fish the major site of calcium metabolism is not the bones, rather occurring at the gills, oral epithelia and fins (Lall 2002). Therefore the role of all forms of vitamin D and its metabolites in the mineral

homeostasis of fish is limited. Teleost fish possess two hypocalcaemic hormones: Stanniocalcin (STC- secreted by the Corpuscles of Stannius), and calcitonin (CA-secreted by the ultimobranchial gland). Stanniocalcin is the predominant hormone that regulates calcium and P homeostasis in freshwater fish (Wagner, Fenwick, Park, Milliken, Copp and Friesen 1988), with oestrogen concentration increasing during bone resorption rather than decreasing as is the case with mammals. An increase in the plasma concentrations of ionic calcium (Ca^{2+}) stimulates STC secretion, and this hormone acts on the gills, skin, gut and kidney to restore normocalcaemia (Flemming 1967). An overview of the presumed interactions of hormones and vitamins on the mineral homeostasis of teleost fish is shown in Figure 1-3 (p.13). It should be noted that the role of vitamin D_3 and K in teleost skeletogenesis is at present unclear but it is thought that the Vitamin D_3 metabolite ($1.25\text{-}(OH)_2D_3$) stimulates absorption of calcium and phosphate in the intestine and resorption of the same nutrients in the kidney. It may also stimulate osteoblasts in bone to secrete osteocalcin, which depends on vitamin K for carboxylation of γ-glutamic acid (Lall and Lewis-McCrea 2007).

It is essential to note that skeletogenesis in fish is dependent on the supply of dietary phosphorus rather than the supply of calcium as in terrestrial animals. This is largely due to the aquatic environment having abundant calcium ions but being limited in phosphorus. Thus, while calcium deficiency is uncommon in teleosts, the provision of phosphorus must be balanced between the requirements of the fish and the minimisation of potentially negative environmental inputs.

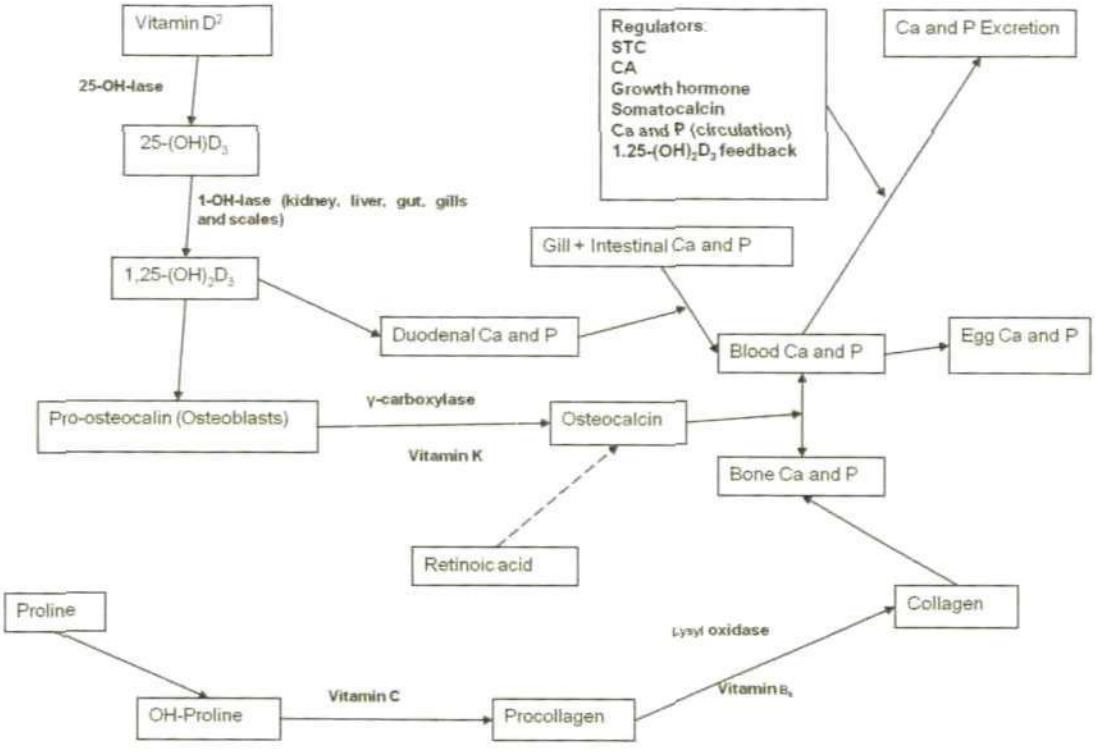

Figure 1-3: Proposed role of calcium, phosphorus, and vitamins in teleost bone metabolism. Redrawn from (Lall and Lewis-McCrea 2007).

1.3 The teleost immune system

Fish, like all other organisms, are constantly exposed to pathogens in their environment. This has led to the evolution of defensive immune systems consisting of a variety of tissues, cells and molecules. The basic requirement of these different systems is to distinguish between 'self' and 'non-self' and then eliminate (or neutralise) 'non self' (Bone and Moore 2008). Two types of immune system are distinguished: innate and acquired, with both requiring cell-mediated and humoral responses. Cell mediated responses rely directly on the involvement of specialised cells that phagocytise or attack pathogens. Humoral immunity refers to those responses that are delivered by bodily fluids such as blood, interstitial fluids, or exocrine secretions including the antibody response.

Innate immunity exists in virtually all plants and animals and is believed to be the earliest form of immunity. It is non-specific, that is, it acts against many different types of organisms and does not become more effective following repeated exposure to the same pathogens. Mechanisms of innate immunity include physical barriers that prevent entry of micro-organisms (e.g. skin and mucosal lining of the digestive tract), chemicals such as cytokines, agglutinins, precipitins (opsonins, primarily lectins), and interferon may be contained in secretions (mucus/ digestive juices) or generalised responses such as inflammation, or phagocytosis by macrophages or non-specific cytotoxic cells.

In contrast the acquired (adaptive) immunity permits the immune system to respond to specific antigen molecules carried on pathogens despite prior exposure. This acquired immunity then provides additional protection against re-exposure to the same antigen. In most fish the acquired immunity systems include two types of lymphocytes: B-cells which produce immunoglobulin (antibody) and both effector and regulatory T-cells involved in cell-mediated immunity. In mammals B-cells originate in the bone marrow while T-cells in the thymus; fish lack bone marrow and jawless fish lack a thymus however the same functions occur in equivalent tissues in both of these examples.

Though the two 'arms' of the immune system are presented separately here there is considerable communication between the two with the systems often acting sequentially with the innate acting as the first line of defence, limiting the severity of attack until the acquired defence can develop.

Table 1-1: Comparison of the mammalian and fish immune systems. Adapted from Tort, Balasch et al. (2003)

	Teleosts	Mammals
Biotic constrictions		
Temperature range	-2 to 35°C	36.5 to 37.5°C
Primary environment	Water	Air
Metabolism	Poikilothermic	Homeothermic
External interfaces	Mucous, skin, gills	Respiratory tree
Humoral diversity		
Ig isotypes	IgM, IgD?	IgM, IgA, IgD, IgE, IgG
Ig gene rearrangement	Multicluster	Translocon
Non-specific diversity	Several C3 isoforms	No C3 isoforms
Overall performance		
Antibody affinity	Low	High
Antibody response	Slow	Fast
Memory maturation	Weak	Strong
Affinity maturation	Low or absent	High
Low temperatures	High dependence, immunosuppressive response	Low dependence
Lymphoid organs		
Haematopoietic tissue	Head kidney	Bone marrow
Thymus	Involution species-dependant, influenced by seasonal changes and hormonal cycles	Involution with age
Lymphoid nodes	Absent	Present
Gut associated lymphoid tissue	Not organised, lymphoid aggregates	Organised, Peyer patches
Germinal centres	Absent, dendritic cells probably present	Present

Tissues of the teleost immune system

The kidney is an organ important both in immunity and haematopoiesis. It is the site of blood cell differentiation as well as the development of early immune responses in fish embryos. With maturity the anterior portion (head kidney) has no renal functions instead it contains lymphomyloid tissues that produce lymphocytes (specifically B-cells) and adrenal-like endocrine tissues. Where present the spleen

also contains lymphocytes and macrophages and is involved in immune reactivity. In teleosts the size, shape and development of the spleen can vary but it generally consists of masses of red and white pulp (respectively sites of red blood cells and white blood cells production) surrounded by numerous small capillaries. The major immune function of the liver is thought to be phagocytosis and presentation of particulate antigens to other lymphoid cells (Pastoret, Griebel, Bazin and Govaerts 1998).

Cells of the immune system

The cells of the immune system can be either fixed or migratory. Fixed macrophage and dendritic cells are found in the skin and mucosal membranes as well as the various lymphoidal organs. Migratory cells, also known as leukocytes occur in both blood, and in interstitial tissue fluid. Although the total number of leukocytes varies with stress, diseases, and environmental conditions, fish generally have a greater white blood cell count than mammals (Yasutake 1983).

Nomenclature of fish leukocytes is based on their similarities to those found within mammals however it should be noted that it is widely agreed that there is no absolute correspondence (Bone and Moore 2008).

The principal cells involved in teleostean innate immunity are phagocytic granulocytes (neutrophils) and macrophages as well as non-specific cytotoxic cells (NCC) or natural killer cells (NKC) while acquired immunity depends on the activities of different types of lymphocytes.

Physical barriers

The mucus producing skin of fish represents an almost unique first line of defence against foreign invaders. Mucus contains both specific and non-specific immunoglobulin (IgM), complement proteins, lectins, pentraxins, and lysozyme. The internal mucosal surface area is approximately 400 times that of the external surface area as well as being the principal site of gas and nutrient exchange.

Non-specific humoral components

A summary of the known non-specific humoral factors involved in the natural resistance of fish is presented in Table 1-2.

Table 1-2: Non-specific humoral molecules and their mode of action in the serum of fish. (Shoemaker, Klesius and Lim 2001)

Molecule	Composition	Mode of action
Lectins	Specific sugar binding proteins	Recognition, precipitation, agglutination, opsonin activity
Lytic enzymes	Catalytic proteins Lysozyme Complement components	Hemolytic and antibacterial properties
Transferrin/Lactoferrin	Gylcoprotein	Iron binding
Cerulopasmin	Acute-phase protein	Opsonization or activation of complement
C-reactive protein	Acute phase protein	Opsonization or activation of complement
Interferon	Protein	Aid in resistance to viral infection

The factors deemed pertinent to the program of investigation are further detailed below.

Lysozyme

Lysozyme is one of the most studied innate responses in fish (Grinde 1989; Mock and Peters 1990; Tort *et al.* 2003; Saurabh and Sahoo 2008). Lysozyme acts upon

the peptidoglycan layer of bacterial cell walls resulting in the lysis of the bacteria. Lysozyme has been found in mucus and ova, with serum lysozyme from peritoneal macrophages and blood neutrophils, having been used as an indicator of non-specific immune response (Mock and Peters 1990; Kiron, Watanabe, Fukuda, Okamoto and Takeuchi 1995; Krogdahl, Bakke-McKellep, Roed and Baeverfjord 2000; Panigrahi, Kiron, Puangkaew, Kobayashi, Satoh and Sugita 2005; Sitja-Bobadilla, Pena-Llopis, Gomez-Requeni, Medale, Kaushik and Perez-Sanehez 2005; Bakke-McKellep, Koppang, Gunnes, Sanden, Hemre, Landsverk and Krogdahl 2007b; Subramanian, MacKinnon and Ross 2007). The lysozyme response has been found to be variable in its potency depending on the species and the tissue location but is present in all species studied (Grinde 1989).

Complement

The complement system appears to be one of the central immune responses in fish; with the classic, alternative and lectin-mediated pathways having been described in several teleost groups (Sakai 1992). Additionally in evolutionary terms, it has been suggested that the lectin-mediated pathway may have preceded the immunoglobulin-mediated pathway and therefore may be more important in fish (Tort *et al.* 2003). There are some relevant differences between the complement systems of mammals and fish: Mammals have only one isoform of the key activation molecule C3, whereas fish express several functionally active C3 isoforms; three in trout (*O. mykis*), five in seabream (*Sparus aurata*) and carp (*Cyprio carpio*) (Nonaka, Irie, Tanabe, Kaidoh, Natsuume-Sakai and Takahashi 1985; Sunyer, Zarkadis, Sahu and Lambris 1996; Nakao, Mutsuro, Obo, Fujiki, Nonaka and Yano 2000). Teleost C3 share the two chain structure although showing variation in the catalytic residues of the protein, they maintain a

differential affinity for several substrates and they are probably products of several polymorphic genes (Slierendrecht, Jensen, Hørlyck and Koch 1993; Sahu and Lambris 2001). The biological meaning of the variability may be the specialisation to bind to specific surfaces and to increase efficiency to eliminate pathogens; however this has yet to be demonstrated (Tort *et al.* 2003).

Specific humoral response

The teleost acquired immune response is poorly developed compared to higher vertebrates with only a single class of immunoglobulin (IgM) compared to the five classes in mammals (IgG, IgE, IgM, IgA, IgD). The lymphocyte is responsible for initiating and mediating the three aspects of specific immunity: humoral immunity, cell-mediated immunity, and memory. Humoral immunity refers to the production of immunoglobulin while CMI refers to responses that are mediated by a variety of cells including lymphocytes and other types, especially macrophages, which are recruited by lymphocyte products. Memory refers to the ability of the lymphoid cell populations to change following exposure to an antigen so that the following exposure produces a reduced latency period and an enhanced magnitude of response. Lymphocytes are found in peripheral blood, the lymphoid organs and other tissues especially during inflammation events.

The acquired immune response utilises both B and T lymphocytes to generate a response to a specific antigen; B-Lymphocytes produce specific Ig on their cell surface membrane, while T-lymphocytes do not express Ig. T-lymphocytes respond to antigen presented by phagocytes and once stimulated they induce B-lymphocytes to produce specific antibody to the presented antigen. Generally B lymphocytes are dependent upon presented antigen and cytokines produced by T lymphocytes to

initiate antibody production; however there are T lymphocyte independent immune reactions where B lymphocytes are stimulated directly.

Non-specific cellular response

Granulocytes and monocytes/macrophages are key cells involved in the cellular part of the non-specific defence mechanism in fish. In mammals granulocytes consist of three morphologically distinct cell types; neutrophils, eosinophoils, and basophils. Only neutrophils have been conclusively demonstrated in teleost fish, with debate over the presence of basophils and eosinophoils (Suzuki and Iida 1992; Plouffe, Hanington, Walsh, Wilson and Belosevic 2005). The precise function of fish neutrophils in inflammation is still debated though it is thought that phagocytosis and subsequent killing, mediated by enzymes and reactive oxygen radicals, may be of primary importance (Ainsworth 1992; Suzuki and Iida 1992; Ellis 1999).

The term macrophage is used to describe cells with one or more separate nuclei, that have the ability to engulf particles and to modify their environment by secretion (Dalmo, Ingebrigtsen and Bogwald 1997). Phagocytes are pivotal in the animal immune response, with cells located in most tissues and organs of the vertebrate body. In mammals macrophages recognise and destroy organisms via specific receptors or immunoglobulin and C3 when these factors have coated the surface of bacteria during an immune response.

In response to such states as inflammation, macrophages secrete many bioactive products including enzymes, antimicrobial peptides, oxygen metabolites and arachadonic acid metabolites and cytokines. In addition to secretory activities, mammalian macrophages govern immunological responses by the recognition and

digestion of foreign substances and presentation of antigen in the context of MHC molecules on the macrophage cell surface, the regulation of B- and T-cell functions and control of their own growth and replication and that of other cells (Dalmo *et al.* 1997).

The sudden increase in any macrophage function following a known stimulation is often called activation. When macrophages are activated the production of reactive oxygen intermediates, nitric oxides, lysosomal enzymes, cytokines, and other signalling molecules (for example prostaglandins, leucotrienes and thromboxanes) is often modulated. The macrophages change their morphological characteristics, produce elevated levels of different molecules and possess enhanced phagocytic and pinocytic capacity. Phagocytes produce large quantities of superoxide (O_2^-) during phagocytosis or upon stimulation with a variety of agents. This response results in a large increase in oxygen consumption and has therefore been termed the 'respiratory burst'. This process together with the production of reactive nitrogen intermediates are key phagocytic killing mechanism of teleosts.

1.4 Rainbow trout aquaculture

Formerly known as *Salmo gairdneri* (Richardson 1836), rainbow trout was considered to be a closer relative of the Atlantic salmon (*S. salar*) and the brown trout (*S. trutta*) until the late 1980s. Based on fossil evidence and molecular genetic studies its taxonomic status was changed to *O.mykiss*. Two forms of the species are recognised; the rainbow trout, a permanent freshwater resident and the anadromous form known as 'steelhead' or ocean trout. Rainbow trout is the main species of *Oncorhynchus* that is farmed with at least 64 countries reporting rainbow trout culture production. Commercial rainbow trout production targets two markets;

large fish over 3kg, farmed mostly in Chile, Norway, Sweden and Finland and 200-300g (pan size) cultured in freshwater in France, Italy, and Denmark. These countries produced in 2006 close to 50% (89 300 t) of all freshwater rainbow trout produced in Europe (FAO 2009).

1.5 Alternative protein sources

There are a number of different protein sources utilised in aquafeeds. Broadly speaking these can be divided into proteins from animals, plants or single celled organisms. Animal and plant sources are discussed in more detail below.

1.5.1 Plant protein sources

Most of the potential, alternative, plant-derived protein sources are known to contain a wide variety of antinutritonal factors (ANF) in soybean meal this includes protease inhibitors, lectins, phytoestrogens, anti-vitamins and allergens (Francis, Makkar and Becker 2001). ANF's have been defined as substances which by themselves, or through metabolic products arising in living systems, interfere with food utilization and affect the health and production of animals (Makkar 1993). Despite the problems associated with the inclusion of elevated levels of plant based proteins in carnivorous fish feeds it has been demonstrated, under research conditions, that it is possible to replace 100% of the fishmeal in feeds for Rainbow trout (*O.mykiss*) with suitable pre-treatment of the raw materials and the appropriate amino acid supplementation; the limiting factors of inclusion being the cost of these pre-treatments and the high unit cost of supplementation with crystalline amino acids (Morris 2004). These high levels of inclusion are possible due to the favourable EAA profile of vegetable protein sources and the reduction of

ANFs either through pre-treatment or the development of genetically modified strains.

1.5.2 Animal by-product proteins

In the EU, approximately 17 million tonnes (mt) of slaughter by-products are produced by the meat industry every year, with the majority of this (14.5mt) from animals that are declared fit for human consumption. Of this total 1.5 mt of fat and 3mt of protein are produced, which are then used in a wide variety of products including pet foods, livestock feeds and fertilisers, soaps and oleochemicals for use in feed applications. These by-products must first be processed in order to avoid spoilage, conventionally achieved by heating to sterilise and reduce the high water content. This process is known as "Rendering".

Rendered products have been identified as having considerable potential for replacing fishmeal in aquafeeds due to relatively high protein concentrations, low phosphorus and other minerals, and high digestibility of nutrients. Considerable research has been aimed at identifying the optimum inclusion level of a wide variety of protein sources including blood meal, feather meal, and meat and bone meal. For most species a 30% inclusion level produced no detrimental effects on growth or subsequent flesh taste. However the use of animal by-products in the human food chain was discontinued in the European Union after the outbreak of BSE (bovine spongiform encephalitis) in the United Kingdom in 1996. Since that time all animal by-products entering the human food chain must be treated to guarantee the inactivation of prions.

Currently the use of animal by-products in Europe is controlled by the animal by-product regulation (ABPR 1774/2002) together with the TSE regulation (999/2001)

which defines conditions and restrictions attached to the current and future use of processed animal protein (PAP). This regulation lays down key points in the handling, processing and marketing of animal by-products including the categorisation of raw material, traceability, and importantly that there is no intra-species recycling (Woodgate 2004). There are three categories of raw materials which must be segregated according to ABPR 1774/2002:

- ***Category 1***: High risk materials from animals that have been destroyed due to disease.
- ***Category 2***: Material from deadstock which must be processed separately and used for specific applications such as fertilisers and bio-energy.
- ***Category 3***: Material derived from animals declared fit for human consumption, which can be used in a range of applications when processed to proscribed standards. These applications include animal feeds provided the species barrier is maintained.

Since 2003 non-ruminant haemoglobin and blood meal have been permitted again in fish feeds produced in the EU (commission regulation 1234/2003; an amendment to the TSE regulation 999/2001). However since 1989, in response to media publicity and retail concerns about potential cross-species transmission of BSE, all three UK based fish feed manufacturers agreed to voluntarily remove materials of bovine and ovine origin from all fish feed formulations. Feather meal, and porcine or avian origin continued to be used up until March 1996. In March 1996 when the probable link between BSE and variant Creutzfeldt-Jacob disease was announced media interest resulted in pressure from retailers for the exclusion of all terrestrial animal proteins including blood meal and feather meal. This led to

the progressive, voluntary withdrawl of blood and feather meals before the end of 1996. Since that time the sole source of animal proteins in uk-produced fish feeds has been fish and crustacean meals (Friedland, Petersen and Rubenstein 2009). In USA, Canada, South America, Asia and Australia there is no legislative constraints and animal by-products are widely used in aquaculture with successful results.

Chapter 2 : Animal by-products and the health of fish; a review of current literature

2.1 How is health defined?

In humans it is generally accepted that malnourishment impairs immune function (Grimble 2007) and the same is true of other vertebrates including fish. The two determinant factors in the profitability of modern intensive aquaculture operations are the number of fish reaching market size and the quality of the animals. Thus the profitability is governed by the disease resistance of the animal and the quality is directly affected by the incidence of deformity: Both of which can be influenced by diet.

Nutritional modulation of resistance to infectious diseases, based upon the information on fish and terrestrial animals, is divided into five major groups:

- A proper balance of macro- and micro-nutrients including amino acids, polyunsaturated fatty acids, vitamins and trace elements which are essential for the development of the immune system at the larval stage.
- Adequate nutrition is essential for the cells of the immune system for lymphocyte proliferation and synthesis of both effector molecules (immunoglobulins, lysozyme, complement etc.) and communication molecules (cytokines, chemokines and eicosanoids). The quantitative requirements for nutrients to maintain a normal immune function is relatively small compared to the requirements for growth and reproduction.
- Some nutrients provide essential substrates for the multiplication of pathogens and their presence in body fluids at low concentrations may limit the growth of ubiquitous pathogens.
- Disease resistance can also be regulated indirectly via the endocrine system.

- Diet composition can modify the microorganisms in the gastro-intestinal tract and affect the integrity of the intestinal epithelium. The presence of oxidised lipid, plant ANF's and fibre can all affect gut physiology and gut microflora.

A summary of the factors affecting the nutritional status, health and immune function of fish is illustrated in figure 1.

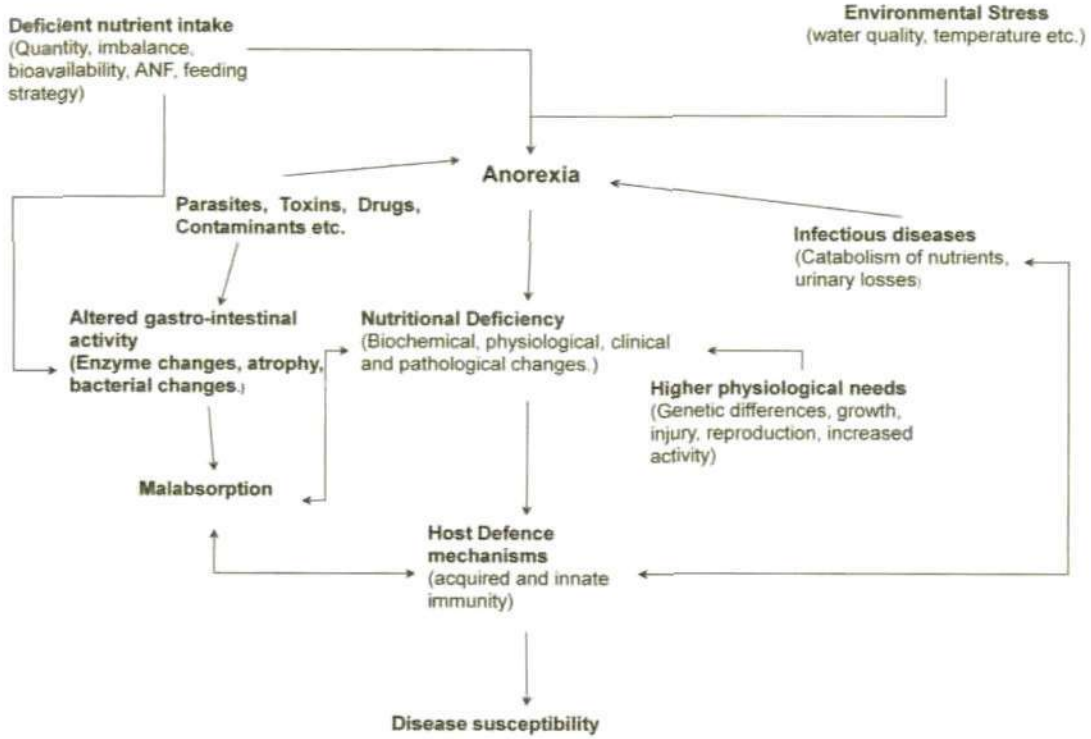

Figure 2-1: Factors affecting nutritional status and immune function of fish (Lall 2000).

2.2 The effects of diet on health

The minimum dietary requirement for protein is a balanced mixture of amino acids and is of primary concern to aquaculture because satisfying this requirement is necessary to ensure adequate growth and health of fish, while providing excessive levels is generally uneconomical, as protein is the most expensive dietary component (Gatlin 2002). Protein is essential for maintenance, growth,

reproduction and may also be used for energy; however there have been limited investigation of proteins effect on disease resistance. Kiron (1995) investigated the modulation of non-specific and specific immune mechanisms by dietary protein in rainbow trout (*Oncorhynchus mykiss*). Fish fed a protein deficient diet (10%) had reduced lysozyme activity and C-reactive protein in serum compared to fish fed diets with 35 and 50% protein. However antibody production was conserved in all diet treatments. Mortality associated with a challenge to *Aeromonas salmonicida* was also elevated in fish fed diets containing 10 and 50% protein compared to those fed 35% protein. In another study Thomas and Woo (1990) observed reduced parasitism in rainbow trout fed a protein deficient diet (19% crude protein) compared to a control diet with 38% protein. All proteins are not identical in their nutritional value and their biological value is a function of its amino acid content: Arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine are the essential amino acids that are the most critical factors in the nutritional value of a protein. Under normal circumstances essential amino acids of dietary origin are conserved. If however amino acid intake is restricted or imbalanced they may be metabolised to form non-essential amino acids. Thus nutritionally satisfactory diets for cultured fish are those which have a sufficiency of both essential and non-essential amino acids for growth and maintenance. Deficiency symptoms may occur, even in an apparent excess of amino acids, due to the protein either not being digested properly or because certain amino acids have become biologically unavailable during processing. The most frequent indicator of an amino acid deficiency is impairment of growth but several deficiency syndromes have also been recorded experimentally. These include dorsal fin erosion associated with lysine deficiency, spinal deformities associated

with tryptophan, lysine, leucine, arginine, or histidine deficiency and a lenticular cataract associated with methionine and tryptophan deficiency (Roberts 1989). In addition the synthesis of nitric oxide via nitric oxide synthase, which is inducible in fish (Schoor and Plumb 1994) has been shown to be an L-arginine dependant metabolic pathway in both channel catfish and goldfish cell cultures (Neumann, Fagan and Belosevic 1995; Buentello and Gatlin 1999). However Buentello (1999) was unable to reproduce the result *in vivo*; hypothesised by the author to be due to the partial regulation of the intracellular availability of arginine by plasma arginine in order to prolong the nitric oxide production from stimulated macrophages.

2.2.1 Lipid

In homeotherm nutrition research, dietary lipids have been studied as modulators of immunity and non-specific disease resistance. The fatty acid composition of cell membranes is especially important in disease resistance because many of the mechanisms and reactions are membrane associated. The fluidity of the membrane depends on the un-saturation of the phospholipids and can influence the function of receptors, receptor binding affinities, cell-cell interactions, permeability and enzyme activities (Blazer 1991). A further factor is that in mammals the fatty acid profile of the cell membrane is primarily influenced by diet; however fish can also change the composition in response to temperature (Abruzzini, Ingram and Clem 1982). Dietary fatty acid composition influences the availability of precursor molecules for the production of eicosanoids; prostagladins, thromboxanes, and leucotrienes. Eicosanoids are oxygenated derivatives of polyunsaturated fatty acids formed by the metabolism of membrane phospholipids by the action of phospholipases. The principal substrate is arachidonic acid (AA; 20:4, n-6) with a 20-carbon backbone, but both eicosapentaenoic acid (EPA; 20:5, n-3) and

docosahexaenoic acid (DHA; 22:6, n-3) are also important substrates in fish. There is limited information on the effect of these soluble mediators in fish; though there is some evidence that all of the groups are produced by fish leukocytes (Rowley 1992).

In general the essential fatty acid requirements of freshwater fish can be met by a supply of the 18:3n-3 and 18:2n-6 in the diet, which can then be elongated and desaturated. Whereas marine fish have low 5-desaturase activity and require the long chain polyunsaturated fatty acids (PUFAs) (20:5n-3 and 22:6n3) (Tocher, Bendiksen, Campbell and Bell 2008). The requirements and deficiency signs in a number of species has been documented however the functional role of n-3 and n-6 PUFA in non specific and specific humoral and cellular immunity is not clear with conflicting results from different authors (Erdal, Evensen, Kaurstad, Lillehaug, Solbakken and Thorud 1991; Secombes 1996). Eicosanoids may be involved in the regulation of the immune system by their direct effects on cells (macrophages and lymphocytes) or their indirect effect via cytokines (Rowley, Knight, Lloydevans, Holland and Vickers 1995). A summary of the known immunomodulatory properties of eicosanoids in *O.mykiss* is shown in Table 2-1.

The effect of essential fatty acid class has been shown by a number of authors to influence the class of eicosanoids produced both *in vivo* and *in vitro*. In general a diet high in n-6 PUFAs (vegetable lipid sources) produce relatively higher levels of the pro-inflammatory 2 series prostaglandins, 5 series leukotrienes and lipoxins derived from eicosapentaenoic acid (EPA 20:5n-3). The situation regarding n-3 derived eicosanoids is more complex with different authors having seemingly contradictory results, though in general there is more support for dietary n-3 fatty acids producing an immunosupressive effect (Knight and Rowley 1995).

Table 2-1: Immunomodulatory properties of eicosanoids in rainbow trout *O.mykiss*.

Eicosanoid		Effect	Reference
Lipoxins	LXA_4	Chemotactic/ chemokinetic for leukocytes	(Rowley 1992)
	LXA_4 LXB_4	Inhibition of lymphocyte proliferative response	(Secombes, Clements, Ashton and Rowley 1994)
	LXA_4	Stimulated the generation of plaque forming cells in splenocytes	(Knight and Rowley 1995)
	LXA_4	Enhanced migration of neutrophils	(Sharp, Pettitt, Rowley and Secombes 1992)
Leukotrienes	LTB_4 LTB_5	Stimulated lymphocyte proliferation	(Secombes *et al.* 1994)
	LTB_4	Enhanced migration of neutophils	(Sharp *et al.* 1992)
Prostaglandins	PGE_2 PGE_3	Suppressed lymphocyte proliferation	(Secombes *et al.* 1994)
	PGE_2	Inhibited respiratory burst activity in macrophages	(Novoa, Figueras, Ashton and Secombes 1996)

The functional role of n-3 and n-6 PUFA in bone lipid metabolism of fish remains to be investigated (Lall and Lewis-McCrea 2007); in terrestrial animals several localised compounds act on bone cells with prostaglandins appearing to be the principal mediator of bone cell functions (Watkins and Seifert 2000). Studies in humans and animals have linked a high intake of saturated fats to low bone density through depressed collagen synthesis (Corwin, Hartman, Maczuga and Graubard 2006).

2.2.2 Carbohydrate

Fish have no dietary requirement for carbohydrate with some carnivorous species unable to tolerate high levels (Hemre, Mommsen and Krogdahl 2002).

Carbohydrate is normally included in aquafeeds as a cheap source of energy or to overcome the problem of bulk/density in high energy feeds i.e. nutrient dense materials such as high quality fishmeal only compromise 50-60% of the bulk required. High levels of carbohydrates have a detrimental effect on the health of carnivorous fish, especially salmonids, causing enteritis and other pathological changes in the gut and liver (Ingh, Krogdahl, Olli, Hendriks, Koninkx and Van den Ingh 1991; Kaushik, Cravedi, Lalles, Sumpter, Fauconneau and Laroche 1995; Baeverfjord and Krogdahl 1996; Ingh, Olli, Krogdahl and Van den Ingh 1996; Bakke-McKellep, Press, Baeverfjord, Krogdahl and Landsverk 2000; Krogdahl *et al.* 2000; Krogdahl, Bakke-McKellep and Baeverfjord 2003; Refstie, Glencross, Landsverk, Sorensen, Lilleeng, Hawkins and Krogdahl 2006; Bakke-McKellep, Froystad, Lilleeng, Dapra, Refstie, Krogdahl and Landsverk 2007a; Bakke-McKellep *et al.* 2007b). Waagbo, Glette, Sandnes and Hemre (1994) found that increasing proportions of carbohydrate only marginally affected the humoral immune responses of Atlantic salmon (*Salmo salar*) following vaccination with *Vibrio salmonicida*, although the cumulative mortality following exposure to this pathogen was lowest in the fish fed 10% carbohydrate. In general while high levels of carbohydrate have been shown to have localised effects on gut integrity these effects have also been shown to be reversible (Baeverfjord and Krogdahl 1996) with Lall (2000) commenting that the effects of carbohydrate on fish health and immune function "are not conclusive".

2.2.3 Vitamins

Vitamin deficiency leads to aberrant biochemical functions and consequent cellular and organ dysfunction (Lall 2000). Several gross morphological and functional changes have been reported in fish deprived of vitamins (NRC 1993) which may be

caused by the low content of vitamins in feeds, environmental or physiological stresses, and by diseases. Vitamins C and E have received the most attention in the literature; this is due to the fact that while they both have distinct metabolic functions they also both have anti-oxidant properties (Blazer and Wolke 1984; Erdal *et al.* 1991). These vitamins have been shown to affect complement and antibody production, as well as macrophage functions including enzymes involved in respiratory burst, intracellular killing and protective mechanisms to prevent tissue damage from free radicals (Blazer 1991). Vitamin A, C, D and vitamin K are considered the determiners of the quality of bone matrix during bone formation in fish (Roy and Lall 2007) and it is these will be discussed further.

Vitamin A activity refers to β-ionone derivatives having biological activity of all-*trans*-retinol. The most active pro-vitamin A form is the carotenoid precursor beta-carotene, which is cleaved by intestinal enzymes to yield 2 moles of all-*trans*-retinol per mole of β-carotene (Lall and Lewis-McCrea 2007). Vitamin A has essential roles in vision, growth, reproduction and normal maintenance of epithelial tissues in all vertebrates, and it also regulates skeletogenesis and cartilage development by controlling chondrocyte function, maturation and proliferation of cells. For *O.mykiss* the vitamin A requirements is listed as 2500 IU Kg^{-1} in NRC (1993) however hypervitaminosis A is associated with lordosis and scoliosis in the mid to anterior hemal regions of the vertebral column (Hilton 1983) and induces toxicity signs (reduced growth, increased mortality and abnormal vertebral growth) in Atlantic Salmon (Ornsrud, Graff, Hoie, Totland and Hemre 2002). In bone, retinoid toxicity reduces collagen synthesis, bone formation, increases the number of osteoclasts causing a net bone loss as well as increasing skeletal turnover (Lall and Lewis-McCrea 2007).

Vitamin C is a water soluble vitamin with diverse functions including as a co-factor in collagen synthesis and also as a reducing agent and antioxidant. It has been shown to be required for fish as they are unable to synthesize (or synthesize enough) to meet that requirement (Halver and Hardy 1989). Adequate vitamin C must be supplied for the formation of bone matrices, collagen synthesis and connective tissue; ascorbic acid is a cofactor in the hydroxylation of proline and lysine which is necessary for the conversion of procollagen to mature collagen (Lall and Lewis-McCrea 2007). Thus skeletal malformations such as lordosis and scoliosis have been recorded in scorbutic rainbow trout (Dabrowski, Elfiky, Kock, Frigg and Wieser 1990). These severe skeletal abnormalities are common in scorbutic fish although the signs of deficiency may not become apparent until after a significant amount of time (15-20 weeks) (Roberts 1989).

The exact physiological role of vitamin D in fish is not clearly defined. Vitamin D_3, which has the greatest effect on intestinal calcium and phosphate absorption in mammals, has been shown to lack biological activity in *O.mykiss* (Hayes, Guillandcumming, Russell and Henderson 1986) with Graff, Hoie, Totland and Lie (2002) reporting no effect on bone formation in Atlantic salmon fry of increased levels of dietary 1,25-$(OH)_2D_3$ (calcitriol). Nonetheless 1,25-$(OH)_2D_3$ receptors have been identified in calcium regulatory tissues (gills and intestine) and increased calcium absorption following 1,25-$(OH)_2D_3$ administration has been observed in the Atlantic cod (*Gadus morhua)* (Sundell, Bishop, Bjornsson and Norman 1992; Sundell, Norman and Bjornsson 1993). As mentioned in Chapter 1 in general it is thought that 1,25-$(OH)_2D_3$ may stimulate absorption of calcium and phosphate in the intestine, resorption in the kidney and may have a direct effect on osteoblasts.

Vitamin K is naturally found in to two forms; phylloquinone and menaquinnone and are essential for fish (Udagawa 2000). In terrestrial vertebrates, vitamin K is an essential micronutrient required for the addition of an extra carboxyl group in the γ-carboxyglutamate (Gla) formation. Gla is essential for the production of osteocalcin, a calcium binding protein synthesised by human osteoblasts and serves as a ground substance for bone mineralization. There is little information on the role of vitamin K in teleost skeletogenesis; Krossøy, Waagbø, Fjelldal, Wargelius, Lock, Graff and Ørnsrud (2009) found vitamin K supplementation (in the form of menadione nitcotinamide bisulphite) did not alter bone strength or morphology. Roy and Lall (2007) reported that dietary vitamin K deficiency in haddock did not cause any detectible effect on the quantity of vertebral bone but was shown to increase the amount of osteoid compared to mineralised bone thus reducing the quality of the bone. However the same authors state: "Practically, almost all aquaculture companies maintain a sufficient amount of vitamin K in their commercial diet". At present the vitamin K requirement for salmonids is not determined, nevertheless the recommendation for growth in salmonids is to include 10mg vitamin K Kg^{-1} dry diet, which far exceeds the mammalian requirement of 0.05-1.5mg Kg^{-1} (Krossøy *et al.* 2009). Thus while vitamin K is essential for the proper functioning of bone, deficiency syndromes would be very rare.

2.2.4 Minerals

Fish require similar minerals to terrestrial animals and imbalances or excesses can lead to disease and pathology. The numerous and varied effects of all the minerals will not be covered in depth here but focus will be paid to those pertinent to the use of animal protein sources and mineralised tissues namely; calcium, phosphorus, and iron.

Calcium and Phosphorus

Calcium is the most abundant cation found in the body of bony fish. Despite the importance of calcium in bone formation and maintenance of skeletal tissues, calcium ions are widely distributed in soft tissues and are needed for a diverse range of physiological processes; blood clotting, muscle contraction, nerve transmission, maintenance of cell membrane integrity, and enzyme activation. In the cell membrane, calcium is closely bound to phospholipid, where it controls the permeability of the membrane and thus regulates the uptake of nutrients into the cell (Lall 2002). The calcium requirement for fish is met in a large part by their ability to absorb these ions directly from the aquatic environment thus deficiency syndromes are uncommon. However it is widely accepted that high concentrations of dietary calcium and phosphorus interfere with the absorption and retention of certain trace elements including magnesium (Sugiura *et al.* 2000).

Fish have a dietary requirement for phosphorus for proper bone mineralization due to the low availability of phosphorus in the aqueous environment (NRC 1993). However excess phosphorus increases phytoplankton growth, aquatic weed growth and algal blooms that cause wide fluctuations in the dissolved oxygen levels of the surrounding water. Thus there is a clear need to establish low phosphorus diets that maintain health and growth while reducing environmental outputs (Hua and Bureau 2006). Phosphorus is stored, together with calcium, in the mineralised tissues (bone and scales) as hydroxyapatite. Scales are a major ion reserve and have been shown to account for 19-24% of the dry mass of tilapia (Garrod and Newell 1958). Phosphorus is also important as it is needed for ATP production. In terms of phosphorus sources the major problem in aquafeeds is the use of plant protein sources where phosphorus is sequestered by the presence of phytate. This is not a

problem in the use of animal proteins but the high ash levels found in meat and bone meal have been shown to be detrimental to phosphorus absorption (Bureau *et al.* 1999). Clinical signs of phosphorus deficiency include reduced growth, anorexia, skeletal deformity, reduced bone mineralisation, curved and enlarged spongy vertebrae (Lall 2002). The effect of phosphorus on the immune system of the channel catfish has also been investigated by Eya and Lovell (1998), who found that resistance to *E.ictaluri* challenge and antibody production were both negatively affected by inadequate phosphorus intake. The dietary requirement for maximum resistance to the bacterial challenge was 0.4% of the diet, and was similar to that required for maximum mass gain, while 0.5% of the diet was needed to maximise antibody production.

Iron

Iron deficiency causes microcytic anemia in several fish species (NRC 1993) however, iron deficiency is not seen as a problem in aquaculture because often the feed and water supply sufficient quantities to meet the fishes physiological needs (Lall 2000). The availability of iron does directly influence the ability of micro-organisms to proliferate and thus their ability to overwhelm the host's immune system and cause disease. Therefore, a number of studies have investigated irons effect directly and indirectly on fish with divergent results. Atlantic salmon supplemented with 400mg iron/kg to a basal diet containing 160mg/kg had no effects on serum total protein, serum antibody, haemolytic complement activity or lysozyme activity in serum, head kidney, or spleen (Andersen, Lygren, Maage and Waagbo 1998). Iron supplementation was seen to increase catalase activity in the head kidney. Channel catfish fed iron deficient diets had increased mortality due to *Edwardsiella ictaluri* infection and reduced chemotactic migration of peritoneal

macrophages (Lim and Klesius 1997; Sealey, Lim and Klesius 1997) but antibody production was not affected by dietary iron level (Sealey *et al.* 1997). Iron is of particular concern when diet formulations include blood meal due to the high availability of haem compounds to both the animal and the pathogen (Lall 2000).

2.3 Do APS affect fish health?

In order to restore confidence in the perception of animal by-products as viable alternatives to fishmeal the question of if they affect the health of fish can be seen to have two distinct parts;

- Could the feeding of animal by-products to fish result in transmissible prion like diseases?
- Do animal by-products influence the welfare of fish in aquaculture operations through altering the ability of the animal to resist disease and deformity?

Although the first question is outside the scope of this work a review of the possible effects of feeding animal by-products to fish would not be complete with a brief review of current fish prion research.

2.3.1 Prion diseases in fish

As previously mentioned prion (proteinaceous infectious agent) was the name given to the presumed agent of the transmissible spongiform encephalopathies (TSE), a group of neurodegenerative diseases which affect the central nervous system of humans and other mammals; scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt-Jakob Disease (CJD) in humans. All those prion proteins have in common the fact that they present two isoforms: a

cellular or normal conformation (PrP) which is apparently benign and a protease resistant conformation (PrP^{Sc}) that according to the "prion hypothesis" would have the capacity to transmit its abnormal conformation to PrP resulting, in an accumulation of PrP^{Sc} in neural cells and subsequently in the disease. Joly, Nguyen and Bourrat (2001) state that a fish PrP gene is probably very divergent from those characterized in mammals and that it would be unlikely to share the same pathological properties. More recently prion protein homologies have been demonstrated in zebrafish, pufferfish, and both salmon and trout (Friedland *et al.* 2009) however in all cases the sequence homology is low in fish. Ingrosso, Novoa, Valle, Cardone, Aranguren, Sbriccoli, Bevivino, Iriti, Liu and Vetrugno (2006) force fed fish scrapie infected material and concluded that the risk of transmission of BSE to humans through fish was negligible. More recent research by Valle, Iriti, Faoro, Berti and Ciappellano (2008) found that TSE prion proteins were unable to cross the intestinal barrier in trout force fed prion infected material, and conclude that TSE like diseases would be unlikely to affect freshwater fish. However the same authors found that there was an accumulation of the PrP^{sc} in the *stratum compactum* of the pyloric ceacae for 7 days following force feeding with PrP^{sc} infected material. Thus while there is still the possibility of fish representing an asymptomatic carrier of prion like diseases the point is, to a certain extent, moot as only animals fit for human consumption (category 3) are utilised in animal feeds and therefore other tissues are already in the human food chain.

2.3.2 Effects of Animal by-products on the health of farmed fish

Historically in the original salmonid diets, devised in the 1870's, animal offal played a significant role though when large proportions of raw horse spleens or livers were included in the diet characteristic bi-lateral cataracts regularly occurred

and the practice was therefore discontinued (Roberts 1989). The majority of recent investigations have examined the use of animal by-products as fishmeal replacements, with optimum inclusion levels determined for a number of animal proteins for *O.mykiss* (Bureau *et al.* 1999; Bureau *et al.* 2000; Serwata 2007) however the focus of these investigations has been the measurement of growth rather than health; thus there is scant mention of any health effects at the higher inclusion levels. The few investigations that have specifically examined if animal protein sources impact the health of fish are listed in Table 2-2.

Table 2-2: Summary of heath investigations in farmed fish using animal by-products. Unless otherwise stated N=3.

Animal Protein source (Inclusion %)[1]	**Species**	**Parameters measured**	**Recorded Effects**	**Reference**
EFM[2] (14%) PMM[3] (22%) SDH[4] (5%) SHFM[5] (17%)	*Oncorhynchus mykiss*	Serum haematocrit and haemoglobin.	Reduced haematocrit in SHFM only. No other differences	Serwata (2007)
EFM (5%) PMM (25-75%) SDH (10%)	*Sparus aurata*	Gut histology, differential leukocyte counts, liver histology, hepato-somatic index.	No significant effects.	Laporte (2007)[9]
SHFM (23%) SHFM/LPN[6] (11/22%)	*Salmo salar*	Plasma lysozyme, antiprotease, respiratory burst, total immunoglobulin, protein and glucose. Mortality after *Virbio anguillarum* challenge.	No significant effects.	Bransden, *et al* (2001)
MBM[7] (6/12%)	*Ictalurus puncatus*	Mortality after *Ewardsiella ictaluri* challenge.	No significant effects.	Li *et al* (2003)[8]

1. Inclusion as % of diet
2. Enzyme treated feathermeal
3. Poultry meat meal
4. Spray dried haemoglobin
5. Steam hydrolyzed feathermeal
6. Lupin meal
7. Meat and bone meal
8. Compared to a 48% soybean meal reference diet. This study also investigated the effect of difference protein levels (28, 32, 36%) with no differences found between experimental and reference diets.
9. N=2

The above investigations almost all show no significant deviation from reference fed fish, with only Serwata (2007) finding lower haematocrit levels in fish fed animal by-products. Nonetheless animal by-products must be validated as lacking

in negative health consequences as there are a number of possible mechanisms for sub-clinical health effects. For example the increased ash content of PMM which has been proven to reduce phosphorus availability and utilisation (Bureau *et al.* 1999; Bureau *et al.* 2000; Sugiura *et al.* 2000; Wang *et al.* 2006). However at present the available literature is too meagre to draw conclusions; while studies that have used animal by-products have seen no major differences in the health parameters assessed, the individual components of the protein sources have all been shown, in some way, to affect disease resistance and/or mineral homeostasis. In general there is still a paucity of information on the nutritional modulation of disease resistance and deformity in farmed fish due to the difficulties of conducting controlled experiments utilising ingredients rather than singular nutrients, therefore there is an obvious need to further corroborate the use of animal by-products in aquafeeds at all production levels.

2.4 Aims

The over-arching aim of this programme of work was to establish if the feeding of animal by-products impairs the health of cultured trout as a model salmonid. Due to the broad definition of health, innate immune function and bone physiology were seen as paramount. Secondary aims were to investigate the effect of diet and physical factors on bone physiology.

Chapter 3 : General Methods

3.1 Fish and husbandry

Female rainbow trout (*O.mykiss*) from Hatchlands Trout Farm and Fishery, Rattery, Devon were used in all experiments. All fish were acclimatised for a minimum of three weeks to the recirculation system at the University of Plymouth during which time the fish were fed reference diets.

Fish in all the experiments were fed twice a day with a minimum of six hours between each feeding. Temperature, pH and dissolved oxygen levels were measured once daily using a HACH HQ 40d multi probe, and pH (7.0±0.5) adjusted using sodium bicarbonate ($NaHCO_3$). Flow rates into each tank of the system were 25 L min^{-1} and remained constant throughout each trial. The entire recirculation system received daily partial water changes in order to prevent the increase of nitrate ions in the water. Ammonia, nitrite and nitrate were measured using HACH Lange GMBH vials (Ammonia 0.015-2.0 mg L^{-1}, Nitrite 0.015-0.6 mg L^{-1}, and Nitrate 5-35mg L^{-1}) and photo spectrometer (Hach Lange DR2800), and recorded weekly. For all experiments water quality parameters were kept within the following ranges Ammonia 0.02-0.1 mg L^{-1},Nitrite 002-0.5mg L^{-1} and Nitrate 15-30mg L^{-1}. The culture system had a set photoperiod of 12L:12D with light provided by fluorescent lights positioned above the culture tanks (80 Watts m^{-3}).

In order to quantify growth the fish were group weighed, following twenty four hours starvation, at appropriate time intervals (7-14 days). All fish fed a fixed body weight percentage per day received food according to compound feeding tables based either upon an assumed FCR of 1 or the feeding model of Bailey and Alanara (2006) with the models adjusted post weighing. Where appropriate experiments

that utilised feeding to apparent satiation detail the methodology used in the relevant sections.

3.2 Diets

3.2.1 Specification of ingredients

A description of the major ingredients utilised throughout the nutritional investigations is listed below. All the processed animal proteins were obtained from UK rendering plants (via Prosper de Mulder, Doncaster UK). All processed animal proteins provided are classified as category 3 as defined in the ABPR 1774/2002 (Chapter 2). A brief description of the processing techniques used in the manufacture of these ingredients is also included as there is evidence that these treatments affect the final product quality (Allan, Williams, Smith and Barlow 1999; Gatlin, Barrows, Brown, Dabrowski, Gaylord, Hardy, Herman, Hu, Krogdahl, Nelson, Overturf, Rust, Sealey, Skonberg, Souza, Stone, Wilson and Wurtele 2007).

***Shetland '70' Fishmeal (United Fish Products, Hull, UK)*:** A free flowing light brown coloured meal produced from whole fish and fish trimmings which may include salmonid species. The raw chilled fish and fish trimmings are cooked (20 minutes, >83°C), dried (1-2 hours, >80°C) and cooled with a typical declared composition of: Protein (69%), Oil (8.0%), Ash (16%) and Moisture (8%).

***Low temperature (LT) Icelandic Fishmeal*:** The production of LT meal implies a reduced drying temperature (<90°C). The result of this lower drying temperature is reduced protein damage and lipid peroxidation.

Plate 4-1: Recirculation system E at the University of Plymouth Fish Nutrition research unit. Note the fibreglass tanks, inlets and lights positioned above all the aquaria to ensure uniformity.

Plate 4-2: Expanded shot of a selected aquarium; note the inlet (far left) stand pipe for water removal (mid shot, background) and net covering to prevent the fish jumping. The water inlet flow has been reduced to aid photography.

The standard specification for an LT grade fishmeal is minimum 72% crude protein and an oil content of 10% and 9% ash.

Steam hydrolysed feathermeal (SHF): Poultry feathers are steam hydrolysed at up to 550 kpa pressure for approximately 30 minutes in a continuous hydrolyser. The hydrolysed feathers are then dried in an indirect steam heated drier (Rota-disc drier) to ~5% moisture, cooled, and milled.

***Poultry meat meal (PMM)*:** mixed species poultry material is reduced in size by mincing to <30mm, introduced into a continuous process dryer (Rota-disc) that evaporates water in the presence of natural fat levels and sterilises the components. The residence time is approximately 90 minutes and the maximum temperature reached is 125°C. On leaving the process, dried components are separated into a protein fraction, and excess fat is removed by an expeller press. The protein fraction (PMM) is cooled, milled and treated with an antioxidant.

***Spray dried haemoglobin (SDH/BM)*:** the raw material is obtained from whole porcine blood. Blood is chilled and separated into plasma and red blood cells by centrifugation. The red blood cell fraction (Haemoglobin) is spray dried to produce a dry (< 5% moisture) haemoglobin powder.

***Standard hydrolysed feathermeal*:** Mixed species poultry feathers are hydrolysed for approximately 30 minutes. The hydrolysed feathers are dried in an indirect steam heated drier (Rota-disc drier) to ~5% moisture, cooled, and milled.

***Soya meal*:** The soya meal used throughout the experiments was a defatted soya with a 44% crude protein content. This was obtained from ISCA Kinver and Kinver, Crediton Devon.

***Vitamin/ Mineral premix*:** The vitamin/ mineral premix used in all the experiments (PnP VitMin) was supplied by Premier Nutrition Products Limited (The Levels, Rugeley, Staffs. WS151RD) and included at manufacturer recommended levels (2%). The premix had the following composition:

Calcium 12.090%, Ash 78.708%, Sodium 8.858%, Vitamin A 1.00 MIu Kg^{-1}, Vitamin D_3 0.1 MIu Kg^{-1}, Vitamin E (α-tocopherol) 7000mg Kg^{-1}, Copper 250.00mg Kg^{-1}, Magnesium 1.560%, Phosphorus 0.519%.

***Barax Antioxidant*:** A commercial anti-oxidant included at manufacturer recommended levels in diets where rapid oxidising of lipid, causing rancidity, was found to be a problem e.g. diets containing blood meal at levels greater than 2% were found to go rancid within 5 days of manufacture when kept at 4°C.

The composition of the diet ingredients is shown in Table 3-1.

3.2.2 Formulation

All experimental diets were formulated using Feedsoft © (Feedsoft Corporation, USA) linear least cost formulation software.

3.2.2 Preparation

All diets were cold pressed without steam in a PTM 6 (Plymouth Tropical Marines, Plymouth, Devon, UK). All the ingredients were ground and sieved to less than $1mm^2$ before being thoroughly mixed. The oil and water fractions were added and the diet extruded through the appropriate die (2-4mm) dependent upon fish size. The resultant pellets were dried in a temperature controlled cabinet at 45°C until total moisture <10%. All diets were then stored at 4°C until use. All diet

ingredients and diets were analysed for proximate composition to ensure accurate formulation and confirm subsequent composition.

Table 3-1: Proximate and mineral composition of diet ingredients: All values represent the mean (N=3), no standard deviations are shown as only the mean value was used to formulate the diets.

	Wheat meal[1]	Soya[2]	Maize gluten[3]	Blood Meal[4]	Feather meal[4]	Poultry meat meal[4]	Fishmeal[5]
Dry matter (%)	87.20	91.08	92.10	95.95	94.29	97.22	92.24
Crude Protein (%DM)	10.43	46.65	63.26	94.03	81.43	65.39	69.49
Lipid (%)	1.36	1.56	1.56	0.09	8.75	11.56	7.28
Ash (%)	2.04	5.09	1.03	2.96	2.35	12.76	12.92
Calcium (%)	0.23	0.28	0.00	0.01	0.52	3.04	2.95
Potassium (%)	0.39	1.82	0.07	1.05	0.15	0.72	1.00
Magnesium (%)	0.09	0.25	0.02	0.03	0.04	0.13	0.22
Sodium (%)	-	-	-	0.34	0.07	0.45	1.38
Phosphorus (%)	0.27	0.57	0.33	0.35	0.36	2.13	2.03
Sulphur (%)	0.12	0.33	0.71	0.41	1.50	0.68	0.80
Manganese (mg kg^{-1})	61.87	52.44	2.09	0.37	8.19	13.00	4.08
Zinc (mg kg^{-1})	60.32	52.21	11.38	18.40	83.75	89.57	49.68
Copper (mg kg^{-1})	8.87	20.82	5.63	2.10	4.24	8.81	2.81
Iron (mg kg^{-1})	59.54	75.86	46.43	2296.30	125.03	179.48	178.27
Crude Protein ADC (%)[6]	76	83	87	100	60	78	90
Phosphorus ADC (%)[7]	58	36	44	81	77	81	52

[1]Kinver Feeds, Crediton, Devon.
[2]Hi-Pro soya, ISCA KInver, Crediton, Devon, UK.
[3]Roquette UK, 9-11 Swallow Road, Weldon North Industrial Estate, Corby, Northamptonshire NN17 5JX.
[4]Animal proteins supplied by Prosper De Mulder, Greenleigh, Kelmarsh Rd, Clipston Mkt. Harborough, LE169RX, UK.
[5]Shetland fishmeal, United Fish Products, Hull, UK.
[6]Apparent digestibility coefficients from Serwata (2007) .
[7]Apparent digestibility coefficients from Snellgrove (2003).

3.3 Growth and performance measurements

A list of the equations used to generate growth and performance data are listed below.

Specific growth rate (SGR):

$$SGR\ (\%\ Bm\ day^{-1}) = \frac{(\ln(Bm_f) - \ln(Bm_i))}{t} \times\ 100$$

Equation 3-1: Specific growth rate equation; Bm_i=initial body mass (g), Bm_f = final body mass (g) t= time (days).

Thermal growth co-efficient (TGC):

$$TGC = \left|\left(\sqrt[3]{\mathrm{Bm}_t} - \sqrt[3]{\mathrm{Bm}_0}\right)/(Txt)\right| \times 1000$$

Equation 3-2: Thermal growth co-efficient; Bm_t= final mass, Bm_0= initial mass, T = temperature (^{0}C), t= time (days).

Fultons condition factor (K):

$$K = \frac{Bm}{\mathrm{SL}^3}$$

Equation 3-3: Fultons condition factor (K). Bm= body mass (g). SL = standard length (cms).

Feed conversion ratio (FCR):

$$FCR = feed\ intake\ (g)/wet\ mass\ gain\ (g)$$

Equation 3-4: Feed conversion ratio (FCR).

Food conversion efficiency (FCE):

$$FCE = (wet\ mass\ gain/dry\ feed\ fed)\ \times 100$$

Equation 3-5: Food conversion efficiency (FCE).

Hepato-somatic index (HSI):

$$HSI = Mass\ of\ liver\ (g)/Whole\ body\ mass\ (g)$$

Equation 3-6: Hepato-somatic index (HSI).

Apparent net protein utilisation (ANPU):

$$ANPU = (carcass\ protein\ (g)/total\ protein\ fed\ (g)) \times 100$$

Equation 3-7: Apparent net protein utilisation (APNU)

Protein efficiency ratio

$$PER = mass\ gain\ (g)/total\ protein\ fed\ (g)$$

Equation 3-8: Protein efficiency ratio (PER).

Apparent net mineral utilisation (ANMU)

$$\text{ANMU} = \frac{(\text{Bm}_\text{f} \times \text{Conc}_\text{min}) - (\text{Bm}_0 \times \text{Conc}_\text{min})}{\text{Total feed intake} \times \text{Conc}_{\text{min}}} \times 100$$

Equation 3-9: Apparent net mineral utilisation equation. ($Conc_{min}$ = minimum concentration of selected mineral, Bm_0 = initial body mass, Bm_f= final body mass).

3.4 Analytical techniques

3.4.1 Proximate analysis

For all experiments the proximate analysis of whole carcass and diets were performed at the University of Plymouth by AOAC (1990) standard analysis; the methods of which are detailed below.

Moisture

All samples were dried at 105°C, in a fan assisted exhaust Pickstone E70F Oven, until a constant mass was achieved. Moisture content was calculated from the difference between wet and dry mass of the sample.

Protein

The protein content was determined using the Kjeldahl method. 1 gram samples were added to with 10mL of concentrated sulphuric acid (H_2SO_4) in the presence of titanium dioxide (TiO_2) and copper sulphate ($CuSO_4$) catalysts and heated at 265°C for 45 minutes and 380°C for a further 60 minutes. The resulting ammonia was distilled and titrated with 0.1M hydrochloric acid (HCl). The protein content was then determined using the following equation:

$$\%\ Crude\ Protein = \frac{(Sample\ titre\ (ml) - Blank\ titre\ (ml)) \times 0.1 \times 14 \times 6.25}{Sample\ \text{mass}\ (mg)} \times 100$$

Equation 3-10: % crude protein calculation where: 0.1= molarity of acid, 14= Relative molecular mass of nitrogen, 6.25 = Animal protein: Nitrogen constant.

It should be noted that following current convention the nitrogen constant used for all calculations is 6.25, rather than the technically correct 5.71 for vegetable protein sources.

Lipid

Lipid composition in both feed, and carcass were determined by the Soxtec method using a Soxtec System HT 1043, (Foss, Warrington UK). The samples were finely ground, and the lipid fraction separated by refluxing petroleum ether through porous thimbles containing 1-3g of sample. The increase in the mass of the extraction cups equates to the lipid fraction of the sample.

Ash

This was determined by combusting the sample for 12 hours in a Carbolite GLM 11/7 muffle furnace (Carbolite Furnaces Ltd., Bamford, Sheffield, U.K.) heated to 550°C. The mass of the remaining residue is the total ash content of the sample.

Gross Energy (Adiabatic bomb calorimeter)

The gross energy content of the diets was determined using a Parr 1356 adiabatic bomb calorimeter (Scientific and Medical products LTD, Cheadle, Cheshire, UK). Approximately 1g of sample was combusted in the presence of oxygen in a pressurised bomb. The increase in temperature of the water surrounding the bomb allowed the energy content of the feed to be calculated (MJ/kg).

Amino acid determination

All amino acid determination was performed at Sciantec Analytical, Cawood, North Yorkshire, UK, using HPLC. As replicates were pooled together, a single value per treatment was obtained and used.

3.4.2 Mineral analysis of feedstuffs and tissues

In order to quantify the mineral content of feed ingredients and selected tissues the samples were dehydrated with body tissues being freeze dried (Edwards Supermodulyo Model 12K, West Sussex, UK: vacuum chamber evacuated to 0.8x 10^{-3}mbar, 48hrs, condensation trap @ -50^{o}C) while feed ingredients/complete diets oven dried @ 110^{o}C until constant mass. Following dehydrating all samples were ground to a uniform powder before being digested in 2N nitric acid, diluted and analysed using inductively coupled plasma optical emission spectrophotometer (ICP-OES).

Digestion

A sample of 100-250mg was placed into a nitric acid (10%) cleaned Kjeldahl boiling tube. 10mL concentrated nitric acid was then added to the tube and the solution heated to the following protocol in a Kjeldtherm digestion block: 60^{o}C for

one hour, 90°C for one hour, 110°C for 30 minutes, 135-140°C for 4 hours. Digestion was confirmed visually by the presence of a clear pale green solution. The solutions were then allowed to cool and diluted to 50mLs with deionised water.

ICP-OES

The mineral composition of the diet ingredients was analysed using a Varian 725-ES Inductively coupled plasma optical emission spectrophotometer (ICP-OES) after sample digestion. ICP-OES was utilised due to the lower detection limits compared to the flame atomic absorption spectrophotometer and the greater range. The following settings were used on the ICP as advised by M.Foulkes (personal communication, March 16th 2007) for optimum mineral determination:

Power (KW)	1.4
Plasma Flow (L min^{-1})	15.0
Auxiliary Flow (L min^{-1})	1.50
Nebulizer Flow (L min^{-1})	0.68
Viewing height (mm)	8.00
Replicate read time (s)	2.00

Where appropriate yttrium oxide Yt_2O_3 (1mg L^{-1}) was included as an internal standard and all samples were standardised to this, therefore exonerating dilution errors. Standard solutions containing 10/5/1/0.5/0 mg L^{-1} of calcium, phosphorus, potassium, magnesium, sodium, sulphur and 1/0.5/0.1/0.05/0 mg L^{-1} of copper, iron, manganese and zinc were used to create the standard curves. Iridium was used as standard in the faecal samples from digestibility studies due to the presence of yttrium for digestibility coefficient calculations.

3.4.3 Serum Protein concentration

Serum protein concentration was determined using a commercially available test kit (Bio-Rad, Hemel Hempstead, UK) which uses a modification of the Bradford method (Bradford 1976) with bovine serum albumen (BSA) used to construct a standard curve.

3.5 Bone histomorphometry

Resin embedding

Initially the specimens were cleaned of soft tissue placed into a labelled histo-cassette where they were fixed in IMS for a minimum of 48 hours before being de-fatted in chloroform for a further 24 hours. After washing in IMS (24 hours) the samples were then incubated with LR Resin (Fluka) for a minimum of 48 hours, with shaking, prior to a second LR resin incubation for 48 hours. The infiltrated samples were then orientated onto resin stubs, covered with LR resin and left to polymerise at 57°C for 24-48 hours. The calcified samples were sectioned (9μm thick) using a Leica SM2500 microtome at the Cellular and Molecular Medicine Unit at St George's hospital London. The mounted sections were then visualised at the University of Plymouth using a Leica DMR fluorescent microscope coupled with an imaging system (JVC TK-C136OB video camera, Image Pro-Plus software). The digital photographs were then analysed using Image J and the following parameters quantitatively assessed according to Parfitt *et al* (1988).

$$Mineralising\ perimeter(B\,Md.Pm/B.Pm,) = \frac{((double\ labelled\ surface + (0.5 \times single\ labelled\ surface))}{Bone\ surface}$$

Equation 3-11: Mineralising perimeter per bone perimeter (%).

$$Mineral\ apposititon\ rate\ (MAR) = \frac{Interlabel\ thickness}{Interlabel\ time}$$

Equation 3-12: Mineral apposition rate (MAR, μm day^{-1}) calculation

$$Bone\ formation\ rate\ (BFR) = MAR\ \times (\frac{MS}{Bone\ surface})$$

Equation 3-13: Bone formation rate (BFR, μm^2 day^{-1}).

Paraffin embedding

Histological samples were dissected and stored in 10% neutral-buffered formalin until processing via standard histological protocols into wax blocks. The wax blocks were cut into 8μm sections using a Leica microtome (Reichert Jung) and mounted on slides prior to staining. Where appropriate vertebral samples were decalcified in 10% EDTA [w/v] (ethylenediaminetetraacetic acid: Sigma Aldrich, UK) with the decalcification time dependant on the size of sample. Decalcification was deemed complete when the sections could be easily cut with a scalpel blade.

Staining protocol

Prior to staining the paraffin embedded samples were sectioned using a Leica microtome (Reichert Yung) at 6-8 microns, dried, cleared with xylene, and hydrated though a series of graded alcohols (100,90,70%) before being rinsed in distilled water for 5 minutes. The staining protocol for the sections prepared in this way is described below.

Mallory's Trichrome stain

The slides were stained with Mallory's trichrome stain following the protocol of Handy, Sims, Giles, Campbell and Musonda (1999). This stain results in

erythrocytes staining orange, nuclei stain red, collagen stains blue and muscle staining red.

3.5.1 Connectivity of trabecular structures

The mechanical integrity of cancellous bone is determined by its volume and microstructure, the composition of matrix and mineral and the balance between fatigue damage and repair. The microstructure can be visualised as a network of plates and bars with variable orientation (Compston 1994). Qualitatively connectivity describes the presence of multiple connections between points, so that some points are connected to each other by more than one pathway. Quantitatively it is an index of the multiplicity of connections and can be defined as the maximum number of connections which can be broken before the network is interrupted. Classically the microstructure of bone was assessed using two dimensional approaches however bone and therefore connectivity is obviously a three dimensional structure. It should be noted that true connectivity of cancellous bone can only be determined via three dimensional approaches therefore knowledge of the mechanical properties must be sought in addition to the two dimensional connectivity.

From the binary image a histogram was used to quantify the ratio of white:black pixels and thus measure trabecular bone area (B.Ar) and cancellous tissue area (T.Ar) as well as bone perimeter (B.Pm).

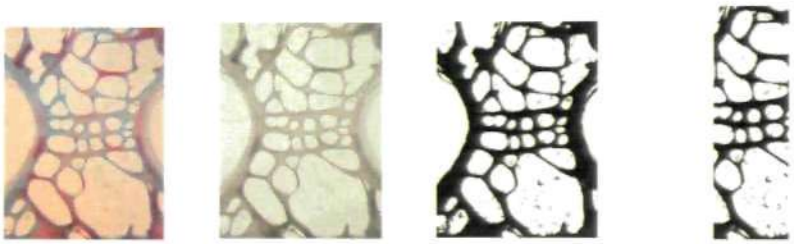

Figure 3-1(A-D): Image processing in Image J for quantitative analysis. A= standard image, B= 8-bit greyscale image, C= B/W picture produced using the threshold function, D= cropped picture used for histogram/ connectivity analysis. Note due to the decreasing connectivity of the trabeculae at the innermost layers of the vertebrae, connectivity was assessed using sections where a minimum of 250μm between cortical surfaces were used.

From these measurements the following indices were derived:

$$\textbf{Trabecular thickness (Tb.Th)} = 4/\pi \times B.Ar/2/B.Pm$$

Equation 3-14: Trabecular thickness (Tb.Th) where B.Ar= bone area, B.Pm = bone perimeter

$$\textbf{Trabecular number (Tb.N)} = Tb.Ar \times 10/Tb.Th$$

Equation 3-15: Trabecular separation (Tb.N) where Tb.Ar = cancellous tissue area, Tb.Th = trabecular thickness.

$$\textbf{Trabecular separation (Tb.Sp)} = 1000/Tb.N - Tb.Th$$

Equation 3-16: Trabecular Separation (Tb.Sp) where Tb.N = trabecular number and Tb.Th is trabecular thickness.

A further method used to evaluate the connectivity of cancellous bone is the Euler Poincare's number, which is a topological property based on the number of holes and the number of connected components in an object and is defined by Equation 3-17.

$$Euler\ Poincare\ number\ (E) = n - m$$

Equation 3-17: Euler Poincare number. Where n = the total number of (disconnected) trabecular profiles and m= the number of marrow cavities.

The more connected the trabecular network, the lower the value of E, with negative values in highly connected systems.

3.5.2 Image J

The slide images were captured using a Nikon Coolpix 990 digital camera attached to either an Olympus SZH stereomicroscope or a Leica DMIR8 inverted microscope. To obtain scaling information a photograph of a 1mm graticule (Graticules Ltd. Trowbridge, UK.) was taken after any changes in focal length were performed. Images were processed using Image J software (Image processing and analysis in Java: available Online: http://rsb.info.nih.gov/ij/ [accessed 27/07/08]).

3.5.3 Intra-observer error

Due to the subjective nature of the histomorphometric measurements the intra-observer error was quantified by calculating the percentage technical error of measurement as used by Ulijaszek and Kerr (1999).

$$A: Technical\ error\ of\ measurement\ (TEM) = \sqrt{\frac{\sum D^2}{2N}}$$

$$B: \%TEM = (TEM/Mean) * 100$$

Equation 3-18A,B: Technical error of measurement (TEM) where D= the difference between measurements made on a given object on two separate occasions, and N = the total number of measurements made on the two occasions.

The technical error of measurement of the author was calculated to be 9.4 %.

3.6 Mechanical properties of vertebrae

The frozen vertebrae were defrosted and allowed to equilibrate to room temperature, the diameter and height recorded and then the sample compressed, in the dorso-caudal plane, using an Instron universal testing machine with a 500N load cell with parallel stainless steel plates. The Young's modulus (N/mm^2), compressive extension (mm), and the load to initial peak (Kg F) (that is where a

10% plastic deformation occurred) were automatically determined and recorded using the Bluehill 2 (Instron, High Wycombe, Bucks, UK) materials testing software. A 5N preload was used on all vertebrae to negate the effects of soft tissue on the plane of compression. Compressive force application was performed at a rate of 2mm min^{-1} and was terminated after the initial elastic deformation had reached a plateau.

3.7 Bone Ash Content

Three vertebrae of the trunk-caudal area of the spine as defined by Kacem and Meunier (2003) were pooled, dried for 12 h at 110°C, and weighed (W_{dry}) to the nearest mg prior to incineration for 12 h at 550°C. The ashes were then weighed (W_{ash}) to the nearest milligram. The vertebral bone mineralization (BM, %) was calculated from the following equation:

$$\text{Bone mineral content (\%)} = (W_{ash} / W_{dry}) \times 100$$

Equation 3-19: Bone mineral content calculation. W_{ash} = wight of ash after incineration. W_{dry}= weight of dried sample.

3.8 Statistics

In all the experiments, unless otherwise stated, data is shown as mean ± standard deviation or median ± 25-75 percentile. The unit of replication for all measurements including growth (plus all subsequent expressions; K factor, HSI) and feed intake was considered to be the tank (group) of fish. All data was analysed using Sigmaplot 11.0 (Systat software Inc. 2008). Percentage data was arcsine transformed before subsequent analysis. The statistical tests used to describe the data are listed next to the relevant data figure.

Chapter 4 : Effect of fishmeal replacement by selected animal by-products in trout feeds on mineralised tissues, immuno-competence and stress.

4.1 Introduction

As previously stated the immune system functions to recognise and eliminate pathogens. The current knowledge of the teleost immune system is summarised in Table 4-1. As can be seen from this diagram, similar to mammals, the recognition of antigens by phagocytes (macrophages and neutrophils in *O.mykiss*) is one of the fundamental steps in the elimination of pathogens. Additionally macrophages act as accessory cells by presenting antigens to lymphocytes in order to facilitate the production of specific antibody. However a major difference between mammalian and teleost immune systems is that in teleosts the innate immune system is considered the most important due to its greater thermal independence (Press and Evensen 1999).

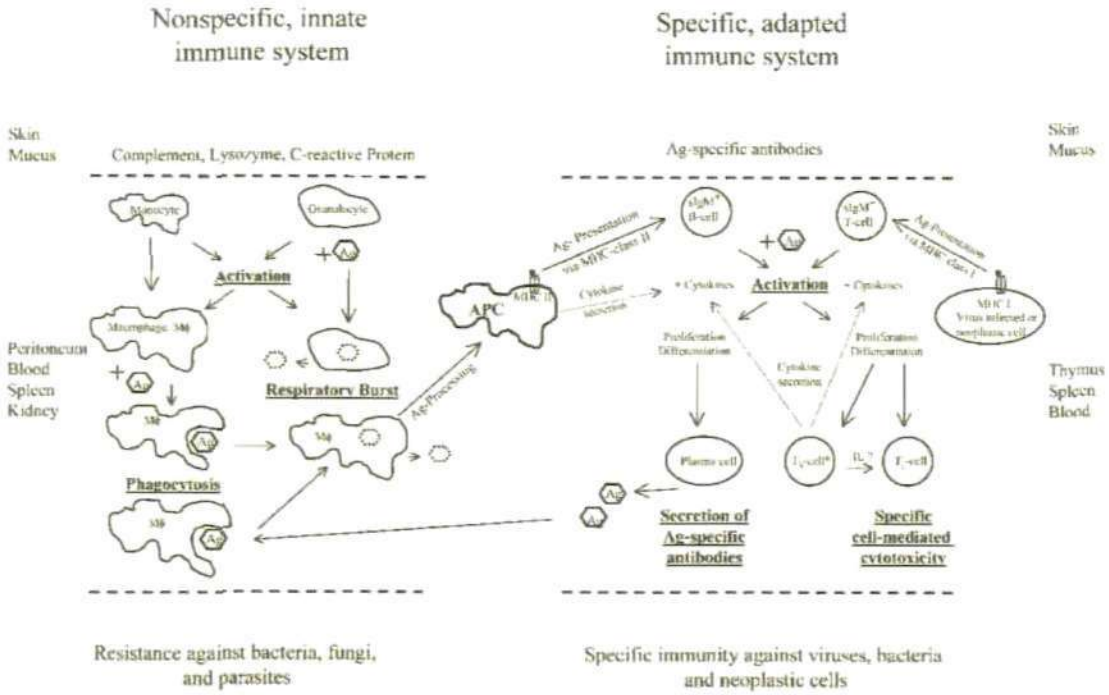

Figure 4-1: Schematic overview of known immune functions of bony fish. Dotted arrows indicate functions which are predicted from proliferation assays only. Abbreviations: Ag, antigen; APC, antigen presenting cell; MHC, major histocompatibility complex; M, macrophage; sIgM, surface immunoglobulin M; Th-cell, helper T lymphocytes; Tc-cell, cytotoxic T-lymphocytes (*Both are predicted from functional assays only, not shown as specific cell population). From (Kollner, Wasserrab, Kotterba and Fischer 2002).

The intensive nature of the modern aquaculture places the animal in inherently stressful situations. Plytycz and Seljelid (2002) define stress as "... a state of threatened homeostasis or disharmony and is counteracted by a complex repertoire of physiologic and behavioural adaptive responses that establish homeostasis." Physiological stress can be induced by a great number of factors including water quality, pollution, stocking density, handling, breeding cycles, and transport.

The effects of stressors in teleost fish have been well defined (Barton and Iwama 1991) with the stress response differentiated into acute and chronic patterns. The effects of stress, in turn, are divided in three categories: primary, secondary and tertiary (Wendelaar Bonga 1997). One of the tertiary effects is the suppression of the immune response. According to Harris and Bird (2000), the immunomodulatory effects of stress and corticosteroids in mammals are well documented and comparable effects have been observed in fish. Cortisol administration to fish has been shown to have a number of effects including, a reduction in the number of circulating T- and B-like lymphocytes, increased phagocytosis, reduced alternative complement and serum lysozyme activity (Pickering and Pottinger 1989a; Mock and Peters 1990; Pulsford, Lemaire-Gony, Tomlinson, Collingwood and Glynn 1994; Espelid, Lokken, Steiro and Bogwald 1996; Kollner *et al.* 2002).

In humans it is generally accepted that malnourishment impairs immune function (Grimble 2007). Similarly in fish the influence that dietary factors may have on disease outbreaks has also been recognised (Blazer 1992). For example Pacific salmon (*Oncorhynchus* spp.) fed dry diets with corn gluten increased mortalities from bacterial kidney disease while substituting cottonseed meal conferred protection (Wood 1968).

Deformities are an increasing problem for aquaculture and impact both profitability and fish welfare (Branson and Turnbull 2007). Severe deformities can affect swimming ability, competition for food and ultimately growth (Hansen, Fjelldal, Yurtseva and Berg 2010) with imbalanced mineral nutrition cited as one of the possible causes for this (Lall and Lewis-McCrea 2007). Plant protein source are known to contain a significant proportion of phytate phosphorus that is indigestible to fish, while animal proteins have a significant ash content that can impair phosphorus utilisation.

The aim of this experiment was to investigate if the feeding of animal by-product proteins, compared to fishmeal and soya reference diets, was detrimental to the health of juvenile trout. More specifically these aims were:

Hypothesis 1: The feeding of alternative proteins decreases disease resistance through a disruption of innate immune responses.

Hypothesis 2: Feeding of alternative proteins may decrease P availability and therefore negatively affect bone formation and mineral apposition in the skeleton leading to weaker bones that are unable to resist the forces applied to them.

Hypothesis 3: Feeding alternative proteins reduces the ability juvenile trout to mount an effective immune response following normal husbandry stressors.

4.2 Methods

4.2.1 Fish and husbandry

Six hundred juvenile (*O.mykiss*) with a mean initial body weight of 31.12±1.68g (mean ± standard deviation) were randomly stocked into 20 replicate 130 L fibreglass tanks therefore resulting in 4 replicate tanks for each experimental

treatment (Chapter 3). During the acclimation period of three weeks, the fish were fed 2% BM day^{-1} of the fishmeal reference diet after which the fish were then fed 2% BM day^{-1} one of the five experimental diets according to a compound feeding chart with an assumed FCR of 1. The fish were weighed every two weeks after 24 hours starvation, and feeding rates were adjusted accordingly. At the termination of the growth trial all sampled fish were individually weighed and measured for determination of the Fulton's condition factor (K) (Chapter 3) before the liver was dissected for hepato-somatic index determination. After an initial sampling period had concluded the experimental animal where then subject to stress, two stressors were used throughout the experimental periods: low water and confinement stress. Both of these have been shown to have a negative effect on the immune response of fish (Tort, Balasch and MacKenzie 2004) and in order that the animals not become accustomed to either of the stressors the application of each was given on alternate days. The exact methodology for both is given below.

Low water stressor protocol

The depth of the water in the experimental tank was lowered to 5cm therefore exposing the dorsal fin of the fish while maintaining the same flow rate of incoming water. This low water treatment was applied for 2 hours per stressor replicate.

Confinement/Hypoxia stressor protocol

The animals were confined within a hand net (50x50x8cm) and the hand net removed from the water for 30 seconds every minute for five cycles (5 minutes) after which the animals were released.

4.2.2 Diet formulation

Five diet formulations were used to condition the fish. These consisted of a fishmeal reference diet (100% of crude protein from fishmeal), a 50% fishmeal replacement with either poultry meat meal or soya, or two 'blended diets' (50% fishmeal, 45-48% poultry meat meal and either 5% enzyme treated feather meal or 2% spray dried hemoglobin) were formulated using FeedSoft™ with the restrictions used representing the NRC (1993) guidelines for the appropriate nutrient class. A 40% digestible protein level was used so that all amino acids would be available in excess of NRC requirement. Apparent digestibility coefficients for rainbow trout from Serwata (2007) and Snellgrove (2003) were used for digestible protein and phosphorus calculations.

All diets were stored at 4°C until use. Proximate analysis on the resultant diets confirmed correct formulation and manufacture (Table 4-1). Table 4-2 shows the mineral content of the experimental diets.

Table 4-1: Specification of experimental diets including diet proximate analysis.

Ingredients g kg^{-1}	Poultry meat meal/ Blood meal (BM)	Poultry meat meal/ Feathermeal (FE)	Fishmeal (FM)	Poultry meat meal (PMM)	Soya (S)
Fishmeal[1]	300.00	300.00	544.50	300.00	416.98
Soya [2]	-	-	-	-	321.29
Poultry meat meal[3]	277.35	267.09	-	315.85	-
Steam hydrolyzed feathermeal[3]	-	50.00	-	-	-
Spray dried hemoglobin[3] (blood meal)	20.00	-	-	-	-
Maize gluten[4]	80.00	80.00	80.00	80.00	40.00
Fish Oil[5]	122.47	122.27	136.10	118.15	143.33
Wheat meal	174.68	155.64	214.30	162.52	50.00
Vitamin/Mineral Premix[6]	20.00	20.00	20.00	20.00	20.00
Carboxymethycellulose[7]	5.00	5.00	5.00	5.00	5.00
Di-calcium phosphorus[8]	-	-	-	-	3.46
Barax anti-oxidant[9]	0.5	-	-	-	-
Proximate analysis (%) (N=3) Values are shown as mean± standard deviation.					
Moisture	**4.99**±0.98	**5.71**±0.67	**4.34**±0.64	**4.09**±0.80	**3.78**±0.44
Protein	**47.04**±0.93	**47.14**±1.53	**45.94**±0.45	**47.35**±0.71	**46.98**±0.57
Lipid	**18.20**±0.52	**19.56**±0.21	**19.96**±0.65	**19.65**±0.64	**18.85**±0.36
Ash	**9.59**±0.41	**9.52**±0.33	**9.13**±0.78	**10.02**±0.71	**8.97**±0.53
Energy (MJ Kg^{-1})[10]	**21.83**±0.00	**22.09**±0.04	**21.41**±0.12	**22.03**±0.02	**22.28**±0.07

1. Shetland '70' fishmeal. United Fish Products, Hull, UK.
2. Hi-Pro Soya (49%) ISCA Kinver and Kinver, Crediton, Devon, UK.
3. All animal by-product meals supplied by Prosper De Mulder, Greenlleigh, Kelmarsh Rd, Clipston Mkt. Harborough, LE169RX, UK.
4. Roquette UK, 9-11 Swallow Road, Weldon North Industrial Estate, Corby, Northamptonshire NN17 5JX.
5. Epanoil, high iodine fish oil. Seven Seas Limited, Hedon Road, Hull, HU9 5NJ.
6. PnP VitMin Premix: Calcium 12.090%, Ash 78.708%, Sodium 8.858%, Vitamin A 1.00 Mlu Kg^{-1}, Vitamin D_3 0.1 Mlu KG^{-1}, Vitamin E (α-tocopherol) 7000mg Kg^{-1}, Copper 250.00mg Kg^{-1}, Magnesium 1.560%, phosphorus 0.519%. Premier Nutrition Products Limited, The Levels, Rugeley, Staffs. WS151RD.
7. Carboxymethylcellulose, Sigma Chemicals UK.
8. Sigma Chemicals UK
9. Barax liquid anti-oxidant. Kemin Industries, UK.
10. (N=2)

Table 4-2: Macro- and micro-mineral content of diets. Values are mean ± standard deviation (N=3).

		BM	FE	FM	PMM	S
Macro minerals (%)	Calcium	**2.08** ±0.12	**2.08** ±0.11	**1.89** ±0.10	**2.21** ±0.10	**1.66** ±0.11
	Potassium	**0.59** ±0.00	**0.56** ±0.00	**0.63** ±0.00	**0.59** ±0.01	**1.03** ±0.03
	Magnesium	**0.15** ±0.00	**0.15** ±0.00	**0.17** ±0.00	**0.15** ±0.00	**0.20** ±0.00
	Sodium	**0.72** ±0.03	**0.71** ±0.03	**0.93** ±0.04	**0.73** ±0.04	**0.75** ±0.03
	Phosphorus	**1.33** ±0.19	**1.32** ±0.12	**1.20** ±0.07	**1.42** ±0.09	**1.14** ±0.09
	Sulphur	**0.51** ±0.01	**0.57** ±0.01	**0.51** ±0.02	**0.53** ±0.01	**0.47** ±0.01
Micro minerals (mg kg^{-1})	Manganese	**15.62** ±1.02	**14.72** ±1.07	**15.64** ±0.98	**15.52** ±1.01	**21.72** ±1.65
	Zinc	**52.04** ±2.96	**53.77** ±2.03	**40.88** ±2.89	**54.63** ±3.02	**40.96** ±2.53
	Copper	**10.39** ±1.11	**10.24** ±0.98	**8.88** ±0.92	**10.54** ±0.92	**13.52** ±1.01
	Iron	**163.30** ±10.79	**120.65** ±6.69	**113.54** ±6.83	**123.73** ±7.30	**103.53** ±7.43

4.2.3 Sampling

At the termination of the experiment three fish per tank were euthanized by destruction of the brain and their proximate composition determined by AOAC (1990) methods (see Chapter 3).

Haematology

A total of 6 fish per tank were anesthetized with tricane sulphonate (MS222: 60mgL^{-1}) and 1mL of blood was removed by caudal venipuncture. The following analyses were then performed.

Glucose

Serum 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].

Haematocrit

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.

Lysozyme

Serum lysozyme activity was determined using the turbidometric method of Ellis (1990). Serum (50µl) was added to 950 µl of 0.2 mg mL^{-1} of *Micrococcus lysodeikticus* in 0.05 M Na_2HPO^4 (pH 6.2). One unit of lysozyme activity being defined as creating a decrease in absorbance of 0.001 absorbance units per min.

Complement

Haemolytic activity driven by the alternative complement pathway was measured using rabbit red blood cells (RBC) as target cells in a Gelatin Veronal Buffer (GVB) (0.01M EGTA, 0.01M MgCl, pH 7.0) as described by Tort et al. (1996). Dilutions of trout serum were made in GVB in 1.7 mL micro centrifuge tubes to give the following concentrations in a total volume of 100 µl : 100%, 80%, 60%, 40%, 20%,

10%, (v/v). Rabbit RBC in Alsever's solution (TCS Biosciences Ltd, Buckingham, UK) were washed three times in GVB and the concentration adjusted to 2 x 10^8 cells mL^{-1}. To each serum dilution tube, 25 µl of the washed rabbit RBC suspension was added and tubes incubated at 20°C for 90 min with manual shaking. After incubation 1 mL ice cold GVB was added to the tubes to stop the reaction. Tubes were then centrifuged at 1600 x g for 5 minutes. The supernatant (200µl) from each tube was transferred to a 96-well plate. The extent of haemolysis was determined by measuring the optical density of the supernatant at 414 nm using an Optimax microplate reader with the Kinetic software SOFTmax® (Molecular Devices, USA). Complete haemolysis (100%) was determined by adding 25 µl of the washed rabbit RBC suspension to 1100 µl of distilled water. Zero haemolysis was determined by adding 25 µl of the washed rabbit RBC suspension 1100 µl GVB buffer. The alternative complement pathway haemolytic activity was reported as the reciprocal of the serum dilution causing 50% lysis of rabbit RBC (ACH50).

Differential leukocyte counts

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 (6%) [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 Yasutake (1983) and the total number of each leukocyte class was expressed as a percentage of the total leukocyte population.

Isolation of trout leukocytes

Three animals per tank were euthanized with an overdose of MS222 (150mg L^{-1} Pharmaq Ltd) and exsanguinated by caudal veinipuncture. Under sterile conditions the head kidney was teased through a 100μm cell separator (Becton Dickinson, UK) moistened with L-15+10% FBS. The resultant suspension was then layered onto a discontinuous Percoll gradient (51%/34%) and centrifuged at 400g for 45 minutes. The leukocyte layer was removed, washed twice in an excess of complete media (L-15+ 10%FBS/TP+ Gentamicin + Fungizone) prior to counting and being adjusted to a density of 10^7 cells mL^{-1}. The cellular suspension (100μl) was then added to six wells on each of three replicate 96 well plates and the cells incubated at 18°C until use. All cellular suspensions where visually verified before use and were stimulated within seven days following Secombes (1990).

Phagocytosis

Each well, of the 96 micro-well plates, containing cells was washed twice with L-15 media and 100μl of neutral red-zymosan solution (1.5×10^8 particles mL^{-1}) added to each of the wells. The suspension of neutral red-stained zymosan was prepared according to the protocol of Pipe, Coles, Carissan and Ramanathan (1999). Briefly 10 g of zymosan particles (Z4250, Sigma Aldrich Ltd, UK.) was mixed with 100 ml of 1% neutral red solution in boiling, distilled water for 1h. The suspension was then centrifuged at 300g for 5 min and resuspended in 1.8% phosphomolybdic acid for 30 min at 4°C, followed by 6% ammonium heptamolybdate for 1 h at 4°C. The particles were then spun six times at 300g for 5 min, the supernatant discarded and the zymosan resuspended in Tris buffered saline, pH 7.6, containing 2% NaCl.

After incubation with the zymosan solution for four hours at 18°C the cells were washed twice (100µl PBS) and then fixed with Bakers formal calcium, BFC, (2% NaCl, 1% calcium acetate, 4% v/v formaldehyde), prior to being washed twice with PBS. A standard curve was generated by serial two fold dilutions of zymosan solution in acidified ethanol (1% [v/v] acetic acid, 50% ethanol in distilled water) with the BFC fixed cells being a negative control. The cells were then lysed with acidified ethanol (1% [v/v] acetic acid, 50% ethanol in distilled water) and the neutral red concentration determined at 550nm in a spectrophotometer (Optimax, Molecular Devices, Sunnyvale,CA). Phagocytic values were normalized by adherent cell number, from one well per fish, determined following lysis with 0.1M citric acid, 1% Tween 20 and 0.05% crystal violet as per Secombes (1990).

Intracellular respiratory burst

The intracellular superoxide anion production of the isolated head kidney leukocytes was determined according to the protocol of Secombes (1990). The only modification being the optimal incubation time of leukocytes with PMA determined to be one hour in our laboratories. This method is based on the reduction of Nitroblue tetrazolium (NBT) by O_2^- to a blue colour. The O_2^- is formed during the 'respiratory burst' of phagocytes in order to kill pathogens. The oxidative burst causes a marked increase in the oxygen uptake, which is mainly channelled to a pathway initiated by the reduction of oxygen to superoxide anion (O_2^-) using NADPH or NADH as the electron donor. The O_2^- is subsequently converted to hydrogen peroxide (H_2O_2) with the aid of the enzyme superoxide dismutase (SOD).

T-cell proliferation

Isolated cells were then stimulated with Concanavalin A; a T cell specific mitogen according to the protocol of Daly et al (1995). Isolated cells from plates prepared as previously, and were stimulated with 25μg mL^{-1} Con A for 10 days, with 20μl of complete media added every three days to replace evaporation. The proliferative response was then measured using MTT ((3-(4,5-DImethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) Sigma Aldrich M5655)). Twenty micro-litres of MTT solution (5mg mL^{-1} w:v in PBS) was added 24 hours prior to termination of the experiment with the MTT being reduced to a purple formazan crystals by the action of mitochondrial reductase enzymes in living cells. After removing the supernatant, the well was washed (150μl PBS) to remove any remaining intracellular MTT, and the formazan crystals resolubilised with acidified iso-propanol (4% 1M HCl in propan-2-ol). The plate absorbance was read at 550nm and the absorbance subtracted from a reference reading at 620nm using a microplate spectrophotometer (as previously). The values are shown as a stimulation index compared to un-stimulated controls.

4.2.4 Mineralised tissue physiology

Dynamic bone histomorphometry

At days 39 and 80 of the trial all fish were anaesthetised (60mg L^{-1} MS222; Pharmaq) and injected with 0.1mL calcein (30 mg mL^{-1} in 0.9%NaCl + 0.2% w/v $NaHCO_3$, pH 7.4) for the determination of dynamic bone histomorphometry parameters.The embedding, sectioning, visualisation and measurement of the fluorescent markers was performed as per Chapter 3.

Scale loss

The scale loss rate was determined by counting the number of scales displaying only one fluorescent label and less than the number of circuli found between labels on the double labelled scales. The scale loss rate is calculated as a percentage of the total number of scales taken per fish.

Whole body mineral content

Whole body mineral content was determined by ICP-OES after nitric acid digestion of the sample (see Chapter 3).

4.3 Results

4.3.1 Growth and feed utilisation

The experimental diets were accepted well by the fish with all treatments recording a minimum of a tripling in body mass within the experimental feeding period. After the 12 weeks of dietary conditioning highly significant differences were observed in the growth of the experimental animals, with the soya protein fed fish having a lower final body mass compared to the other diets. This trend was further reflected in all other expressions of growth including body mass gain, specific growth rate and thermal growth coefficient (Figures 4-1,4-2). In addition the soya fed fish displayed an increased feed conversion ratio (Figure 4-4) and a concomitant reduction in feed conversion efficiency (Table 4-3). Similar significant trends were observed for the protein efficiency ratio, and apparent net protein utilization. No significant differences were observed between the proximate analyses of the final fish carcasses (Table 4-3).In addition no significant differences were observed between the condition factor of the fish with all treatments producing animals of a similar body

conformation. This was repeated for both the hepato-somatic index, a ratio of the size of the liver to the size of the animal, also the haematocrit of the blood. The ANMU of selected minerals is illustrated in Figure 4-5.

The apparent net mineral utilisation of phosphorus was between 30-45% for all the diets tested with significant differences observed between treatments. The soya and fishmeal fed animals had higher phosphorus ANMU than the blood meal and poultry meat meal fed animals, with feathermeal not significantly different from the two extremes. Much lower figures were recorded for the remaining macro minerals however there were differences between treatments. Soya fed animals had a lower sulphur and magnesium ANMU ($P<0.001$, $P<0.003$ respectively) compared to all other diets.

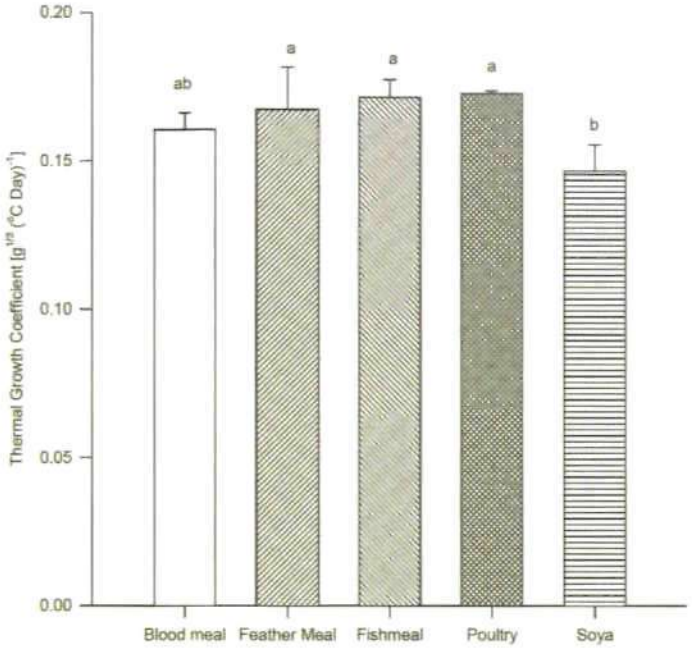

Figure 4-2: Mean thermal growth coefficient of fish fed animal by-products for 12 weeks. Different superscripts denote significant differences (ANOVA, Holm-Sidak, P<0.05).

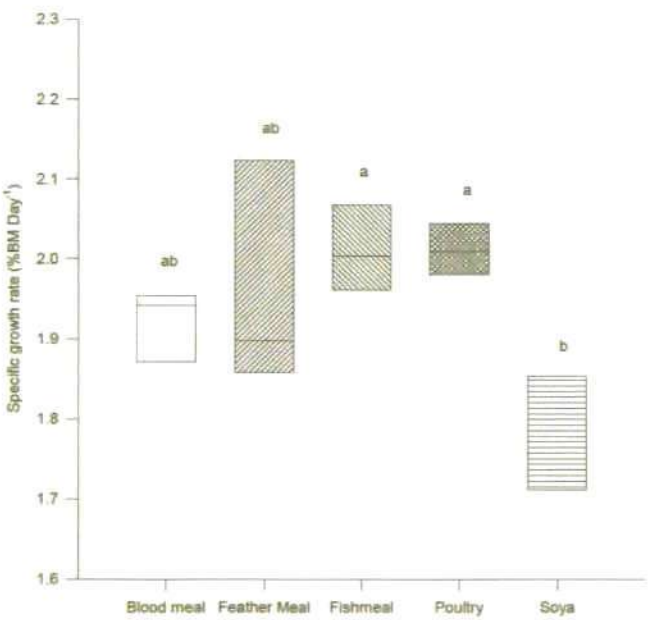

Figure 4-3: Median specific growth rate (±25-75 percentile) of fish fed animal by-products for 12 weeks. Different superscripts denote significant differences (Kruskal Wallis, Tukeys, P<0.05).

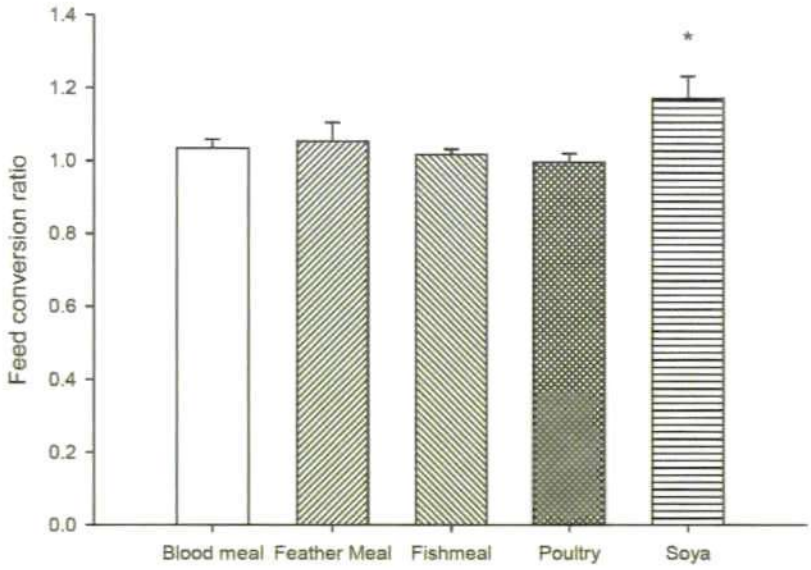

Figure 4-4: Mean feed conversion ratio of fish fed animal by-products for 12 weeks. * denote significant differences from all other values (ANOVA, Holm-Sidak P<0.05).

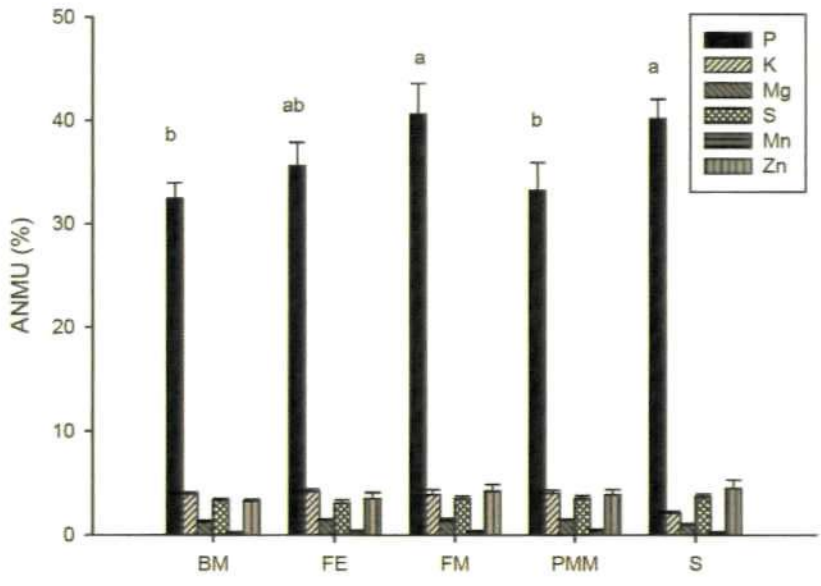

Figure 4-5: Apparent net mineral utilisation (%) of phosphorus (P), potassium (K), magnesium (Mg), sulphur (S), manganese (Mn) and zinc (Zn) from experimental diets fed to rainbow trout for 12 weeks. Different letter denote significant differences (P<0.05, ANOVA).

Table 4-3: Growth and health of trout fed experimental diets for 12 weeks. Data shown as mean± standard deviation for normally distributed data analyzed by ANOVA, or median (inter-quartile range) for non parametric (Kruskal Wallis) analyzed data. All percentage data was arcsine transformed before analysis. Different superscripts denote significant differences. NS = no significant differences between treatments.

		BM	FE	FM	PMM	S	Statistical Test	Significance
IBM (g)[1]		**33.67**±0.50	**35.58**±1.91	**34.77**±1.14	**35.57**±2.50	**33.27**±1.15	ANOVA	NS
FBM (g)[2]		**117.56**±5.12^{b}	**127.27**±9.13^{b}	**128.52**±5.67^{b}	**131.34**±6.49^{b}	**106.70**±7.92^{a}	ANOVA	P<0.001
BMG (%)[3]		253.42^{ab} (242.54-255.58)	243.42^{a} (235.41-282.30)	267.88^{a} (258.14-281.20)	269.09^{a} (263.12-276.13)	223.48^{b} (210.53-230.42)	Kruskal-Wallis	P=0.01
Specific Growth rate (%BM day^{-1})		1.94^{ab} (1.89-1.95)	1.89^{ab} (1.86-2.06)	2.00^{a} (1.96-2.06)	2.00^{a} (1.98-2.04)	1.80^{b} (1.74-1.83)	Kruskal-Wallis	P=0.01
Thermal Growth coefficient (TGC)		**0.16**±0.00^{ab}	**0.17**±0.01^{a}	**0.17**±0.00^{a}	**0.17**±0.00^{a}	**0.15**±0.00^{b}	ANOVA	P=0.03
Feed conversion ratio (FCR)		**1.03**±0.02^{a}	**1.05**±0.05^{a}	**1.01**±0.02^{a}	**0.99**±0.02^{a}	**1.17**±0.06^{b}	ANOVA	P<0.001
Feed conversion efficiency (FCE)		**96.79**±2.33^{a}	**95.07**±4.74^{a}	**98.44**±1.48^{a}	**100.48**±2.35^{a}	**85.56**±4.38^{b}	ANOVA	P<0.001
Protein efficiency ratio (PER)		**2.05**±0.04^{a}	**2.01**±0.10^{a}	**2.14**±0.03^{a}	**2.12**±0.05^{a}	**1.82**±0.09^{b}	ANOVA	P<0.001
Apparent net protein utilization (ANPU)		**47.49**±0.78^{a}	**46.11**±1.44^{a}	**45.75**±0.46^{a}	**45.00**±0.72^{a}	**42.01**±1.94^{b}	ANOVA	P<0.001
Fulton's factor		1.17 (0.97-1.22)	1.20 (1.18-1.24)	1.22 (1.19-1.26)	1.26 (1.24-1.27)	1.19 (1.16-1.20)	Kruskal-Wallis	NS
Hepato-somatic index		**1.06**±0.03	**1.19**±0.05	**1.14**±0.12	**1.20**±0.06	**1.06**±0.05	ANOVA	NS
Carcass proximate analysis (%)								
	Initial Fish	BM	FE	FM	PMM	S	Test	Significance
Moisture	**74.88**±1.29	**69.58**±0.36	**69.83**±0.57	**69.70**±0.70	**69.66**±0.69	**70.83**±0.55	ANOVA	NS
Protein	**14.72**±0.86	**16.59**±0.99	**16.28**±0.29	**15.44**±1.18	**15.47**±0.63	**15.88**±1.62	ANOVA	NS
Lipid	**5.42**±1.10	**11.03**±0.26	**11.19**±0.67	**11.16**±0.99	**11.79**±2.39	**10.83**±0.83	ANOVA	NS
Ash	**1.96**±0.17	**2.51**±0.16	**2.11**±0.21	**2.05**±0.26	**2.21**±0.18	**2.04**±0.30	ANOVA	NS
NFE[4]	3.14±1.16	**0.12**±0.11	**1.12**±0.17	**0.53**±0.57	**0.88**±0.67	**0.88**±0.04	ANOVA	NS

1. Initial body weight.
2. Final body weight.
3. Body mass gain.
4. Nitrogen free extract (by calculation).

4.3.2 Dynamic bone histomorphometry

Examples of the double labelled calcein stained scale and vertebrae are shown in Figure 4-6 and Figure 4-7 respectively.

Typically the inner scale marking (A) consisted of a continuous band appearing at approximately two thirds of the diameter of the scale. The mineralisation front at this time appeared to be found over the apex of two circuli at some points. The outer band (B) was rarely continuous, with some examples, displaying only a few small areas of actively mineralising tissue. In general the marking was confined to the apex of the outermost circuli with most mineralisation occurring at the dorsal and ventral scale surfaces. Little fluorescent marking was observed on the anterior portion of the scale, found externally to the scale pocket.

In scales that had been replaced between injections only one band was visible, commonly on the outer perimeter. Due to the regular spacing of the circuli, which has been used to age fish, the possibility of the marking being band A was excluded.

For the vertebrae both calcein bands were clearly defined in the apical portion of the growing bone, becoming closer and eventually indistinguishable from each other behind the growth areas. Where present the bands were continuous in cortical bone, however appeared fragmented in trabecular bone due to the large number of inter-trabecular spaces.

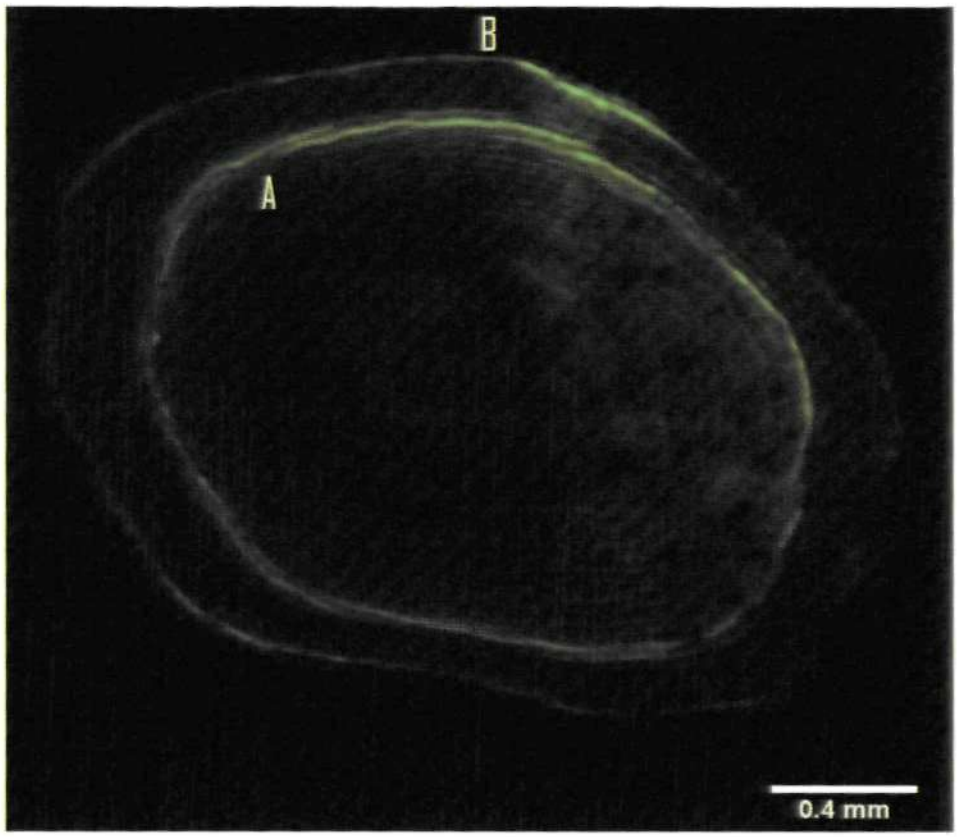

Figure 4-6: Double labelled scales viewed under fluorescent light. A= initial label, B= final label.

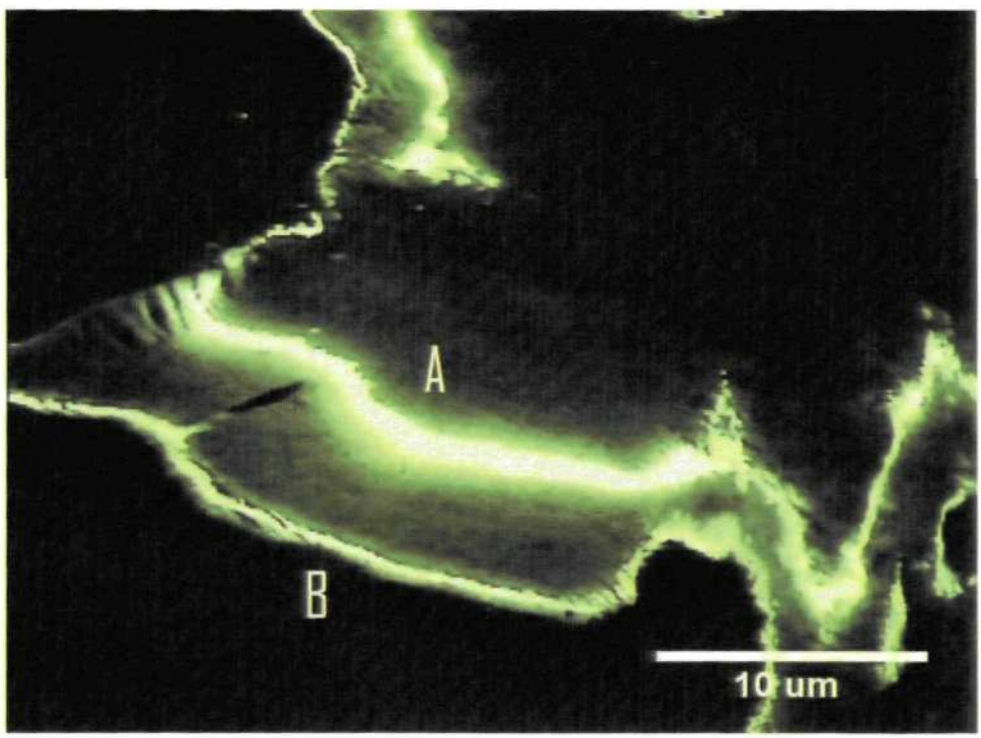

Figure 4-7: Double calcein labelled trabecular bone from trout vertebra. A= initial label, B= second label.

A summary of the data is shown in Table 4-4. No differences were found between the scale loss and the scale/ bone histomorphometry parameters quantified of the fish fed the different diets.

As no significant differences were found between treatments the mineral apposition rate and bone formation rate was compared between scales and vertebrate. No differences were found between the bone formation rates (Students T-test P=0.172), though the mineral apposition rate was significantly different (Students T-test, P <0.001) with scales having a much greater apposition rate than bones.

4.3.3 Immunology

No significant differences were observed between the peripheral blood leukocyte populations of the fed fish.

No significant interaction between stress and dietary treatment were elucidated for the cellular immune mechanisms, the only exception being the intracellular respiratory burst of head kidney leukocytes *in vitro*. A two way ANOVA revealed a significant interaction between diet and stress and therefore all permutations were analysed individually. Feather meal, fish meal and poultry meat meal fed animals had a significantly reduced intracellular super oxide anion production after stress compared to pre stress levels (Figure 4-13). There were no differences between pre and post stress anion production for the soya or blood meal fed fish. Serum lysozyme levels were unaffected by stress or diet (Figure 4-9) as was alternative complement with no differences found between any of the treatments both pre and post stress (Figure 4-10). The application of stress had a number of highly significant effects including a reduction in circulating serum glucose levels, reduced mitogenesis, more

avidly phagocytic adherent cells but with a reduced intra cellular super oxide anion production (Figure 4-13).

Table 4-4: Scale and vertebrae dynamic histomorphometry data (scales N=4, bones N=2) of fish fed diets containing blood meal (BM), feather meal (FE), fish meal (FM), poultry meat meal (PMM) or soya meal (S) based diets for 12 weeks. Due to inhomogenity of variance all vertebrae data was analysed utilising the rank transformed, non-parametric Kruskal- Wallis test, therefore vertebrae data is presented as mean and inter quartile range. NS = no significant differences between dietary treatments.

		BM	FE	FM	PMM	S	
Scales	Scale Loss (%)	0.08±0.07	0.11±0.10	0.05±0.06	0.09±0.11	0.10±0.04	NS
	Md.Pm/B.Pm (%)[1]	0.81±0.22	0.65±0.09	0.94±0.06	0.82±0.04	0.75±0.04	NS
	Mineral Apposition Rate (µm d^{-1})	3.37±0.08	3.28±0.12	3.77±0.37	3.38±0.06	3.33±0.15	NS
	Bone formation rate (nm/mm/ d)	4.78±1.18	3.63±0.41	5.62±0.89	4.58±0.26	4.16±0.19	NS
Vertebrae	Md.Pm/B.Pm (%)[1]	1.07 0.88-1.25	0.86 0.80-0.93	1.06 0.87-1.25	0.95 0.75-1.14	1.10 0.86-1.35	NS
	Mineral Apposition Rate (im d^{-1})	1.06 0.99-1.12	1.30 0.66-1.95	0.72 0.64-0.80	1.33 1.08-1.57	1.11 0.98-1.23	NS
	Bone formation rate (nm/mm/ d)	5.40 4.00-6.76	6.06 3.37-8.75	5.72 5.02-6.42	4.12 2.35-5.89	5.00 3.43-6.57	NS

[1]Mineralizing perimeter as % of bone perimeter or scale perimeter.

Table 4-5: Haematocrit and differential leukocyte counts of pre-stress peripheral blood leukocytes expressed as a percentage of total leukocytes from fish fed experimental diets containing animal by-product or plant protein sources for 12 weeks. NS= no significant differences. Data is mean ± standard deviation (N=4).

	BM	FE	FM	PMM	S	Significance
Haematocrit (%)	38.65 ±1.86	37.73 ±5.13	34.33 ±4.72	37.58 ±1.49	35.35 ±2.59	NS
Lymphocytes (%)	96.20 ±2.12	94.96 ±1.92	97.18 ±1.30	96.22 ±1.86	96.46 ±0.96	NS
Monocytes (%)	0.74 ±0.47	1.33 ±1.09	0.37 ±0.35	0.89 ±0.39	0.57 ±0.13	NS
Neutrophils (%)	2.32 ±1.13	2.17 ±0.55	2.19 ±1.14	1.67 ±0.65	2.51 ±0.73	NS
Thrombocytes (%)	0.65 ±0.61	0.65 ±0.51	0.25 ±0.27	1.06 ±0.57	0.22 ±0.27	NS

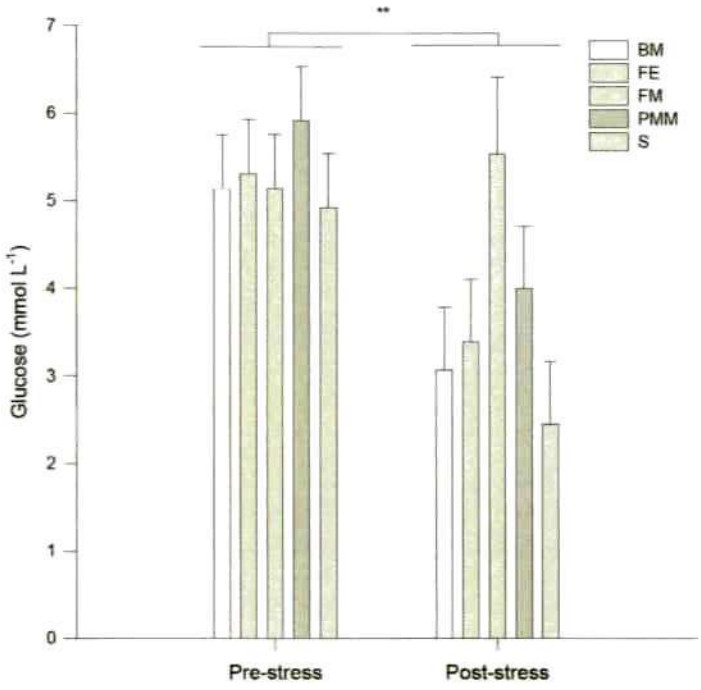

Figure 4-8: Pre and post stress serum glucose levels of fish fed animal or plant alternative protein based diets for 12 weeks. No significant differences were found between dietary treatments however stress application caused a highly significant reduction in the serum glucose level. **= highly significant differences between treatment means (P<0.001). Data is mean ± standard deviation (N=4).

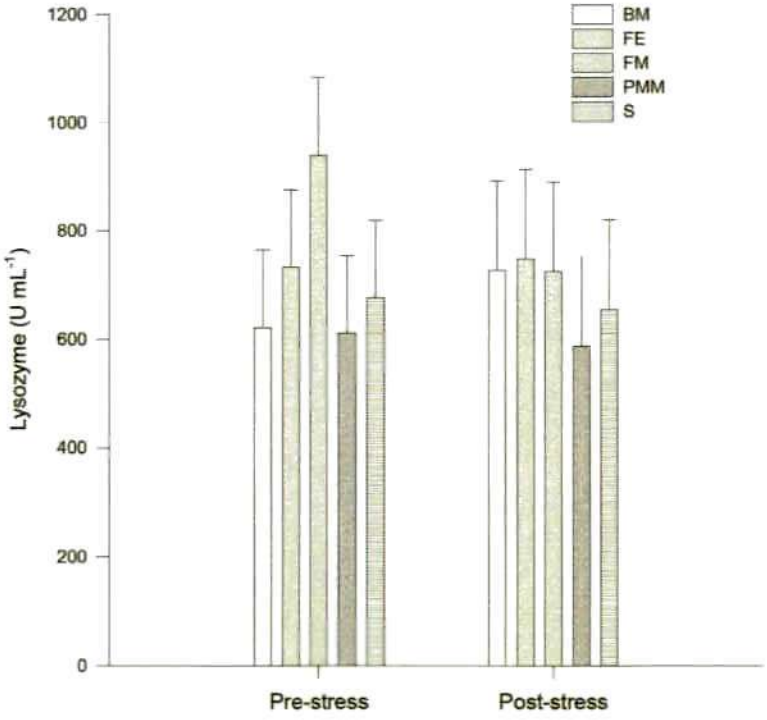

Figure 4-9: Serum lysozyme levels pre and post stress application for fish fed experimental diets for 12 weeks. No differences were determined for either dietary treatment or stress application. (Two Way ANOVA, P>0.05). Data is mean ± standard deviation (N=4).

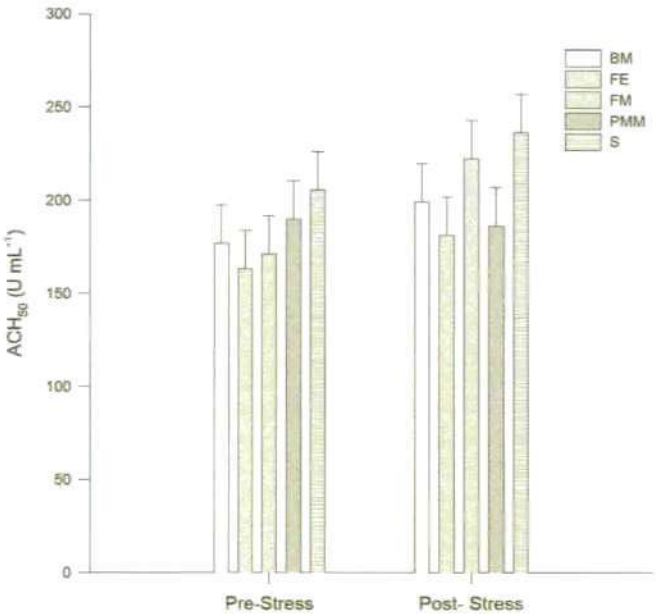

Figure 4-10: Serum alternative complement activity pre and post stress for fish fed animal or plant based diets for 12 weeks. No significant differences were found between treatments before or after stress (AONVA, Holm-Sidak P>0.05). Data is mean ± standard deviation (N=4).

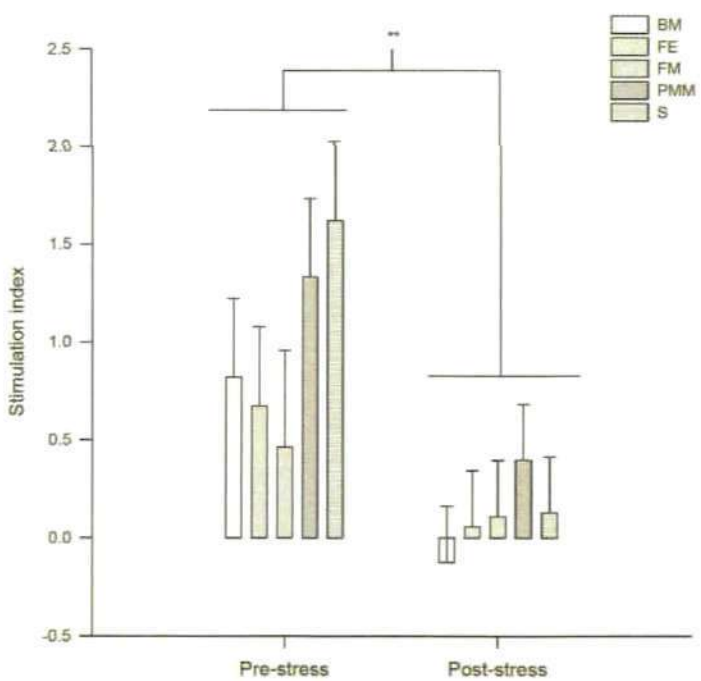

Figure 4-11: T-Cell stimulation index of isolated leukocytes from animal by-product or plant based diet fed fish after 12 days *in vitro*. No differences between treatments were found however the mean stimulation values were decreased by stress.= highly significant differences (P<0.001) between treatment means. Data is mean ± standard deviation (N=4).**

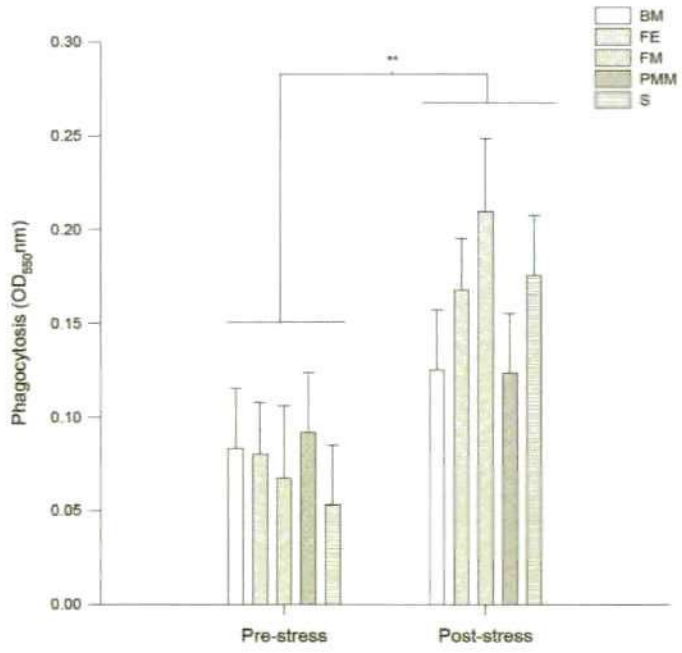

Figure 4-12: Phagocytic ability of head kidney leukocytes from fish fed animal or plant based diets for 12 weeks was significantly increased by stress (Two Way ANOVA, P<0.001). No differences were determined between dietary treatments pre or post stress. **= highly significant differences (P<0.001). Data is mean ± standard deviation (N=4).

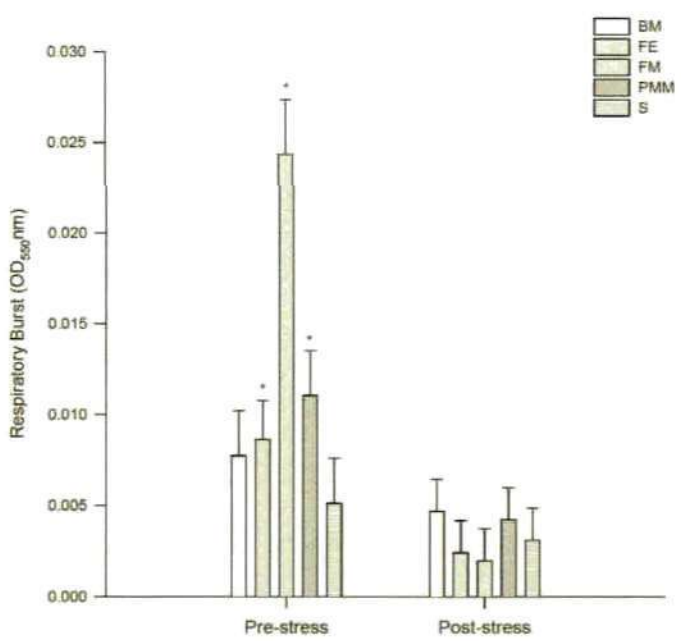

Figure 4-13: Intra-cellular respiratory burst activity of leukocytes of fish fed animal or plant based diets for 12 weeks. FE, FM, and PMM fed fish all had significant reductions in respiratory burst activity following stress application (Two Way ANOVA, Holm-Sidak P<0.05), no differences were found between BM and S fed animals pre and post stress. It should also be noted that no differences were found between BM, FE, PMM, and S fed fish pre stress. *= significant differences. Data is mean ± standard deviation (N=5).

4.4 Discussion

The thermal growth coefficients of the animal by-product fed fish in the current study (0.16-0.17) are similar to those of Bureau (1999) and indicated that the diets were well accepted and utilised. The feed conversion ratio achieved (approximately 1.0) is higher than that of other authors (Serwata 2007 and Morris 2005) for animal by-product fed trout but comparable to figure given by Naylor et al (2009) for salmonid aquaculture as a whole. In addition the condition factor of 1.17-1.26 compares well with the optimum reported figure for wild salmon (Baxter, Vallis and Quinlan 1991) and the hepato-somatic index is also similar to that reported by Panserat *et al* (2009) for trout fed diets containing fish oil or plant oil sources. Thus all diets can be stated as supporting the stringent growth requirements of modern aquaculture.

In our experiment the fish fed plant protein (soya) exhibited a reduced growth rate and feed conversion efficiency (Table 4-2), this is in keeping with a number of other investigations that have found soya elicits a localized inflammation of the gut, leading to reduced growth (Baeverfjord and Krogdahl 1996; Bakke-McKellep *et al.* 2007a; Bakke-McKellep *et al.* 2007b).

Protein retention, as measured by Apparent Net Protein Utilisation (ANPU), was high (42-48%) which indicates that protein deposition rates were consistently high during the rapid growth phase of trout in this nutrition trial. High ANPUs are associated with a balanced amino acid profile, optimal dietary protein level and an adequate intake and use of non-protein energy in the form of oil or available carbohydrate (Young, Morris, Huntingford and Sinnott 2006). However the increased ANPU of the animal by-product fed fish compared to the soya fed animals

did not translate to any significant differences in the proximate composition with no differences found between dietary treatments. Significant differences were observed between the ANMU of the fed fish with PMM and BM fed animals having a lower phosphorus utilisation than fishmeal or soya. This is possibly due to the marginally higher ash content of the diet which has been previously shown to decrease phosphorus absorption (Ye, Liu, Tian, Mai, Du, Yang and Niu 2006).

The dynamic histomorphometry was not significantly different between treatments, for both scales and vertebrae however the lowest bone formation was seen in the PMM fed animals. The vertebral bone formation rates recorded in the current study were much lower than those measured in humans; Compston, Vedi, Stephen, Bord, Lyons, Hodges and Scammell (2002) measured the bone histomorphometry of UK gulf war veterans and found the control group to have a bone formation rate of $0.087\mu m^2/mm/day$. The mean value for the vertebrae, irrespective of treatment, in the current study was $0.0052\mu m^2/mm/day$ however this should be expected due to the poikilothermic nature of fish. Due to the novel nature of this work there is at present no bone formation rate data available for teleosts therefore comparisons with other fish are not possible. The higher mineral apposition rate found in the scales ($3.42\mu m/day$) compared to $1.104\mu m/day$ in vertebrae is probably due to the two dimensional nature of scales, with the mineralised portion of the scale consisting of a thin layer on top of collagen fibres (Kobayashi *et al.* 1972; Persson, Bjornsson and Takagi 1999).

The proportion of leukocyte class was unaffected by diet with all values falling within the ranges quoted by Yasutake (1983) for juvenile *O.mykiss* and no significant differences found between the dietary treatments. Post stress leukocyte proportion data is unavailable.

While circulating cortisol is the most commonly used indicator of stress in fish, plasma cortisol levels reach their highest levels approximately one hour after stress, and return to basal levels after 6 hours, which has been suggested by some authors to be an adaptation to avoid tissue damage (Barton and Iwama 1991; Wendelaar Bonga 1997). Cortisol has also been shown to be a poor indicator of stress status in chronic experiments due to adaptation (Barton, Ribas, Acerete and Tort 2005). In addition to this fact some authors have found plasma glucose to be dependent on diet and therefore plasma glucose was used in the current study (Wedemeyer, Barton and McLeay 1990).

Pre-stress serum glucose levels were within the normal range for *O.mykiss* quoted by Manera and Britti (2006) and are comparable to those quoted by Gagnon, Jumarie and Hontela (2006) and Miller, Wang, Palace and Hontela (2007) for un stressed animals. However both authors observed a hyperglycaemic response following stress, which agrees with the currently accepted view of the teleost stress response. Nevertheless the highly significant hypoglycaemia in the current study does agree with Wood, Walsh, Thomas and Perry (1990) who found a hypoglycaemic response in trout stressed through exhaustive exercise. It is probable therefore that the response in the current research follows the general adaptive response, as defined by Selye (1936) and therefore, dependent upon the stressor employed, may result in eventual substrate depletion in chronic or repeated stress situations (Martinez-Porchas, Martinez-Cordova and Ramos-Enriquez 2009).

As previously stated lysozyme activity has been proven to be highly variable between conspecifics (Grinde 1989) and therefore the results of the current experiment should be viewed conservatively, however Saurabh and Sahoo (2008) quote the normal range of *O.mykiss* lysozyme as 600-1000 U mL^{-1} and the values

obtained in the current investigation concur with this. Some authors have found serum lysozyme to increase post stress application (Mock and Peters 1990; Binuramesh, Prabakaran, Steinhagen and Michael 2005) this phenomenon was not observed in the current study, possibly due to the chronic rather than acute stress application.

The proliferation of ConA stimulated HK derived leukocytes was significantly reduced by stress application but not influenced by diet. Stress greatly reduced the stimulation index, a finding that is repeated in the literature (Espelid *et al.* 1996) with the suggestion that elevated cortisol levels disrupt cytokine production, and therefore leukocyte mitogenesis, as is the case in mammals (Weyts, Cohen, Flik and Verburg-van Kemenade 1999).

The phagocytic ability of the HK derived phagocytes was increased by stress, although diet had no effect. Previous studies have found divergent effects of stress upon phagocytosis, with some authors reporting an increase (Pulsford, Lemairegony, Tomlinson, Collingwood and Glynn 1994), some a decrease (Narnaware, Baker and Tomlinson 1994) and some no effect (Ainsworth, Dexiang and Waterstrat 1991). The apparent discrepancies within the published literature may arise from the fact that under stressful situations leukocytes are known to migrate out from haematapoetic tissues, and therefore the phagocytic index must be normalised to adherent cell number (Secombes 1990). It would appear that our data supports the accepted paradigm that stress activates macrophages, increasing their phagocytic potential but that the actual number of phagocytes is reduced in the HK following stress therefore causing the reduction in killing activity. The large decrease in the super oxide anion production was found to be significantly affected by diet with feather meal, fish meal and poultry meat meal fed animals all reducing, while blood

meal and soya fed fish were equivocal. This finding may suggest that the phagocytic killing ability of neutrophils from soya and blood meal fed animals are already at a maximum and therefore unable to respond to a further stimulus. However until the mechanism behind the differences is found this interpretation should be viewed with caution. Comparisons between studies are difficult due to the widely differing methodologies employed; with some authors using whole blood rather than isolated leukocytes, and some reporting data that does not appear to have been normalised to adherent cell number, as recommended by Secombes (1990). Nevertheless the fact that stress significantly disrupts super oxide anion production is reported in the available literature (Novoa, Figueras, Ashton and Secombes 1996; Kollner *et al.* 2002).

The alternative complement activity of the serum was within known ranges of *O.mykiss* (Panigrahi *et al.* 2005) with no differences found between dietary treatments or stress application. It therefore appears that chronic stress, in this instance, did not affect humoral immunity.

In summary repeated stress has been shown to cause immuno-suppression in agreement with a number of different investigations (Pickering and Pottinger 1989b; Espelid *et al.* 1996; Weyts *et al.* 1999; Tort *et al.* 2003; Tort *et al.* 2004; Whyte 2007; Fast, Hosoya, Johnson and Afonso 2008) but while fishmeal replacement by animal by-products cannot be entirely exonerated from impacting disease resistance or the ability of animals to cope with husbandry stressors, it would appear the effects are limited. Indeed, Bransden *et al* (2001) examined the effect of the inclusion of feathermeal on immune function in *S.salar* and found no difference compared to the fishmeal based control diet in lysozyme, antiprotease, neutrophil oxygen radical production and plasma total immunoglobulin. The mortality following *Vibrio*

anguillarum infection was also not significantly different to control fed animals. Plus Li, Wise, Manning and Robinson (2003) also found no increase in mortality from *Edwardsiella ictaluri* challenged fed *Ictalurus punctatus* due to the feeding of animal by-products. A direct pathogen challenge of animal by-product fed animal may give a more realistic indication of immuno-competence compared to selected *in vitro* studies therefore it would appear that the influence of animal by-products on the immune system, both pre and post stress, is minimal.

In conclusion animal by-products, at current non-EU levels, have therefore been proven to not affect bone physiology and have minimal affects on innate immune responses both pre and post stress. Furthermore stress application has been proven to have a mixture of immune-suppressive and stimulatory affects.

Chapter 5 : The effect of increased substitution of fishmeal with poultry meat meal on immune function and mineralised tissues.

5.1 Introduction

Poultry meat meal (PMM) has been proven to be a valuable partial fishmeal replacement for a number of different fish species. PMM has been successfully included in the diets of Chinook salmon, silver sea bream, Australian snapper, gilthead sea bream, red drum, Nile tilapia, gibel carp, gilthead sea bream, and cuneate drum. (Fowler,1991; El-Sayed,1994; Quartararo et al., 1998; Nengas et al., 1999; Kureshy et al.,2000;Fasakin et al., 2005; Yang et al., 2005; Hu et al., 2008; Guo et al., 2007). It has great potential to be incorporated in the diet of carnivorous fish species, including salmonids, due to its high protein content and lower price compared to fish meal (Fishmeal price ≈ 710 Eu/ tonne, compared to approximately 310 Eu/ tonne for PMM: source www.hammersmithltd.blogspot.com [accessed 12/06/09]). In addition, studies on the apparent digestibility of PMM revealed that this product is well-digested by several fish species (Bureau *et al.* 1999; Serwata 2007). However advances in processing technology have allowed an increase in PMM's use from approximately 50% of the crude protein in the 1980-1990's up to 100% in recent investigations (Nengas, Alexis and Davies 1999; Takagi, Hosokawa, Shimeno and Ukawa 2000; Gaylord, Rawles and Gatlin 2004; Yang, Xie, Cui, Zhu, Lei and Yang 2006). Current salmonid aquafeeds, manufactured outside of the European Union have reduced fishmeal usage to 24% of the crude protein with the remainder from plant and animal protein sources (Naylor, Hardy, Bureau, Chiu, Elliott, Farrell, Forster, Gatlin, Goldburg, Hua and Nichols 2009). However while there are both economic and sustainability drivers behind this increasing usage there is a scarcity of data on the sub-clinical health effects of using these protein sources at elevated levels. Of the studies mentioned above (Nengas *et al.* 1999; Takagi *et al.* 2000; Gaylord *et al.* 2004; Yang *et al.* 2006) none have investigated, or reported,

major effects of elevated PMM on disease resistance or the incidence of deformity. Nevertheless PMM does contain a significant proportion of saturated fat which, in isolation, has been demonstrated to affect both in mammals (Seifert and Watkins 1997; Watkins, Li, Allen, Hoffmann and Seifert 2000; Corwin *et al.* 2006; Berge, Witten, Baeverfjord, Vegusdal, Wadsworth and Ruyter 2009; Trichet 2010).

Essential fatty acids have also been shown to play an important role in the function of the immune system in fish (Bell, Farndale, Dick and Sargent 1996). A range of cardiac diseases, autoimmune disorders and inflammatory disorders are exacerbated with an increase in the dietary n-6 to n-3 ratio (Terano, Salmon, Higgs and Moncada 1986; Calder 2001). Elevation of the n-6 to n-3 fatty acid ratio in Atlantic salmon post-smolts also had marked effects on the fish, including an increased incidence of atherosclerotic lesions, decreased resistance to bacterial infection and an altered ability of the liver to detoxify xenobiotics (Thompson, Tatner and Henderson 1996) without any discernable effect on growth. The relative levels of n-3 fatty acids are lower in chicken fat (see table below)

Table 5-1: Omega 3/6 content (%) of selected lipids used in salmonid aquafeeds.

	% fatty acid		
Lipid Source	n-6 Fatty acids	n-3 fatty acids	n-3 HUFA
Herring oil	<5	>15	5-15
Anchovy oil	<5	>15	>15
Menhaden oil	<5	>15	>15
Chicken Fat	5-15	<5	<5

It has been suggested that certain pathologies and immunological parameters can be elicited in fish by elevating the ratio of n-6 to n-3 fatty acids (Montero, Kalinowski, Obach, Robaina, Tort, Caballero and Izquierdo 2003).

However PMM is an ingredient and not a nutrient thus there will be a multitude of factors that can influence health. In terms of mineral homeostasis the Ca:P ratio, linked to the ash content of the diet, has also been proven to influence bone physiology (Fontagne, Silva, Bazin, Ramos, Aguirre, Surget, Abrantes, Kaushik and Power 2009).

The aim of this experiment was to investigate if increasing dietary PMM inclusion affects innate immune responses and increases the risk of deformity due to an altered bone physiology. Specific hypotheses are below:

Hypothesis 1: Elevated PMM will decrease P availability and therefore lead to reduced growth, inferior bone morphology, decreased vertebral strength when compared to fishmeal fed animals.

Hypothesis 2: High levels of PMM will impair innate immune responses through a greater intake of saturated fats and a reduced P availability.

Hypothesis 3: Increased saturated fats from PMM will prevent fillet drip loss through the ability of saturated fats to resist oxidation.

5.2 Methods

5.2.1 Fish and Husbandry

Six hundred juvenile *O.mykiss* were stocked into 18 tanks of the recirculation system described in Chapter 3, giving three replicate tanks per treatment. After an acclimation period of three weeks where the fish were fed to apparent satiation twice a day with the fishmeal based reference diet (see Table 5-2). The fish (26.78±0.66g) were then fed one of six diets formulated to contain an increasing proportion (0%, 30%, 40%, 50%, 60% and 70%) of the digestible crude protein from poultry meat

meal (see Table 5-2). All fish were fed to apparent satiation twice a day with a minimum of six hours between feeds. Feeding was deemed complete when the fish stopped accepting food within a five minute period. The fish were group weighed every two weeks, after 24 hours starvation to quantify growth. A random sampling of initial point fish was removed for carcass composition (N=6) and 3 fish from each tank at the end of the experiment were also removed for proximate analysis. At days 44 and 66 of the trial all fish were anaesthetised (60mg L^{-1} MS222; Pharmaq Ltd, Hampshire, UK) and injected with 0.1mL calcein (30 mg mL^{-1} in 0.9%NaCl + 0.2% w/v $NaHCO_3$, pH 7.4) for the determination of dynamic bone histomorphometry parameters.

5.2.2 Diet formulation

Six iso-caloric iso-nitrogenous diets were formulated with increasing proportions of the digestible protein from poultry meat meal. The composition of the diets is shown in Table 5-2. All diets were made as described in Chapter 3.

5.2.3 Sampling

At the termination of the experiment three fish per tank were removed for proximate analysis as described in Chapter 3. In addition a further six fish were anaesthetised and serum collected as in Chapter 3, for determination of serum lysozyme and protein. Three fish were filleted and the fillets weighed and the drip loss over 7 days determined for each fish. The vertebrae from the dissected fish were then collected for determination of mineral apposition rates, connectivity of trabeculae, mechanical properties and bone mineralisation using a minimum of three consecutive vertebrae from the dorso-caudal region (see Chapter 3).

Table 5-2: Composition of experimental diets.

	% digestible PMM inclusion					
Component (g/kg)	0	30	40	50	60	70
Fishmeal	58.25	40.55	34.66	28.16	22.86	16.96
PMM[1]	-	22.91	30.55	38.18	45.82	53.46
Wheat meal	21.78	17.87	16.57	15.28	14.84	14.41
Fish Oil	17.46	16.15	15.72	15.27	13.97	12.66
PnP premix[2]	2.00	2.00	2.00	2.00	2.00	2.00
CMC[3]	0.50	0.50	0.50	0.50	0.50	0.50
Proximate analysis (n=2) data shown as mean ± standard deviation						
Moisture	2.70±0.8	3.48±0.6	3.13±1.0	3.26±0.6	3.43±1.1	3.07±0.9
Protein	42.54±3.75	44.42±0.43	45.05±1.92	45.68±0.87	46.30±0.65	46.93±0.41
Lipid	20.91±1.39	22.68±0.47	21.59±2.60	22.31±1.34	20.97±1.71	23.43±2.71
Ash	9.54±1.69	10.19±1.99	10.40±1.38	10.62±2.03	10.83±0.94	11.05±0.13
Phosphorus	1.25±0.64	1.41±0.93	1.46±0.89	1.51±0.43	1.79±0.65	1.61±0.63
Calcium	2.00±0.74	2.24±0.51	2.31±0.49	2.39±0.50	2.47±0.67	2.54±0.87

1= Poultry meat meal 2= Premier nutrition vitamin/mineral premix (see Chapter 3 for full specification) 3= carboxymeythlcellulose (Sigma Aldrich, UK).

Drip loss

For evaluating drip loss, loins of white muscle tissue from the epiaxial part of the fillet were cut into approximately 15g pieces, weighed (W_0), and placed within an airtight plastic bag containing a pad of absorbent paper. After 168h of storage, at 4°C, the loins where unwrapped and weighed again (W1), the drip loss (DL) could be calculated after following formula:

$$Drip\ Loss\ (DL) = [(M_0 - M_1)/M_0] \times 100$$

Equation 5-1: Drip loss (DL) equation where M_0= initial mass (g), M_1= final mass (g) of fillet.

Lysozyme

As per Chapter 4.

Serum protein

As per Chapter 3.

Isolation of head kidney leukocytes

As per Chapter 4.

Phagocytosis assay

As per Chapter 4.

Connectivity of vertebral trabeculae

See Chapter 3.

Mechanical properties of vertebrae

See Chapter 3.

Bone mineral content

The bone mineral content was determined by the ash method. The bone samples were dried until constant weight ($110^{o}C$) and then combusted in a muffle furnace.

5.2.4 Statistical Analysis

All data is shown as mean ± standard deviation. Due to the dose/response nature of the experiment normally distributed data was analysed by linear regression using Sigmaplot 11.0 (Systat software Inc. 2008). Where regression was not applicable due to in-homogeneity of variance a non-parametric test was utilised and is detailed next

to the relevant data. All regression graphs include both 99 and 95% confidence intervals (dashed and dotted lines respectively). Percentage data was arcsine transformed prior to subsequent analysis. A difference of $p<0.05$ was considered significant.

5.3 Results

5.3.1 Growth

All fish grew well throughout the trial with no significant mortalities observed (Figure 5-1). After ten weeks all experimental animals had recorded a body weight gain of approximately 350 percent or more, indicating the diet was well accepted and palatable. There were no outward signs of ill-health in any of the experimental animals and mortalities throughout the trial were less than 1%, regardless of treatment.

Increasing the proportion of dietary poultry meat meal did not affect any measure of growth (FBM/%BMG/SGR/ TGC) however the TGC was seen to non-significantly decrease after 50% PMM inclusion level (Table 5-3). The total feed intake of the fish was not affected by diet, though the FCR was observed to increase proportionally to the PMM inclusion level ($P=0.022$, $R^2= 0.287$). Body shape (Fulton's coefficient: K factor) was unaffected by dietary treatment. The ANMU of phosphorus displayed a highly significant inverse relationship with %PMM inclusion in the diet ($R^2= 0.61$, $P<0.001$). The protein efficiency ratio, a measure of how well the ingested protein is converted to protein gain, was significantly affected by diet, however due to the experimental design whereby the diets were formulated to the digestible protein this was expected.

Table 5-3: Growth and feed utilisation of experimental animals after 10 weeks of dietary conditioning. Data = mean± standard deviation.

PMM Level	0	30	40	50	60	70	Linear regression
Initial body weight (g)	27.0 ±1.5	27.0 ±0.2	26.0 ±0.2	27.0 ±0.5	27.0±0.5	27.0 ±0.5	NS
Final Body weight (g)	128.0 ±10.3	130.0 ±11.8	128.0 ±6.6	129.0 ±11.2	120.0 ±10.2	123.0 ±8.8	NS
%Body weight gain	371	379	385	383	348	361	NS
SGR[1]	2.59 ±0.04	2.58 ±0.2	2.58 ±0.02	2.61 ±0.16	2.45 ±0.07	2.66 ±0.04	NS
TGC[2]	0.22 ±0.01	0.23 ±0.01	0.23 ±0.01	0.23 ±0.01	0.21 ±0.01	0.21 ±0.01	NS
Feed fed (g)	1488 ±68	1611 ±211	1640 ±137	1669 ±220	1510 ±130	1515 ±80	NS
FCR[3]	1.01 ±0.03	1.07 ±0.05	1.06 ±0.03	1.16 ±0.03	1.09 ±0.11	1.14 ±0.10	p=0.02
FCE[4]	99.07 ±3.38	94.02 ±4.38	94.30 ±5.65	86.12 ±2.39	92.08 ±9.64	88.11 ±8.10	p<0.01
PER[5]	2.33 ±0.08	2.12 ±0.09	2.09 ±0.12	1.89 ±0.05	1.99 ±0.21	1.88 ±0.17	p<0.001
K factor[6]	1.22	1.22	1.20	1.23	1.24	1.24	NS

1= Specific growth rate (%Bm Day^{-1}) (see chapter 3 for definition).
2= Thermal growth coefficient (see chapter 3 for definition).
3=Feed conversion ratio (see chapter 3 for definition).
4=Feed conversion efficiency (see chapter 3 for definition).
5= Protein efficiency ratio (see chapter 3 for definition).
6= Condition (K) factor (see chapter 3 for definition).

5.3.2 Immunology

No evidence of a significant effect of increased PMM usage was observed upon phagocytosis or lysozyme production (Table 5-4). No relationship between fillet drip loss and diet was elucidated.

Table 5-4: Immunological and fillet quality measures of fish fed diets containing 0-70% of the digestible protein from poultry meat meal for 10 weeks. Data is shown as mean± standard deviation.

Parameter	% Poultry meat meal inclusion						Linear regression
	0	30	40	50	60	70	
Phagocytosis (AU)	0.015± 0.005	0.017± 0.008	0.016± 0.007	0.019± 0.011	0.024± 0.001	0.014± 0.003	NS
Lysozyme (IU mL^{-1})	901.57± 377.96	498.95± 251.88	831.52±5 44.00	596.22± 194.56	400.30± 179.69	1103.55± 820.71	NS
Drip Loss (%)	6.59±0.74	6.67±0.53	7.48±1.80	6.64±1.26	7.10±0.26	6.70±0.51	NS

5.3.3 Bone Connectivity

The trabecular bone was not affected by diet with no significant relationship determined for all measures: trabecular bone area, trabecular number, trabecular separation, or trabecular thickness.

Table 5-5: Vertebral trabecular connectivity measurements for fish fed diets containing 0-70% of the digestible protein from poultry meat meal for 10 weeks. (N=3). Data is mean± standard deviation.

Parameter	%Poultry meat meal inclusion						Linear regression
	0	30	40	50	60	70	
Bone area	37.43 ±4.80	47.12 ±10.54	41.34 ±4.36	37.46 ±9.03	37.76 ±2.86	45.33 ±3.87	NS
Trabecular thickness	3.61 ±1.40	5.28 ±4.1	3.69 ±0.70	4.19 ±0.99	3.02 ±0.60	4.97 ±0.88	NS
Trabecular number	119.32 ±45.63	120.42 ±59.24	114.20 ±15.82	90.13 ±10.73	128.31 ±23.29	93.36 ±17.49	NS
Trabecular separation	5.83 ±1.85	5.20 ±2.19	5.20 ±0.64	7.07 ±1.5	5.00 ±0.99	6.08 ±1.57	NS

5.3.4 Dynamic bone histomorphometry

No relationship was found between the scale/ bone dynamic histomorphometry parameters (mineralising perimeter, mineral apposition rate and bone formation rate) and diet. It should also be noted that the mineral apposition rate is greater in the scales however the bone formation rate is greater in the vertebrae compared to the scales due to the greater mineralising perimeter of the bone.

Table 5-6: Dynamic histomorphometry measurements of fish fed increasing levels of poultry meat meal for 12 weeks. (Scales N=3, Bone N=2). Data is mean± standard deviation.

		Poultry meat meal inclusion (%)						
		0	30	40	50	60	70	Regression[1]
Scale	Scale Loss Rate (%)	0.08 ±0.11	0.12 ±0.09	0.07 ±0.10	0.09 ±0.08	0.09 ±0.11	0.10 ±0.10	NS
	Md.Pm/B.Pm (%)[2]	0.60 ±0.04	0.61 ±0.11	0.62 ±0.06	0.64 ±0.03	0.73 ±0.01	0.48 ±0.07	NS
	MAR (im day^{-1})[3]	7.78 ±0.81	7.55 ±1.81	7.16 ±1.17	6.55 ±0.59	7.55 ±0.27	6.55 ±0.80	NS
	BFR (nm day^{-1})[4]	0.79 ±0.09	0.72 ±0.21	0.71 ±0.14	0.69 ±0.05	0.82 ±0.11	0.54 ±0.11	NS
Bone	Md.Pm/B.Pm (%)[2]	0.98 ±0.17	1.26 ±0.04	0.78 ±0.13	0.56 ±0.23	1.29 ±0.15	1.14 ±0.21	NS
	MAR (im day^{-1})[3]	3.82 ±0.91	4.90 ±1.90	4.62 ±0.74	2.88 ±1.04	4.88 ±1.46	3.83 ±1.33	NS
	BFR (nm day^{-1})[4]	17.16 ±5.04	33.23 ±13.21	16.55 ±5.02	14.47 ±11.86	38.23 ±10.80	29.40 ±8.03	NS

1= Linear and polynomial regressions.
2= Mineralising perimeter/ bone perimeter (see Equation 3-11).
3= Mineral apposition rate (see Equation 3-12)
4= Bone formation rate (see Equation 3-13)

Bone mechanical properties and mineral content

Vertebral bone ash was affected by diet with increasing PMM level reducing mineral content resulting in a highly significant relationship ($P=0.007$, $R^2=0.37$). In addition the mechanical properties of the vertebrae were influenced by diet with the Young's modulus increasing equating to stiffer, less elastic bones, with increasing PMM content ($P=0.04$, $R^2=0.30$). The Compressive extension was also affected and decreased with increasing PMM content ($P=0.04$, $R^2=0.22$) however the force

needed to permanently deform the vertebrae, in the dorso-caudal plane, was unchanged by dietary conditioning.

Table 5-7: Effect of increasing level of digestible poultry meat meal on the ash content and mechanical properties of trout vertebrae. (Also see Figures 5-4 to 5-6). N=3.

Parameter	Diet PMM inclusion (%)						Linear regression
	0	30	40	50	60	70	
Bone ash (%)	30.93 ±2.86	32.41 ±1.84	29.97 ±0.82	28.95 ±0.82	26.81 ±2.80	25.51 ±3.23	p=0.007
Young's Modulus (N mm^{-2})	127.83 ±15.51	183.44 ±17.65	130.83 ±28.02	131.49 ±37.14	180.82 ±8.91	213.82 ±8.20	p=0.03
Compressive extension (mm)	0.58 ±0.07	0.49 ±0.13	0.57 ±0.06	0.57 ±0.314	0.31 ±0.03	0.33 ±0.04	p=0.04
Compressive load (kgF)	7.06 ±0.89	9.18 ±0.99	7.75 ±0.93	7.49 ±1.33	5.96 ±0.74	6.95 ±1.78	NS

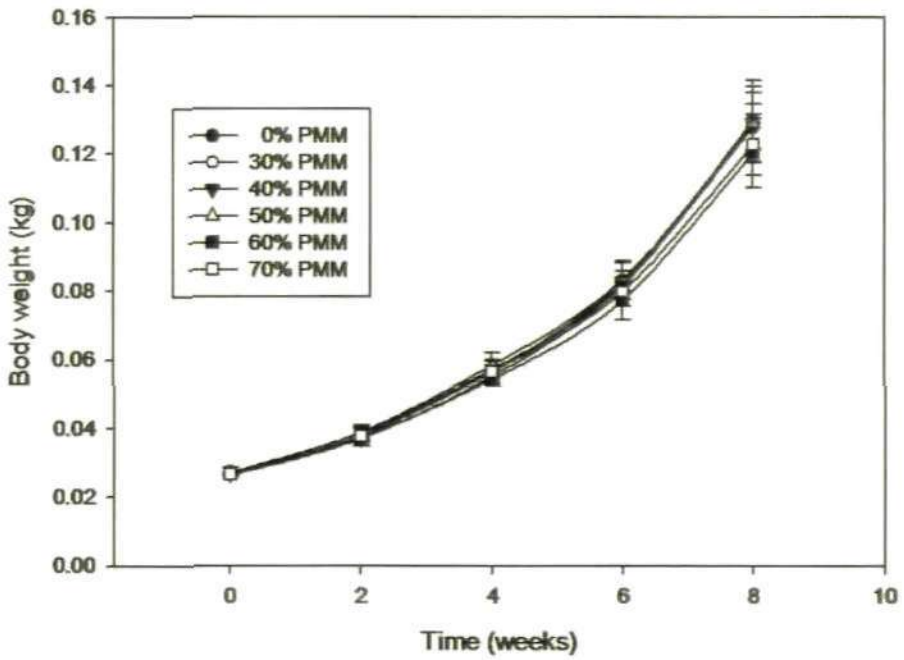

Figure 5-1: Body mass (kg) of fish fed diets incorporating increasing levels of poultry meat meal (0-70% digestible protein from poultry meat meal (PMM)). No significant differences were observed between treatments. Data is shown as mean± standard deviation of three replicates.

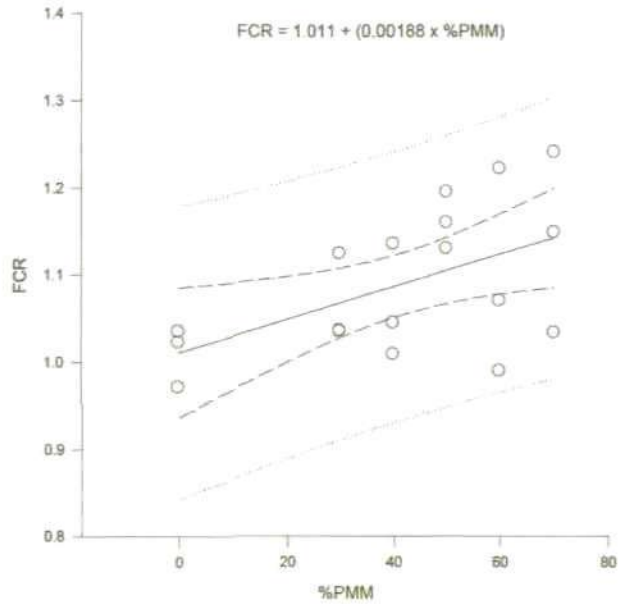

Figure 5-2: Linear regression of feed conversion ratio of fish fed 0-70% digestible protein from poultry meat meal (PMM) and %PMM inclusion (R^2=0.287, P=0.022). Dashed lines = 95% confidence interval, dotted = 99% confidence interval. Each value represents the tank mean. (N=3).

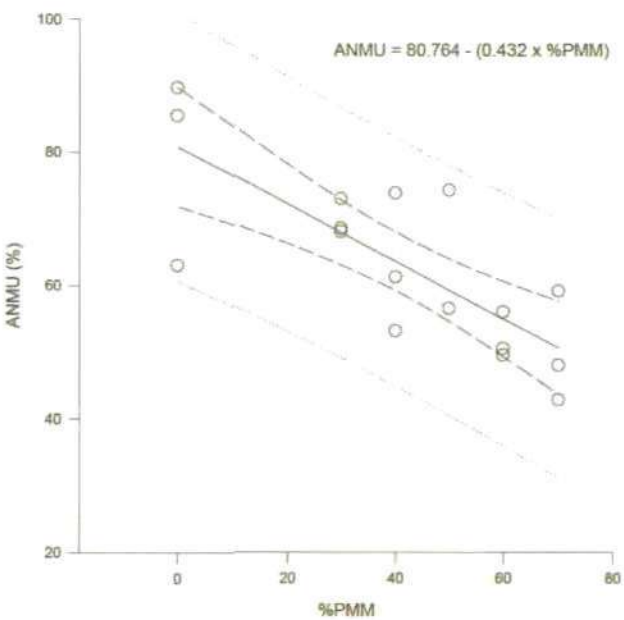

Figure 5-3: Apparent net mineral (phosphorus) utilisation of trout fed increasing proportions of poultry meat meal (0-70% substitution) for 66 days. (R^2= 0.61, P<0.001). Dotted lines = 99% confidence interval, dashed lines = 95% confidence interval. Each value represents the tank mean. (N=3).

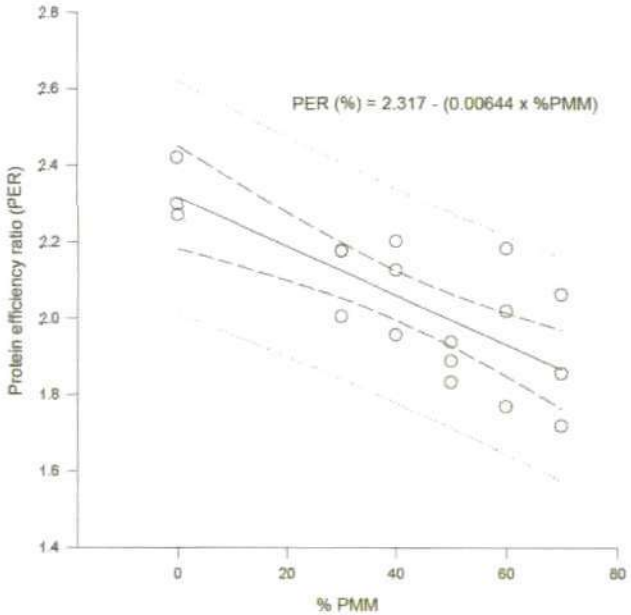

Figure 5-4: Linear regression of the protein efficiency of trout fed deits containing 0-70% digestibly protein from poultry meat meal for 66 days and %PMM inclusion. (N=3, R^2= 0.57 P<0.001). Dotted lines = 99% CI, dashed lines = 95% CI. Each value represents the tank mean. (N=3).

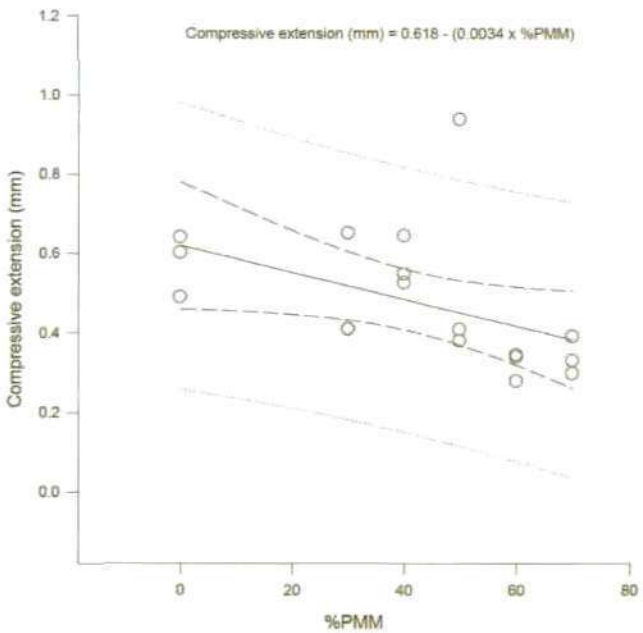

Figure 5-5: Linear regression of the compressive extension of vertebrae from fish fed increased proportions of poultry meat meal (0-70%) and %PMM inclusion. (N=3, R^2= 0.22, P=0.04). Dotted lines = 99% CI, dashed lines = 95% CI. Each value represents the tank mean. (N=3).

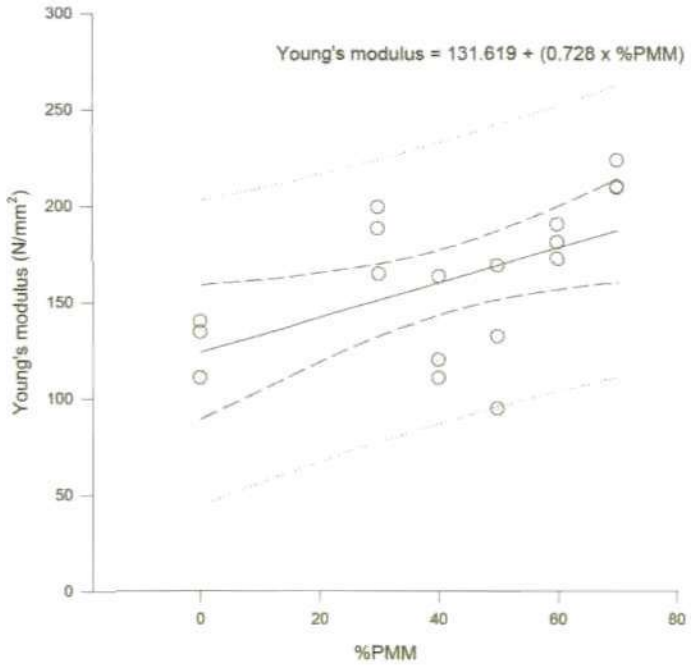

Figure 5-6: Young's modulus of trout vertebrae after dietary conditioning with increasing levels of poultry meat meal (0-70%) for 66 days. (N=3, R^2=0.30, P=0.03). Dotted lines = 99% CI, dashed lines = 95% CI. Each value represents the tank mean. (N=3).

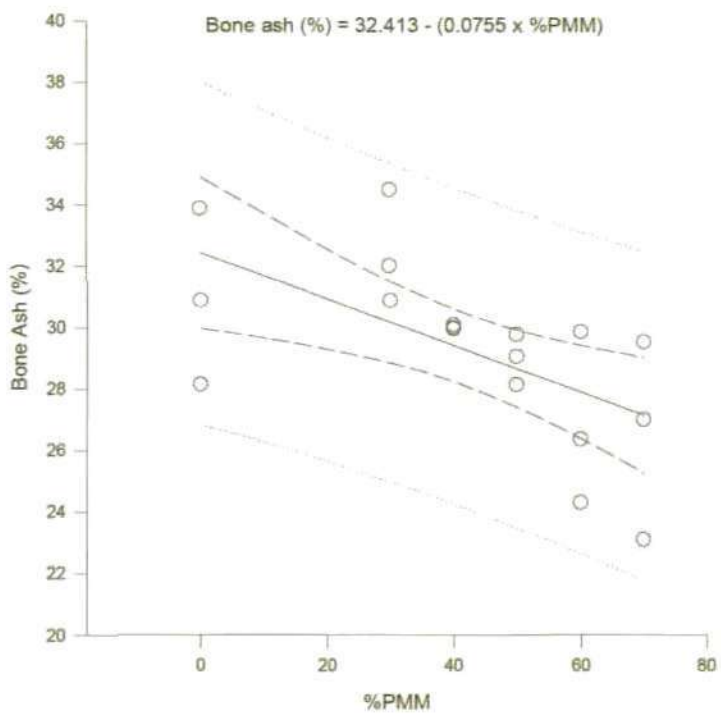

Figure 5-7: Linear regression of trout vertebral bone ash content and dietary inclusion (%) of digestible protein from PMM. (N=3, R^2=0.38, P=0.007). Dotted lines = 99% CI, dashed lines = 95% CI. Each value represents the tank mean. (N=3).

No significant differences were found in the proximate analysis (protein, lipid, ash, moisture) of the final experimental animals.

Table 5-8: Proximate composition of final animals (N=3). Different superscripts in the same column denote significant differences (P<0.05). Nitrogen free extract (NFE) by substitution.

		%PMM inclusion					
	Initial fish	0	30	40	50	60	70
Moisture	74.18 ±1.55	68.87 ±2.09	69.55 ±1.71	69.16 ±1.20	68.12 ±2.11	69.18 ±1.87	68.36 ±1.61
Protein	14.92 ±0.62	15.21 ±0.87	15.82 ±1.01	15.71 ±0.83	15.46 ±1.10	15.94 ±0.96	15.92 ±1.00
Lipid	5.42 ±1.09	12.74 ±0.65	11.21 ±0.92	11.75 ±0.44	13.40 ±1.19	11.87 ±0.88	12.57 ±0.79
Ash	1.92 ±0.12	2.08 ±0.06	2.37 ±0.11	2.35 ±0.09	2.13 ±0.13	2.24 ±0.42	2.30 ±0.09
NFE	3.56	1.1	1.05	1.03	0.89	0.77	0.85

5.4 Discussion

No effect of the increased poultry meat meal was observed on growth (figure 5- 1) though there is a non-significant trend for the thermal growth coefficient to decrease above 50% digestible PMM inclusion. However there was a significant effect on the feed conversion ratio which was evidenced to increase, indicating a reduced utilisation of ingested food, with higher proportions of PMM. This fact implies that the reduced costs of the diet formulation due to the greater PMM usage will be offset by an increase in the amount of diet needed for a specified weight gain. The FCRs achieved in the current study, 1-1.2, nevertheless are comparable with those quoted by Naylor et al. (2009) for salmonids. The apparent net mineral utilisation of the diet was proven to be affected by PMM inclusion

No obvious signs of ill health or deformity were observed with minimal mortalities recorded throughout the trial. No significant effect was found of increased PMM use and the measured innate immune responses (phagocytosis and lysozyme) indicating that even at very high levels of fishmeal substitution PMM does not significantly

disrupt the innate immune functions measured. However the lack of significance is attributable to the large individual variance found in the genetically out-bred rainbow trout. For the lysozyme this produced a coefficient of variation of approximately 62% therefore while no differences were observed in the current study the negative result should still be viewed conservatively. These variations are a normal component of fish nutrition experiments and highlight the difficulties of integrating the contrasting experimental designs needed for immunological and nutritional studies. No significant correlation between diet and drip loss was resolved either with all treatments recording 6-8% loss. However drip loss is commonly assessed over less than the 7 days post slaughter used in the current investigation (Roth, Slinde and Arildsen 2006; Wilkinson, Paton and Porter 2008) thus any initial changes will have been masked by subsequent loss.

The decreasing apparent net phosphorus utilisation with increasing PMM% indicate less mineral was utilised and therefore may account for the reduced compressive extension, vertebrate ash content and the increased stiffness of the bones. This suggests a greater occurrence of osteoid bone, or less mineral in the mineralised element, however this assertion cannot be confirmed with the current data set. Light microscopy of the osteoid seam may elucidate differences as would differential staining of mineralised and non-mineralised bone. Alternatively halastatic demineralisation, that is the reduction of bone mineral content without apparent osteoclastic activity, has been recorded in teleosts (Kacem and Meunier 2003) and therefore the possibility remains that an increased proportion of osteoid could be found away from the mineralisation front. In addition a mineralising perimeter of the vertebrae was in some cases greater than 100%; this being due to the fact that the single labelled surface is often highly convoluted due to the intramembraneous

ossification of the bone tissue (Nordvik *et al.* 2005). As mentioned previously (Chapter 4) the bone formation rate is considerable lower than that found in mammals and while the mineral apposition rate appears lower in the vertebrae the two dimensional nature of the scales would act to increase the apparent mineral apposition.

Increased dietary PMM was therefore found to decrease P availability, and thus reduce bone ash, resulting in increased stiffness of the vertebrae. However no effect on the innate immune response, fillet quality or growth was found.

In conclusion up to approximately 90% substitution of fishmeal with PMM may increase the probability of deformities through a reduction in vertebral mineral content leading to an alteration in the mechanical properties of the bone. While the mechanism for this is not resolved from the data presented here, it would seem plausible that this is due to a reduction in mineral utilisation caused by an increase in ingested ash and thus concurring with previous experiments (Ye *et al.* 2006; Fontagne *et al.* 2009). However it should be stressed that the data is only suggestive, as no increase in the incidence of obvious deformities was observed and, at present, there is no evidence that the changes in mechanical properties leads to an increased risk of malformation. Therefore it would appear that PMM could be utilised at much greater levels than currently used, and thus reduce the reliance on unsustainable marine derived proteins, without a negative impact on disease or deformity.

Chapter 6 : The effect of exercise on rainbow trout caudal vertebrae.

6.1 Introduction

The concept that bone form reflects the mechanical loading history during life is well recognised (Forwood 2008). The phenomenon of bone adaptation to imposed mechanical loadings is often referred to as 'Wolff's law' (Wolff 1892). Wolff's law states that bone in a healthy animal will adapt to the loads it is placed under, therefore if the loading on a particular bone increases, the bone will remodel itself over time to resist that load. Though the specifics of the 'law' are subject to considerable debate the general concept of bone functional adaptation is widely accepted (Ruff, Holt and Trinkaus 2006). For example short term resistance training increases bone turnover in rats, while long term exercise increases both bone strength and mass (Notomi, Lee, Okimoto, Okazaki, Takamoto, Nakamura and Suzuki 2000). Lanyon (1982) proposed the simple feedback loop shown in Figure 6-1, where increased strain, through increased activity, leads to deposition of more bone tissue with decreased activity leading to a resorption of bone tissue.

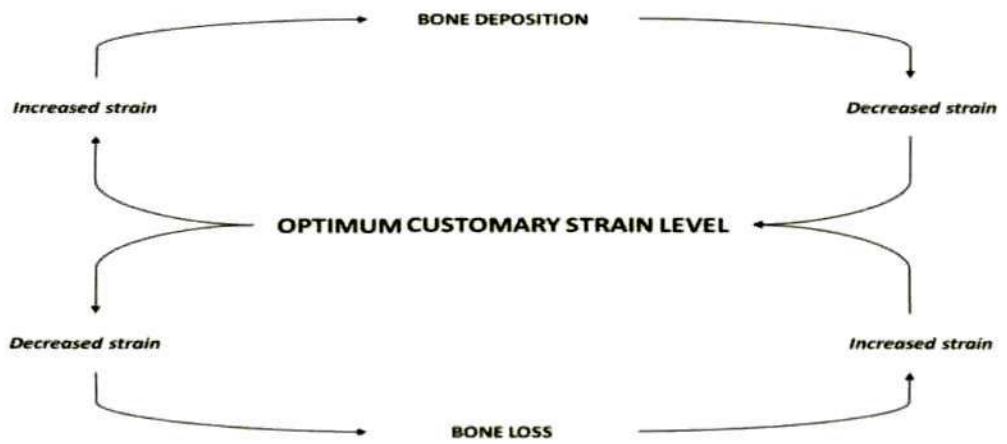

Figure 6-1: Simple feedback model of bone function adaptation (from Lanyon 1982).

The ability of bone to adapt to mechanical loads is brought about by continuous bone resorption and bone formation. If these processes occur at different locations, the

bone morphology is altered. Frost defined this as modelling (Frost 1990a). In a homeostatic equilibrium resorption and formation are balanced. In that case old bone is continuously replaced by new tissue. This ensures that the mechanical integrity of the bone is maintained but it causes no global changes in morphology. Frost defined this as remodelling (Frost 1990b).

Fish farms use water flow to maintain water quality (oxygen, pH, ammonia, nitrite etc.) with an increase in the water flow and velocity resulting in an increase in the speed at which fish must swim in order to maintain position (Kihara, Ogata, Kawano, Kubota and Yamaguchi 2002). The increased water velocity elevates the frequency of tail beats and therefore the physical stresses imposed on muscle ligaments and ultimately bone (Kranenbarg, Waarsing, Muller, Weinans and van Leeuwen 2005). However it is generally assumed that at favourable swimming speeds, exercise training, especially in salmonids leads to increased growth, increased feed conversion efficiency, increased protein turnover, higher plasma haematocrit, increased growth hormone and thyroxine, reduced circulating catecholamines and lower blood cortisol (Davison 1997).

The vertebral body of the salmonid backbone consists of four layers or compartments, two of which are formed through mineralization of preformed collagenous tissue, (the notochordal sheath and the intervertebral ligament) and two of which are formed through ossification. The two ossified layers consist of both cortical (compact) bone which forms the bulk of the amphicoel and the trabecular bone. The three inner layers have ordered lamellar collagen matrixes, which alternate perpendicularly from layer to layer, whereas the outer layer of trabeculae has a woven matrix (Nordvik *et al.* 2005). Early reports considered most teleost bones incapable of remodelling and therefore unable to repair micro-fractures (Moss

1962), however more recent studies have found that the trabeculae of the lower pharyngeal jaw of the cichlid *Astatoreochromis alluaudi* have been shown to adapt to diet, with fish fed on molluscs having more abundant trabeculae than those fed a soft diet (Huysseune, Sire and Meunier 1994). High current velocity has been linked to the development of lordosis in carp (Backiel, Kokurewicz and Ogorzalek 1984), and has been implicated in the occurrence of deformities in sea bass and red sea bream (Divanach, Papandroulakis, Anastasiadis, Koumoundouros and Kentouri 1997; Kihara *et al.* 2002).

Modern fish farms must obtain maximum return (gain in biomass) per unit input (feed intake). In order to achieve this some authors have advocated the use of feeding models based on the daily energetic requirements, of their own fish, to predict growth and reduce waste (Alanara, Kadri and Paspatis 2001; Bailey and Alanara 2006). The digestible energy need (DEN) model has been shown to accurately predict the growth of salmonids, but the enhanced growth rates required in modern aquaculture have been implicated by some authors in the incidence of deformities (Waagbo 2008). One hypothesis is that this is due to soft tissue (muscle and organ) mineral requirements outpacing that of bone (mineralised tissue) resulting in increased resorption and/or a decrease in bone mineral content (Lall and Lewis-McCrea 2007; Deschamps, Kacem, Ventura, Courty, Haffray, Meunier and Sire 2008). Therefore the following study utilises the DEN feeding model to reflect modern aquaculture practice.

6.1.2 Aims

The main aim of the experiment was to determine if increased flow rate and therefore increased exercise affects bone modelling in fish. Thus the hypothesis

tested was that exercise will increase the strength of the vertebrae as the vertebral bone morphology adapts to the greater forces applied. A secondary aim of the experiment was to evaluate the DEN feeding model as an example of current industry practice.

6.2 Methods

6.2.1 Fish and husbandry

One hundred and twenty *O.mykiss* (68.71±3.33g) were randomly stocked into 6 replicate 130 L fibreglass tanks as part of the recirculation system F at the University of Plymouth. After an acclimation period of one month the fish were fed a commercial diet (EWOS Sigma 50P) for a further 52 days (2 meal portions/ day, six hours apart) according to the digestible energy need (DEN) of the fish as proposed by Bailey and Alanara (2006). The fish were group weighed every two weeks, after 24 hours starvation, and the model adjusted accordingly. The tanks were randomly assigned to one of two treatments; high current (25L min^{-1}, directional/ laminar flow, at pressure) and low current (25L min^{-1},uni-directional flow, low pressure). These two treatments were experimentally induced by using a PVC end cap drilled with an 8mm hole pointing at 45^{0}, 15cm above the water surface; and by directing the 'low' flow vertically into the tank from 32mm inlet diameter and using 6" PVC pipes as baffles. A random sampling of initial point fish was removed for carcass composition (N=6). After 52 days of dietary conditioning a sample of fish (N=3) was removed, anaesthetised and a sample of blood (1mL) removed by caudal veinipuncture. The blood was allowed to clot for 18 hours (4^{0}C) prior to being centrifuged (13,000 rpm, 5 minutes) and the supernatant serum removed and snap frozen in liquid nitrogen before storage at -80^{0}C until analysis. A further three fish

were also removed and the tenth to thirteenth vertebrae in reverse order from the urohyle, were removed and placed into neutral buffered formalin prior to being decalcified (2M EDTA, 7 days) and processed by standard techniques for paraffin embedding. Further samples of three vertebrae were also removed for bone ash and bone strength determinations.

6.2.2 Mechanical testing

The mechanical testing was conducted as per the methodology in Chapter 3.

6.2.3 Bone mineral content

As per Chapter 3.

6.2.4 Alkaline phosphatase and serum protein

Blood (1mL) was removed by caudal puncture, allowed to clot overnight at 4^0C and the serum removed and stored at -80°c until serum alkaline phosphatase and protein levels were determined following the protocol of Deschamps, Labbé, Baloche, Fouchereau-Péron, Dufour and Sire (2009). Briefly 100 µl of plasma sample, in triplicate was incubated at 37°C for 1 h with *p*-nitrophenyl phosphate (pNPP: 1 mg mL^{-1}, Sigma N9389) in an alkaline buffer (1.5M, pH 10.3,[Sigma Aldrich, A9226]). The reaction was stopped by adding 50 µl of 3N NaOH. Absorbance was measured at 405 nm against a blank and converted into the amount of produced *p*nitrophenyl (pNP) using a standard dilution-curve (pNP: 10 mM, Sigma N7660). Serum protein concentration was determined using a commercially available test kit (Bio-Rad, Hemel Hempstead, UK) which uses a modification of the Bradford method (Bradford 1976).

6.2.5 Histological sections

As per Chapter 3,

6.2.6 Statistical Analysis

All data is shown as mean ± standard deviation. Where appropriate data was analysed by one way analysis of variance (ANOVA) and *post hoc* Tukeys test, after Anderson-Darling normality test, using Sigmaplot 11.0 (Systat software Inc. 2008). Percentage data were arcsine transformed prior to subsequent analysis. A difference of $p<0.05$ was considered significant.

6.3 Results

The DEN feeding model predicted the final body weight, irrespective of treatment, to be 131.83±7.82g. The actual final body weights were in excess of this with a mean weight of 141.85±8.61g, again irrespective of treatment. The feed conversion ratio, SGR and TGC were unaffected by treatment and were approximately 0.8, 1.65 and *0.18 respectively*.

A summary of the results can be seen in Table 6-2.

Flow rate did not have a significant effect on growth with no difference in final body weight, % body weight gain, specific growth rate, or thermal growth coefficient. Nor did flow rate significantly affect the body shape or feed conversion. However effects were seen in bone area, trabecular thickness, cortical width and bone ash content.

The total bone area was seen to decrease with a higher flow rate as did the trabecular thickness, however the cortical bone width was seen to increase. No discernable effect was found on the trabecular separation or the Euler number. In terms of the

vertebrae's mechanical properties, there was no statistically significant effect however the Young's modulus, and to a lesser extent, compressive load were higher in the high flow rate exposed fish. Additionally the compressive extension that is the elasticity of the vertebrae was also reduced in exercised fish although non-significantly.

No significant differences were found between the proximate analysis (moisture, protein, lipid, ash) of the two treatments (Table 6.1).

Table 6-1: Proximate composition of experimental animals kept in either high or low flow rate tanks for 52 days and fed a commercial diet. *= calculated. Different superscripts denote significant differences.

	Moisture	Protein	Lipid	Ash	NFE*
Initial Fish	**74.18**±1.87	**14.92**±0.77	**5.42**±1.06	**1.92**±0.11	**3.56**
High Flow	**68.94**±2.64	**17.04**±0.82	**11.61**±0.79	**2.20**±0.18	**0.21**
Low Flow	**66.71**±1.90	**17.43**±0.91	**13.32**±1.10	**2.25**±0.07	**0.29**

Table 6-2: Summary of growth, haematology, vertebral connectivity and mechanical properties for fish subjected to either high or low flow rates for 52 days. (N=3). NS= no significant differences between treatments. All data is shown as mean ± standard deviation.

Parameter		High Flow	Low Flow	ANOVA
Initial body weight (g)		**64.57** ±4.38	**62.84** ±2.51	NS
Final body weight (g)		**140.82** ±11.61	**136.76** ±6.81	NS
% Body weight gain		**123.33** ±12.41	**122.10** ±7.45	NS
Specific growth rate (%BW day^{-1})		**1.63** ±0.06	**1.63** ±0.11	NS
Thermal growth co-efficient		**0.18** ±0.01	**0.17** ±0.01	NS
Feed conversion ratio		**0.78** ±0.04	**0.80** ±0.04	NS
K factor		**1.26** ±0.02	**1.26** ±0.05	NS
Serum protein (g dL^{-1})		**4.04** ±0.53	**3.94** ±0.61	NS
Serum alkaline phosphatase (µmol L^{-1} min^{-1})		**82.72** ±22.16	**83.44** ±19.98	NS
Bone mineral content (ash, %)		**30.50** ± 0.45	**27.77** ±1.35	p=0.02
Vertebrae cancellous bone connectivity	**Bone area (%)**	**38.30** ±3.63	**43.80** ±1.14	p=0.04
	Cortical width (µm)	**17.00** ±0.24	**15.30** ±0.65	p=0.04
	Trabecular thickness (µm)	**9.17** ±0.30	**10.28** ±0.37	p=0.01
	Trabecular number (mm^{-2})	**42.23** ±3.23	**42.90** ±1.23	NS
	Trabecular separation (µm)	**14.88** ±2.04	**13.18** ±0.62	NS
	Euler number	**-4.56** ±2.80	**-8.63** ±2.99	NS
Mechanical properties	**Young's modulus (N mm^{-2})**	**181.38** ±23.74	**158.39** ±25.76	NS
	Compressive extension (mm)	**0.54** ±0.02	**0.57** ±0.21	NS
	Compressive load (KgF)	**6.91** ±0.31	**6.88** ±1.06	NS

6.4 Discussion

The DEN feeding model accurately predicted the growth of the fish under our conditions with the projected weights approximately 8% less than those actually achieved. The lower figure predicted is probably a function of the fact that the model uses the daily digestible energy need and therefore makes no account of the days in which the experimental animals were starved prior to weighing or other experimental procedures. The FCR achieved in the current study (approximately 0.8) is much lower than the 1.3 salmonid economic FCR (total feed fed/total species group biomass increase) (Naylor *et al.* 2009). However it should be noted that while the FCR of the current study is comparable to the best achievable in modern aquaculture it is acknowledged that in practical terms feeding models are a balance between achieving a low FCR and the need to maintain a high growth rate.

Resistance training induced no negative effects on growth, proximate composition or body condition of the fish. However the bone area (B.Ar/T.Ar) and trabecular thickness (Tb.Th) were both reduced, while bone mineralisation and the width of the cortical bone were increased. Due to the major similarities of the current study with that of Deschamps *et al* (2009), a number of direct comparisons with that investigation will be drawn and for the rest of this chapter Deschamps *et al* (2009) will be referred to simply as Deschamps.

Deschamps investigated the effect of sustained exercise on bone histomorphometry (total vertebral bone area in the cross sectional plane), plasma ALP/TRAP levels, plasma thyroid hormones and bone mineral content. The author did not directly asses the mechanical properties of the bone. Similar to this study Deschamps found that sustained exercise increased bone density (through a reduction in the bone cross

sectional area and an increase in bone mineralisation) and hypothesised that the increased mineralisation would result in stronger bones that are more able to resist the higher forces arising from the increased exercise. In the current study the trabecular bone area was observed to decrease by approximately 15% with exercise while the cortical width increased by 10%. This suggests the increased strain from exercise acts to decrease poorly mineralised, woven, trabecular bone in favour of compact cortical bone and therefore the total bone mineral content. It should also be noted that while no significant differences in the mechanical properties of the vertebrae were found in the current study the Young's modulus mean value was 14% greater in the exercised fish. The Young's modulus, determined from the stress/strain curve of a material, is a measure of stiffness, i.e. a materials ability to withstand plastic deformation, with brittle materials having higher values. Thus the increased cortical bone may have served to increase stiffness but the experimental design was unable to resolve these differences due to the large variability between treatments. These findings suggest that exercise induces modelling changes that serve to increase the stiffness of the bone but there is no effect on overall strength in the dorso-caudal plane, however the strength of the bone in this plane is unlikely to be representative of the strain patterns *in vivo*. Indeed the sub-carangiform locomotion of the trout is defined by the movement of between one half and two thirds of the muscle mass (Sfakiotakis, Lane and Davies 1999) resulting in sinusoidal movement, thus the strain patterns are unlikely to be running parallel in the anterior posterior direction. Therefore this represents a limitation of the methodology currently employed as the mechanical assessment will not be generating loads akin to those engendered by muscular action, which the modelling response has tried to normalise, thus greater changes may be noted if similar loads were applied experimentally.

Deschamps concluded the increased bone mineralisation to be a positive attribute based on the fact that other authors had found correlations between bone strength and mineral content (Kranenbarg *et al.* 2005). The data of the current study does not support this assertion and thus more evidence is required to determine the practical implications of the increased bone density. Indeed it could be argued that the loss of elasticity in the bones as a result of exercise may increase the risk of deformities due to the inability of the bone to resist plastic deformation, alternatively the stiffer bones may transmit forces more readily to the inter-vertebral disc which has been implicated in the aetiology of deformities in Atlantic Salmon (Witten, Gil-Martens, Hall, Huysseune and Obach 2005).

Deschamps also stated that as the bone area was reduced in exercised fish, bone resorption was of greater importance than bone deposition but experimentally the author found no variation in plasma TRAP or ALP between treatments. While both plasma TRAP and ALP are widely regarded as poor indicators of osteoclast and osteoblast function, compared to standard histomorphometry (Leung, Fung, Sher, Li and Lee 1993; Ballanti, Minisola, Pacitti, Scarnecchia, Rosso, Mazzuoli and Bonucci 1997), the author provided no evidence for this assumption. As the bone will have adapted as the exercised animals have grown the changes observed in both studies were the result of bone modelling, rather than remodelling as defined by Frost (1990b; Frost 1990a).

Furthermore some authors have implied a relationship between the incidence of deformities and the rapid growth rates required in modern aquaculture, citing the soft tissue growth may be at the expense of mineralised tissue (Lall 2002). In the current study high SGR's and low FCR's were induced but no increase in the number of obvious deformities was observed, indeed no differences in the number of

deformities in exercised and control fish were found in Deschamps study either. Thus there appears to be little scientific evidence for this hypothesis in fish past larval stages. Similarly Deschamps states that during rapid growth in active conditions dietary uptake and absorption of minerals from the surrounding water may not fulfil the entire physiological requirement and lead to a reduction of bone area. This argument is flawed on two counts; scales have been shown to be a significant sink of minerals in fish with some authors regarding them as the most labile source (Witten and Huysseune 2009). Also for more minerals to be available for soft tissue biological processes a loss of bone tissue would coincide with either a maintenance or loss of bone mineralisation, however in both Deschamps study and this bone mineralisation increased in exercised fish. Thus there is a need to identify where minerals are resorbed from; bone, scales, or both, under mineral deficient conditions.

In both Deschamps study and this, the bone area was reduced in exercised fish however the plane of quantification was perpendicular in the former. Deschamps measured in cross section whereas this author utilised sagittal sections. While neither can be confirmed to be correct without the determination of the plane of force, bone mechanical adaptation theory stipulates that the trabeculae will be aligned in the direction of the major force. Thus as the trabeculae occur as inter-connected plates in the dorso-caudal plane (see Nordvik *et al.* 2005) for standardisation of future teleost bone histomorphometric studies I suggest that bone area, trabecular thickness and trabecular separation be determined in the sagittal plane at spatially defined points. In addition quantification of vertebral bone area in sagittal sections removes the requirement for vertebrae size normalisation of cross sectional bone areas.

In conclusion trout vertebrae will adapt to greater forces by increasing the thickness of the autocentrum and reducing the trabecular thickness resulting in an increased bone mineralisation. It is possible that this increase in autocentrum bone serves to maintain the mechanical integrity of the reduced bone tissue area while resisting the increased forces imposed upon it from the increased tail beat frequency needed to maintain position in the water column.

Chapter 7 : Characterisation of Rainbow trout RANKL/ OPG expression and their role in mineralised tissue resorption.

7.1 Introduction

Deformities in farmed fish are a major problem in modern aquaculture directly impacting the welfare of fish and consumer acceptance (Branson and Turnbull 2007). Skeletal deformities have been shown to lead to decreased survival, slow growth, and poor nutrient utilisation (Fontagne *et al.* 2009). As well as obvious spinal deformities such as platyspondylia (compressed vertebrae) and fused vertebrae, sub-clinical deformities such as 'hyper-dense' vertebrae and abnormal inter-vertebral distance have been found in up to 22% of normally shaped individuals (Witten, Villwock, Peters and Hall 2000; Deschamps *et al.* 2008).

The primary function of bone is to provide support for the body and protection for vital organs. These are principally mechanical functions and in order to fulfil these, the bone matrix must have the right combination of stiffness and strength to withstand the forces imposed upon it. These forces are dynamic in nature and mineralised tissues must be able to adapt; therefore the skeleton is subject to constant reshaping (cartilage), remodelling (bone) and replacement (teeth) (Witten and Huysseune 2009). This remodelling is mediated by osteoblasts, cells that synthesize new bone, and osteoclasts, which resorb bone (Fox, Evans and Lovibond 2008).

Receptor activator of nuclear factor -κβ ligand (RANKL) is a membrane bound protein which is expressed on the surface of osteoblasts and is responsible for the formation of bone resorptive osteoclasts. RANKL stimulates the fusion of pre-osteoclasts, the attachment of osteoclasts to bone, their subsequent activation and their survival (Boyce and Xing 2008). Overproduction of RANKL is linked to debilitating degenerative bone disorders in a number of species due to the increased resorption reducing bone micro-architecture and therefore the ability of the bone to

with stand the forces placed upon it (Kostenuik 2005). RANKL has been identified in trout as a member of the tumour necrosis factor super-family of cytokines but its relationship to bone quality has not been examined (Glenney and Wiens 2007).

The counter regulatory factor osteoprotegerin (OPG) is a soluble decoy receptor that binds to RANKL and prevents it from binding to, and activating receptor activator of nuclear factor kappa-beta (RANK) thus inhibiting the formation, attachment, activation and survival of osteoclasts (Hofbauer and Heufelder 2001).OPG rapidly reduces osteoclast numbers but has no effect on osteoblasts and therefore determines the rate of bone formation. OPG genes have been identified and cloned in the medaka (*Oryzias latipes*) but not in other teleost species at present (Renn, Seibt, Goerlich, Schartl and Winkler 2004).

The ratio of RANKL: OPG, may be the ultimate determinant of bone resorption and in many situations, bone resorption is stimulated by both increased RANKL, and decreased OPG, which can amplify the pro-resorptive signals. A schematic representation of the actions of RANKL and OPG can be seen in Figure 7-1.

In fish mineralised tissues are not restricted to the internal skeleton but also represented externally in the scales. Scales can contain up to 20% of the total body calcium and are thought to be a better potential mineral reservoir than the internal skeleton (Garrod and Newell 1958). Furthermore the teleost scale contains osteoblasts and osteoclasts similar to those found in mammalian and avian bones with osteogenesis of the regenerating scale analogous to that of mammalian membranous bone (Yoshikubo, Suzuki, Takemura, Hoso, Yashima, Iwamuro, Takagi, Tabata and Hattori 2005). Goldfish scales have previously been shown to be good sources of osteoclastic markers with both tartrate resistant acid phosphatase

(TRAP) and cathepsin K (cath K) expression increasing during the reproductive season when the calcium content of the serum increases (Azuma, Kobayashi, Nakamura, Suzuki, Yashima, Iwamuro, Ikegame, Yamamoto and Hattori 2007).

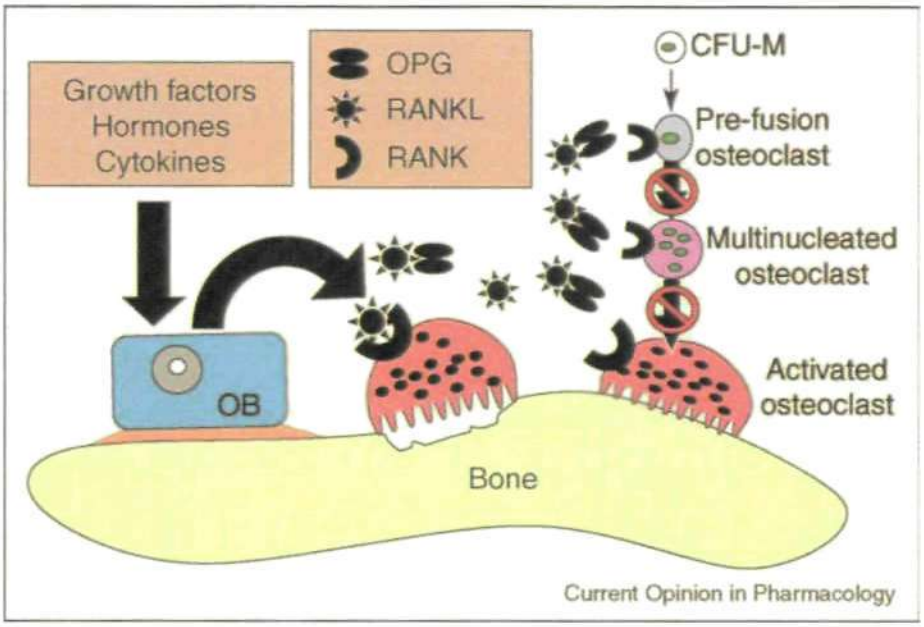

Figure 7-1: Mechanism of action for OPG and RANKL. Pro-resorptive hormones, cytokines and growth factors act via their cognate receptors on osteoblasts (OB) and other cells to induce the production of RANKL. Some of these factors also suppress osteoblast production of OPG, which further increases the RANKL:OPG ratio. When this ratio is high, free RANKL is able to activate RANK on osteoclast precursors (CFU-M, or colony forming unit–macrophage) and stimulate their fusion and differentiation into mature osteoclasts. Free RANKL also activates mature multinucleated osteoclasts to resorb bone and protects them from apoptosis. When RANKL is bound by OPG there is rapid cessation of osteoclast formation, activation and survival (Kostenuik 2005).

Unlike mammals where the growth of mineralised tissue is dependent upon dietary calcium, in the aquatic environment phosphorus (P) is limiting, therefore bone and scale growth requires dietary P intake. However excess P has been shown to negatively impact the environment thus the challenge for nutritionists is to formulate diets that meet the stringent requirements of fast growing fish while minimising excess P output (Coloso, King, Fletcher, Hendrix, Subramanyam, Weis and Ferraris 2003). Traditionally the assessment of optimum nutrient inclusion levels in formulated diets has been through the use of classical growth studies where maximum growth rate is correlated to optimum nutrient inclusion. On the basis of

these studies the NRC (1993) proposed a minimum requirement of 0.6% phosphorus for *O.*mykiss. However studies by Rodehutscord (2000) found that 1.0% dietary P resulted in the greatest bone mineral content. Thus an integrated approach is now needed that encompasses the health and welfare of the farmed animal; thus the cellular and molecular mechanisms that underpin the utilisation of P within trout must also be elucidated. Current consensus is that there is a paucity of scientific knowledge on the determinants of bone quality in fish (Lall and Lewis-McCrea 2007) with additional knowledge required to understand the aetiology of skeletal deformities.

The aim of the experiment was to determine if a lack of dietary phosphorus induces changes in the mRNA expression of RANKL/OPG and to examine the relationship between these osteoclastic markers and the strength and micro-architecture of teleost vertebrae. A secondary goal is to evaluate scales as a non-invasive methodology for studying osteoclast differentiation and therefore mineral homeostasis. Therefore the hypothesis tested were:

Hypothesis 1: RANKL mRNA expression would increase during P deficiency in trout, while OPG expression would decrease, due to increased osteoclast and reduced osteoblast activity.

Hypothesis 2: The greater bone resorption, from increased osteoclast activity, will reduce trabecular bone area and result in a decrease in bone strength.

7.2 Methods

7.2.1 Fish and husbandry

One hundred and twenty juvenile rainbow trout (*O.mykiss*) from Hatchlands trout farm, Rattery, Devon were stocked into replicate 130 L fibreglass tanks as part of a larger recirculation system (total system volume 6 m^3, flow rate 800 L h^{-1}, O_2= 90-95% saturation, NH_3= 0-0.2 mg L^{-1},NO_2^-= 0-0.2 mg L^{-1}, NO_3^-=0-0.25mg L^{-1}, Temperature = 15°C, pH=7.0±1, photoperiod = 12 hours light: 12 hours dark). After an acclimation period of three weeks the fish were fed either a phosphorus sufficient (excess of NRC 1990 guidelines) or phosphorus deficient (50% of requirement) diet for a further 8 weeks. Both diets were formulated to be iso-nitrogenous and iso-energetic (Table 1). The diets were cold pressed through a 3mm die, without steam, using a PTM 6 (Plymouth Tropical Marines, Plymouth, Devon, UK) cut to appropriate lengths and dried at 45°C in a temperature controlled cabinet until total moisture was less than 9%. All diets were stored at 4°C until use. The fish were fed 1% BW day^{-1} over two equal meal portions with feeding rates adjusted every two weeks following group weighing.

Table 7-1: Diet ingredients for phosphorus sufficient (P+) and phosphorus deficient diets (P-). Different subscripts in the same row indicate significant differences between means.

Ingredients (g Kg^{-1})	P+	P-
Acid washed casein[1]	390.90	390.90
Cornstarch[2]	331.80	362.98
Fish oil[3]	171.10	171.10
Fishmeal[4]	50.00	50.00
Di-calcium phosphorus[5]	31.20	0.00
Vitamin/ mineral premix[6]	20.00	20.00
CMC[7]	5.00	5.00
Proximate and mineral analysis (%, N=3). Vales shown as mean ± standard deviation		
Moisture	**4.01**±1.22	**5.13**±0.87
Crude protein	**39.80**±1.09	**38.83**±0.24
Lipid	**17.83**±0.82	**18.01**±0.64
Ash	**6.29**±0.61[a]	**3.52**±0.46[b]
Calcium	**1.04**±0.05[a]	**0.29**±0.08[b]
Phosphorus	**0.95**±0.02[a]	**0.35**±0.01[b]

[1]Acid washed Casein: Bacarel & Co Ltd. Unit 8, Part 2, Whitebridge Estate, Stone, Staffs. ST15 8LQ. S4126,
[2]Sigma Aldrich Ltd. UK.
[3]Seven Seas, Hull, UK.
[4]Skretting, Preston, UK.
[5]20% diclacium phosphorus. Premier Nutrition Products Limited The Levels, Rugeley, Staffs. WS151RD.
[6]PNP low P vitamin/mineral premix (Calcium 12.090%, Ash 78.708%, Sodium 8.858%, Vitamin A 1.00 MIu Kg^{-1}, Vitamin D_3 0.1 MIu KG^{-1}, Vitamin E (α-tocopherol) 7000mg Kg^{-1}, Copper 250.00mg Kg^{-1}, Magnesium 1.560%, Phosphorus 0.36%). Premier Nutrition Products Limited The Levels, Rugeley, Staffs. WS151RD.
[7]Carboxy-methyl cellulose C4888, Sigma Aldrich Ltd. UK.

7.2.2 Sampling

At the termination of the experiment the fish were euthanized by an overdose of methane tricane sulphonate (MS222: 150mgL^{-1} Pharmaq Ltd) followed by destruction of the brain. Blood (1mL) was removed by caudal puncture, allowed to clot overnight at 4^{0}C and the serum removed and stored at -80°c until serum alkaline phosphatase and protein levels were determined following the protocol of (Deschamps *et al.* 2009). Briefly 100 µl of serum sample, in triplicate was incubated at 37°C for 1 h with *p*-nitrophenyl phosphate (pNPP: 1 mg/mL, Sigma N9389) in an alkaline buffer (1.5M, pH 10.3 [Sigma Aldrich, A9226]). The reaction was stopped by adding 50 µl of 3N NaOH. Absorbance was measured at 405 nm against a blank and converted into the amount of produced *p*nitrophenyl (pNP) using a standard dilution-curve (pNP: 10 mM, Sigma N7660). Serum protein concentration was determined using a commercially available test kit (Bio-Rad, Hemel Hempstead, UK) which uses a modification of the Bradford method (Bradford 1976).

A sample of scales, including epidermis and mucus was removed and immediately frozen in liquid nitrogen, prior to storage at -80^{0}C. The spinal column was then dissected out and a sample of three vertebrae (vertebrae number 20 counting in reverse order from the urohyale, identified as the trunco-caudal area in (Kacem and Meunier 2003)) were then fixed in 10% formal saline prior to histological processing. Vertebrae from this particular area of the spinal column were selected as preliminary studies had identified these to be not only being the largest morphometrically but also to have the least intra-animal variation when mechanically tested. A further three vertebrae were immediately frozen in liquid nitrogen and stored at -80°C for mRNA expression analysis with a further three stored at -20°C for the determination of the bones mechanical properties.

7.2.3 Histology

Performed as per Chapter 3.

7.2.4 Mechanical testing

As per Chapter 3.

7.2.5 Quantification of mRNA Expression

RNA extraction

Scale and vertebrae total RNA was extracted using a Sigma GenElute mammalian total RNA extraction kit (RTN70, Sigma Aldrich LTd, Poole, Dorset, UK) with the following modifications to the manufacturers recommendations. For scales 500µl of lysis buffer was added to approximately 50mg of sample, in a 1.5mL micro-centrifuge tube and the mixture sonicated (Misonix microsin XL, output frequency: 22.5Khz, probe diameter: 3mm), on ice, for 10 seconds at a maximum of 5 watts RMS. Individual vertebrae (100-200mg) were crushed using an RNAse free pestle and mortar, with a small volume of liquid nitrogen, before being sonicated as above. All samples were then subject to the manufacturer's protocol for extraction including a DNA digest step. RNA quantity and quality was evaluated using a Nanodrop spectrophotometer with samples having an A260:280 ratio of less than 1.8 discarded. The RNA samples were then pooled by tank prior to subsequent analysis.

Quantitative PCR

Generation of plasmid standards

Primers for *O.*mykiss RANKL and B-actin were obtained in Primer 3/ BLAST (**www.ncbi.nlm.nih.gov/tools/primer-blast/**) from the following RefSeq mRNA

sequences numbers NM_001124568.1 and NM_0011711871.1 respectively. OPG has not been functionally described in *O.mykiss* therefore OPG orthologs were determined by searching the EST and genomic databases of **www.tigr.org/tdb/tgi/** and **www.ncbi.nlm.nih.gov/BLAST/** using the medaka (*O.latipes*) OPG sequences from Wagner *et al* (2003). First strand cDNA was reverse transcribed from 250ng of total RNA using 1mM dNTPs plus 5μM random nonamers, in a final volume 10μl. Further cDNA was produced using M-MLV (Sigma M1302) and the genes of interest amplified by PCR. Thermal cycling was conducted using a GeneAmp PCR System 9700 (Perkin-Elmer) under the following conditions: 94 ^{0}C for 10 min, then 30 cycles at 94^{0}C for 1 min, 65 ^{0}C for 2 min and 72 ^{0}C for3 min. The PCR product was then subject to TAE agarose gel electrophoresis and the product confirmed by size. The appropriate band was then excised, the DNA extracted (NA1111 GenElute gel extraction kit, Sigma Aldrich, Poole, UK) and ligated into the pGEM-T easy vector (A1360, Promega UK Ltd) followed by transformation into JM109 competent *E.coli* as per manufacturer's instructions. The resultant bacteria were plated onto LB/Ampicillin/Salmon Gal plates and screened visually after incubation overnight at 37°C. Suitable colonies were then cultured in LB broth overnight at 37°C in a shaking water bath and successful incorporation of the construct confirmed by the size after gel electrophoresis of the extracted DNA linearised by EcoR1 (Promega, R6011). Singular colonies obtained from streak plates of the broth cultures were used to culture larger volume of cultures enabling 100ng mL^{-1} standards to be extracted using Sigma midiprep plasmid kits (NA0200, Sigma Aldrich.). Copy number for each plasmid was calculated on these measurements. The linear range of the assay was determined by the amplification of log serial dilutions of plasmids from 500 to 5×10^{6} copies.

qPCR

Quantitative PCR was conducted as per Evans and Fox (2007) with a Applied Biosystems Step One machine and Step One software using the DNA-binding dye SYBR Green (Sigma) for the detection of PCR products. A total of 2µl of external plasmid or cDNA was added to a final reaction volume of 25µl containing 0.05 U/µl Taq, SYBR green and specific primers (0.2µM). The primers used were as follows:
RANKL sense, 5'-GCTTCAGCAGAGCCTAGCAT-3';

RANKL antisense, 5'-GTTGGACACGCTGACGTAGA-3';

OPG sense, 5'-CTGAAGCATTGCACAAAGGA-3';

OPG antisense, 5'-CCCATGGGTCATGTAGAACC-3';

β-actin sense, 5'-CATGGACTCCGGTGACGGCG-3';

β-actin antisense, 5'-ACCGCGCTCCGTCAGGATCT-3',

which yield products of 234, 198 and 136 bp respectively. Reaction conditions were 94°C for 2 min, followed by 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s with a final melt curve to confirm primer specificity. PCR efficiency for all targets and β-actin was calculated to be 95% (±4.29) with β-actin chosen as the housekeeping gene due to the lack of effect of dietary P restriction on its expression (Sugira *et al* 2007). In addition β-actin was not observed to vary between treatments. For each sample copy number relative to 10^6 β-actin copies in the same sample was calculated.

7.2.6 Whole fish/serum/scale mineral content

Whole fish mineral content was determined by the methods outlined in Chapter 3.

For serum mineral determination 100µl of serum from each of the 8 sampled fish was pooled by tank and subject to nitric acid digestion as for whole fish. Scales were washed in distilled water (x2) and ethanol (x1) to remove residual mucus, before being dried, with 20-50mg of sample digested and subject to ICP determination as before.

7.2.7 Bone ash content

As per Chapter 3,

7.2.8 Tartrate resistant acid phosphatase (TRAP)

A sample of 6 scales from 6 fish per tank was evaluated for the presence of TRAP, a biochemical marker of osteoclast activity. Scales samples were placed into a 1.5mL eppendorf tube and residual mucus removed by washing twice with distilled water, one wash with ethanol, and a further two washes with distilled water. After washing the scales were fixed in 8% formaldehyde for 30 seconds, washed with distilled water and then stained according using the leukocyte acid phosphatase kit (Sigma 387A). After staining the slides were evaluated microscopically for the presence of red staining indicative of TRAP. Due to the labile nature of the stain scales were semi-quantitatively scored as TRAP -with no visible staining, or TRAP + (visible areas of resorption seen at x40 final magnification).

7.2.9 Statistical Analysis

Where appropriate data was analysed by one way analysis of variance (ANOVA), post Anderson-Darling normality test, using Sigmaplot 11.0 (Systat software Inc. 2008). The non-parametric Mann Whitney test was used on semi-quantitative data obtained from the determination of the number of fish per tank exhibiting TRAP

positive staining scales. Percentage data were arcsine transformed prior to subsequent analysis. A difference of $p<0.05$ was considered significant.

7.3 Results

7.3.1 Growth, mineral content and vertebral mechanical properties

A summary of the results, apart from the qPCR data can be seen in Table 7-2. The P + diet recorded a specific growth rate of 0.898±0.009 % BW day^{-1}, consistent with the 1% BW day^{-1} fed. However the fish actually lost weight while on the P deficient diet; with a specific growth rate of -0.579±0.29 %BW day^{-1}.

Both the whole fish and serum calcium and phosphorus were significantly reduced by the P- diet but bone mineralisation BM was not influenced by diet. In addition the table shows a highly significant decrease in serum alkaline phosphatase (ALP) levels, between phosphorus sufficient fed fish and phosphorus deficient animals.

No significant differences were found in all the measures of the connectivity of the vertebral cancellous bone between P+ and P- fed fish. There was a non significant reduction in both the trabecular thickness and trabecular separation while the bone area/tissue area and the trabecular number increased though again this was not significant.

The Young's modulus, a measure of the stiffness of the vertebrae, was significantly different ($P=0.018$) with P- fed fish having stiffer, more brittle, bones. The compressive extension at first peak, that is the maximum elastic deformation and the load at this peak (compressive load at first peak) were not altered by dietary conditioning.

The mineral content of the scales was significantly affected by diet with phosphorus deficient fish having a lower Ca and P content than P sufficient animals. Similarly the numbers of fish per tank showing TRAP stained areas, indicative of osteoclastic resorption, was significantly increased in P deficient fish.

Table 7-2: Summary of experimental results excluding q-PCR data for fish fed a phosphorus sufficient (P+) or phosphorus deficient (P-) diet for 8 weeks. Where applicable all data are shown as mean ± standard deviation with significant differences noted in the final column with NS indicating no significant difference between means (N=6). Alpha = 0.05, NS = no significant differences between treatments.

Parameter		Diet		Significance
		P+	P-	
Specific growth rate (% BM day^{-1})		**0.89**±0.00	**-0.58**±0.29	**P=0.008**
Whole fish mineral content	Calcium	**1.59**±0.17	**0.35**±0.07	**p=0.04**
	Phosphorus	**1.32**±0.10	**0.29**±0.03	**p=0.04**
Serum mineral content (mg dL^{-1})	Calcium	**19.27**±4.70	**2.62**±2.31	**p=0.04**
	Phosphorus	**40.93**±4.39	**7.17**±1.46	**p=0.05**
Serum alkaline phosphatase (µmol L^{-1} min^{-1})		**501.99**±256.93	**19.05**±11.85	**p=0.007**
Scale mineral content (%)	Calcium	**8.62**±0.82	**7.86**±0.33	**p=0.04**
	Phosphorus	**4.40**±0.30	**4.04**±0.18	**p=0.04**
Median number of fish per tank with TRAP+ scales		**1**	**3**	**p=0.05**
Bone ash content (%)		**32.79**±1.52	**32.20**±1.76	**NS**
Vertebral cancellous bone connectivity	**Bone Area (%)**	**36.96**±7.25	**41.86**±6.76	**NS**
	Trabecular thickness (µm)	**1.68**±0.39	**1.21**±0.44	**NS**
	Trabecular number (mm^{-2})	**236.70**±100.39	**392.00**±154.19	**NS**
	Trabecular separation (µm)	**3.08**±1.37	**1.85**±1.18	**NS**
Mechanical properties of vertebrae	**Young's modulus (N mm^{-2})**	**150.81**±12.25	**182.91**±8.63	**p=0.05**
	Compressive Extension (mm)	**0.79**±0.27	**0.59**±0.25	**NS**
	Compressive load (kgF)	**16.12**±1.10	**16.93**±3.25	**NS**

7.3.2 q-PCR

No significant differences between the β-actin normalised mRNA expression profiles, of both RANKL and OPG, were found (Figure 7-2) however the relative expression of RANKL in scales was found to be significantly higher (P=0.015) in phosphorus There is a non-significant trend for OPG expression to decrease, in both tissues, with a phosphorus deficient diet. Similarly no differences in expression were observed between internal and external mineralised tissues. It should be noted that in all tissues examined the expression levels of RANKL were particularly low.

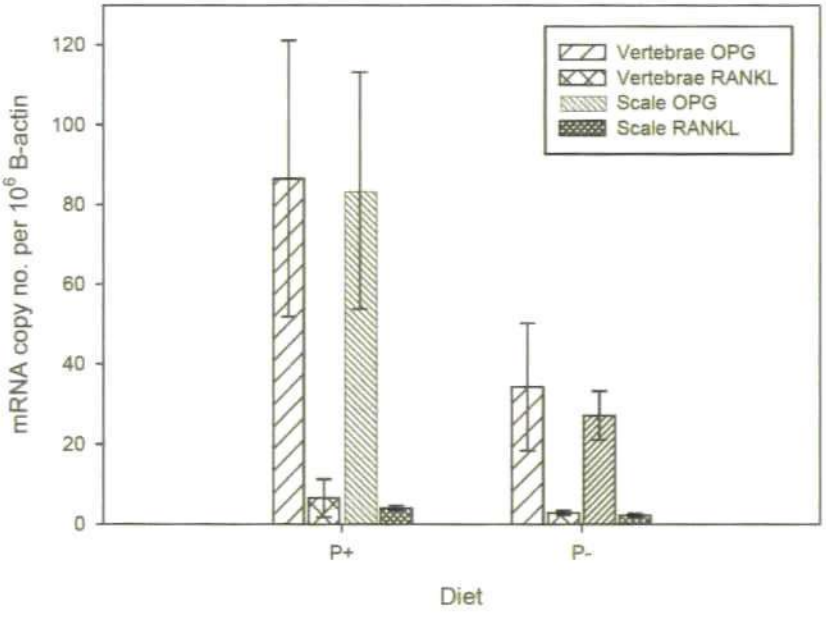

Figure 7-2: mRNA expression of receptor activator of nuclear factor kappa- beta ligand and osteoprotegerin in both vertebrae and scales for phosphorus sufficient and phosphorus deficient fish. Error bars represent ±SEM.

The ratio of RANKL/OPG mRNA expression in the vertebrae was not significantly different between treatments, with growing P+ fish having a ratio of 0.08±0.07 while the P- fish were 0.08±0.03. The ratio in scales was seen to increase from 0.04±0.02 in P+ fish to 0.07±0.15 in P- fed fish; however this was not statistically significant due to the large variation between replicates.

7.4 Discussion

In the current study a significant reduction in the whole fish and serum Ca/P levels and the reduced growth of the P- fed fish indicate they were phosphorus deficient. Subjectively the P- fed fish darkened in colour and appeared listless but no significant mortalities occurred throughout the trial. These observations agree with Sugiura (2004) who found that hypophosphataemic mature fish exhibit reduced activity and feed intake. The fact that the P deficient animals lost weight over the experimental period, coupled with their behaviour indicate, that rather than continuing to grow the animals reduced feed intake to a minimum and thus both bone growth and resorption were retarded. Starvation has been shown to inactivate osteoblast activity in fish (Persson, Johannsson, Takagi and Bjornsson 1997), and in the current study both plasma ALP and bone OPG expression levels were lowered due to being fed the P- diet. Similarly few negative effects of P deficiency in salmonids has been previously reported by other authors (Åsgård and Shearer 1997) but the majority of teleost studies have reported a reduction in bone mineral content (Baeverfjord 1998; Pimentel-Rodrigues and Oliva-Teles 2000; Vielma, Koskela and Ruohonen 2002; Oliva-Teles and Pimentel-Rodrigues 2004; Fjelldal, Nordgarden and Hansen 2007). The discrepancy between investigations is partially a function of differing methodologies and species, but may also be attributed to the fact that the occurrence of growth in experiments that have observed reduced BM, have induced osteomalacia and therefore an increased proportion of poorly mineralised osteoid bone. However no reduction of the bone mineral content (BM) or bone area (B.Ar/T.Ar) was observed in this study indicating there was no significant resorption of the cancellous vertebral bone or halastatic demineralisation of the vertebrae as described by Kacem and Meunier (2003). In addition the ratio of vertebral OPG:

RANKL was unchanged between P sufficient and P deficient fish. In higher mammals bone formation and resorption occur concurrently, on the periosteal and endosteal bone surfaces respectively, and increases in the growth of bone would expected to be correlated with an increase in ALP and RANKL. The current data imply a similar situation in trout with higher RANKL expression in P sufficient, and therefore actively growing, fish.

The P requirement of *O.mykiss* is 0.6% of the diet according to the NRC (1993), in the current investigation the P- diet contained 0.34% P, equating to 56% of the requirement; an increase of the apparent digestibility of limiting nutrients is often reported (Sugiura, Dong, Rathbone and Hardy 1998) thus the increased digestibility may not have resulted in a deficiency of sufficient severity for increased vertebral resorption in mature fish. This can be addressed in future studies either by increasing the P requirement, by exercise, or by utilising juvenile animals with a concomitant higher growth rate.

The fact that the OPG expression was reduced in P- fish may indicate decreased remodelling, with this assertion supported by the highly significant decrease in serum alkaline phosphatase, a common marker of osteoblastic activity. However the expression levels of RANKL were low even in growing fish this is due to both the bones and scales containing a large proportion of non-target cells that will have reduced the apparent expression levels during normalisation. Additionally this may be a function of the comparatively acellular nature of teleost bones compared to mammals, with low RNA recoveries from fish bone found by other authors (Krossoy, Ornsrud and Wargelius 2009). A further possibility is that, although RANKL homologues have been described in trout, RANKL has little role in the regulation of osteoclast formation in fish. This is indicated by the lack of an increase

in RANKL mRNA expression but an increase in the number of TRAP stained scales described in the current experiment.

The suitability of β-actin as a housekeeping gene has been challenged frequently in both mammalian and fish studies (Cheung *et al* 2002, Olsvik *et al* 2005), however in the current study no differences between P deficient and sufficient fish, in both scales and vertebrae, were observed in agreement with Sugiura *et al* (2007) who found that β-actin expression was unaffected by dietary P intake and therefore the housekeeping gene of choice is P requirement studies.

The Youngs Modulus (YM) is a measure of the ability of a material to withstand plastic deformation and is determined from the slope of the stress-strain curve. Elastic materials have a low YM while hard brittle materials have a higher YM. In this study a higher YM was observed in the P- treatment indicating a loss of elasticity in the vertebrae. Spongy, soft vertebrae and scales are a commonly reported finding in P deficiency studies and where reported the mechanical properties of this bone are a reduced strength and YM (Baeverfjord 1998; Fjelldal *et al.* 2007). The fact that no decrease in the compressive extension, compressive load nor bone mineralisation occurred makes this increased YM difficult to explain within the confines of the current experiment.

In summary no increase in β-actin normalised RANKL mRNA expression was observed, nor was there an increase in OPG mRNA expression during P deficiency in both scales and vertebrae. Serum TRAP/ALP levels were also unaffected. No reduction in bone strength was observed with no evidence of increased internal bone resorption, however scale TRAP+ areas did increase indicating scales may be an important site of P exchange in deficient animals. In addition the relative (non-

normalised) expression of RANKL was seen to increase threefold however in the scales of phosphorus deficient fish.

A recent manuscript by De Vrieze, Metz, Von den Hoff and Flik (2010) confirmed the presence of ALP, TRAP and cathepsin K in the scales of carp (*C.carpio*) however while the authors found scales to be a good model of mineralisation the effect of diet was not investigated. Scales have been cited as the most labile source of minerals in teleosts (Garrod and Newell 1958; Witten and Huysseune 2009) however there has been little firm evidence of this affirmation presented in the scientific literature. In the current study it is apparent that the scales were being resorbed in preference to the bone; evidenced by the increase in the number of fish with TRAP positive scales, and the loss of minerals from these scales, but not from the bones. This finding is supported by the increased ratio of RANKL: OPG compared to P sufficient animals. However the fact that this increase was non-significant may indicate a limited capacity for resorption in coldwater salmonids. Given that the majority of scientific studies investigating P deficiency in teleosts induce osteomalacia, determination of osteoblastic functions may prove to be more pertinent. Thus future work could investigate if fish bone remodelling influenced more by Cbfa1/RunX2, an essential osteoblastic differentiation factor, rather than the osteoclastic factors examined here. The results presented here imply that bone remodelling is linked to growth but that dietary factors do not induce resorption in internal mineralised tissues to preserve soft tissue functions as is the case in mammals. This has important welfare implications as reduced soft tissue P affects a large number of physiological mechanisms notably the immune response and cellular energy production via ATP (adenosine triphosphate). The limited capacity for remodelling means that defects in osteoblast function, at any stage of production, due

to biotic or abiotic factors, will have a cumulative effect throughout the animal's life on the quality of the bone and therefore the incidence of deformities. It is exactly this cumulative effect that requires the monitoring of P status throughout the cultured animals life cycle to ensure requirements for proper mineralisation and health are met; especially in light of the drive to increase the use of plant based protein sources containing large amounts of biologically unavailable P. Scales therefore may offer the ability to monitor individual animals, at all life stages, to ensure their stringent needs are met and thus improve the welfare of farmed fish.

Chapter 8 : Discussion

Historically animal offal inclusion in aquafeeds resulted in negative effects upon the health of salmonids, most prominently the occurrence of characteristic bi-lateral cataracts (Roberts 1989). More recently the majority of studies investigating the use of animal by-products have focussed on the determination of the inclusion level for optimum growth, however given the link between diet, disease and deformity this program of work investigated the effect of the feeding of selected animal by-products on the health of *O.mykiss*. Of the published growth studies only sparse information is available on the possible effects of feeding these proteins on immune function and none have examined possible effects on bone physiology. As previously stated in chapter 2 of the available studies that have investigated the use of animal by-products and disease resistance no differences were found between the various treatments (Bransden *et al.* 2001; Li *et al.* 2003). However the scarcity of information does not allow robust conclusions to be drawn. Thus validation of animal by-products as effective replacements for unsustainable marine derived proteins was required. Thus the experiments presented within this thesis expand on the growing body of evidence to support the fact that animal by-product proteins are not only viable, but valuable, replacements for un-sustainable marine derived proteins.

Chapter 4 investigated the effect of animal by-product inclusion, at levels consistent with non European Union aquafeeds, on bone and scale physiology, the innate immune response, and stress challenge. No significant differences were found in the growth and feed conversion ratio when compared to fishmeal, and the growth was significantly greater in animal by-product fed animals compared to soya fed animals. In addition the protein efficiency ratio and apparent net protein utilisation was reduced in soya compared to both animal by-product and fishmeal fed animals.

Significant differences were also found in the apparent net mineral retention of the fish with PMM/ BM fed fish having a lower P utilisation possibly due to the increased ash content of the diet which has been shown to decrease phosphorus absorption (Ye *et al* 2006).

It was also of interest to note that the bone formation rate measured is considerably lower than the figures quoted for humans, due to the poikilothermic nature of fish. The mineral apposition rate of the scales was found to be much greater than that of the vertebrae however this is due to the structure of scale where the mineralised layer is essentially a two dimensional rather than three dimensional structure.

Stress was found to have a number of significant effects on the fish including reduced serum glucose, increased phagocytosis and reduced respiratory burst activity by head kidney derived phagocytes and reduced T-cell proliferation. All of these responses are reported within the available literature and fit the accepted paradigm of the immunosuppressive effects of the teleost chronic stress response (Pickering and Pottinger 1989a; Barton and Iwama 1991; Espelid *et al.* 1996; Fletcher 1997; Wendelaar Bonga 1997; Martinez-Porchas *et al.* 2009).

Diet was observed to affect the intra-cellular respiratory burst activity of head kidney leukocytes with feathermeal, fishmeal and poultry meat meal fed animals, all recorded a significant reduction in post stress respiratory burst activity; soya and blood meal fed animals did not. While the mechanism for this is outside of the scope of this thesis it would appear that the soya and blood meal fed animals had an already depressed respiratory burst activity. The production of the super oxide anion, the respiratory burst, is one of the key pathogen clearance mechanisms for fish which are more reliant upon innate immune response compared to mammals (Press and

Evensen 1999). In addition phagocytic killing is a pre-cursor of antigen presentation and initiation of an adaptive immune response. No effect of diet or stress was observed on the humoral innate immune responses measured. Therefore while ABP usage cannot be entirely exonerated from affecting the trout immune response the effect was only observed where blood meal was utilised. In addition the use of poultry meat meal or a blended diet of poultry meat meal and feather meal compares favourably to the soya based diet which also recorded a reduced pre-stress respiratory burst activity compared to fishmeal fed animals.

Outside of the European Union animal by-products are commonly utilised for aquaculture feeds however, to date, no significant increase in mortality or deformity has been recorded. Thus while the respiratory burst is vital in pathogen clearance the results contained within this thesis should be viewed conservatively. This is primarily due to the fact that *in vitro* measures of immune function were used rather than whole animal challenge type investigations which would provide a better indication of the animal's ability to cope with ubiquitous pathogens outside of the controlled research environment. It should also be remembered that due to the extensive nature of this experiment the possibility of a surrogate outcome should not be overlooked, and future work should seek to confirm this finding under practical conditions.

In chapter 5 trout were fed an increasing proportion of PMM. Outside of the EU approximately 24% of the crude protein in a typical 'grower' feed comes from PMM (Matthews and Cooke 2003). Economics and sustainability are the two issues driving the progressive reduction of fishmeal in aquafeeds, and the concomitant increase in alternative protein usage therefore this study investigated if PMM at much greater inclusion levels than those currently used, affect innate immune responses, flesh

quality or bone physiology. No correlation between dietary PMM inclusion and serum lysozyme or phagocytosis was found. In addition the drip loss, a measure of fillet quality, was unaffected by diet; though the validity of this result should be questioned due to the extended measurement period when compared to other experiments (Lie 2001). Growth was not affected due to the methodology of the experiment; however feed conversion was reduced with increasing PMM. An inverse relationship was also found in the apparent net mineral utilisation of phosphorus. This decrease in the ANMU would appear to be the cause for the observed reduction in the vertebrae's mechanical properties (compressive extension) and bone ash content. The stiffness of the bone was observed to increase with increasing PMM inclusion; however the histo-morphology of the trabecular bone had no significant correlation with PMM inclusion. Therefore it appears the substitution of fishmeal with PMM at elevated levels may present a risk factor for deformities through a reduction in phosphorus utilisation leading to reduced bone ash and consequently alteration of the mechanical properties of the vertebrae. Nevertheless it should be noted that at present this work is suggestive and that no increase in obvious malformations was observed.

To overcome this reduction in the availability of minerals, research could be directed towards the fractionation of PMM to reduce ash content, or alternatively the use of feed additives, such as citric or formic acid which have been used to improve the digestibility of the endogenous minerals (Vielma, Ruohonen and Lall 1999). Future work should also investigate the role of saturated fat in bone homeostasis, given the link between dietary fat source and bone physiology in salmonids found by Berge et a*l* (2009).

Throughout all the current experiments no significant effects were observed on growth, body conformation or the incidence of obvious malformations of the animal by-product fed fish. Significantly reduced growth was only observed where soya based diets were used. This finding is in common with a large body of scientific evidence; however, the production cycle for the rainbow trout is considerably shorter than that of the Atlantic salmon where deformities represent an even greater problem. Thus the differences in the mechanical properties of the vertebrae may result in increased deformities over the extended production cycle of the salmon and therefore warrant further investigation.

One of the difficulties of this program of investigation has been the lack of consistent and reliable indicators of bone cell and tissue function. Therefore experiments were also performed to help understand teleost skeletal tissue physiology.

Exercise was found to have a profound effect on bone ash and both the trabecular and cortical bone; though these effects did not result in a measurable difference in the mechanical properties of the vertebrae. Increased exercise therefore translates to an increased cortical width and a reduction in trabecular width leading to an increased bone ash content. Given that cortical bone forms though intra-membranous mineralisation of preformed tissue (Nordvik *et al.* 2005) and that the osteoclasts are absent from the periosteal surface of the cortical bone it would appear the bone models itself to the increased load through a differential between compact bone growth and trabecular bone growth rather than remodelling as stated by Deschamps *et al* (2009). This profound effect has important implications for salmonid welfare as exercise will result in stiffer bones, lacking elasticity, thus reducing the ability of the animal to cope with sudden trauma. Moreover this effect may be exaggerated in fish species that require longer production cycles.

In order to investigate osteoblast and osteoclast function both static and dynamic bone histomorphometry techniques were used. While these are accepted methodologies in mammalian biology they have, up to now, not been utilised in teleost bone research. Flourochrome labelling has been used to mark fish both as part of capture/release programmes but also for co-habitation challenge studies (Monaghan 1993; Leips, Baril, Rodd, Reznick, Bashey, Visser and Travis 2001) and also for the determination of skeletogenesis in larval fish (Haga, Du, Masui, Fujinami, Aritaki and Satoh 2010). Currently, this author is unable to find any use of double labelling in adult fish for the determination of bone formation rate. Thus the data presented herein represents a novel approach for teleost physiology, but in order for the methodology to gain wider acceptance a number of difficulties must be overcome. Due to the low bone formation rate of fish care must be taken to ensure a sufficient inter-label period to allow resolution of the different marks for quantitative analysis. It should also be remembered that in mammalian mineralised tissue physiology double flourochrome labelling is used to not only measure formation rates but also determine resorption dynamics, however the period of time required for resolution of the two fluorescent labels in fish may preclude the use of this method for that purpose. Interestingly no differences were found between the bone formation rates of the scales and vertebrae in chapter 4, raising the possibility of the use of scales as a non-invasive methodology for mineral homeostasis determination, however further corroboration of this is required due to the subjective nature of the measurements.

Chapter 7 examined if the mRNA expression of receptor activator of nuclear factor kappa beta ligand and osteoprotegerin were affected by an induced phosphorus deficiency in adult fish. Both RANKL and OPG are used in mammalian physiology

as biomarkers of osteoclast differentiation. No difference was found in their expression between phosphorus sufficient and phosphorus deficient animals, indicating there was no increase of osteoclast numbers in the bones however TRAP staining indicated an increase in osteoclast activity in the scales. It would appear from this study that the induced phosphorus deficiency was severe enough to reduce whole body, serum and scale mineral content but not to affect bone ash and trabecular morphology.

When this finding is viewed in light of the findings of chapter 6, that exercise has a significant effect on bone, it would appear that trout bone remodelling is of limited importance in the actively growing fish used, and that changes in osteoblast activity may be of greater relevance. This evidence has important implications for monitoring the welfare of cultured salmonids as any defect in bone tissue formation during rapid growth, where dietary provision is critical, would not be readily remodelled and therefore result in bone tissues that are unable to resist changes in the mechanical forces applied to them.

The fact that resorption of scales appears to be of greater importance during phosphorus deficiency raises interesting evolutionary questions. If scales are the most labile source of mineral in coldwater salmonids, is the same true of tropical species where temperature elevates homeostatic mechanisms, and is there a difference between species where scaled and non-scaled varieties are found, for example in the scaled and 'mirror' variants of the common carp (*Cyprinus carpio*).

In conclusion animal by-products have been shown to be effective, valuable replacements for unsustainable marine derived proteins, with minimal effects on the trout immune system and the ability of the animal to cope with common husbandry

stressors. In addition increased levels of PMM may represent a heightened risk of malformations unless measures are taken to increase the phosphorus utilisation of the ingredient. Therefore the current non EU inclusion levels of 5-10% feather meal, 2-5% blood meal, and 20-30% PMM has little effect on the risk of diseases or deformity, with the possibility to increase PMM levels to approximately 90% of the crude protein content without significant impact on immunity or bone physiology. The limiting factor in the studies presented here, being the economics of PMM inclusion due to the reduced feed conversion at higher levels and the applicability of the work to other salmonids with a longer production cycle.

Furthermore this program of work further corroborates the conclusion drawn by De Vrieze *et al* (2010) that scales are a viable non-invasive measure of teleost mineral homeostasis.

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