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The assessment of selected novel feed ingredients to replace fishmeal on the nutrition and health status of ornamental fish

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by

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ABSTRACT

Four investigations were conducted to assess the effects of feeding selected novel feed ingredients on the nutrition and health status of ornamental fish. Each dietary ingredient was assessed by determining the effects on growth performance, feed utilisation, haematological status, serological status and immuno-competence of mirror carp, a model ornamental fish species.

The first experiment was conducted to evaluate the effect of feeding a tropical earthworm meal (*Perionyx escavatus*) and soybean meal on the haemato-immunological response and growth performance of mirror carp (*Cyprinus carpio*). Fish were fed diets for a total of 88 days, fishmeal served as the main protein source in the control diet. Two remaining diets consisted of fishmeal fixed at 33 % provision of protein and the remaining 66 % protein was provided by soybean meal (SBM diet) or *P. excavatus* meal (EW diet). After 60 days of feeding fish fed EW diet showed a significant elevation in final body weight compared to fish fed a fishmeal diet and fish fed a SBM diet. Similar improvements were observed in feed utilisation efficiency. After intraperitoneal injection with heat inactivated *Aeromonas hydrophila* and 28 days of feeding EW diet to mirror carp showed decrease in some aspects of the innate immune response. Contrary to this, fish fed soybean meal showed signs of inflammation.

The second experiment was conducted to evaluate the effect of feeding two plant protein concentrates and a combination of whey protein concentrate and casein protein on the growth performance, haematological and serological responses of mirror carp (*C. carpio*). Fish were fed diets for a total of 84 days, fishmeal served as the main protein source in the control diet. Three remaining diets consisted of fishmeal fixed at 33% provision of protein and the remaining 66% protein was provided by rice protein concentrate (RPC diet), corn protein concentrate (CPC) and a combination of whey protein concentrate (8%) and casein protein (58%) (WPC diet). After 84 days of feeding fish fed WPC diet showed a significant elevation in final body weight compared to fish fed a fishmeal diet and fish fed RPC and CPC diets. Similar improvements were observed in feed utilisation and protein efficiency. At the end of the trial feeding fish fed WPC diet showed a significant elevation in mean corpuscular haemoglobin levels compared to fish fed fishmeal and RPC diets. Serological analysis showed that feeding carp the WPC showed a significant increase in serum albumin and protein concentrations compared to fish fed fishmeal diet.

The final experiment was conducted to evaluate the effect of feeding selected exotic ingredients on immune responses and expression of immune related genes in mirror carp (*C. carpio*). Fish were fed diets for a total of 63 days. Fishmeal served as the main protein source in the control diet and two experimental diets consisted of fishmeal fixed at 34% provision of protein and the remaining protein was provided either by earthworm meal (EW diet) or a

combination of whey protein concentrate (8%) and casein (58 %) (WPC diet). At the start of the trial fish were injected intraperitoneally with *A. hydrophila* bacterin. Compared to fish fed fishmeal, a significant increase in mRNA expression of the pro-inflammatory cytokines IL-1β (24 h post injection) and TNFα (at 12 h and 48 h post injection) was observed in fish fed EW. Moreover a similar trend was observed for complement 3 (C3) gene, where fish fed EW showed significant elevations in mRNA expression values at both 12 and 48 h post injection compared to control fed fish. In contrast, fish fed WPC showed a significant decrease in C3 and TNF-α mRNA expression compared to fish fed fishmeal (48 h post injection). Fish fed EW and WPC diet showed a significant increase leukocyte levels compared to fish fed fishmeal 14 days post injection. Fish fed fishmeal presented significantly higher circulatory IgM levels at 7 d post injection compared to fish fed EW and WPC diets. In contrast, fish fed EW and WPC showed a significant increase in IgM levels at 28 d post injection.

This study concludes that feeding fish non-plant based feed commodities had a positive effect on carp growth response, feed utilisation and immuno-competence. These findings are both novel and highly relevant for the ornamental industry where high value feed commodities are more acceptable.

All experimental work involving animals was carried out in accordance with the 1986 Animals Scientific Procedures Act under Home Office project license # 30/2135 and personal license # 30/2644

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Author's Declaration

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

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To the sea for always being there!

Dedication

I would like to dedicate this thesis to my mother, Elizabeth Rawling and my father Geoffrey Rawling for their unconditional love and support over the last 30 years.

Abbreviation List

AA Amino acids

ANOVA Analysis of variance

ANC Antinutritional compounds

AOAC Association of Official Analytical Chemists

ANF Antinutritional factors

BSA Bovine serum albumin

BCG Bromocresol green

CPC Corn protein concentrate

CFU Colony forming unit

cDNA Copy Deoxyribonucleic acid

C3 Complement 3 protein

DMSO Dimethy sulfoxide

EAA Essential amino acids

ELISA Enzyme Linked Immunosorbent Assay

EGTA Ethylene glycol-bis(2-aminoethylether)-N,N,N′,N′-tetraacetic acid

EW Earthworm meal

FCR Feed conversion ratio

FM Fishmeal

GVB Gelatin veronal buffer

HSP Heat Shock protein

IP Intraperitoneal cavity

IL-1β Interleukin 1 beta

IgM Immunoglobulin

MCV Mean corpuscular volume

MCH Mean corpuscular haemoglobin

MCHC Mean corpuscular haemoglobin concentration

MS222 Tricaine methanesulfonate

Mab-HRP monoclonal antibody – horse radish peroxidase

NRC National research council

NBT Nitroblue tetrazolium

OD Optical Density

PAMPS Pathogen associated molecular patterns

PER Protein efficiency ratio

PBS Phosphate buffer saline

PMA Phorbol myristate acetate

PCR Polymerase chain reaction

RNA Ribonucleic acid

RT-PCR Real time polymerase chain reaction

RBA Respratory burst activity

RBC Red blood cells

RPC Rice protein concentrate

SE Standard error

SBM Soybean meal

SGR Sepcific growth rate

SPC Soybean protein concentrate

TSB Tryptone soy broth

TNFα Tumor necrosis factor alpha

WG Weight gain

CHAPTER 1.

INTRODUCTORY CHAPTER

1.1 Aquaculture and the ornamental industry

Aquaculture is generally considered to be the rearing or husbandry of aquatic organisms for commercial purposes (Landau, 1992). It is differentiated from capture fisheries in so much that there is ownership of stock, and there is deliberate human intervention in the production cycle of the aquatic organism (Naylor *et al*., 2000). The contribution of aquaculture to the global supply of fish for ornamental value and food has increased significantly over the last decade, where according to the Food and Agriculture Organisation of the United Nations (FAO), aquaculture is now recognised as the fastest growth sector of agribusiness on a global scale with an estimated annual increase in production amounting to over 10%. Nearly 50% of total seafood production is now derived from various culture systems (FAO, 2012).

Likewise the growth in trade of ornamental fish has increased at a similar rate to food aquaculture where the global wholesale value of live ornamental fish in 2009 was estimated by the FAO to be US$ 750 million with a retail value of US$3 billion (see Figure 1.1). Approximately 1 billion ornamental fish were exported annually involving more than 100 countries. In 2009, the top five exporting countries were Singapore (19% by value), Spain (14%), Japan (7%), Czech republic (5%) and Israel (4%). In the same period, the top five importing countries were USA (13%), Italy (12%), United Kingdom (7%); Singapore (6%) and Germany (6%) (F.A.O. 2012). The ornamental fish industry produced an average annual growth rate of 14% since 1985 and the entire value of the industry inclusive of retail sales, associated materials, wages and non-exported product was estimated to be approximately US$15 billion (Bartley, 2000). Indeed, in the UK market research conducted by the Ornamental Aquatic Trade Association (OATA, 2004) estimated that 14% of all households

own an aquarium or pond, making fish the third most popular household companion animal after cats and dogs. In fact Davenport (2001) calculated that in 1999 the UK ornamental fish trade was worth approximately £12million.

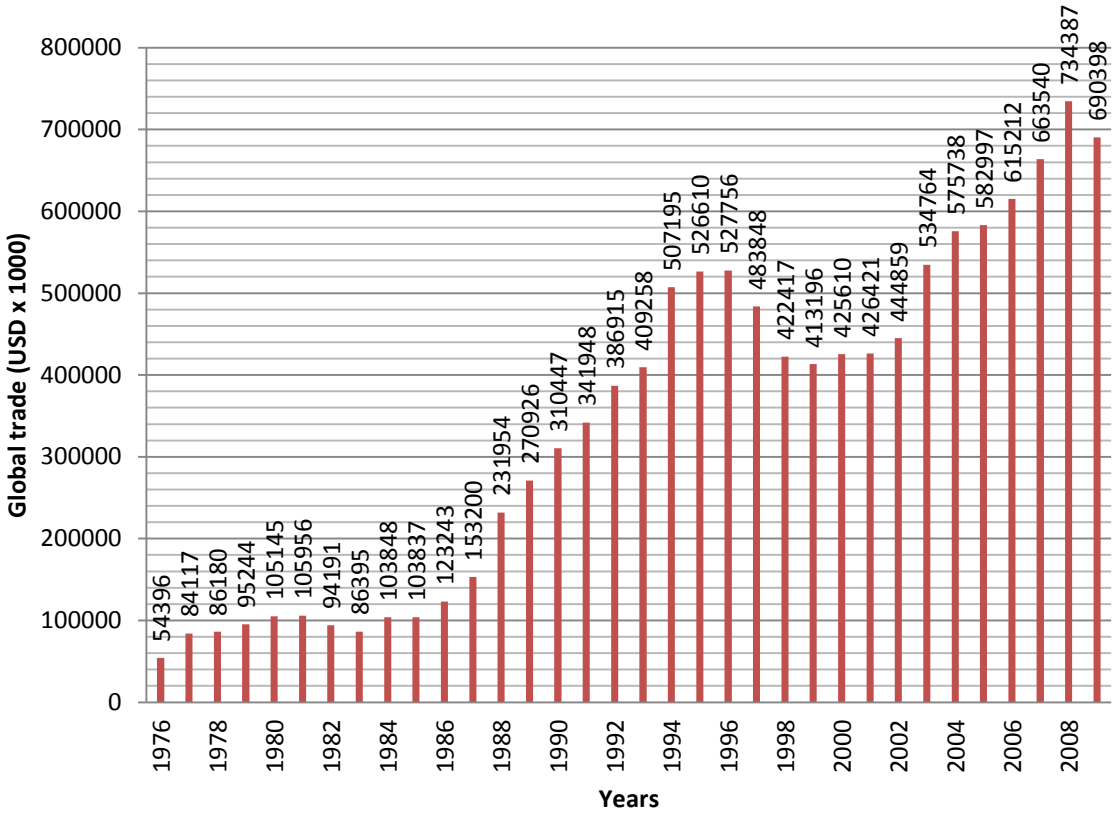


Figure 1.1. Total global wholesale value of ornamental fish species from 1976 to 2009 (F.A.O. 2012)

Advances in culture techniques and introduction of new species have contributed to the rapid growth of the aquaculture sector, resulting in a global initiative to introduce the concept of sustainable practice. Indeed, Norway one of the leading aquaculture producers in continental Europe has already ratified such measures as eco-friendly and stable production to mitigate public perceptions of non-sustainable practice (Norwegian Ministry of Fisheries and Coastal

Affairs, 2009). Although an important step from a legislative perspective, in practice the increase in global demand of fish for both food and aesthetic value has presented a significant problem for the aquafeed industry. For nearly a decade the challenge has been how to fuel such rapid growth. Up until recently fishmeal and fish oil have been heavily utilised in feeds primarily because they fulfil the nutritional requirements of nearly all farmed and pet fish (N.R.C. 2011). Indeed, in 2006 alone, the aquaculture sector consumed 3724 thousand tonnes of fish meal and 835 thousand tonnes of fish oil constituting nearly 85% of the total annual consumption of these feed commodities (Tacon and Metian, 2008; Tacon *et al*., 2010). Many factors including good amino acid profile, high nutrient digestibility and lack of anti-nutritional factors have been well documented and compiled to demonstrate the quality of fish meal and oil (N.R.C. 2011). Subsequently, fishmeal has become the 'gold standard' to which alternative feedstuffs must be compared.

Dietary protein is the major and most expensive component of practical aquafeeds (Wilson *et al*. 2002). With a growing interest in non-fishmeal protein sources for aquafeeds, it is important to sustain comparable levels of feed intake, feed conversion efficiency, as well as growth rate and survival in fishes. Therefore, establishment of optimal dietary requirements of amino acids (AA) and characterization of alternative protein/AA sources have been a major focus of fish nutrition research. Of particular interest in the commercial sector is the principle of ''least-cost'' aquafeed formulation that has been evolving in recent years towards customer-based aquafeeds, which has led to the concepts of ''functional aquafeeds'' and ''environmentally oriented aquafeeds''. Despite the efforts to substitute away from fishmeal and fish oil in aquafeeds many consumers, farmers, purchasers and policy makers remain unclear about the suitability and sustainability of alternative commodities. Indeed to be a viable alternative protein a feed commodity must possess certain characteristics including: wide availability, ease of handling and shipping and more

importantly nutritional characteristics such as low level fibres, high protein content targeting approximately 48-80% crude protein favourable amino acid profile, high nutrient digestibility and palatability (Gatlin *et al*., 2007; Naylor *et al*., 2009).

Contrary to mainstream aquaculture in the ornamental industry fish hold more profit in their inherent aesthetic value therefore the emphasis for feeds is not to improve growth performance *per se*, but to deliver nutrients for maintenance and disease resistance. Indeed because of the high mark-up price of most ornamental aquafeeds compared to commercial feeds, there is more potential to use high value dietary specifications. In this respect such dietary specifications have been commonly termed non-conventional feed resources (NCFRs) that include insect proteins and by-products from industrial operations such as single cell proteins and whey/ casein proteins. These feed commodities although would be too expensive to use in practical feed formulations, they have more value in the ornamental aquafeed industry to improve both longevity and perhaps more importantly the quality of ornamental fishes.

1.2 Potential feed commodities to replace fishmeal

Conventional aquaculture diets, particularly diets for carnivorous fish species, are nutrient dense and may contain up to 450 g crude protein (CP)/kg (N.R.C. 2011). Such diets preclude the use of ingredients with only moderate CP content, such as pulses including peas and faba beans or oilseed meals including canola/rapeseed meal and flax. As a result a number of candidate sources have been shortlisted, based on the criteria as outlined above after Gatlin *et al*., (2007), as potential non-fish protein sources for use in ornamental aquafeeds including earthworm protein, a number of plant protein concentrates and whey/ casein protein.

Indeed, since Lawrence and Miller (1945) first reported on the protein content of earthworm, many nutritional evaluations have been published (French, 1957; Needham,

1957; McInroy, 1971; Sabine, 1978; Stafford and Tacon, 1985; Blair, 1985; Boushy, 1986; Edwards and Neuhauser, 1988; Pereira and Gomes, 1995; Zhenjun *et al*., 1997). Of particular note Sabine (1978) reported that *Eisenia foetida* was considered to be a species with relatively high protein content of 58-71% dry weight. Similarly, Zhenjun *et al.*, (1997) reported that *E. foetida* had a similar crude protein level to Chinese and Peruvian fishmeal; however the actual nutritive value of a feed protein is determined essentially by the content, proportion and availability of its amino acid profile. In this context, Sabine (1983) reviewed earthworm amino acid profiles from a number of authors and concluded that earthworm protein was high in essential amino acids including the sulphur amino acids methionine and cysteine (Table 1.1).

In fish Stafford and Tacon (1984) were the first to report that rainbow trout fed worm meal from *Dendrodrilus subrubicundus* at 70 and 360 g/kg replacement of fishmeal showed similar growth performance to fish fed fishmeal diet. Likewise, Stafford and Tacon (1985) fed rainbow trout *E. foetida* at inclusion levels of 5 - 30% and reported no adverse effects towards growth performance. Furthermore Nandeesha *et al*. (1988) fed earthworm meal to common carp at inclusion levels of 200 and 250 g/kg replacement of fishmeal and found no adverse effects on growth rates. Since the late 1980's this potential feed commodity has received little research focus for reasons that are currently unknown. Despite this, earthworm protein has a high biological value and in addition has the potential to confer health benefits to ornamental fishes through the recognition that earthworm ceolomic fluid contains a number of immunogenic properties including bacteriostatic, proteolytic, cytolitic and mitogenic (Vetvicka *et al*., 1994; Cooper *et al*., 2002; Cooper and Roch, 2003). In this context the recognition that a feed commodity can add value at no extra cost is an important feature for the ornamental industry. Indeed the emphasis of modern aquafeeds is develop the

concept of immunonutrition and deliver high quality feeds that may support optimal growth as well as improve fish welfare, this aspect will covered in more detail later in the review.

Table 1.1. Essential amino acid composition of selected earthworm species compared to fishmeal (g 100 g of protein) taken from Sabine (1983)

	McInroy (1971)[a]	Taboga (1980)[b]	Sabine (1981)[a]	Graff (1981)[a]	Graff (1981)[c]	Fishmeal (herring meal)[d]
Arginine	6.1	7.3	6.8	6.1	6.1	6.7
Histidine	2.2	3.8	2.6	2.3	2.6	2.0
Isoleucine	4.6	5.3	4.2	4.7	4.5	3.5
Leucine	8.1	6.2	7.9	8.2	7.9	6.4
Lysine	6.6	7.3	7.1	7.5	7.1	6.9
Methionine	1.5	2.0	3.6	1.8	2.0	1.5
Phenylalanine	4.0	5.1	3.7	3.5	4.1	3.5
Threonine	5.3	6.0	4.8	4.7	4.8	3.3
Tryptophan		2.1				0.5
Valine	5.1	4.4	4.9	5.2	5.0	4.7

[a]*Eisenia foetida* species
[b]Mixture of *E. foetida* and *Lumbricus rubellus*
[c]*Eudrilus euginiae*
[d] Fishmeal (taken from Morrison, 1957).

Contemporary research focus has been skewed towards using plant based feedstuffs in animal feeds typically reflecting the low cost of production and wide availability (for reviews see Gatlin *et al*., 2007; Naylor *et al*., 2009; Hardy, 2010). Plant proteins are increasingly being used in commercial feeds for salmonids to offset the use of fishmeal mainly because manufacturing costs and accessibility are in line with that of fishmeal (Figure 1.2). The most commonly researched plant based feed commodity are soybean meal (SBM) and soy protein concentrate (SPC) which have high protein content and contain the majority of essential amino acids to support conventional fish growth rates. Soybean is the leading oilseed crop produced globally with a projected production to exceed 200 mmt (Gatlin et al. 2007). Indeed trade routes already exist for the global distribution soybean making this feed commodity and ideal candidate to replace fishmeal. Storebackken *et al*. (2000) highlighted that soybean has a

relatively low cost compared to fishmeal, comparible digestible protein levels, comparible AA levels and protein content. A large part of the production of soybean is the extraction of the oil content which yields a cake of high quality protein. This cake is then processed further using various techniques, including heat treatment and alcohol extraction, to remove or deactivate antinutritional compounds (ANC), oligosaccharides and fibre (for review see Drew *et al*., 2007). Subsequently this yields a wide array of soybean products such as soy flour, SBM, SPC and soybean protein isolate (SPI) which are all products of processed soy flour and have all been evaluated to a certain extent as dietary ingredients for fish.

Research has shown that SPCs, produced mainly through aqueous ethanol or methanol extraction of defatted soy flakes, supports better growth than soybean meal or soy flour when fed to salmonids (Murai *et al.,* 1989; Shimeno *et al.,* 1993; Olli *et al.,* 1994; Kaushik *et al.,* 1995). Mambrini et al. (1999) demonstrated that a soy protein concentrate (Soycomil™) could replace up to 50% of the dietary protein from fishmeal without any negative effects towards growth performance when fed for 90 days to rainbow trout (Oncorhynkuss mykiss). Likewise, Storbakken *et al*. (1998, 2000) fed diets to Atlantic salmon replacing 75% of the dietary protein from fishmeal with Soycomil™ and reported that after 84 days of feeding there was no adverse effects towards growth performance. Also Kaushik *et al*. (1995) reported that 84 days of feeding SPC at levels ranging from 33 to 100% replacement of fishmeal had no adverse effects on rainbow trout growth performance or nutrient utilisation. Similarly, Ollie *et al*. (1994) on comparison of four soybean products fed to rainbow trout (solvent-extracted soybean meal, de-hulled and solvent-extracted soybean meal, de-hulled full-fat soybean meal and SPC), concluded that the nutritive value of SPC is comparable to that of fishmeal.

Escaffre *et al*. (1997) reported that replacement of fish meal with up to 40% of dietary SPC had no adverse effect on growth performance or survival rate of common carp larvae.

Likewise, Jackson *et al*. (1982) reported that tilapia fed diets containing 250 g/kg soybean meal (SBM) inclusion had no adverse effects on growth performance. On the contrary Stickney *et al.* (1996) reported that rainbow trout fed diets at 70 and 100% replacement of fish meal with SPC had reduced growth performance. Similarly, Kissil *et al*. (2000) reported that in seabream fed diets replacing 60 and 100% of fishmeal protein with soy protein concentrate significantly reduced growth performance. However, as no evaluation of the amino acid profiles of the soybean products was investigated it is difficult to establish the actual biological value of each soybean products. Despite this there have been several recent investigations where the use of plant protein concentrates have allowed for fishmeal replacement in excess of 50 % without any adverse effects towards fish growth performance and feed utilisation (Burr *et* al., 2012; Collins *et al*., 2013). Certainly research in this area will continue to improve the quality of plant protein source for use in aquafeeds for food fish and the ornamental sector.

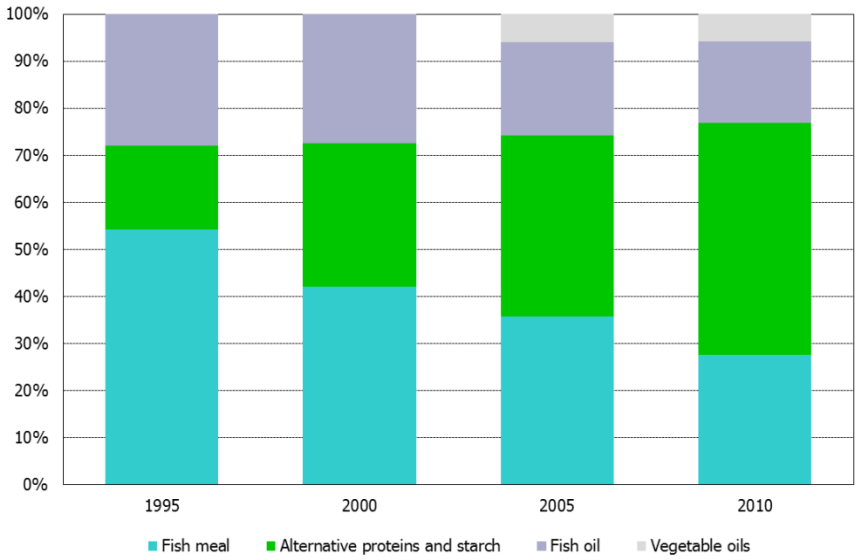


Figure 1.2. Changes in composition of salmon feeds over time with substitution of fishmeal and fish oil (Tacon *et al*., 2011)

Whey representing ~20% of milk protein is a co-product of cheese making and casein manufacture in the dairy industry. Following the casein the curd separates from the milk, after coagulation of the casein proteins through the action of chymosin (rennet), the remaining watery thin liquid is known as whey (Zadow, 1994). Research to date has demonstrated that whey protein is highly digestible and has high crude protein content (~70-80% dry matter), for these reasons it is the protein of choice for elite athletes and immuno-suppressed patients (Buckley *et al*. 1998; Playford *et al*. 1999; Coombes *et al*. 2000; Ha and Zemel, 2003; Rankin and Darragh, 2006). Currently whey protein concentrates are manufactured through modern membrane processing technologies including industrial applications of microfiltration and ultra/diafiltration (Smithers, 2008). Current knowledge of the use of whey derived ingredients for use in animal feeds is limited. Despite this whey protein concentrates and casein proteins have been extensively used in semi-purified diet formulations in fish vitamin and mineral investigations for several decades (Halver, 1982, Halver, 1989). A comprehensive review of milk derived bioactive factors, including those from whey and casein proteins, has been published by Korhonen (2009), thus this section will only focus on key milestones that could have an impact on the use of these commodities in ornamental aquafeeds.

Research to date using mammalian models has demonstrated that whey and casein proteins are complete proteins whose biologically active components provide additional benefits which enhance immune function, see Table 1.2 (Playford *et al*., 2004). Specific bioactive proteins such as insulin-like growth factor (IGF-I and II), platelet- derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor (TGF-β) and betacellulin have all been identified and all play major roles in cell growth, proliferation and differentiation (Rogers *et al*., 1995; Francis *et al*., 1995; Pakkanen and Aalto, 1997; Dunbar *et al*., 1999, Elfstrand *et al*., 2002). In addition whey proteins have been shown to contain

significant levels of immunoglobulins in the range of 300-600 mg/ l (Smithers, 2004; Ayers *et al*., 2003). These immunoglobulin-rich isolates could impart a passive immunity to the target animal and evidence suggests they play a major role in combating infections and even enhance gut health (Buckley *et al*. 1998; Coombes *et al*. 2000; Mehra *et al*. 2006; Mero *et al*., 1997; Playford *et al*. 1999; Kunwar and Kunwar, 2009). Indeed, Kunwar and Kunwar, (2009) concluded that Enprocal, a whey protein concentrate (WPC) supplement, through down regulation of proinflammatory cytokines TNF-α and IL-1ß, which are cytokines involved in inflammation and are members of a group of cytokines that stimulate the acute phase reaction, appears to be beneficial in reducing the effects of gut inflammatory responses which could have significant implications to alleviating symptoms of irritable bowel disease and Chrons disease in humans. As of yet the application of whey protein isolates and concentrates on animal digestive disorders is still to be elucidated.

Casein is the major component of protein found in bovine milk accounting for nearly 70-80% of its total protein and is responsible for the white colour of milk. It is the most commonly used milk protein in the industry today and is isolated from milk by acid or by rennet precipitation. The acid, or isoelectric, precipitation is performed at pH 4.6, where the caseins precipitate and the whey proteins remain soluble. Caseins are flexible and heat stable proteins that are of significant physiological importance to the body for functions relating to the uptake of nutrients, vitamins and they are a source of biologically active peptides (Hambraeus, 1992). Casein also contains the minerals calcium and phosphorous which are important minerals for the development and maintenance of the skeletal system and participate in several other physiological processes such as muscle contraction, blood clot formation, nerve impulse transmission, the maintenance of cell integrity and acid-base equilibrium.

The amino acid composition is the most important factor in defining food protein quality, followed by the digestibility of the protein and the bioavailability of its amino acids. Because of their amino acid composition the main bovine milk proteins, caseins and whey proteins, can be regarded as a complete source of amino acids (Hambraeus, 1992)

Table 1.2. Target Functions, Mechanisms, and significant of Action of Whey Components taken from Walzem *et al*. (2002).

Target functions	**Bioactive compound**	**Mode of action**	**Significance**	**Reference**
Growth stimulant	IGF-1	Cell cycle of intestinal cells	Promotes tissue repair	Corkins *et al.* (1999)
Maturation of cells	TGFβ	Bind to and activate natural receptors on enterocytes	Maintains intestinal integrity	Donnet-Hughes *et al.* (2000)
Protection from disease	Lactofericin	Disrupt pathogenic bacterial membrane	Pathogen inhibition	Yamauchi *et al.* (1993)
Prevention of disease	Lactoferrin	Stimulate beneficial bacteria	Prebiotic effects	Petschow *et al.* (1999)
Elimination of foreign molecules	Immunoglobulins	Endotoxin binding	Toxin excretion	Boes *et al.* (1998)

1.3 Disease in Aquaculture sector

Reports by FAO consider disease outbreaks as a significant constraint to the development of the aquaculture sector, with a global estimate (made by the World Bank in 1997) of disease losses in the range of US$3 billion per year. This significant problem has been facilitated by anthropogenic translocation of living or fresh-frozen aquatic organisms where in cases the emergence of serious infectious diseases have manifested in regions with no prior evidence of disease due to a particular pathogen (Farley, 1992; Ganzhorn *et al*., 1992; Lightner *et al*.,1992). Examples include the introduction of *Gyrodactalus, Nebendenia* and pilchard herpesvirus to Norway, Japan and Australia (Gaughan, 2001; Gaughan *et al*., 2000; Johnsen and Jensen, 1991; Ogawa *et al*., 1995). Moreover, major economic losses to aquatic stocks followed the intercontinental translocation of whit spot virus (Lightner *et al*., 1992) in commercial prawns, whirling disease in salmonids (Hederick *et al*., (2003) and koi herpesvirus in ornamental and food species of koi and common carp (St-Hilaire *et al*., 2005).

Despite measures such as increased quarantine controls, better risk assessment and hazard indication by countries currently trading in the aquaculture sector, additional measures need to be implemented to prevent further transmission of disease. One approach would be to emulate what has already been implemented in human medicine where patients under conditions of severe stress such as after major surgery, are placed on enhanced recovery after surgery (ERAS) protocols. ERAS is based on robust evidence where patients receive through oral intervention an increased calorie intake and excess supply of important exogenous nutrients to support necessary functions to restore and repair damaged tissues (Grimble, 1996; Lewis *et al*., 2001; Basse *et al*., 2002; Grimble, 2007; Lewis *et al*., 2009). The introduction of ERAS has led to significant reductions in patient's recovery periods post-surgery, thus reducing hospital patient costs (Eskicioglu *et al*., 2009; Lassen *et al*., 2009; Melnyk *et al*., 2011). Built upon the successes of the ERAS programme the concept of

introducing enhanced nutrition could have significant implications in reducing mortality rates in all aspects aquaculture practice. Therefore further research is required to develop enhanced feeds that could help mitigate pathogenesis and subsequently improve fish quality pre and post stressful situations such as when fish are being transported.

1.4 Linking fish nutrition and immunology

The notion you are what you eat is a familiar phrase and so this idea is largely based on our comprehension of the linkage between nutrition and immunology. In well- nourished humans, qualitative changes in macronutrients as well as supplementation of single nutrients influence immune functions (Albers *et al*., 2005). So, besides genetic and environmental factors, the nutritional status of the fish can be considered as a major factor that influences the resistance to infection. The application of nutrients for this purpose is referred to as immunonutrition and has been defined in human medicine as 'modulation of the activities of the immune system, and the consequences on the patient of immune activation, by nutrients or specific food items fed in amounts above those normally encountered in the diet' (Grimble, 1996).

The teleost immune system is well-developed to operate an efficient defence mechanism in the wild thus the endogenous sources of nutrient supply the basic requirements to augment an immune response. In contrast under such conditions where fish are kept in close confinement the opportunity for a disease is increased (see Figure 1.2), thus the energy and nutrient requirements increase and so the exogenous nutrient supply becomes critical to maintaining homeostasis.

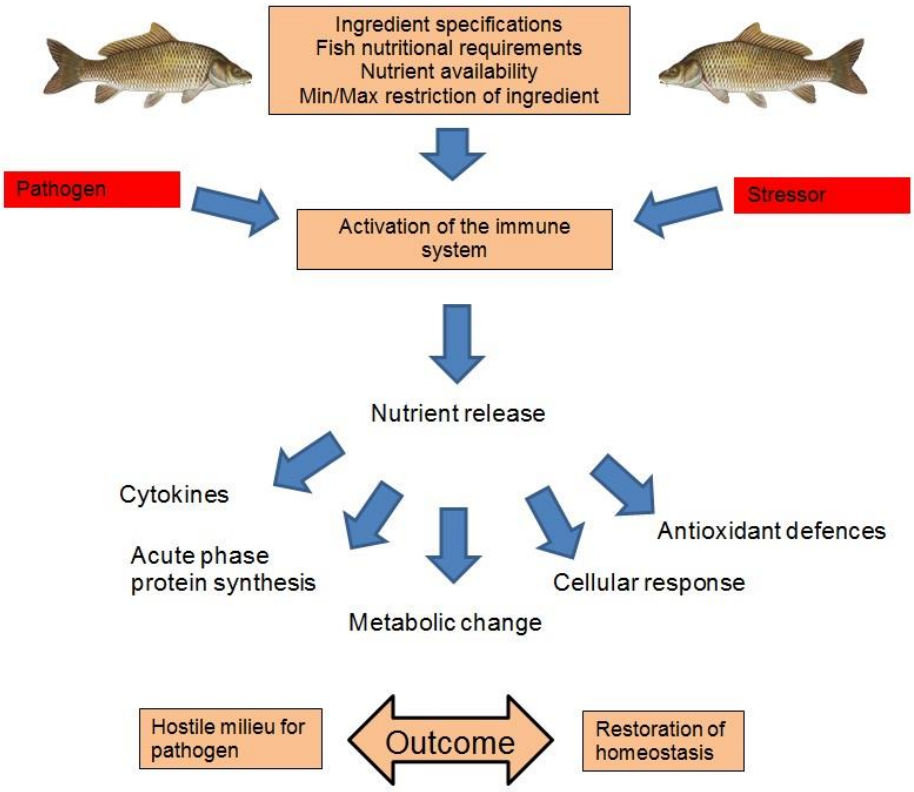


Figure 1.3. The concept of immunonutrition in preventative health adapted from Kiron (2012).

Not until recently has it been widely accepted that nutrition has major health implications in fish (Trichet, 2010), as such the interaction of amino acids, lipids and vitamins/minerals are important in cellular division and the synthesis of key soluble factors such as cytokines, complement and acute phase proteins (Trichet, 2010; Calder, 2006). As feeds probably represents the single largest expense to all aquaculture research and development in this area is necessary to continue to advance understanding and facilitate through nutritional intervention more effective strategies to combating disease. In order to understand the evolution of the immune system and in its interaction with macro-ingredients the next section

will briefly introduce the main functions of the immune system and how the fish immune system has evolved and adapted to living in a highly antigenic environment.

1.5 Overview of the immune system

There are two main recognised arms of the immune system the innate (non-specific) and the acquired (adaptive) immune responses. However, an increasing body of evidence, both from mammalian and fish immunology indicate that both systems operate synergistically to combat disease. Innate responses in vertebrates and invertebrates are thought to precede the adaptive responses in so much that the innate responses activate and determine the nature of the adaptive response. Thus cooperating in the maintenance of homeostasis during development, growth and following tissue damage (see Figure 1.3; Fearon and Locksley, 1996; Fearon,1997).

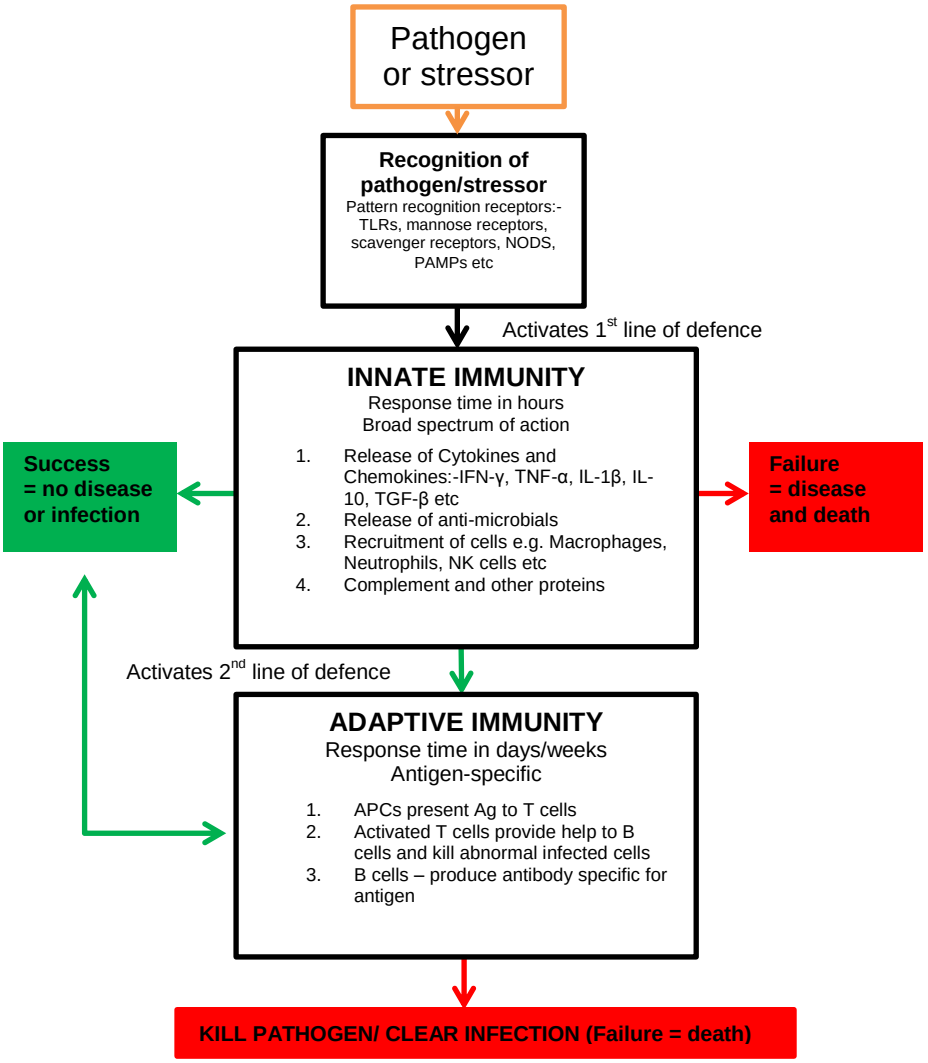


Figure 1.4. Simple schematic showing the immune response to an infection threat. The response can be divided into three stages as outlined above in the black boxes.

1.5.1 Innate immunity

The innate immune system is an evolutionary ancient system present in both invertebrates and vertebrates and so is characterised by being non-specific. It is mediated by germ-line encoded parameters namely pattern recognition proteins or receptors (PRP/R). These parameters identify conserved molecular patterns called pathogen associated molecular patterns (PAMPs) associated with microbes and inherent danger signals from malignant tissues or apoptotic cells (Medzhitov and Janeway, 2002). Typical PAMPs include polysaccharides, glycoproteins such as bacterial lipopolysaccharides (LPS), peptidoglycans, DNA CpG motifs and virus associated double stranded RNA (dsRNA) see Figure 1.4, (Janeway, 1989; Medzhitov and Janeway, 2002). The advantage of the innate system through the process of being inducible by external molecules allows for a rapid response, which has been tailored by environmental factors and pathogenic associations. As a result the specificity of the innate defence is an inheritable trait that provides a preliminary line of defence (Medzhitov and Janeway, 1998; Tort *et al*., 2003;Alverez-Pellitero, 2008).

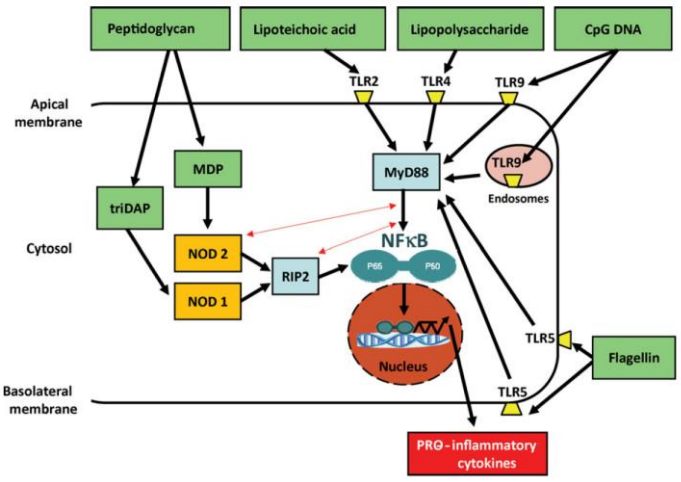


Figure 1.5. Sensing of molecular moieties produced by invading microbial pathogens (PAMPs) mediated by PRRs. These PRRs include toll like receptors (TLRs) which are triggered by bacterial moieties. MyD88 is an essential core intermediary molecule for most TLRs leading to activation of transcription factor NFκB the key cell signalling molecule for inflammation. Figure was taken from Knight *et al*. (2008)

1.5.2 Adaptive immunity

The adaptive arm is relatively recent in development and as the name suggests it is acquired during early stages of development. It is assumed to appear in jawed vertebrates about 400-500 million years ago (Tort *et al*., 2003). The key components in the evolution of the adaptive system are the appearance of the thymus, the B- and T-lymphocytes and the RAG (recombination activation gene) enzymes, which through the process of gene rearrangement can generate the observed diversity of the immunoglobulin superfamily (B- and T-cell receptors and the major histocompatibility complex). Unlike the innate system the components of the adaptive system are not germ-line encoded; however it has an impressive capacity to recognise and respond to very specific structures presented by pathogens (Agrawal *et al*., 1998). This results in an unlimited diversity of pathogen recognition and so the specific activity reflects the disease history of the individual.

1.5.3 The teleost immune system

From an evolutionary perspective fish are considered as the earliest class of vertebrates having both innate and adaptive immunity. The fish immune system operates at the crossroads between the innate and adaptive responses and is habituated to the environment and the piokilothermic nature of the fish (Tort *et al*., 2003). The aquatic environment is highly antigenic and thus the external barriers of the fish such as skin, gills and digestive tract play an important role in controlling potential infectious routes. Such protective barriers are reinforced by the production of mucus containing a number humoral soluble compounds such as lectins, pentraxins, lysozymes, complement proteins, antibacterial peptides and immunoglobulin (IgM, IgT, IgZ), which have an important role in inhibiting the entry of pathogens (Alexander and Ingram, 1992; Rombout *et al*., 1993; Boshra *et al*., 2006; Magnodottir, 2006). Subsequently the innate immune response in fish is commonly divided into three compartments: epithelial/mucosal barrier, the cellular components and humoral components. In contrast the adaptive responses of fish, predominated by a humoral IgM antibody response are generally recognised to be slower to develop when compared to the mammalian counterparts (Ellis, 2001, Magnadottir, 2010, Trichet, 2010). So, when confronted by a highly variable and antigenic environment, the fish immune response is predominated by a broader range of innate responses characterised by a lack of antigen specificity and memory compensating for a relatively slow reacting and adaptive immune response.

In the wild, fish have a well-developed and complex innate system that maybe constitutive or responsive (Ellis, 2001, Magnodottir, 2010). In contrast, in a farm or even a tank the infection pressure is much greater due to the physical constraints. Upon infection systemic innate immune responses can provide an early defence against the pathogen; however as is in most cases pathogens are adept at evading these responses and infecting fish

that are generally weak (Magnodottir, 2010). Consequently the immune response of the fish operates at two distinct levels local and systemic (Table 1.3).

Table 1.3. The components and functions of the immune system of fish adapted from Scheley and Field (2002)

Immune system	Type of defence	Physical components	Modes of action
Innate immune system	Physical barriers	Skin, gills, scales, mucus membranes	Prevent the entry of antigens from entering systemic circulation e.g. pathogenic bacteria, parasites
	Cell mediated barriers	Phagocytic cells e.g. neutrophils, macrophages	Phagocytosis, secretion and activation, cytokine production, T-lymphocyte stimulation
		Inflammatory cells e.g. mast cells, basophils	Release of inflammatory mediators e.g. histamine, prostaglandins
		Natural killer cells	Induce apoptosis of infected or malignant cells. Synthesise and secrete IFN-γ
	Humoral mediated barriers (soluble factors)	Complement system	Complement activation. Cause apoptotic cell death
		Interferons/ Mx- proteins	Inhibit virus replication
		Transferrin	Chelates iron- inhibits growth of bacteria. Activates macrophages
		Chemokines	Activate/recruit other cells to site of infection
		Acute phase protein	Promote the repair of damaged tissues
		Lytic enzymes	Modulation of surface charge of bacteria to facilitate phagocytosis
		Antiproteases	Restrict bacteria to grow *in vivo*
		Antimicrobial peptides	Induce precipitation and agglutination reactions. Activate complement. Induce cytokine release
Adaptive immune response	B-lymphocytes	Plasma cells	Secrete antibodies
	T-lymphocytes	CD4+T-cells	Induce activation of lymphocytes
		Th1 cells	Promote cell mediated responses
		Th2 cells	Promote humoral (antibody) responses
		CD8+ T-cells	Cytotoxic action – destroy infected and malignant cells Suppress activity of lymphocytes

1.5.4 Immune tissues of fish

Despite the obvious differences between mammals and fish, fish depend on both cellular and humoral immune responses and have specialised organs dedicated to the immune response. However the morphology of the immune system is quite different between fish and mammals. Perhaps the most obvious difference is the fact that fish lack bone marrow and lymph nodes. Instead the head kidney serves as a major lymphoid organ, in addition to the thymus and the spleen (Press and Evensen, 1999). Despite this, the secondary lymphoid organs seen in mammals are also seen in fish (see Figure 1.4; Tort *et al*., 2003).

The foremost part of the teleost kidney, commonly referred to as the head kidney (pronephros), is alomerular, bifurcates at the anterior part and penetrates beneath the gills (Zapata *et al*., 1997). The head kidney is an important hematopoietic organ, with morphological similarities to the bone marrow in higher vertebrates (Meseguer *et al*., 1995). Moreover the head kidney is a major producer of antibodies and contains sinusoidal macrophages that act synergistically with the endothelial cell layer in the sinusoids to trap passing particles and molecules from the blood stream (Dannevig *et al*., 1994; Brattgjerd and Evensen, 1996). Subsequently trapped antigens may then be retained for long periods of time by aggregations of pigment-containing melano-macrophages, also known as melano-macrophage centra (Agius and Roberts, 2003). Indeed, it has been suggested that these aggregations of melano-macrophages, are primitive analogues to the germinal centres of the lymph nodes in mammals (Ferguson, 1976; Ellis, 1980). This makes the head kidney serve not only as a primary, but secondary lymphoid organ (Kaattari and Irwin, 1985). Further, the head kidney serves as an endocrine organ releasing cortocosteriods and other hormones and so plays a key role in the immune-endocrine interactions.

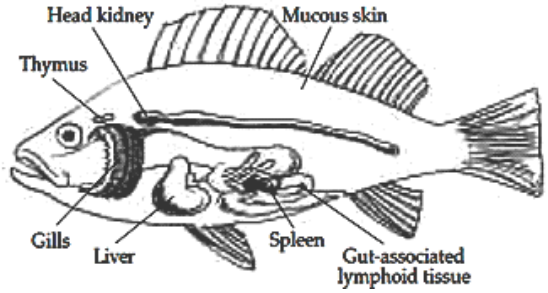


Figure 1.6. Immune structures in the teleost fish taken from Tort *et al*. (2003).

1.6 Nutrients in aquafeeds and effects on immune responses

Nutrients, essential or non-essential, are well documented to have a significant effect on stress tolerance and health (Calder and Kew, 2002; Trichet, 2010; Kiron, 2012). Evidence from mammalian studies has demonstrated there are a number nutrients that are important in maintaining an efficient immune system including essential amino acids (EAAs), the essential fatty acid linoleic acid and micronutrients such as Zn, Cu, Fe and Se (for reviews see Gross & Newberne, 1980; Chandra, 1991; Kuvibidila *et al*., 1993; Scrimshaw & San Giovanni, 1997; Calder & Jackson, 2000). Current understanding on the role of dietary nutrients on fish health is limited to vitamin C and E and lipids. This may partly be due to the fact that commercial aquafeeds generally contain essential nutrients in excess of the dietary needs of the animal. However, from a physiological perspective, fish growth and immune response to nutrients is a complex process that apart from genetic background depends on other mutually interdependent processes, such as development, nutrition, metabolism and physiological stress. Moreover these processes are mediated by multiple tissue contributions (e.g. liver, intestine, pancreas and muscle, head kidney, spleen), so in order to establish a complete view a more global and multidisciplinary approach is required. Despite this there are a number of studies that have already established basic knowledge regarding the effect of

feeding selected macronutrient sources on the immune responses of fish, therefore the following section attempts to summarise these studies.

1.6.1 Protein

Proteins are composed of up to 20α - amino acids linked into chains by peptide bonds. The chains are cross- linked by disulphide bridges, hydrogen bonds and van der Waals forces. The amino content of proteins, particularly current protein sources used in aquafeeds can differ markedly. Consequently the capacity of different feedstuffs to meet the amino acids requirements of fish will differ considerably for example in Table 1.5, the concentration of lysine in all three plant feedstuffs is deficient compared to fishmeal. This is reflected in the fact that lysine is known to be the first limiting AA in most vegetable proteins (Gomez-Requeni *et al*., 2011). Thus, lysine availability could have significant implications towards achieving optimal growth and immuno-competence of fish.

In the context of animal nutrition, protein generally refers to crude protein (CP); that is, N x 6.25 (1/0.16 =6.25), a definition based on the assumption that proteins contain 16 percent N. In this context the requirement of fish for dietary protein can be broken down into two components; firstly, a need for the essential amino acids that the fish cannot synthesise either at all or at a rate proportionate with its requirements for protein deposition or equal with the synthesis of a variety of other compounds with metabolic functions; and secondly, a supply of either non-essential amino acids or sufficient amino nitrogen to enable fish to synthesise these amino acids. On this basis fish, as with all monogastric animals, do not have specific protein requirement as such, but require the AA that compose proteins (Wilson, 2002). In fish nutrition the protein requirement includes both the essential EAA and non-essential amino acids that provide undifferentiated nitrogen for the synthesis of nitrogenous compounds that are of physiological and immunological importance.

Table 1.4 The 10 essential amino acid composition of common aquafeed ingredients vs the amino acid requirements for two common aquaculture fish species (% as fed basis; taken from N.R.C. 2011)

	Fushemal (menhaden)	Soybean meal (Solvent extracted)	Corn gluten meal	Wheat gluten meal (VITEN®)	Common carp	Rainbow trout
Arginine	3.82	3.67	2.02	3.00	4.30	3.50
Histidine	1.45	1.22	1.31	1.85	2.10	1.60
Isoleucine	2.66	2.14	2.54	2.90	2.50	2.40
Leucine	4.48	3.63	10.20	5.60	3.30	4.40
Lysine	4.72	3.08	1.11	1.45	5.70	5.30
Methionine	1.75	0.68	1.63	1.50	3.10	0.84
Phenylalanine	2.41	2.44	3.96	4.00	6.50	5.20
Threonine	2.50	1.89	2.07	2.15	3.90	3.40
Tryptophan	0.65	0.69	0.43	0.80	0.80	0.50
Valine	3.22	2.55	3.09	3.20	3.60	0.00

Notes: N.R.C (2011) Nutrient requirements of fish

To clarify protein requirement is not an absolute value but is dependable on the bioavailability of the protein source, its AA profile and its digestibility to the target animal. Lower protein requirement is only achieved with a highly digestible protein source, a well-balanced EAA profile and adequate digestible protein to energy ratio (DP/DE). As previously mentioned fishmeal protein sources have delivered on all three levels, however with the increasing awareness that fishmeal and fish oil stocks are reaching their maximum exploitation contemporary research is focusing on alternative protein sources that can at the very least reduce the dependency of this feed commodity. It is now accepted that vegetable sources of both protein and oil may help to sustain comparable levels of feed intake, feed conversion efficiency, as well as growth rate and survival of fishes (Gatlin *et al*., 2007; Naylor *et al*., 2009; Hardy, 2010). Hence most of the focus regarding the effect of protein source on immune function in fish is using examples from studies involving plant sources.

Plants contain a number of anti-nutritional factors (ANFs) as a part of their inherent defence mechanism including lectins, saponins, phytic acid and protease inhibitors amongst others (for reviews see Francis *et al*., 2001; Krogdhal *et al*., 2010). As a result when fed to fish they have been shown to cause a number of severe pathologies including metabolic dysfunction in fish liver and liver steatosis (Martin *et al*., 2003; Sitjà-Bobadilla *et al*., 2005), inflammation in the intestine (Sitjà-Bobadilla *et al*., 2005; Bakke- Mckellep *et al*., 2007; Uran *et al*., 2008; Uran *et al*., 2009), reduced protein deposition (Gomez-Requeni *et al*., 2003) and an impaired resistance to infection (Krogdahl *et al*., 2000). To remove the harmful ANFs feed manufacturing companies have employed several methods including alcohol extraction which have facilitated the use of soybean based products in feeds for salmonids without causing enteritis or other gross morphological changes (Escaffre *et al*., 2007). Removal of heat-labile secondary compounds may be accomplished by extrusion or other heat treatment. However, elimination of heat-stable secondary compounds, and increasing the biological value of diets, requires fractionation of crops. Fractionation technologies range from low technology processes such as de-hulling to medium technologies such as air classification to sophisticated technologies such as aqueous and solvent protein purification as highlighted in Section 1.2. Currently replacement of up to 50 % of fishmeal with a mixture of plant proteins is possible in rainbow trout (*O. mykiss*) without affecting fish growth or immuno-competence (Escaffre *et al*,. 2007; Santigosa *et al*., 2008). Moreover other plant protein sources such as wheat gluten meal, corn gluten, sunflower, and pea meal when fed in excess of 30% fishmeal replacement show no adverse effects towards performance or disease (Hardy, 1996; Mente *et al*., 2003; Thiessen *et al*., 2003; Gill *et al*., 2006). Indeed studies on the nutritional value of processed plant proteins in various fish species have consistently shown improved digestibility and growth compared to feeding unprocessed ingredients (Hardy, 2010).

However, there are limits to use of plant protein in diets for fish particularly carnivorous species. Sitja-Bobadilla and co-workers reported that feeding gilthead seabream (*Spaurus aurata*) with diets containing high levels of plant protein, above 50% fishmeal replacement caused approximately a 66 % decrease in alternative complement activity and severe liver steatosis, in comparison to control fed fish. Moreover they reported that leucocyte head kidney respiratory burst activity and plasma myeloperoxidase activity was significantly elevated in fish fed diets with high plant protein sources indicative of an inflammatory response (Sitja –Bobadilla *et al*., 2005). Likewise in an earlier study by Krogdahl and co-workers they reported a higher level of mortality in Atlantic salmon (*S. salar*) when fed diets with high plant protein >50 % fishmeal replacement, following challenge with *Aeromonas salmonicida* (Krogdhal *et al*., 2000). Also significant increases were observed in lysozyme activity and IgM levels in the mid and distal intestine suggestive of a localised inflammatory response caused by the high inclusion of soybean meal and soy molasses. Despite the fact that research effort has focused on the effect of plant protein sources it is still important to explore the possibility of using other alternative feed commodities and this is particularly important in the development of feeds for the ornamental industry. As previously mentioned for ornamental aquafeeds there is potential for inclusion of high value dietary specifications such as insect and invertebrate meals, particularly in diets of cyprinids and salmonids (Satfford and Tacon, 1984; Rawling *et al*. 2012). This could have an immediate impact on improvement of fish quality, but before incorporation of such exotic ingredients into practical fish feed formulations challenges must be met by candidate ingredients in the form of availability, environmental impact and the potential presence of organic and inorganic contaminants.

Proteins *per se* are antigenic, and so animals and humans regulate this by having immune tolerance mechanisms at the level of the intestinal mucosa (Macdonald and

Monteleone, 2005; Rescigno *et al*., 2008; Sakaguchi *et al*., 2008; Hadis *et al*., 2011). This system of regulation works well in higher vertebrates, where gut associated lymphoid tissue (GALT) consists of both organised lymphoid tissues, such mesenteric lymph nodes (MLN) and Peyer's patches (PP), and more diffusely scattered lymphocytes in the intestinal lamina propria (LP) and epithelium including a large number of IgA^+ plasmablasts (Forchielli and Walker, 2005) . In contrast it is generally accepted that fish lack an organised GALT, thus do not contain follicular lymphoid structures, but rather a more diffusely distributed gut-associated lymphoid tissue containing many lymphoid cells, macrophages, eosinophilic and neutrophilic granulocytes (Zapata and Amemiya, 2000; Bernard *et al*., 2006a, Huttenhuis *et al*., 2006). Fundamental knowledge regarding the interaction of nutrients and gut immune responses of fish are very limited (Rombout *et al*., 2010). However it is apparent that protein source can have a significant impact on intestinal inflammatory responses as shown by numerous investigations regarding the use of plant proteins (Bakke-Mckellep *et al*., 2007; Uran *et al*., 2008, Krogdhal *et al*., 2010). Perhaps this reflects to some degree that if fed to fish in high inclusion levels there is still enough presence of ANF's in plant sources to attenuate growth performance and immuno-competence. As previously stated, if the initiative is to include plant protein sources to offset the use of fishmeal a multidisciplinary approach is required in order to establish a more holistic view.

Currently, the application of genomics and transcriptomics has risen as powerful integrative tools to reconstruct the pathways and functional networks that govern the process of growth and immunity in fish. Recently, Panserat and Kaushik (2010) highlighted how genomic and post-transcriptomic tools have expanded the knowledge base of specific metabolic pathways and nutritional control of gene expression affected by dietary changes. In particular, Tacchi and co-workers pioneered the use of an Atlantic salmon "salar_2" microarray platform to study the changes in expression of genes associated with liver and

protein metabolism and immune function in Atlantic salmon. Specifically the gene ontology analysis, in the mid intestine of fish fed the plant protein compared to fish fed marine protein showed higher expression in a number of genes that are associated with enteritis. For example the gene for MyD88, which is an essential core intermediary molecule for most TLRs leading to activation of transcription factor NF-κß the key cell signalling molecule for inflammation, showed a 2-fold increase in expression. Moreover genes relating to protein and energy metabolism, mitochondrial activity/kinases and transport showed higher expression. In contrast genes relating to cell proliferation and apoptosis showed a lower expression in fish fed plant protein vs fish fed marine protein. This type of approach allowed for a more holistic perspective to understanding the metabolic pathways affected by fishmeal and fish oil replacement, thus complementing the basic husbandry techniques, which continue to be a valuable tool for assessing *in vivo* responses (Tacchi *et al*., 2012).

1.6.2 Amino acids

Amino acids are essential components of cells and tissues, so the right balance of amino acids in the diet for the target animal is important to achieve optimal growth performance and maintain a healthy immune system. Similar to mammals with the exception of arginine, fish have absolute requirements for 10 EAA including arginine, histidine, isoleucine, leucine, lysine, methionine, phenyalanine, threonine, tryptophan, and valine (N.R.C. 2011; Wilson 2002). Their role in immunity has been extensively studied in mammals where in fact it is known that isoleucine, is oxidised by human neutrophils and lymphocytes providing a source of N and energy for cellular proliferation and protein synthesis (Burns, 1975). Also glutamine, arginine, leucine, and valine have received much attention (Suchner *et al*., 2000; Roth, 2007). Interestingly, Glassy and Furlong (1981) studied the uptake of glutamine, valine and isoleucine by a B cell line as a function of progress through the cell cycle and showed

that the order of the rate of uptake was leucine > isoleucine >> valine. In addition they reported that the highest rate of uptake of the three amino acids was during the S-phase (DNA synthesis), with a progressive decline in uptake through the G2 and M phases coinciding with mitosis, followed by an increase in uptake through the G1 phase. The authors concluded that the rate of uptake most likely reflects the timing of protein synthetic activity (Calder, 2006).

Glutamine is the one of the most abundant free non-essential AA in the human body (Suchner *et al*., 2000). This amino acid is a precursor for other AA such as glutamate, histidine, proline and arginine as well as many other biologically important molecules such as proteins, nucleic acids (Boza *et al*., 2000), amino sugars (Ghosh *et al*., 1960), and glutathione (Higashiguchi *et al*., 1993; Roth *et al*., 2002). Also glutamine is used in mammals as major metabolic fuel for cells of the gastrointestinal tract and rapidly dividing cells such fibroblasts and lymphocytes (Buchman, 1996; Wiren *et al*., 1998; Curi *et al*., 1999). Likewise in fish glutamine is required as a major energy substrate for leukocytes and is a key modulator of cytokine and nitric oxide (NO) production (Buentello and Gatlin, 1999; Li *et al*., 2007). Currently compared to mammals (Anderson *et al*., 2002),the intracellular and intercellular pathways for glutamine synthesis and degradation in fish are still to be fully elucidated; however dietary glutamine supplementation has been shown to enhance weight gain, feed intake, intestinal development and digestive enzyme activities when fed to Jian carp (Yan and Zhou, 2006). Although glutamine in many senses is considered a non-essential AA it's actually very important to a number of cellular functions and so in human medicine is considered a conditional AA. As early evidence has shown from human research the application of glutamine as a supplement to the traditional diets certainly warrants attention.

Arginine is a dibasic AA which is obtained mainly from dietary sources. In mammals arginine is metabolised within the enterocyte via the arginase pathway to orthinine and urea (Suchner *et al*., 2002). However in fishes the metabolic pathway of arginine is still to be fully

elucidated. Despite this fish have a particularly high requirement for dietary arginine in comparison to other amino acids because firstly it is abundant in protein (as a peptide bound AA) and tissue fluid and secondly its *de novo* synthesis is limited or even completely absent in fish. Subsequently fish are completely reliant on exogenous sources of arginine to fuel the synthesis of a number of important proteins including proline and polyamines. Also arginine is an important immunomodualtory compound and two pathways of arginine metabolism have been identified in mammals as being crucial to this action: firstly, the 'arginase' pathway, where arginine is converted to urea and orthinine, thus generating polyamines by the action of orthinine decarboxylase (Eq. (1) taken from Satriano *et al*., 1999). This route of polyamine synthesis has been shown to augment lymphocyte mitogenesis (Klein and Morris, 1978).

$$\text{arginine} \xrightarrow{\text{arginase}} \text{ornithine} + \text{urea} \xrightarrow{\text{ornithinedecarboxylase}} \text{putrescine}$$
$$\xrightarrow{\text{supermidine synthase}} \text{spermidine} \xrightarrow{\text{spermine synthase}} \text{spermine} \quad (1)$$

Secondly, arginine is the sole substrate for nitric oxide (NO) production in all higher vertebrates. NO is synthesised through the oxidation of L-arginine by nitric oxide synthase (NOS) Eq. (2) taken from Auvinen *et al*., 1992), of which both inducible (iNOS) and constitutive (cNOS) have been identified in terrestrial mammals (Stuehr and Griffith, 1992). Inducible NOS has been found in numerous cells including macrophages, neutrophils and fibroblasts and is activated to produce NO when stimulated by cytokines, bacterial lipopolysaccharides (LPS) or parasites (Nathan, 1992). Indeed, Arginine indirectly has been shown to have numerous beneficial effects on T-cell mediated immunity in a number of animal models including rats and mice (Madden *et al*., 1988). For this reason inducible NO is thought to play a major role in the resistance to intracellular pathogens (Brunet, 2001). In fish

Wang and co-workers (1995) were the first to demonstrate the role of arginine in the production of inducible NO by fish macrophages.

$$\text{arginine} \xrightarrow{\text{NOS}} \text{N}^{\text{G}} - \text{hydroxy} - \text{L} - \text{arginine} \xrightarrow{\text{NOS}} \text{NO} + \text{citrulline} \quad (2)$$

Since, it has been reported that fish can produce NO by tetrahydorbiopterin- dependant NO synthase (see Eq. 2; Buentello and Gatlin, 1999). Recently, Aragao and co-workers showed that Senegalese sole (*Solea senegalensis*) when subjected to acute stress had decreased plasma concentrations of arginine and orthinine, despite the fact that no difference in growth performance was reported compared to unexposed fish (Aragao *et al*., 2008). They concluded that the differences in plasma AA profiles maybe attributed to the fish having extra requirements to synthesise proteins and other specific compounds related to the stress response. Also Buentello and Gatlin (1999 & 2001) have completed extensive investigations on channel catfish showing that inducible NO production is increased with dietary inclusion of excess arginine. Moreover they reported that the ability of the fish to survive a challenge with *Edwardsiella ictaluri* was increased by dietary arginine availability.

Comparable evidence from studies involving both mammals and fish suggest that AA regulate the key metabolic pathways that are crucial to maintenance, growth, reproduction and immune responses. As mentioned previously fish don't have an absolute protein requirement, however do require the AA that comprise the protein. So it would be important to explore the possibility of supplementing existing aquafeeds with key AA and utilise advanced tools such as nutrigenomics to help provide a molecular insight into the effects of feeding AA on these interdependent processes. This approach will undoubtedly help develop biomarkers to promote better growth and disease prevention in fish.

1.6.3 Lipids and fatty acids

Lipids are dense macronutrients that provide both the metabolic energy and supply of essential fatty acids (EFA) in aquafeeds (Kaushik and Medale, 1994). In general the biological active forms of EFAs are C20 and C22 fatty acids derived from the C18 PUFA. The two most important families of PUFAs that have the most nutritional significance are linoleic acid (LA) 18:2n-6 (omega-6), the parent acid of the n-6 family, and α-linolenic acid (ALA) 18:3n-3 (omega-3), the parent acid of the n-3 family (Sargent *et al*. 2002). Similar to terrestrial mammals, with the exception of marine fish, fish are able to convert C18 PUFA to C20 or C22 by a series of elongation and desaturation reactions producing important compounds such as docosahexanoic acid (DHA, 22:6*n* - 3), eicosapentaenoic acid (EPA, 20:5*n* - 3) and arachidonic acid (ARA, 20:4*n* – 6); all of which are involved in maintaining cell membrane structure and immune function (Sargent *et al*., 1993a, 1995, 1997). Contrary to terrestrial animals in fish DHA and EPA are the major PUFAs of cell membranes and are commonly termed '*n* – 3 HUFAs'. In comparison to terrestrial animals fish tissues have in general much higher concentrations of DHA and EPA than ARA and so have correspondingly high dietary requirements for *n* - 3 PUFAs (Sargent *et al*., 1999).

PUFAs contained in membrane phospholipids (PL) are precursors for synthesis of eicosanoids which bind to specific G-protein-coupled receptors and signal cellular physiological responses to inflammation, vasodilation, blood pressure, pain and fever (for reviews see Funk, 2001; Sargent *et al*., 2002; Schmitz and Ecker, 2008; Wall *et al*., 2010). Central to this role is the regulated, dioxygenase-catalysed oxidation of ARA and EPA to produce highly bioactive eicosanoids with a short half-life produced by cells to act immediately (Schmitz and Ecker, 2008). The types of eicosanoids (i.e. prostaglandin, leukotriene and thromboxanes) are generally thought to be affected by source where eicosanoids derived from *n* – 6 PUFAs (e.g. ARA) have a pro-inflammatory effect. Whereas

eicosanoids derived from *n* - 3 PUFAs (e.g. EPA or DHA) are recognised to attenuate inflammatory responses mainly through their ability to inhibit the formation of *n* - 6 PUFA derived eicosanoids (Calder, 1998; Bell and Sargent, 2003; Wall *et al*., 2010). In addition recent evidence has shown that source of PUFA can influence peptide mediators through modulating the activity of nuclear factor-κ ß (NF-κß) and/or peroxisome proliferator activated receptor (PPAR)- γ (Novak *et al*., 2003; Zhao *et al*., 2004). As of yet it is still unclear as to the exact underlying mechanisms responsible for modulating NF-κß activity, nevertheless eicosanoid action is predominantly determined by the inter alia ratio of ARA:EPA. A high tissue ratio of ARA: EPA can result in enhanced eicosanoid actions that could lead to a number of conditions such as irritable bowel syndrome and other such inflammatory ailments (see Figure 1.6). In contrast, high tissue ratios of EPA:ARA can suppress the inflammatory response such as found in fish oil and some plant oils e.g. corn oil (for reviews Simopoulous, 1999; Sargent *et al*., 2002; Schmitz and Ecker, 2008; Wall *et al*., 2010).

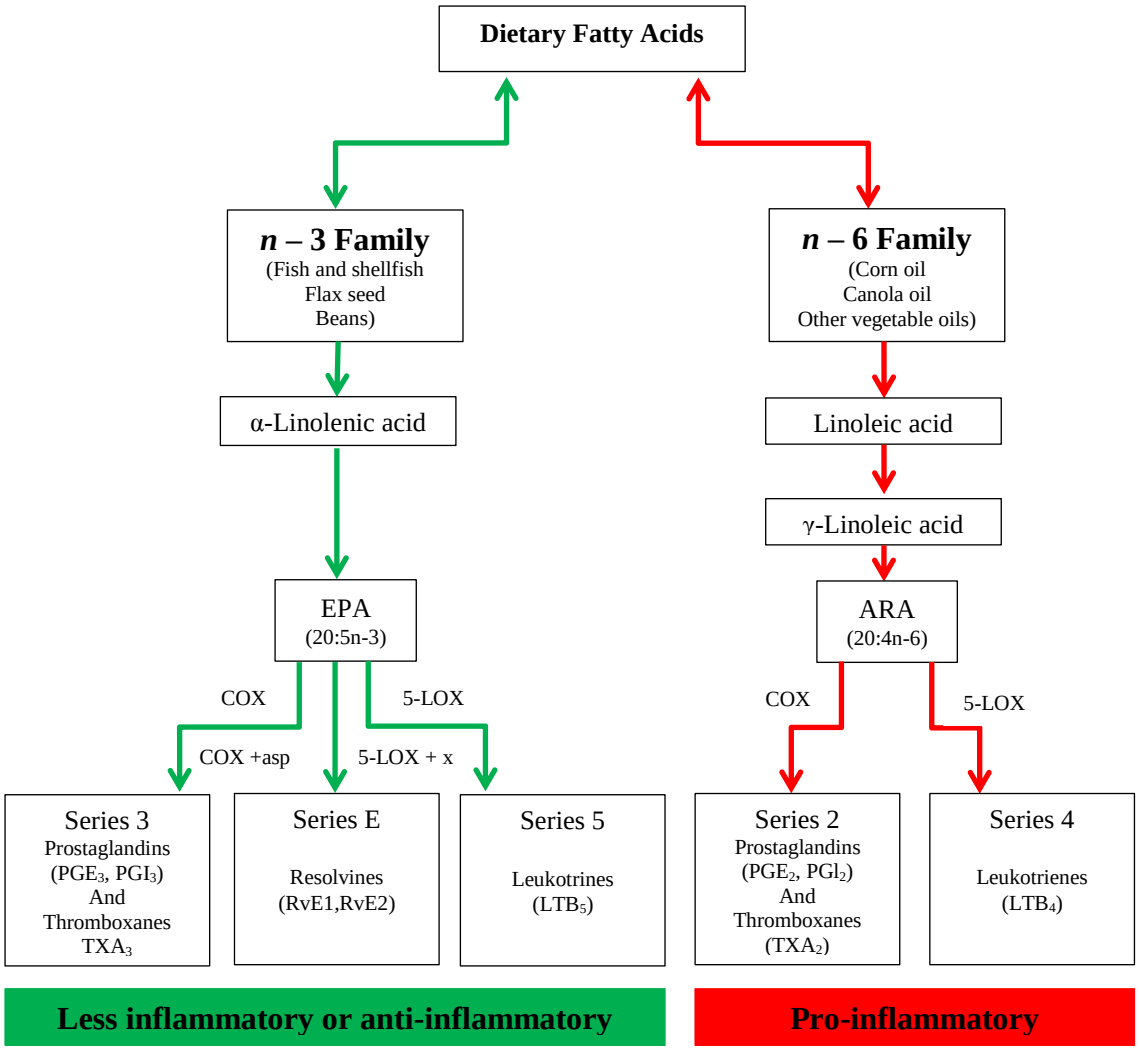


Figure 1.7. Sources of dietary fatty acids and an outline of eicosanoid production pathways with free ARA, EPA released from cell membranes as substrates for cyclooxygenase (COX), 5-lipooxygenase (5-LOX), and other reactions (x).The term series refers to the different biologically active molecules such as the prostaglandins, thromboxanes and leukotrines derived from either ARA or EPA. The numbers after the series refers to the number of double bonds in the molecule. Adapted from Glaser *et al*. (2010).

Fish oil is the main lipid source fed to fish for several reasons: firstly it is an excellent source of *n* – 3 HUFA and secondly it does not compromise the lipid composition of the fish carcass. To date fish oil is the only commercial source of *n* – 3 HUFA for human consumption and is essential in diets of truly marine fish species. For these reasons fish oil has been intensively used in aquafeeds and/so caused a shortfall in this limited commodity. Plant products are the obvious candidate source to replace fish in aquafeeds for reasons synonymous to the fishmeal story. A number of studies have recently shown that salmonids can be grown with 100% of the dietary fish oil being replaced by a blend of vegetable oils and marine fish can be grown with up 60% replacement of fish oil, without any adverse effects on growth rates (Bell *et al*. 2005; Izquierdo *et al*., 2005; Torstensen *et al*., 2005). However fish represent a virtually unique source of *n* - 3 HUFA (omega – 3) which are known to confer a number of health benefits and a range of human diseases and conditions that respond to the consumption of *n* – 3 HUFA continues to grow (Brouwer *et al*., 2006; Calder, 2006 and 2007; Torrejon *et al*., 2007; Nagao and Yanagtia, 2008). Although it appears that salmonids and marine fish can be grown with high levels of vegetable oil without compromising overall fish performance, all fish fed high levels of vegetable oils are characterised by reduced levels of EPA and DHA in their flesh subsequently affecting their nutritional benefit (Regost *et al*., 2003; Torstensen *et al*., 2004, Bell and Waagbo, 2008).

The issue for future supply of *n* - 3 HUFA for marine fish and human population has emphasised the need to continue to search for alternative sources of HUFA supply and to address the lack of knowledge of EFA requirements and PUFA metabolism in fish. Through the use of modern technologies such as nutrigenomics may help to add to the understanding of the complex metabolic pathways of PUFA utilisation in fish. Despite this future provisions of HUFA may come from the use of microorganisms where marine protists and dinoflagellates, such as species of *Thraustochytrium, Schizochytrium* and *Crypthecodinium*

are the rich sources of DHA (Singh and Ward, 1997). Whereas microalgae like *Phaeodactylum* and *Monodus* are good sources of EPA (Yongmanitchai and Ward, 1993). Increased research focus will undoubtedly improve yields through conventional selection, enhanced culture techniques and/or screening of genetically modified organisms will ultimately make up for the shortages of these key dietary components (Ward and Singh, 2005). Hypothetically it may be possible to engage with the biofuel industry and combine production waste containing useful protein biomass and HUFA as an alternative to fishmeal and oil usage in animal feeds (Olsen *et al*., 2008). From an aquaculture perspective, strides can be made in further boosting immunity of different fish species via dietary lipid manipulation, particularly with respect to optimizing species-specific balances for *n* - 6: *n* - 3 FA. In addition the development of such diets could have significant implications in improving the health and welfare of ornamental fish.

1.6.4 Micro/trace nutrients and feed additives

The definition on immunonutrition by Grimble (1996) highlights that no one specific nutrient is solely responsible for influencing the immune response and so it would be unreasonable to ignore the importance of vitamins, minerals and feed additives to fish growth and immuno-competence. On this note micro and trace nutrients such as vitamins A, C and E, carotenoids and trace minerals such as selenium, zinc, copper, manganese and iron have received the most attention because of their recognised antioxidant ability in fish. Thus, protecting cell membranes from oxidative damage, therefore playing an important role in maintaining immune responses (Amar *et al*., 2004; Hung *et al*., 2007a,b). Vitamin and mineral premixes included in aquafeeds generally meet the requirements of the fish (N.R.C. 2011; Halver, 2002). Nevertheless a dearth of information exists reporting that over supplementation with certain vitamins and minerals in diets particularly vitamin C and E have improved stress

tolerance, immunological responses and disease resistance in fish (for reviews see Lall and Lewis-McCrea, 2007; Lim *et al*., 2008a; Lim *et al*., 2010a).

The main role of feed additives is for intentional inclusions to animal feeds to modulate immune responses and could be either immuno-potentiators (positive influence) or immuno-suppressors (negative influence; but not necessarily 'negative' as in the case of regulatory functions) that attenuate stress and pathogenesis. The term feed additives encompasses a wide range of substances that could include intact microbes (e.g. probiotic organisms), microbial cell components (e.g. Lipopolysaccharide, muramyldipeptide), fungal polysaccharide (e.g. zymosan, β-glucan), phytotherapeutic agents commonly termed 'phytobiotics' (e.g. rosemary, astragalus root) and nucleotides (e.g. laltide). Indeed the use of pre/probiotic applications in aquaculture practices has received much attention within the last decade where numerous reviews have highlighted the rapid progress in understanding the effect of feeding bacteria to fish immunity and growth performance (for reviews see Merrifield *et al*., 2010; Nayak, 2010; Ringø *et al*., 2010). Specifically the changes in the gut milieu resulting from the presence of supplemented beneficial microorganisms (e.g. lactic acid bacteria) including the production of inhibitory compounds responsible for antimicrobial activity, competition with potential pathogens for binding sites or stimulation of epithelial barrier function, augmentation of immune responses such as that of the regulatory cytokines, inhibition of virulence gene or protein expression in gastrointestinal pathogens, and improvement of gut structure and digestion. Erudition of the use of microbes in aquaculture research has made it plausible to believe that this maybe one of the best approaches to preventative health care in aquatic organisms.

The recognition that immunostimulants can have significant effects on fish growth and immuno-competence has been well documented since the late 1980's. Sakai (1999) was the first major review to categorise the use of immunostimulants based on source in

aquaculture. Since a dearth of information has been published regarding the use of immunostimulants as potential agents to induce defence pathways in fish (for reviews see Bricknell and Dalmo, 2005; Dalmo and Bøgwald, 2008; Trichet, 2010). The rationale underlying the use of immunostimulants is that receptors on the target immune cells recognize the immune-stimulatory substances as high-risk molecules and induce defence pathways. Subsequently these immune-potentiating substances are now generally included as dietary supplements during stressful aquaculture operations, such as grading, transfer, vaccination or during crucial life stages to attenuate pathogenesis and maintain good health.

1.7 Conclusion

Aquatic feeds have evolved over the last half a century and the feed industry is constantly aiming to optimize the quality of their products in a more cost effective manner. The realisation that existing resources for fishmeal and fish oil are limited is a major driving force to development of novel alternative feed ingredients that could replace such limited commodities without compromising fish growth performance and immuno-competence. Indeed, as more tools such as nutrigenomics and bio-markers become available for major fish species, it would be possible to have a more robust appraisal on the benefits of inclusion of novel alternative feed ingredients to fish health and growth performance.

Likewise the use functional ingredients as supplements are being increasingly employed to add value to feed at no extra cost. However vigilance should be exercised on those that are labelled immune-modulators as extensive and rigorous research is still required to validate the benefits of such ingredients to each target fish species. Nevertheless, through the application of this knowledge it should be possible to implement preventive health care strategies based on nutritional principles for aquaculture operations including control of

disease and maintaining fish quality which are essential factors for the success of keeping fish for their ornamental value.

The aim of this research programme was to address the current agenda towards meeting the goals of finding suitable 'sustainable' feed ingredients to offset the demand of fishmeal usage in aquafeeds for the ornamental industry. This research programme was designed to incorporate the concept of linear least cost modelling to produce nutritionally sound and economic formulations based on a mixture of various exotic ingredients to potentially reduce fishmeal dependence. As a part of the experimental tool kit to investigating the effects of feeding novel ingredients towards fish quality the integration of classical nutrition and immunological research methodologies was employed. This perhaps reflects the current direction in the research agenda of global aquaculture where there is an increase in the number of studies integrating the research methodologies of both nutrition and immunology as highlighted in Section 1.4. This aspect to the PhD programme is both novel and is an important goal aligned to the philosophy of the ornamental industry which is to improve fish quality and increase the longevity of fish. Although a dearth of information exists regarding the use of 'functional' feed ingredients (see Section 1.6.4), such as immunostimulants, prebiotics and probiotics there is a lack of information regarding the effects of macro-nutrient profile changes to fish immune response. This knowledge will be imperative to the development of future aquafeeds that provide a more integrative strategy to combating disease through nutritional intervention. Indeed, classical evaluations of novel feed ingredients are based mainly on their qualities to improve fish growth performance with no reference to the effects on the fish immune response. Within this research programme due to the ornamental nature of the fish species used and the policy of the Waltham Centre for Pet Nutrition, it was mandatory to use only mild, non-invasive techniques for both the assessment of growth performance and immune status. This explicitly limits sampling to only using

whole blood of fish. With these restrictions in place understanding the effect of feeding alternative protein sources to fish growth performance and immuno-competence was assessed through the sampling of fish whole blood. In this regard a number of alternative potential feed ingredients as highlighted in, were investigated to assess the effect of feeding such sources at a high replacement of fishmeal >60 % in each experiment on the fish growth performance and welfare. The selection criteria for the chosen protein sources were based principally on the nutritional profile and sustainability of the protein source (see Section 1.2). Due to the non-invasive nature of this research programme it was not possible to challenge the fish with a live pathogen; therefore a bacterin was developed to stimulate infection.

CHAPTER 2

GENERAL METHODOLOGIES

2.1 Overview

In the present study the following analytical procedures were fundamental to the experimental analysis. Other methods unique to specific trials (including diet formulation) are described in the relevant experimental chapters. Unless stated all materials, chemicals and reagents were sourced from Sigma-Aldrich ltd (Poole, Dorset, UK) and Fisher Scientific ltd (Loughborough, Leicestershire, UK). All experimental work involving fish fully conformed to the UK Animal Scientific procedures Act of 1986 with the required project licence # 30/2135 and personal licence # 30/2644. Approval was given by the institutional Animal Ethics Committee and the Ethical review process of the Waltham Centre for Pet Care and Nutrition.

2.2 Rearing facilities and maintenance of water quality

All experimental trials were conducted within a freshwater recirculation system (RS), system 'A', at the Aquatic Health and Nutrition Research Aquarium (see plate 2.1). In order to effectively function and provide a suitable environment for the rearing of fish, water quality parameters in a RS must be effectively managed to remain within the optimal ranges for the rearing of mirror carp (*Cyprinus carpio*).

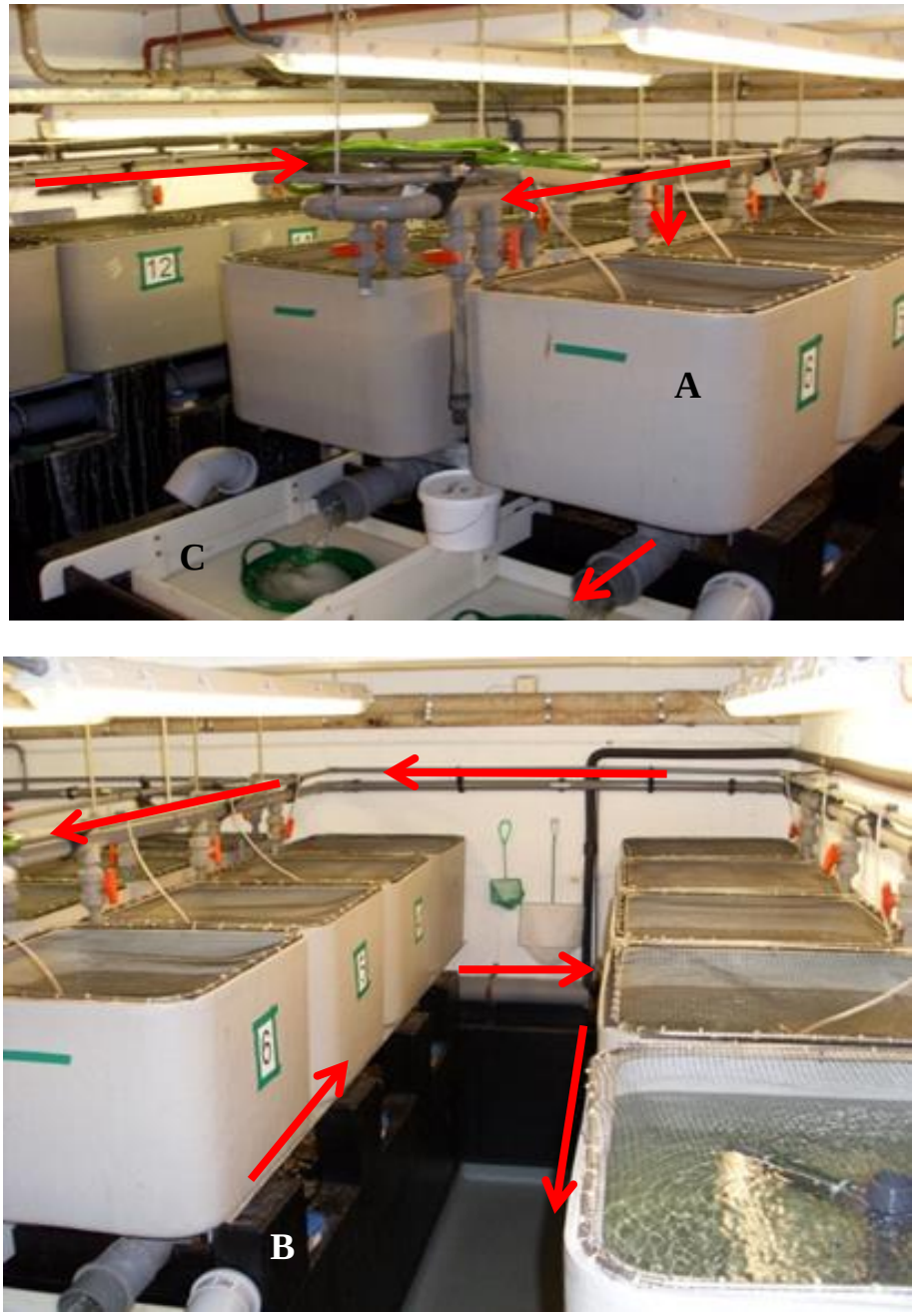


Plate 2.1 Annotated pictures of recirculation system 'A' located at the University of Plymouth. Red arrows indicate the direction of water flow in and out of the experimental tanks (A) and returning to the water pump via the mechanical filter (C) and the sump containing the biological filter (B).

The facility was a closed freshwater RS with a total volume of ~6000 l. Eighteen experimental 150 l fibreglass tanks received water at a rate ~800 l/ h. An automated 12 h dark and light regime was maintained throughout all experimental trials. Each experimental tank received a constant ~30 l/ h flow of municipal water to maintain losses from evaporation and alleviate any potential build-up of nitrates and trace elements. An activated carbon filter (Commandomatic TCF, Waterco Ltd, Sittingbourne, Kent, UK) removed chlorine and organic compounds from the incoming water. As a by-product of the breakdown of proteins, fish excrete ammonia gas (NH_3) via the gills, which is highly toxic to fish if left untreated. Ammonium ions (NH_4^+) are less toxic to fish and are also always present in amounts relative to water pH. The biological oxidation of ammonia to nitrate (NO_3^-) is a two stage process; ammonia is firstly oxidised to less toxic nitrite (NO_2^-) by the *Nitrosomonas* species, which is subsequently converted to nitrate by the *Nitrobacter* species. All nitrogenous compounds were monitored on a weekly basis using an automated discrete analyser (model: AQ2, Seal analytical, UK). The following levels of nitrogenous compounds were considered acceptable; ammonia between 0.04 – 0.08 mg/ l, nitrite between 0.02 – 0.06 mg/ l and nitrate between 54 – 58 mg/ l. If required, nitrogenous compounds were controlled by partial water exchange.

Particulate material coming from fish faecal material, undigested feed and sloughed bacteria from the bio-filter was constantly removed by the crude mechanical filtration system as shown in Plate 2.1. A commercial fibre was used in the mechanical filter to trap material and was replaced every 2-3 days. The saturation of dissolved oxygen needs to be maintained >60 % for both fish and bio-filter (Masser *et al*., 1999) and was maintained at >80% by a side supply of compressed air (compressor; Rietschle, UK) delivered to the experimental tanks via air-stones and perforated pipes to each sump water. Temperature was maintained at 26 ± 1ºC with an in-line thermostatically controlled heating unit. Typically there is a natural decrease of pH in a RS, as nitrification both consumes OH^- ions and produces acids, and carbon

dioxide produce by fish forms carbonic acids (H_2CO_3) in the aqueous environment. The pH of recirculating water was maintained between 6.8 – 7.5 by use of sodium bicarbonate ($NaHCO_3$) as required. Oxygen saturation, pH, and temperature were monitored daily with an electronic meter (Hach, HQ4d).

2.3 Experimental fish

Mirror carp fry (*C.carpio*) were obtained from Hampshire carp hatcheries, Hampshire, U.K for all experimental trials. Fish were transported in well aerated polythene bags and upon arrival fish were gradually acclimated to the temperature of the aquarium facility for ~2-3 h. The bags containing the fish were then opened and all fish were initially screened for any pathogens before being dispersed equally into two experimental tanks. All fish were monitored daily and fed *ad libitum* on a commercial diet until grading (EWOS commercial diet, crude protein: 48 %; crude lipid: 23 %). A period of at least four weeks was allowed until the fish were randomly distributed into experimental tanks prior to experimental trials. Any anaesthetisation of fish fully complied with Home Office procedures using tricane methanesulphonate (MS-222, Pharmaq Ltd, Fordingbridge, Hampshire, UK). The dose of MS-222 was dependant on the size of the fish but mostly used at a concentration of 0.15 g/ l and buffered with sodium bicarbonate.

2.4 Feeding and weighing

All fish in each experimental tank were batch weighed at t = 0 and fed relative to % biomass per day spread over three feeding times (~0900 h, ~1200 h, ~1700 h). All fish in individual tanks were batch weighed on a weekly basis following a 24 h starvation period throughout the experimental trials. Feed input was adjusted accordingly in the event of any mortality.

2.5 Feed formulation and diets

Experimental diets were formulated to be isonitrogenous and isolipidic using feed formulation software (Feedsoft®, USA). Moreover each experimental diet was formulated to contain approximately 38 % crude protein and 8 % lipid to meet the known requirements of juvenile common carp (N.R.C. 2011). Each diet was produced by mechanically stirring the ingredients into a homogenous mixture using a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 – 10STDA mixer). Warm water was added to reach a consistency suitable for cold press extrusion to form 1 mm pellets (PTM Extruder system, model P6, Plymouth, UK). Fishmeal served as the main protein source in each control diet for all trials (see Figure 2.1). The remaining experimental diets consisted of fishmeal fixed at 33 % provision of protein and the remaining 66 % protein was provided by an experimental ingredient.

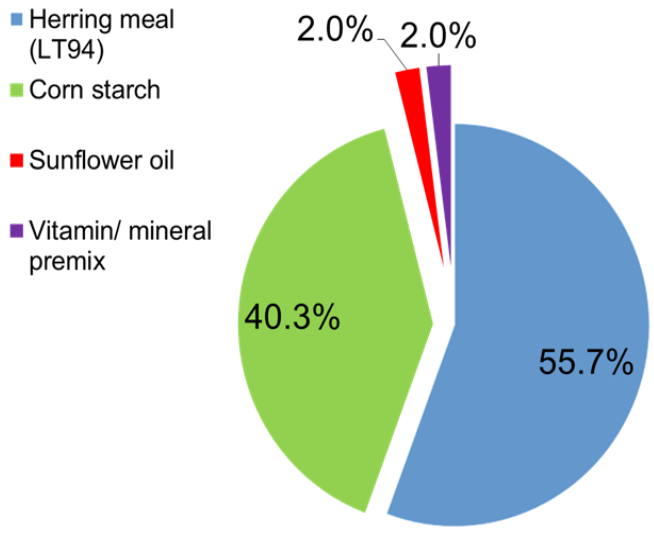


Figure 2.1. Major components of the basal diet used in each trial.

2.6 Growth and feed utilisation

Growth performance and feed utilisation was assessed by net weight gain (NWG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER). Calculations were made using the following formulae:

$$NWG\ (g/\ fish) = Final\ wt.(g) - Initial\ wt.(g)$$

$$SGR\ (\%) = 100 \times ((Ln\ Final\ wt.(g) - Ln\ Initial\ wt.(g)) / (Days\ fed))$$

$$FCR = Feed\ intake\ (g) / live\ weight\ gain\ (g)$$

$$PER = live\ weight\ gain\ (g) / protein\ intake\ (g)$$

2.7 Proximate analysis of diets and feed ingredients

Diets and feed ingredients were subjected to analysis for determination of moisture, protein, lipid, ash and gross energy. All diets were ground by use of a grinding machine and analysed on a dry matter basis. Analysis was conducted in duplicate according to protocols of the AOAC (2007) as described in the following sub sections.

2.7.1 Moisture

Diets were weighed and air dried at 105 ºC with a fan assisted oven (Genlab Ltd, UK) until a constant weight was achieved. Percentage moisture was determined by the following equation:

$$Moisture\ (\%) = ((wet\ wt.(g) - dry\ wt.(g)) / (wet\ wt.\ (g))) \times 100$$

2.7.2 Crude protein

Determination of total crude protein in diets and feed ingredients was achieved in duplicate by the Kjeldhal method, which determines the protein content from the total nitrogen content of samples. Total nitrogen is multiplied by a factor of 6.25 (5.95 for proteins of plant origin) to calculate the crude protein content. Briefly, ~100 mg of sample was weighed directly into a micro Kjeldahl tube along with one catalyst tablet (3 g K_2SO_4, 105 mg $CuSO_4$ and 105 mg TiO_2; BDH Ltd. Poole, UK) and 10 ml concentrated sulphuric acid (H_2SO_4)(Sp. Gr. 1.84, BDH Ltd. Poole, UK). Digestion of samples was performed with a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) with the following protocol; 100 ºC for 30 min, 225 ºC for 45 min (1 h if samples had high lipid content) and 380 ºC for 1 h. Once digestion was complete and following a cooling period, the samples were distilled using a Vodapest 40 automatic distillation unit (Gerhardt Laboratory Instruments, Bonn, Germany), the distillate was neutralised with concentrated H_2SO_4 and from the titration value the crude protein value was determined using the following formula:

$$\% \text{ Nitrogen} = \frac{(\text{mls Sample Titrant} - \text{mls Blank Titrant}) \times (\text{Acid Normality}) \times (\text{MW of Nitrogen})}{\text{Sample wt.}}$$

2.7.3 Lipid

Lipid content was determined in duplicate using the Soxhlet extraction method. Briefly, diets and feed ingredients were weighed (~3 g) and placed into a cellulose thimble lightly plugged with cotton wool and inserted into the condensers and raised into the 'rinsing' position of a SoxTec™ extraction system (Tecator Systems, Högnäs, Sweden; model 1043 and service unit 1046). Pre-weighed glass cups containing 40 ml of petroleum ether were clamped into the condensers and extraction levers were moved to 'boiling' position for 30 min. Following this the extraction levers were set to 'rinsing' position for 45 min. The glass cups containing

the extracted lipid were then left to cool for 30 min and weighed. Total lipid was determined by the following formula:

Total lipid (%) = ((final weight of beaker – intial weight of beaker) / intial sample wt.)) x 100

2.7.2 Ash

Ash (total mineral or inorganic content) content was determined in duplicate by adding a known sample weight (~500 mg) to a pre-weighed ceramic crucible. The crucibles and samples were then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550ºC for 12h. After cooling in a dehumidification chamber, percentage ash was determined from the sample residue by:

Ash (%) = ((Sample residue (g) – crucible wt. (g)) / (initial sample wt. (g))) x *100*

2.7.3 Gross energy

Gross energy was determined in duplicate in MJ/ kg with a Parr Adiabatic Bomb Calorimeter model 1356 (Parr Instrument Company, IL, USA). Briefly, ground diet and feed ingredients were compressed in 1 ± 0.5 g pellet and weighed. The pellet was then placed into a nickel crucible with a 10 cm length fuse wire, which was formed into a 'U' shape to touch the pellet. The crucible was then carefully placed into the chamber and filled with oxygen to complete the loading process. The bomb was then placed in a vessel containing 2 kg of water to determine the released heat energy. Prior to initiating the reaction the sample weight was keyed into the calorimeter for determination of MJ gross energy per kg as calculated by the instrument algorithm.

2.8 Trace element analysis

2.8.1 Sample preparation and digestion of feed ingredients

All trace element analysis was carried out in triplicate nitric acid digested samples unless otherwise stated. Feed ingredients were analysed by dry weight and all dried samples were homogenised using a grinder prior to digestion. For analysis of all elements except Se ~100 mg sample was weighed directly into each Kjheldhal tube and digested in 7 ml of nitric acid (70% ANALAR grade). Samples were digested using a Gerhardt Kjeldatherm 40 tube digestion block (Gerhardt laboratory Instruments, Bonn, Germany) using the following protocol; 60 ºC for 1 h, 100 º C for 1 h, 120 ºC for 30 min, 135 ºC for 2 h (an additional hour was given for samples containing high lipid content). After a cooling period of 30 min, 2 ml 30 % (w/w) hydrogen peroxide was added and samples further digested at 135ºC for 1 h. Samples were then transferred into a pre weighed 50 ml polypropylene vials and diluted ~50 ml with ultra-pure Milli-Q water (Millipore Corp, MA, USA) and total weight was determined. Duplicate blanks compromising of only nitric acid and hydrogen peroxide were also analysed in each digestion.

2.8.2 Trace element analysis of digests

All samples were analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) (Varian 725-ES OES spectrometry, Varian Inc. CA, USA) using the following wavelengths for each element (nm); Cu 324.75, Ir 224.628, Fe 238.204, Mn 257.610, Zn 213.857 and Y 360.074. Concentrations of each element in digests were determined in parts per million (ppm and mg/ kg) against an external standard calibration and concentration in original samples was calculated using the following formula:

$$\textit{Sample conc. (mg/ kg)} = \textit{((digest wt. (g)) / (sample wt.(g)))} \times \textit{digest conc. (mg/ kg)}$$

2.9 Haematological and serological analysis

2.9.1 Haematocrit

Haematocrit (packed cell volume) of whole blood was determined in duplicate as described by Brown (1988). Whole blood was collected into 300 µl heparinised haematocrit tubes and subsequently separated using a Centurion haematocrit centrifuge at 10,500 g for 5 min. Haematocrit was determined as the total percentage packed cell volume using a Hawskley haematocrit reader (% PCV).

2.9.2 Haemoglobin

Haemoglobin was determined based on Drabkin's cyanide-ferricyanide solution (Sigma-Aldrich Ltd. Poole, UK). Briefly, 4 µl of blood was added to 1 ml of Drabkin's solution (1/200 dilution factor, d.f.) mixed and measured after 5 minutes of incubation using a spectrophotometer at 540 nm. The sample haemoglobin levels (g/ dl) were determined against a standard curve of haemoglobin porcine lyophilized powder (Sigma-Aldrich Ltd. Poole, UK) and calculated using the following formula:

$$\textit{Haemoglobin concentration (g/ dl)} = \textit{(Absorbance of sample / Absorbance of standard)} \times \textit{d.f.}$$

In addition to calculating haemoglobin levels of fish whole blood mean corpuscular haemoglobin (MCH) levels and mean corpuscular haemoglobin concentration (MCHC) were calculated using the following formula:

$$\textit{MHC (pg)} = \textit{(haemoglobin conc. (g/ dl))} \times \textit{10) / total red blood cell count } (10^6\ mm^3)$$

$$\textit{MCHC (g/ dl) = (haemoglobin conc. (g/ dl)) / heamtocrit conc. (l/l)}$$

2.9.3 Total Erythrocyte and Leukocyte counts

Leukocyte and erythrocyte counts were determined according to Dacie and Lewis (1975). Briefly, 20 µl of whole blood was aliquoted into 980 µl of Dacies solution and mixed for 60 sec to ensure a homogenous solution. A 5 µl sample of this solution was then aliquoted onto a haemacytometer and a minimum of 500 cells were counted for a statistically valid result. From the total red blood cell counts the mean corpuscular volume (MCV) was calculated using the following equation:

$$MCV\ (fl) = ((haematocrit\ conc.\ (l/l)) \times 1000) / total\ red\ blood\ cell\ count\ (10^6\ mm^3)$$

2.9.4 Differential blood cell counts

In order to quantify the circulatory levels of lymphocytes granulocytes and monocytes, 5 µl of whole blood was smeared onto microscope slides. Slides were air-dried, fixed in 95 % methanol for 1 min, stained with Giemsa (BDH, Laboratory supplies Poole, UK) for 10 min and washed twice with distiller water. Once dried the slides were mounted in DPX (BDH Laboratory supplies, Poole, UK). Lymphocytes, granulocytes and monocytes were identified following the descriptions of Rowley (1990) see Plate 2.2 for examples. A minimum of 200 cells per sample were counted and the values were expressed as a percentage of the total leukocytes.

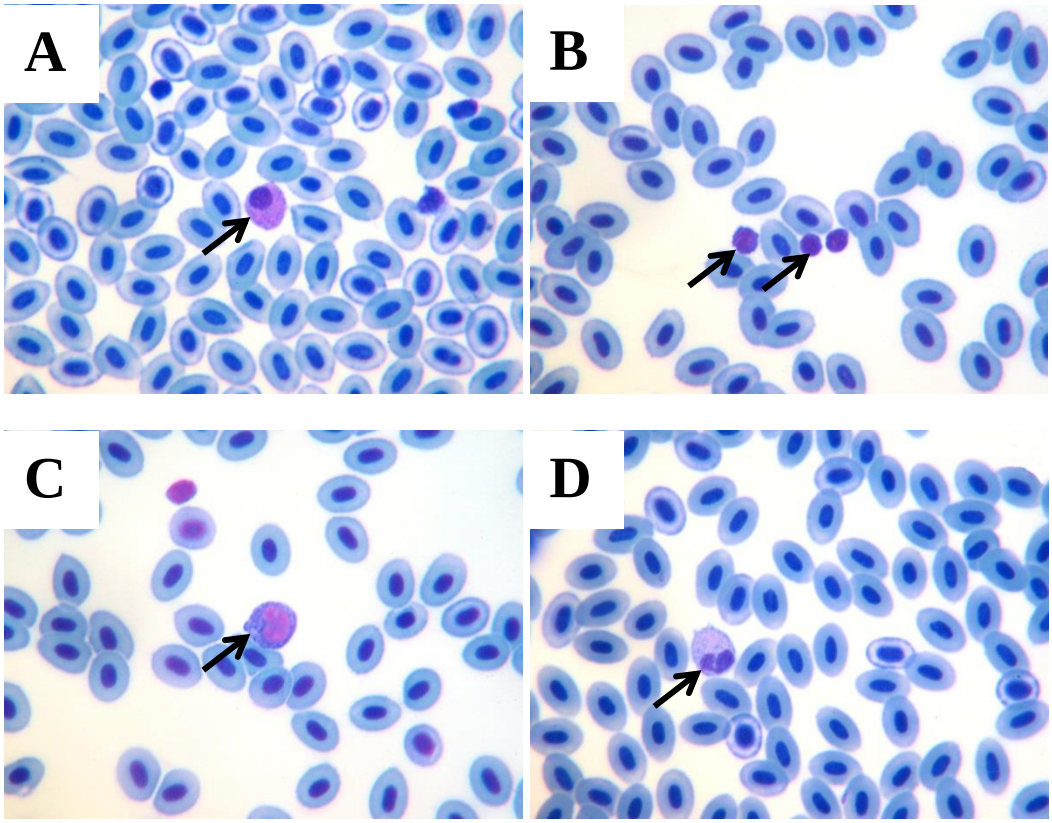


Plate 2.2. Different cell types according to Rowley (1990). Arrows indicate cells of interest in images: (A) Basophilic granulocyte, (B) Lymphocytes, (C) Monocyte, (D) Neutrophilic granulocyte

2.9.5 Serum Albumin and globulin

Serum albumin was determined according to the methods of Doumas *et al.* (1997). The assay measures serum albumin by the use of the anionic dye bromocresol green (BCG). BCG is a blue divalent anion in neutral or alkaline pH conditions, whereas in acidic pH, below its pk_a value (4.7), the dye is present in an undissociated form (yellow colour). The binding of the dye to albumin at acidic pH unbalances this equilibrium of undissociated and dissociated forms, resulting in increased absorbance at 630 nm. Briefly, 20 µl of serum was added to 4 ml of buffered BCG dye reagent (1 N NaOH, 11.8 mg/ ml $C_4H_6O_4$, 20 % w/v NaN_3 pH 4.1-4.2) and allowed to stand at 25 ºC for 10 minutes. The serum samples were then measured against a reagent blank and read at 630 nm using a spectrometer (V-530 UV/VIS spectrometer, Jasco, Easton, MD, USA). Sample albumin was determined in mg/ dl against a standard curve of bovine serum albumin (BSA) using the following equation:

Serum albumin (g/ dl) = (absorbance of test sample x 4) / (absorbance of standard).

Determination of serum globulin content was calculated by subtracting the albumin content from the total serum protein concentration as shown in the equation below:

Serum globulin conc. (g/dl) = Total serum protein conc. (g/dl) – Serum albumin conc. (g/dl)

2.9.6 Serum protein

Serum protein was determined by the Biuret method according to Grant and Kachmer (1976) (Sigma-Aldrich T 1949). The assay measures protein by use of a copper/protein complex that forms relative to the amount of protein present. Briefly, 1 part sample was added to 50 parts total protein reagent (0.6 M NaOH, 12 mM $CuSO_4$, 31.9 mM $KNaC_4H_4O_6 \cdot 4H_2O$, 30.1 mM KI). In a clear 1 ml cuvette, 20 µl of test sample, deionised water and protein standard was added to 1 ml of total protein reagent mixture. Each cuvette was incubated at room temperature for 10 min and immediately read at 540 nm (V-530 UV/VIS spectrometer, Jasco, Easton, MD, USA). Sample protein was determined in mg/ ml against a standard curve of bovine serum albumin (BSA). The following equation was used to determine the concentration of protein in test samples:

$$\textit{Total Protein (mg/ ml)} = A_{540}\ \textit{(sample) x Conc. of Satndard (mg/ ml)} / A_{540}\ \textit{(standard)}$$

2.10 Statistical analysis

All statistical analysis was carried out using SPSS for Windows version 18. Unless otherwise stated all means are reported a standard error of the mean (SE). Prior to analysis all data was tested for homogeneity of variance (Levene test) and normality of distribution (Shapiro-Wilk test). Unless otherwise indicated, differences across treatments were either modelled by one-way ANOVA or a two-way ANOVA. Where appropriate, a post hoc Tukey's honestly significant difference (HSD) test was performed to make pair wise comparisons between individual treatments. Data violating the assumptions of parametric tests after log transformation were tested with the equivalent non-parametric Kruskal-Wallis test and or Mann-Witney *U* test. All percentage data was transformed using arcsine function prior to

analysis. Unless otherwise stated, differences were considered significant at a value of $P \leq 0.05$.

CHAPTER 3A.

HAEMATOLOGICAL AND GROWTH RESPONSE OF MIRROR CARP (*C. CARPIO*) FED A TROPICAL EARTHWORM MEAL AND COMMERCIAL SOYBEAN MEAL IN EXPERIMENTAL DIETS

Abstract

An investigation was conducted to evaluate the effect of feeding a tropical earthworm meal (*P. excavatus*) on the haemato-immunological response and growth performance of mirror carp (*C. carpio*). Fish were fed diets for a total of 88 days, fishmeal served as the main protein source in the control diet. Two remaining diets consisted of fishmeal fixed at 33 % provision of protein and the remaining 66 % protein was provided by soybean meal (SBM diet) or *P. excavatus* meal (EW diet). After 60 days of feeding fish fed EW diet showed a significant elevation in final body weight compared to fish fed a fishmeal diet and fish fed a SBM diet. Similar improvements were observed in feed utilisation efficiency. After 28 days of feeding EW diet to mirror carp showed decrease in some aspects of the innate immune response, but at the same time gives rise to significant enhancement of growth and feed utilisation efficiency. Compared to control and SBM fed fish (7.69 ± 0.28 and 5.92 ± 0.31 g/ dl, respectively), a significant increase in haemoglobin was measured in EW fed fish (9.57 ± 0.24 g/ dl). Consequently significant elevations were also observed in mean corpuscular haemoglobin (MCH; 79.13 ± 4.59 pg) and mean corpuscular haemoglobin concentration (MCHC; 22.69 ± 0.54 pg) in EW fed fish. On the contrary, compared to control and SBM fed carp total leukocyte levels (2.72 ± 0.17 and 3.10 ± 0.17 $x10^4$ mm^3, respectively) were significantly decreased in the EW group (2.15 ± 0.14 $x10^4$ mm^3).

3A.1. Introduction

Historically fishmeal has been the preferred protein source in aquafeeds for many reasons including; concentrated form of high quality proteins, excellent amino acid profile, high nutrient digestibility and lack of anti-nutritional factors. The continued use of fishmeal in aquafeeds makes this material the 'gold standard' to which alternative feedstuffs must be compared. While the supply of fishmeal and fish oil is arguably sustainable in present times, the prognosis for expected growth in the international demand for aquafeed is expected to exceed global supply (Tacon and Metian, 2008). Indeed this expressed concern has been on-going since the early 1990's, where New and Wijkström, (1990) first coined the phrase 'fishmeal trap' which became common parlance within the world of aquaculture. Southgate (2003) suggested fishmeal would become too expensive to use in aquafeeds at current inclusion levels, consequently the inclusion of non-fishmeal sources of protein in animal feeds is a topic of great interest (Naylor *et al*., 2009; Olsen and Hasan, 2012).

To be a viable alternative protein source a potential ingredient must possess certain characteristics including: wide availability, competitive price, ease of handling and shipping and more importantly nutritional characteristics such as low level fibres, high protein content targeting approximately 48-80% crude protein (Gatlin *et al*., 2007), favourable amino acid profile, high nutrient digestibility and palatability. Although some plant derived ingredients such as soybean meal and wheat gluten meal possess most of the aforementioned characteristics historically their high price relative to fishmeal have precluded their use in aquafeeds and properties such as anti-nutritional factors (see Section 1.2), amino acid imbalances and palatability have been recognised to interfere with feed utilisation affecting animal production and fish immunity (reviewed by Francis *et al*., 2001; Hardy, 2010, Trichet, 2010). More recently the refinement of plant feedstuffs (refer to Section 1.2) has made these

feed commodities more economical and as a result it is likely that a combination of novel plant and animal derived feedstuffs would be required to successfully replace fishmeal.

Approaches employed for the partial or total replacement of fishmeal in commercial feeds fall into two broad categories, namely the use of conventional feed ingredients by maximising their nutritional value; and the use of a new generation of unconventional feed ingredients. In the ornamental sector the use of unconventional feed commodities is more acceptable mainly because of the high mark-up price of ornamental feed compared to commercial feeds. Subsequently this allows for more research opportunity to explore exotic ingredients that would otherwise be too expensive to use in commercial feeds. Such feed ingredients are commonly referred to as non-conventional feed resources (NCFRs) and encompass a wide range of ingredients as outlined in Section 1.2. The emphasis of the current investigation was to focus on the potential of earthworm meal to replace fishmeal in ornamental fish diets. Previously research has demonstrated that replacement of fishmeal with earthworm meal irrespective of worm species used has no adverse effects towards fish growth and feed utilisation including rainbow trout (*O.mykiss*; Stafford and Tacon, 1984 and 1985), common carp (*C.carpio*; Nandeesha *et al*., 1988; Rawling *et al*., 2012). However in the far-east, Asia and Australasia considerable research effort has been made to develop earthworms for bioremediation of organic wastes and as biomass for animal consumption (Sinha *et al*., 2010). Many earthworm species have been investigated for use as potential animal feed ingredients. However, to date little is known about the effect of feeding *Perionyx excavates*, a tropical epigeic worm species, to fish. *P. excavatus* has high fecundity high biomass production rates and a low environmental footprint which makes it ideally suited for worm meal production for potential use in feeds for the animal industry. Consequently for the first time an earthworm meal made from *P. excavatus* was fed to mirror carp and in comparison to earthworm meal a commercially available soybean meal was used

to assess the effects of each dietary specification on the growth performance, feed utilisation and haematological responses of mirror carp, a model ornamental fish species.

The specific hypotheses tested were as follows:

$H_0$1 - The replacement of fishmeal with earthworm meal will elevate growth performance and feed utilisation

$H_0$2 - Feeding fish soybean meal will have a negative effect on the fish growth performance and feed utilisation compared to fish fed fishmeal

$H_0$3 - The haematological and serological responses of the fish will remain unaffected by inclusion of earthworm meal

$H_0$4 - Replacement of fishmeal with soybean meal will have a negative effect on the fish haematological and serological responses

3A.2 Materials and methods

3A.2.1. Fish and experimental protocol

The experiment was carried out at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Mirror carp (*C.carpio*) fry were obtained from Hampshire carp hatcheries, Hampshire, U.K. After four weeks acclimation 360 fish (11.74 ± 0.9 g) were randomly distributed into 12 x 150 l fibreglass tanks (30 fish per tank) containing aerated recirculated freshwater. Fish were fed diets to a fixed regime of 3% body weight per day spread across three feeding times for 70 days. Fish were batch weighed on a weekly basis following a 24 h starvation period and reared at 26 ± 1 ºC with a 12:12 h light:dark photoperiod. Water pH was maintained between 6.8 – 7.5, dissolved oxygen between 7.5 – 8 mg/ l, ammonium between 0.04 – 0.08 mg/ l, nitrite between 0.02 – 0.06 mg/ l and nitrate between 54 – 58 mg/ l. All water quality parameters were measured daily using an automated discrete analyser (model: AQ2, Seal analytical, UK). The experiment fully conformed to the UK Animal Scientific procedures Act of 1986 with the required project and personal licence. Approval was given by the institutional Animal Ethics Committee and the Ethical review process of the Waltham Centre for Pet Care and Nutrition.

3A.2.2 Feed formulation and diets

Three isonitrogenous and isolipidic diets were formulated using feed formulation software (Feedsoft®, USA) to contain approximately 38 % crude protein and 8 % lipid to meet the known requirements of juvenile common carp (N.R.C. 2011). Fishmeal served as the main protein source in the control diet (FM diet). Two remaining diets consisted of fishmeal fixed at 33 % provision of protein and the remaining 66 % protein was provided by soybean meal (SBM diet) or *P.excavatus* meal (EW diet). Each diet was produced by mechanically stirring the ingredients into a homogenous mixture using a Hobart food mixer (Hobart Food

Equipment, Australia, model no: HL1400 – 10STDA mixer). Warm water was added to reach a consistency suitable for cold press extrusion to form 1 mm pellets (PTM Extruder system, model P6, Plymouth, UK). The nutritional profile was determined according to AOAC (2007) official protocols see Section 2.7 for details (see Table 3.1).

Table 3.1. Formulation and chemical composition of experimental diets. Each ingredient component is expressed as g/ kg per diet.

	FM	**EW**	**SBM**
Ingredients			
Herring meal LT94[1]	553.94	180.00	180.00
Corn starch	401.06	345.18	247.13
P. excavatus meal[2]		411.17	
Soybean meal[3]			494.77
Fish oil[4]		18.65	33.11
Sunflower oil	20.00	20.00	20.00
Vitamin/ mineral premix [5]	20.00	20.00	20.00
Antioxidant[6]	0.50	0.50	0.50
Molasses	5.00	5.00	5.00
Proximate analysis			
Dry matter (%)	93.4	92.0	91.7
Crude Protein (%)[7]	38.1	39.7	40.7
Crude lipid (%)[7]	7.8	6.3	8.2
Ash (%)[7]	8.9	6.7	7.6
NFE[8]	38.6	35.5	35.2
Gross energy (MJ kg $^{-1}$)	19.2	19.8	19.0

Dietary codes: FW = control diet (Fishmeal LT94), EW = earthworm, SBM= soybean protein meal,
[1]United fish products, Aberdeen, Scotland, U.K.
[2]Anphu Earthworm Farm, Vietnam. (crude protein 65.8 %; ash 6.3 %; moisture 8.3 %; lipid 8.7 %).
[3]HP-110, Hamlet Protein, U.K (crude protein 57.5%; ash 6.8 %; moisture 6.5 %; lipid 2.5 %).
[4]Epanoil (Seven Seas Ltd, U.K)
[5]Premier nutrition vitamin premix; each 1kg of premix contains: 12.1 % calcium, Ash 78.7 %, Vit A 1.000 µg/kg, Vit D3 0.100 µg/kg, Vit E (as alpha tocopherol acetate) 7000.0 mg/kg, Copper (as cupric sulphate) 250.000 mg/kg, Magnesium 1.56 %, Phosphorous 0.52 %
[6]Barox antioxidant liquid
[7]Values are given based on a dry matter basis
[8]Nitrogen free extracts (NFE) = dry matter – (crude protein + crude lipid + ash)

3A.2.3 Growth and feed utilisation

After 70 days of feeding on experimental diets all growth and feed utilisation of fish was determined according to the calculations outlined in Section 2.6.

3A.2.4 Haematological and serological analysis

At the end of the trial five fish per tank were anaesthetized with tricane methanesulfate (MS222) at 150 mg/ l. Blood was sampled from the caudal vein using a 25 gauge needle and 1 ml syringe. Sub samples of whole blood were left to clot for a period of 12 h (at 4 ºC) and then centrifuged at 3,600 g for 6 minutes to recover serum. Serum was removed and stored at -80 ºC until analysis of albumin, globulin and protein.

3A.2.5 Haematocrit

Haematocrit was determined according to the protocol outlined in Section 2.9.1.

3A.2.6 Haemoglobin

Haemoglobin concentration of whole blood was determined according to the protocol outlined in Section 2.9.2.

3A.2.7 Total Erythrocytes and Leukocyte counts

Total leukocyte and erythrocyte counts were determined according to the protocol outlined in Section 2.9.3.

3A.2.8 Serum Albumin, globulin and Protein

After 70 days of feeding on experimental diets fish serum was collected and all serological parameters were determined according to methods outlined in Section 2.9.5 and 2.9.6.

3A.3 Statistical Analysis

All data are presented as means ± standard error (SE). All growth data and haematological data was transformed where necessary and statistical analysis was conducted using SPSS statistics version 18 for windows (SPSS Inc., Chicago, Il, USA) and accepted at the $P < 0.05$ level. Data were analysed using a one-way ANOVA and Significant differences between control and experimental groups were determined using post-hoc Tukeys HSD test.

3A.4 Results

3.4.1A Growth performance and feed utilisation

After 70 days of feeding fish fed earthworm meal displayed an elevation by ca. 15 % in average fish weight compared to fish fed fishmeal diet (Figure 3.1). In contrast fish fed soybean meal showed a decrease by ca. 22 % in average fish weight compared to fish fed fish meal. A high growth performance was observed in all groups (Table 6); fish biomass increased by over 500% after 70 days of feeding with FCR ⩽ 1.4 and SGR > 2.5. SGR %/ day improved significantly from 2.97 ± 0.03 %/ day in the control fed fish to 3.26 ± 0.03. %/ day in fish fed EW diet. However, when compared to control fed fish, fish fed on SBM diet demonstrated a significantly reduced SGR (2.55 ± 0.14 %/ day). Compared to control and SBM fed fish (58.26± 5.94 and 42.70 ± 4.59 g/ fish, respectively), average weight gain for fish fed the EW diet (71.28 ± 0.97 g/ fish) was significantly higher. Equally protein efficiency ratio was significantly higher in fish fed EW diet (2.04 ± 0.03) compared to fish fed control and SBM diet (1.83 ± 0.06 and 1.37 ± 0.06, respectively). Indeed fish fed the control (1.19 ± 0.05 g/ g) and SBM diets (1.40 ± 0.07 g/ g) displayed significantly higher FCR compared to fish fed the EW diet (1.08 ± 0.02 g/ g).

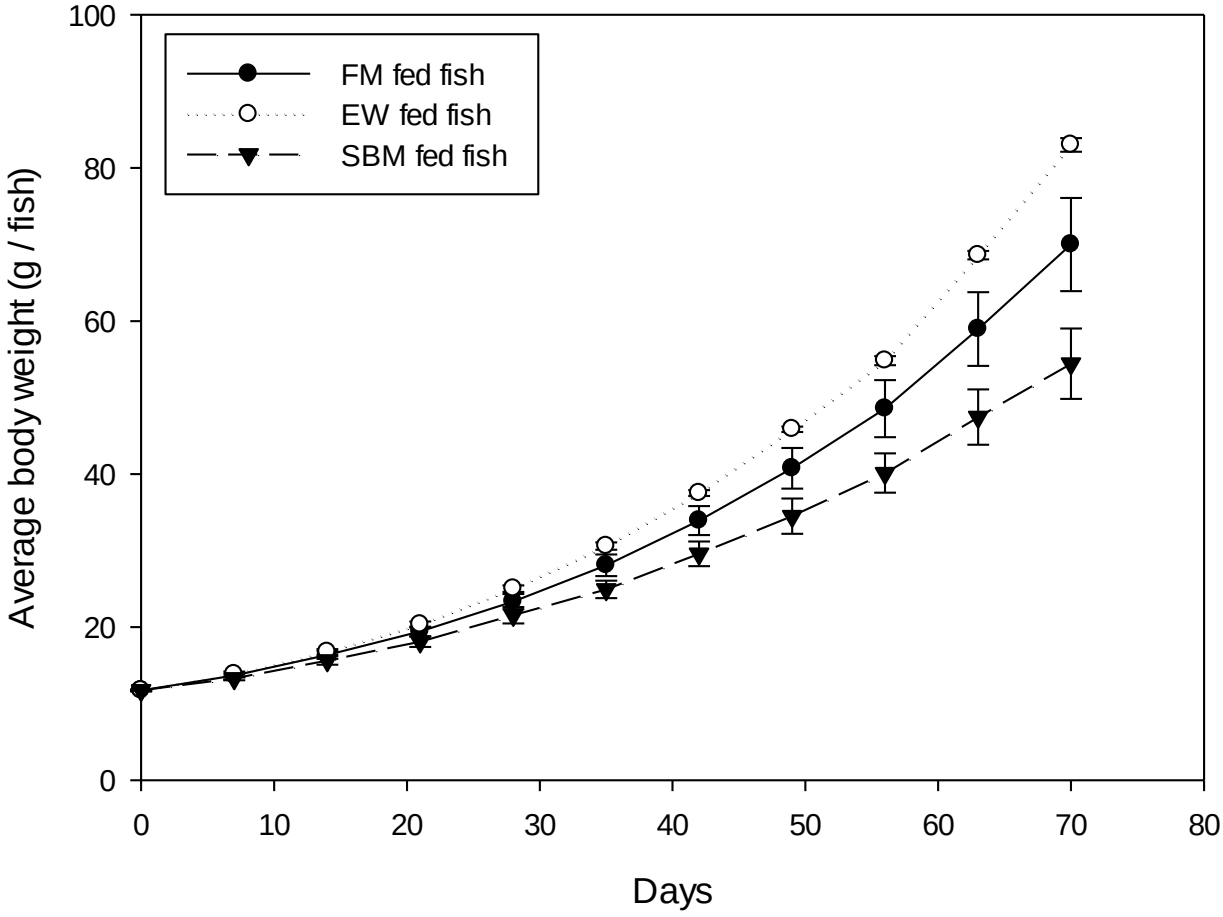


Figure 3.1. Average body weight gain of fish after 70 days of feeding on experimental diets.Data values are means ± standard deviation (*n* = 3). Dietary codes: FM = Fishmeal fed fish, EW = Earthworm fed fish, SBM = Soybean meal fed fish.

Table 3.2. Growth performance indexes of Common carp after 70 days of feeding on experimental diets. Values expressed as means ± SE (*n* = 3). Dietary codes: FM = Fishmeal fed fish; EW = Earthworm meal fed fish; SBM = Soybean meal fed fish.

Parameters	**Diets**			***P* - value**
	FM	**EW**	**SBM**	
Initial body weight (g/ fish)	11.74 ± 0.08	11.73 ± 0.05	11.73 ± 0.03	0.987
Final body weight (g/ fish)	70.00 ± 3.51[a]	83.01 ± 0.51[b]	54.43 ± 2.66[c]	0.001
Weight gain (g/ fish)	58.26± 3.43[a]	71.28 ± 0.56[b]	42.70 ± 2.65[c]	0.001
Protein efficiency ratio	1.83 ± 0.06[a]	2.04 ± 0.03[b]	1.37 ± 0.06[c]	<0.001
Specific growth rate (%/ day)	2.97 ± 0.02[a]	3.26 ± 0.02[b]	2.55 ± 0.08[c]	0.001
Feed conversion ratio (g/ g)	1.19 ± 0.02[a]	1.08 ± 0.01[b]	1.40 ± 0.04[c]	<0.001

[a-c] Significant differences between groups are indicated by difference in superscript letters

3A.4.2 Haematological and serological analysis

All values for haematology, serum and plasma data is presented in Table 3.3. The haematocrit level (% PCV) for fish fed EW diet (42.20 ± 1.14 %) was significantly elevated compared to control and SBM fed fish (40.72 ± 1.62 and 37.00 ± 1.15 %, respectively). Furthermore compared to control and SBM fed fish, the haemoglobin, mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration levels for fish fed the EW diet were significantly elevated (P <0.001). On the contrary, the level of circulatory leukocytes was significantly elevated (P = 0.001) in fish fed the SBM and control diet (3.10 ± 0.17 and 2.72 ± 0.17 $x10^4$ mm^3, respectively) compared to carp fed EW diet (2.15 ± 0.14 $x10^4$ mm^3). There were no significant differences between total numbers of erythrocytes (RBC), mean corpuscular volume, serum globulin levels, or serum albumin levels between treatments (P > 0.05). However, serum protein was significantly lower (P = 0.037) in fish fed SBM diet (3.15 ± 0.06 g/ dl) compared to fish fed fishmeal (3.40 ± 0.11 g/ dl). The albumin/ globulin ratio was significantly (P = 0.010) elevated in fish fed the SBM diet (2.08 ± 0.06) compared to fish fed the EW and control diet (1.86 ± 0.08 and 1.78 ± 0.07, respectively). No significant differences were observed in the differential cell counts.

Table 3.3. Haematological, serological indexes of Common carp after 70 days of feeding on experimental diets. Values expressed as means ± SE (*n* =15). Dietary codes: Control = Fishmeal fed fish; EW = earthworm fed fish; SBM = Soybean meal fed fish.

Parameter	**Diets**			***P* - value**
	FM	**EW**	**SBM**	
Haematocrit (% PCV)	40.72 ± 1.62[a]	42.20 ± 1.14[a]	37.00 ± 1.15[b]	0.021
Haemoglobin (g/ dl)	7.69 ± 0.28[a]	9.57 ± 0.24[b]	5.92 ± 0.31[c]	<0.001
RBC (10^6 mm^3)	1.34 ± 0.08	1.27 ± 0.08	1.30 ± 0.08	0.660
Leucocytes (10^4 mm^3)	2.72 ± 0.17[a]	2.15 ± 0.14[b]	3.10 ± 0.17[a]	0.001
Lymphocytes (%)	95.83 ± 0.39	94.27 ± 0.77	95.93 ± 0.32	0.110
Granulocytes (%)	3.43 ± 0.37	5.00 ± 0.67	3.60 ± 0.30	0.075
Monocytes (%)	0.34 ± 0.13	0.73 ± 0.23	0.47 ± 0.12	0.628
MCV (fL)	309.79 ± 14.41	349.73 ± 19.02	295.20 ± 13.16	0.054
MCH (pg)	59.30 ± 2.90[a]	79.13 ± 4.59[b]	47.06 ± 2.86[a]	0.001
MCHC (g/ dl)	19.21 ± 0.50[a]	22.69 ± 0.54[b]	16.08 ± 0.83[c]	<0.001
Serum albumin (g/ dl)	0.51 ± 0.003	0.50 ± 0.003	0.54 ± 0.002	0.053
Serum globulin (g/ dl)	2.89 ± 0.10	2.78 ± 0.11	2.61 ± 0.057	0.115
Serum protein (g/ dl)	3.40 ± 0.11[a]	3.28 ± 0.11[ab]	3.15 ± 0.06[b]	0.037
Albumin / globulin Ratio	1.78 ± 0.07[a]	1.86 ± 0.08[a]	2.08 ± 0.06[b]	0.010

[a-c] Significant differences between groups are indicated by different superscript letters
RBC – Red Blood Cells
MCV – Mean Corpuscular Volume
MCH – Mean Corpuscular Haemoglobin
MCHC – Mean Corpuscular Haemoglobin Concentration

3A.5 Discussion

3.A.5.1 Growth performance and feed utilisation

Arguably contemporary research strategies are mainly investigating the use of plant based alternative protein sources to replace fishmeal (Gatlin *et al*., 2007; Hardy, 2010). Research into the use of non-conventional feed resources (NCFRs) to replace fishmeal and fish oil in aquafeeds is still in its infancy. In this context research into the potential use of insect meal including invetebrate earthworm meal to replace fishmeal in aquafeeds is poorly studied. Earthworms are well known for their ability to breakdown organic waste and so have been supported for use in the far east and Australia (Dynes, 2003), in relation to its role in turning 'waste to wealth' through the process of bioremediation, vermicomposting and production of earthworm biomass for potential use in the animal feed industry (Kale, 1998; Garg and Kaushik, 2005; Benitez *et al*., 2005). Many earthworm species have been advocated for the process of bioremediation and vermicomposting however it appears that the earthworm species *P. excavatus* a tropical epigeic worm species, has high fecundity and biomass production rates which makes it ideally suited for the potential use in feeds for the animal feed industry (Sharma *et al*., 2005; Sinha *et al*., 2010).

The nutritional evaluation of earthworm protein has shown that this commodity has a biological value regardless of the earthworm species (see Section 1.2) (Sabine, 1983; Tacon *et al*., 1983; Zhenjun *et al*., 1997). In fish the utilisation of earthworm meal as a protein source is poorly studied. Only a small number of studies have been conducted within the last decade to investigate the effect of feeding earthworm meal to fish growth performance and feed utilisation including common carp (*C.carpio*; Tuan and Focken, 2009; Rawling *et al*., 2012) and vundu fingerlings (*Heterobranchus longifilis*; Sogsbean and Madu, 2008). Indeed Tuan and Focken (2009) reported that fish fed diets where the fishmeal was replaced with earthworm meal at levels 30, 70 and 100 % had similar or higher growth rate, protein

efficiency, and energy retention than fish fed fishmeal based control diet. Likewise in the current study fish fed earthworm meal at 66 % replacement of fishmeal showed a higher specific growth rate, protein efficiency and lower feed conversion, indicative of better growth performance and feed utilisation than fish fed fishmeal diet.

On the contrary fish fed soybean meal at 66 % replacement of fishmeal showed a significant decrease by ca. 22 % in average body weight at day 70 of the experimental period when compared to fish fed fishmeal diet. Moreover compared to the fishmeal fed fish, significant decreases were observed in protein efficiency ratio ($P < 0.001$), specific growth rate ($P = 0.004$) and a significant increase in feed conversion ratio ($P = 0.029$). Similarly, Stickney *et al*. (1996) reported that rainbow trout fed diets at 70 and 100 % replacement of fish meal with soybean protein concentrate had reduced growth performance. Likewise, Kissil *et al*. (2000) reported that in seabream fed diets replacing 60 and 100 % of fishmeal protein with soy protein concentrate had significantly reduced growth performance. Indeed more recent studies have shown negative effects towards fish growth performance when fed soybean meal at the expense of fishmeal including rainbow trout (*O. mykiss*; Romarheim *et al*., 2006; Collins *et al*., 2012) juvenile cobia (*Rachycentron canadum*; Chou *et al*., 2004) Atlantic salmon (*Salmo salar*; Krogdahl *et al*., 2003). Moreover, in a recent review by Collins *et al*. (2013) highlighted that a number of other plant proteins have similar negative effects in salmonids. However as outlined in Section 1.4 there are a number of studies that have reported no adverse effects to growth performance of fish when fed modified plant proteins so currently plant proteins are increasingly being used in commercial feeds for salmonids (Naylor *et al*., 2009; Hardy, 2010; Zhang *et al*., 2012).

It is evident from recent literature and the current study that there are varying results in relation to growth performance and feed utilisation of fish when fed soybean meals and soybean protein concentrates. Despite this with the advancement in technology allowing for

the refinement and development of novel plant feed resources, where many of the undesirable characteristics have been removed, the future is promising for the inclusion of higher levels of plant protein sources in aquafeeds. Indeed in experimental Chapter 4 the efficacy on carp growth performance and feed utilisation of two plant protein concentrates is tested.

3A.5.2 Haematological and serological analysis

There are a number of specific haematological parameters recognised as valuable tools for monitoring fish health and physiological responses to environmental stress (Bhaskar and Rao, 1984; Schuett *et al*., 1997; Jawad *et al*., 2004). Svobodova *et al*. (2005) suggested that icthyo-haematology is a useful tool in the assessment of feed composition and nutritional status in relation to environmental conditions affecting fish. The present study demonstrated that compared carp fed fishmeal diet and commercial soybean meal diet, fish fed earthworm diet showed significantly elevated haemoglobin, MCH and MCHC levels (P = 0.001 and P = <0.001, respectively). This observed elevation could be due to higher levels of iron (Fe) in the earthworm meal compared to fishmeal and soybean meal; indeed inductively coupled plasma optical emission spectrometry (ICP-OES, ISO9001-2000SGC) showed that Fe levels within the earthworm meal (499.47 mg/ kg, dry weight) was significantly higher than fishmeal and soybean meal Fe levels (245.74 and 91.90 mg/ kg dry weight, respectively). Fe is a vital element for all living organisms because it is essential for multiple metabolic processes to include oxygen transport. Moreover, Fe is central to the quaternary structure of haemoglobin, DNA synthesis and electron transport and so increased levels of iron in the earthworm meal may have contributed the observed results. It must be noted that compared to previously published haemoglobin levels for cyprinids (7 – 10 g/ dl) both fish fed fishmeal and earthworm diets were in line with these values; however, fish fed soybean meal diet showed ca. 18 % reduction in haemoglobin level (Smeda and Houston, 1979; Schwaiger *et*

al., 2000; Svobodova *et al*., 2005; Walencik and Witeska, 2007). This study is the first to report the effects of feeding soybean meal on fish haematology and shows that fish fed this dietary regime had a reduction in erythrocyte haemoglobin concentration which could affect respiratory gas exchange and transport. This could have significant implications to the fish under periods of high stress such as transportation, when oxygen consumption rates are reported to be higher (Wendelaar Bonga, 1997; Sloman *et al*., 2000); this should be a topic of interest in future studies if plant sources are to be included at higher inclusion levels in future diets for ornamental fish.

Leukocytes play a major role in the innate immunity where they are thought to scout the organism for foreign invaders or danger signals marked by pattern recognition proteins or receptors. For this reason their circulatory levels can be considered as a bio-indicator of health status in fish (Whyte, 2007; Magnadottir, 2010). In the present study compared to fishmeal and soybean meal fed fish, fish fed earthworm meal showed a significant reduction, by ca. 22 %, of total leukocyte levels. However the levels shown in the current study are in line with those previously reported for cyprinids (Groff and Zinkl, 1999). In contrast the highest value for total leukocyte levels was shown in fish fed soybean meal, perhaps confirming the elevated levels in the RBA response of fish fed this diet compared to fish fed earthworm meal. There were no differences observed in the differential blood counts for all experimental groups. This said there is a trend towards significance in granulocyte levels where fish fed earthworm meal had higher levels of circulatory granulocytes compared to all other experimental treatments (P = 0.075). In fish the main immunes cells of the innate immune system are granulocytes (in fish mainly neutrophils) and monocytes/ macrophages and both cell types are recognised to have phagocytic activity (Magnodottir, 2010). In this regard although there was a significant decrease in total leukocyte levels in fish fed earthworm meal, within the population of lymphocytes there seems to be an apparent trend

towards an increase in the proportion of granulocytes which could be beneficial to eliminating any potential pathogen. Perhaps the reason why there was no observed significant difference could be due to the high variation within the cohorts of fish. Serological analysis showed that serum protein levels in fish fed soybean meal was significantly lower compared to fish fed fishmeal ($P = 0.037$). This may be related to a lower protein digestibility of soybean meal compared to fishmeal. Previously Degani *et al*. (1997) reported that the protein digestibility of soybean meal when fed to carp was significantly lower compared to fishmeal. This observation could also be related to the disruption of liver protein metabolism due to the damage to liver tissue. There are a number of reports pertaining to the effects of feeding soybean meal and oil on disruption of fish liver tissue through increasing lipid accretion, commonly termed liver steatosis (Sitjà-Bobadilla *et al*., 2005; Wassef *et al*., 2007).

3A.5.3 Conclusion

The present investigation demonstrated that replacing standard herring fishmeal with earthworm meal at high inclusion rates (i.e. 66 % replacement of fishmeal; 411 g/ kg dietary inclusion) can have positive effects on growth and feed utilisation of mirror carp. In contrast it appears that feeding carp soybean meal at high level of replacement of fishmeal (66 % replacement, 494 g/ kg dietary inclusion) caused a significant decrease in growth performance and feed utilisation. In addition the fish fed soybean meal showed a high level of circulatory leukocytes which could indicate an inflammatory response in fish fed this dietary regime. The soybean meal used in this study was a standard commercial meal produced commercially and although this product was processed to remove some of the anti-nutritional factors (ANFs) not all may have been removed. Likewise in fish fed soybean meal the serum protein level was lower compared to fish fed fishmeal, this may be as a result of a lower protein digestibility of the soybean meal compared to fishmeal in carp or it could be as a

result of damage to liver tissue through lipid accretion. Subsequently these attributes may have contributed to the observed adverse effects in carp growth performance and haematological responses in this study. Indeed a strong link between inflammatory responses and the use of soybean meal has been well established in salmonids and cyprinids (see Section 3B.5).

On the contrary, in relation to the improvement in growth response and feed utilisation in fish fed earthworm meal this could have significant implications for the ornamental aquatic industry were high value diet specifications based on exotic ingredients are more acceptable. Furthermore fish fed earthworm meal showed elevated haemoglobin levels compared to fish fed fishmeal. However this result may be offset by the fact that a reduction in circulatory leukocytes was observed in fish fed earthworm compared to fish fed fishmeal. Chapter 5 provides a more detailed evaluation of the effects of feeding earthworm meal to fish immune responses and so current feed formulations need to take these findings in to account when using this commodity in diets for cyprinids. In addition future studies should evaluate lower inclusion levels to see if elevated growth performance can be achieved using less earthworm meal, this perhaps would reflect a more practical diet formulation for cyprinids.

CHAPTER 3B.

IMMUNOLOGICAL RESPONSE OF MIRROR CARP (*C. CARPIO*) FED A TROPICAL EARTHWORM MEAL AND COMMERCIAL SOYBEAN MEAL IN EXPERIMENTAL DIETS

Abstract

A preliminary experiment was conducted to investigate the effects of feeding an earthworm meal (EW diet) and a commercial soybean meal (SBM) on mirror carp immune response. Fish were injected intraperitoneally with a bacterin from *Aeromonas hydrophila* and at day 0, 7, 14 and 28 fish blood was collected from the caudal vein for further analysis. At day 14 and 21 post injection fish fed the EW diet demonstrated a significant reduction in respiratory burst activity (RBA) compared to control and SBM fed fish. At day 7 post injection fish fed fishmeal diet demonstrated a significant elevation in serum IgM levels compared to fish fed EW and SBM diets ($P < 0.001$).

3B. 1 Introduction

The aquatic environment is highly antigenic and thus the external barriers of fish such as skin, gills and digestive tract play an important role in controlling potential infectious routes. The fish immune system is required to function in a broad range of environmental conditions, thus the fish immune response is predominated by a broader range of innate responses characterised by a lack of antigen specificity and memory (Tort *et al*., 2003; Engelsma *et al*., 2003; Magnadottir, 2010). In healthy fish pathogens and other disease causing agents can be neutralised or prevented from entering the host by the mucosal layer covering the physical epithelial barrier and the innate immune system. Although the fish innate immune system is becoming well characterised as outlined in Section 1.5 (Magnadottir, 2010; Rebl *et al*., 2010), many factors including genetics, age, nutritional status and gut microflora all contribute to

the observed variation in immune responses of fish (Lorenzen *et al*., 2009). Consequently a new area of research is developing that integrates nutrition and immunology research methodologies to define a role for nutrients in the metabolism and function of fish immune cells at molecular, cellular, tissue and whole body levels (Calder *et al*., 2002).

Up until recently it is now widely accepted that nutrition has major health implications in fish (Trichet, 2010) and so the interaction of amino acids, vitamins and minerals are required in cell division and the synthesis of important proteins by immune cells (Calder, 2006; Trichet, 2010). Thus it is important that dietary formulations meet known nutritional requirements because aquafeeds represent the largest component of production costs. The awareness that fishmeal and fish oil are reaching their maximum exploitation levels, as outlined in Section 1.1, are key factors driving research focus towards the development of new feeds that ensure fish are of the highest quality whilst reducing the use of these limited commodities (Tacon and Metian, 2008; Tacon *et al*., 2010). Improvement in fish quality is particularly important in the ornamental industry because the value of fish is largely based on their aesthetic value; this is particularly the case in the koi carp industry where fish have been sold for up to $500,000.

Arguably, current understanding of the effects on health and growth performance of fish subjected to alternative protein sources is limited to plant protein sources (Rumsey *et al*., 1994; Krogdahl *et al*., 2000; Gatlin *et al*., 2007; Fjelldal *et al*., 2010, Krogdhal *et al*., 2010). Many investigations have noted the effects of feeding plants proteins to fish and have concluded that feeding plant sources in excess of 30% fishmeal replacement causes enteropathy in the intestine of salmonids (reviewed by Krogdhal *et al*., 2010) and cyprinids (Uran *et al*., 2008). Indeed many studies have modelled and induced enteropathy using soybean meal mainly in salmonids. The pathology of soybean meal enteropathy in fish has been well characterised where histological analysis of the intestinal tissue has shown

shortening of the primary and secondary villi and widening of the lamina propria (Baeverfjord & Krogdahl,1996; Bakke-McKellep *et al*., 2000; Chikwati *et al*., 2013). Moreover several recent studies have characterised the effects of soybean enteropathy using RT-PCR assays and have targeted an array of pro-inflammatory cytokines including TNF-α, IL-1β and IL-17, as biomarkers to demonstrate the acute inflammatory response of salmonids and cyprinids when fed soybean meal in excess of 20% replacement of fishmeal (Uran *et al*., 2008; Marjara *et al*., 2012; Sahlmann *et al*., 2013). Currently to the authors knowledge little is known about the effect of feeding non-conventional feed resources to fish (NCFRs) immune response.

As demonstrated in Section 3A earthworm protein as potential protein source has no detrimental effect towards growth and feed utilisation when fed to carp at 66% replacement of fishmeal. In this context the biological value of earthworm meal is high compared to conventional plant protein sources currently being investigated (Refer to Section 1.2). In the present study the aim was to understand the effect of feeding earthworm meal and soybean meal to immune response of mirror carp. To induce an infection a bacterin was developed based *Aermonas hydrophila* and the fish were injected via the intraperitoneal cavity.

The specific hypotheses tested were as follows:

$H_0$1 - Feeding fish earthworm meal will decrease the fish respiratory burst response of carp whole blood compared to fish fed fishmeal.

$H_0$2 - Feeding soybean meal will increase the fish respiratory burst response of carp whole blood compared to fish fed fishmeal.

$H_0$3 - Replacement of fishmeal with either earthworm meal or soybean meal will have no effect on the fish circulatory IgM response compared to fish fed fishmeal.

3B.2 Materials and methods

3B.2.1. Fish and experimental protocol

The experiment was carried out at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Mirror carp (*C.carpio*) fry were obtained from Hampshire carp hatcheries, Hampshire, U.K. Following the nutrition trial 80 stock fish (20.37 ± 0.7 g) were randomly distributed into 4 x 150 l fibreglass tanks (20 fish per tank) containing aerated recirculated freshwater. Fish were fed diets to a fixed regime of 3 % body weight per day spread across three feeding times for 28 days. For rearing conditions see section 3A.2.1.

3B.2.2 Feed formulation and diets

Fish were fed the same diet composition as fed in trial 3A. All diets were analysed according to official AOAC (2007) protocols see Section 2.7 for details.

3B.2.3 Intraperitoneal injection of bacterin from *A. hydrophila* and immunological analysis

3B.2.3.1 Bacterin preparation

To determine the concentration of bacteria used for bacterin a standard concentration curve was prepared from an *A. hydrophila* isolate (NCIMB, strain 1137/ 47) by using the viable drop counts technique (Miles and Misra, 1938). Briefly, the bacteria were grown overnight in tryptic soy broth (TSB; Oxoid, UK) at 30 °C and the pellets were harvested and washed twice with phosphate buffered saline (PBS, pH 7.3) by centrifuging at 1600 × g for 15 min. Cells were resuspended in PBS at a concentration of 1 $x10^8$ cells/ ml as determined from a standard

curve of absorbance at 610 nm versus colony forming units. The bacterin was prepared for immunisation by heat-killing bacterial cells in PBS (pH 7.3) at 70 ºC for 1 h. Before injection the bacterial cell suspension was checked for sterility by aliquoting a 100 µl on TSA plates and incubating overnight at 30 ºC.

3B.2.4 Immunisation assay

Following the growth phase of the trial fish were kept on the same feeding regime as before for a further 28 day period. Before injection of bacteria, fish were anaesthetised with MS-222 at 150 mg/ l. Each experimental group was administered 100 µl of whole cell bacterin (1 x 10^8 cfu/ ml) intraperitoneally (I.P.) and control fish were injected I.P. with PBS. At day 7, 14, 21 and 28 days blood samples were collected by caudal venipuncture. Sub samples of whole blood was left to clot for a period of 12 h (at 4 ºC) and then centrifuged at 3,600 g for 6 minutes to recover serum. Serum was removed and stored at -80 ºC until analysis.

3B.2.5 Respiratory burst activity

The respiratory burst activity (RBA) of phagocytes from fish I.P. injected with *A. hydrophila* was quantified using the reduction of nitroblue tetrazolium (NBT) to formazon as a measure of superoxide anion (O2-) production. The assay was carried out according to the method of Secombes (1990) as modified by Stasiack and Bauman (1996). Blood samples were collected by caudal venipuncture using heparin at 100 USP U/ ml of sample as anticoagulent. 50 µl of blood was aliquoted into 'U' bottom well microtitre plates, incubated at 37 ºC for 2 h to facilitate adhesion of cells, and PMA was added to the wells at 1µl/ ml (Phorbol 12-Myristate 13-Acetate, Sigma Aldrich, UK). The supernatant was then removed and the adhered wells were washed thrice with PBS. After washing, 50 µl of 0.2 % NBT (Sigma Aldrich, UK) was added and the resulting solution was incubated at room temperature for 2 h. The cells were

then fixed with 100 % methanol for 2-3 minutes and again were washed thrice with 30% methanol. The microtitre plates were left to air dry and then 60 µl of 2N KOH and 70 µl of DMSO were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue solution was measured using and ELISA plate reader at 540 nm.

3B.2.6. Enzyme Linked Immunosorbent Assay

A direct ELISA was used to measure the antibody titre in fish serum samples according to Adams and Thompson (1990). Briefly, a suspension of *A. hydrophila* (corresponding strain used for raising serum) with a concentration of 1×10^8 cells/ ml was added to the microtitre plate at 100 µl/ well. The plate was incubated overnight at 4 °C, after which the bacterial cells were fixed to the bottom of the well using 50 µl/ well of 0.05 % (v/v) glutaraldehyde in PBS (pH 7.2) incubating for 20 min at 20-22 °C.

For fish serum, two fold dilutions of the serum from 1/8 - 1/1024 were made in antibody buffer (1 % BSA solution, Sigma Aldrich, UK) and 100 µl/ well was added to the ELISA plate. The serum from the control fish was added to the last row as a positive control. Anti-common carp/ Koi carp IgM monoclonal antibody labelled with horseradish peroxidase (MAbs, Aquatic Diagnostics Ltd., Stirling, U.K.) was used to detect IgM fish titre levels following the manufacturer's instructions. One hundred µl/ well of reconstituted anti-fish Mab-HRP was added to the microtitre plate and incubated for 1 h at 22 ºC. One hundred µl/ well of chromogen (42 mM TMB dihydrochloride solution) in substrate buffer (Sodium acetate / citric acid buffer, pH 5.4) were added to the wells. The microtitre plate was then incubated for a further 10 min at 20-22°C and the reaction was stopped with 50 µl/ well of 2 M H_2SO_4. Finally, the plate was read in an ELISA plate reader at 540 nm (Dynex technologies, UK) and the results were compared between control and sample wells. Values

three times higher than the negative control value were considered positive. The positive values at the lowest dilution were considered as a titre value for the serum examined.

3B.3 Statistical analysis

All data are presented as means ± standard error (SE). Antibody titres are presented as the average antibody titre (-Log2 + 1 ± Standard error) for ten fish. Data was transformed where necessary and statistical analysis was conducted using SPSS statistics version 18 for windows (SPSS Inc., Chicago, Il, USA) and accepted at the $P < 0.05$ level. Data was analysed two-way ANOVA. Significant differences between control and experimental groups were determined using post-hoc Tukeys HSD test.

3B.4 Results

3B.5.1 Respiratory burst activity and circulatory IgM response to I.P. injection with bacterin

The RBA of neutrophils is presented in Figure 3.2. The NBT assay showed significant differences ($P < 0.001$) in RBA among groups. The highest RBA activity was recorded in fish fed SBM 14 days post injection (0.205 ± 0.07). Compared to fish fed the EW diet, RBA for fish fed control diet was a significantly higher at day 21 post immunisation ($P < 0.001$). Circulatory fish IgM response to I.P. injection is presented in Table 3.4. A significant increase ($P < 0.001$) in IgM titre levels was shown in fish fed fishmeal (5.50 ± 1.27 –log2 +1), compared to all other treatment groups at day 7 post injection. Moreover the lowest recorded value at this time point was observed in fish fed soybean meal (4.00 ± 1.04 –log+2). No differences were observed between dietary groups throughout the other sampling points.

Figure 3.2. RBA of fish after intraperitoneal injection with heat inactivated *A. hydrophila*. Data expressed relative to fish injected with PBS ± SE (*n* = 10). Dietary codes = fishmeal fed fish; EW = earthworm fed fish; SBM = soybean meal fed fish. * - denotes significant difference between fish fed control and EW fed fish at P< 0.05. # - denotes significant difference between SBM fed fish and EW fed fish

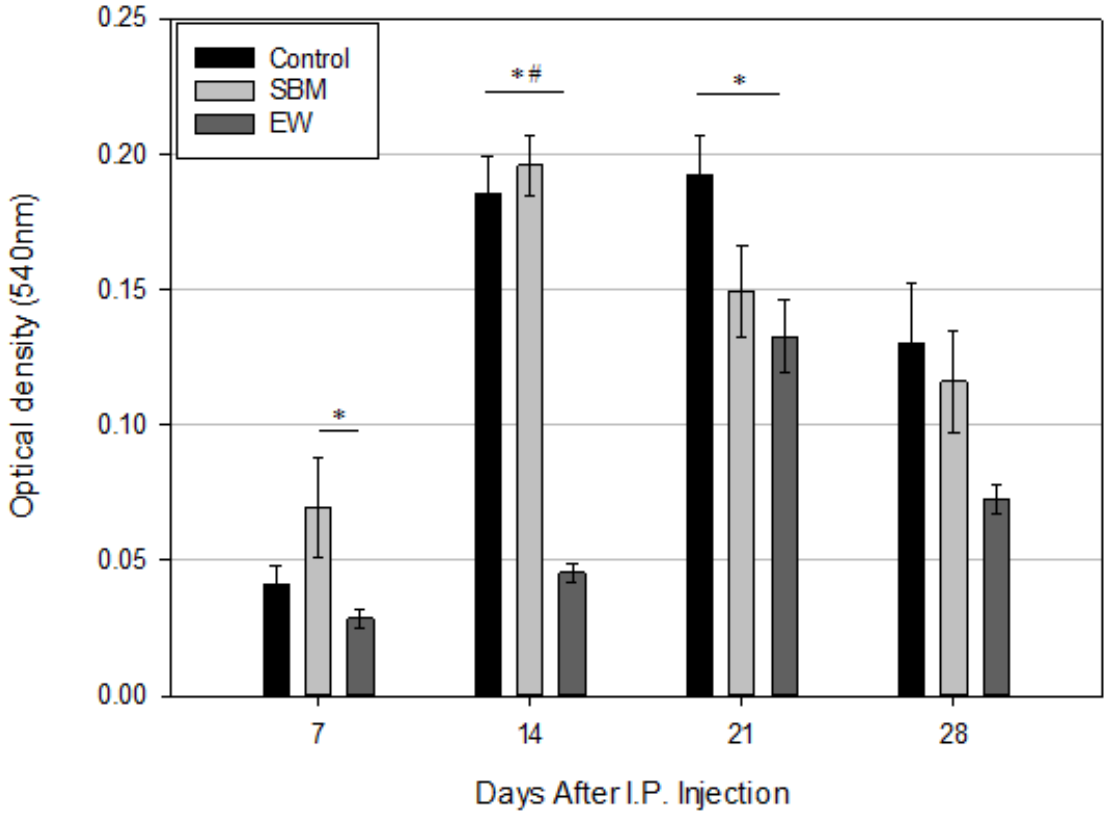


Table 3.4. Response of fish circulatory antibody titre levels after intraperitoneal injection (I.P.) with heat inactivated *A. hydrophila*. Values expressed as means (-log2+1) ± SE (*n* = 10). Dietary codes: fishmeal fed fish; EW = earthworm meal fed fish; SBM = soybean meal fed fish. All values were expressed compared to PBS control serum samples.

Days after I.P. injection	**Diets**			***P*- value**
	Control	**EW**	**SBM**	
7	5.50 ± 1.27[a]	4.30 ± 0.64[b]	4.00 ± 1.04[c]	<0.001
14	10.70 ± 1.55	11.0 ± 2.61	10.70 ± 1.42	0.931
21	10.30 ± 1.27	10.50 ± 1.87	9.10 ± 1.20	0.078
28	11.30 ± 1.35	11.60 ± 1.94	11.20 ± 1.80	0.876

[a-c] Superscripts denote a significant difference using Tukey's HSD.

3B.5 Discussion

With the rise in awareness that fish oil and fishmeal resources are becoming critically low it is important to find alternative protein sources that not only meet the nutritional requirements of the target species but to ensure high fish quality to maintain aesthetic value and to control disease outbreak. Therefore, it is important for potential protein sources to fulfil the nutritional requirements of the immune system. Hence in this investigation the aim was to determine the effect of feeding earthworm meal on mirror carp, immunological responses. As the mirror carp is a model ornamental fish species, this allowed one to draw conclusions with both relevance to the ornamental and the aquaculture sector.

3B.5.1 Respiratory burst activity (RBA) response

In fish the production of superoxide and hydrogen peroxide known as RBA forms the basis of a highly potent antibacterial system (Anderson, 1994; Secombes, 1997). RBA responses in fish can be strongly activated by pathogen associated molecular patterns (PAMP) and so increased RBA can be correlated with increased activity of phagocytic cells to kill pathogens (Sharp and Secombes, 1993). Most of the studies pertaining to the activation of RBA have focused on LPS, which has been shown to induce respiratory burst in goldfish (*Carassius auratus*; Stafford *et al*., 2002; Reiger *et al*., 2010) rainbow trout (*O.mykiss*; Boltana *et al*., 2009; Nya and Austin, 2010) Atlantic Salmon and gilthead seabream (*S.aurata*; Sepulcre *et al*., 2007). However other stimuli including important fish pathogens have also been shown to induce strong RBA such as *Aeromonas* spp in goldfish (Katzenback and Belosevic, 2009) and carp (Ardo *et al*., 2010) and *Vibrio Anguillarium* in gilthead seabream infections (Chaves-Pozo *et al*., 2005). In the present study fish were injected with *A.hydrophila* to stimulate the RBA of phagocytes, as measured by means of NBT reduction by reactive oxygen species (ROS). The results showed a 4-fold higher increase in RBA of fish fed fishmeal and SBM

diets compared to fish fed earthworm diet 14 days post injection. Moreover at day 21 post injection, fish fed the control diet demonstrated significantly elevated RBA compared to fish fed the soybean and earthworm meal. The higher RBA values can be correlated with higher circulatory leukocyte counts for both fish fed fishmeal and soybean meal compared to fish fed earthworm diet.

One hypothesis for the observed decrease in RBA and leukocytes levels (refer to Section 3A.4.2) of carp fed earthworm meal could be due to the antibacterial activity of the earthworms in the diet. It has been well characterised that within the earthworm's ceolomic fluid contains a number of antimicrobial proteins that exert cytolytic functions against pathogenic Gram negative bacteria (e.g. *A. hydrophila*) and Gram positive bacteria (e.g. *Bacillus megaterium*). These peptides include fetidins 40-45 kDa, lysenins 41kDa and eiseniapore 38kDa (Valembois *et al* 1982; Cooper and Roch, 2003). These cytolytic functions may have acted directly on sequestering the presence of *A.hydrophila*, thus the fish phagocytes may have remained in a pre-stimulated state displaying low levels of RBA. Previously in comparison to ayu (*Plecoglossus altivelis*) it has been demonstrated that carp neutrophils display low levels of respiratory burst in resting cells (Serada *et al*., 2005). Further work would be required to elucidate whether these proteins remain active through the process of monogastric digestion.

In contrast compared to fish fed earthworm meal both fish fed soybean meal and fishmeal exhibited a significant increase in RBA, where the highest recorded value was in fish fed soybean meal. In a recent study on the functional induction of the respiratory burst response in fish it was suggested that inflammatory cytokines could play an important role (Reiger *et al*., 2010). These include cytokines tumor necrosis factor (TNF) α-1 and -2 (Grayfer *et al*., 2008; Grayfer and Belsovic, 2009a) interferon (IFN) γ (Grayfer and Belosevic, 2009b; Arts *et al*., 2010; Grayfer *et al*., 2010), colony stimulating factor- 1

(Grayfer *et al*., 2009) and interleukin (IL)-8 (Omania Harun *et al*., 2008). Interestingly recent reports suggest that in some teleost fish including common carp (Forlenza *et al*., 2009a) and seabream (Roca *et al*., 2008) the cytokine TNF-α does not directly activate phagocytes, but activates endothelial cells leading to an indirect activation of phagocytes. In this context the fact that it has been noted during soybean enteropathy there is an acute up-regulation in the expression of pro-inflammatory cytokines in cyprinids and salmonids (Uran *et al*., 2008; Marjara *et al*., 2012; Sahlmann *et al*., 2013); this could have indirectly induced the observed increase in respiratory burst response in fish fed soybean meal compared to fish fed earthworm meal.

3B.5.2 Circulatory IgM response

In comparison to human immunoglobulins in general the IgM is the main immunoglobulin present in teleost fish where it is thought that its structural flexibility may compensate for a lack of diversity (Ellis, 1998; Watts *et al*., 2001). Recently other classes of immunoglobulins have been identified in fish namely IgD, IgT and IgZ. Although there has been number of recent studies indicating the presence of IgT in mucosal gut tolerances to bacteria, as of yet there are still more questions than answer to role and function of these molecules in fish immune responses (Randelli *et al*., 2008, Zhang *et al*., 2011). In contrast the presence of IgM has been well characterised in fish where it is an important effector molecule linking the innate immune response with the adaptive immune response (Magnadottir *et al*., 1995; Kachamakova *et al*., 2006). IgM has been found to exist in two forms in fish, where B cells exhibit both membrane bound IgM as a monomer and soluble IgM as a tetramer (Grove *et al*., 2006). In the present study the levels of soluble IgM were measured using a direct ELISA assay. At 7 days post injection with *A.hydophila* fish fed fishmeal demonstrated higher IgM titre levels compared to all other experimental treatments. Apart from this time point all other

time points showed no effect of protein source towards IgM titre responses in carp. Interestingly, similar titre levels were reported by Yin and colleagues (2009) where they vaccinated common carp with a vaccine developed against *A. hydrophila/A. salmonicida*.

3B.5.3 Conclusion

In summary, the current investigation has shown that feeding earthworm meal to fish at high level replacement of fishmeal can have a negative effect towards respiratory burst response of carp. This can be correlated with and equal significant decrease in circulatory levels of leukocytes as shown in Section 3A.4.2. In contrast this study showed that when fish are fed high level of soybean meal in the diet they presented classical inflammatory symptoms as previously characterised in salmonids and cyprinids, where the highest recorded respiratory burst response was in fish fed soybean meal. Taking into account the response of the fish in relation to growth performance and feed utilisation it would appear that feeding earthworm meal to fish at high level replacement of fishmeal can have a positive effect to carp. However as shown by the results of this study this may be offset by the reduction in respiratory burst response thus implying that fish have a reduced ability of phagocytic cells to augment this type of response to *A.hydrophila*. As suggested this observed response could be attributed to the fact that earthworm meal contains a number for antimicrobial peptides that may have passively been utilised by the fish. This important finding should be taken into consideration when formulating aquafeeds and should assess the innate immune response in more detail upon exposure to live pathogens, to discern which component of the earthworm meal causes the negative effects and to what extent this impacts fish disease resistance. In fact, determining the level of survival during challenge is one of the best criteria for defining the level of resistance because it reflects the cumulative effects of all host-pathogen interactions during production (Wiegertjes *et al.,* 1996). Unfortunately, due to the nature of this kind of

work it was no possible to adopt this approach working under the current constraints of the sponsors Waltham pet nutrition's ethical council.

CHAPTER 4.

GROWTH AND HAEMATOLOGICAL RESPONSE OF MIRROR CARP FED PLANT PROTEIN CONCENTRATES AND A COMBINATION OF WHEY PROTEIN CONCENTRATE AND CASEIN PROTEIN IN EXPERIMENTAL DIETS

Abstract

An investigation was conducted to evaluate the effect of feeding two plant protein concentrates and a combination of whey protein concentrate and casein protein on the growth performance, haematological and serological responses of mirror carp (*C. carpio*). Fish were fed diets for a total of 84 days, fishmeal served as the main protein source in the control diet. Three remaining diets consisted of fishmeal fixed at 33% provision of protein and the remaining 66% protein was provided by rice protein concentrate (RPC diet), corn protein concentrate (CPC) and a combination of whey protein concentrate (8%) and casein protein (58%) (WPC diet). After 84 days of feeding fish fed WPC diet (27.80 ± 0.53 g/ fish) showed a significant elevation in final body weight compared to fish fed a fishmeal diet (25.13 ± 0.40 g/ fish) and fish fed RPC and CPC diets (16.77 ± 0.45 and 14.09 ± 0.054 g/ fish, respectively). Similar improvements were observed in feed utilisation and protein efficiency. At the end of the trial feeding WPC diet (95.23 ± 0.72 %) to mirror carp showed a significant decrease in the proportion of lymphocytes in the blood (P = 0.012), compared to fishmeal fed fish and RPC diets (97.37 ± 0.40 and 97.60 ± 0.34 %). In contrast there was a significant elevation (P = 0.008) elevation in the proportion of granulocytes in fish fed WPC diet (4.13 ± 0.61 %) compared to fish fed fishmeal and RPC diets (1.90 ± 0.34 and 2.37 ± 0.39, respectively). Likewise in fish fed WPC diet a significant elevation (P = 0.008) was shown in mean corpuscular haemoglobin levels (82.11 ± 5.21 pg) compared to fish fed fishmeal and RPC diets (69.58 ± 3.13 and 65.45 ± 3.26 pg, respectively). Serological analysis showed that feeding carp the WPC showed a significant increase in serum albumin and protein

concentrations compared to fish fed fishmeal diet ($P < 0.001$). Indeed this led to a significantly higher ($P < 0.001$) albumin/ globulin ratio in fish fed WPC compared to carp fed fishmeal and RPC diets.

4.1 Introduction

Several decades ago fishmeal was used almost exclusively in feeds for poultry and swine. However, with the increased price of fishmeal and the rapid growth of other industry producing sectors such as aquaculture forced the swine and poultry industries to rapidly reduce the use of fishmeal. Subsequently this stimulated an extensive evaluation of nutritional and other feed related research to identify alternative protein sources to fishmeal and today very little or in some feeds anything at all of fishmeal is used in grower or finishing feeds for broilers and pigs (New and Csavas, 1995). In recent years, the same developments are being observed within the aquaculture and ornamental feed industry and so the inclusion levels of fishmeal are being substantially reduced in feed for many important fish and crustacean species. This reduction has come about through extensive research into improving the quality of existing feed commodities already in use in terrestrial agriculture such as plant proteins and by-products from the dairy industry such as whey protein (see Section 1.2 for details).

Historically whey and casein proteins have been considered by the cheese manufacturing industry as waste products. However within the last two decades research in this area has discovered that whey proteins consist of many components including β-lactoglobulin, α-lactalbumin, proteose peptones, blood proteins, lactose, and important essential minerals such as calcium that have been exploited as highly nutritious food supplements for humans and feeds for the swine industry. By virtue of the content of essential amino acids, the biological value of whey proteins is high compared with that of other dietary proteins such as plant proteins. Whey proteins have proportionally more sulphur-containing

amino acids (cysteine, methionine) compared to other protein sources, which contribute to the observed higher protein efficiency ratio (PER) of whey protein (3.2) compared to that of casein (2.6) in human nutritional studies (Bounous and Gold, 1991; Korhonen and Pihlanto, 2006). Given these attributes casein and whey proteins have been used as principle protein sources in semi-purified diets in many fundamental nutritional evaluations of vitamin and mineral requirements of fin fish for decades (Halver, 1982; Halver 1985; Torrissen and Christiansen, 1995; Yamamoto *et al*., 2008). To date little is known about the effects of feeding a combination of whey protein concentrate and casein meal to fish growth performance and feed utilisation.

Plant feedstuffs would be the noticeable choice to replace fishmeal particularly cereal grains where global markets already exist for these particular feed commodities and trade routes are already established for increased availability if required (Gatlin *et al*., 2007). Indeed, in 2008 it was reported that cereal production was close to 2,525 million tonnes a 33.1 % increase in production when compared to data from 1995. Moreover in 2008 of this total cereal production total maize was produced at 822.7 million tonnes (32.6 %), followed by wheat at 689.9 million tonnes (27.3 %) and rice at 685.0 million tonnes (27.1 %) (F.A.O, 2012). From this corn and rice would appear to be major candidates to offset the use of fishmeal in aquafeeds. However as outlined in Section 1.2, plant protein sources consist of a number of anti-nutritional factors, significant deficiencies in essential amino acids (lysine and methionine) and contain other undesirable qualities such as high crude fibre content (Francis *et al*., 2001; Kaushick and Hemre, 2008; Krogdhal *et al*., 2010).

Nonetheless substantial improvements in the nutritional quality of plant feed commodities have been well documented through chemical and mechanical processing. Subsequently this has eliminated or reduced the concentration of certain undesirable characteristics including the removal of anti-nutrients (see Section 1.2) (Hardy, 2010), non-

soluble carbohydrates which are of little use to fish (Stone, 2003) leaving a cake with high protein content (Barrows *et al*., 2007; Krogdhal *et al*., 2010). These particular high protein products were coined by Hardy (2010) as protein concentrates and present the most promising alternate protein sources to use in aquafeeds for a number of reasons. Firstly the crude protein content of most protein concentrates range between 70 – 80 % making them suitable for both carnivorous and omnivorous feeds without the known side effects of high level substitution such as reduced growth rate, lower plasma cholesterol (Kaushik *et al*., 1995; Stickney *et al*., 1996; Refstie *et al*., 2000, 2001; Storebakken *et al*., 2000). Secondly, the production levels of protein concentrates could potentially meet the demands of the aquafeed industry without causing an economic shortfall. In fact several cereal concentrates made from maize gluten and wheat processing have already been investigated in experimental diets for turbot (*Psetta maxima*; Regost *et al*., 1999), Atlantic salmon (*Salmo salar*; Opstvedt *et al*., 2003), carps (Kaur and Saxena, 2005) and European sea bass (*Dicentrarchus labrax*; Robaina *et al*., 1999) with no detrimental effect to growth performance. In effect replacing fishmeal with plant protein concentrates to supply approximately half of dietary protein has been relatively easy; however when substituting higher percentages of fishmeal with plant protein concentrates is proving to be more difficult (Naylor *et al*., 2009; Hardy, 2010). But, several recent reports have concluded that when carefully formulating with plant protein concentrates into feeds for Atlantic salmon, at 90% replacement of fishmeal protein, it was possible in fish larger than 30g to get similar growth performance when compared to fish fed fishmeal (Bendiksen *et al*., 2011; Burr *et al*., 2012).

The current investigation aims to assess the effects feeding two commercially available plant protein concentrates from rice and corn and a combination of whey protein concentrate and casein meal on fish growth performance and feed utilisation. Additional

haematological analysis will be assessed to determine the effects of feeding such novel feed ingredients to fish health status for each dietary regime.

The specific hypotheses tested were as follows:

$H_0$1 - Feeding fish a combination of whey protein concentrate and casein meal will have a positive effect on the fish growth performance and feed utilisation compared to fish fed fishmeal.

$H_0$2 - Feeding rice protein concentrate will have a negative effect on the fish growth performance and feed utilisation compared to fish fed fishmeal.

$H_0$3 - Replacement of fishmeal with corn protein concentrate will have a negative effect on the growth performance and feed utilisation.

$H_0$4 - Replacement of fishmeal with a whey protein concentrate and casein meal will have no detrimental effect on the fish haematological and serological profiles.

$H_0$5 - Feeding fish the plant protein concentrates will have a detrimental effect on the fish haematological and serological profiles compared to fish fed fishmeal.

4.2 Materials and methods

4.2.1. Fish and experimental protocol

The experiment was carried out at the Aquaculture and Fish Nutrition Research Aquarium, University of Plymouth, UK. Mirror carp (*C. carpio*) fry were obtained from Hampshire carp hatcheries, Hampshire, U.K. After four weeks acclimation 360 fish (7.42 ± 0.1 g) were randomly distributed into 12 x 150 l fibreglass tanks (30 fish per tank) containing aerated recirculated freshwater. Fish were fed diets to a fixed regime of 3 % body weight per day spread across three feeding times for 84 days. Fish were batch weighed on a weekly basis following a 24 h starvation period and reared at 26 ± 1 ºC with a 12:12 h light:dark photoperiod. For details on rearing conditions refer to section 3A.2.

4.2.2 Feed formulation and diets

Three isonitrogenous and isolipidic diets were formulated using feed formulation software (Feedsoft®, USA) to contain approximately 38 % crude protein and 8 % lipid to meet the known requirements of juvenile common carp (N.R.C. 2011). Fishmeal served as the main protein source in the control diet. Three remaining diets consisted of fishmeal fixed at 33 % provision of protein and the remaining 66 % protein was provided corn protein concentrate (CPC diet), rice protein concentrate (RPC) and whey protein concentrate at 8 % and casein meal at 58 % (WPC diet). Each diet was produced by mechanically stirring the ingredients into a homogenous mixture using a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 – 10STDA mixer). Warm water was added to reach a consistency suitable for cold press extrusion to form 1 mm pellets (PTM Extruder system, model P6, Plymouth, UK). The nutritional profile was determined according to AOAC (2007) official protocols see Section 2.7 for details (see Table 4.1).

Table 4.1. Formulation and chemical composition of experimental diets. Each ingredient component is expressed as g/ kg per diet.

	FM	**CPC**	**RPC**	**WPC**
Ingredients				
Herring meal LT94[1]	553.94	180.00	180.00	180.00
Corn starch	391.06	400.20	377.81	432.58
Corn protein concentrate[2]		334.84		
Rice protein concentrate[3]			356.23	
Whey protein concentrate[4]				50.00
Casein (Bacarel)[5]				244.39
Fish oil[6]		29.96	30.96	38.03
Sunflower oil	20.00	20.00	20.00	20.00
Vitamin/ mineral premix[7]	20.00	20.00	20.00	20.00
Antioxidant[8]	0.50	0.50	0.50	0.50
Molasses	5.00	5.00	5.00	5.00
CMC – binder[9]	10.00	10.00	10.00	10.00
Proximate analysis				
Dry matter (%)	93.0	94.2	95.5	93.3
Crude Protein (%)[10]	39.6	40.4	40.4	39.3
Crude lipid (%)[10]	5.9	7.5	7.4	7.0
Ash (%)[10]	10.1	4.9	5.0	4.7
NFE[11]	40.1	41.4	42.7	42.3
Gross energy (MJ kg $^{-1}$)	19.2	19.8	19.7	19.4

Dietary codes: FM = control diet (Fishmeal LT94), CPC = corn protein concentrate, RPC = Rice protein concentrate, WPC = Whey protein concentrate and casein
[1]United fish products, Aberdeen, Scotland, U.K.
[2]Corn protein concentrate, Empyreal® 75 (crude protein 76.7 %; ash 1.2 %; moisture 7.7 %; lipid 2.6 %).
[3]Rice protein concentrate, Remy Pro (crude protein 72.0 %; ash 1.5 %; moisture 7.0 %; lipid 2.2 %).
[4]Bacarel casein (crude protein 89.9 %; ash 2.0 %; moisture 9.6 %; lipid 0.3 %)
[5]Whey protein concentrate (carbelac) (crude protein 79 %; ash 3.6 %; moisture 6.6 %; lipid 0.6 %).
[6]Epanoil (Seven Seas Ltd, U.K)
[7]Premier nutrition vitamin premix; each 1kg of premix contains: 12.1 % calcium, Ash 78.7 %, Vit A 1.000 µg/ kg, Vit D3 0.100 µg/kg, Vit E (as alpha tocopherol acetate) 7000.0 mg/ kg, Copper (as cupric sulphate) 250.000 mg / kg, Magnesium 1.56 %, Phosphorous 0.52 %
[8]Barox antioxidant liquid
[9]Carboxy-methyl cellulose (Sigma Aldrich, Poole, UK)
[10]Values are given based on a dry matter basis
[11]Nitrogen free extracts (NFE) = dry matter – (crude protein + crude lipid + ash)

4.2.3 Growth and feed utilisation

After 84 days of feeding on experimental diets all growth and feed utilisation of fish was determined according to the calculations outlined in Section 2.6.

4.2.4 Haematological and serological analysis

At the end of the trial five fish per tank were anaesthetized with tricane methanesulfate (MS222) at 150 mg/ l. Blood was sampled from the caudal vein using a 25 gauge needle and 1 ml syringe. Sub samples of whole blood were left to clot for a period of 12 h (at 4 ºC) and then centrifuged at 3,600 g for 6 minutes to recover serum. Serum was removed and stored at -80ºC until analysis of albumin, globulin and protein.

4.2.5 Haematocrit

Haematocrit was determined according to the protocol outlined in Section 2.9.1.

4.2.6 Haemoglobin

Haemoglobin concentration of whole blood was determined according to the protocol outlined in Section 2.9.2.

4.2.7 Total Erythrocytes and Leukocyte counts

Total leukocyte and erythrocyte counts were determined according to the protocol outlined in Section 2.9.3.

4.2.8 Serum Albumin, globulin and Protein

After 84 days of feeding on experimental diets fish serum was collected and all serological parameters were determined according to methods outlined in Section 2.9.5 and 2.9.6.

4.3 Statistical Analysis

All data are presented as means ± standard error (SE). All growth data and haematological data was transformed where necessary and statistical analysis was conducted using SPSS statistics version 18 for windows (SPSS Inc., Chicago, Il, USA) and accepted at the $P < 0.05$ level. Data were analysed using a one-way ANOVA and significant differences between control and experimental groups were determined using post-hoc Tukeys HSD test. Data violating the assumptions of parametric tests after log transformation were tested with the equivalent non-parametric Kruskal-Wallis test and or Mann-Witney *U* test. All percentage data was transformed using arcsine function prior to analysis. Unless otherwise stated, differences were considered significant at a value of $P \leqslant 0.05$.

4.4 Results

4.4.1 Growth performance and feed utilisation

After 84 days of feeding on experimental diets the final fish weight was elevated by ca. 10.6 % in fish fed WPC compared to fish fed FM diet (Figure 4.1). In contrast, fish fed rice protein concentrate showed a decrease by ca. 33.3 % in final fish weight compared to fish fed fish meal. Moreover compared to fish fed fishmeal diet, fish fed corn protein concentrate showed the largest decrease in final fish weight by ca. 43.9 %. A moderate growth performance was observed in all groups (Table 4.2); in fish fed FM and WPC diet fish biomass increased by over 300% after 86 days of feeding with FCR $\leqslant$ 1.0 and specific growth rate (SGR) > 1.7. SGR improved significantly from 1.70 ± 0.07 %/ day in the FM fed fish to 1.83 ± 0.04. %/ day in fish fed WPC diet. However, compared to FM fed fish, fish fed RPC diet demonstrated a significantly reduced SGR (1.13 ± 0.07 %/ day). Moreover fish fed CPC diets (0.89 ± 0.06 %/ day) showed the lowest SGR which was significantly lower compared to FM fed fish ($P < 0.001$). Compared to fishmeal fed fish (0.99 ± 0.03 g/ g), both

fish fed RPC and CPC (1.67 ± 0.11 and 2.17 ± 0.15 g/ g) demonstrated a significant increase in feed conversion ratio (FCR). Indeed fish fed WPC diet showed the lowest FCR (0.92 ± 0.01 g/ g). The highest protein efficiently (PER) ratio was shown in fish fed WPC (2.03± 0.07). In comparison to fish fed FM diet (1.84 ± 0.07), both fish fed RPC and CPC showed a significant decrease in PER (0.83 ± 0.08 and 0.54 ± 0.07).

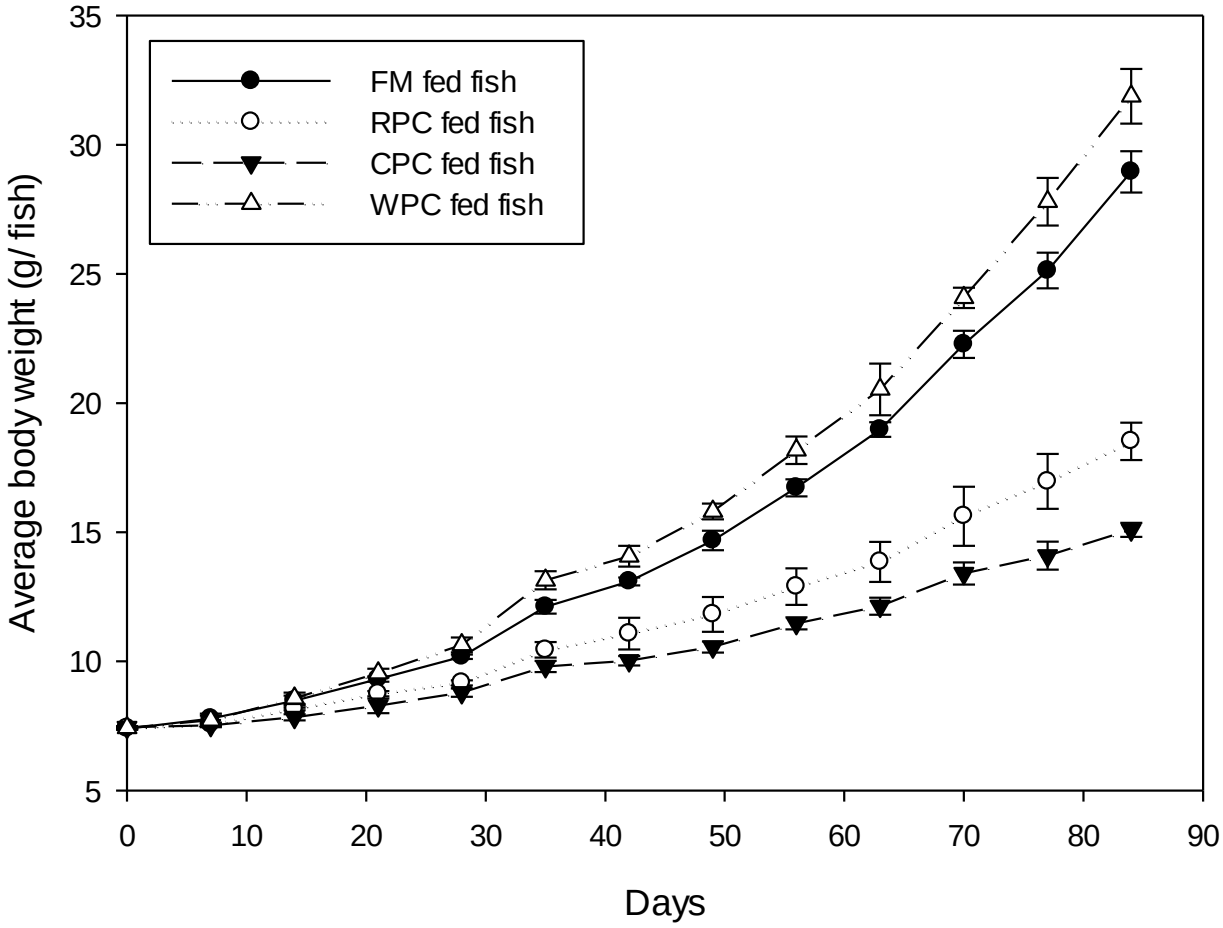


Figure 4.1. Average body weight gain of fish after 84 days of feeding on experimental diets (*n* = 3). Dietary codes: FM = Fishmeal fed fish, RPC = Rice protein concentrate fed fish, CPC = Corn protein concentrate fed fish, WPC = whey protein concentrate and casein fed fish.

Table 4.2. Growth performance indexes of Common carp after 84 days of feeding on experimental diets. Values expressed as means ± SE (*n* = 3). Dietary codes: FM = Fishmeal fed fish; RPC = Rice protein concentrate fed fish, CPC = Corn protein concentrate fed fish, WPC = whey protein concentrate and casein fed fish.

Parameters	**Diets**				***P* - value**
	FM	**RPC**	**CPC**	**WPC**	
Initial body weight (g/ fish)	7.39 ± 0.02	7.41 ± 0.03	7.44 ± 0.03	7.42 ± 0.02	0.534
Final body weight (g/ fish)	25.13 ± 0.40^{a}	16.77 ± 0.45^{b}	14.09 ± 0.54^{c}	27.80 ± 0.53^{d}	<0.001
Weight gain (g/ fish)	17.74 ± 0.38^{a}	9.36 ± 0.46^{b}	6.64 ± 0.34^{c}	20.38 ± 0.51^{d}	<0.001
Protein efficiency ratio	1.84 ± 0.04^{a}	0.83 ± 0.05^{b}	0.54 ± 0.04^{c}	2.03 ± 0.04^{a}	<0.001
Specific growth rate (%/ day)	1.70 ± 0.02^{a}	1.13 ± 0.04^{b}	0.89 ± 0.04^{c}	1.83 ± 0.02^{a}	<0.001
Feed conversion ratio (g/ g)	0.99 ± 0.02^{a}	1.67 ± 0.06^{b}	2.17 ± 0.09^{c}	0.92 ± 0.01^{a}	<0.001

$^{a-d}$ Significant differences between groups are indicated by difference in superscript letters

4.4.2 Haematological and serological analysis

All values for haematology are presented in Table 4.2 and all serological data is presented in Figure 4.2 A, B and C. No significant differences were observed in haematocrit, haemoglobin, total erythrocyte counts and total leukocyte count ($P > 0.05$). In contrast compared to both fish fed FM and RPC diets (97.37 ± 0.40 and 97.60 ± 0.34 %, respectively) a significant ($P = 0.012$) decrease in the proportion of lymphocytes was shown in fish fed WPC diet (95.23 ± 0.72 %). On the contrary there was a significant ($P = 0.008$) elevation in the proportion of granulocytes in fish fed WPC diet (4.13 ± 0.61 %), compared to both fish fed FM and RPC diets (1.90 ± 0.34 and 2.37 ± 0.39 %, respectively). Likewise fish fed WPC diet showed a significant ($P = 0.014$) increase in mean corpuscular haemoglobin level (82.11 ± 5.21 pg), compared to both fish fed FM and RPC diets (69.58 ± 3.13 and 65.45 ± 3.26 pg, respectively).

A significant increase ($P < 0.001$) in serum albumin levels was observed in fish fed WPC diet (1.22 ± 0.002 g/ dl), compared to both fish fed FM and RPC diets (0.51 ± 0.012 and 0.51 ± 0.017 g /dl, respectively). Likewise fish fed WPC diet (3.50 ± 0.08 g/ dl) showed a significant elevation ($P < 0.01$) compared to fish fed fishmeal and RPC diet (3.32 ± 0.09 and 3.17 ± 0.07 g/ dl, respectively) in serum protein levels. No significant differences were observed in the data for serum globulin levels. As a result of the elevated albumin levels observed in fish fed WPC diet, the A/G ratio in fish fed WPC diet showed a significantly higher value compared to fish fed FM and RPC diets ($P < 0.001$)

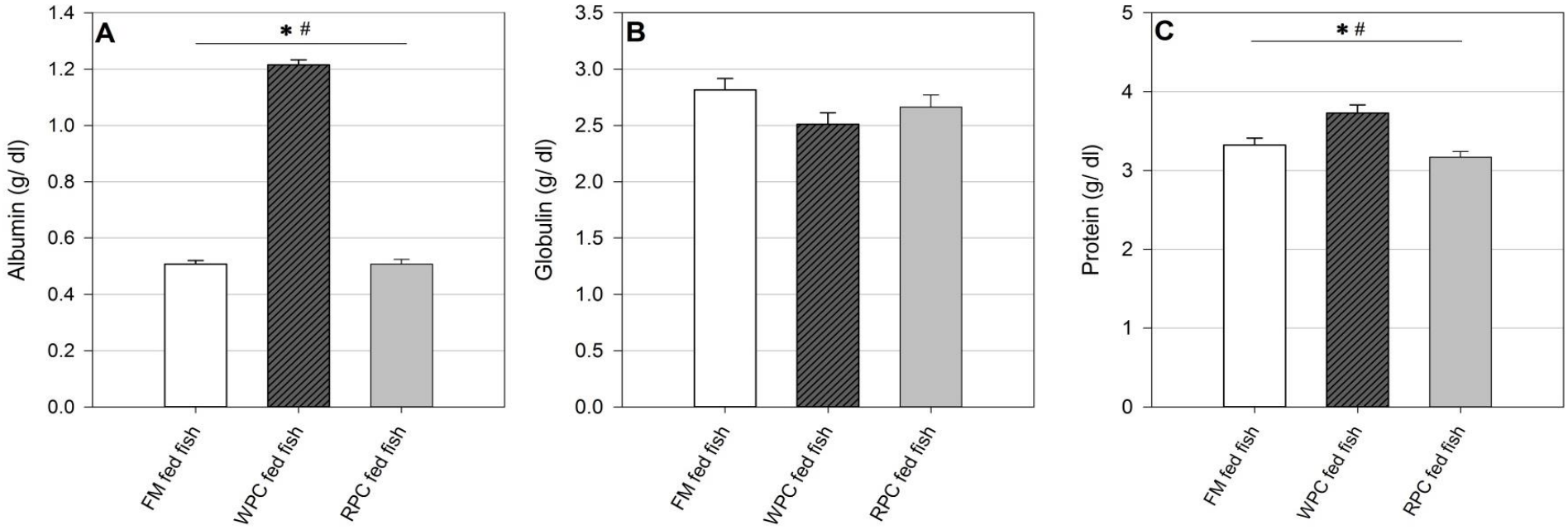
Table 4.3. Haematological indexes of Common carp after 84 days of feeding on experimental diets. Values expressed as means ± S.E. (*n* =15).

Dietary codes: FM = Fishmeal fed fish; RPC = Rice protein concentrate fed fish, WPC = whey protein concentrate and casein fed fish

Parameter	**Diets**			***P* - value**
	FM	**RPC**	**WPC**	
Haematocrit (% PCV)	32.40 ± 1.63	32.33 ± 1.28	34.67 ± 1.63	0.393
Haemoglobin (g/ dl)	7.17 ± 0.30	6.92 ± 0.41	7.90 ± 0.46	0.204
RBC (10^6 mm^3)	1.04 ± 0.04	1.06 ± 0.03	0.97 ± 0.03	0.148
Leucocytes (10^4 mm^3)	2.59 ± 0.04	2.54 ± 0.06	2.51 ± 0.08	0.658
Lymphocytes (%)	97.37 ± 0.40[a]	97.60 ± 0.34[a]	95.23 ± 0.72[b]	0.012
Granulocytes (%)	1.90 ± 0.34[a]	2.37 ± 0.39[a]	4.13 ± 0.61[b]	0.008
Monocytes (%)	0.50 ± 0.14	0.27 ± 0.08	0.63 ± 0.19	0.363
MCV (fL)	314.31 ± 11.66	309.46 ± 15.25	360.04 ± 17.67	0.057
MCH (pg)	69.58 ± 3.13[a]	65.45 ± 3.26[b]	82.11 ± 5.21[a]	0.014
MCHC (g/ dl)	22.21 ± 0.73	21.65 ± 1.30	23.03 ± 1.27	0.689
Albumin/ globulin ratio	1.86 ± 0.08[a]	1.78 ± 0.07[b]	6.38 ± 0.27[c]	<0.001

[a-c] Significant differences between groups are indicated by different superscript letters
RBC – Red Blood Cells
MCV – Mean Corpuscular Volume
MCH – Mean Corpuscular Haemoglobin
MCHC – Mean Corpuscular Haemoglobin Concentration

Figure 4.2. Serological indexes of Common carp after 84 days of feeding on experimental diets, A) Albumin, B) Globulin and C) Protein. Values expressed as means ± S.E. (*n* =15). Dietary codes: FM = Fishmeal fed fish; RPC = Rice protein concentrate fed fish, WPC = whey protein concentrate and casein fed fish. * - denotes a significant difference between control fed fish and WPC fed fish ($P < 0.05$). # - denotes a significant difference between WPC fed fish and RPC fed fish ($P < 0.05$).



4.5 Discussion

4.5.1 Growth and feed utilisation

It is important to ensure that diet composition meets the essential nutrient requirements of the fish to provide optimal conditions for growth and to maintain fish quality. In the ornamental industry the latter is perhaps more important because the true value of the fish is in its aesthetic value. As suggested previously many contemporary nutritional evaluations of alternative feed ingredients have focused on plant feed ingredients (reviewed by Galtin *et al*., 2007; Hardy, 2010; Collins *et al*., 2013). Given the success of plant protein concentrates already in use in aquafeeds (Naylor *et al*., 2009; Hardy, 2010; Zhang *et al*., 2012), two plant protein concentrates were selected based on their nutritional characteristics as potential protein sources to replace fishmeal. Furthermore a third diet was formulated based on a combination of whey and casein proteins that are considered as by-products from the dairy industry. Previously many vitamin and mineral requirement studies for fish have used whey and casein in semi-purified dietary formulations as the principle protein source reflecting the high biological value of these proteins (Halver, 1982; Halver 1989; Yamamoto *et al*., 2008). In the current study fish were fed a combination of whey protein concentrate and casein meal (50 and 244 g/ kg, respectively) at 66 % replacement of fishmeal protein level. The original formulation for this diet was based on whey protein concentrate only, however due to the physico-chemical properties of whey protein being highly hydroscopic it was not feasible to include this protein source at the intended inclusion levels. Therefore casein was added to the diet and compared to fishmeal fed fish a significant elevation in final body weight by ca. 11% was observed in fish fed this diet. Likewise a similar trend was observed for weight gain where feeding fish a combination of whey and casein protein had the highest weight gain which was significantly higher than fish fed fishmeal diet ($P < 0.001$). These findings probably reflect the fact that the essential amino acid profile of both casein and whey protein

concentrate in the experimental diet are in surplus to all requirements of cyprinids and the bioavailability of these proteins are higher compared to that of fishmeal. Most importantly though due to the high success of feeding the a combination of whey and casein protein on cyprinid growth performance and feed utilisation, this novel finding provides preliminary evidence for future research to investigate the use of both whey and casein proteins as feed commodities in the ornamental industry because high value dietary specifications are more acceptable in this area.

This experiment for the first time demonstrates the use of RPC in experimental diets for mirror carp. Previously low to moderate inclusion of plant protein concentrates from soybean, lupin, potato and cowpea has been reported to have no adverse effect on growth performance in several fish species including tilapia (*Oreochromis niloticus*, Olvera-Novoa *et al*., 1997) rainbow trout (*Oncorhynchus mykiss*, Xie and Jokumsen, 1997; Thiessen *et al*., 2004; Glencross *et al*., 2006) and sea bass (*Dicentrarchus labrax*; Guroy *et al*., 2013). The results of the present experiment indicate that replacing 66% dietary fishmeal with RPC (356 g/ kg) lead to adverse effects towards fish growth performance and feed utilisation. Specifically fish fed RPC demonstrated a significant reduction in SGR ($P < 0.001$) and FCR ($P < 0.001$) compared to fish fed fishmeal. Guroy *et al.* (2013) also observed a similar result when sea bass where fed RPC in excess of 50 % replacement of fishmeal. The conclusion from this study was that it was acceptable to feed RPC at 25 % replacement of fishmeal with no adverse effects to sea bass growth performance. Similarly, Palmegiano *et al*. (2007) reported that when feeding blackspot seabream (*Pagellus bogaraveo*) with RPC in excess of 50 % replacement of fishmeal caused a negative effect towards fish growth performance; they concluded that a 20 % replacement of fishmeal could be used without affecting growth performance. Likewise, Palmegiano *et al*. (2006) reported that 20 % of RPC, as a partial replacement of fishmeal did not affect growth performance of rainbow trout. But when fish

were fed higher levels of RPC (35 % and 53 %) caused a significant reduction in weight gain by ca. 33%. Similarly in the current study compared to fish fed fishmeal fish weight gain was significantly reduced in RPC fed fish by ca. 54 %.

Fish were also fed another plant protein concentrate derived from corn. The corn protein concentrate used in the study was designed to be a high energy feed commodity that was highly digestible to fish (crude protein 76.7 % and lipid 2.6 %). This study for the first time reports the effects of feeding CPC to mirror carp growth performance and feed utilisation. The results suggest that feeding CPC (335 g/ kg) at 66 % replacement of fishmeal caused a negative effect towards fish growth response and feed utilisation. Similar to fish fed the RPC diet, fish CPC diet showed a significant decrease in both SGR ($P < 0.001$) and FCR ($P < 0.001$) compared to fish fed fishmeal. This result may be due to a deficiency in lysine and methionine or the fact that the digestibility of the protein is not as high as fishmeal in cyprinid fish.

The findings from this study are consistent with previous investigations using other plant protein concentrates, which show a decrease in growth performance with substitution levels above 50 % inclusion. These plant sources include soybean protein concentrate (Deng *et al*., 2006; Collins *et al*., 2013) and potato concentrate meal (Refstie and Tiekstra, 2003) pea protein concentrate (Penn *et al*., 2011). In contrast, several studies have reported considerable success in partial (40 – 75 %) and/or total replacement of fishmeal with plant protein concentrates (Mambrini *et al*., 1999; Refstie *et al*., 2001; Bendiksen *et al*., 2011; Burr *et al*., 2012). As stated in Section 3A it is evident that the substitutions of fishmeal with plant protein sources albeit as a meal or as a more refined protein concentrate results in variable success depending on the fish species, the protein source, processing treatment and inclusion level. It is well established that the most limiting amino acids of plant protein sources are methionine and lysine refer to Section 1.5 (Kaushik and Hemre, 2008). A study by Guroy *et*

al. (2013) supplemented both the 50 and 75 % replacement of fishmeal RPC diet with crystalline methionine and lysine and reported some improvements in growth performance of fish. But this was still below the level of performance observed in fish fed fishmeal diet. They suggested that these reported reductions might be due to inadequate provision of other amino acids (e.g. tryptophan), anti- nutritional factors or reduced nutrient digestibility. This may be the case in the current study and should be a topic for future investigation if high levels of plant protein sources are to be included in ornamental aquafeed formulations. In addition future experiments should consider the use of enzymatic pre-treatment or even dietary inclusion may be a means of elevating the digestibility of such plant protein sources.

4.5.2 Haematological and serological analysis

Icthyo-haematology is a useful tool useful tool in the assessment of feed composition and nutritional status in relation to environmental conditions affecting fish (Svobodova *et al*., 2005). The results of the present study demonstrated that fish fed RPC diet showed a significant decrease in mean corpuscular haemoglobin ($P < 0.014$), compared to fish fed fish meal and WPC diet. Therefore it could be suggested that the amount of haemoglobin per red blood cell is lower in fish fed RPC diet. This could have implications on the oxygen carrying capacity of the red blood cells and result in inadequate oxygen supply to vital organs. In particular in periods of high stress such as transportation or netting stress, as oxygen consumption rates are known to increase in these situations such low levels of haemoglobin could lead to fish mortality (Wendelaar Bonga, 1997; Sloman *et al*., 2000). Although the circulatory leukocyte levels remained unaffected by dietary treatments the proportions of lymphocytes was significantly lower in fish fed WPC diet compared to fish fed fishmeal ($P = 0.017$). However the proportions of granulocytes was higher in fish fed WPC diet ($P = 0.006$) compared to fish fed fish meal. Granulocytes are classified as neutrophils, eosinophils or

basophils on the basis of their cellular morphology and cytoplasmic staining characteristics (refer to plate 2.2). In fish granulocytes are important effector cells in response to pathogenesis and have phagocytic capabilities as well as releasing a variety of antimicrobial agents (Magnadottir, 2010). In this regard feeding fish WPC diet may be beneficial to increasing the levels of granulocytes in the blood of carp compared to fish fed fishmeal.

Serum proteins including albumin and globulin are valuable bio-indicators of fish health status (Rehulka, 1993). Indeed, the most abundant serum protein in mammals and fish is albumin which is also used as an indicator of liver impairment (Branch *et al*., 1973; Peters, 1996; Kemath *et al*., 2001; Metcalf *et al*., 2007). In the present study feeding fish WPC diet caused a significant 2- fold increase in serum albumin concentration ($P < 0.001$) compared to fish fed fishmeal. This result could be due to higher bioavailability of proteins in WPC diet which may have caused a subsequent increase in liver protein metabolism. In keeping with the ethical and technical constraints of the current study further work should aim to characterise serum protein responses in more detail using proteomic approaches to give a more holistic perspective to the effects of feeding whey/casein proteins and other modified plant sources on serum protein metabolism in carp.

No dietary effect was observed in the data for serum globulin. The globulin levels reported in this study were in line with the controls reported by Misra *et al*. (2006). The albumin/ globulin ratio was significantly higher in fish fed WPC diet compared to fish fed fishmeal ($P <0.001$). This result may be due to the fact a high level of albumin was observed in fish fed WPC diet. Serum protein level was significantly higher in fish fed WPC diet compared to fish fed fishmeal ($P = 0.001$) and RPC diet ($P < 0.001$). This result may reflect the fact that the bioavailability of proteins in the WPC diet was higher than that of fishmeal; future studies should determine the protein digestibility of this diet in carp. The observed

levels for fishmeal fed fish were in line with the optimal levels reported for healthy cyprinids by Rehulka (1993) (>25 - 33 g/ l).

4.5.3 Conclusion

The present experiment indicates that feeding a combination of whey protein concentrate and casein meal has the potential as a novel feed commodity for the inclusion in diets for ornamental aquafeeds, where high value specifications are more acceptable. Dietary inclusion at 66 % replacement of fishmeal (8% whey protein concentrate and 58 % casein) meal can be used without detrimental effects on the growth performance and feed utilisation of mirror carp. In contrast feeding rice protein concentrate and corn protein concentrate at 66 % replacement of fishmeal as a single ingredient caused significant reductions in both growth performance and feed utilisation. This may be as a result of deficiencies in essential amino acids lysine and methionine in the plant protein sources (Kaushik and Hemre, 2008). Consequently, future studies should consider using lower levels of inclusion of the selected plant protein concentrates as it is evident that lower levels of inclusion at ca. 20 – 25 % replacement of fishmeal is more acceptable, regardless of fish species. Unfortunately the assessment of the haematological profiles of fish fed CPC diet was not determined in this experiment due to the fish being too small to successfully take a blood sample. Despite this it was evident that fish fed a combination of whey protein concentrate and casein meal showed an elevation in mean corpuscular haemoglobin, serum albumin and albumin/ globulin ratio compared to fish fed fishmeal indicating that fish health was no compromised.

As outlined in Section 1.4, contemporary research strategies are integrating both nutritional and immunological research methodologies to develop novel feed commodities that not only meet the physiological requirements of the fish but may have beneficial effects on the immune responses of fish. Therefore in summary of both the finding from the

successful inclusion of the earthworm meal (Chapter 3) and a combination of whey protein concentrate and casein meal to mirror carp growth performance and feed utilisation it was important to test the efficacy of each dietary regime on the innate and adaptive immune responses. This will allow for further conclusions as to the efficacy of each dietary regime to improve fish quality and welfare regarding immuno-competence. The aspect will be the focus of Chapter 5 and will aim to provide preliminary evidence using gene expression profiling of selected inflammatory cytokines and complement protein 3 as the primary biomarkers of immune responses. Additional biomarkers will be used to determine the effect on cellular and adaptive responses of mirror carp fed each dietary regime.

CHAPTER 5.

DIETARY MODULATION OF IMMUNE RESPONSE AND RELATED GENE EXPRESSION PROFILES IN MIRROR CARP (*C. CARPIO*) USING SELECTED EXOTIC FEED INGREDIENTS

Abstract

An investigation was conducted to evaluate the effect of feeding selected exotic ingredients on immune responses and expression of immune related genes in mirror carp (*C.carpio*). Fish were fed diets for a total of 56 days. Fishmeal served as the main protein source in the control diet and two experimental diets consisted of fishmeal fixed at 33% provision of protein and the remaining 66% protein was provided either by earthworm meal (EW diet) or a combination of whey protein concentrate (8%) and casein (58 %) (WPC diet). At the start of the trial fish were injected intraperitoneally with *Aeromonas hydrophila* bacterin. Compared to fish fed fishmeal, a significant increase in mRNA expression of the pro-inflammatory cytokines IL-1β (24 h post injection) and TNFα (at 12 h and 48 h post injection) was observed in fish fed EW. Moreover a similar trend was observed for complement 3 (C3) gene, where fish fed EW showed significant elevations in mRNA expression values at both 12 and 48 h post injection compared to control fed fish. In contrast, fish fed WPC showed a significant decrease in C3 and TNF-α mRNA expression compared to fish fed fishmeal. Fish fed EW and WPC diet showed a significant increase leukocyte levels compared to fish fed fishmeal 14 days post injection. Fish fed fishmeal presented significantly higher circulatory IgM levels at 7 d post injection compared to fish fed EW and WPC diets. In contrast, fish fed EW and WPC showed a significant increase in IgM levels at 28 d post injection. The present study showed an up-regulation of all immune related genes in fish fed EW, compared to fish fed fish meal, which is indicative of an acute inflammatory response. In contrast it appears

feeding WPC has an immunosuppressive effect towards TNFα and C3 expression in carp blood. This may be offset by the fact that feeding WPC to carp appears to aid in the increased production of circulatory IgM and leukocyte levels compared to fishmeal fed fish.

5.1 Introduction

It is well established that traditional feed formulations based on high levels of good quality fishmeal satisfy the essential amino acid requirements of fish (N.R.C. 2011). However, with a decline in fishmeal availability the search for alternative sources is becoming the focus of contemporary research. Since dietary protein is fundamental to growth and development this has been a priority area for evaluation. Indeed, a number of recent investigations have shown that protein sources (and amino acids) can have an impact on disease resistance and immunity in fishes reviewed by (Trichet, 2010; Krogdhal *et al*., 2010; Olivia-Teles, 2012; Kiron, 2012). Consequently this is driving a new area of research that integrates nutrition and immunological research methodologies (refer to Section 1.4).

As previously mentioned the aesthetic value of the fish in the ornamental industry is very important, so the emphasis for feeds is not to improve growth performance *per se*, but to deliver nutrients for maintenance and disease resistance. Although there is still an initiative in the ornamental aquafeed industry to reduce the dependency of fishmeal usage there is equally a focus towards the concept of 'functional feeds'. The term 'functional feeds' is used to describe feed items that provide a specific health benefit to the animal above and beyond its nutritional value and it is hoped these diets will improve both health status and growth of the animal (Tacchi *et al*., 2011) In this context there is more potential to use high value diet specifications including whey proteins, insect proteins and other exotic feed commodities, commonly termed non-conventional feed resources (NCFRs). Some areas of the world, especially China and Australasia, have already invested considerable effort towards research

and development of suitable insect proteins. In particular earthworm species have been targeted for their capacity to degrade organic wastes and provide potential biomass for the animal feed industry (Dynes, 2003; Sinha *et al*., 2010). Indeed, as suggested in Section 1.2 Sabine (1983) reviewed the amino acid profiles of several earthworm species and concluded that earthworm protein was high in the ten essential amino acids for teleost fish including the sulphur amino acids methionine and cysteine. Therefore by virtue of its amino acid profile the biological value (BV) of earthworm meal is high. In fact, feeding earthworm meal at levels of 20-30 % replacement of fishmeal has improved growth performance in a number of fish species including tilapia (*Oreochromis* spp; El-Sayed, 1999), vundu catfish (*Heterobranchus longifilis*; Sogsbean and Madu, 2008), rainbow trout, (*O. mykiss*; Stafford and Tacon, 1985) and common carp (*C. carpio*; Nandeesha *et al*., 1988). Previously we reported that feeding mirror carp at 66 % replacement of fishmeal with earthworm meal significantly improved fish growth performance and feed utilisation (Rawling *et al*., 2012). The classical nutritional approach to understanding the benefits of earthworm meal to fish growth performance is therefore well reported (refer to Chapter 3A); however little evidence has been reported evaluating the potential functional aspect of earthworm meal in terms of fish welfare and health status.

Historically whey proteins and casein have been considered by the cheese manufacturing industry as waste products. However within the last two decades research in this area has revealed that whey/ casein proteins consist of many components including β-lactoglobulin, α-lactalbumin, proteose peptones, blood proteins, lactose, and important essential minerals such as calcium that can be exploited as highly nutritious food supplements for humans and feed for the swine industry. By virtue of the content of essential amino acids, the BV of whey/casein derived proteins is high compared with that of other dietary proteins such as plant proteins; furthermore they have proportionally more sulphur-containing amino

acids (cysteine, methionine) compared to other protein sources (Bounos and Gold, 1991; Walzem *et al*., 2002; Korhonen, 2009). For these reasons many classical vitamin and mineral requirement studies in fish have included whey and casein proteins as the principle protein source in semi-purified diets (Halver, 1982; Halver, 1989; Woodward, 1994). Indeed the results in Chapter 4 demonstrated that feeding a combination of whey protein concentrate and casein meal significantly increased carp growth performance and feed utilisation in comparison the fish fed fishmeal. However, little is known about the effects of feeding these commodities on fish immune responses. Given the attributes of these ingredients the present study investigated the effects of feeding earthworm meal and a combination of whey/casein meal in separate diets to assess their effects on the innate and adaptive immune responses of mirror carp as a model ornamental fish species. The expression of pro-inflammatory cytokines (TNF-α and IL-1β) and complement protein 3 (C3) following injection with heat inactivated *Aeromonas hydrophila* were the primary biomarkers monitored in this study. In addition respiratory burst activity, haematology and antibody titre were used to measure the cellular and humoral responses of mirror carp for each dietary regime.

The specific hypotheses tested were as follows:

> $H_0$1 - Feeding fish earthworm meal will cause a significant up-regulation in the expression profiles of all immune related genes compared to fish fed fishmeal diet
>
> $H_0$2 - Feeding fish a combination of whey protein concentrate and casein meal will cause a down-regulation in the expression profiles of all immune related genes compared to fish fed fishmeal.

$H_0$3 - Replacement of fishmeal in the diet will have no effect on fish cellular responses, haematological parameters and all serum responses.

$H_0$4 - Replacement of fishmeal with a combination of whey protein concentrate and casein meal will increase the circulatory IgM levels of the fish

$H_0$5 - Feed a combination of whey protein concentrate and casein meal will have no effect on fish cellular, haematological and serum responses compared to fish fed fishmeal.

5.2 Materials and Methods

5.2.1. Fish and experimental protocol

The experiment was carried out at the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK. Mirror carp (*C. carpio*) fry were obtained from Hampshire carp hatcheries, Hampshire, U.K. After four weeks acclimation 120 fish (77.57± 1.02g) were randomly distributed into into 8×150 l fibreglass tanks (20 fish per tank) containing aerated recirculated freshwater. Therefore 40 fish were used pertreatment with four experimental treatments. Fish were fed experimental diets to a fixed regime of 2 % body weight per day spread across three feeding times (0900 and 1600) for 63 days and reared at 26 ± 1 ºC with a 12:12 h light:dark photoperiod. For rearing conditions and ethical approval see section 3A.2.

5.2.2. Feed formulation and diets

Three isonitrogenous and isolipidic diets were formulated using feed formulation software (Feedsoft®, USA) to contain approximately 38 % crude protein and 8 % lipid to meet the known requirements of juvenile common carp (N.R.C. 2011). Fishmeal served as the main protein source in the control diet (diet FM). Two remaining diets consisted of fishmeal fixed at 34% provision of protein and the remaining protein was provided by earthworm meal (diet EW) or a combination of whey protein concentrate at 8% and casein at 58 % (diet WPC) (Table 5.1). Each diet was produced by mechanically stirring the ingredients into a homogenous mixture using a Hobart food mixer (Hobart Food Equipment, Australia, model no: HL1400 – 10STDA mixer). Warm water was added to reach a consistency suitable for cold press extrusion to form 1 mm pellets (PTM Extruder system, model P6, Plymouth, UK). The nutritional profile was determined according to AOAC (2007) official protocols (Table 5.1).

Table 5.1. Formulation and chemical composition of experimental diets. Each ingredient component is expressed as g/ kg per diet.

	FM	**EW**	**WPC**
Ingredients			
Herring meal LT94[1]	553.94	180.00	180.00
Corn starch	401.06	345.18	432.58
P. excavatus meal[2]		411.17	
Casein (Bacarel)[3]			244.39
Whey protein concentrate[4]			50.00
Fish oil[5]		18.65	38.03
Sunflower oil	20.00	20.00	20.00
Vitamin/ mineral premix[6]	20.00	20.00	20.00
Antioxidant[7]	0.500	0.500	0.500
Molasses	5.00	5.00	5.00
CMC-binder[8]	10.00	10.00	10.00
Proximate analysis			
Dry matter (%)	92.4	93.2	95.1
Crude Protein (%)[9]	39.0	40.2	41.2
Crude lipid (%)[9]	8.8	9.8	10.6
Ash (%)[9]	8.9	6.6	4.6
NFE[10]	35.7	36.6	38.7
Gross energy (MJ kg $^{-1}$)	18.3	19.3	19.8

Dietary codes: FM = control diet (Fishmeal LT94), EW = earthworm meal, WPC = whey protein concentrate / casein diet
[1] United fish products, Aberdeen, Scotland, U.K.
[2] Anphu Earthworm Farm, Vietnam. (crude protein 65.8 %; ash 6.3 %; moisture 8.3 %; lipid 8.7 %).
[3] Bacarel casein (crude protein 89.9 %; ash 2.0 %; moisture 9.6 %; lipid 0.3 %)
[4] Whey protein concentrate (carbelac) (crude protein 79 %; ash 3.6 %; moisture 6.6 %; lipid 0.6 %).
[5] Epanoil (Seven Seas Ltd, U.K)
[6] Premier nutrition vitamin premix; each 1 kg of premix contains: 12.1 % calcium, Ash 78.7 %, Vit A 1.0 µg/ kg, Vit D3 0.1 µg/ kg, Vit E (as alpha tocopherol acetate) 7000 mg/ kg, Copper (as cupric sulphate) 250.000 mg/ kg, Magnesium 1.56 %, Phosphorous 0.52 %
[7] Barox antioxidant liquid
[8] Carboxy methyl cellulose (Sigma Aldrich, Poole, UK)
[9] Values are given based on a dry matter basis
[10] Nitrogen free extracts (NFE) = dry matter – (crude protein + crude lipid + ash)

5.2.3 Bacterin preparation and Immunisation assay

The bacterin used for the experimental injection was prepared according to Rawling *et al.* (2012). Briefly, to determine the concentration of bacteria used for bacterin a standard concentration curve was prepared from an *Aeromonas hydrophila* isolate (strain B2/12, obtained from the University of Stirling, UK) by using the viable drop counts technique (Miles and Misra, 1938). After 7 days of feeding on the experimental diets each experimental group was administered 100 µl of whole cell bacterin (1×10^8 CFU/ml) intraperitoneally (I.P.) and control fish were I.P. injected with PBS. For more details on methods refer to section 3B.2.3.1.

5.2.4 Sample collection for gene expression analysis

Following I.P. injection with bacterin fish were anaesthetised with MS-222 at 150 mg/ l and samples of whole blood were collected from carp at time 0, 12, 24, 48 h post injection (five fish per treatment). All fish were only sampled once during the experimental period. Whole blood was collected via the caudal vessels and 100µl was immediately placed in RNA later at a ratio 1:5 (whole blood: RNA later). Samples were kept at room temperature until analysis.

5.2.4.1 RNA extraction and cDNA synthesis

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions, with some modifications. Briefly, 100 µl of whole blood was removed from RNA later by centrifugation at 12,000 x *g* for 10 min (4º C) and aliquoted into 1 ml of TRIzol reagent for 10 min. Following this 200 µl of chloroform was added and after mixing, samples were centrifuged at 12,000 x *g* for 15 min. The upper aqueous phase was transferred into a tube containing an equal volume of isopropanol. Mixtures were vortexed

and centrifuged at 14,000 x *g* for 15 min. Supernatants were discarded and the precipitated RNA pellets were washed using 1 ml of 75% ethanol. Total RNA was dissolved in diethylpyrocarbonate (DEPC) water which was treated with DNase (TURBO DNA-free™, Ambion) following the manufacturer's instructions, to remove any contaminating genomic DNA. The concentration and quality of RNA in each sample were determined by measuring 260/280 nm and 260/230 absorbance ratios (NanoDrop Technologies, Wilmigton, USA). The integrity of RNA was confirmed by running samples in a 1 % agarose gel, samples were stored at -80 °C. A total amount of 1 µg of RNA was used for cDNA synthesis, employing iScript cDNA synthesis kit (Bio-Rad). The iScript cDNA synthesis kit contains a combination of oligo dTs and random hexamers to work with a wide variety of targets. The reaction was placed at 25 °C for 5 min, then 42 °C for 30 min and inactivated at 85 °C for 5 min.

5.2.4.2 Real-time PCR

PCRs were performed with the SYBR green method in an iQ5 iCycler thermal cycler (Bio-Rad). Duplicate PCR reactions were carried for each sample analysed. Each PCR reaction was set on a 96 well plate by mixing 1 µl of diluted (1/10) cDNA with 5 µl 2 x concentrated iQ™ SYBR Green Supermix (Bio-Rad), containing SYBR Green as a fluorescent intercalating agent, 0.3 µM forward primer and 0.3 µM reverse primer. The primer used and their sequences are presented in Table 5.2. The thermal profile for all reactions was 3 min at 95ºC and then 45 cycles of 20 s at 95 ºC, 20 s at 60 ºC and 20 s at 72 ºC. Fluorescence monitoring occurred at the end of each cycle. Additional dissociation curve analysis was performed and showed in all cases one single peak.

β-actin and 40S were used as housekeeping genes in each sample in order to standardise the results by eliminating variation in mRNA and cDNA quantity and quality

(Bustin *et al.* 2009). No amplification product was observed in negative controls and no primer-dimer formations were observed in the control templates. The data obtained was analysed using the iQ5 optical system software version 2.0 (Bio-Rad) including Genex Macro iQ5 Conversion and genex Macro iQ5 files. The calculations in this spread sheet are derived from the algorithms outlined by Vandesompele *et al.* (2002) and from the GeNorm manual and associated calculations (http://medgen.ugent.be/~jvdesomp/genorm/). GeNorm calculates the stability value "*M*" of the reference genes by comparing the variation in expression for all other target genes. Modification of gene expression is represented with respect to the controls being sampled at the same time as the treatment.

Table 5.2. Primers used for real-time PCR analysis

Gene	GenBank accession no.	Product length (bp)	Forward primer	Reverse Primer
TNF-α	AJ311800	109	GTGTCTACAGAAACCCTGGA	AGTAAATGCCGTCAGTAGGA
IL-1β	AJ245635	89	TTACAGTAAGACCAGCCTGA	AGGCTCGTCACTTAGTTTGT
C3	AB016211; AB016212; AB016213; AB016214; AB016215	113	GTCGGTCCTGGACTGTCTCT	AGTGCACTGCTTCTCCTGCT
β-actin	M24113	204	CTGGTATCGTGATGGACTCT	CAGAGCTTCTCCTTGATGTC
40S	AB012087	146	CAGAACGAGAGGGCTTATCA	AGAATACGGCCTCTGATGGA

5.2.5 Culture media for isolation of peripheral blood leukocytes (PBLs)

Carp peripheral blood leukocytes were cultured essentially as described by Scharsack *et al*. (2004). Briefly, culture media for cell separation, cultivation and PBS were diluted with sterile distilled water to adjust the osmotic pressure according to carp osmolality (270 mOsmol/ kg, cRPMI). A heparinised medium was used for the collection of blood, where sodium heparin was diluted at 50,000 IU/ ml (Sigma Aldrich, Poole, UK) in RPMI 1640 media (Invitrogen, UK). For washing isolated PBLs (wash medium), RPMI 1640 media was diluted with 10,000 IU/ ml of sodium heparin (Sigma Aldrich, Poole, UK). For carp PBL cultures, RPMI was supplemented with 1 % (v/v) carp serum (PBL medium). Carp serum for PBL medium was isolated and pooled from 15 healthy fish. The pooled serum was heat inactivated for 30 min at 56 ºC, 0.2 µM filtered and stored at -22 ºC until use.

5.2.6 Isolation of PBLs

At day 7, 14, 28 and 56 days 6 fish per tank were anaesthetized with MS-222 at 150 mg/ l. Blood was sampled from the caudal vessel using a 25 gauge needle and 1 ml syringe and immediately diluted in heparinised medium (1:1 ratio of blood to medium). Cell suspensions were layered on Ficoll paque-plus (1.071 g/ cm^3, GE healthcare, UK) and centrifuged at 450 x g for 25 min at 4ºC without brakes. Isolated PBLs were washed thrice with wash medium (450 x g for 10 min) and resuspended in PBL medium to a density of 1 x 10^7 cells/ml. The numbers of viable cells were enumerated (using trypan blue exclusion) using a haemocytometer.

5.2.7 Respiratory burst activity of PBLs

The respiratory burst activity (RBA) of phagocytes from fish I.P. injected with *A. hydrophila* was quantified using an assay based on the reduction of nitroblue tetrazolium (NBT) to

formazon as a measure of superoxide anion (O2-) production. The assay was carried out according to the method of Secombes (1990) as modified by Stasiack and Baumann (1996). Briefly, isolated PBLs were incubated in 100 µl of PBL medium in the presence of phorbol myristate acetate (PMA, 1µg/ ml) for 2 h. The supernatant was then removed and the adhered wells were washed three times with PBS. After washing, 50 µl of 0.2 % NBT was added and the resulting solution was incubated at room temperature for 1 h. The cells were then fixed with a 100 % methanol for 2-3 minutes and again washed thrice with 30 % methanol. The microtitre plates were then left to air dry and then 120 µl of 2M KOH and 140 µl of DMSO were added into each well to dissolve the formazon blue precipitate formed. The OD of the turquoise blue solution was measured using and ELISA plate reader (Dynex technologies, UK) at 630 nm. The number of adhered cells was calculated using lysis buffer reagent to lyse cells and subsequently stained nuclei of cells was enumerated using a haemocytometer and O.D. were adjusted to 10^5 cells/ well.

5.2.8 Haematological analysis and serological

Blood was sampled from the caudal vessels using a 25 gauge needle and 1 ml syringe. In order to quantify total leukocyte counts, 5 µL of whole blood was smeared on to microscope slides. Slides were air-dried, fixed in 95 % methanol, stained with Giemsa (BDH Laboratory Supplies Poole, UK) and mounted in DPX (BDH). The levels were quantified as total number of leukocytes per 1000 blood cells (Merrifield *et al*., 2010). Lymphocytes, thrombocytes and granulocytes were identified following the descriptions of Rowley (1990). A minimum of 200 cells per sample were counted from 5 fish per tank (n = 15) and the values were expressed as a percentage of the total leukocytes after (Rawling *et al*. 2012).

5.2.9 Enzyme Linked Immunosorbent Assay

A direct ELISA was used to measure the antibody titre in fish serum samples as described elsewhere Adams and Thompson (1990) and Rawling *et al.* (2012). Briefly, a suspension of *A. hydrophila* (corresponding strain used for raising serum) with a concentration of 1 $\times 10^8$ cells/ ml was added to the microtitre plate at 100 µl/ well. The plate was incubated overnight at 4 °C, after which the bacterial cells were fixed to the bottom of the well using 50 µl/ well of 0.05 % (v/v) glutaraldehyde in PBS (pH 7.2) and incubation for 20 min at 20-22°C.

For fish serum, two fold dilutions of the serum from 1/64 - 1/4096 were made in antibody buffer (1 % BSA solution, Sigma Aldrich, UK) and 100 µl/ well was added to the ELISA plate. The serum from the control fish was added to the last row as a positive control. Anti-common carp/ Koi carp IgM monoclonal antibody labelled with horseradish peroxidase (MAbs, Aquatic Diagnostics Ltd., Stirling, U.K.) was used to detect IgM fish titre levels following the manufacturer's instructions. One hundred µl/ well of reconstituted anti-fish Mab-HRP was added to the microtitre plate and incubated for 1 h at 22 ºC. One hundred µl/ well of chromogen (42 mM TMB dihydrochloride solution) in substrate buffer (Sodium acetate / citric acid buffer, pH 5.4) were added to the wells. The microtitre plate was then incubated for a further 10 min at 20-22 °C and the reaction was stopped with 50 µl/ well of 2 M H_2SO_4. Finally, the plate was read in an ELISA plate reader at 540 nm and the results were compared between control and sample wells. Values three times higher than the negative control value were considered positive. The positive values at the lowest dilution were considered as a titre value for the serum examined.

5.2.10 Haemolytic complement activity (alternative complement pathway)

Haemolytic activity driven by the alternative complement pathway was determined by using rabbit red blood cells (RaRBC) as target cells in the presence of 10 mM EGTA-Mg-GVB (veronal-buffered saline containing10 mM ethylene glycoltetraacetic acid (EGTA), 10 mM MgCl2 and 0.1 % gelatine, pH 7.0) as described by Tort *et al*. (1996). Briefly, two-fold serial dilutions were made with 50 µl of carp serum in 10 mM EGTA-Mg-GVB buffer in a 96-well plate. To this 25 µl of RaRBC suspension (2 x 10^8 cells/ ml) in 10mM EGTA-Mg-GVB was added to each well and incubated at 20 ºC for 90 min. The haemolytic reaction was stopped by transferring the suspension in a micro-centrifuge containing 1000 µl of 10 mM of EDTA-GVB (veronal-buffered saline containing 10 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 % gelatin, pH 7.0). The supernatant (200 µl) from each micro-centrifuge tube was transferred to a new 96-well plate. The amount of haemolysis (y) was determined by measuring the optical density of the supernatant at 414 nm using an ELISA plate reader (Dynex technologies, UK). Complete (100 %) and no (0 %) haemolysis were determined by adding 25 µl of RaRBC suspension to 100 µl of distilled water and 25 µl of RaRBC suspension to 100 µl of gelatin veronal buffer (GVB, pH 7.0), respectively. The value y/(1-y) and the reciprocal of the serum dilution were plotted on a log-log graph and the ACH50 (U/ ml), the reciprocal dilution giving 50% haemolysis (y/(1-y) = 1), was read from the graph following Yano (1992).

5.3. Statistical analysis

All data are presented as means ± standard error (SE). Antibody titres are presented as the average antibody titre (-Log2 + 1 ± SE) for 6 fish. Data was transformed where necessary and statistical analysis was conducted using SPSS statistics version 18 for windows (SPSS Inc., Chicago, Il, USA) and significance was accepted at the $P < 0.05$ level. Gene expression data was analysed using two-way ANOVA. Significant differences between control and experimental groups were determined using post hoc Tukeys HSD test. All other data were analysed using Kruskal-Wallis test and/or Mann-Witney *U* test.

5.4. Results

5.4.1 Gene expression of carp whole blood

Gene expression analysis for C3, TNF-α and IL-1β are presented in Figure 5.1A, B and C respectively. At 12 h and 48 h post injection compared to fish fed fishmeal, a significant 3- and 4-fold up-regulation in C3 expression was observed in EW fed fish ($P = 0.03$; $P < 0.001$, respectively). Likewise, at 12 h and 48 h post injection compared to fish fed fishmeal, a similar significant up-regulation in cytokine TNF-α was observed in EW fed fish ($P < 0.001$ and $P = 0.015$, respectively). In contrast, a significant down regulation of C3 and TNF-α expression was observed in fish fed the WPC diet compared to fish fed fishmeal at 48 h post injection ($P < 0.001$ and $P = 0.001$, respectively). Moreover compared to PBS-injected controls, C3 and TNF-α expression was significantly lower in fish fed fishmeal at 24 h post injection ($P = 0.024$ and $P < 0.001$, respectively). The expression of cytokine IL-1β was significantly up-regulated in fish fed the WPC diet compared to fish fed fishmeal at 12 h and 24 h post injection ($P = 0.034$ and $P < 0.001$, respectively). In addition, compared to fish fed fishmeal a significant up-regulation in IL-1β expression was observed in fish fed earthworm meal 24h post injection ($P = 0.02$).

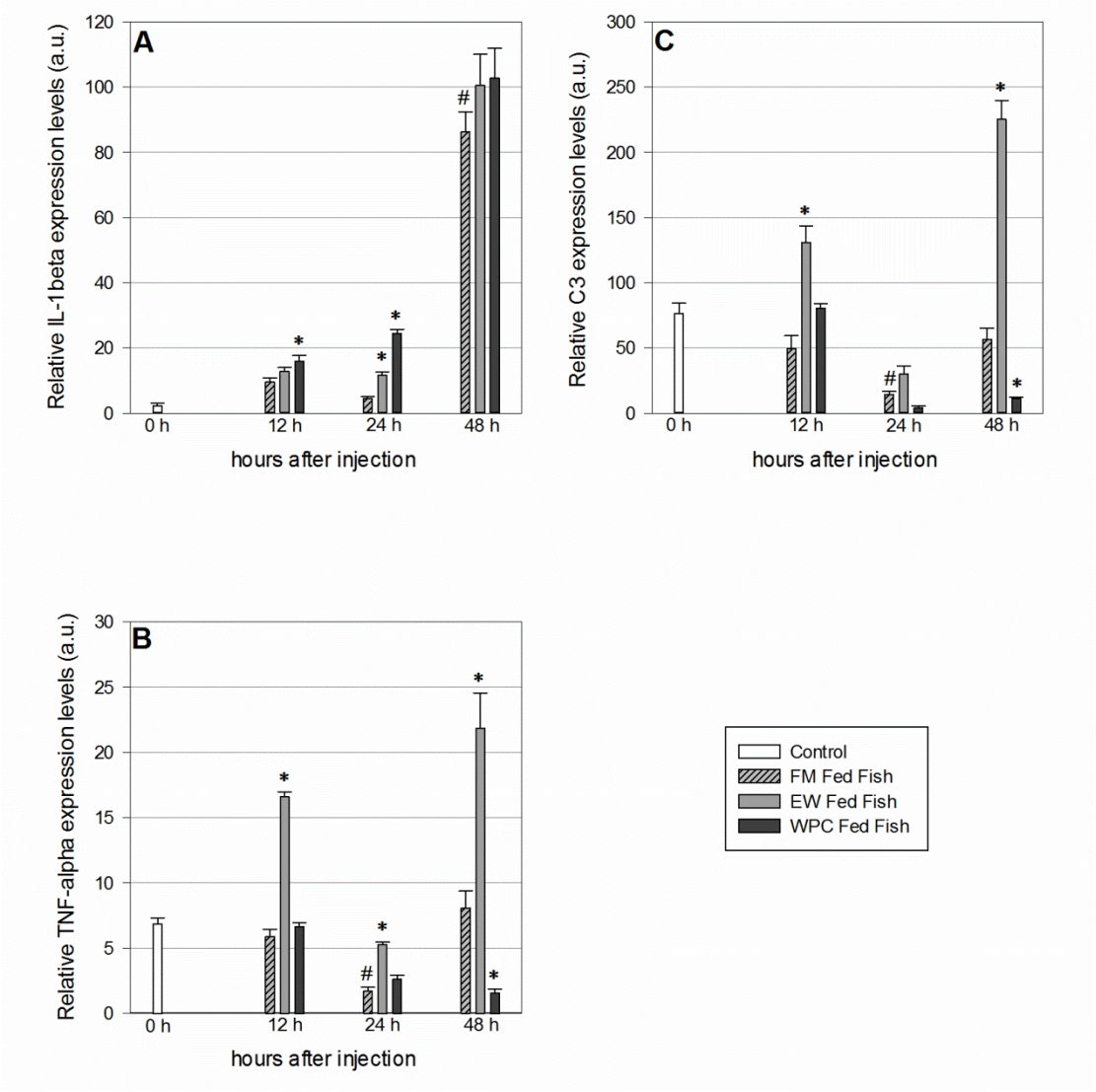


Figure 5.1. Real-time PCR expression in carp blood of (A) IL-1β; (B) TNFα and (C) C3 genes following I.P. injection with heat inactivated *A. hydrophila*. The expression values are presented relative to the expression of endogenous controls 40S and β-actin. Values expressed as means ± SE (*n* = 3/ time point). Dietary codes: FM = fishmeal fed fish (LT94); EW = earthworm meal fed fish; WPC = whey / casein protein fed fish. * - denotes significant difference in expression compared to fish fed fishmeal ($P < 0.05$). # - denotes significant difference in expression compared to control fish injected with PBS only ($P < 0.05$).

5.4.2 Respiratory burst activity of PBLs and haematological analysis

The data for respiratory burst activity of PBLs, total leukocyte and differential cell counts are presented in Table 5.2. No differences were observed in respiratory burst activity of PBLs or differential cell counts between the dietary groups throughout the experimental period. Nevertheless, compared to fish fed fishmeal, there was a trend towards increased respiratory burst activity of PBLs 14 days post injection in fish fed EW and WPC. Compared to control fed fish (23.17 ± 1.64 / 1000 blood cells), fish fed EW and WPC diet that fish fed showed a significant increase in the leukocyte counts (41.67 ± 4.14 and 41.17 ± 7.13 / 1000 blood cells, respectively) at 14 days post injection. In contrast compared to control and WPC fed fish (45.67 ± 3.66 and 55.50 ± 3.40 /1000 blood cells, respectively), fish fed EW (35.33 ± 3.12/ 1000 blood cells) showed a significant decrease in leukocyte levels at 56 days post injection.

Table 5.3. Respiratory burst activity of peripheral blood leukocytes (RBA), differential cell counts and leukocyte counts of fish after intraperitoneal injection (I.P.) with heat inactivated *A. hydrophila*. Values expressed as ± SE (n = 6). Dietary codes: FM = control (Fishmeal LT94); EW = earthworm meal fed fish; WPC = whey / casein protein fed fish

Parameters	**Diets**			***P-value***
	FM	**EW**	**WPC**	
RBA (optical density)				
7 dpi*	0.370 ± 0.044	0.411 ± 0.030	0.362 ± 0.016	0.397
14 dpi	0.106 ± 0.008	0.161 ± 0.022	0.150 ± 0.020	0.085
28 dpi	0.075 ± 0.004	0.088 ± 0.015	0.084 ± 0.004	0.357
56 dpi	0.066 ± 0.024	0.089 ± 0.020	0.119 ± 0.030	0.343
Leukocytes[1]				
7 dpi	67.67 ± 7.89	55.17 ± 4.90	76.17 ± 6.86	0.203
14 dpi	23.17 ± 1.64[a]	41.67 ± 4.14[b]	41.17 ± 7.13[b]	0.027
28 dpi	59.50 ± 6.87	34.67 ± 6.76	60.83 ± 5.48	0.096
56 dpi	45.67 ± 3.66[a]	35.33 ± 3.12[b]	55.50 ± 3.40[a]	0.022
Lymphocytes (%)				
7 dpi	97.08 ± 0.746	96.17 ± 1.046	97.92 ± 0.455	0.494
14 dpi	95.58 ± 1.165	94.08 ± 2.107	94.75 ± 2.479	0.766
28 dpi	95.42 ± 1.841	94.83 ± 1.691	96.58 ± 1.012	0.723
56 dpi	95.42 ± 1.497	96.82 ± 0.746	97.25 ± 0.834	0.650
Monocytes (%)				
7 dpi	0.83 ± 0.279	1.50 ± 0.428	0.83 ± 0.333	0.409
14 dpi	0.83 ± 0.279	0.67 ± 0.279	0.58 ± 0.327	0.627
28 dpi	0.58 ± 0.154	0.75 ± 0.281	0.33 ± 0.105	0.423
56 dpi	0.42 ± 0.154	0.17 ± 0.167	0.33 ± 0.247	0.347
Granulocytes (%)				
7 dpi	2.08 ± 0.507	2.33 ± 0.667	1.25 ± 0.403	0.254
14 dpi	3.58 ± 1.091	5.25 ± 1.874	4.67 ± 2.186	0.715
28 dpi	4.00 ± 1.722	4.42 ± 1.599	3.08 ± 0.995	0.885
56 dpi	4.17 ± 1.509	2.92 ± 0.597	2.42 ± 0.870	0.796

*dpi = day post injection

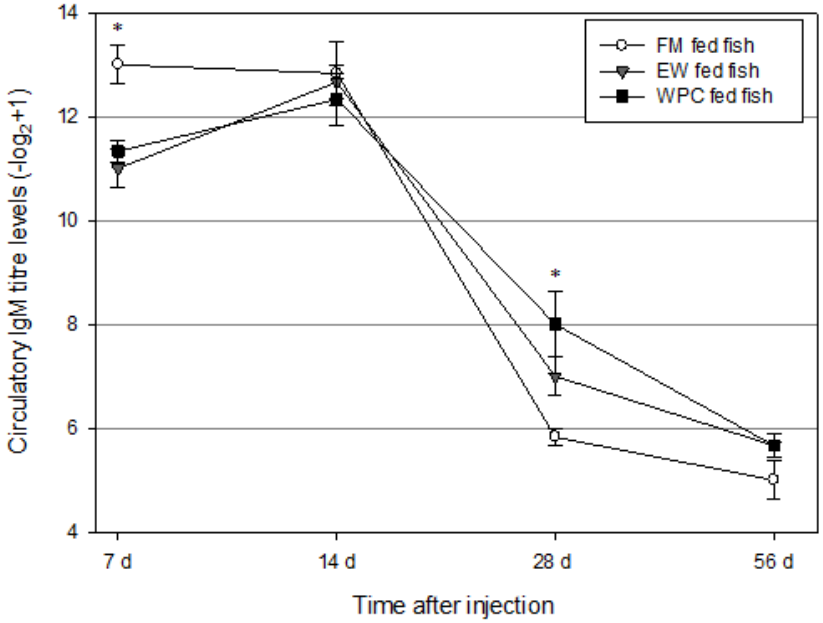
[1] number of cells per 1000 blood cells

[a-b] Superscripts denote a significant difference at $P < 0.05$.

5.4.3 Circulatory IgM response

The data for circulatory IgM response is presented in Figure 5.2. At day 7 post injection fish fed fishmeal (13.00 ± 0.37 -log +1) showed a significant increase in circulatory IgM titre levels compared to fish fed EW and WPC (P = 0.007). In contrast at day 28 post injection fish fed EW and WPC (7.00 ± 0.37 -log +1 and 8.00 ± 0.63 -log +1, respectively) showed a significant increase in IgM titre levels compared to fish fed fishmeal (5.83 ± 0.17 -log +1; P = 0.006).

Figure 5.2. Response of fish circulatory antibody titre levels after intraperitoneal injection (I.P.) with heat inactivated *A. hydrophila*. Values expressed as means ($-\log_2+1$) ± SE (n = 6). Dietary codes: FM= fishmeal fed fish (LT94); EW = earthworm meal fed fish; WPC = whey / casein protein fed fish. * - denotes significant difference between fish fed fishmeal and both EW and WPC fed fish at P< 0.01.



5.4.4 Haemolytic complement activity

All values for haemolytic complement activity are present in Table 5.3. At day 7 post injection fish fed fishmeal showed a significant decrease ($P = 0.016$) in complement activity (8.77 ± 1.15 U/ ml) compared to fish fed EW and WPC diet (42.34 ± 12.07 and 33.12 ± 0.55 U/ ml, respectively). In contrast at day 14 post injection fish fed fishmeal diet showed a significant increase ($P < 0.001$) in complement activity (120.21 ± 10.24 U /ml) compared to fish fed EW diet (4.37 ± 0.83 U /ml). Indeed fish fed earthworm diet showed the lowest response and compared PBS injected fish (23.50 ± 3.60 U/ ml) was significantly lower ($P = 0.026$). At day 28 post injection fish fed WPC diet showed the lowest response (23.53 ± 11.09 U/ ml) and compared to fish fed fishmeal (123.47 ± 16.41 U/ ml) was significantly decreased ($P = 0.011$). After 56 days post injection fish fed earthworm diet showed the lowest response (16.41 ± 5.49 U/ ml) when compared to fishmeal fed fish (138.50 ± 17.17 U/ ml) this was significantly lower ($P = 0.003$). Indeed fish fed fishmeal with the exception of day 7, showed the highest alternative complement activity across the whole sampling period.

Table 5.4. Haemolytic complement activity (ACH_{50} U/ ml) after intraperitoneal injection (I.P.) with heat inactivated *A. hydrophila*. Values expressed as ± SE (*n* = 3). Dietary codes: Control fishmeal fed fish injected with PBS only; FM = control (Fishmeal LT94); EW = earthworm meal fed fish; WPC = whey / casein protein fed fish

Parameter	**Diets**				***P-value***
	Control (PBS)	**FM**	**EW**	**WPC**	
ACH_{50} (U/ ml)					
7 dpi*	25.89 ± 6.04^{ab}	8.77 ± 1.15^{b}	42.34 ± 12.07^{a}	33.12 ± 0.55^{a}	0.016
14 dpi	23.50 ± 3.60^{a}	120.21 ± 10.24^{b}	4.37 ± 0.83^{c}	46.27 ± 15.99^{ab}	0.001
28 dpi	50.80 ± 6.78^{ab}	123.47 ± 16.41^{b}	64.67 ± 15.79^{ab}	23.53 ± 11.09^{a}	0.016
56 dpi	33.30 ± 0.65^{a}	138.50 ± 17.17^{b}	16.41 ± 5.49^{ac}	69.33 ± 16.62^{ab}	0.004

*dpi = day post injection

[a-c] – superscripts denote a significant difference in data ($P < 0.05$).

5.5 Discussion

In the ornamental industry the emphasis for aquafeeds is not to improve growth *per se*, but to deliver nutrients for maintenance and improve the aesthetic value of ornamental fish. In this regard the basis for contemporary research strategies is to integrate the methodologies of both nutrition and immunology to develop new feeds that will enhance both welfare and health of ornamental fish. In the current study a series of RT-PCR assays were used as a primary biomarker to characterise the effects of feeding *P. excavatus* meal and a combination of whey protein concentrate and casein meal on innate and adaptive immune responses of mirror carp after I.P. injection of heat inactivated *A. hydrophila*. In addition a number of other end-points were used including respiratory burst activity, haematology and a direct ELISA to determine the effect of feeding each dietary regime to the cellular and humoral responses of the fish.

5.5.1 Gene expression of carp whole blood

IL-1β is a prototypic pro-inflammatory cytokine that can affect nearly every cell type often in concert with another pro-inflammatory cytokine, TNF reviewed by (Dinarello, 1997; Bird *et al*., 2002; Huising *et al*., 2004; Savan and Sakai, 2006). In fish, as in humans, IL-1β is one of the earliest cytokines released during inflammation (Engelsma *el al*., 2002). The current study showed constitutive expression IL-1β cytokine in the blood of carp similar to the levels reported previously in carp and rainbow trout exposed to *Ichthyophthirius multifiliis* (Sigh *et al*., 2004; Gonzalez *et al*., 2007). This observation is in accordance with mammalian studies in which mononuclear cells from healthy humans showed no significant levels of mRNA encoding for IL-1β (Dinarello, 1991). In the present study a significant up-regulation in IL-1β expression was observed in fish fed whey protein concentrate and casein at 12 h and 24 h post injection compared to fishmeal fed fish. Likewise in fish fed earthworm meal a significant up-regulation in this cytokine was observed 24 h post injection. Although there

were no differences observed in all fish 48 h post injection ($P = 0.384$), probably due to high variation in sample data, there was a strong up-regulation in IL-1β expression compared to expression levels at 12 h and 24 h post injection. A similar level of IL-1β expression in carp blood was reported by Gonzalez and colleagues (2007) at 36 h post injection with *I. multifiliis*, suggesting a strong link between infection and expression of this cytokine in carp blood.

The pleiotropic cytokine TNFα has been shown in fish to be an important component of the innate immunity and pro-inflammatory response of fishes (Secombes *et al*., 2001; Grayfer *et al*., 2008; Roca *et al*., 2008). In this regard several gene expression studies have shown increased levels of expression of TNFα and other immune related genes in relation to feeding soybean meal to salmonids and cyprininds in various tissues including distal intestine and head kidney (Urán *et al*., 2008; Lilleeng *et al*., 2009; Marjara *et al*., 2012). The present study reports for the first time the effects of feeding earthworm meal and a combination of whey protein concentrate and casein meal on mirror carp whole blood TNFα cytokine expression. The investigation showed a significant up-regulation in TNFα expression of fish fed earthworm meal was observed at 12 h and 48 h post injection, compared to fish fed the fishmeal diet. The expression levels observed for TNFα in fish fed earthworm meal in the present study are in accordance with results reported by Gonzalez and colleagues (2007) where the authors observed a similar up-regulation in TNFα in whole blood of common carp 48 h after infection with *I. multifiliis*. In mammals both TNFα and IL-1β act in concert regulating the inflammatory response and it appears in this study that fish fed earthworm meal showed this synergistic effect indicative of an acute inflammatory response (Fehrenbacher *et al*., 2005). In contrast fish fed the whey protein concentrate and casein diet showed a significant down regulation in TNFα expression 48 h post injection (Figure 5.1B). Indeed, Kanwar and Kanwar (2009) reported that a mixture of whey protein concentrate and

other milk derived proteins down-regulated the expression of TNFα in a co-culture transwell model using both human Th1 macrophages and Caco-2-cell lines. This preliminary finding could be beneficial in reducing the effects of chronic enteropathy as seen in several commercial fish species including salmonids and cyprinids when fed high levels of plant proteins reviewed by Krogdhal *et al*. (2010); however further work would be required to validate this.

The complement of fish, like higher invertebrates can be activated through all three pathways (classical, alternative and lectin pathway) and in fish it appears the alternative complement pathway is the most well studied, perhaps reflecting its importance in fish (for review see Boshra and Sunyer, 2006). In contrast to mammals, complement system in fish is active at very low temperatures due to their poikilothermic nature, and their alternative complement pathway titres are several orders of magnitude higher (Suyner and Tort., 1995; Sunyer *et al*., 1998). Interestingly it has been reported that through the alternative pathway, fish complement mediates the lysis of erythrocytes (RBCs) from a number of animal species, whereas in humans this activity is mainly restricted to the lysis of rabbit RBCs (Sunyer and Tort, 1995). This property of fish complement may suggest that this allows for a wider recognition of foreign antigens when compared to mammals. This would seem plausible due to the fact that fish live in a highly antigenic environment and so a broader recognition of foreign antigens allows the fish to recognise a broader range of microorganisms (Sunyer *et al*., 1998). In teleost fish it is recognised that some key complement proteins such as C3 exist in multiple isoforms that are the products of different genes (Sunyer *et al*., 1996; Sunyer *et al*., 1998; Nakao *et al*., 2000). For this reason the PCR primers used in this study for the C3 gene were designed to be consensus for the five recognised isoforms of this complement protein (C3-H1, C3-H2, C3-S, C3-Q1, and C3-Q2) (Sunyer *et al*., 1998).

In the present study after the fish were injected with heat-inactivated *A. hydrophila*, a strong up-regulation in C3 expression was observed in fish fed earthworm meal compared to fish fed fishmeal at 12 h and 48 h post injection (Figure 5.1C). Analogous to the current study, Saeij and colleagues (2003) reported a similar profile in C3 and TNFα expression. The authors attributed the induction of C3 mRNA expression to the synergistic effects of TNFα during the inflammatory response. In human macrophages, TNFα is critical in the regulation of complement factor B (*Bf/C2*) gene expression (Lake *et al*., 1994) and more recently it has been shown in fish that *Bf/C2* expression plays an important role in activating the alternative complement pathway (Sunyer *et al*., 1998, Nakao *et al*., 2002; Wei *et al*., 2009; Zhou *et al*., 2012), thus in this context the expression of TNFα in the current study maybe regulating *Bf/C2* expression and subsequently the C3 mRNA expression. However the exact mechanism of this apparent synergistic effect of TNFα to induce *Bf/C2* expression in fish effector cells is still to be elucidated. In comparison to the expression of complement 3 protein the measured alternative complement activity was the highest in fish fed fishmeal, with the exception of day 7 post injection, across the whole sampling period. Indeed fish fed earthworm meal showed the lowest complement activity at day 14 post injection which was lower than control injected fish at this time point. The exact reason for this response remains to be elucidated; however throughout the data set there was a lot of variation within the fish per treatment which may have contributed to the sporadic results. Future trials should consider using higher sample numbers to attempt to reduce this observed variation.

5.5.2 Respiratory burst activity of PBLs and haematological response

Leukocytes play a major role in the innate immunity and their circulatory levels can be considered as a bio-indicator of health status in fish (Whyte, 2007). In the present study the leukocyte levels were quantified in relation to total blood cell levels and showed a significant

elevation 14 days post injection in fish fed earthworm and whey protein concentrate and casein diets compared to fish fed fishmeal. In contrast, after 56 days post injection fish fed earthworm meal exhibited a significant decrease in leukocyte levels compared to fish fed fishmeal and a combination of whey protein concentrate and casein meal. Previously, it was reported that feeding earthworm meal to fish for 60 days can cause a reduction in the circulatory leukocyte levels (Rawling *et al*., 2012), and it appears the result from the present study confirms this response. The differential cells counts revealed no difference between lymphocytes, monocytes and granulocytes proportions between treatments. Likewise the extracellular respiratory burst activity of isolated peripheral blood leukocytes showed no difference across the experimental groups indicating that feeding the experimental diets had no effect towards respiratory burst activity in carp PBLs.

5.5.3 Circulatory IgM response

As key effector molecules of jawed vertebrate's adaptive immune system, immunoglobulins are produced by B lymphocytes, either as a secretory form (antibody) or as a membrane form (B cell receptor) (Zhang *et al*., 2011). The immunoglobulin IgM has been well characterised in fish and appears to be specialised in the systemic immune response (Zhang *et al*., 2010). In the current study, to determine the systemic levels IgM a direct ELISA assay was used. Following injection with heat inactivated *A. hydrophila* the IgM titre levels were significantly higher in fish fed fishmeal at 7 d post injection. In contrast at 28 d post injection the titre levels in fish fed earthworm meal and the whey protein concentrate/ casein diet were significantly elevated compared to the control. The results from this study confirms those reported by Yin *et al*. (2009) whom also observed similar titre levels response in common carp when fish were vaccinated against *A. hydrophila/A. salmonicida*.

5.5.4 Conclusion

The results of this investigation demonstrated that replacing standard herring fishmeal with earthworm meal at high inclusion rates (i.e. 66 % replacement of fishmeal; 411 g/ kg dietary inclusion) can have an immunomodulatory effect on mirror carp post vaccination with bacterin. An up-regulation in expression of all immune related genes in fish fed earthworm meal compared to fish fed fish meal is indicative of an acute inflammatory response to injection with heat inactivated *A. hydrophila*. In contrast it appears that feeding whey protein concentrate and casein meal to carp (50 and 244 g/ kg, respectively) at 66 % replacement of fishmeal caused a significant down-regulation in TNFα and C3 expression. However, feeding a combination of whey protein concentrate and casein to fish appears to increase the production of circulatory IgM and leukocyte levels compared to fishmeal fed fish. It remains to be elucidated as to what extent this finding could impact disease resistance in cyprinids, but future studies should focus on using live challenge which reflects the cumulative effects of all host-pathogen interactions during production (Wiegertjes *et al*., 1996). Future studies should consider using lower inclusion levels to evaluate the effect of earthworm meal, whey protein concentrate and casein meal in more practical feed formulations for ornamental fish. These commodities could potentially have positive effects towards the immune response of high value species leading to premium diets incorporating exotic ingredients which could enhance the health and welfare of ornamental fish.

CHAPTER 6.

GENERAL DISCUSSION

6.1 Overview of findings

For nearly a decade much of the research focus in food aquaculture has been to identify alternative protein sources to offset the demands on fisheries resources that supply the mainstay raw materials required to manufacture fishmeal. This particular sector alone in 2006 consumed 3,274 million tonnes of fishmeal and nearly 835,000 fish oil (Tacon *et al*., 2010). The food aquaculture sector is now growing at a compound rate of ~10 % per annum which is faster than all other sectors of agribusiness. Indeed future projections of fishmeal usage show that there will be a shortfall in this feed commodity if continued to be utilised in aquafeeds at current inclusion levels (Tacon *et al*., 2010). Moreover the average annual growth rate of the ornamental industry is ~14% since 1985 (Bartley, 2000). Therefore the objective of this research programme was to identify potential novel ingredients to replace fishmeal in diets for fish to ensure the future feed security to fuel an ever expanding market The realisation that fishmeal demand is exceeding supply is a major factor driving research in this area to identify alternate protein sources that will deliver adequate supply of nutrients to support optimal growth performance without any deleterious effects towards fish immuno-competence.

Contrasting to classical nutritional approaches to the assessment of novel feed ingredients, this study endeavoured to integrate the methodologies of both nutrition and immunology to give a more robust assessment of potential selected feed commodities. This aspect is important to the future development of ornamental aquafeeds to meet increasing consumer demands of fish, establish healthy fish stocks and help provide a more preventative strategy to mitigating the global spread of disease. As outlined in Section 1.3, the transportation of live animals such as in ornamental trade has contributed to the considerable

rise in disease manifestations in areas of the world with no prior evidence of disease (Whittington and Chong, 2007). Therefore through nutritional influence it may be possible to develop novel feed formulations that not only integrate a preventative strategy to disease control but tailor feeds for a specific purpose such as to improve the longevity and welfare of the fish. On this basis the emphasis of each investigation was to assess the effects of feeding selected novel ingredients on the growth performance and health status of mirror carp, a model ornamental fish species. The results showed that it is possible to replace fishmeal with earthworm meal with no detrimental effects on the growth performance and immuno-competence of carp (Table 6.1). Likewise feeding a combination of whey protein concentrate and casein protein showed positive effects on the growth performance, haematological responses and immuno-competence of carp. These particular feed commodities are novel and with rising concerns of feed security these results champion the use of earthworm meal and whey/casein proteins in future ornamental aquafeed formulations. In addition this study demonstrated that feeding the selected plant sources as single replacement of fishmeal above 50 % had negative effects on the growth performance and health status of cyprinids.

Table 6.1. Synopsis of fish growth performance and feed utilisation after feeding with selected novel ingredients compared to fish fed fishmeal.

Protein source	**Ranking**	**Ingredient Description**	**Growth performance outcome**
Earthworm powder	Primary	Earthworm powder made from freeze dried *P. escavatus* (Indian blue worm).	↑ WG, ↑ SGR ↓ FCR ↑ PER
WPC (Casein and whey protein)	Primary	of the total diet g/ kg 24.4 % was casein (Bacarel) and 5 % was whey protein concentrate (Carbolec 80)	↑ WG,↑SGR
Soybean meal	Secondary	Hamlet protein HP110 finely ground non-GMO soya protein	↓WG, ↓PER, ↓SGR, ↑FCR
Rice protein concentrate	Tertiary	Remypro N70 extracted from rice	↓WG, ↓PER, ↓SGR, ↑FCR
Corn protein concentrate	Tertiary	Empyreal™ 75 extracted from corn kernel and is a high-energy corn protein concentrate	↓WG, ↓PER, ↓SGR, ↑FCR

Key: WG = weight gain; SGR = specific growth rate; FCR = feed conversion ratio; PER = Protein efficiency ratio

6.2. Growth performance and feed utilisation of plant sources

To date much of the research focus has been skewed towards using vegetable protein sources to replace fishmeal usage in aquafeeds perhaps reflecting the economic and production stability of these feed commodities (Gatlin *et al*., 2007; Hardy, 2010). Indeed given these positive attributes in the current study each plant source was carefully selected on the basis of a favourable nutritional profile, as determined by ICP-OES analysis and standard AOAC protocols. The utilisation of soybean meal in the first experiment (Chapter 3A) presented a crude protein level (53 %) that was suitable for the inclusion in aquafeeds according levels outlined by Gatlin *et al*. (2007). Likewise both the rice protein concentrate and the corn protein concentrate used in the second experiment (Chapter 4) presented an acceptable crude protein content between 45-80 % (> 72 % dry matter basis) as recommended by Gatlin *et al*. (2007).

The results of the present study show that replacement of fishmeal with high levels (>50 %) of selected plant sources as a sole protein source gave rise to negative effects on the growth performance and feed utilisation of mirror carp. Specifically in the first experiment (Chapter 3A), carp were fed a commercial soybean meal at > 60% (411 g/ kg dietary inclusion level) replacement of fishmeal and showed negative effects towards SGR and FCR. Likewise in the second experiment (Chapter 4) both fish fed both plant concentrates showed a negative effect towards SGR and FCR (Table 6.1). Previous studies have shown that chronic exposure (> 4 weeks) to plant sources above 50 % dietary inclusion can have deleterious effects to fish growth performance (Hasan *et al*., 1997; Refstie and Tiekstra, 2003; Deng *et al*., 2006; Penn *et al*., 2011; Collins *et al*., 2013). The findings in this study are in agreement and may be as a result of deficiencies in specific essential amino acids such as lysine or methionine (Kaushik and Hemre, 2008; Gomez-Requeni *et al*., 2011) or poor digestibility (Degani *et al*., 1997). Moreover studies have shown that plant sources contain a number of

anti-nutritional factors (ANFs) as a part of their inherent defence mechanisms, which can interfere with digestive processes and intestinal tissue (Francis *et al*., 2001; Krogdahl *et al*., 2010; Chikwati *et al*., 2013). Indeed pathologies such as shortening of the primary and secondary mucosal folds and widening of the lamina propria in both salmonid and cyprinind intestine have been attributed to presence of ANFs (reviewed by Krogdhal *et al*., 2010). Subsequently these qualities may have contributed to the observed reduction in growth performance and feed utilisation in the present study.

Despite these obvious deleterious effects towards the nutritional status of salmonids and cyprinids future technological developments in extraction and processing of plant materials could yield a feed commodity that is more suitable for use in aquafeeds. As it has recently been shown that feeding a blend of plant sources in diets fed salmonids as can give rise to comparable growth performance to that of fishmeal in (Bendiksen *et al*., 2011; Burr *et al*., 2012; Penn *et al*., 2012).

6.3 Growth performance and feed utilisation of earthworm meal

The impact of using insect and invertebrate based protein in animal feeds and foods for human consumption is fast becoming a topical area for research focus to improve food security (Oonincx and de Boer, 2012; van Huis, 2013). In fact in January 2013, the EU supported a 3 million Euro grant proposal entitled ‘Enabling the exploitation of Insects as a Sustainable Source of Protein for Animal Feed and Human Nutrition’ (project reference: 312084). The consortium, aptly named ‘PROTEINSECT’, aims to facilitate the exploitation of insects as alternative protein sources for animal and human nutrition. Equally there seems to be a global initiative to drive research focus in this area to promote insect proteins as a viable alternate protein sources to be utilised in feeds for fish, poultry and pigs and even as food commodities for human consumption. The technical consultation meeting entitled

"Assessing the Potential of Insects as Food and Feed in assuring Food Security" held from 23-25 January 2012 at FAO Rome (http://www.fao.org/forestry/edibleinsects/74848/en/) suggested a number of major challenges must be considered to implement the use of insects in animal feeds including: selecting suitable insect species and strains, finding cheap rearing substrate (if possible by utilizing organic waste side-streams, but assuring feedstock safety when rearing insects on organic waste and manure), managing diseases and setting up sanitation procedures, producing a constant supply of high quality insects (including quality assurance), developing innovative and cost-effective production systems, increasing automation/mechanization, safeguarding animal welfare (ethical concerns), establishing a regulatory framework, and elaborating an industrial code of practices/standards. Although these aspects still require considerable research and development, the success of the findings in the first experiment (Chapter 3A) can only champion the use of earthworm meal for use in in diets for cyprinids. The earthworm meal used in this study was freeze dried whole *P.excavatus. P. excavatus* has a high fecundity and is easily grown in most organic waste substrates making it an excellent candidate for bioremediation and for use as biomass in aquafeeds (Edwards *et al*., 1998). In Vietnam it is produced in vast quantities and is already being distributed in the Asian market.

Results from the first experiment (Chapter 3A) showed that feeding the selected earthworm meal was highly successful supporting the growth performance and feed utilisation of carp in comparison to fishmeal fed fish. In fact fish fed earthworm meal show the best growth performance and feed utilisation compared to all other test ingredients, where compared to fishmeal fed fish significant elevations in SGR, and PER was observed (Table 6.1). Fish fed earthworm meal showed a significant decrease in FCR compared to fishmeal fed fish indicating feed utilisation was better in carp fed earthworm meal. Therefore on this basis it may be possible to replace fishmeal with earthworm meal in excess of 60 % inclusion

in diets for cyprinid fish. However caution must be taken to ensure the amino acid profile of the selected earthworm species is compatible to carp requirements. Despite this, these findings are both novel and highly relevant for the ornamental industry where high value dietary specifications are more acceptable.

6.4 Growth performance and feed utilisation of dairy sources

Fish fed a combination of whey protein concentrate and casein protein showed significant elevations in all growth performance and feed utilisation indices compared to fish fed fishmeal (Table 6.1). These findings probably reflect the high bioavailability of amino acids of the selected dairy based proteins. Typically, lysine, methionine and threonine are identified as the first limiting amino acids in fishmeal replacement diets and the order of limitation among these three will depend on the mix and the composition of the proteins in the formula. In this regard the fact that compared to the lysine and threonine requirements for carp both casein and whey protein contain higher levels and show a high amino acid digestibility similar to egg protein (Hambraeus, 1992), may have contributed to the observed increase in growth performance and feed utilisation.

Interestingly, the physicochemical property of casein which in milk exists in the form micelle formation plays a pivotal role in its bioavailability as a protein source. The casein micelle is a large colloidal particle that has been shown to form clots in the human intestine (Frühbeck, 1998). The ability of this commodity to form clots in the human intestine makes it very efficient in nutrient supply because it is able to provide a sustained slow release of amino acids into the blood stream (Boirie *et al*., 1997). This provides for better nitrogen retention and utilisation by the body and/so may explain further why fish fed the dairy based diet showed increased growth performance and feed utilisation compared to fish fed fishmeal. This could have significant benefits in future aquafeed formulations where supplementation

of casein protein could provide a pool of essential amino acids in diets that have known deficiencies such as plant based feeds.

6.5 Haematological and serological response of fish fed plant proteins

Icthyo-haematology is a useful diagnostic tool in the assessment of feed composition and nutritional status in relation to environmental conditions affecting fish. In the present study the measured haematological indices showed that feeding fish plant proteins at >60 % (330 and 360 g/ kg dietary inclusion level) replacement of fishmeal regardless of source had a detrimental effect. In particular it appears that fish fed the plant protein sources showed lower haematocrit levels compared to fish fed fishmeal which could be an indication that fish health may be compromised. Indeed the haemoglobin levels in the fish fed soybean meal (Chapter 3A) were significantly lower compared to fishmeal fed fish and although not significant fish fed pant sources in the first and second experiment showed the lowest mean corpuscular volume (MCV) levels. Previously, Hemre *et al*. (2005) reported significant reductions in MCV levels of fish fed increasing inclusion of plant proteins in salmon diets. This result was correlated with an increased organ index value for the spleen which could be as a result of activation and increased proliferation of leukocyte populations (Hemre *et al*., 2005). However further work would be required to validate this hypothesis in carp. Similarly, Kumar *et al*. (2010) reported that feeding soybean meal at 75 % replacement of fishmeal (51 % dietary inclusion rate /kg) caused significant reductions in MCV and mean corpuscular haemoglobin. They hypothesised that trapping of damaged erythrocytes in the spleen because of an inflammatory response caused by the presence of anti-nutritional factors (ANFs) could lead to a compensatory release of immature erythrocytes resulting in a decrease in MCV. Furthermore certain ANFs (e.g. lectins) have been shown to bind to enterocytes thus affecting nutrient metabolism and absorption in the gut (Hendericks *et al*., 1990). This could have led to deficiencies in amino acids, minerals and vitamins important for the proliferation of

erythrocytes and leukocytes resulting in aberrant MCV levels. Further work would be required to elucidate this hypothesis.

The findings from the serological analysis showed that fish fed soybean demonstrated a significant decrease in serum protein concentration compared to fish fed fishmeal. Although no appraisal of this was undertaken this may be a result of lower digestible protein in the soybean diet or it could be associated with adverse effects to liver integrity which would have affected protein metabolism. The liver receives nutrients and compounds from the intestine and/so needs to respond to any substances that may have a detrimental effect to the fish. In this context, Tacchi *et al*. (2012) fed a soybean protein concentrate to Atlantic salmon at 18 % inclusion (180 g/ kg dietary inclusion level) and showed through transcriptomic analysis that genes involved with the oxidative stress response in the liver were significantly elevated compared to fish fed control diet. They concluded that the induction of these genes (MPV17 protein, amine oxidase and HSP 70 kDa protein) may indicate a diet-induced stress response in the liver of fish fed plant sources. Indeed a number of other studies have shown that feeding plant sources can have a detrimental effect on the metabolic functions of the liver in fish and even disrupt liver tissue causing pathologies such as liver steatosis (Bakke-McKellep *et al*., 2007a; Wessel *et al*., 2009).

In summary caution must be taken when formulating with plant sources as they contain a number ANFs that can interfere with protein digestion and metabolism. In addition through the induction of a localised inflammatory response, feeding plant sources to fish could have a negative impact on fish haematological status and gut integrity which could lead to the release of immature dysfunctional erythrocytes and acute enteropathy.

6.6 Haematological and serological response of fish fed earthworm meal

This study has shown that feeding earthworm meal at 66 % replacement of fishmeal (411 g kg dietary inclusion level) to carp had no deleterious effects on the haematological and serological profiles of the fish. Specifically haematocrit, haemoglobin and mean corpuscular haemoglobin (MCH) showed significant elevations compared to fish fed fishmeal, but all values were still within the biological ranges reported for cyprinids by Groff and Zinkl (1999). Despite this, these observed elevations could be due to the fact that the selected earthworm meal presented a higher concentration of iron (Fe) compared to fishmeal and all other test ingredients (Chapter 3A.5). The element iron is central to the quaternary structure haemoglobin, DNA synthesis and electron transport. Interestingly feed is considered the major source of iron for fish because natural waters usually contain low amounts of soluble iron. The iron requirement for cyprinids is 150 mg/ kg of the diet (Satoh, 1991). The levels reported using ICP-OES analysis showed that the iron concentration of the earthworm meal was 499 mg/ kg. This value was 2 times higher than fishmeal and 4 times higher than soybean meal (Desjardins *et al*., 1987). The reason why there was such a high iron content in the earthworm meal compared to all other ingredients remains to be elucidated; however this particular attribute may have contributed to the observed increase in cell haemoglobin concentration which can be beneficial to the process of respiratory gas exchange in carp erythrocytes. All other values for haematological analysis were in line with values reported by Groff and Zinkl (1999) for common carp. These results are both novel and important in supporting the potential use of earthworm meal in future ornamental feed formulations.

6.7 Haematological and serological response of fish fed dairy sources

In the second experiment it was apparent that a significant elevation in MCH was shown in fish fed whey/ casein protein diet compared to fish fed fishmeal suggesting there was an

increase in haemoglobin concentration per cell in fish fed this dietary regime. This apparent elevation could be due to an increase in the uptake of iron (Fe) or the presence of a number of bioactive proteins as such as insulin-like growth factor 1 and 2, platelet –derived growth factor each of which may have contributed to the increased *in situ* proliferation of erythrocytes however this remains to be elucidated.

A significant decrease in the proportion of lymphocytes was observed in fish fed whey/casein protein diet compared to fishmeal; however this may be offset by an elevation in the proportion of granulocytes. In fish granulocytes are important effector cells in response to pathogenesis (in particular neutrophils) and so play a pivotal role in the front line defence mechanisms in fish. In this context feeding a combination of whey/ casein protein may be beneficial to carp by increasing the basal level of circulatory granulocytes thus acting as an immunopotentiator.

In fish serum albumin is a good indicator of liver impairment and in this regard fish fed whey/ casein protein showed a significant 2-fold increase in albumin concentration compared to fish fed fishmeal. This result may indicate liver function was not compromised by the inclusion of whey/ casein protein. Indeed, a similar trend was observed in serum protein level where fish fed a combination of whey/ casein protein showed a significant elevation compared to fishmeal fed fish. This result is both a positive bio-indicator of health status and consistent regarding the high bioavailability of whey and casein proteins compared to other protein sources (Korhonen, 2006).

6.8 Immune response of fish to alternative proteins sources

Contemporary research strategies are now focusing on the integration of nutritional and immunological methodologies to provide a more robust assessment of novel feed commodities. In this study a bacterin was developed from heat inactivated *A. hydrophila* to

simulate infection and was injected into the intraperitoneal cavity of the fish. A number of biomarkers including respiratory burst activity (RBA) of phagocytes, immunoglobulin levels (IgM), gene expression profiles of inflammatory cytokines and complement protein C3, serum alternative complement activity and haematological responses were employed to determine the effects of feeding each dietary regime to fish immuno-competence.

Phagocytes are important effector cells of the innate immune defence in teleost fish and can be strongly induced by the presence of pathogen associated molecular patterns (PAMPs). As a part of the antibacterial response to the presence of PAMPs fish phagocytes produce superoxide and hydrogen peroxide ions of which are considered to be a hallmark of the inflammatory response (Matsuyama *et al*., 1999) Recently it has been reported that inflammatory cytokines could play an important role in the functional induction of the respiratory burst response in fish, which futher confirms the integral link between RBA and the inflammatory response (Rieger *et al*., 2010). On account of this, the RBA was used as a biomarker for inflammatory responses during this study and was measured by mean of NBT reduction. The results from the first experiment showed that feeding fish soybean meal at 66 % replacement of fishmeal (495 g/ kg dietary inclusion) induced a significantly higher response compared to fish fed earthworm meal. In fact the highest recorded level for RBA was in fish fed soybean meal 14 days post injection with *A. hydrophila*. This finding may be indicative of a systemic inflammatory response which is in agreement with previous studies reporting induction of systemic and local inflammatory responses of tissues in salmonid and cyprinid fed diets with moderate inclusion of soybean meal (Krogdhal *et al*., 2000; Uran *et al*., 2008; Marjara *et al*., 2012). Contrary to this fish fed earthworm meal in the first experiment (Chapter 3B) displayed a significant reduction in RBA of whole blood 14 days post injection with *A. hydrophila*. However in the third experiment (Chapter 5) there was no observed dietary effect on the RBA of isolated peripheral blood leukocytes of fish after

injection. As inferred in the discussion section of the first experiment (Chapter 3B.5) the presence of antibacterial peptides in the earthworm diet may have caused a reduction in the activity of phagocytes. Whether or not these isolated peptides can remain active through monogastric digestion and whether they directly reduce fish phagocytic activity remains to be determined

Interestingly fish and chicken erythrocytes have been observed to actively form rosettes to facilitate the clearance of pathogens by macrophages (Passantino *et al*., 2002) and could produce cytokines or specific signalling molecules in response to binding (Passantino *et al*., 2004; Passantino *et al*., 2007). In 1999, Bishlawy *et al*. hypothesized a relationship between erythrocytes, haemoglobin and the immune system suggesting an active role in the immune response to pathogens (Bishlawy *et al*, 1999).Several studies have shown that haemoglobin is an important source of bioactive peptides that participate in the innate immune response (Liepke *et al*., 2003; Jiang *et al*., 2007). The antimicrobial activity of the respiratory globins is likely one of the most ancient anti-microbial mechanisms conserved across the invertebrates and vertebrates (Iwanaga, 2002). These respiratory protein-derived peptides exhibit antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria and yeast (Fogaça *et al*., 1999; Liepke *et al*., 2003). Although little attention has been given to these observations it remains clear that vertebrate haemoglobins and associated molecules in invertebrate species have bactericidal properties and participate in the killing of invading microbes. On account of this it may be inferred that increased haemoglobin levels in the fish fed earthworm meal may have contributed to increased capacity of carp erythrocytes to sequester the presence of the PAMP presented by *A.hydrophila*, thus reducing the activity of carp phagocytes leading to a cessation in respiratory burst response in fish fed this dietary regime. However this hypothesis requires further attention.

As a part of the adaptive arm to the teleost immune system immunoglobulins are important effector molecules produced by B lymphocytes, either as a secretory form (antibody) or as a membrane form (B cell receptor) (Zhang *et al*., 2011). In this study systemic IgM responses of carp to experimental dietary regimes were measured using a direct ELISA, post injection with heat inactivated *A. hydophila*. The results of both the first and third experiments (Chapter 3B and 5) revealed that at day 7 post injection a significantly higher level of circulatory IgM was evident in fish fed fishmeal compared to all other dietary regimes. The identity of the individual component(s) of fishmeal that cause this response remains uncertain; although as stated earlier in Chapter 5 it was not an aim of the study to pin-point the specific causal components. In contrast at day 28 post injection, fish fed a combination of whey and casein proteins showed the highest level of serum IgM, which was significantly higher compared to fish fed fishmeal This could be due to the fact that whey and casein proteins are known to contain a high level of immunoglobulins in the range of 300 – 600 mg/ l (Ayers *et al*., 2003; Smithers, 2004), which could have passively contributed to the observed increase in serum IgM levels. Indeed this mechanism of passive transfer is well characterised in mammalian systems, where it has been reported that purified milk proteins when added to cultures of murine spleen cells significantly increased cell proliferation and production of immunoglobulin M (Wong *et al*., 1998; Stelwagen *et al*., 2009). This further supports the hypothesis that the presence of bioactive peptides in milk proteins can stimulate the production of IgM, which could be beneficial to the recipient host.

On account of the success of fish fed both earthworm meal and a combination of whey and casein protein to fish growth performance and haematological status in the final experiment (Chapter 5) these dietary commodities were used to ascertain their effect on selected gene expression profiles of fish following injection with heat inactivated *A. hydrophila*. The results revealed that fish fed earthworm meal had an acute inflammatory

response where both the mRNA expression of pro-inflammatory cytokines IL-1β and TNFα and complement protein C3 was higher compared to fish fed fishmeal. This was the first experiment to show this result in carp blood, therefore it could be postulated that feeding *P. excavatus* earthworm meal from has the potential to augment a systemic immune response to pathogen challenge.

Contrary to this, fish fed a combination of whey protein concentrate and casein protein showed significant decreases in the mRNA expression of both TNFα and C3 at 48 h post injection with *A. hydrophila*. Interestingly in the murine system it has been reported that whey/ casein proteins exhibit immunosuppressive effects on the activation and proliferation of lymphocyte cultures (Barta *et al*., 1991). Furthermore Kanwar and Kanwar (2009) observed that exposing whey protein to human cell cultures decreased the protein expression of TNFα and IL-1β. On account of these findings it could be suggested that feeding whey proteins may help to augment mucosal tolerances to feed antigens. As suggested in Section 1.6.1, in some cases such as feeding high levels of plant sources can be deleterious to fish intestinal mucosal surfaces causing inflammatory associated pathologies. In this regard supplementation of the diet with whey /casein proteins could potentially have a positive effect on alleviating these symptoms and certainly warrants attention.

6.8 Further Research

6.8.1 Evaluation of growth performance and feed utilisation

Since feed formulation should be based on nutrient bioavailability, reliable data on the digestibility of different ingredients used in this study must be considered in future studies as a necessary prerequisite (Gomes *et al*., 1995). In view of this future investigations should aim to investigate a blend of plant sources in diets for ornamental fish as this may reflect a more practical diet formulation. In addition this may help to mitigate nutrient deficiencies and

improve protein digestibility. However, in the current study because of the ethical and technical constraints imposed by the sponsors it was not possible to determine the digestible protein levels of each test ingredient.

Contrary to this the inclusions of earthworm meal and whey /casein proteins in diets for cyprinid fish show great promise for future use in practical diets for ornamental fish. Indeed this study was the first to report such success of feeding both diets to carp at such high inclusion levels (411 and 348 g/ kg dietary inclusion level). However, as the physiology of the gastrointestinal (GI) tract is different amongst fish species and the rearing conditions of ornamental fish are so diverse, an assessment of the effects of feeding earthworm meal and whey /casein proteins to different species is required.

Although not a feature of this study, future investigations built upon the successes of these experiments should aim to address the effects of feeding the selected commodities on lipid profiles of carp. As outlined in Section 1.6.3, lipids and fatty acids have a pivotal role in the stability of plasma membrane structures and the differentiation and expression of cell surface characteristics important to many physiological responses such as inflammation, vasodilation, blood pressure, pain and fever (Wall *et al*., 2010). This data is necessary to validate the effects of feeding future novel feed ingredients to the health and welfare of ornamental fish.

6.8.2 Evaluation of haematological and serological responses

It is apparent that feeding carp different selected protein sources showed significant effects on the haematological profiles of carp. These affects are both novel and add value to the future use of these commodities in ornamental aquafeeds. However future investigations should aim to identify why there was a significant increase in cell haemoglobin concentration in fish fed earthworm meal and whey /casein proteins. It was postulated that this observed

increase may be due to the increase Fe content of the feed and future experiments should determine the post-prandial plasma concentrations of iron to confirm these results.

On account of increased serum protein levels in fish fed whey/ casein protein (Chapter 5) and a decrease in serum protein level in fish fed soybean meal (Chapter 3A), much of the fishes humoral components of the innate arm exist as serum proteins including antimicrobial substances, acute phase proteins (APRs), complement proteins and immunoglobulins that are mainly induced by plasma-borne signals commonly known as the acute phase response (for reviews see Ellis, 2001, Bayne and Gerwick, 2001). Pro-inflammatory cytokines such as IL-1, IL-6 and TNFα and their synthesis and release are provoked by stimuli such as those that result from wounding (e.g. eicosanoids, tissue factors and kinins) and as a consequence of the presence of PAMPs (e.g. lipopolysaccharides). The presence of pro-inflammatory cytokines induces altered rates of plasma/serum protein synthesis, where the majority of plasma proteins are synthesised in the hepatocytes. These proteins include acute phase proteins, proteins associated with fighting infections and pathogen clearance (i.e. complement proteins C2, factor B, C3, C4, C5 and C9, lysozyme and IgM), and in restoring the healthy state (Hatherill *et al*., 1999). Consequently it would be important for future studies to characterise the effects of feeding whey/casein protein commodities to serum protein profiles in healthy and diseased fishes using proteomic analysis. This knowledge would contribute to a better understanding of the effects of feeding such commodities to serum protein profiles which are integral to the development of an intact host immunological function.

6.8.3 Evaluation of dietary influences on immune responses

Previously a whole plethora of information has been published regarding the effects feeding soybean meal to salmonid and cyprinid intestinal and liver responses at transcript, proteomic

and physiological levels (Rumsey *et al*., 1994; Krogdhal *et al*., 2000; Martin *et al*., 2003 Bakke-McKellep *et al*., 2007; Uran *et al*., 2008; Marjara *et al*., 2012; Tacchi *et al*., 2012). Contrary to this, the results in the present study show for the first time that feeding the selected non-plant based feed commodities can significantly affect fish systemic immune responses at both transcript level and physiological levels. As the genomic information regarding carp stress and immune response biomarkers increase future investigations should aim to assess these responses to gain a better insight into the effects of feeding these commodities to fish health and welfare of ornamental fish.

Despite the great advances achieved in the use of transcriptomics to identify bio-markers and provide a more detailed insight into the minor genomic changes in metabolic pathways (Panserat and Kaushik, 2010; Tacchi *et al*., 2012), in this process of discovery an important aspect is to determine how gene expression is correlated to protein translation. To date there is little comparative evidence to show the response of the transcriptome in relation to the proteome upon dietary changes in fish studies, perhaps this reflects the point that research in this area is still in its infancy (Kolditz *et al*., 2008). Still it is evident that several factors such as the transcriptional/translational regulation, proteins post translational modifications, analytical methods used in studies and temporal scale can influence the relationship between genome and proteome (Pandey and Mann, 2000; Greenbaum *et al*., 2002). Most studies from the mammalian literature show a poor correlation between protein expression and changes in transcript level (Pandey and Mann, 2000). So the future development of understanding how changes in nutrient profiles affect complex metabolic pathways associated with fish growth and immunity rely on the integration of systems biology (i.e. genotyping, transcriptomics, metabolomics and proteomics). The integration of systems biology into nutritional research has been coined ‘nutrigenomics’ and through the application of this discipline future studies will be able to undertake high throughput

screening of thousands of genes. This will undoubtedly improve the molecular insight into nutrient metabolism and consequently revolutionise biomarker development in relation to health promotion and disease prevention of ornamental fish.

6.9 Conclusion

Fish growth and immune responses to modulation in the nutrients is a complex process that apart from genetic background depends on other mutually interdependent processes such as development, nutrition, metabolism and physiological stress. Moreover these processes are mediated by multiple tissue contributions. So in order to establish a more complete view a global and multidisciplinary approach is required. In this context this study through the integration of nutritional and immunological research methodologies revealed that feeding non plant based feed commodities to carp had a positive effect on growth performance and immuno-competence. These findings are both novel and highly relevant for the ornamental fish industry where high value dietary specifications are more acceptable. Recent developments in transcriptomic and proteomic analysis and their application will make it possible to further evaluate these commodities to fish metabolism, stress response and immuno-competence. This knowledge will be very important to the development of optimal diets that will reduce the dependency of fishmeal in modern ornamental aquafeeds and improve the longevity and welfare of ornamental fish.

7. APPENDIX

7.1. Mineral composition of raw ingredients

Ingredients	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
Fishmeal (LT94)	22.46	0.00	245.74	11097.15	1770.73	8.69	9855.50	19148.95	9037.80	83.60
Soybean meal	2.08	0.01	91.90	18299.85	2585.88	21.79	454.18	5221.34	3403.27	44.47
Corn protein concentrate	0.21	0.01	68.25	1167.21	649.83	5.97	2450.21	1953.12	9430.89	46.91
Rice protein concentrate	0.30	0.02	82.51	1335.50	2021.20	8.21	2600.00	3300.00	10545.74	51.52
Earthworm meal	3.71	0.02	499.47	7850.16	1598.97	39.03	2278.73	8478.27	8810.50	129.01
Whey protein concentrate	4.03	0.00	15.72	3316.83	505.18	0.84	1732.35	2867.66	9356.98	12.02
Casein protein	131.89	0.28	8.78	0.00	16.00	0.11	108.66	5724.82	6581.92	42.66

Table 7.1. Mineral composition of raw ingredients used in experimental chapters (%)

7.2. Proximate composition of raw ingredients

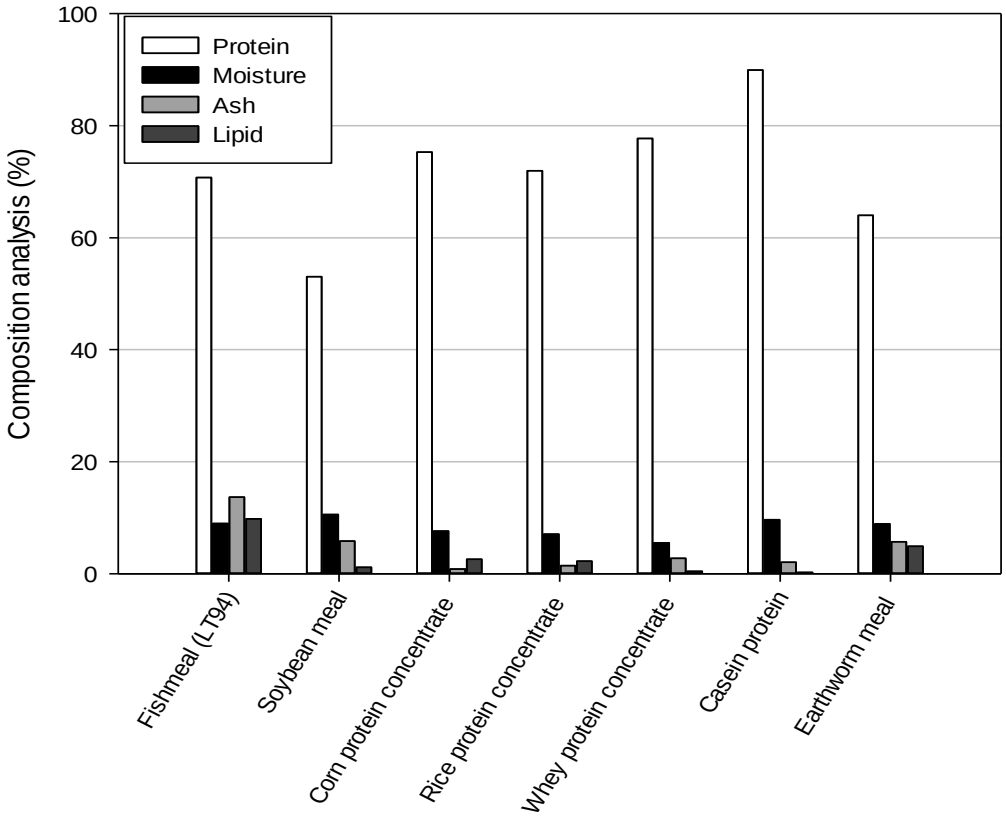


Figure 7.1. Proximate composition (%) analysis of raw ingredient used in experimental chapters.

7.3. Standard curve for development of bacterin

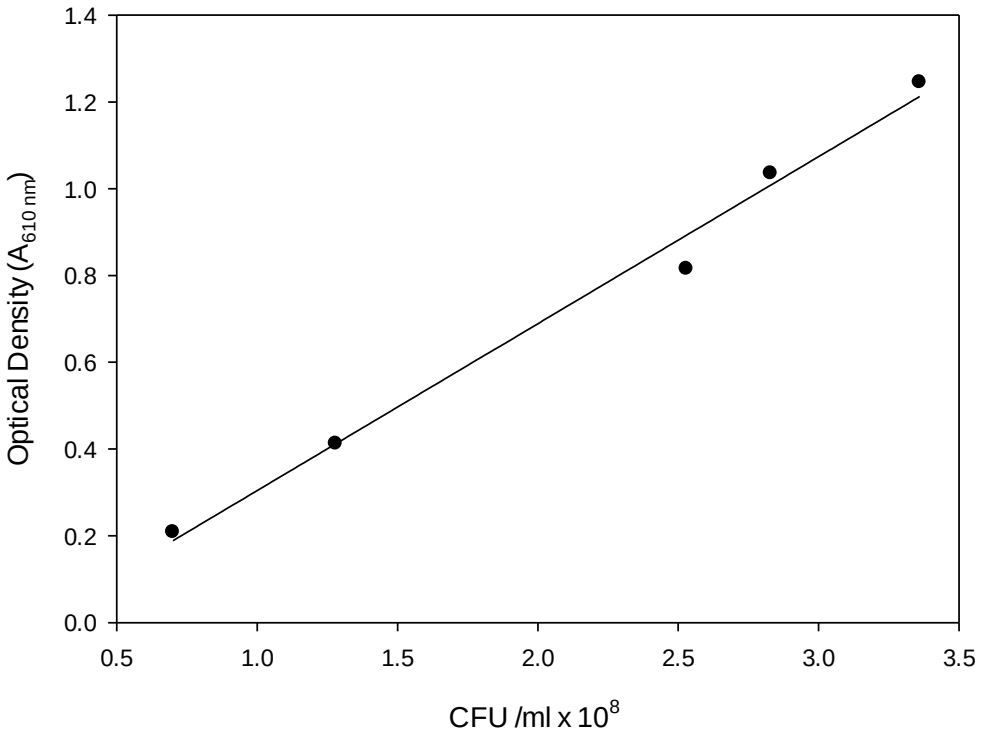


Figure 7.2. Standard curve for of *Aeromonas hydrophila* for development of bacterin. Data values are colony forming units versus optical density (R^2 = 0.99, y = 0.3848x-0.0805).

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