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The Bioavailability and Assimilation of Dietary Zinc in Rainbow Trout (Oncorhynchus mykiss)

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By

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ABSTRACT

This study examines three possible methods for improving the digestibility and bioavailability of zinc to rainbow trout (*Oncorhynchus mikiss*). The first method was to examine the availability of the zinc utilisation from commonly used protein sources; the second was to assess the efficacy of the upstream use of an enzyme treatment of the raw materials; the third was to assess the use of organically complexed mineral supplements as opposed to the inorganic salts widely used at present.

The first section indicated that the zinc from the soyabean meal was the most available (49.4%). The zinc digestibilities of the animal based protein used in this current study were 15.1% for LT94 fish meal, 26.6% for the Provimi 66 white fishmeal and 15.8% for poultry meat meal. The zinc in the maize/corn gluten meal was 31.9% digestible and from the NuPro 26.1%. Gram for gram maize gluten meal supplied the least amount of zinc to the fish (3.66 mg per kg). Based on these results the diets for the subsequent supplementation trials were formulated. The liver, eye and caudal fin were identified biomarkers of a severe zinc deficiency.

The second part of the study revealed a soybean product, treated by exogenous enzymes, had a higher phosphorus digestibility, (49.0%, vs. 36.6%) and zinc digestibility (30.7% vs. 7.9%) The treatment did not improve the protein digestibility (85%).

The third part of the study showed the organic source proved more digestible than the inorganic, 37.4% and 26.9% respectively. The fish fed the organic source maintained a higher level of zinc in both the eye and caudal fin. The liver zinc levels were unaffected by both dietary level and zinc source. Analysis of the liver for a zinc dependant protein showed that under stress conditions only the organic supplemented fish were able to synthesis this protein. The analysis of the mRNA levels coding for this protein indicate the fish on both zinc forms up regulated the production of the mRNA to the same extent when stressed.

Finally this study also examined the viability of using a stable isotope to identify different ‘preferences’ for one form of supplementation over the other in different tissues. This method illustrated a tissue dependant difference to how the fish attempted physiologically to compensate for zinc deficiency. The rate of turnover was fastest in the liver, then the caudal fin and then the eye, and also showed that when the diet was more deficient there was an increased ability for the tissues to take up the organic form.

Home Office Statement

All experimental work involving animals was carried out in accordance with the 1986 Animals Scientific Procedures Act under Home Office project license #30/2644 and personal license #30/8705

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Publications

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- Leeming, D.J., and Davies, S.J., (in prep). *Effect of phytase pre-treatment of soyabean meal on mineral availability in rainbow trout* (Oncorhynchus mykiss).

List of Abbreviations

ADC	Apparent digestibility coefficient
ANF	Anti-nutritional factor
cDNA	Complimentary deoxyribonucleic acid
DDGS	Distillers dried grains with solubles
DNA	Deoxyribonucleic acid
EAA	Essential amino acid
FCR	Feed conversion ratio
G6PD	Glucose-6-phosphate dehydrogenase
GCLC	Glutamate cystene ligase catalytic
GI	Gastro-intestinal
GST	Glutathione -S- transferase
ICP MS	Inductively Coupled Plasma Mass Spectrometry
ICP OES	Inductively Coupled Plasma Optical Emissions Spectrometry
LMA	Low melting agarose
MRE	Metal Responsive Elements
MS-222	Tricaine methanesulfonate
MTA	Metallothionein A
MTB	Metallothionein B MTF-1 Metal regulatory Transcription Factor 1
NMA	Normal melting agarose
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
Redox	Reduction-oxidation
RNA	Ribonucleic acid

ROS	Reactive oxygen species
SBM	Soybean Meal
SCGE	Single cell gel electrophoresis
SD	Standard deviation
SEM	Standard error of the mean
SGR	Specific growth rate
SOD	Super oxide dismutase
SPC	Soy Protein Concentrate
SPI	Soy Protein Isolate
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative real time polymerase chain reaction
ZIP	Zrt- and Irt-like proteins
Zn-AA	Zinc chelated or complexed amino acids
Zn-Met	Zinc methionine
Zn-pr	Zn-proteinate
ZnT	Zinc transporter

CHAPTER 1. Introduction

1.1 General overview

The increasing demand for food from the marine environment over the last decade has been well documented, as has the predicted reliance on the aquaculture industry to meet this demand due to the limitations of increasing wild capture fisheries which have plateaued since the early 1990's (fig 1.1, FAO 2012). If it is to be assumed that industry will meet this demand using currently farmed species, a major reform of current practices will be essential. The unsustainable requirement of fishmeal has already been identified as a major concern and the efforts to replace substantial amounts have been largely successful by turning to plant-based proteins as an alternative. This course of action brings with it its own problems with regards to amino acid composition of plant-based diets and possible pathological side effects (Gatlin et al, 2007), many of which have to be overcome with innovative processing techniques and inventive diet formulations leading to the reduced reliance on fishmeal.

Some commercial feed manufacturers now use as little as 30% fishmeal in their salmonid diets (personal observations), a level previously closer to 70-80% (Scottish Executive, 2002). The complications involved with this increased utilisation of plant proteins are not limited to balancing proteins and removing toxins but also include a plethora of secondary complications involving mineral availability. Many of the plant proteins contain anti-nutritional factors (ANFs) which may drastically reduce the digestibility of di and tri-valent cations such as zinc, copper and iron. A notable example is phytate in soyabean-based products (Budavari et al., 1989). The simplest way to overcome this problem is to supplement the diet with the minerals affected by the ANFs, for example, with zinc this has

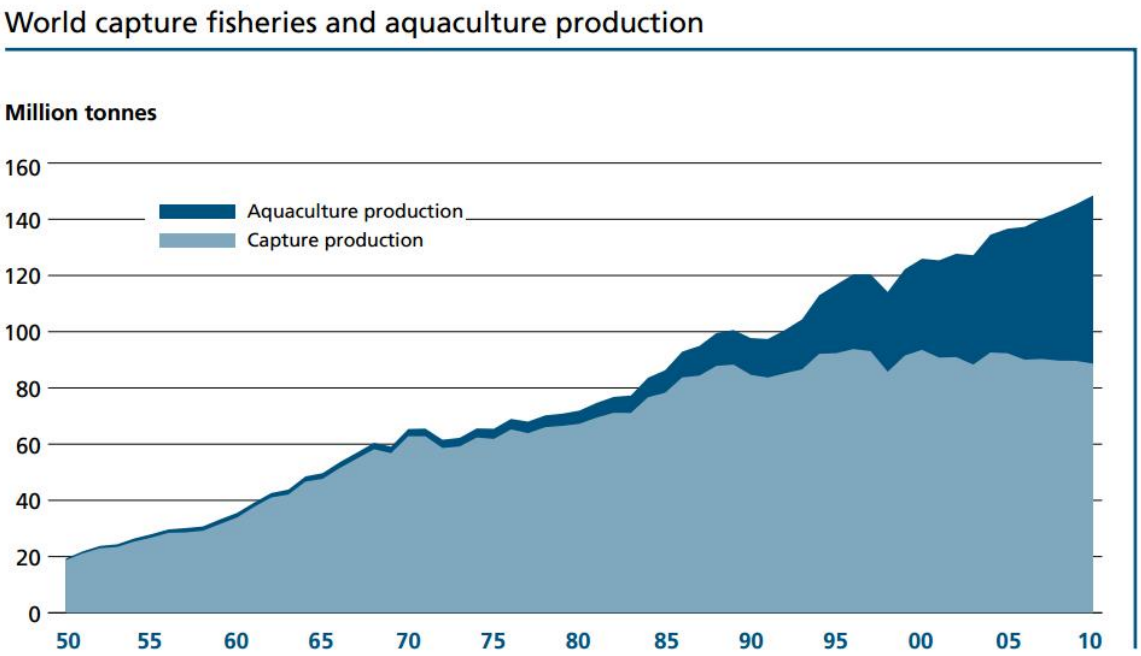

Figure 1.1 Past a predicted future contributions of capture fisheries and aquacultures contribution to the global seafood demand (FAO, 2012)

led to some diets containing over five times the fish's requirements as its digestibility may be as low as 16% (Storebaken et al., 1998). This 'loading' of the diet with highly indigestible minerals is deleterious to both the environment and profit as the remaining 84% of this relatively expensive and potentially toxic natural resource is effectively thrown into the sea.

In 2011 the Scottish salmon industry produced 158,018 tonnes of Atlantic salmon (Scottish Salmon Producers' Organisation, 2013); at a conservative estimate, assuming a general feed conversion rate (FCR) of 1.0, this took an equal amount of feed, containing on average 180 mg Zn/kg, to produce. That equates to approximately 28.5 tonnes of zinc used, which if only 16% digestible means that nearly 24 tonnes of zinc was wasted and released into the environment. At current market value ($2057/t, Infomine) that's a waste of $48,380, not including the environmental cost, just for zinc, not including copper, phosphorus, iron etc. The environmental impact of this has received very little attention. One study by Russell et al.

(2011) reported sediment concentrations around sea cages of 409 mg Zn/kg; another by Dean et al. (2007) reports 410 mg Zn/kg in the sediment compared to a background level at clean sites of 17 mg Zn/kg. Licences for sea cages in Scotland are awarded by SEPA (Scottish Environment Protection Agency) and the assessment criteria for them include sediment concentrations for zinc and copper. The upper boundary for zinc in this criteria is 410 mg Zn/kg, a level above which, it states that 'adverse effects will be observed in >50% of the benthic animals in that environment' (Delvalls et al., 2004). If the utilisation of plant proteins high in ANFs like phytase continues and the digestibility of these minerals is not improved, as the aquaculture industry continues to expand the sediment concentration will surely increase, potentially resulting in the refusal of new licenses and current licences being revoked.

1.2 The Role of Zinc in Biological Processes

The chemistry of zinc lends itself particularly well for use in biological processes. Zinc's stable valency of 2+ is unchanged under physiological conditions making it unsuitable to undergo free radical-producing redox reactions (Reilly, 2004). Zinc is a Lewis acid (an electron acceptor), which combined with its concentrated charge (due to small size) makes it the redox stable ion of choice for metallocomplexes (McCall, 2000). Given this it is far from a coincidence that zinc is ubiquitous in cellular metabolism and widespread in its importance in biological systems.

Zinc is most commonly incorporated into proteins, either as a structural tie modifying the proteins tertiary structure by cross-linking between regions of polypeptides, or as a Lewis acid (an electron pair acceptor) in the active site of enzymes when its role is described as catalytic. When acting as the latter, as in Carboxypeptidases for example (Folk *et al*, 1960),

one zinc ion is coordinated by a number of ligands (4 in the case of carboxypeptidase B). The structural role of zinc is not limited to enzymes but is also incorporated into structural proteins however the vast majority of the literature concentrates on its role in the suite of enzymatic proteins. The chemical property, most import in this role, is its high electronegativity (affinity for electrons). Consequently a Zn^{2+} ion is capable of bonding to several amino acid side chains. Histidine, glutamic acid, aspartic acid and cysteine are common ligands to zinc (cysteine more commonly, purely as structural rather than catalytic) (Chesters, 1997). It is amino acids containing nitrogen or sulphur that are particularly susceptible to forming ligands with zinc (Reilly, 2004). One example is the tetrahedral coordination of zinc by four cysteine residues in alcohol dehydrogenase (Vallee and Falchuk, 1993).

Enzymes are not limited to incorporating only one metal ion into their makeup, nor are the metals limited to performing only one function per enzyme. In the case of Cu/Zn superoxide dismutase, which incorporates zinc ions for a structural purpose, its activity in the dismutase of the superoxide to form hydrogen peroxide and oxygen comes from the copper ion in its active site (Vallee and Falchuk, 1993).

Any review of zinc dependant proteins would not be complete without mentioning two groups of metalloproteins which have the most diverse roles in biology; the zinc finger proteins and the metallothioneins. Zinc finger proteins are vital for the nuclear components of the cell to undergo transcription, repair (both nucleotide and base excision repair) and replication (Chesters, 1997). A zinc finger protein is a nucleoprotein that links to the double helix of the DNA at a site named ‘zinc twists’ (fig 1.2) and initiates transcription processes. Multiple zinc fingers are incorporated to form a zinc finger protein by a complex web of polypeptide loops and beta sheets configured by zinc ligands (Vallee et al., 1991).

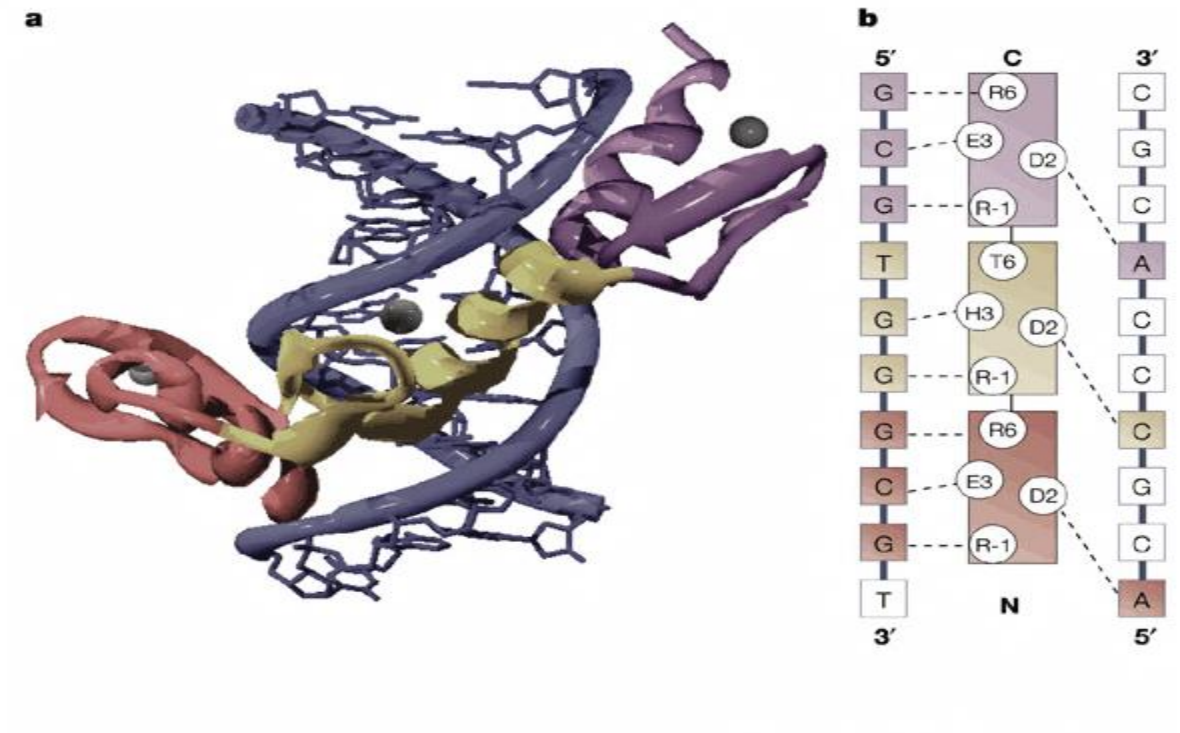

Figure 1.2. a) The Zif268–DNA complex showing the three zinc fingers bound in the major groove of DNA5. The DNA is blue and fingers 1, 2, and 3 are red, yellow, and violet respectively. Zinc ions are shown as grey spheres. b) A diagram showing the sequence specific protein-DNA interactions Zif268 and its DNA binding site

The involvement of zinc in the transcription of genetic information is not restricted solely to genes implicated with zinc utilisation or homeostasis. The mechanism of zinc-stimulated transcription is well documented and involves the activation of the Metal regulatory Transcription Factor -1 molecule (MTF-1) by a Zn^{2+} ion. Activated MTF-1 binds to the Metal Responsive Elements (MREs) located on the 5' flanking region of the target gene (Andrews, 2001) stimulating transcription. The discovery of this mechanism has since led to the identification of a much wider involvement of zinc in many more non-zinc incorporating proteins. In the puffer fish (*Fugu rubripes)* genome 28 antioxidant genes have been found to have the sequence for the MRE binding site at least twice within close proximity to their open reading frame (Chung, 2005). These included Glutathione -S- transferase (GST),

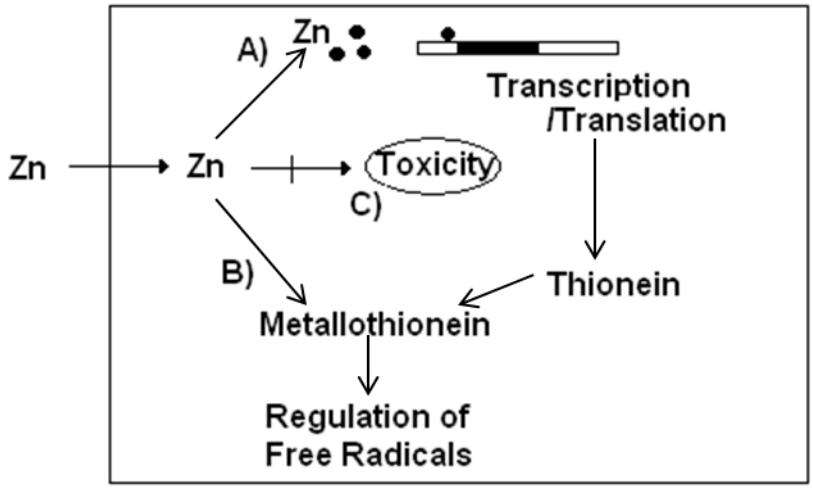

Figure 1.3 The synthesis of metallothionein. A) the synthesis of thionein. B) the incorporation of a metal ion into metallothionein, C) the cause of toxicity by free Zn ions.

Glutamate cystene ligase catalytic subunit (GCLC) and Glucose-6-phosphate dehydrogenase (G6PD).

One of the most common metalloproteins is metallothionein. Zinc is one of many metallic inducers of metallothionein synthesis and up to six zinc molecules can also be incorporated into its structure via metal-thiolate linkages (fig 1.3). Zinc is not the only metal that is incorporated by metallothionein which has led to metallothionein being thought to be one of the main heavy metal detoxification compounds in cells (Hogstrand, 1991). Although there is no doubt about the ability of metallothioneins to bind potentially dangerous free metal ions there is some argument over whether this is its main function in the cell. Olssan et al (1987) reported increased hepatic metallothionein content during the sexual maturation of female rainbow trout *(Oncorhynchus mykiss)*. With zinc also being required for proteins like DNA-RNA polymerase (Wu et al, 1987) it has been postulated that the ability of metallothionein to

scavenge free zinc allows it to act as a zinc donor for the polymerases at times of reproduction and as an acceptor or storage unit for zinc before or after reproduction.

Measuring zinc status is difficult, complicated by an organism's ability to regulate zinc, by both controlling its dietary absorption and also their endogenous excretion (Clearwater et al., 2002; Salguerio et al., 2000). In fish, biliary excretion and intestinal sloughing is considered to be the main mechanism of zinc homeostasis (Bury et al, 2003; Hardy et al., 1987).

Dietary zinc absorption occurs in the anterior gastrointestinal tract. At environmentally unrealistic levels of dietary zinc the uptake is dominated by a passive absorption (Bury et al, 2003) however, in reality, at dietary levels (50 mg/kg) a saturatable active transport mechanism involving zinc transporter proteins is used (Glover and Hogstrand, 2002). At these lower concentrations the use of zinc transporter proteins is crucial for zinc absorption due to its ionic charge and therefore water solubility it cannot freely pass through membranes. In humans the zinc concentrations in the cytosol and lumens of organelles is regulated by groups of transporter proteins known as ZIP and ZnT transporters. Analogous transporters have also been identified in fish (Feeney et al, 2005).

Zinc is transported around the body bound to protein in the plasma. In mammals 60-70% of this is bound to either albumin or α-2-globulin. Approximately only 0.1% of the total body zinc is found in the plasma which has led to the postulation that the plasma is purely a carrier of zinc between site of absorption, storage and metabolism (Salguerio et al., 2000).

Due to its essential role in a large number of proteins, zinc is the second most abundant trace element, after iron, in the human body. A consequence of this abundance is the number of functions requiring zinc which are essential for the maintenance of optimal health. Deficiency of zinc can lead to impaired immunity (Paripatananont and Lovell, 1995);

oxidative stress (Hidalgo et al., 2002); recurrent infections; delayed wound healing; gustatory abnormalities; DNA damage; mental lethargy; and increased risk of abortion (Salguerio et al., 2000) among many other effects .

The role of zinc in the maintenance of oxidative stress has been investigated *in vitro* by adding zinc to cells which are exposed to Hydrogen Peroxide (Chung et al, 2005). It was shown that zinc played a role as an indirect antioxidant when the cells were pre-treated with zinc sulphate as a result of the increased transcription of metallothionein, GST and G6PD; however the zinc also acted as a pro-oxidant when the cells were exposed to H_2O_2 and zinc sulphate simultaneously. It was proposed that the H_2O_2 treatment, which is known to be capable of releasing Zn^{2+} ions from metallothioneins and other proteins (Zhang *et al*, 2003) disrupted the cellular zinc homeostasis and then the simultaneous addition of more zinc resulted in the inability of cells to regulate the free Zn^{2+} ion concentration which is potentially very toxic. In rat's deficient of zinc, DNA damage in the liver manifested if the form of single strand breaks was observed to increase. This may have been caused by a decreased level of protection against reactive oxygen species due to a lack of zinc dependant protective enzymes or by a reduced ability to repair DNA damage due to a lack of zinc dependant repair enzymes i.e. fapy glycosylase (Castro et al., 1992). Zinc has also been shown to protect against lipid peroxidation in rats supplemented with dietary zinc. (Al-Damagh, 2007).

1.3 Zinc in fish

1.3.1 Zinc requirements

Table 1.1 Zinc requirement levels and deficiency symptoms in salmonids

Species	Requirement Level	Diet type	Deficiency symptom	Reference
Rainbow Trout	15-30 mg/kg	Purified	Reduced growth	Ogino and Yang (1979)
Rainbow Trout	40 mg/kg	Fishmeal	Reduced growth	Satoh et al. (1987)
Rainbow trout	150 mg/kg	Soyabean	Reduced growth	Satoh et al (1997)
A. Salmon	65 mg/kg	Fishmeal	Reduced whole body Zn	Lorentzen & Maage (1999)

A nutritional requirement is defined as the amount of a nutrient needed by an organism to sustain healthy life. The definition of a healthy life for fish in commercial aquaculture if open to interpretation; optimal growth is considered adequate by some; the ability to survive suboptimal conditions and disease challenges is adequate for others. It is becoming a more common practice to regard the dietary level at which either the whole body or selected organs become saturated of that nutrient (table 1.1). This practice assumes that once the animal cannot store any more of the nutrient it will have sufficient supplies to meet all its requirements and may be considered a very conservative approach however this potentially leads to using more of the nutrient than is actually required. To further complicate the issue, the raw materials used for the macro nutrients (fishmeal or plant-based products) may also play a significant role in altering the dietary levels required to achieve the selected target.

As discussed in the previous section, zinc is known for its essential role in growth, health and DNA replication. The majority of research in essential minerals, both into their biological

role and their nutritional requirements, has predominately concentrated on higher organisms. Trace element research in fish has generally been disjointed and sporadic, and mineral requirements have been defined for only the most commercially valued species. Even this is based on limited research to date with conflicting evidence.

Ogino and Yang (1978) calculated the zinc requirement for salmonids to be 15-30 mg/kg. This was calculated using a purified egg albumin diet. In reality though, even in a fish meal diet containing 65 mg/kg zinc, an Atlantic salmon (*Salmo salar)* could not maintain a normal whole body zinc status (Lorentzen and Maage 1999). In the same trial the authors calculated that an additional supplementation of zinc to reach dietary levels of 140 mg/kg was required to achieve a normal zinc status for this species. Similar experiments have shown the same in rainbow trout (*Oncorhynchus mykiss*), requiring an additional supplementation of 40 mg/kg to a white fish meal diet in order to achieve normal growth, never mind to maintain zinc status (Satoh et al., 1987).

Results from experiments like these using practical diets indicate that there is some aspect of the diet which does not allow the fish to utilize all the zinc present. Satoh et al (1987) attributed this to high levels of hydroxyapatite in the fish meal, especially tricalcium phosphate. A very similar effect on zinc availability is also seen in plant based diets, a soybean based diet required 150 mg/kg zinc supplementation in order to obtain optimal growth in rainbow trout (Satoh et al 1997), this was attributed to the presence of phytates (Storebakken et al., 2000). These anti-nutritional factors were not present in the purified egg-based diet of Ogino and Yang's, so meeting the zinc requirements of fish, which on the face of it did not seem to be of concern becomes a very real issue, especially with the present need to replace high quality fish meal with alternate protein sources in practical diet formulations.

1.3.2 Zinc Uptake

Fish have two routes of zinc uptake, first from the diet and second from the surrounding water. There is the potential for waterborne zinc to be absorbed in both the gut, from the swallowed external water, and also directly from the external aqueous environment via the gills. The drinking of water by freshwater species, especially salmonids, is very low and freshwater zinc levels are usually <10 µg/l. This is considered too low to make any significant contribution to whole body zinc (Spry et al., 1988) from either branchial or gastric uptake however the gills affinity for zinc is extremely high (Glover et al., 2003). Bury et al (2003) calculated the maximum uptake rate for zinc in both the gut and the gills. They reported a gut uptake rate of 933 nmol/kg/h and a gill uptake rate of only 240-410 nmol/kg/h. These results support claims that despite the high affinity for zinc in the gill, dietary uptake is the major contributor to the body zinc status (Glover and Hogstrand, 2002; Spry et al., 1988). Spry et al (1988) also reported no correlations between dietary and waterborne zinc uptake. The uptake mechanism in fish is described as high affinity low capacity in the gills and low affinity but high capacity in the gut.

Only a very small fraction of the total zinc in the environment is in the free form. As previously mentioned free zinc ions are potentially very toxic to many biological processes, zinc therefore is adsorbed to organic and inorganic compounds, be that in the sediments or in other organisms. For the zinc to become available to other organisms these compounds need processing in some way. In fish it is only by digestion in the GI tract that these compound are broken down. The gills are incapable of sequestering zinc from these compounds and can therefore only absorb the very limited free zinc in the water column.

1.3.3 Zinc Excretion

Zinc excretion in fish has had relatively little attention in the scientific literature to date. Until recently it was known that very little zinc was excreted via the urine (<1%) (Hardy et al., 1987), and that zinc was actively transported from the blood to the gills (Maage and Julshamn, 1993), where some dietary zinc is excreted from the body. Not until Clearwater et al (2002) and Bury et al (2003) both claimed that endogenous zinc was excreted by billiary excretions and intestinal sloughing did it become documented that the GI tract was also involved with not only the uptake but also the storage and regulation of zinc. Before this time any zinc in the GI tract or faeces was assumed to be principally unabsorbed from the diet. One further possible route of zinc excretion is the integument and its associated mucosal layer due to the high levels of zinc located in this tissue (Spry et al., 1988).

1.3.4 Zinc storage and regulation

Zinc is very highly regulated in all aspects of the fish's body: its uptake from the water or the diet; its excretion by the gills, the intestine, the urine or the integument; and also by its distribution within the body. This regulation means that even a dietary level of 1700 mg/kg $ZnSO_4$ is still non-toxic to the fish (Wekell et al., 1983).

Both the intestine and the skeletal tissue respond to differing levels of dietary zinc, indicating that they are involved with zinc storage. Maage and Julshamn (1993) and Sun and Jeng (1998) showed that zinc accumulates fastest and to the greatest extent in the intestine when dietary zinc levels are high. Maage and Julshamn (1993) also showed that when dietary levels of zinc were low the intestine level decreased, indicating that the zinc can also be mobilized

when required. Similar effects have been documented in the vertebrae and other skeletal tissues, with zinc levels increasing with high dietary zinc and decreasing when the dietary zinc was deficient (Maage and Julshamn, 1993; Do Carmo e Sa et al., 2004). Do Carmo e Sa et al (2004) reported that a zinc deficiency for 4-5 weeks resulted in mobilization of zinc from the vertebrate in juvenile Nile tilapia.

There is some debate in the literature about the role of the kidney with regards to zinc metabolism and excretion. Maage and Julshamn (1993) report that as dietary zinc increases from 17-97 mg/kg the levels of zinc in the kidney also accumulate. This, as with the intestines and the skeletal tissue, would indicate that the kidney has some kind of storage role for this mineral. The obvious critique of this hypothesis would be that the kidney is used as an excretory organ, however, the low zinc levels in the urine of fish would indicate that this is not the case in practice. On the other hand Overnell et al (1998) found the kidneys to be unresponsive to dietary zinc levels in turbot *(Psetta maxima)*. More investigation into the role of the kidneys is required, especially when there are conflicting reports about whether or not the levels of zinc in the kidney are affected by the level of zinc in the diet.

The gills are another organ in which the zinc levels respond positively to an increase in dietary zinc concentration (Wekall et al, 1983). The general consensus here is that this is probably due to their excretory role. The other visceral tissues, including liver, do not seem to respond to dietary zinc. The liver is mentioned specifically because of its known role in mammals. The mammalian liver is used to store zinc as metallothioneins (Salguerio et al., 2000), however in fish this does not appear the case. Fish hepatic tissue seems to perform only a metabolic role. As mentioned in the first section of this review zinc is water soluble and cannot therefore pass freely through cell membranes and requires ZIP or ZnT transporters to facilitate this process. Feeney et al (2005) reported that dietary zinc did not

stimulate transcription for these transporters in the liver. In order for there to be a positive correlation between dietary zinc and zinc levels in the liver an increase in these transporters would need to occur. Since this is not the case then this supports the theory that the liver is not being used for zinc storage in fish.

Plasma zinc and the muscular tissues do no display any significant correlation with dietary zinc levels (with the exception of the muscular tissues in Nile tilapia (Do Carmo E Sa et al.,2004)) (Overnell et al.,1998; Maage and Julshamn, 1993). Plasma is considered simply as a carrier of zinc between the intestine and either the organs for storage or metabolism, or to the gills for excretion.

Regardless of the ability of the fish to regulate zinc within its body the turnover of zinc is relatively fast (~1% per day). This means that in order to avoid deficiency a continual supply of dietary zinc is essential.

1.4 Alternative protein sources

1.4.1 Soybean

Soybean is arguably the alternative protein source most likely to provide an answer to the increasing problem of fish meal replacement in aquafeeds. The role of the global soybean industry (seemingly led by the US soybean industry) in developing this potential cannot be underestimated. Since 1995 promising research, well-funded from the United Soybean Board, has increased the demand for soybean meal in the aquaculture industry from almost nothing to approximately 5 mmt in 2005. Undeniably this has been a great marketing venture and has

identified and overcome numerous nutritional hurdles, however the product is far from perfect and there are still many obstacles to clear.

Global soybean production is huge (>200 M metric tonnes per annum), the primary product being oil. The by-product from the oil industry is a high protein cake which is further processed to produce soy flour, soybean meal (SBM), soy protein concentrate (SPC) or a soy protein isolate (SPI). SPC and SPI are both products of yet further processing of the soybean meal, this increases the cost and means that these products are not yet economical for large scale inclusion in commercially produced aquafeeds. However with development of new technologies combined with the increasing price of fish meal prices a time may come when it is cost effective and SPC/SPI become viable commodities.

Soybean meal has a crude protein content of ~48%, this is increased in SPC to ~64% (NRC, 1993), both of which meet the requirement of fish (Gatlin III et al., 2007). However, the nutritional inadequacies of soy based products are numerous but not insurmountable: they provide an inadequate supply of essential amino acids (EAA); have low ash and fat concentrations; have surplus carbohydrates; and the limited minerals that are present are tightly bound to phytic acid.

The low mineral concentrations can be overcome simply mineral premix supplementation, as can the deficiency of the EAAs to some extent. The limiting EAAs are all sulphur based (methionine and cysteine). The high cysteine concentration present in soybean products can meet 40-60% of the sulphur based EAA requirements but methionine is thought to be the first limiting amino acid for growth (Takagi et al., 2001). Supplementation of DL-methionine when SPC was the primary protein source demonstrated a positive effect (Mambrini et al., 1999). Further supplementation of lysine, arginine and tryptophan to soybean based diets

have also been shown to increase growth rates in fish (Furuya et al., 2004; Floreto et al., 2000).

Most carnivorous fish have a reduced capacity to assimilate dietary carbohydrates due to their limited ability to metabolise polysaccharides such as starch. Non-starch polysaccharides particularly present a problem when soybean products are incorporated into aquafeeds. Raw soybeans contain ~20% non-starch polysaccharides, a considerable amount to be concerned about when their presence is not only unusable by the fish but also negatively effects other nutrient utilisation and feed efficiency. A second carbohydrate fraction presenting negative consequences for the fish are oligosaccharides. Consisting up to 15% of soybean meal oligosaccharides have been reported to increase the viscosity of the chime in the digestive tract, interfering with the uptake of nutrients, especially the fat and minerals in salmonids (Refstie et al., 1998). Refstie *et al*. (1998) also linked oligosaccharide concentration with reduced growth performance, and Bureau et al. (1998) reported soybean meal induced enteritis, both in salmonids spp. The level of oligosaccharides could be reduced by using SPC rather than SBM, SPC recording only 3% oligosaccharides as opposed to 15% in SBM. Bacterial or fungal fermentation may also reduce the negative effects of oligosaccharides.

As with many plant based protein sources phytic acid is another problem with soybean products (~40 mg/kg in SBM and further concentrated to 70-100 mg/kg by the purification of SBM to SPC). The majority (~66%) of phosphorus is present as phytic acid, in this form virtually none of the phosphorus is available to fish (Riche and Brown, 1996). A second property of phytic acid, a property of upper most relevance to this review, is the ability of phytic acid to bind divalent cations, especially zinc. Just like the phosphorus incorporated in phytic acid, once the divalent cation is bound it is virtually completely unavailable, passes through the gut and out into the environment. Not only is this detrimental to the fish, which

present signs of mineral deficiencies, but it is also a large source of pollution, especially under the sea/lake cages, and requires extra expense to the farmers who need to further supplement minerals to the diet. Unfortunately there is no processing treatment that will remove phytic acid from soybean products, the only options are to either use mutant varieties of soybean low in phytates, which although do exist are not widely available especially for use in livestock feed ingredients; or to add phytase to hydrolyse the phytic acid. However even the addition of phytase presents problems due to the heat sensitivity of the enzyme during extrusion. One other possible way of reducing the effects of phytic acid is to use minerals in forms unavailable to the acid, proteinate minerals could possibly improve mineral availability in phytic acid rich diets.

1.4.2 Corn/Maize

Corn unlike soybean is mainly produced to be an energy source for livestock, only a small percentage is used for human consumption. Corn oil is the primary food product of corn cultivation but corn starch is also used in over 400 products, from paper coatings to food sweeteners. An increasing amount of corn starch is now also being used to produce ethanol. The main components of corn are bran/fibre, germ, gluten, and starch. Bran/fibre is of little or no use to the aquafeed industry; the germ is used for corn oil extraction leaving corn germ meal; the gluten is concentrated and dried to form corn gluten meal; and the starch can be fermented to produce ethanol.

Corn gluten meal is a promising ingredient for inclusion in aquafeeds however it does have some undesirable properties which currently limit its inclusion to ~25% of the diet. Corn gluten meal has a minimum of 60% crude protein content, this can reach 70% in more refined products but further refinement increases the cost and borders on being uneconomical. Corn gluten meal is highly digestible but is lacking in lysine, however at the current common

inclusion levels of ~15% of the diet lysine can be supplied from other ingredients, potentially soybean meal or concentrate (SPC) as both are relatively high in lysine. A minor concern with corn gluten meal is the high content of xanthophylls. Animals fed diets containing corn gluten meal have produced yellow coloured musculature, a quality often desired in chickens, demanding a higher price by implying some form of higher welfare standards. Yellow fillets of trout on the other hand are not so appealing to the consumer. This can be overcome by supplementing astaxanthin or canthaxanthin, a practice already commonly used in trout and salmon diets to produce redder fillets.

One area for potentially developing a cheap and possibly sustainable source of protein is the refinement of the by-products from the ethanol based bio fuel industry. The current waste product of ethanol production is DDGS (distillers dried grains with solubles), a product with ~30% crude protein and high fibre content, neither of which are desirable characteristics for aquafeed ingredients. However as only the carbohydrate is required for ethanol production it may be possible to separate the components of corn starch prior to the ethanol production and obtain a corn protein concentrate which is usable in aquafeeds.

1.4.3 Cottonseed

Cottonseed is the third highest produced legume seed, after soybean and rapeseed, worldwide. Cottonseed meal has a protein content of ~41% and compared to legume seed based meals and fish meal it is cheap to produce. Having a low market price, a reasonable protein level, and being mass cultivated globally, cottonseed meal has great potential for inclusion into high protein fish diets. Inclusions of up to 30% of the diet have shown no negative growth effects but a study by Lee et al. (2002) stated that the source and the previous processing of cottonseeds influenced the utilisation limits of cottonseed meal in fish diets.

Two possible health problems may arise if high levels of cottonseed meal are incorporated into fish diets. One is caused by gossypol and the other by quercitin (and its metabolites). Cottonseed meal typically contains between 400-800 mg/kg of free gossypol. Herman (1970) identified histopathological changes in the liver and kidneys of rainbow trout when exposed to levels >95 mg/kg gossypol however no effect on growth was seen until ~300 mg/kg. Lee et al. (2002) using juvenile rainbow trout reported that depending on cottonseed meal source, fish can absorb approximately 35-50% of dietary gossypol, and the majority of the absorbed gossypol seemed to be excreted. Free gossypol levels of 60, 150 and 225 mg/kg (dependant on the source of the cottonseed) did not depress growth rates.

Isorhamnetin, a metabolite of quercitin, is known to have oestrogenic effects and accumulate in animals (Piskula and Terao, 1998). Quercitin accumulation in fish has not been well studied however traces have been found in the liver and body tissues of tilapia. On the contrary rainbow trout supplemented with cottonseed meal have not recorded any quercitin in their plasma, indicating that it is not available to them. Whether tilapia have an increased ability to absorb quercitin has yet to be studied.

1.4.4 Canola

Produced by rapeseed cultivars canola comes from crops bred to have low levels of eurcic acid and glucosinolates (anti-nutritional factors for most fish). The primary product of canola crops is canola oil, similarly to soybeans the by-products left after oil extraction can be turned into a meal which can also be further purified into a protein concentrate. The basic canola meal has crude protein levels of 35% whereas the canola protein concentrate reaches protein levels more similar to high quality fish meals, around 65-70%.

There are the familiar problems with canola based fish diets as with soybean based diets: the majority of the phosphorus and the divalent minerals can be locked into the phytic acid; and there are limiting EAAs. A typical canola meal contains about 4% phytic acid which will probably increase as the protein is purified to form the protein concentrate. Methionine and lysine are also the limiting EAAs as with many of possible plant protein sources. In addition to these usual problems there is the added presence of toxic eurcic acid and glucosinolates. Eurcic acid is a cardiotoxin, causing heart lesions in rats at low levels of inclusion. It does however seem that the solvent extraction of the oil and the pressing of the seeds reduces the levels of eurcic acid below the levels causing pathologies in fish.

Glucosinolates are thyrotoxins, inhibiting the uptake of iodine leading to iodine deficiencies even when additional iodine is supplemented. Fortunately, as mentioned above, canola is a specifically bred strain of rapeseed developed for its low levels of glucosinolates. Standard rapeseed meal would contain ~5% glucosinolates but canola meal contains <0.2%, at such low levels no pathological effects are seen in fish.

1.5 Rationale

The rationale for this study has been derived from the four sections above, in summary they are:

1. There is an ever increasing need to improve the use of the natural resources wastefully used in aquaculture at the present time
2. Zinc needs to be focused on because of its biological importance in a vast number systems regulating fish health and performance and also its potential environmental impact if used in excess

3. The formulation of practical diets using proteins from a growing list of sources, alien to the natural diet of a salmonid species, brings with it a plethora of complications and interactions between the nutrients provided by them. A deeper understanding of these interactions is required
4. The fundamental research behind the bioavailability of minerals is confused and contradictory. Very few studies, if any, use the genetically coded transcriptome of an animal combined with analysis of the synthesised end products to quantify its requirements. The use of which may add some clarity to this area of research.

1.6 Study objectives

This PhD thesis examines three possible methods for improving the digestibility and bioavailability of zinc from salmonid diets.

1. The utilisation of other protein sources (Chapter 3);
2. The upstream use of enzyme treatment of the raw materials (Chapter 4);
3. The use of organically complexed mineral supplements as opposed to the inorganic salts widely used at present (Chapter 5).

Throughout this study the biological availability of these supplements was investigated and how the various forms are utilised by different tissues was assessed. In the later chapters emphasis was placed on defining the minerals availability by their biological function within the proteins they are required for. This includes quantifying the amount of the active protein and comparing this to its coding in the transcriptome. A direct comparison between the uptake of an organic and an inorganic form by the use of stable isotope labelled compounds has also been made.

CHAPTER 2: General Methodologies

2.1 Overview

The analytical procedures outlined in the following section were the fundamental techniques undertaken throughout the present study. Diet formulation and individual experimental designs unique to particular trials are described in the relevant experimental chapters. Unless otherwise stated, all materials, chemicals and reagents were sourced from Fisher Scientific Ltd. (Loughborough, Leicestershire, UK) and Sigma-Aldrich Ltd. (Poole, Dorset, UK). All experimental work involving fish was carried out under the Home Office project licence # 30/2135 and personal license # 30/8705.

2.1.1 Rearing Facilities

All experimental trials were conducted at the University of Plymouth within a freshwater recirculation system, number 'F', part of the Aquaculture and Fish Nutrition Research Groups Aquarium (see fig 2.1.). The water quality parameters of a recirculation system, in order to provide a suitable environment for the rearing of fish, must be maintained daily and kept within the optimal ranges for the rearing of trout. The following levels of nitrogenous compounds were considered acceptable; ammonia (un-ionized) <0.1 mg l^{-1}, nitrite < 1.0 mg l^{-1} and nitrate < 50 mg l^{-1}. Water was tested for nitrogenous compounds on a twice weekly basis using a Hach Lange DR 2800 and coded cuvettes for ammonia (Lange LCK 304), nitrite (Lange LCK 341) and nitrate (Lange 340) (Hach Lange Ltd., Salford, UK). Partial water exchanges were used, if required, to control the levels of the nitrogenous compounds.

Figure 2.1. Recirculation system 'F', University of Plymouth. Letters correspond to (A) Outgoing water to chiller; (B) incoming water from carbon filter and chiller; (C) Sump, containing bio-media for bacterial filter; (D) Drum filter; (E) Waste water pipe. Arrows indicate the direction of flow from experimental tanks to drum filter.

Temperature was maintained at 15.1 ± 1 °C with a thermostatically controlled chiller (Optipac R407C, PSA, Saint Barthelemy, D'Anjou, France). Dissolved oxygen levels, pH, and temperature were monitored daily using an electronic meter (Hach HQ4d). There is a natural decrease of pH in a recirculation system caused by the nitrification consuming OH^- ions and produces acids, and also the carbon dioxide produced by fish forms carbonic acid (H_2CO_3) in the aqueous environment. The pH of recirculating water was maintained between 6.5 and 8.0 with the addition of sodium bicarbonate ($NaHCO_3$) as required. The saturation of dissolved oxygen was maintained above 80 %, for both fish and bio-filter (Masser et al.,

1999), by a continual supply of compressed air (compressor: Rietschle, UK) delivered via air-stones within each tank and a perforated pipe to the sump.

The system (tanks, sump and pipework) had a total volume of ~6000 l. Twenty experimental 130 l fibreglass tanks each received water at a rate of ~800 l h^{-1}. A 12 h light/dark photoperiod was maintained throughout all experimental trials with an automated T5 lighting system. A continual top up at 10 l h^{-1} of municipal water, treated by an activated carbon filter (Commandomatic TCF, Waterco Ltd., Sittingbourne, Kent, UK) to remove chlorine and organic compounds, was used to maintain water volume and counteract any loss from evaporation.

The mechanical filtration, used to remove particulate material arising from faecal material, undigested feed and sloughed bacteria from the bio-filter was provided by a rotating drum screen filter (Aquasonic DF100, Aquasonic Ltd, Wauchop, Australia).

2.1.2 Experimental Fish

All experimental work was carried out on juvenile all female triploid (XXX) rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) obtained from Torr Fisheries (North Devon, UK). Fish were transported directly from the fishery to the aquarium facility in a 1000 l tank supplied with pure oxygen (BOC, UK); transport time was approximately two hours. On arrival fish were gradually acclimated to the temperature of the aquarium facility over a period of one hour. All fish were checked on arrival, monitored daily and fed *ad libitum* a commercial trout diet (EWOS, West Lothian, UK) for a period of at least three weeks until graded into experimental tanks. Fish were randomly allocated into the experimental tanks 2-3 days prior to commencing the experimental trial. Anaesthetisation of fish was carried out

within Home Office procedures with tricane methanesulfonate (MS-222) (Pharmaq Ltd. Fordingbridge, Hampshire, UK) at a dose rate of 80 mg l^{-1}, buffered with sodium bicarbonate until the pH was equal to the system water.

2.1.3 Feed and Weighing

Feeds were formulated using FeedSoft Pro™ which requires the input of the nutrient profile of each raw material. For this all the raw materials were analysed for moisture, oil, protein and ash. Calcium, phosphorus, sodium, potassium, magnesium, manganese, copper, zinc, sulphur and iron were also analysed and data is presented in the appendices (sections 8.1 and 8.2)

Feed was calculated as a percentage of the total biomass in each tank. The biomass was taken at $t = 0$ and throughout the trials every two weeks. Within this two week period the feed input was adjusted daily, based on a predicted feed conversion ratio (FCR) of 1. The fish were fed two equal rations twice daily at ~09:00 and ~18:00 hours. Feed input was adjusted accordingly in the event of any mortality.

2.1.4 Growth and Feed Performance

Growth performance and feed utilisation was assessed by specific growth rate (SGR) and FCR. Calculations were as follows:

$$SGR = ((Ln\ final\ wt.\ (g) - Ln\ initial\ wt.\ (g)) / (Days\ fed)) \times 100$$

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

(Ln is the natural log)

2.2 Proximate Analysis

Diets and carcass were analysed for moisture, ash, and lipid. All diets were ground by use of a high speed blender and analysed on a wet weight basis. Analysis was conducted in triplicate according to protocols of the AOAC (1990) which are outlined below.

2.2.1 Moisture

Percentage moisture was calculated using:

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

Diets and carcasses (in triplicate, whole with peritoneal cavity opened) were weighed and dried at 105°C with a fan-assisted oven (Genlab Ltd., UK) until a constant weight was achieved. Percentage moisture was then determined.

2.2.2 Ash

Percentage ash was calculated using:

$$Ash\ (\%) = Sample\ residue\ (g) / initial\ sample\ wt.\ (g) \times 100$$

Ash content was determined in triplicate by adding approximately 500 mg to a pre-weighed ceramic crucible. The samples were then incinerated in a muffle furnace (Carbolite, Sheffield, UK) at 550°C for 12 hours. After cooling in a desiccator, percentage ash was determined.

2.2.3 Lipid

Percentage lipid was calculated using:

$$Total\ lipid\ (\%) = ((cup\ (incl.\ lipid)\ wt.\ (g) - cup\ wt.\ (g)) / (initial\ sample\ wt.\ (g))) \times 100$$

Lipid content was determined in triplicate using the Soxhlet extraction method. Approximately 3 g of the diet was added to a cellulose thimble with a cotton wool bung and placed in the thimble holder suspended over a beaker of known weight containing 40 ml of petroleum ether and inserted into the condenser. The condenser was raised into the 'rinsing' position on the SoxTec™ extraction system (Tecator Systems, Högnäs, Sweden; model 1043 and service unit 1046). The beakers, petroleum ether and thimbles were then clamped into the condensers and the unit set to 'boiling' for 30 min, after which the unit was set to 'rinsing' for 45 min to wash out all lipid present in the samples. The petroleum ether was then evaporated and the beakers containing extracted lipid were then transferred to a fume cupboard, cooled for 30 min and weighed. Total lipid content was then determined.

2.2.4 Protein

Determination of total crude protein in diets, feed ingredients and carcasses was measured in triplicate by the Kjeldhal method. The Kjeldahl method measures protein from the total nitrogen content of samples. The nitrogen content is then multiplied by a factor of 6.26 (5.71 for proteins of plant origin) to calculate apparent protein content (AOAC, 1990). In brief, ~100 mg of sample was weighed directly into a micro Kjeldahl tube along with one catalyst tablet (3g K_2SO_4, 105 mg $CuSO_4$ and 105 mg TiO_2; BDH Ltd., Poole, UK), 10 ml concentrated sulphuric acid (H_2SO_4) (Sp. Gr. 1.84, BDH Ltd. Poole, UK) was added and digestion was performed with a Gerhardt Kjeldatherm digestion block (Gerhardt Laboratory Instruments, Bonn, Germany). The thermo-cycle schedule followed; 30 min at 100 °C, 45 min (1 h if samples had high lipid content) at 225 °C, and 1 h at 380 °C. Once digestion was complete and following a cooling period, the samples were distilled into excess boric acid to form an ammonium borate complex using a Vapodest 40 automatic distillation unit (Gerhardt

Laboratory Instruments, Bonn, Germany), the distillate was neutralised with concentrated H_2SO_4 and the titration value obtained. The efficiency of the nitrogen recovery was also measured by including triplicate samples of acetanilide which has a known nitrogen content of 10.36 and sample (and blanks) nitrogen contents were corrected for the efficiency prior to use in the calculation. Analyses with efficiencies of less than 95% were discarded and repeated.

Percentage protein was calculated using:

$$Crude\ protein\ (\%) = (((T_s - T_b) \times N_A \times MW_N \times CF) / SW) \times 100$$

Where T_s *= sample titration volume (ml),* T_b *= blank titration volume (ml) and SW = sample weight (mg).* N_A *= the acid normality of the acid used (*H_2SO_4*=0.20)* MW_N *= the atomic weight of nitrogen (1400.67mg) and CF = the conversion factor of nitrogen to protein (6.25 animal and maize/ 5.71 plant).*

2.3 Trace Element Analysis

2.3.1 Digestion

All trace element analysis was carried out in triplicate on nitric acid digested samples. For the analysis of individual tissues, samples were freeze dried (Super Modulyo freeze-drier; Girovac, UK) and percentage moisture determined from initial wet weight and final dry weight. For the analysis of the whole body, samples were oven dried at 105ºC and percentage moisture determined (see: section *2.2.1 Moisture*). Dietary ingredients were analysed by wet weight. All the samples were homogenised using a household blender or pestle and mortar prior to digestion. Approximately 100 mg sample was weighed directly into each micro

Kjeheldahl tube and digested in 10 ml nitric acid (70 % ANALAR grade). Samples were digested using a Gerhardt Kjeldatherm 40 tube digestion block (Gerhardt Laboratory Instruments, Bonn, Germany) and the following thermo-cycle was used; 1h at 60 °C, 1h at 90 °C, 30 min at 110 °C, and up to 4h at 135 °C. After a cooling period of 1h, samples were then transferred into 50 ml polypropylene vials and diluted to ~ 50 ml with ultra-pure Milli-Q water (Millipore Corp., MA, USA). Duplicate blanks comprising of only nitric acid and hydrogen peroxide were also analysed in each digestion run.

2.3.2 ICP OES/MS

All minerals were analysed by Inductively Coupled Plasma Optical Emissions Spectrometry (ICP OES) (Varian 725-ES OES spectrometer, Varian Inc. CA, USA) using the following wavelengths for each element (nm); Ca 422.67, Cu 327.39, Fe 238.20, K 766.49, Mg 285.21, Mn 257.61, Na 589.59, P 213.61, S 181.97, Y 371.03 and Zn 213.857. For samples containing Y (as an inert internal marker of digestion), a 1 ppm internal indium (In) standard was added to all sample digests (measured at 230.61nm). Zinc isotopes (66, 67, 68 and 70) were analysed by high resolution Inductively Coupled Plasma Mass Spectrometry (ICP-MS) (VG Axiom sector field ICP-MS, VG Elemental, Winsford, UK). Zinc isotope 64 was not measured because of the inevitable polyatomic interference of $^{32}S_2$ molecules being measured as 64. Germanium 72 was also analysed in order to prove the absence of Germanium from the samples as germanium also has an isotope of 70 which would lead to the misinterpretation of ^{70}Zn isotope ratios. Mass bias calculations were applied to all results prior to ratio calculations to eliminate the instrumental artefact associated with the detection of lighter isotopes.

The ICP -OES operating conditions were as follows:

Power (KW)	1.40
Plasma Flow (L/min)	15.00
Auxilliary Flow (L/min)	1.50
Nebulizer Flow (L/min)	0.68
Veiwing Height (mm)	8.00
Replicate Read Time (s)	2.00

The ICP-MS operating conditions were as follows:

Power (KW)	1.40
Plasma Flow (L/min)	13.00
Auxiliary Flow (L/min)	0.70
Nebuliser gas flow (L/min)	0.86
Collision cell gas flow (mL/min)	3.50
Collision cell gas	7% hydrogen in helium

Concentrations of each element in the digests were determined in parts per million (ppm) against an external standard calibration and concentrations in original samples calculated using the following calculation:

$$\textit{Sample conc. (ppm)} = \frac{\textit{digest vol. (ml)}}{\textit{sample wt. (g)}} \times \textit{digest conc. (ppm)}$$

2.3.3 Digestibility and Retention

The determination of net apparent digestibility (ADC) of trace elements and protein, the inert marker yttrium oxide was added to diets at 1 g kg^{-1} yttrium oxide as recommended by Ward

et al. (2005). Under light anaesthetic, faecal material was stripped from every fish in each experimental tank by hand. Pressure was gently applied on the hind portion of the peritoneal cavity. After one sample was taken the fish were allowed to recover for at least four days during which they were fed *ad libitum* before being sampled again. Faecal material was pooled for each replicate tank, dried at 105°C for 24 hours, and homogenised prior to trace element analysis. Percentage apparent digestibility was determined from the faecal and dietary concentrations of the element or protein relative to reciprocal concentrations of Y using the following equation (Paripatananont and Lovell, 1997):

Apparent digestibility coefficient (ADC) (%)

$$= 100 \times (1 - ([^{Y}\ diet] \times [^{Element}\ faeces]) / ([^{Y}\ faeces] \times [^{Element}\ diet]))$$

Where [^{Y} diet]is the concentration of yttrium in the diet, [^{Y} faeces]is the concentration of Yttrium in the faeces, [^{Element} diet] is the concentration of the element in the diet and [^{Element} faeces] is the concentration of the element in the faeces

For the determination of the digestibility from individual ingredients with in the diet the ADCs were incorporated into the following equation:

$$ADC_{Ing}\ (\%) = [ADC_{test} - (1\text{-}i) \times ADC_{ref}]/i$$

Where *i* is the proportion of the mineral supplied by the test ingredient.

Nutrient retention was determined from dietary and whole body element concentration relative to feed and element intake concentration by the following equation:

Element retention (%)

$$= 100 \times \frac{(final\ body\ wt. \times [final\ whole\ body\ nutrient] - initial\ body\ wt. \times [initial\ whole\ body\ nutrient])}{(Feed\ consumed \times [dietary\ nutrient]))}$$

Where [final whole body nutrient] is the concentration of the nutrient in the whole body at the end of the trial, [initial whole body nutrient] is the concentration of the nutrient in the whole body at the start of the trial and [dietary nutrient] is the concentration of the nutrient in the feed.

2.4 Determination of Zinc-Dependent Enzymes

Activities of zinc dependent enzymes were assayed in triplicate and normalised to sample protein concentration or tissue weight in the case of metallothionein.

2.4.1 Protein

Sample protein in homogenates was determined by the method as described by Smith et al. (1985), in which a spectrophotometric bicinchoninic acid (BCA (Kit: Sigma; BCA1-1KT) is used. The assay uses a copper/protein complex that forms relative to the amount of protein present. Tissue homogenates were diluted 1:10 with distilled water. 1 part reagent A (copper (II) sulphate) was mixed with 50 parts reagent B (BCA solution). 25µl of sample homogenate was added to 200 µl reagent mixture in a clear 96 well microplate. The plate was then incubated for 30 min at 37 °C (Heraeus, Kendo Laboratory Products, Hanau, Germany) before being read at 550 nm (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA). Sample protein was determined in mg/ml against a standard curve of bovine serum albumin (BSA).

$$\textit{Sample protein (mg ml}^{-1}\textit{)} = ((A_{550}\ \textit{change over 30 min)} \times \textit{(slope of absorbance vs. mg protein)} - \textit{intercept of absorbance vs. mg protein)} \times \textit{dilution factor}$$

2.4.2 Superoxide Dismutase

A Fluka SOD determination kit (Kit 19169, Sigma, Poole, UK) was used to measure hepatic superoxide dismutase (SOD). The reaction was based on the inhibition of the formation of 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1). Liver samples were homogenised in 1 in 9 in 0.25 M sucrose buffer adjusted to pH 7.4 (0.25 M sucrose, 10 mM Tris-BASE, 1 mM EDTA) and further diluted 1 in 20 with buffer, centrifuged at 10,000 g for 15 min at 4 °C and the assay was performed according to the manufacturer's instructions. Briefly, 20 µl of sample was added to each plate well. 200 µl WST working solution and 20 µl of enzyme working solution were added and the plate mixed thoroughly. The plate was then incubated at 37 °C (Heraeus, Kendo Laboratory Products, Hanau, Germany) for 20 min and then read at 450 nm with a microplate reader (Optimax Tuneable Microplate Reader, Molecular Devices, CA, USA).

SOD activity (% inhibition rate) was determined by the following calculation:

$$SOD\ activity = \frac{((Abs\ blank\ 1 - Abs\ blank\ 2) - Abs\ sample)}{(Abs\ blank\ 1 - Abs\ blank\ 2))} \times 100$$

Where; 'Abs blank 1' is the absorbance of a blank where sample is substituted for distilled water; 'Abs blank 2' is the absorbance of a blank which includes enzyme dilution buffer instead of enzyme working solution. 'Abs sample' is the absorbance of the sample.

Specific enzyme activity (unit SOD) was determined from a standard curve of units SOD vs. % inhibition and normalized to sample protein (mg).

Units SOD/ mg protein =

((% inhibition × slope of % inhibition vs. units SOD) - intercept of % inhibition vs. units SOD) / mg sample protein

2.4.3 Metallothionein

Tissue levels of metallothionein were measured using the silver saturation assay (Scheuhammer and Cherian, 1991). Silver is used as it has a higher affinity for the protein thiols than any other metals found in association with metallothionein. It displaces all other metals bound to the protein and binds itself to the thiols in their place (as well as to cytosolic proteins and ligands). The addition of haemoglobin will then bind to any excess silver and take the silver of all other ligands apart from metallothionein. Heating then causes the haemoglobin bound silver to precipitate leaving only the heat stable metallothionein bound silver in the supernatant, which can subsequently be measured with ICP OES and the concentration of silver in µg can be converted to concentration of metallothionein by using:

$$\mu g\ MT/g\ tissue = (([Ag^{Sup}] - [Ag^{Blank}]) \times 3.55 \times V_T \times DF) / S_V$$

where $[Ag^{Sup}]$ = concentration of silver in the final supernatant, $[Ag^{Blank}]$ = concentration of silver in a blank, 3.55 = the ratio of silver to metallothionein, V_T = the total volume of the assay sample, DF is the sample dilution factor and S_V = Sample volume.

2.4.3.1 Sample preparation

Livers were removed from the fish and halved, each half was frozen separately; one for the determination of metallothionein concentration and one for the metallothionein gene expression analysis (see section 2.6). Approximately 350mg of the frozen tissue was homogenised in 4 volumes of 0.25M sucrose solution. The homogenate was centrifuged at 18,000g for 20 min and the resulting supernatant removed and frozen at -80 until required for metallothionein determination.

2.4.3.2 Haemolysate preparation

A red blood cell haemolysate was prepared according to Onosaka and Cherian (1982). Briefly, 10ml of mammalian blood was added to 20ml of heparinised isotonic KCl (1.15%, w/v) and centrifuged a 500g for 5min. The supernatant was discarded and the pellet resuspended in 30ml of the heparinised 1.15 KCl solution. The centrifugation/resuspension was repeated twice and the final pellet was lysed for 10 min in 15ml of Tris base buffer (30mM, pH 8.0) at 20ºC. The lysate was centrifuged at 8000g for 10min and the supernatant (red blood cell haemolysate) was divided into 2ml aliquots and frozen at -80ºC until required.

2.4.3.3 Assay procedure

The frozen tissue supernatant was thawed and 250 µl was added to an acid washed glass test tube. Triplicate samples were taken from each liver homogenate supernatant. 550 µl of glycine buffer (0.5M, pH 8.5 adjusted with NaOH). A $AgNO_3$ stock solution (9.27 mM; 1000µg Ag^+/ml) was diluted to a 20 µg/ml Ag^+ working solution and 500 µl was added to

glycine/supernatant mix and incubated at 20ºC for 5 min. 100µl of the haemolysate was added and mixed on a vortex mixer the heated in a boiling water bath for 1.5 min then centrifuged at 1200g for 5 min. The addition of 100 µl haemolysate, the heating and the centrifuge were repeated once more making the total volume 1.5 ml. The resulting clear supernatant was removed and added to another acid washed centrifuge tube and spun at 15,000g for 5 min. The final supernatant was analysed for silver concentration using ICP OES (328.07 nm) and the concentration per g of liver calculated.

2.5 Single Cell Gel Electrophoresis

2.5.1 Sample preparation

The SCGE or 'Comet' assay Single can identify strand breaks and oxidised nucleotides (Singh et al. 1988) in erythrocytes. 100µl samples of whole blood were mixed with 900 µl freezing medium (20 % (v/v) bovine serum albumin (Invotrogen, Gibco, Paisley, UK), 40 % (v/v) RPMI-1640 medium (Sigma, Poole, UK) and 20 % DMSO (v/v) for cryopreservation at -80 °C.

2.5.2 Assay procedure

Microscope slides were coated with 1.5 % normal melting agarose (NMA) and left to air dry. Whole blood/freezing medium was further diluted 1 in 5000 with $CaCl_2$ and $MgCl_2$-free DPBS physiological saline solution (Gibco, Invitrogen, Paisley, UK) to give a cell count of approximately $2.4x10^5$ cells ml^{-1}. Cells were resuspended in 0.75 % low melting agarose (LMA). In duplicate, two 85 µl spots of the cell/agarose suspension (four spots per sample, five fish per tank, three tanks per treatment) were placed on to each slide, covered with 22

mm^2 cover slips and left to set at 4 °C for one hour. Once the gel had set, the cover slips were then removed and slides were immersed in lysis solution (2.5 mM NaCl, 100 mM Na_2EDTA, 10 mM tris-BASE, 10 % DMSO, 1 % Triton X-100, 1 % *n*-lauroyl-sarcosinate and adjusted with NaOH to pH 10) for one hour. Slides were then placed in a horizontal electrophoresis chamber in electrophoresis buffer (0.3 M NaOH and 1 mM EDTA) to a level <1 mm above gels and DNA left to unwind for 10 min. Electrophoresis was performed for 20 min using a current of 300 mA and 25 V (Apelex ST 1006T electrophoresis unit). After electrophoresis slides were washed in neutralisation buffer (0.4 M Tris-chloride pH adjusted to 7.5 with HCl) for 10 min followed by distilled water for 10 min. Slides were then left to air dry for 24 hours (to align DNA on the same focal plane for ease of scoring) before staining with 80 µl 2 µg ml^{-1} ethidium bromide solution and covering with 24/50 mm cover slips,. Stained slides were scored immediately using a fluorescence microscope (Leica DMR) and Komet 5.0 image analysis software (Kinetic Imaging Ltd., Liverpool, UK). Percentage tail DNA, was measured in 50 nucleoids per gel (100 per slide) (Kumaravel & Jha, 2006).

2.6 Gene Expression

2.6.1 RNA extraction

Total RNA was extracted using an RNeasy MiniKit for animal tissue (Qiagen, Manchester, UK). Briefly, approximately 20mg of liver (taken from the other half used for metallothionein analysis) was homogenised by sonication (3-5 sec pulse) on ice in 350 µl RLT buffer. The resulting homogenate was added to a shredder column (QuiShredder, Qiagen) centrifuged for 3 min at 13,000 g and supernatant collected in a DNA free tube and centrifuged again. The final supernatant was mixed with 1 vol 70% ethanol and added to an RNeasy column and centrifuged for 15 sec at 13,000 g. The column was washed with 350 µl

of RW1 buffer. A 15 min DNase treatment was conducted and the column washed again with 350 µl RW1 buffer. Two further washes of 500 µl RPE buffer were carried out and the RNA was eluted into 30 µl RNA free water. The quality and quantity of total RNA was determined by spectrophotometry (Nanodrop, ND-1000 Spectrophotometer) reading of 260/280 nm ratio and 260/230 nm ratio. Only samples with a 260/280 ratio of 1.9-2.2 and 260/230 ratio >1.5 were considered acceptable. All samples were diluted to 100 ng/µl and frozen at -80ºC until reverse transcription into cDNA was to be carried out.

2.6.2 cDNA Synthesis

Reverse transcription of the RNA to cDNA was carried out using ImProm-II™ reverse transcription system (Promega, Southampton, UK). Briefly, 800ng (8 µl of eluted RNA) was added to a working solution of ImProm-II 5X reaction Buffer (0.83X; Promega, UK), $MgCl_2$ (2.5mM; Sigma-Aldrich, UK), deoxynucleotide mix (0.36mM; Sigma-Aldrich, UK), Hexanucleotide primers (0.021 µg/µl; Sigma-Aldrich, UK) and ImProm-II reverse transcriptase (4.17% v/v; Promega, UK) made up with RNA free water. cDNA was synthesised by PCR (GeneAmp® PCR System 9700, Applied Biosystems) with one thermo-cycle of annealing for 5 min at 25ºC, extension for 60 min at 42ºC and heat inactivation for 15min at 70ºC. cDNA was stored at -80ºC until qRT-PCR analysis.

2.6.3 qPCR

Lyophilised primers were obtained (Eurofins MWG Operon, Ebersberg, Germany) and were rehydrated with RNase free water to a stock concentration of 100µmol. Forward and reverse primers were mixed with SYBR Green JumpStart *Taq* ReadMix (20mM tris-HCl, 100mM

KCl, 7mM $MgCl_2$, 0.4mM dNTP mix, 0.05 unit/µl *Taq* polymerase, Jumpstart *Taq* antibody, SYBR Green 1) to give a working solution concentration of 375 nmol. 6 µl of liver cDNA was added to the primer/SYBR Green mix and fluorescence was detected over 40 cycles of PCR using a StepOne Real-Time PCR system (Applied Biosystems, USA). Thermo-cycling conditions were; denaturing at 94ºC for 15 sec, primer specific annealing at 53-55 ºC (table 5.2) for 1min and extension at 72 ºC for 1min with fluorescence detection switch on. The cycle threshold was set at 25,000 for all runs and a standard curve of pooled cDNA was run on each plate for normalisation between each plate run.

2.7 Statistics

All statistical analysis was carried out using SPSS for Windows versions 17-20. Unless otherwise stated all means are reported ± standard error of the mean (SEM) or pooled SEM. Prior to analysis all data was tested for homogeneity of variance (Levene test) and normality of distribution (Kolmogorov-Smirnov test). Differences across treatments were modelled by one-way ANOVA and where appropriate, either a *post hoc* Fisher's least significant difference (LSD) test or a Tukey's HSD range test was performed to make pair wise comparisons between individual treatments. Data violating the assumptions of parametric tests (SCGE data) after log transformation were tested with the equivalent non-parametric Kruskal-Wallis and Mann-Whitney *U* tests. All percentage data was arcsine transformed prior to analysis. In instances where n=2 due to restrictions on tank space (chapter 3), to allow statistical evaluation, measurements used in the statistical analyses were taken from individual fish as opposed to whole tank averages. This method, although not allowing for tank effects, was necessary to allow some meaningful comparisons between treatments to be made. Unless otherwise stated, differences were considered significant at a value of $p \leq 0.05$.

CHAPTER 3. Digestibility and bioavailability of endogenous zinc in potential alternative protein sources

3.1 Introduction

The inclusion of plant protein, terrestrial animal protein and co-products of fermentation processes into fin fish feeds is of great interest to the aquaculture industry (Alexis 1997; Alexix and Nengas, 2001; Espe et al 2006; FAO 2002; Kaushik et al, 1995; Watanabe et al, 1998). Extensive research in this area has been driven by feed manufacturers, wanting to reduce the increasingly expensive fishmeal in their diets, and by producers of 'alternative' protein sources who now find that their products and by-products are economically viable as fishmeal increases in price (New and Wijkstrom, 2002; Tacon and Metian, 2008). Public perception and environmental issues with regard to overfishing wild stocks are also applying further incentive to reduce the amount of fishmeal in carnivorous fish species diets (Naylor 2000). However, fishmeal in these diets cannot simply be replaced by substituting a product with an equal protein concentration. Each protein source will have its own amino acid profile, the deficiencies of which are well documented in the literature (Aslaksen et al 2007; NRC 1993; NRC 2011) as are the feed formulation methods of avoiding these deficiencies (Barrows et al, 2007; Barrows et al 2008; Gatlin et al 2007). Each protein source also has its own, specific, mineral profile. For the majority of products, these profiles, on a weight to weight basis, often exceed the published requirements for salmonids (NRC 2011). However, running a laboratory based analysis of the product does not fully provide insight into the actual biological availability of the minerals. Many plant proteins and animal proteins derived from trimmings contain anti-nutritional factors (ANFs) which further reduce the biological availability of the minerals (Gatlin et al 2007). These factors should be taken into account when formulating diets but there is currently limited information about the

biologically available levels of minerals in different protein sources, and what information there is has often been calculated using different methods.

Zinc is a mineral of great biological importance to fish, second only to iron (Kiron 2012; Lall 2002; Watanabe et al 1997). Zinc is responsible for the formation, structure and activity of over 300 proteins involved DNA repair (Ho and Ames, 2002), antioxidant activity (Luo et al, 2011), eye function (Nusetti et al 2010), and immune function (Lall, 2002). The dietary requirement of zinc for rainbow trout is published as 15-30 mg/kg (Ogino and Yang, 1979), Atlantic salmon 37 mg/kg (Maage and Julshamn, 1993), hybrid tilapia 26-29 mg/kg (Lin et al 2008) and common carp 15-30 mg/kg (Ogino and Yang, 1979). The average zinc concentration in high quality fishmeal is 64 mg/kg, soyabean meal 49 mg/kg, maize gluten meal 16 mg/kg, poultry meat meal 76 mg/kg, low quality white fishmeal 129 mg/kg and yeast 146 mg/kg. Diets containing 65 mg Zn/kg fed to Atlantic salmon could not maintain normal whole body zinc status and required additional zinc supplementation up to 140 mg Zn/kg (Lorentzen and Maage, 1999). Rainbow trout fed a low quality white fishmeal diet required an additional 40 mg Zn/kg supplementation to maintain normal growth (Satoh et al, 1987) and when fed a soyabean based diet required 150 mg Zn/kg to be supplemented.

Despite the quantity of material published concerning the zinc supplementation requirements and availability of minerals from frequently used protein sources there is very little consistency in the literature regarding experimental designs or the interpretation of results.

The first criticism is that zinc transport, including its uptake, is very highly regulated in fish. Dietary levels of 1700 mg Zn/kg diet has no effect on the whole body zinc content of rainbow trout (Wekell et al, 1983) indicating zinc uptake conforms to a non-toxic saturation model and the excess dietary zinc is not absorbed from the GI tract. With the knowledge of both this highly regulated uptake and the relatively low zinc requirements why the majority of zinc

digestibility studies conducted with rainbow trout still use zinc levels multiple times higher than the animals' requirements is unclear.

A second criticism of the literature on this topic is that few papers use the same end point or marker of zinc status. Maage and Julshamn (1993) use whole body zinc, Hernandez et al (2012) used growth parameters, and Rider et al (2010) used tissue zinc levels. This lack of focus on one consistent endpoint may explain many seemingly contradictory reports.

The final criticism of the literature is of the use of the equation to calculate the apparent digestibility coefficient (ADC) for minerals from individual protein sources (section 2.3.3). It seems common practice to use the proportion of the test protein source incorporated in the diet as '*i*' in the equation even when the proportion of the mineral supplied by the test protein source is different to the proportion of the protein supplied.

The aim of this experiment is to calculate the ADC for zinc from six commonly used protein sources by a) using diets that do not meet the zinc requirements of rainbow trout, this will ensure maximum uptake of digestible zinc; and b) using diets that are formulated to have 60% of the zinc supplied by the test protein source. This investigation will also establish markers of zinc deficiency (as opposed to indicators of zinc transport) by measuring the effect of chronic zinc deficiency on the metabolic and exchangeable/storage zinc pools throughout the body.

3.2 Methods

3.2.1. Experimental design

Two separate feeding trials were conducted with a reference casein based diet and six subsequent test diets containing either maize gluten meal (MGM), soyabean meal (SBM), poultry meatmeal (PMM), high quality brown fishmeal (LT94), low quality white fishmeal (WFM) or NuPro® (NP). All ingredients were analysed for protein, lipid, ash, moisture and mineral composition (see 8.1 and 8.2). Diets were formulated to be isonitrogenous, isolipidic and have 60% of the zinc supplied by the test protein source and be deficient in zinc (Table 3.1).

Trial 1: Digestibility

A total of 210 rainbow trout (*O.mykiss*) weighing 300 ±15.8g were divided into 14 x 126 litre tanks (15 fish per tank). Fish were acclimated for two weeks and fed an EWOS commercial diet (43% protein, 24.9% lipid) at 1% biomass per day. Each diet was randomly assigned to two tanks. Experimental diets were fed for two weeks at 2% body weight per day, followed by one week being fed to apparent satiation twice daily (09:00 and 18:00) leading up to sampling in week 4.

Trial 2: Bioavailability

A total of 480 rainbow trout (*O.mykiss*) weighing 40 ± 3.5g were divided into 16 x 126 litre tanks (15 fish per tank). Fish were acclimated for two weeks and fed an EWOS commercial diet (43% protein, 24.9% lipid) at 1% biomass per day. Each diet was randomly assigned to two tanks. Experimental diets were fed for five weeks at 3% body

Table 3,1 Diet composition (%), Protein and Lipid content (%) and final zinc concentration (mg/kg)

	Ref.	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6
Casein	44.0	0.1	17.5	21.0	17.8	28.0	33.5
Maize Gluten Meal	-	60.3	-	-	-	-	-
Soyabean Meal	-	-	41.5	-	-	-	-
Poultry Meatmeal	-	-	-	31.0	-	-	-
LT94 Fishmeal	10.0	10.0	10.0	10.0	44.0	10.0	10.0
Provimi 66 Fishmeal	-	-	-	-	-	21.5	-
NuPro	-	-	-	-	-	-	21.5
Starch	26.9	10.5	11.9	18.9	19.1	21.4	15.9
Fish Oil	13.0	13.0	13.0	13.0	13.0	13.0	13.0
Squid Attractant	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Vit/Min Premix (Zn Free)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Protein (%)	46.8	47.2	47.4	47.2	47.3	46.9	47.4
Lipid (%)	13.4	13.2	13.2	12.8	13.1	13.0	13.4
Zinc (mg/kg)	23.1	18.3	22.2	26.2	25.7	33.4	37.6

weight per day (three feeds per day at 09:00, 13:00 and 18:00). The EWOS commercial diet was fed in addition to the experimental diets. The fish were required to double in weight before tissue sampling could begin.

3.2.2. Sampling regime

Trial 1

Faecal sampling for digestibility commenced in week 4 of the trial. Faeces were collected by stripping the fish by applying gentle manual pressure on the posterior section of the GI tract after the fish had been sedated in 40 mg/l MS 222 (Pharmaq, UK). The faeces were collected in aluminium trays and dried for 24 hours at 105ºC. The samples from each tank were pooled and ground into a fine powder for protein and mineral analysis.

Trial 2

The initial sampling was conducted prior to the fish being fed the experimental diets and 16 fish were sampled (one per tank). The fish were pooled, eight fish were used for whole body analysis and eight fish were dissected for their liver, muscle, fin, bone (vertebra), eye, and mid-intestine. Serum and bile were also collected.

Once all fish had doubled in weight the liver, muscle, fin, bone (vertebra), eye, mid-intestine, serum and bile were collected. Four fish from each tank were anaesthetised using 120 mg/l MS 222 (Pharmaq, UK). Serum was obtained by the collection of 1 ml of blood from the caudal vein which was left to clot overnight at 4ºC. The sample was then spun at 1800 g for 5 min, the supernatant removed and then spun again at 1800 g for 2 min. The serum was then immediately digested in nitric acid ready for mineral analysis.

After the blood had been sampled from the anaesthetised fish it was killed by destruction of the brain (schedule 1 method, Home Office). The tissues were removed. The intestines were rinsed in distilled water to remove any faecal matter and along with the other tissues were freeze dried (Edwards Supermodulyo Model 12K West Sussex, UK: vacuum chamber evacuated to 08 x 10^{-3} mbar, 48hrs condensation trap @ -50ºC). The four samples from each tank were pooled and ground down into a fine powder for the nitric acid digest and mineral analysis.

The bile was collected by holding the intact gall bladder over a 2ml centrifuge tube and pierced with a scalpel blade. The bile was frozen at -80°C and later thawed and digested for mineral analysis.

Four fish were also removed from each tank, anaesthetised with MS 222 and killed by destruction of the brain (schedule 1 death, Home Office) for whole body analysis.

3.2.3. Analytical Procedures

Protein, lipid, ash and moisture content were determined in each diet (section 2.2), ICP-OES was used to perform trace element analysis on all diets (table 3.1), faeces and tissues (section 2.3). Levels of Ca, Cu, Fe, K, Mg, Mn, Na, P, S and Zn were analysed for their digestibility and tissue levels and Y was analysed as an indigestible dietary marker for use in the ADC calculations.

3.2.4. Calculations

The ADCs of dry matter, crude protein and minerals were calculated as the fraction net absorption of nutrients from diets based on yttrium as a non-absorbable indicator (Austreng et al.,2000). The ADCs of dry matter was first calculated, using the formula:

$$ADC_{DM}\,(\%) = 100 \times [1-(\text{conc. of yttrium in diet}/\ \text{conc. of yttrium in faeces})]$$

This was further expanded to calculate the ADCs for minerals in the diet by multiplying the (conc. of yttrium in diet/ conc. of yttrium in faeces) by (mineral concentration in the faeces/mineral concentration in the diet):

$$ADC_{diet}\,(\%) = 100 \times [1-(\text{conc. of yttrium in diet}/\ \text{conc. of yttrium in faeces}) \times (\text{mineral concentration in the faeces/mineral concentration in the diet})]$$

The ADCs for the nutrients in the test ingredients were calculated by incorporating the percentage of each mineral supplied by the alternative protein source:

$$ADC_{Ing}\ (\%) = [ADC_{test} - (1\text{-}i) \times ADC_{ref}]/i$$

where ADC_{Ing} is the ADC of the alternative protein source; ADC_{test} and ADC_{ref} are the ADCs for the experimental and reference diets respectively; and *i* is the proportion of the mineral supplied by the test ingredient.

The SGR was calculated as:

SGR = 100 x (Ln. final body weight – Ln. initial body weight)/no. days in trial

The FCR was calculated as:

FCR = feed intake (g)/body weight gain (g)

The net retention of zinc was calculated as:

100 x (amount of Zn fed (g)/amount of whole body zinc gained (g))

3.2.5. Statistics

Statistical analysis was performed using analysis of variance. A Tukeys multiple range test was applied to rank significantly different means. Computer software, SPSS v17 was used to perform the statistical calculations; $p<0.05$ was considered statistically significant for all analyses. Where n=2 individual fish measurements were used for statistical analysis.

3.3 Results

3.3.1 Apparent Digestibility Coefficients

Table 3.2 displays the ADCs for Zn, Ca, Cu, Fe, K, Mg, Mn, Na, P, and S from the casein reference diet and the 6 test protein sources.

The apparent digestibility coefficients (ADCs) for zinc from the test ingredient range from 15.1% in LT 94 fishmeal to 49.4% in soyabean meal. The amount of zinc available to the fish from each protein source was calculated using the ADCs, and the concentrations analysed in the raw materials (appendix 8.1) are displayed in figure 3.1. Soyabean meal is shown to have the most available zinc. The lower quality Provimi 66 fishmeal (high ash) has more available zinc than the LT94 fishmeal.

Maize gluten, soyabean meal and poultry meatmeal all report negative calcium ADCs, and the other 4 ingredients reported relatively low ADCs. There is no correlation between the dietary level of Ca and the negative ADCs, nor is there between any levels of other minerals.

Table 3.2 Mineral ADCs (%) for the 6 test ingredients and the casein reference diet

Diet	Ca	Cu	Fe	K	Mg	Mn	Na	P	S	Zn
Ref.	16.8^{bcd}	68.9^{dg}	24.1^{d}	84.9^{b}	48.5^{g}	22.3^{beg}	19.4	90.6	85.3	74.3
MG	-15.5	63.4^{g}	23.5^{d}	26.0	31.6^{g}	4.9^{e}	-378.8^{a}	23.1^{a}	73.7	31.9^{a}
SB	-35.8	59.3^{g}	20.6^{d}	91.3^{b}	49.0^{g}	18.1^{beg}	-1923.1^{ab}	57.3^{ab}	25.8^{ab}	49.4^{ab}
PM	-17.5	48.4^{g}	12.2	93.0^{b}	28.7^{g}	10.9^{e}	-38.8ab^{c}	19.9^{ac}	-2.7^{abc}	15.8^{abc}
LT	4.6^{abcd}	68.8^{dg}	20.3^{d}	98.5^{ab}	55.1^{g}	-1.6	68.8ab^{c}	44.4^{abd}	63.6^{acd}	15.1^{abc}
P66	6.4^{adcd}	51.5^{g}	26.1^{d}	94.3^{b}	47.4^{g}	13.7^{be}	2.6^{abcde}	23.8^{ace}	33.3^{abde}	26.6^{acde}
NP	13.4^{bcd}	27.1	19.3^{d}	97.1^{b}	71.9	8.4^{e}	-1116.41^{abcdef}	60.2^{abdf}	94.2^{bcdef}	26.1^{acde}

Suffixed values denotes significant difference from a - Ref, b - maize gluten meal (MG), c - soyabean meal (SB), d - poultry meatmeal (PM), e - LT94 fishmeal (LT), f - Provimi 66 fishmeal (P66), g – NuPro (NP). ANOVA $p<0.05$, LSD post hoc (n = 3)

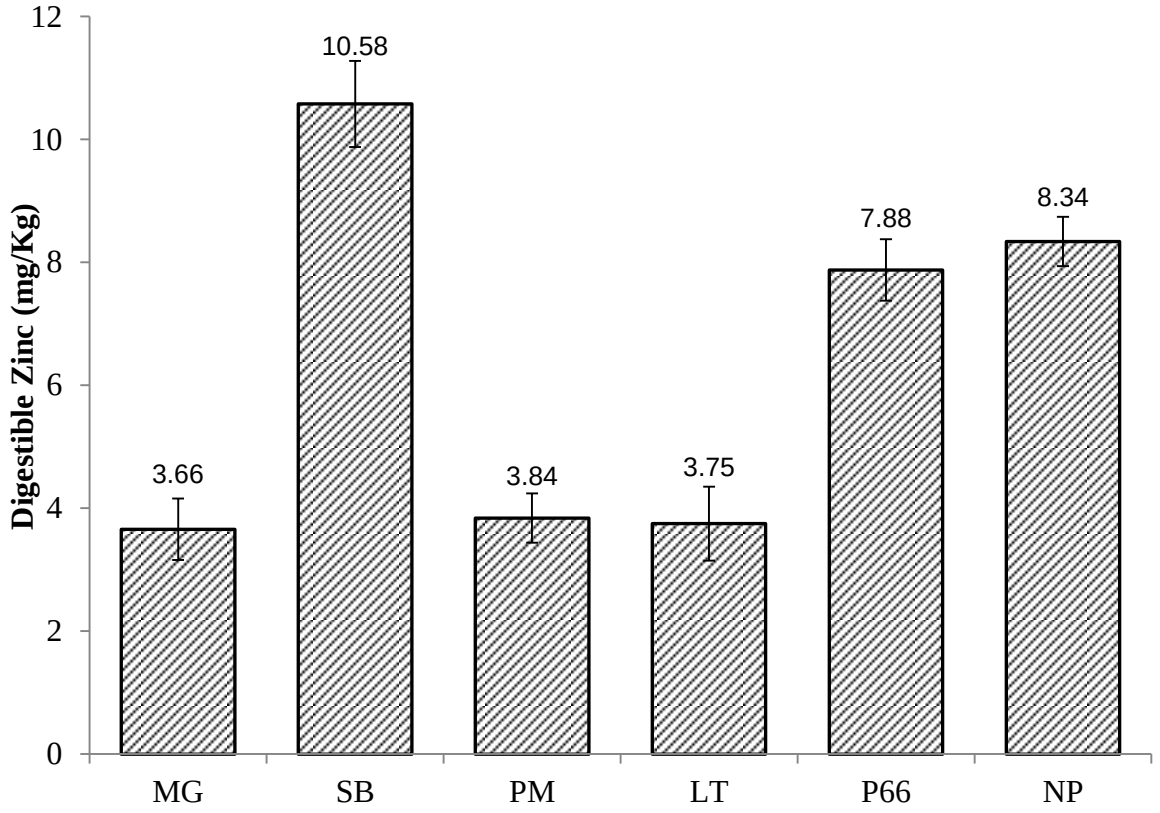

Figure 3.1 Milligrams of zinc available from 1kg of raw material (mean ± S.D., n = 3)

The ADCs for copper are relatively high for all the ingredients evaluated. LT 94 fishmeal and maize gluten display the highest ADCs for Cu. The lowest Cu digestibility is found in NuPro, The ADCs for iron are consistent for all test ingredients except poultry meatmeal which is significantly lower. The ADCs for potassium were consistently high in all ingredients except maize gluten. The ADCs for magnesium show a similar trend to those of potassium apart from in NuPro which has a significantly higher ADC. The ADCs for Mn show variation, soyabean meal provides the best digestibility of 18% and LT 94 negatively affecting the uptake of Mn from the reference fraction of the diet. The ADCs for Na report high variability between ingredients and the majority resulted in a negative value.

The ADC values for P range from 19.9% in poultry meatmeal to 60.2% in NuPro and up to 90.6% in the casein diet. The ADCs for S range from -2.7% in poultry to 94% in NuPro. The two highest ADC values were Nupro and maize gluten which also had the two highest dietary concentrations. There is a significant difference between the two fishmeals, with the S in LT 94 being twice as available as in the Provimi 66 despite the dietary concentrations being equal.

3.3.2 Protein ADC and Zinc/Protein ADC Comparison

The ADCs of the protein for each test protein source and the casein reference diet are displayed in Table 3.3. The highest ADC of the test protein sources was for maize gluten with a value of 85.3% followed by LT94 fishmeal with a value of 80.8%.

No correlation between protein digestibility and mineral availability was found indicating that protein digestibility had no significant effect on the mineral digestibility.

Table 3.3 Protein ACDs (%)

Protein Source	ADC (%)
Casein	97.8 ± 41
Maize Gluten Meal	85.3 ±2.2[a]
Soyabean Meal	76.5 ± 3.0[b]
Poultry Meatmeal	65.3 ±2.7[c]
LT 94 Fishmeal	80.8 ±1.2[ab]
Provimi 66 Fishmeal	69.8 ±5.4[bc]
NuPro	59.1 ±6.1[c]

Values with the same suffix are statistically equal
(ANOVA LSD $p<0.05$, n=3)

Table 3.4 Mean growth performance and feed utilization of rainbow trout fed experimental diets. Values in the same column with different suffix are significantly different ($p<0.05$)

	Weight Gain %	SGR	FCR
Ref.	111 ± 10^{bc}	2.49 ± 0.12^{bc}	1.16 ± 0.05^{bc}
Diet 1	78 ± 9^{e}	1.92 ± 0.10^{e}	1.50 ± 004^{e}
Diet 2	102 ± 7^{c}	2.34 ± 0.11^{c}	1.24 ± 0.04^{c}
Diet 3	121 ± 8^{ab}	2.65 ± 0.09^{ab}	1.08 ± 0.04^{ab}
Diet 4	134 ± 5^{a}	2.83 ± 0.06^{a}	1.00 ± 0.02^{a}
Diet 5	124 ± 6^{ab}	2.69 ± 0.06^{ab}	1.07 ± 0.03^{ab}
Diet 6	92 ± 5^{d}	2.18 ± 0.06^{d}	1.34 ± 0.03^{d}
Commercial	136 ± 11^{a}	2.86 ± 0.13^{a}	0.99 ± 0.04^{a}

3.3.3 Growth Rates and Feed Performance

The fish more than doubled in weight from 50 ± 2g to 112 ± 10g with the exception of the maize gluten diet and the NuPro diet. During the trial the mortality rate was zero. The best growth performance was observed in the commercial diet (FCR=0.99, SGR=2.86) followed by the other two fishmeal diets (LT94 and Provimi 66) with FCRs of 1.0 and 1.07 and SGRs of 2.84 and 2.69 respectively. The two worst performing diets were the maize gluten meal diet (FCR=1.51, SGR=1.92) and NuPro (FCR=1.34, SGR=2.18), both of which were significantly different to the other diets in growth performance (table 3.4). FCRs are artificially high due to the absence of a feed waste collection system.

3.3.4. Tissue Distribution of Zinc

The whole body zinc concentrations ranged from 90.0 µg/g in the fish fed the NuPro diet to 66.7 µg/g in the fish fed the maize gluten meal diet (figure 3.2a). Only the fish fed the maize

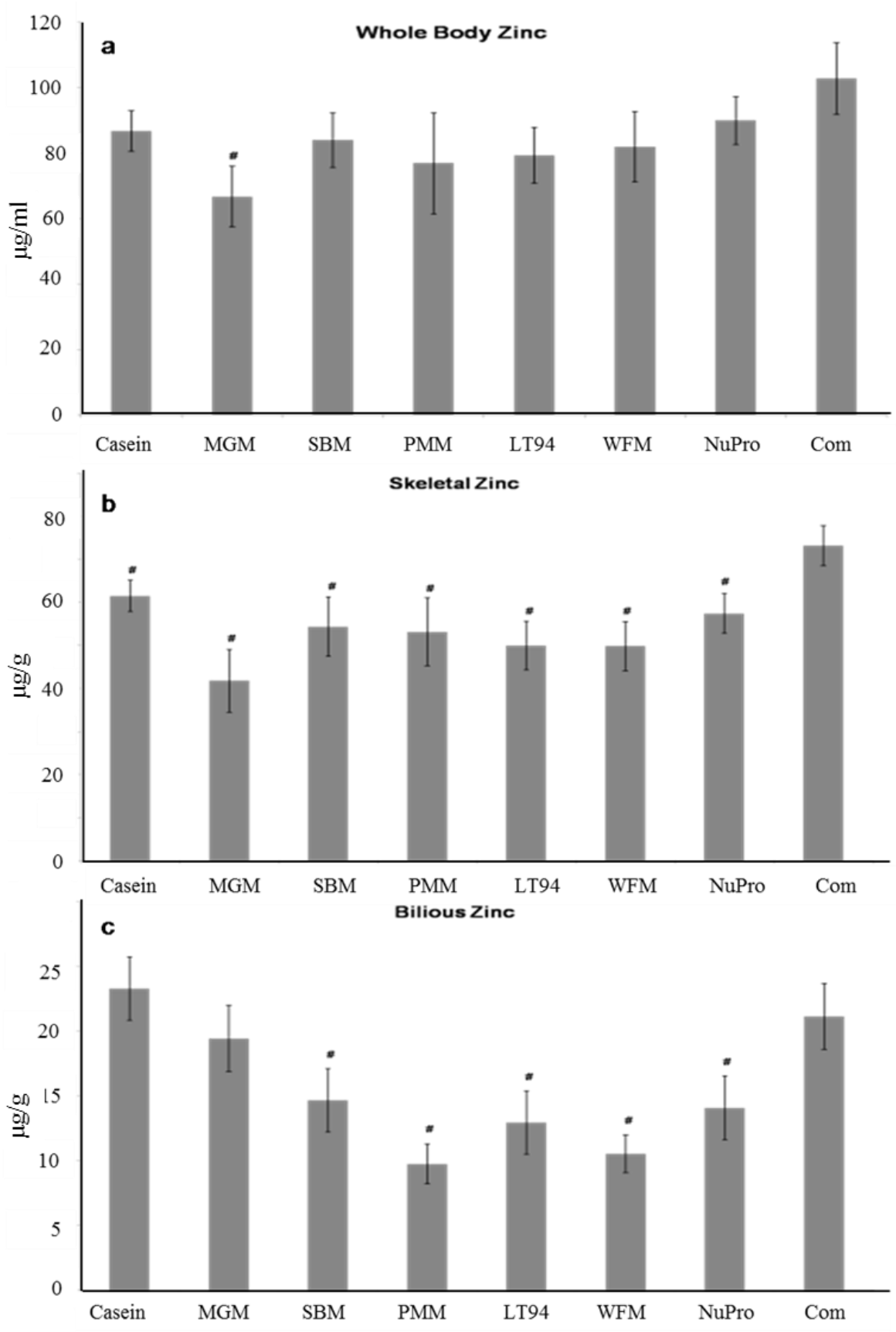

Figure 3.2 Zinc tissue distribution in rainbow trout fed experimental diets. n=2 (individual fish used) error bars display standard deviation. # indicates significant difference from the commercial diet ($p<0.05$)

gluten meal diet had a significantly lower whole body zinc level than the fish fed the commercial diet which contained 3-4 times the amount of zinc. All the test diets did seem to lower the whole body zinc levels but not significantly (using one-way ANOVA, p-value of 0.05), a larger number of repeats may have reduced the apparent variance within each treatment.

The skeletal zinc concentration ranges from 61.5 µg/g in the fish fed the casein diet to 41.8 µg/g in the fish fed the maize gluten meal diet (figure 3.2b). All the experimental diets produced significantly lower skeletal zinc concentrations compared to the fish fed the commercial diet, and followed the same trend as the whole body zinc levels.

The zinc concentration in the bile ranges from 23.3µg/g in the fish fed the casein diets to 9.75µg/g in the fish fed the poultry meat meal diet (figure 3.2c). Neither the casein nor the maize gluten meal diet significantly altered the bilious zinc concentration from the level in the commercial diet fed fish, which were given almost four times the level of zinc. The remaining five diets all produced lower bilious zinc concentrations (compared to the commercial diet) but no other significant differences between themselves. With the exception of the soybean diet, these diets also had the lowest available zinc levels.

The zinc concentration in the livers ranged from 146.8 µg/g in the fish fed the NuPro diet to 72.4 µg/g in the fish fed the maize gluten meal diet (figure 3.3a). Only the maize gluten fed fish had a significantly lower hepatic zinc concentration compared with the fish fed the commercial diet.

The caudal fin zinc concentrations ranged from 84.0 µg/g in the fish fed the poultry meat meal diet to 70.1 µg/g in the fish fed the maize gluten meal diets (figure 3.3b). There was a

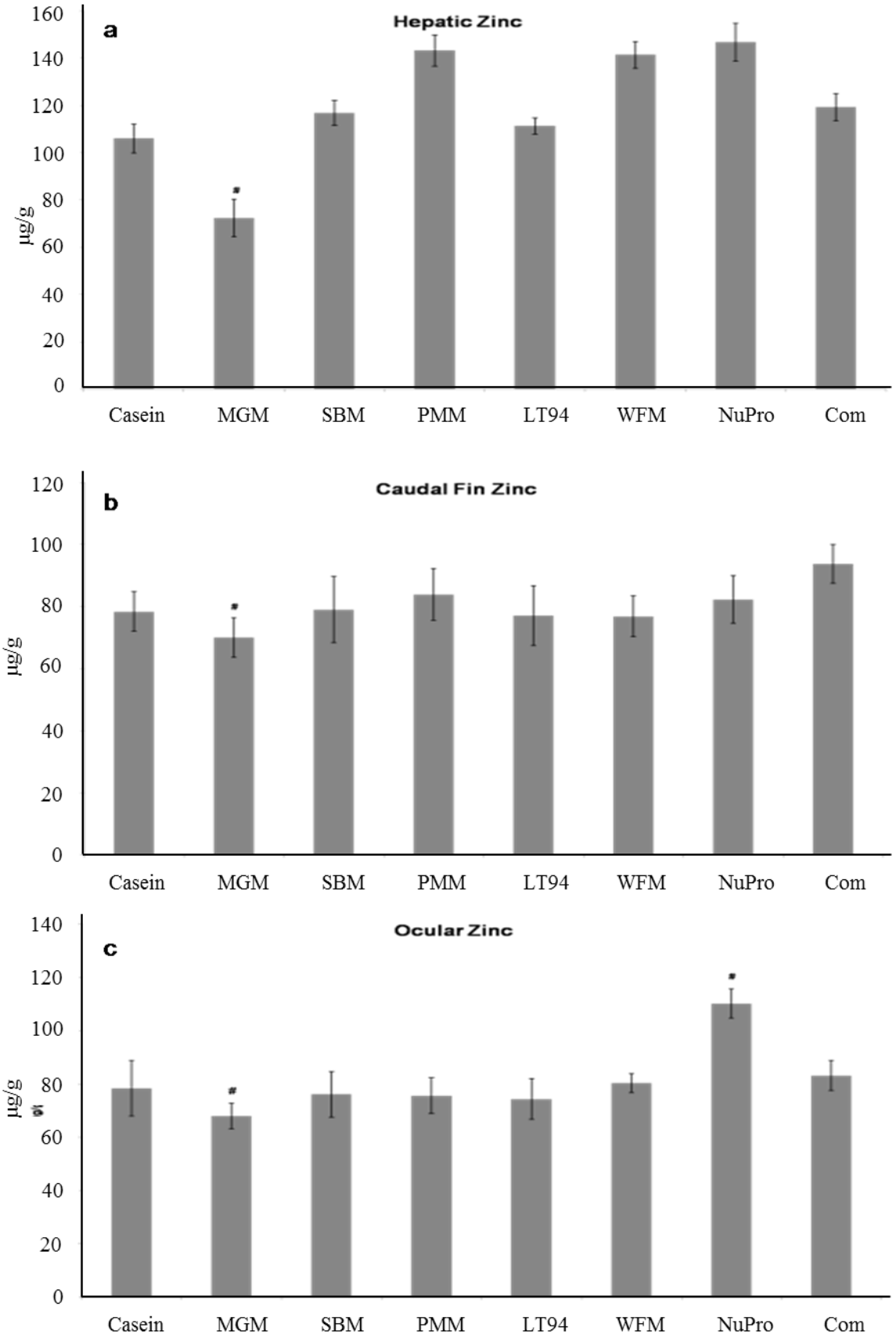

Figure 3.3 Zinc tissue distribution in rainbow trout fed experimental diets. n=2 (individual fish used) error bars display standard deviation. # indicates significant difference from the commercial diet ($p<0.05$)

significant difference between the maize gluten meal fed fish and those fed the commercial diet but not between the fish fed the maize gluten meal and any other diet.

The ocular zinc concentrations ranged from 110.3 µg/g to 68.1 µg/g in the fish fed the NuPro and the maize gluten meal diets respectively (figure 3.3c). Both of which were significantly different to the fish fed the commercial diet but at either extreme. None of the remaining test diets altered the ocular zinc concentration significantly from the commercial diet fed fish.

The serum zinc concentrations ranged from 26.8 µg/g in the casein diet treatment to 11.6 µg/g in the maize gluten meal treatment (figure 3.4a). The zinc concentration in the serum of the fish fed the maize gluten meal was significantly lower than all the other treatment. The serum zinc level in the fish fed the soybean meal diet was also lower than all the treatments except the high quality LT94 fishmeal diet.

The muscular zinc concentrations ranged from 18.4 µg/g in the fish fed the NuPro diet to 12.2 µg/g in the fish fed the soybean meal diet (figure 3.4b). Both the Provimi 66 fishmeal diet and the NuPro diet fed fish had significantly higher muscular zinc concentrations than the fish fed the commercial diet but not compared to the fish fed either the casein or the LT94 fishmeal. The fish fed the soybean meal diet were significantly lower than the all the other treatments except the commercial diet.

The Zinc concentration in the mid intestine ranged from 270.1 µg/g in the fish fed the casein diet to 87.3 µg/g in the fish fed the soybean meal diet (figure 3.4c). The maize gluten meal and the poultry meat meal fed fish also showed significantly lower intestinal zinc levels compared with the other test diets. All the fish fed the test diets were however significantly lower in intestinal zinc than the fish fed the commercial diet.

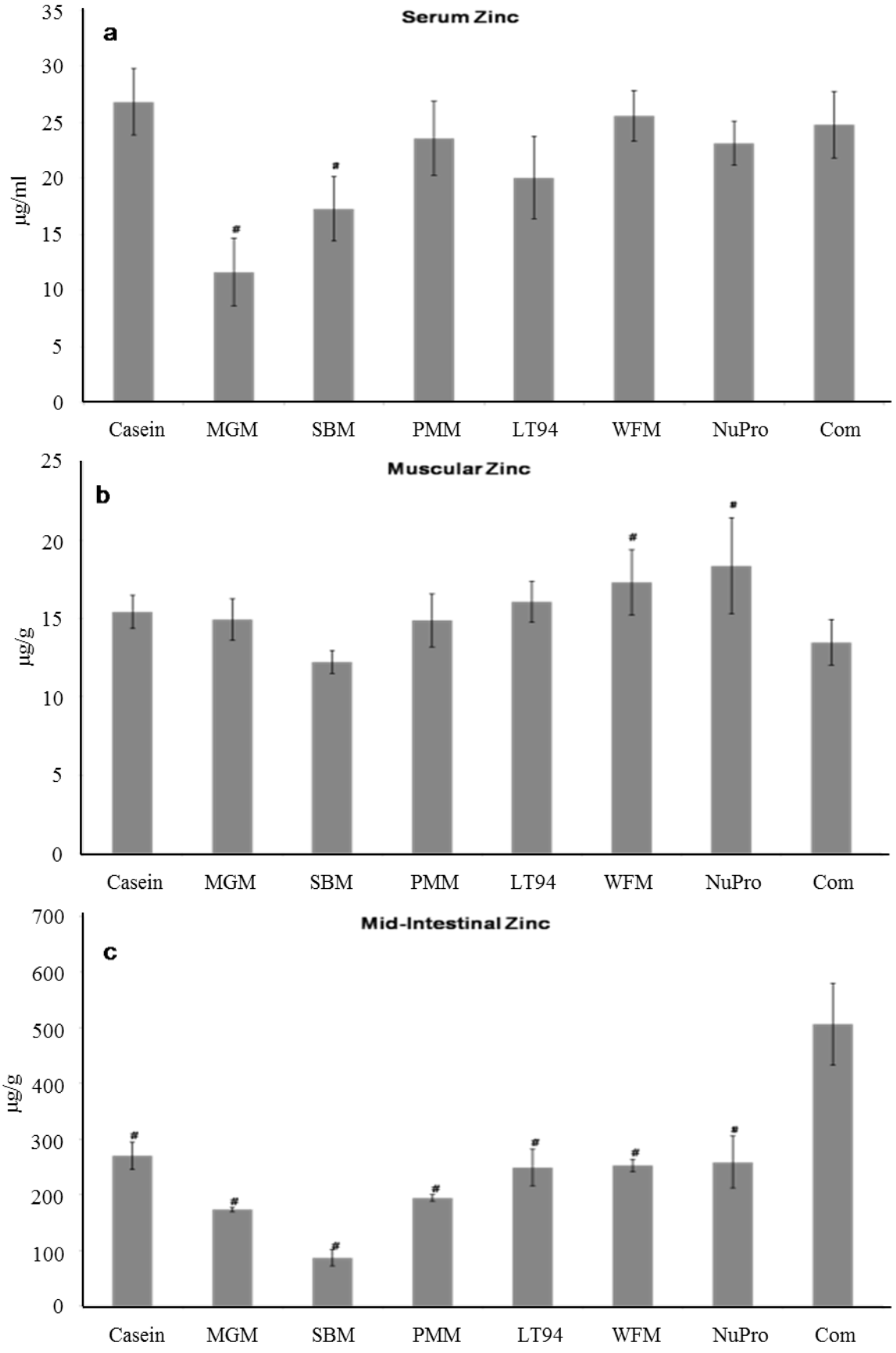

Figure 3.4 Zinc tissue distribution in rainbow trout fed experimental diets. n=2 (individual fish used) error bars display standard deviation. # indicates significant difference from the commercial diet ($p<0.05$).

3.3.5 Tissue distribution of other minerals

Table 3.5 Liver mineral concentration of rainbow trout fed experimental diets. Mean ± SD; n=2 (individual fish used) Values with different suffix in the same column denotes significant differences

	Na (g/1)	S (g/1)	K (g/1)	P (g/1)	Ca (g/1)	Mg (g/1)	Cu (mg/1)	Fe (mg/1)	Mn (mg/1)
Casein	3.64 ± 0.53	11.1 ± 0.5	15.3 ± 0.7	12.5 ± 0.8	0.19 ± 0.05	0.64 ± 0.08	260 ± 21.7	198 ± 17.8	5.1 ± 0.4
MGM	3.40 ± 0.51	11.3 ± 0.4	14.7 ± 0.8	11.7 ± 0.9	0.17 ± 0.08	0.61 ± 0.07	257 ± 14.4	199 ± 19.5	3.9 ± 0.3
SBM	3.06 ± 0.46	10.4 ± 0.5	16.2 ± 0.9	12.7 ± 0.7	0.18 ± 0.03	0.66 ± 0.07	264 ± 22.7	222 ± 24.9	3.5 ± 0.4
PMM	3.43 ± 0.39	10.8 ± 0.3	15.1 ± 1.1	12.0 ± 1.2	0.18 ± 0.04	0.64 ± 0.09	275 ± 13.3	216 ± 11.5	3.8 ± 0.4
LT94	3.26 ± 0.61	11.2 ± 0.5	14.9 ± 0.8	12.1 ± 1.0	0.17 ± 0.08	0.65 ± 0.06	277 ± 19.9	229 ±23.8	3.5 ± 0.2
WFM	3.39 ± 0.53	11.0 ± 0.4	15.4 ± 0.8	12.3 ± 1.1	0.16 ± 0.06	0.64 ± 0.08	260 ± 17.1	194 ±22.1	3.8 ± 0.3
NP	3.42 ± 0.46	11.0 ± 0.3	15.9 ± 0.7	12.2 ± 0.7	0.16 ± 0.06	0.65 ± 0.07	245 ± 24.4	443 ± 35.3	3.1 ± 0.7

In general the distribution of Na, S, K, P, Ca, Mg, Cu Fe and Mn was unaffected by the protein source used in the diets. However fish fed the casein reference diet had significantly raised Mn levels in the liver (table 3.5) and mid intestine (table 3.6) compared to all other diets. The mid intestine also showed significantly elevated Cu levels in this group. There were no significant differences between the casein diet and any of the other diets for any of the minerals in the bile (table 3.7); however there was a significantly higher K level and a significantly low Mg level in the serum (table 3.8).

The diet with 18% NuPro resulted in a significantly raised level of Fe in the liver compared with both the casein reference diet and all the other test diets. There was also an increased level of Ca and Mg in the bile in these fish..

Table 3.6 Mid-intestine mineral concentration of rainbow trout fed experimental diets. Mean ± SD; n=2 (individual fish used) Values with different suffix in the same column denotes significant differences

	Na (g/1)	S (g/1)	K (g/1)	P (g/1)	Ca (g/1)	Mg (g/1)	Cu (mg/1)	Fe (mg/1)	Mn (mg/1)
Casein	4.99 ± 0.65	12.2 ± 0.2	12.9 ± 0.2	9.9 ± 0.3	0.35 ± 0.09	0.62 ± 0.04	7.5 ± 0.9[a]	35.8 ± 2.4	10.5 ± 1.2[a]
MGM	4.59 ± 0.58	12.4 ± 0.3	12.9 ± 0.1	10.1 ± 0.3	0.37 ± 0.12	0.64 ± 0.06	4.5 ± 0.7	32.8 ± 3.5	6.6 ± 0.8
SBM	4.24 ± 0.49	12.3 ± 0.4	13.0 ± 0.3	9.8 ± 0.4	0.30 ± 0.08	0.61 ± 0.02	4.3 ± 0.8	33.4 ± 1.5	4.5 ± 0.7
PMM	4.22 ± 0.63	12.9 ± 0.2	13.4 ± 0.2	10.4 ± 0.2	0.31 ± 0.08	0.66 ± 0.04	3.7 ± 0.6	35.6 ± 3.8	5.5 ± 1.2
LT94	4.38 ± 0.52	12.3 ± 0.5	12.8 ± 0.1	9.4 ± 0.3	0.36 ± 0.11	0.59 ± 0.02	3.8 ± 0.8	32.1 ± 2.7	6.4 ± 0.9
WFM	4.58 ± 0.48	12.8 ± 0.2	13.2 ± 0.2	10.1 ± 0.4	0.31 ± 0.10	0.63 ± 0.03	3.7 ± 0.5	33.9 ± 1.9	6.2 ± 1.0
NP	4.00 ± 0.41	12.1 ± 0.4	12.4 ± 0.3	9.3 ± 0.5	0.29 ± 0.12	0.57 ± 0.02	3.2 ± 1.0	37.8 ± 3.8	5.6 ± 1.3

Table 3.7 Bile mineral concentration of rainbow trout fed experimental diets. Mean ± SD; n=2 (individual fish used) Values with different suffix in the same column denotes significant differences

	Na (g/1)	S (g/1)	K (g/1)	P (g/1)	Ca (g/1)	Mg (g/1)	Cu (mg/1)	Fe (mg/1)	Mn (mg/1)
Casein	6.06 ± 0.15	8.62 ± 0.30	0.29 ± 0.03	0.06 ± 0.03	0.31 ± 0.02	0.02 ± 0.01	1.5 ± 0.3	8.8 ± 0.9	0.1 ± 0.1
MGM	6.32 ± 0.21	9.12 ± 0.27	0.30 ± 0.04	0.06 ± 0.02	0.32 ± 0.01	0.02 ± 0.01	2.1 ± 0.6	10.3 ± 1.5	0.1 ± 0.1
SBM	6.12 ± 0.17	8.62 ± 0.29	0.25 ± 0.02	0.10 ± 0.01	0.31 ± 0.02	0.03 ± 0.01	1.9 ± 0.4	8.4 ± 0.8	0.1 ± 0.1
PMM	5.98 ± 0.22	8.97 ± 0.42	0.27 ± 0.04	0.09 ± 0.02	0.33 ± 0.01	0.02 ± 0.01	0.9 ± 0.3	7.9 ± 1.0	0.1 ± 0.1
LT94	6.24 ± 0.13	9.03 ± 0.22	0.31 ± 0.02	0.06 ± 0.02	0.34 ± 0.02	0.02 ± 0.01	0.9 ± 0.3	8.4 ± 1.3	0.1 ± 0.1
WFM	6.30 ± 0.18	9.11 ± 0.18	0.25 ± 0.03	0.08 ± 0.01	0.41 ± 0.02[a]	0.04 ± 0.01[a]	1.7 ± 0.3	9.2 ± 1.5	0.1 ± 0.1
NP	6.20 ± 0.15	8.77 ± 0.24	0.24 ± 0.02	0.09 ± 0.02	0.42 ± 0.01[a]	0.04 ± 0.01[a]	1.0 ± 0.2	10.5 ± 1.7	0.1 ± 0.1

Table 3.8 Serum mineral concentration of rainbow trout fed experimental diets. Mean ± SD; n=2 (individual fish used) Values with different suffix in the same column denotes significant differences

	Na (g/1)	S (g/1)	K (g/1)	P (g/1)	Ca (g/1)	Mg (g/1)	Cu (mg/1)	Fe (mg/1)	Mn (mg/1)
Casein	3.02 ± 0.23	0.65 ± 0.08	0.14 ± 0.02[a]	0.45 ± 0.08	0.12 ± 0.02	0.01 ± 0.01[a]	0.72 ± 0.22	15.0 ± 1.4	0.1 ± 0.1
MGM	3.12 ± 0.27	0.65 ± 0.03	0.08 ± 0.01	0.47 ± 0.06	0.12 ± 0.01	0.02 ± 0.01	0.32 ± 0.18	15.7 ±2.0	0.1 ± 0.1
SBM	3.18 ± 0.24	0.52 ± 0.04	0.01 ± 0.01	0.40 ± 0.07	0.10 ± 0.03	0.03 ± 0.01	0.35 ± 0.15	14.8 ± 1.3	0.1 ± 0.1
PMM	2.97 ± 0.22	0.58 ± 0.03	0.03 ± 0.01	0.44 ± 0.05	0.10 ± 0.02	0.02 ± 0.01	0.27 ± 0.15	14.4 ± 2.3	0.1 ± 0.1
LT94	3.23 ± 0.29	0.64 ± 0.07	0.02 ± 0.01	0.51 ± 0.07	0.11 ± 0.01	0.02 ± 0.01	0.44 ± 0.22	18.1 ± 2.5	0.1 ± 0.1
WFM	3.31 ± 0.17	0.68 ± 0.06	0.02 ± 0.01	0.49 ± 0.08	0.12 ± 0.03	0.03 ± 0.01	0.48 ± 0.16	18.2 ± 2.8	0.1 ± 0.1
NP	3.41 ± 0.21	0.67 ± 0.04	0.03 ± 0.01	0.46 ± 0.04	0.11 ± 0.01	0.03 ± 0.01	0.51 ± 0.12	19.6 ± 3.0	0.1 ± 0.1

The diet with 18% Provimi 66 fishmeal resulted in an increased level of both Ca and Mg in the bile while the supplementation of LT94 fishmeal, soyabean meal and poultry meatmeal had no significant effect on the mineral levels in either the serum, liver, bile or mid intestine for any of the tissues.

3.3.6 Net Zinc Retention

The net zinc retention ranged from 67.8% from the casein diet to -207.1% from the maize gluten meal diet (table 3.9). Both the LT 94 fishmeal and the commercial diet gave very similar results of 66.3% and 67.5% respectively, which is unsurprising since the LT 94

Table 3.9 Mean net zinc retention of rainbow trout fed experimental diets. Based on wet body weight (n=2). Values with different suffix are significantly different ($p<0.05$).

Diet	Casein	MGM	SBM	PMM	LT94	WFM	NuPro	Com
% Zinc Retention	67.8 ± 3.7^{a}	-207.1 ± 20.6^{d}	23.3 ± 1.9^{c}	20.9 ± 2.5^{c}	66.3 ± 4.8^{a}	46.6 ± 2.8^{b}	21.9 ± 1.2^{c}	67.5 ± 2.2^{a}

fishmeal was supplied from the same manufacturer as the commercial diet. Both the soybean meal, the NuPro and the poultry meat meal recorded similar retentions of 23.3%, 22.0% and 21.0% respectively.

3.4 Discussion

This study was a comparative mineral digestibility study designed around the hypothesis that in order to obtain an accurate ADC for a mineral from a protein source, that mineral must; a) be in a known, fixed ratio between the reference diet and the test protein, and b) must reside at a level close to or below the animal's requirement for that mineral's efficient utilization. Both these stipulations are relatively novel to the approach generally taken. The majority of studies involving ADC calculations use only the ratio of the test protein to reference diet (i.e. "30% of the reference diet was replaced with fishmeal") regardless of the actual levels of mineral in the test protein source (Sugiura et al, 2000; Cheng and Hardy, 2003). The advantage of the conventional approach is that it is possible to use the same ingredient ratio for virtually every mineral, the disadvantage is that there is no way of accurately knowing what amount of the mineral is coming from the test ingredient. With regard to the second stipulation, to the author's knowledge, no other study has specifically formulated diets to be as close to mineral deficiency as possible in order to calculate ADCs. This part is crucial to the hypothesis that because minerals are so highly regulated by fish, if there is more of a mineral than required, the body will either not take them up from the GI tract or will excrete excess back into the GI tract. Both result in a higher concentration in the faeces, undermining the calculation of the ADC.

With the above hypotheses in mind this trial focused on zinc to the possible detriment of the accuracy of the ADC calculations for the other minerals. It was however decided to measure these other minerals in order to allow a comparison to be made between other published data. Table 3.2 displays the ADCs for Ca, Cu, Fe, K, Mg, Mn, Na, P, S and Zn from the casein reference diet and the 6 test protein sources. The ADCs for casein are for the whole diet so cannot be directly compared to the other protein sources, however, an almost negligible

amount of zinc was supplied by any of the other ingredients in that diet so the values are a good approximate. The ADCs for the zinc should be considered the real digestibility values so long as the two fore mentioned hypotheses hold true. With regard to the other minerals, some scepticism may be advisable when comparisons are made between test ingredients and it would possibly not be recommended to rely on these figures alone for diet formulation. Negative results indicate an accumulation of the mineral in the faeces, this could be due to: very poor digestibility; an antagonistic interaction with other nutrients; or the body may be obtaining an excess of the mineral from the diet, water or a combination of both.

3.4.1 Digestibility

The ADCs for zinc from the test ingredient range from 15.1% in LT 94 fishmeal to 49.4% in soyabean meal. As described above, this trial was designed to establish the exact amount of zinc available to the fish from each protein source, therefore these results should be totally comparable between each other but not necessarily between other values in the literature from experiments using the conventional method. There are two surprising results: 1) the fact that the soyabean meal is shown to have the most available zinc when soyabean meal is notorious for containing high concentrations of phytic acid which strongly chelates divalent minerals like zinc (Satoh et al, 1993). However the soyabean meal used in this trial is a very high quality meal; 2) the Provimi 66 fishmeal (high ash) had more available zinc than the LT94 fishmeal. High ash fishmeal like Provimi 66 contain higher levels of hydroxyapatite than higher grade fishmeals like LT94 because of the hard tissue content usually found in them. This anomaly may be explained to some extent by comparing phosphorus levels and availabilities in each diet. P is well known to reduce zinc availability (Porn-Ngam et al. 1993) but the levels of P in the two diets are almost equal, however the availabilities are not and it

may ultimately be Ca that determines that zinc availability. The amount of Ca present is significantly larger in the Provimi 66 fishmeal. Satoh et al (1992, 1993) attributed the mineral binding properties of tricalcium phosphate to the phosphorus released when the tricalcium phosphate is dissolved in the gut of the fish. In the presence of a high concentration of Ca, as in Provimi 66, the Ca may inhibit the phosphorus from binding to the zinc. The lower digestibility of the phosphorus in the Provimi 66 than in the LT94 fishmeal would support this theory.

In the literature there is some disagreement about the availability of zinc from different protein sources. Yamamoto et al (1997) reported ADCs of 30% for a white fishmeal, 74.1% for a soyabean meal and 47.7% for a maize gluten meal. The results from this current trial rank the ADCs for each ingredient in the same order but only the Provimi 66 fishmeal provides a comparable value. Sugiura et al (1998) report ADC values of 17.2% for an anchovy meal and 15.7% for a poultry meatmeal which are very comparable to the current study's LT94 and poultry meatmeal. They also reported ADCs of 0.0% for a soyabean meal and a maize gluten meal. Interestingly the authors of that paper also used a casein based reference diet which reported an ADC of 79.6% compared with the 74.3% recorded in this trial.

In summary the ADCs in this study are comparable with other studies in the literature and the possible anomaly of the zinc in the white fishmeal being more digestible than the zinc from the LT94 fishmeal could be due to the lower availability of phosphorus in the Provimi 66 fishmeal.

Maize gluten, soyabean meal and poultry meatmeal all report negative ADCs, and the other 4 ingredients reported relatively low ADCs. There is no correlation between the dietary level of Ca and the negative ADCs, nor is there between any levels of other minerals. Ca has been

found to be notoriously difficult to assess for digestibility in fish (Yamamoto et al, 1997; Cheng and Hardy, 2003; Sugiura et al, 1998). It is proposed that this is due to high competition between several other minerals for the same uptake sites or because calcium may be obtained from the environment via the gills.

The ADCs for Cu are relatively high for all ingredients. It is well documented in the literature that both Zn and Fe are metabolic antagonists of Cu (Watanabe et al, 1997) and the 3 elements share numerous uptake pathways and even metabolic roles. LT 94 fishmeal and maize gluten display the highest ADCs for Cu and both have either a very low zinc concentration or digestibility. Likewise, the lowest Cu digestibility is found in NuPro, but this contains the highest zinc and second highest Fe levels, which also display above average ADCs. This study provides strong evidence that Cu, Zn and Fe interact with each other with regard to mineral availability which needs to be considered when designing diets for subsequent experiments.

The ADCs for Fe are consistent for all test ingredients except poultry meatmeal which is significantly lower. The ADC values reported here are quite moderate. Negative or negligible availabilities for Fe from fishmeals, poultry meatmeals and some plant meals have been reported in other studies (Sugiura et al, 1998) but much higher values have also been reported. 72.2% was reported for soyabean meal and 78.7% for maize gluten by Cheng and Hardy (2003). Fe, like Cu, can be obtained from the environment and as Fe homeostasis is maintained primarily by adjusting its absorption rather than by excretion (Morris, 1987); this may explain differences in ADC values among the literature. An adequate supply of dietary Fe has however been shown to reduce absorption in the gills (Segner and Storch, 1985). Fe absorption has been shown to be negatively affected by phosphates and in the present study this could also explain the low ADC for poultry. This effect is not reflected in either of the fishmeal diets however there is a high Fe concentration in both the fishmeal diets as well.

The ADCs for K were consistently high in all except maize gluten. Together with the ADC values of Mg, the K ADCs should generally be considered highly reliable due to the extremely low amounts of both these minerals in the reference casein diet, however the low concentration of K in the maize gluten may make its value unreliable. The ADC for the casein diet may also be unreliable due to the small amount of K present in the diet coming entirely from the 5% fishmeal in its formulation. The high digestibility does not necessarily reflect the requirement of the fish as the main excretory role (and therefore homeostatic mechanism) for K is via the kidney and so may passively enter the body from the gut.

The ADCs for Mg show a similar trend to those of K. The values are similar for all ingredients with the exception of maize gluten which is low and Nupro which is significantly higher. The same reasoning for why maize gluten K digestibility was low is also valid for Mg. The high ADC for NuPro could be related to a relatively high level of K in this diet, an effect reflected in the soyabean meal diet but to a lesser extent.

The ADCs for Mn show considerable variation, with soyabean meal providing the best digestibility of 18% and LT 94 actually negatively affecting the uptake of Mn from the reference fraction of the diet. More is known about the excretory pathway of Mn than its uptake mechanisms. Absorbed Mn is almost totally excreted in the bile into the GI tract therefore normal biological behaviour of the body may falsely lower the apparent digestibility. Further work with stable isotope markers may be required if a more realistic digestibility was required.

The ADCs for Na report high variability between ingredients and the majority resulted in a negative value. As discussed previously in the explanation, a negative value implies accumulation of the mineral in the faeces. The negative Na values do coincide with high K values and may be a result either of the body maintaining an ionic charge or as an active,

facilitated uptake of K, Likewise the apparent digestibility of K may be a mechanism to remove excess Na. However LT 94 fishmeal reports both a high K and Na uptake. The mineral analysis of the LT94 diet and the LT 94 fishmeal alone indicate a high Na concentration, possibly responsible for a change in the interactions between the two.

The ADC values for P range from 19.9% in poultry meatmeal to 60.2% in NuPro and up to 90.6% in the casein diet. Poultry meatmeal and Provimi 66 fishmeal display significantly higher Ca levels than the other ingredients (with the exception of LT94 fishmeal) and casein and Nupro possess the least. This supports the theory that P availability is negatively affected by Ca concentrations in many animals including rainbow trout (Porn-Ngam et al, 1993) and carp (Nakamura, 1982). LT 94 fishmeal has an equally high level of Ca as the poultry meatmeal and the Provimi 66 fishmeal, however the ratio of Ca to P is almost equal. Satoh et al (1993) investigated the effects of this ratio on other minerals, however the balance of these two minerals in the LT 94 fishmeal (i.e. nearly equal) may affect the bioavailability of the P by not 'locking it away' as a tri-basic phosphate compound.

The ADCs for S range from -2.7% in poultry to 94% in NuPro. The two highest ADC values were Nupro and maize gluten which also had the two highest dietary concentrations. However there is a significant difference between the two fishmeals, with the S in LT 94 being twice as available as in the Provimi 66 despite the dietary concentrations being equal, which may spoil the trend of the availability of S being concentration dependant. Very little work has been done on S availability so it is not possible to compare these values or even suggest an explanation for these variances.

The highest ADC of the test protein sources was for maize gluten with a value of 85.3% followed by LT94 fishmeal with a value of 80.8%. All the protein ADCs calculated in this trial agree with other Protein ADCs for similar products in the literature (Guimaraes et al,

2008 -Nile tilapia; Sampaio-Oliveira and Cyrino, 2008 - largemouth bass; Sugiura et al, 1998 - salmonids ; Yamamoto et al, 1997 – rainbow trout). No correlation between protein digestibility and mineral availability was found indicating that protein digestibility had no significant effect on the mineral digestibility.

Another method for assessing the availability of a specific nutrient from a raw material is by using the net retention of that nutrient (further discussed in section 3.4.3). To calculate this, the concentration of that nutrient in the diet needs to be known and the amount of the diet ingested needs to be known. The start and end concentrations of that nutrient in the animal also needs to be measured. That advantage of this method is that you can quantify how much of the nutrient has actually been retained by the animal, which is useful for both economic and environmental assessments; however the disadvantages are that you only obtain a retention value for that particular whole diet and over that particular time period. The rate of turnover within the body and the amount excreted will influence the net retention and so provides no information about the availability of the nutrient from that raw material or diet. For formulation purposes the important information is how much of a specific nutrient will be absorbed by the animal from a specific raw material and how does this compare with a different raw material at the same temperature and in the same size fish. This trial allowed the calculation of both the digestibility of zinc from each protein source (table 3.2) and net retention from the whole diets (table 3.9). The digestibility values were used for formulation of the diets for subsequent trials.

3.4.2 Tissue distribution

The elevated level of Mn in the liver and the mid intestine coincide with casein having the highest ADC for Mn of all the diets, despite having the lowest concentration. The elevated level in the intestine may indicate that this is the main region of uptake and the elevated level in the liver may imply that this is a storage pool for Mn as it is expected that storage pools respond fastest to alterations on in dietary levels of minerals (Swinkles et al, 1994).

The elevated level of Cu in the intestine implies the same as for Mn, and again the casein diet contains the lowest level of Cu but displays the highest digestibility.

The high level of K in the serum, when linked with an average digestibility and a significantly low concentration may indicate increased transportation from a storage site to a functional pool but without the use of markers only assumptions can be made. It is surprising however that a similar pattern was not seen with the maize gluten diet as it has an even lower digestibility for K.

The elevated levels of Fe in the liver of the fish fed the NuPro diet cannot be explained by either unusual concentrations of Fe in the diet or a significantly different digestibility. It implies that the availability of the Fe and hence its activity is different. The increase seen may be due to storage of the iron or its metabolism. Further examination of other sites of Fe storage/functionality would be very interesting with this diet.

The increased levels of Ca and Mg in the bile indicate the excess of these minerals is being removed. The ADCs for Ca and Mg are both the highest in the NuPro diet which is obviously linked with this increased excretion. Interestingly, the increased level in the bile would ultimately return to the faeces so the reported ADC levels in this study may have been artificially low.

The increased levels of Ca and Mg in the bile of the fish fed the Provimi 66 fishmeal diet mirrors the effect seen in the NuPro diet, the only difference is that the increased levels of Ca or Mg in the body are probably due to higher dietary concentrations rather than high digestibility.

A 62% supplementation of maize gluten to the casein diet and the corresponding drop in dietary zinc level to 19 mg/kg was the only effective means of significantly altering the zinc status of any of the tissues in the fish. The level of zinc in the serum was significantly lowered and more importantly the level of zinc in the liver also significantly dropped. It is known that with low dietary zinc intakes redistribution of zinc occurs to those pools that are important metabolically. The results from this diet indicate that the liver is only a storage pool and it will be interesting to identify where the functional pools definitely reside in fish. It is an encouraging sign that at these levels, in fish of this size, alterations to the zinc status of the fish are being observed over a defined time frame.

3.4.3 Net zinc retention

The net retention of a mineral is the difference between the total amount of that mineral fed to the fish throughout the duration of the trial and the overall increase in total body zinc. The negative retention observed from the maize gluten meal diet implies that more zinc has been excreted than has been taken up from the diet. This appears to be a nonsensical result, especially in a group of fish which are seemingly so deficient in zinc that important functional pools like the liver, eye and fins are being depleted. Coincidentally, the group of fish fed the maize gluten diet also had a strange result with the amount of zinc in the bile. The large amount of zinc in the bile suggested the excretion of the mineral but why an animal

depleting its metabolic zinc pools would excrete more zinc via the bile is unclear, however it would explain how the fish were getting a negative net retention. A possible explanation for this could involve the hepatoenteric circulation system. In both humans and rats this system has been shown to re-circulate both iron and arsenic from the body into the bile which then is secreted into the duodenum and the minerals can be reabsorbed (Suzuki et al., 2001). The confirmation of this is beyond the remit of this study but it could possibly involve this system.

3.5 Outcome

The zinc digestibilities of the tested ingredients have been calculated and were used to formulate the diet for subsequent zinc supplementation experiments in rainbow trout. This diet will be composed of commercially important ingredients and also be zinc-deficient. The diet will be formulated with a combination of LT94 fishmeal and maize gluten and will have a crude protein level of ~45% and a zinc concentration of ~25 mg/kg. This preliminary trial demonstrated that even over a short period, a dietary zinc concentration of this level, using these ingredients is enough to alter the zinc status of rainbow trout and form a basis for future studies.

CHAPTER 4. The effect of phytase treatment of soyabean meal on protein, phosphorus and zinc digestibility

4.1 Introduction

According to the UN Food and Agriculture Organization (FAO), aquaculture is growing more rapidly than all other animal food production sectors (FAO, 2009). This rapid growth demands an equally rapid expansion of aquafeed production which needs to be economically viable and environmentally neutral. There is debate over the 'sustainability' of fishmeal and fish oil production and with the anticipated growth of aquaculture the demand for fishmeal and oil is expected to exceed the supply in the next 5-10 years, assuming feed formulations stay the same. Unsurprisingly the aquafeed industry has, for many years now, focused on developing a stable alternative source of protein in terms of cost and supply. Plant proteins, particularly soya derived protein, have been at the forefront of this research and seem to have the most potential to satisfy the nutrient requirement of many farmed finfish species.

Soyabean is produced mainly for its oil, yielding a secondary product of a high protein cake. This is processed to a variety of soyabean products; soy flour (SF), soyabean meal (SBM), soy protein concentrate (SPC) or soy protein isolate (SPI). Soybean meal has a protein content of 44-48% which is increased to 60-64% in SPC and further increased in SPI. Soybean meal has a high level of cysteine but low levels (relative to fishmeal) of essential amino acids and tyrosine. Methionine, lysine and threonine are most likely to be the limiting amino acids in high soyabean meal based diets but the further processing to SPC or SPI may concentrated these amino acids to levels exceeding those found in fishmeal. The further processing may however also concentrate anti-nutritional factors/agents such as phytate.

Phytate or phytic acid is the principal storage form of phosphorus in many plant tissues. In soyabean meal 50-80% of the total phosphorus is in the phytate form, the remaining phosphorus is present as a soluble inorganic phosphate or as cellular phosphorus bound in nucleic acids and phosphorylated proteins (Tyagi and Verma, 1998). Each molecule of phytate ($C_6H_{18}O_{24}P_6$) contains 6 phosphate molecules joined by ester bonds to a cyclic alcohol, inositol, and under physiological conditions in the gut, chelates with positively charged cations, reducing their bioavailability (Erdman, 1979). Phytate may also form complexes with proteins in both acidic and alkaline conditions. In acid conditions the phytate can bind to amino groups on lysine, imidazole groups on histidine and guanidyl groups on arginine; in alkaline conditions it will form ternary complexes, all of which are resistant to proteolytic digestion resulting in reduced protein/amino acid digestibility and utilization (Denstadli et al 2006; Riche and Garling, 2004).

Removal or degradation of phytate can increase the nutritional value and bioavailability of many cations naturally present in the meal. Numerous processing techniques have been employed to try and reduce the phytate content. Milling cereals removes phytate (Bohn et al, 2008) but it also removes many of the minerals of interest along with most of the dietary fibre. Heat treatment has a minor effect on phytate levels (Pontoppidan et al, 2007) and cooking results in the leaching of phosphorus to the cooking water. Genetic modification of cereals to have low phytate levels but normal phosphorus levels has been achieved (Raboy, 1997) and this increased the availability of the phosphorus to fish (Sigiura et al, 1998) but this solution raises socio-economic concerns about GM products. The most efficient method of reducing phytate is to use the enzyme phytase. Phytase catalyses the hydrolysis of phytate and sequesters the orthophosphate groups from the inositol ring. Phytase is endogenous to cereals and its activity can be increased by germination or fermentation (Kumar et al, 2012). The addition of extra exogenous phytase, from microbial sources is an effective way of further

reducing phytase without the need for germination or fermentation. Yeasts, fungi and bacteria have all been used as phytase producers for this purpose. The most common used are derived from *A. niger* and *E. coli*, but other sources have been identified (Greiner and Konietzny, 2006).

Early work into the pre-treatment of soyabean meal with exogenous phytase for use in aquafeeds by Cain and Garling (1995) reported increased weight gain and phosphorus utilisation in juvenile rainbow trout. Later work by Storebakken et al (1998) and Vielma et al (2002) also reported that the pre-treatment of soyabean meal with phytase increased weight gain, feed efficiency, protein utilization and zinc, magnesium, and calcium uptake in Atlantic salmon and rainbow trout respectively.

The aim of this experiment was to measure the protein, zinc and phosphorus digestibility of a commercially available soy protein and to assess the effect of a exogenous microbial phytase pre-treatment of the soy protein ingredient.

4.2 Methods

4.2.1 Experimental fish

A total of 225 rainbow trout were acquired (Torr Fisheries, Devon) and acclimated according to method outlined in section 2.1.2. Throughout the acclimation period fish were fed a commercial grower diet (EWOS, West Lothian) at 2% bodyweight per day.

4.2.2 Diets and experimental regime

Two test diets and one reference diet were formulated (table 4.1) and produced 'in house'. The reference diet had fishmeal as its only protein source and the two test diets contained a soy protein concentrate (Hamlet Protein, Denmark) at 50% dry weight inclusion. The 50% dry weight inclusion contributed 62% of the protein to the diet. Two Hamlet Protein SPC products were used, one per test diet. Both SPCs were compositionally identical except one was dephytinized with a microbial phytase (Natuphos 5000L, BASF) reducing the phytic acid from 1.6% to 0.4% but maintaining the total phosphorus at 0.59% (Appendix 8.1). The diets were formulated to be isonitrogenous and isolipidic and both test diets were identical in their mineral composition (table 4.1).

A total of 25 fish per tank, mean weight 50 ± 1.4g, were randomly assigned to a dietary treatment, each consisting of 3 replicate tanks (n=3). Fish were fed the experimental diet at approximately 2% biomass per day, maintained for a 21 day period using a predicted FCR of 1.0 to project a weekly increase in feed ration. On days 22, 25 and 29 the fish were stripped (section 2.3.3) and their faeces pooled by tank. The faeces from each sample day were pooled for analysis.

Table 4.1 Diet formulation (%) and composition

	Reference	HP 100	HP 110
Soyabean Meal (HP 100)	0	50	0
Soyabean Meal (HP 110)	0	0	50
LT94 Fishmeal	65.61	23.69	23.69
Fish Oil	23.12	13.96	13.96
Corn Starch	10.27	11.35	11.35
Vit/Min Premix	1	1	1
Y_2O_3	0.1	0.1	0.1
Protein (%)	43.9[a]	44.2[a]	44.1[a]
Lipid (%)	22.4[a]	22.0[a]	22.1[a]
Calcium (g/kg)	12.2[a]	5.9[b]	6.1[b]
Potassium (g/kg)	6.5[a]	12.5[b]	13.7[b]
Magnesium (g/kg)	1.5[a]	2.1[a]	2.2[a]
Sodium (g/kg)	9.8[a]	4.5[b]	4.5[b]
Phosphorus (g/kg)	9.8[a]	6.6[b]	6.9[b]
Sulphur (g/kg)	3.9[a]	3.9[a]	4.1[a]
Copper (mg/kg)	4.6[a]	9.7[b]	10.6[b]
Iron (mg/kg)	170.8[a]	125.1[b]	122.4[b]
Manganese (mg/kg)	29.7[a]	40.8[b]	42.0[b]
Zinc (mg/kg)	108.9[a]	102.2[b]	99.8[b]

4.2.3 Analytical procedures

Protein, lipid, ash and moisture content were determined in each diet (see 2.2). ICP-OES was used to perform trace element analysis on all diets (table 4.1) and faeces (see 2.3). Levels of P and Zn were analysed for their digestibility and Y was analysed as an indigestible dietary marker for use in the ADC calculations (Austreng et al 2000).

4.2.4 Calculations

The ADCs of dry matter was calculated, using the formula:

$$ADC_{DM}\,(\%) = 100 \times [1-(\text{conc. of yttrium in diet/ conc. of yttrium in faeces}]$$

This was further expanded to calculate the ADCs for minerals in each diet:

$$ADC_{diet}\,(\%) = 100 \times [1-(\text{conc. of yttrium in diet/ conc. of yttrium in faeces}) \times (\text{mineral concentration in the faeces/mineral concentration in the diet})]$$

The ADCs for the nutrients in the test ingredients were calculated by incorporating the percentage of each component (62.1% for protein, 44.3% for P and 24.5% for Zn) supplied by the alternative protein source:

$$ADC_{Ing}\,(\%) = [ADC_{test} - (1-i) \times ADC_{ref}]/i$$

4.2.5 Statistics

Statistical analysis was performed using analysis of variance and a Tukeys multiple range test was applied to rank significantly different means. Computer software, SPSS v17. was used to perform the statistical calculations; $p<0.05$ was considered statistically significant for all analysis.

4.3 Results

4.3.1 Protein Digestibility

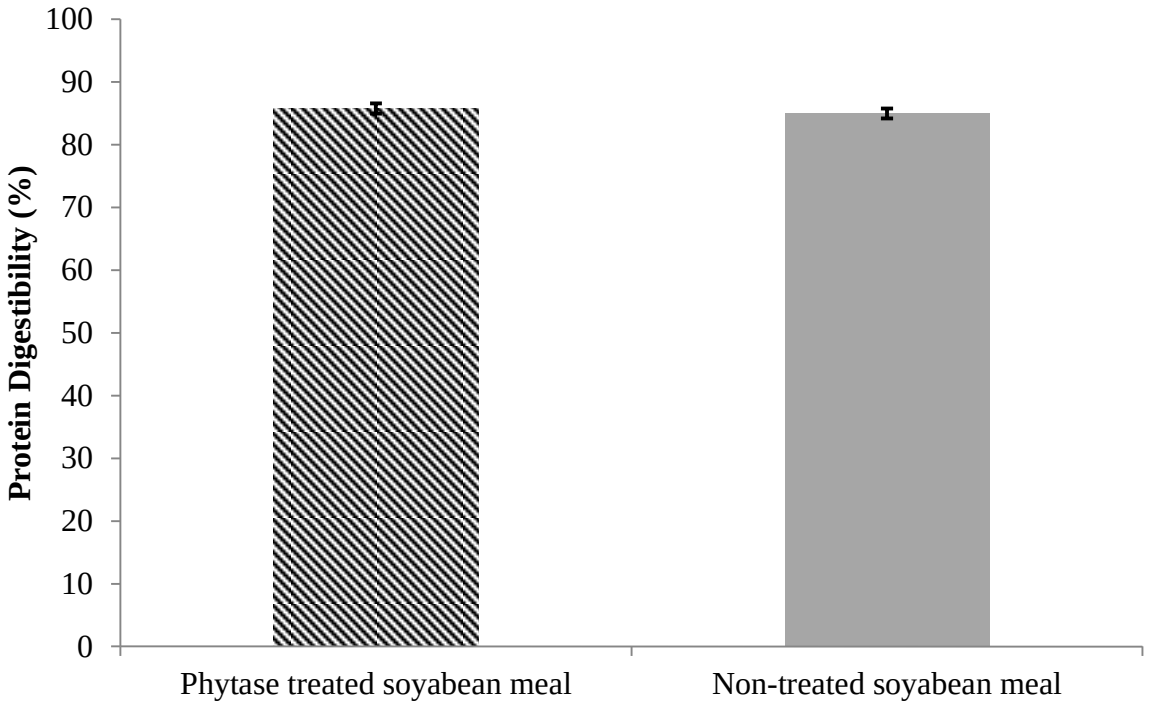

Figure 4.1 Protein Digestibility
(mean ± S.D, n=3. * denotes significant difference between treatments (P<0.05)

The protein (Figure 4.1) in the untreated soyabean meal was 84.9 ± 0.79% digestible and in the phytase treated soyabean meal 85.7 ± 0.82% digestible. A one-way analysis of variance indicated there was no significant difference at p = 0.05.

4.3.2 Phosphorus and Zinc Digestibility

The phosphorus digestibility from the untreated soyabean meal was significantly lower than from the phytase treated soyabean meal with values of 36.6 ± 0.31% and 49.0 ± 0.77%

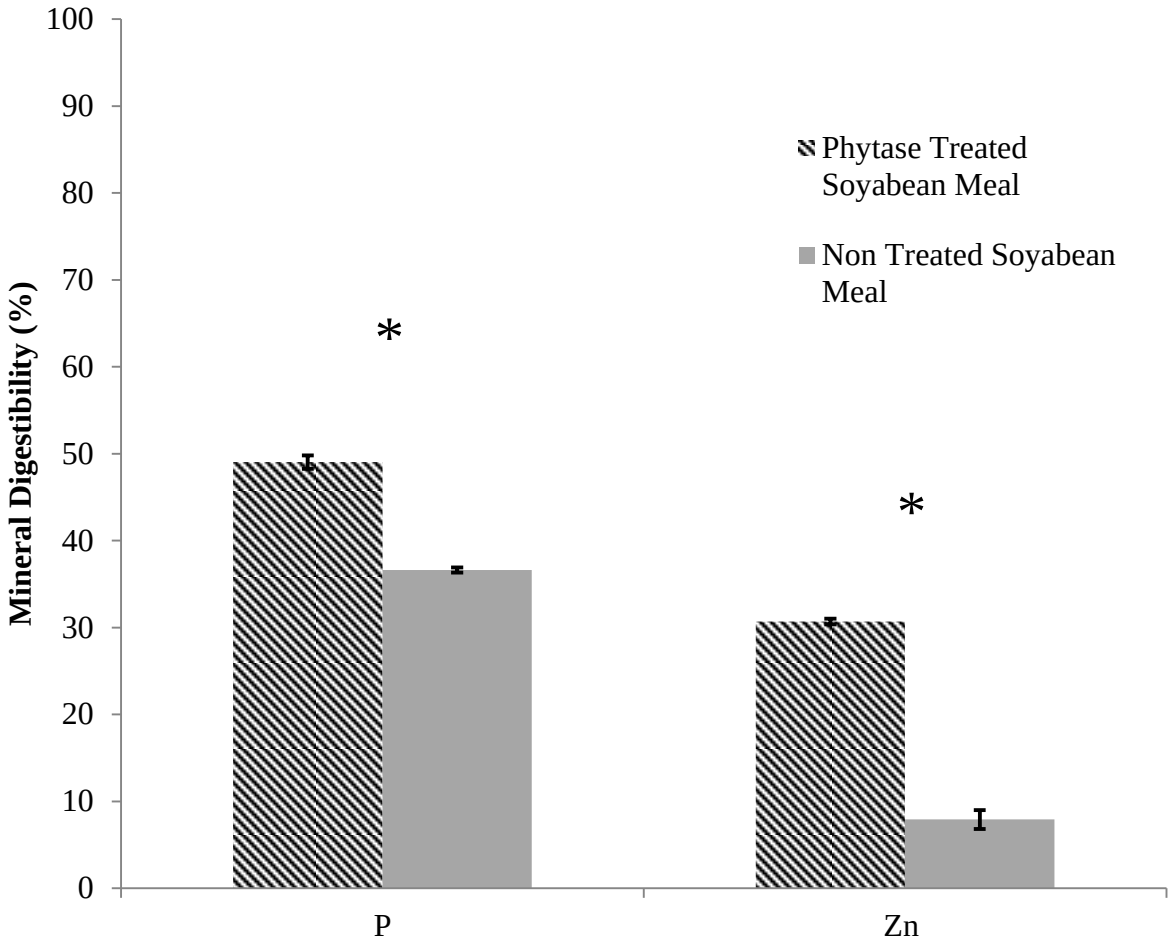

Figure 4.2 Phosphorus and Zinc Digestibility. Solid bar = non-treated; Hatched bar = phytase treated (mean ± S.D, n=3. * denotes significant difference between treatments ($p<0.05$)

respectively. This was also seen with the zinc digestibility which resulted in the digestibility increasing from 7.9 ± 1.09% in the untreated soyabean meal to 30.67 ± 0.33% in the pre-treated meal. Treating the soyabean meal with phytase resulted in 0.82 g more phosphorus and 23.22 mg more zinc being absorbed by each fish per kg of feed ingested.

4.4 Discussion

The results clearly show that the pre-treatment of soyabean meal with phytase makes both the phosphorus and the zinc more available to rainbow trout. Previously similar results have been reported by Storebakken et al (1998) and Cain and Garling (1995). Both of these groups used salmonids and reported a higher digestibility and retention of phosphorus, and a higher whole body phosphorus level respectively. The increased phosphorus digestibility is proposed as evidence that the hydrolysation of phytate was successful. The enhanced uptake of zinc in the present study is further evidence of the successful breakdown of the phytate. It has been well documented that phytate decreases zinc availability in salmonids (Richardson et al, 1985) and, as evidenced by the very low zinc availability from the untreated soyabean meal used in this study, the zinc from soya based feedstuffs is particularly poor (Sugiura et al, 1998). Ramseyer et al. (1999) reported higher bone zinc content and Storebakken et al (1998) reported higher whole body zinc content when dephytinised plant materials were fed to rainbow trout and Atlantic salmon respectively, reinforcing the increased zinc digestibility reported here.

In the present study, unlike Storebakken et al (2000), protein digestibility was not increased by the addition of phytase. However, the authors of that study used a soy protein concentrate which may have a more concentrated level of phytate to start with due to its processing. The protein availability in both this current study and Stoebakken et al (2000) are both contrary to the results in a study by Teskeredzic et al (1995), which reported a negative effect on protein retention when rapeseed protein concentrate was treated with phytase and fed to rainbow trout. It is unclear why these discrepancies occur but it could be due to differences in composition of the soyabean meal, soy protein concentrates and rapeseed protein concentrates, or it could be due to the treatment procedures.

The industrial protocol of adding phytase to the diet may also play an important role in how effective the treatment will be in practice. In the current study and in both the investigations of Vielma et al (2002) and Storebakken et al (2000) the phytase was added to the soyabean product during processing Other studies have top sprayed phytase onto the preformed pellets prior to feeding. Forster et al (1999) top sprayed phytase onto pellets up to levels of 4500U phytase kg^{-1} diet with no observed effect on rainbow trout. Oliva-Teles et al (1998) incorporated 2000U phytase kg^{-1} diet during the pelleting process and also reported no enhanced performance when fed to sea bass (*Dicentrarchus labrax*). In both the above mentioned studies, where the phytase was either top sprayed or incorporated into the pellets with the other ingredients the temperature of the feed storage conditions and/or fish holding water may play an important role. Neither group reports the feed storage conditions but Forster et al held the fish at 11ºC and Oliva-Teles et al held theirs at 20ºC. Both groups used Natuphos® (BASF, Germany) as the phytase, which is obtained by fermentation from an *Aspergillus niger* strain. The temperatures used in these trials are not optimal for phytase activity which is optimal at 50ºC, an issue which is partially avoided when the phytase is incorporated into the processing and drying of the raw material and may explain why no effect of was observed in these studies conducted under controlled experimental conditions and temperatures.

In conclusion, the present data clearly demonstrates the enhanced uptake of both phosphorus and zinc from the diet. It cannot be confirmed from this study whether this has any long term effect on either growth or the health of the fish but it certainly would result in a significantly reduced phosphorus and zinc load in to the water and may result in the need for less of these resources to be used in modern plant-based aquafeeds. The economic feasibility of this potentially expensive treatment also needs investigating.

CHAPTER 5. Organic verses inorganic zinc supplementation; effect on growth, digestibility, tissue levels and protein synthesis.

5.1 Introduction

The type of zinc source used could become of uppermost importance when formulating a diet for aquaculture which requires zinc supplementation because of its protein source composition. There can be considerable differences in both the digestibility and the bioavailability of the zinc depending on whether the zinc is from an inorganic salt, i.e. $ZnSO_4$ or ZnO, or whether the zinc is ligated to an organic compound (Paripatananont and Lovell, 1997; Rider et al, 2009; Satoh et al, 2001). Currently it is common practice to supplement diets with an inorganic salt, mostly $ZnSO_4$, however several organic sources of zinc have been used as nutritional supplements both in mammals and fish (Chesters,1997; Maage et al.,2001; Kucukbay et al.,2006). When a diet is supplemented with an inorganic zinc salt the zinc is considered a 'free' zinc ion while in the gut lumen and is therefore available for uptake out of the gut (providing the element is not bound by another insoluble compound or anti-nutritional factor) but must utilize the tightly regulated and competitive cation uptake mechanisms indicated by the decrease in copper absorption when excess zinc is present and vice versa (Kumar, 2006). These mechanisms are often non-specific for zinc and therefore zinc availability may be influenced greatly by other free cationic elements. By using a zinc source in which the zinc is either simply complexed (where only one atom in the ligand donates an electron pair; monodentate) or chelated (two or more atoms donate an electron pair; polydentate) to either an amino acid or a hydrolyzed protein, the zinc has the potential to be taken up from the gut via a different pathway (Glover et al, 2003). Complexing/chelation may also prevent the zinc being bound by other anions or ligands which may be insoluble and unavailable for absorption. When using an organic form of zinc, the strength of the ligands

bonded to the zinc ion is critical. If the bond is too weak then the acidity of the stomach may cause the zinc ion to dissociate from the compound and effectively have the same properties as the zinc from the usually cheaper inorganic salt. To the other extreme if the organic compound chelates the zinc ion too strongly then free zinc ion may not become available at the site of metabolic requirement, rendering the supplementation useless (Cao et al, 2000).

There is some debate in the literature about whether or not dietary supplementation with an organic source of zinc is more effective than the supplementation with inorganic salts. It has been shown in rainbow trout (Apines et al., 2001) and in catfish (Paripatananont and Lovell, 1995) that supplementation with a zinc-amino acid chelate is more effective than supplementation with $ZnSO_4$. These differences did not always manifest themselves as increased growth or FCRs but more subtly, as increased activity of alkaline phosphatase, superoxide dismutase (both zinc dependant enzymes) and overall zinc retention. Conversely Li and Robinson (1996) observed no difference in skeletal zinc deposition in catfish fed either the organic or inorganic sources. Maage et al. (2001) also reported that the inorganic source was equally efficient as the organic source at raising zinc status in Atlantic salmon. Do Carmo e Sa et al. (2005) reported that the zinc bioavailability was reduced in tilapia fed amino acid-chelated zinc compared with $ZnSO_4$ and ZnO.

Taking all of the above into account this trial proposes to test the null hypothesis that there is no beneficial effect on growth or health when rainbow trout (*O.mykiss*) are fed either an organic of inorganic source of zinc. If there is a difference observed then the null hypothesis will be rejected and the alternative hypothesis that an organic source of zinc is beneficial to the rainbow trout will be accepted.

The trial will take the form of a standard 10 week growth experiment but the fish will also be subjected to a one week stress experiment at the end of the growth trial. Before an after the stress trial the fish will assessed for oxidative status, enzyme activity and tissue mineral content/retention in order to evaluate the more subtle effects which have been observed in some of the studies mentioned above. The diets have been designed based on a zinc deficient diet known to reduce the levels of zinc in the eye, liver and caudal fin as well as reduce the whole body zinc concentration. The basal diet will be supplemented with the two sources of zinc. This is a classical method of evaluating the dose response of a therapeutic treatment i.e. deliberately causing the deficiency and then trying to alleviate the symptoms of the deficiency by adding incremental levels of the deficient substance.

All of the investigations mentioned above, whether advocating organic sources or not, used dietary zinc concentrations above the requirements of the fish and none used this method of trying to alleviate the problems caused by the use of a deficient diet. It could be argued that in reality the fish would not be exposed to such low levels of zinc, however there will be times during production when the fish are exposed to stress from husbandry techniques or exposure to disease when the requirements of the fish are elevated closer to, if not above, the levels provided by the diet. This is becoming more likely with the drive towards higher plant material inclusion in the diet which can reduce the zinc availability (Gatlin et al., 2007). The use of such low levels of zinc, in the authors opinion, are required to illustrate the differences between mineral sources and diets and it is these differences which, although may be hidden at higher zinc levels, may result in better health in times of stress, disease or maturation. There are also potential environmental and economic advantages to using a more available source of zinc.

The range in levels of supplementation has been deliberately chosen to be narrow; this is required because of how tightly rainbow trout regulate zinc. This illustrates the subtlety required when working with dietary zinc and rainbow trout. The rationale for using these levels comes from previous work in which a maize gluten meal diet contained 19 mg/kg zinc and significantly lowered the zinc status and yet a fishmeal diet containing 26 mg/kg zinc did not. In order to visualize the dose response from the supplementation it is important to span this threshold. It has been decided not to supplement the diets above 50 mg/kg because above this level the fish seem to be able to regulate the zinc and very few differences can be deduced.

5.2 Methods

5.2.1 Experimental Design

A 10 week growth trial immediately followed by a one week stress experiment was conducted using a non-zinc supplemented diet and three sets of diets supplemented with increasing zinc levels of two different forms of zinc. All the diets were formulated using Feedsoft Pro™ and were isonitrogenous and isolipidic (Table 5.1). The unsupplemented diet was formulated to have a zinc content of 25 mg/kg, the low supplemented diets to have a zinc level of 30 mg/kg (5 mg/kg supplemented), the medium supplemented diets to have a zinc level of 40 mg/kg (15 mg/kg supplemented), and the high supplemented to have zinc level of 55 mg/kg (30 mg/kg supplemented). A zinc sulphate salt (Sigma, UK) was used as the inorganic zinc form and a yeast-based BioPlex zinc (Alltech, USA) was used as the organic zinc source. At the end of the 10 weeks the remaining fish were subjected to an intense regime of husbandry-like stressors.

5.2.1.1 Growth and Zinc Retention Trial

Fingerling rainbow trout (20-30 g) obtained from Torre Fisheries (Minehead, UK) were acclimated to the system for four weeks and fed a commercial diet (EWOS) to reach an average weight of 67.89 ± 3.37 g. A total of 475 fish were divided into 19 tanks (25 per tank). Each tank was randomly allocated a diet; three tanks for the unsupplemented diet, three tanks for the high supplementation for both zinc forms, three tanks for the low zinc supplemented diets in both zinc forms in and two tanks for both the medium supplemented diets. The fish were fed at 2% body weight per day (calculated every seven days) for 70 days. The initial sampling was conducted on day 0, prior to the first experimental feeding and the final sampling was conducted on day 70. Sampling consisted of initial weight-taking and analysis of the zinc levels in the eye, fin, liver, serum and whole body by ICP-OES (chapter 2.3).

Table 5.1 Diet Formulation (%) and composition

	Non-supplemented	Low $ZnSO_4$	Med $ZnSO_4$	High $ZnSO_4$	Low Zn BioPlex	Med Zn BioPlex	High Zn BioPlex
Casein	14.0	14.0	14.0	14.0	14.0	14.0	14.0
LT 94 Fishmeal	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Maize Gluten meal	37.0	37.0	37.0	37.0	37.0	37.0	37.0
Corn Starch	13.8	13.8	13.8	13.8	13.8	13.8	13.8
Fish Oil	19.0	19.0	19.0	19.0	19.0	19.0	19.0
Vit/min premix	1.0	1.0	1.0	1.0	1.0	1.0	1.0
L-lysine	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Y_2O_3	0.1	0.1	0.1	0.1	0.1	0.1	0.1
$ZnSO_4$	0.0	0.0022	0.0066	0.0132	0.0	0.0	0.0
BioPlex Zn	0.0	0.0	0.0	0.0	0.0041	0.0124	0.0249
Protein (%)	47.0 ± 0.6	46.6 ± 0.3	47.2 ± 0.1	46.5 ± 0.7	47.6 ± 0.5	47.7 ± 0.4	47.0 ± 0.1
Lipid (%)	21.8 ± 0.6	21.6 ± 0.8	22.1 ± 0.3	21.9 ± 0.6	22.0 ± 0.3	22.1 ± 0.5	21.7 ± 0.6
Zinc (mg/kg)	24.2 ± 0.3	29.2 ± 0.1	38.5 ± 1.4	57.8 ± 0.7	29.7 ± 0.5	39.7 ± 0.1	56.8 ± 0.2

5.2.1.2 Stress experiment

The effect of husbandry stress was assessed after seven days of alternate netting (twice a day for 1 min) and confinement (by reduced water levels for 45 min). Prior to the onset of the stress regime five fish per tank were removed from the experiment and blood and livers were sampled as unstressed controls. On day seven of the stress experiment the fish were netted once and then sampled for blood and livers. Analysis of both the stressed and unstressed individuals consisted of DNA strand break analysis by single cell gel electrophoresis (chapter 2, section 2.5), metallothionein levels (chapter 2, section 2.4.3), superoxide dismutase activity (chapter 2, section 2.4.2) and the gene expression of metallothionein A and B (chapter 2.6).

Table 5.2 *O.mykiss* gene specific primers for metallothionein genes (MTA and MTB) and housekeeping gene (18S Ribosomal RNA).

Gene	Forward (5'-3')	Reverse (5'-3')	Product (bp)	An. T (ºC)	Ref # (NCBI)
MTA	catgcaccagttgtaagaaagca	gcagcctgaggcacacttg	74	55	M18103.1
MTB	tcaacagtgaaattaagctgaaatacttc	aagagccagttttagagcattcaca	97	54	M18104.1
18S	cggaggttcgaagacgatca	tcgctagttggcatcgtttatg	62	53	FJ710874.1

5.2.2 Gene expression primers and validation

Primers (Table 5.2) were designed using Primer Blast (NCBI) and were designed to produce amplicons spanning 1 intron junction. Using a DNA calculator (Sigma-Aldrich) and OligoCalc (Northwestern University, USA) primers were designed to avoid secondary structures, hairpin formation and self-annealing complementarity. After RT- PCR (chapter 2, section 2.6.2) cDNA was subjected to 40 cycles of PCR amplification (chapter 2, section 2.6.3) and the amplicons size was verified on a 2% agarose gel with suitable DNA ladder (BioLabs, UK).

5.2.3 Statistics

Statistical analysis was performed using analysis of variance and a Tukeys multiple range test was applied to rank significantly different means. Computer software, SPSS v18 (IBM) was used to perform the statistical calculations; $p<0.05$ was considered statistically significant for

all analyses. Single cell gel electrophoresis data (% Tail DNA) was arcsine transformed prior to statistical testing for significance between means (n = 200 per fish, 5 fish per treatment).

Gene expression data was first assessed for the efficiency ($e = 10^{-1/slope} - 1$) of the qRT-PCR using a standard curve run on each plate and plates with efficiencies outside 0.9-1.1 were rejected for further analysis and the plates repeated/optimized. Comparative quantification ($2^{-\Delta\Delta Ct}$) was used to calculate expression fold-changes and all changes were normalized to the housekeeping gene. Treatments then were run over multiple plates and were standardized by resolving for slope and y-intercept of the standard curve with efficiency closest to 1.0 prior to comparative quantification.

5.3. Results

5.3.1 Growth

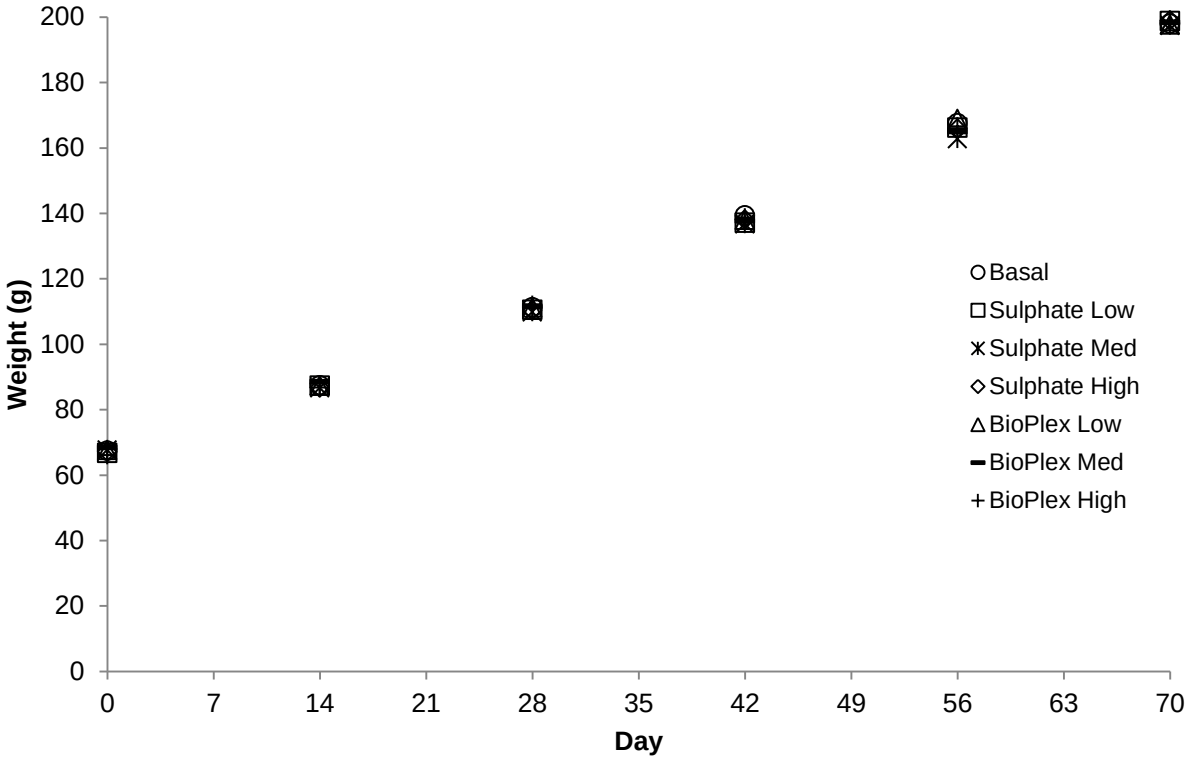

Figure 5.1 Individual fish weights over the 10 week trial period (mean ± S.D., n=3) * denotes significant difference between treatments.

The level of zinc and the form of zinc supplied by the diets had no significant effect on the growth (fig 5.1). The initial average weight of the individual fish ranged from 66.1 ± 1.6 g in the BioPlex high diet to 68.4 ± 1.8 g in the BioPlex medium diet and the final average weight of the individual fish ranged from 197.4 ± 4.3 g sulphate medium diet to 199.0 ±5.7g in the sulphate high diet. The maximum weight gained was 132.4 ± 7.4 g in the BioPlex High diet and the minimum weight gained was 129.7 ± 8.1 g in the sulphate medium diet. This resulted in no significant differences of FCR (which ranged from 0.98 in the high organic to 1.12 in the basal) or SGR (ranged from 2. 06 in the high organic to 1.97 in the basal) (Table 5.3). The apparent digestibility coefficients for each zinc form were statistically different (fig 5.2) the

Table 5.3 Diet performance (weight gain, FCR and SGR) (mean ± S.D., n=3)
Values in the same column with different suffix significant difference (p<0.05)

	Initial Weight (g)	Final weight (g)	Weight Gain (g)	FCR	SGR
Basal	67.5 ± 1.3	198.0 ± 8.1	130.5 ± 6.8	1.12 ± 0.07	1.97 ± 0.06
Low Sulphate	66.8 ± 1.7	198.8 ± 3.5	132.0 ± 1.8	1.12 ± 0.09	1.98 ± 0.09
Low BioPlex	67.7 ± 1.0	197.7 ± 7.2	130.0 ± 6.2	1.07 ± 0.03	1.99 ± 0.05
Med Sulphate	67.7 ± 1.8	197.4 ± 4.3	129.7 ± 8.1	1.05 ± 0.09	2.00 ± 0.07
Med BioPlex	68.4 ± 1.8	198.4 ± 4.6	130.0 ± 2.8	1.06 ± 0.06	1.99 ± 0.05
High Sulphate	67.2 ± 1.2	199.0 ± 5.7	131.7 ± 4.5	0.99 ± 0.08	2.05 ± 0.08
High BioPlex	66.1 ± 1.6	198.5 ± 9.0	132.4 ± 7.4	0.98 ± 0.07	2.06 ± 0.07

organic zinc was 37.4 ± 5.0% digestible and the inorganic zinc was 26.9 ± 4.4% digestible, a relative increase in digestibility of 38.8%.

5.3.2 Tissue Zinc Levels

The serum levels of zinc after 70 days on the respective diets are displayed in figure 5.3. The fish on the base diet, with 0.0 mg/kg zinc supplementation had a serum zinc concentration of 14.4 ± 2.1 mg/l. The 5 mg/kg supplementation of either zinc form did not result in a significant increase in serum zinc concentration, with the fish on the inorganically

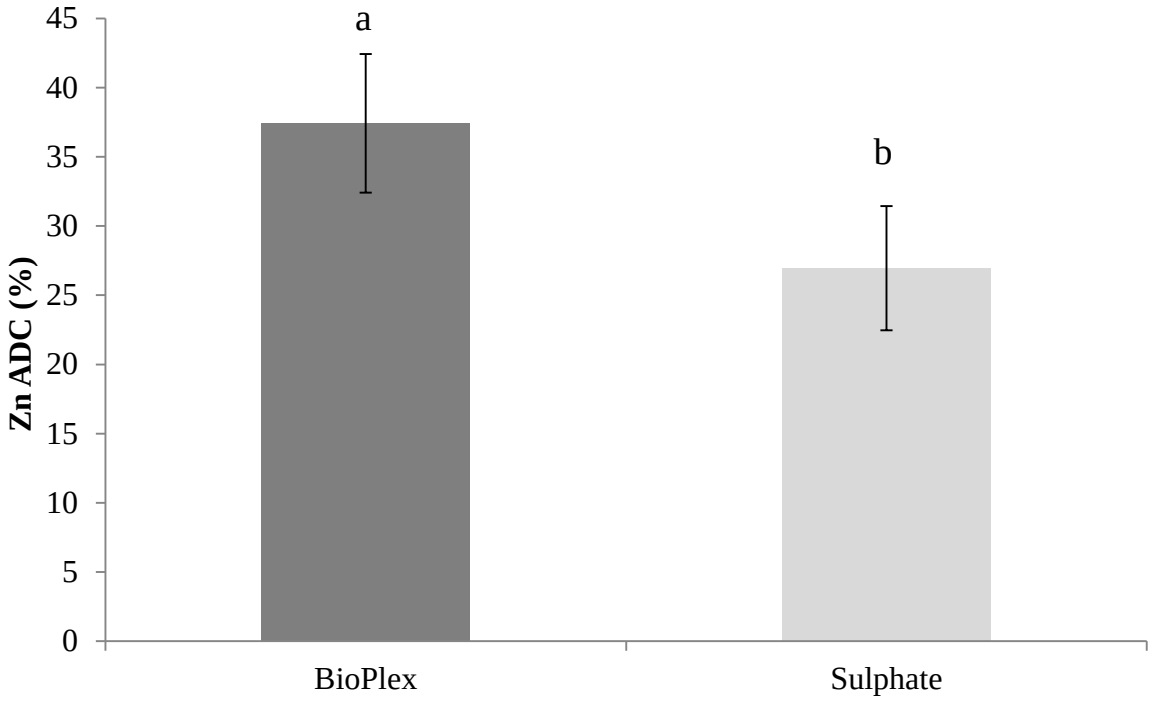

Figure 5.2 Zinc ADCs (%) (mean ± S.D., n=3) Bars with different suffix are statistically different (p<0.05)

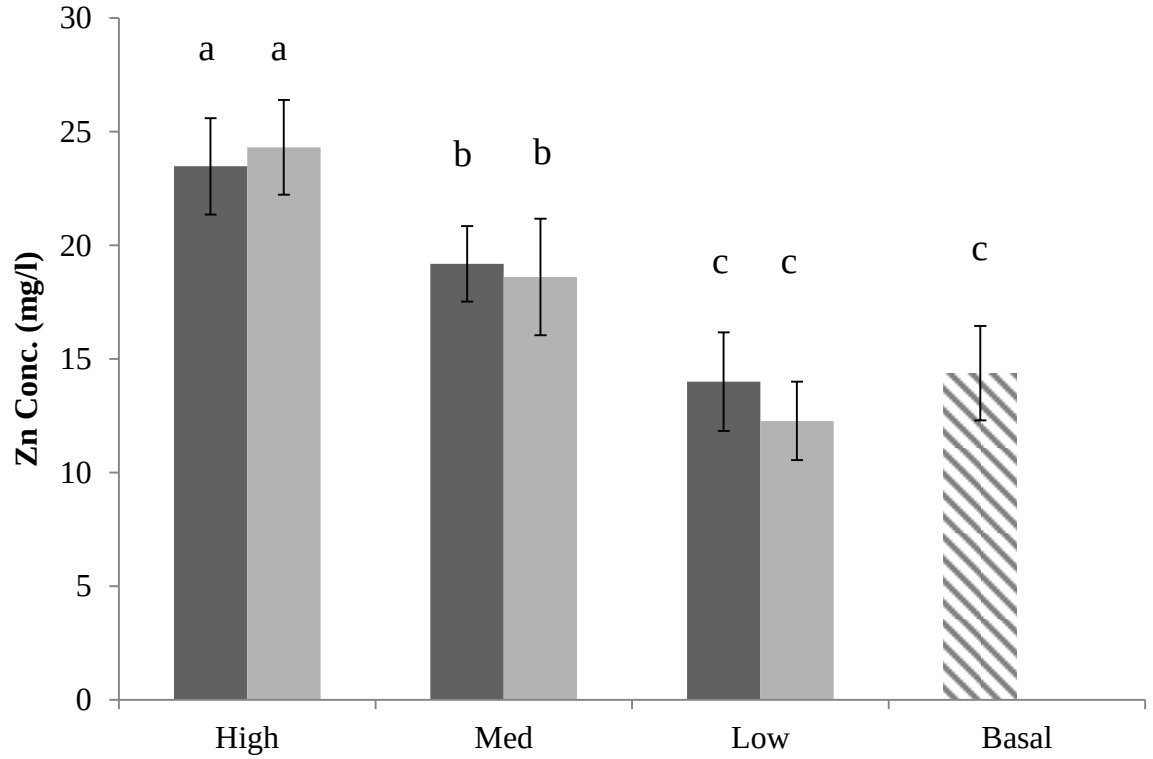

Figure 5.3 Serum zinc levels (mean ± S.D., n=3) Dark grey bar = organic zinc; light grey bar = inorganic zinc; Hatched bar = unsupplemented. Bars with different suffix are statistically different (p<0.05)

supplemented diet having 12.3 ± 1.7 mg/l and the fish on the organically supplemented diet having 14.0 ± 2.2 mg/l in their serum. The medium level supplementation of 15 mg Zn/kg diet did significantly increase the serum zinc concentration from both the base diet and the 5 mg/kg supplemented diet, reporting serum concentrations of 18.6 ± 2.6 mg/l from the inorganic zinc and 19.2 ± 1.7 mg/l from the organic zinc. The 30 mg/kg supplemented diets resulted in a significant increase in serum zinc concentration from all the other 3 levels of supplementation with serum zinc levels of 24.3 ± 2.1 mg/l from the inorganic zinc and 23.5 ± 2.1 mg/l from the organic zinc. The form of zinc fed to the fish had no significant effect on the serum zinc concentration at any of the supplementation levels used.

The ocular zinc concentrations of the fish after 70 days on the respective diets are displayed in figure 5.4. The 0 mg/kg supplemented diet resulted in the ocular zinc level falling to 96.3 ± 7.4 µg/g. The 5 mg/kg and the 15 mg/kg supplementation of the inorganic zinc did not significantly increase the ocular zinc levels from the base, unsupplemented diet, reporting levels of 105.9 ± 7.8 µg/g and 105.4 ± 8.2 µg/g respectively. The organic supplementation at the same levels (5 mg and 15 mg/kg) did significantly increase the ocular zinc concentration from both the unsupplemented diet and diet supplemented with an equal level of zinc in the inorganic form. The 5 mg/kg organic zinc supplementation resulted in levels of 123.7 ± 7.1 µg/g of zinc in the eye and the 15 mg/kg organic zinc supplemented diet resulted in an ocular zinc concentration of 127.0 ± 12.6 µg/g. In the diets supplemented with 30 mg Zn/kg, in either form, there was a significant increase in ocular zinc concentration from the unsupplemented diet, with levels of 138.1 ± 6.2 µg/g and 136.5 ± 19.9 µg/g in the organic and inorganic diets respectively. Within the organically supplemented diets there is no significant difference between the 5mg/kg supplemented diet and the 30 mg/kg supplement diets but all are significantly increased from the unsupplemented diets. In the inorganically supplemented diets the only significant increase in ocular zinc concentration from the

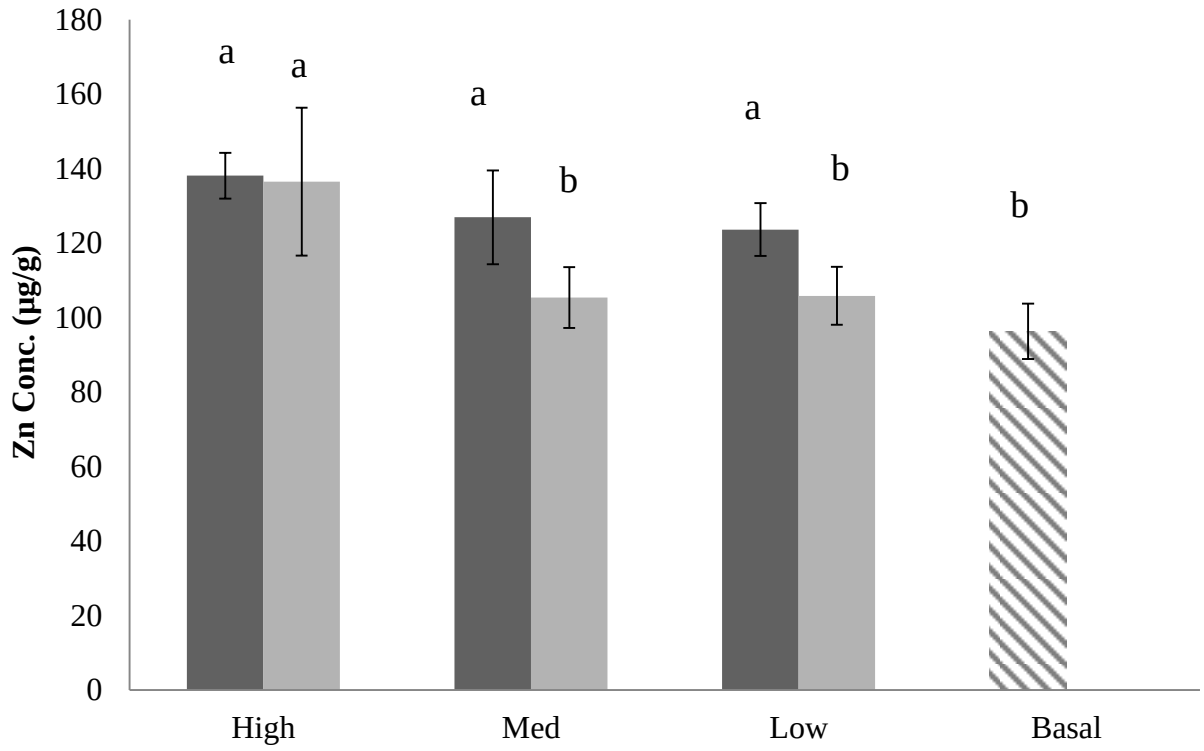

Figure 5.4 Ocular zinc levels (mean ± S.D., n=3) Dark grey bar = organic zinc; light grey bar = inorganic zinc; Hatched bar = unsupplemented. Bars with different suffix are statistically different ($p<0.05$)

unsupplemented diets can be seen in the highest level (30 mg/kg) of supplementation.

The caudal fin zinc concentration in fish fed the unsupplemented diet was 80.5 ± 7.6 µg/g (fig5.5). The 5 mg/kg supplementation of the organic zinc increased the caudal fin zinc levels to 89.9 ± 3.0 µg/g and the 5 mg/kg supplementation of the inorganic zinc increased the caudal fin zinc level to 83.0 ± 3.2 µg/g, neither of which were significantly different to the fish on the unsupplemented diet however the variance in the unsupplemented base diet is significantly larger than in all other groups. The increase in dietary zinc supplementation from 5 mg/kg to 15 mg/kg resulted in an increase in caudal fin zinc levels to 108.2 ± 1.9 µg/g in the organically supplemented fish and 108.1 ± 1.4 µg/g in the fish on the inorganically

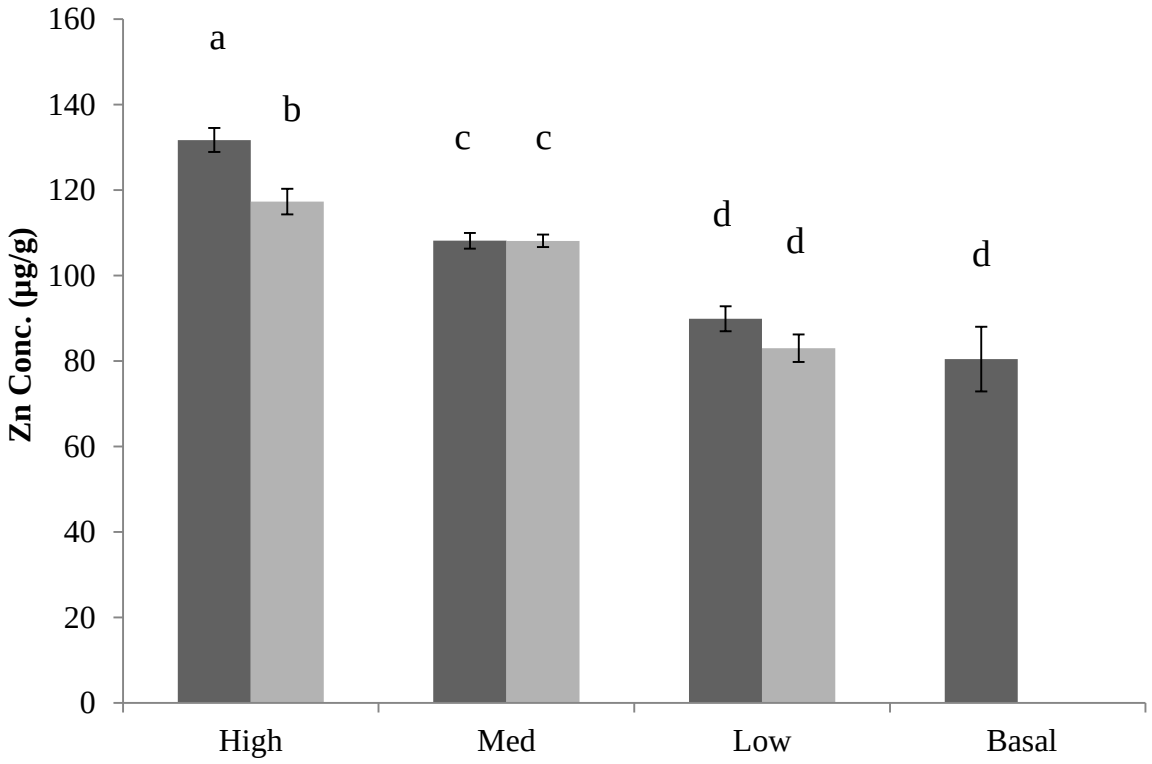

Figure 5.5 Caudal fin zinc levels (mean ± S.D., n=3) Dark grey bar = organic zinc; light grey bar = inorganic zinc; Hatched bar = unsupplemented. Bars with different suffix are statistically different ($p<0.05$)

supplemented diets. Both are significantly higher than in the fish fed the 5 mg/kg supplemented diets but not from each other. The highest level of supplementation resulted in a significant increase in caudal fin zinc levels from the medium level supplementation in both forms of zinc. The organic supplementation also resulted in a further, significant increase over the inorganically supplemented diet, resulting in levels of 131.7 ± 2.8 µg/g and 117.3 ± 3.0 µg/g respectively.

The hepatic zinc concentrations of the fish are displayed in figure 5.6. The fish on the unsupplemented diet had a hepatic zinc concentration of 99.2 ± 6.2 µg/g and the supplementation at all three levels and in both forms of zinc had no significant effect on this. The highest level of supplementation, an additional 30 mg Zn/Kg of diet, resulted in a hepatic

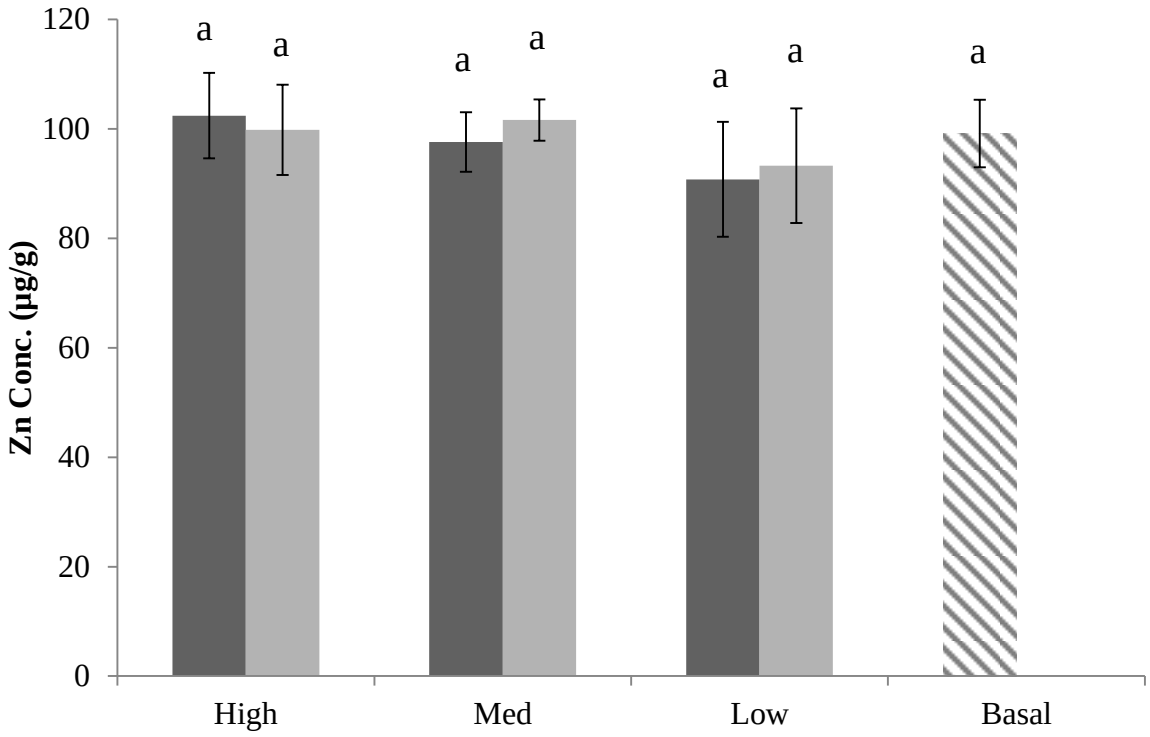

Figure 5.6 Hepatic zinc levels (mean ± S.D., n=3) Dark grey bar = organic zinc; light grey bar = inorganic zinc; Hatched bar = unsupplemented. Bars with different suffix are statistically different ($p<0.05$)

zinc concentration of 102.4 ± 7.8 µg/g and 99.8 ± 8.3 µg/g for the organic and the inorganic zinc respectively. The medium level of supplementation resulted in 97.6 ± 5.5 µg/g and 101.6 ± 3.8 µg/g for the organic and the inorganic zinc and the lowest level of supplementation resulted in 90.8 ± 10.5 µg/g with the organic zinc and 93.3 ± 10.5 µg/g with the inorganic zinc. The form of zinc at any of the levels of supplementation had no effect of the hepatic zinc concentration.

The concentration of zinc in the bile (fig 5.7) was negligible at all dietary levels. The negative results and large relative spread of data indicates that the levels were close to the limits of detection by the ICP OES with the levels of standards used.

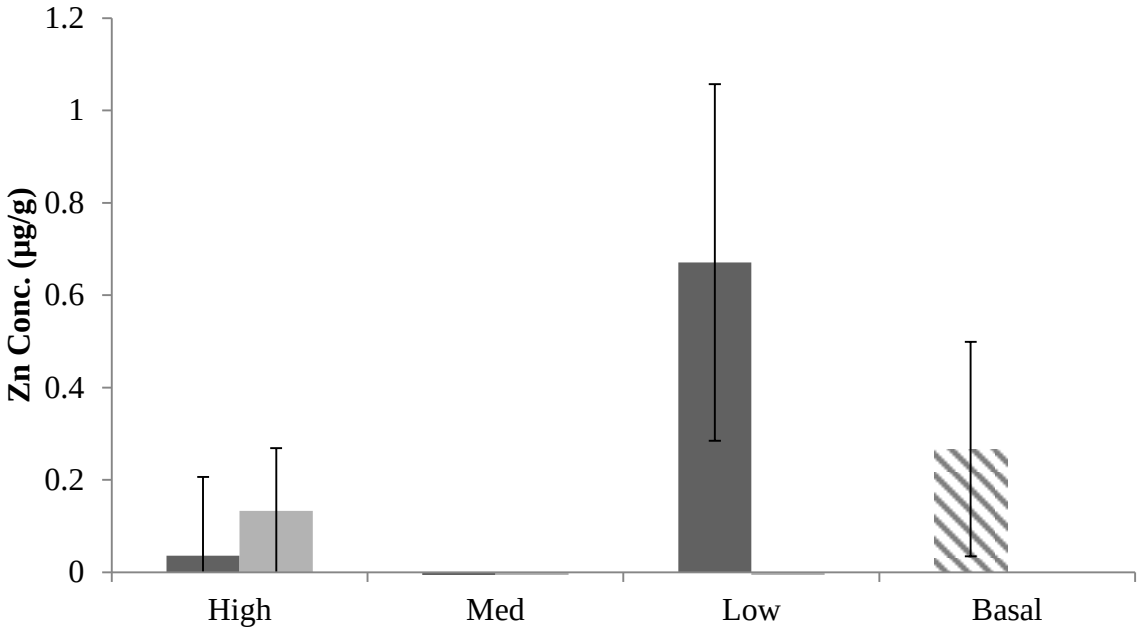

Figure 5.7 Bile zinc levels (mean ± S.D., n=3) Dark grey bar = organic zinc; light grey bar = inorganic zinc; Hatched bar = unsupplemented. Bars with different suffix are statistically different ($p<0.05$)

5.3.3 Zinc dependant proteins

Figure 5.8 displays the % inhibition of a water soluble tetrazolium salt (WST). The higher the activity of the enzyme superoxide dismutase the higher the inhibition of the WST reaction with a superoxide anion will be. With all dietary levels of zinc supplementation, in either form, as well as the unsupplemented diet, resulted in the inhibition of the WST/superoxide anion reaction by 86.89 ± 0.76% in the unstressed fish and 86.29 ± 0.59% in the stressed fish. This indicated that neither dietary zinc level, zinc form nor exposure to the simulated husbandry stressors affected the activity level of the superoxide dismutase.

The level of metallothionein increased in all treatments post stress. Figure 5.9 displays the amount metallothionein increased. The fish on the unsupplemented base diet increased the

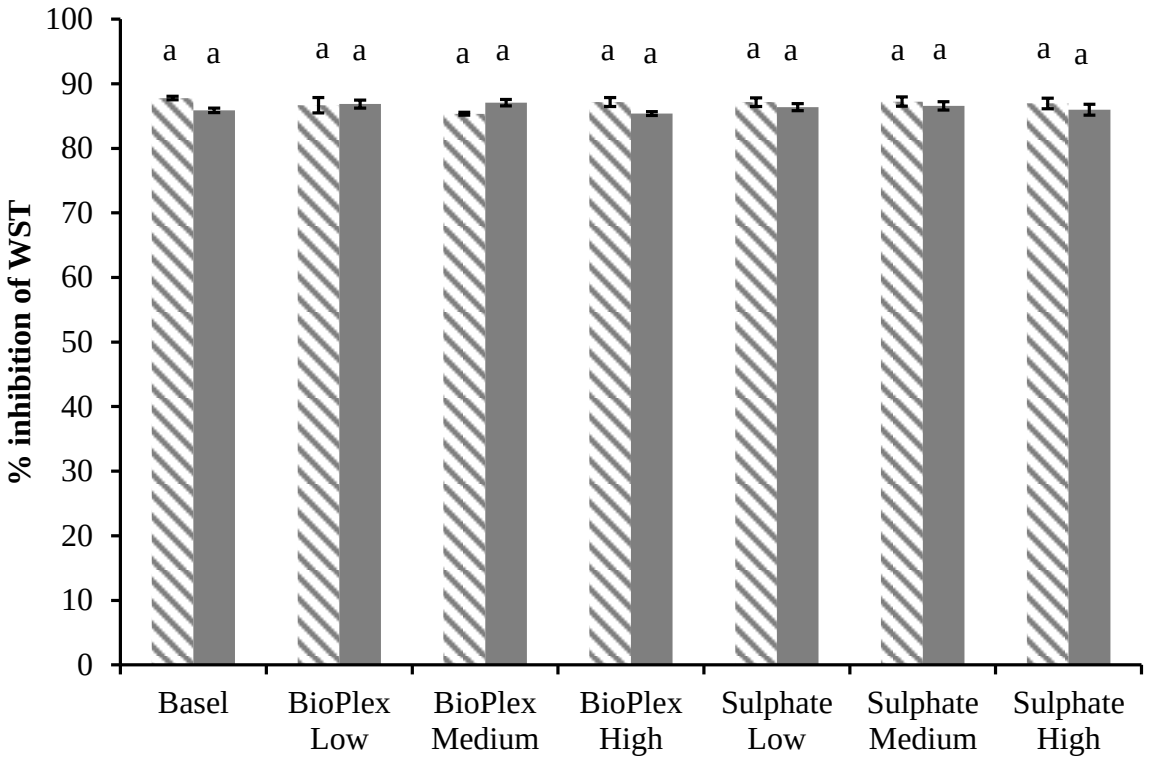

Figure 5.8 Percentage inhibition of the formation of WST as an indicator of superoxide dismutase activity. Hatched bar = unstressed fish; Solid bar = stressed fish. Bars with different suffix are statistically different (mean ± S.D. n = 5, $p<0.05$)

amount of metallothionein by 0.05 µg/g liver. The fish fed the inorganic zinc supplemented diets also increased the amount of metallothionein post stress but this increase was not significantly more than the increase in the fish on the unsupplemented diets nor was it affected by the level of supplementation. The fish on the highest level of inorganic zinc supplementation increased their metallothionein by 0.07 µg/g liver after the stress while the fish on the lowest level of supplementation also increased their metallothionein levels by 0.07 µg/g liver and the stress to the fish on the medium supplementation level also resulted in an increase of 0.07 µg/g. The fish fed the organic zinc showed a zinc mediated dose-dependent response to the stress, increasing the level of metallothionein post stress as the dietary level of zinc increased. The fish on the lowest level of organic zinc supplementation increased their metallothionein levels by 0.10 µg/g liver; the fish on the medium level of organic zinc

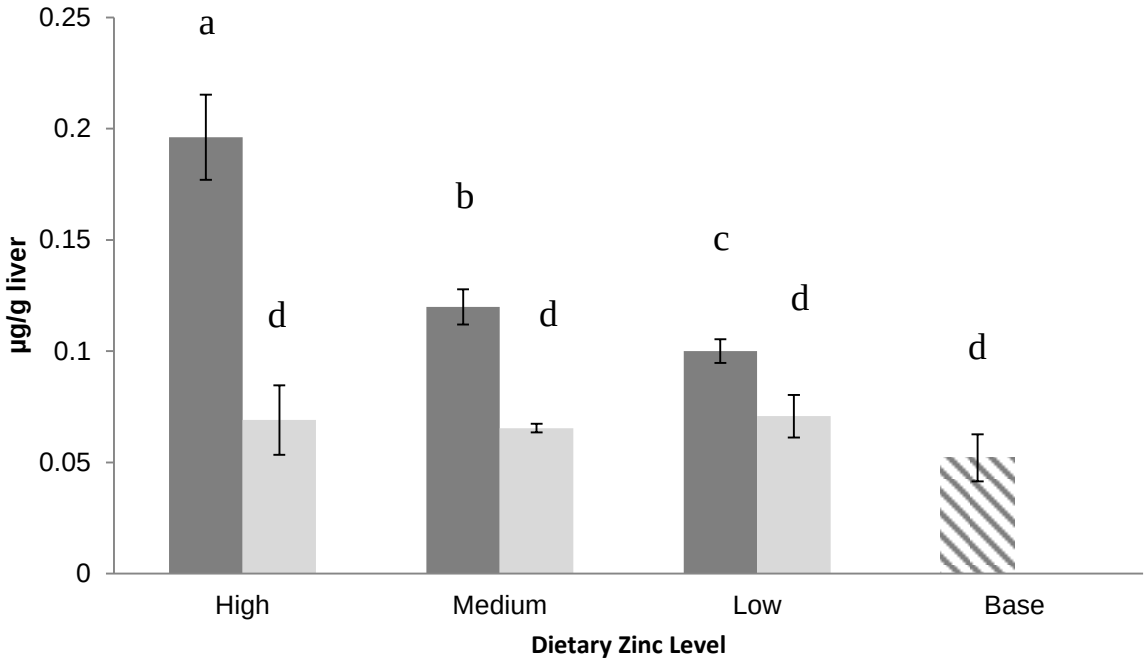

Figure 5.9 Increase in metallothionein levels post stress. Dark grey bar = organic zinc; light grey bar = inorganic zinc; Hatched bar = unsupplemented. Bars with different suffix are statistically different (mean ± S.D. n = 5, $p<0.05$)

supplementation increased their metallothionein levels by 0.12 µg/g liver and the fish on the highest level of organic zinc supplementation increased their metallothionein levels by 0.20 µg/g liver. All increases in metallothionein in the fish fed the organic zinc were significantly different to the fish fed either the level of zinc beneath theirs or the fish fed the inorganic zinc at an equal concentration.

5.3.4 Metallothionein gene expression

Exposure to the husbandry stressors induced expression of both MTA and MTB genes (figures 5.10 and 5.11). There was no significant difference between the expressions of MTA or MTB at any level of zinc supplementation or between MTA and MTB in fish feed either

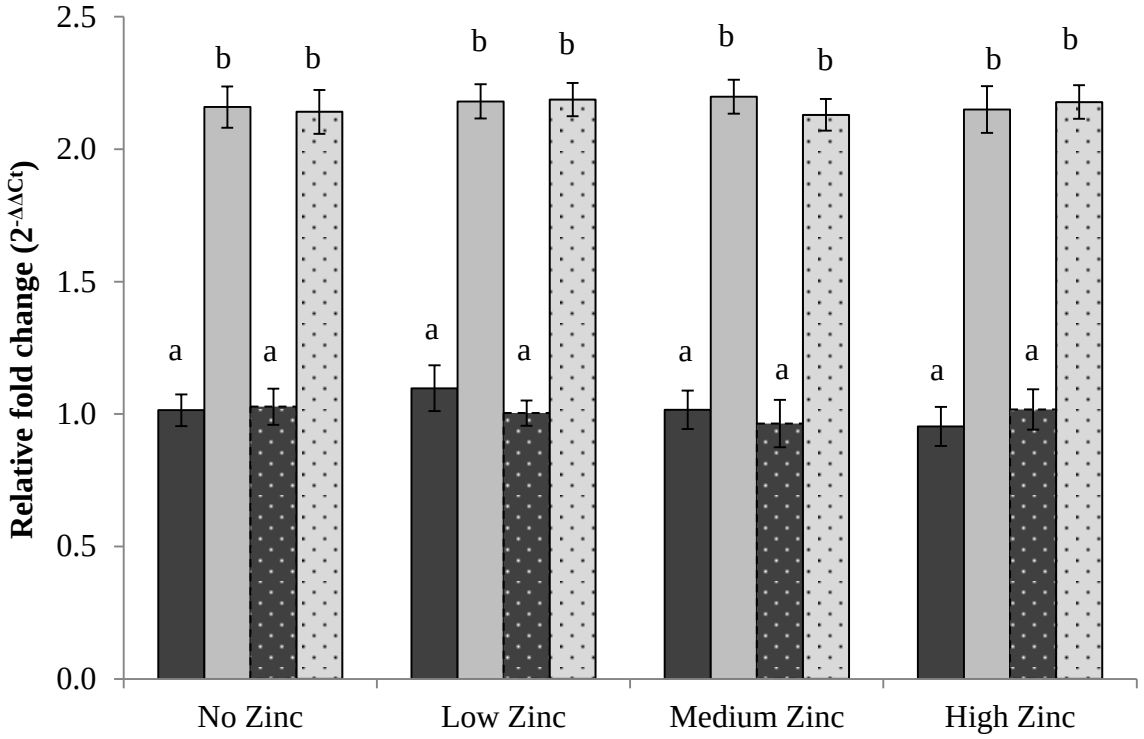

Figure 5.10 Relative fold changes in Hepatic MTA mRNA production ($\Delta\Delta^{CT}$). Solid dark grey = unstressed organic zinc; Solid light grey = stressed organic zinc; Doted dark grey = unstressed inorganic zinc; Doted light grey = stressed inorganic zinc. Bars with different suffix are statistically different (mean ± S.D. n = 5, p<0.05)

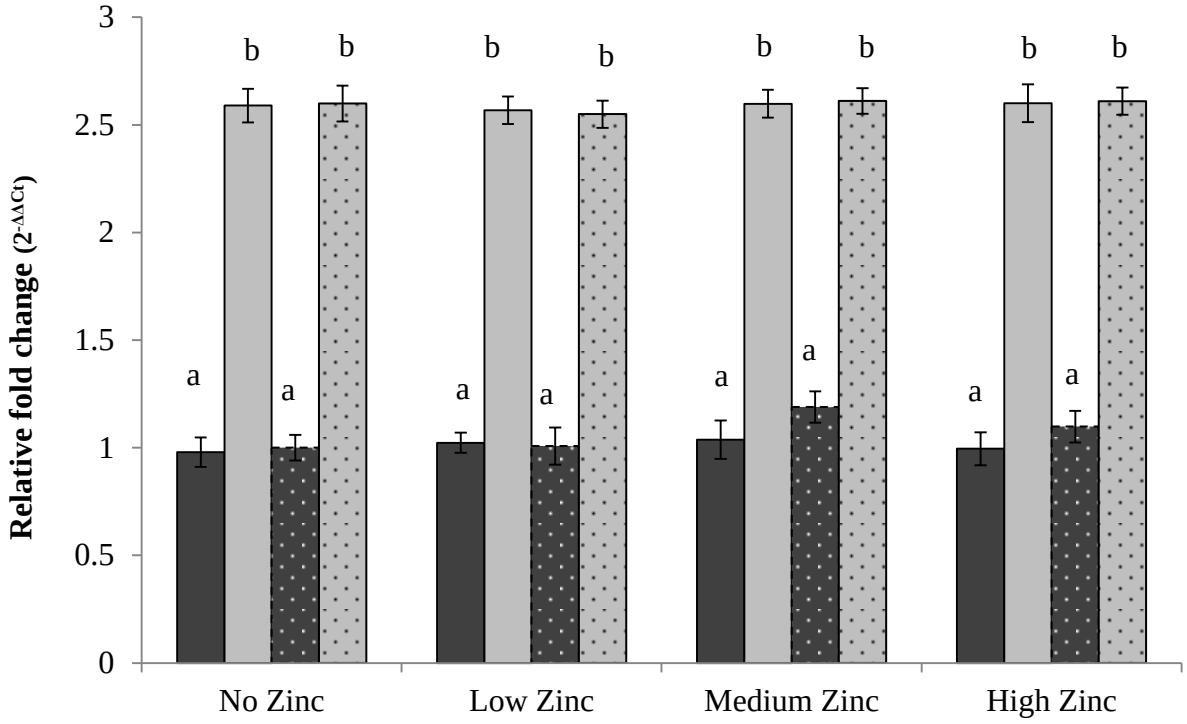

Figure 5.11 Relative fold changes in Hepatic MTB mRNA production ($\Delta\Delta^{CT}$). Solid dark grey = unstressed organic zinc; Solid light grey = stressed organic zinc; Doted dark grey = unstressed inorganic zinc; Doted light grey = stressed inorganic zinc. Bars with different suffix are statistically different (mean ± S.D. n = 5, p<0.05)

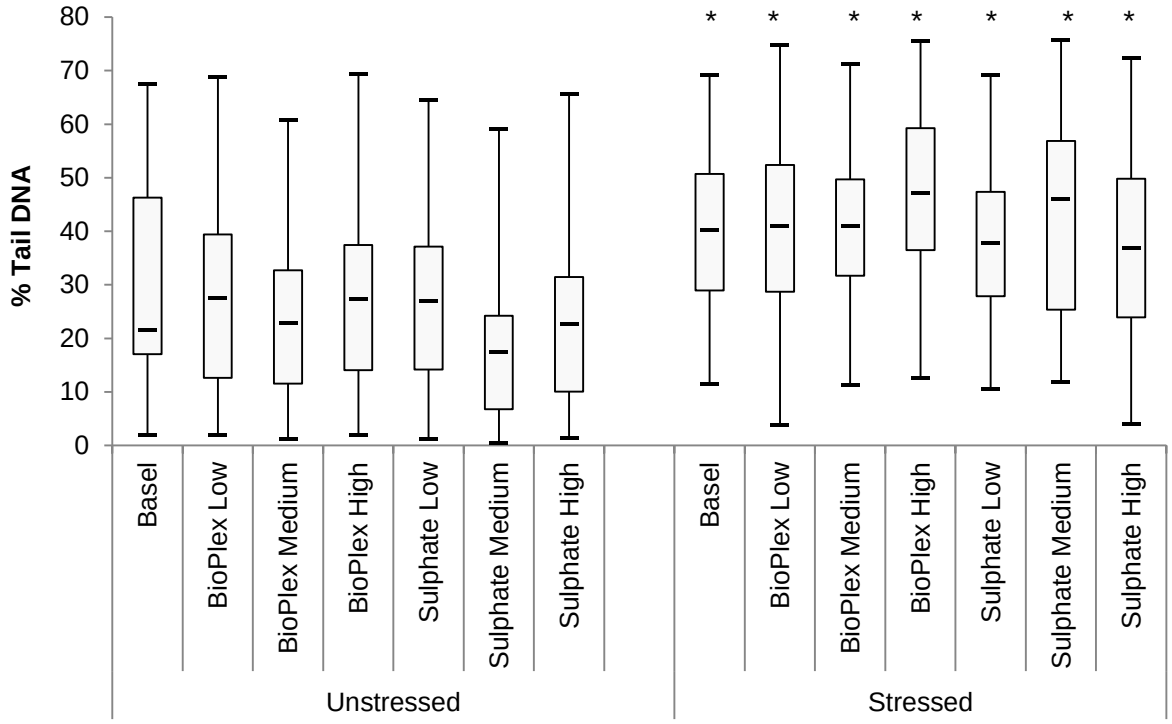

Figure 5.12 Percentage tail DNA. Boxes indicate 1st and 3rd quartile; Whiskers display minimum to maximum range; Markers indicate mean (n=5). * denotes significant differences (Kruskal-Wallis) between stressed and unstressed groups on the same diet.

of the zinc forms with in either stress status. There was a significant difference between the stressed and unstressed fish for both MTA and MTB at all levels of supplementation but not between different dietary levels. On average there was a 2.1 ± 0.03 fold increase in MTA and an average 2.6 ± 0.03 fold increase in MTB when the fish were stressed.

5.3.5 DNA damage

DNA strand breaks in the erythrocytes were induced by the stress regime. Mean control levels of DNA damage in the unstressed fish were between 17.4% (with a 1st - 3rd quartile range of 6.8% to 24.2%) in the medium level inorganic zinc fed fish to 27.4% (with a 1st – 3rd quartile range of 12.7% to 39.4) in the low level organic zinc fed fish. DNA damage

increased significantly in the stressed fish with a range of 36.9% to 47.2% in the high level organic zinc fed fish and the high level inorganic zinc fed fish respectively. There were no significant differences between fish fed different dietary concentrations of zinc in either form within the same stress status.

5.4 Discussion

Neither form of zinc nor the dietary level of zinc had any effect on the growth performance of the fish. The diets were iso-nitrogenous and iso-caloric and the zinc levels ranged from 25 mg/kg in the unsupplemented diet, which is within the 15-30 mg/kg requirement range (Ogino and Yang, 1978) to 55 mg/kg which is well above the requirement for this species i.e. rainbow trout. It should be noted that the zinc in the organic zinc source is not an organo-zinc (i.e Zn-Carbon bond). It is a yeast cultured in a zinc enriched broth in which the zinc is incorporated into the yeast structure, similar to an amino acid chelate but it is not only bound to one type of amino acid as in zinc methionine.

The high level of maize gluten in the diets did not reduce the level of available zinc sufficiently to cause growth impairments and all the diets resulted in good FCRs, comparable with commercial diets containing higher zinc levels. Similarly, Li and Robinson (1996) reported no significant difference in weight gain, FCR or survival of fish fed $ZnSO_4$, zinc methionine or a zinc proteinate at supplementation levels ranging from 15-90 mg/kg, however the base diet seems to already contain 50 mg/kg of endogenous zinc. The authors of that study also reported bone zinc concentrations increasing linearly with increasing dietary zinc which they state indicates a storage role of bone but also indicates the fishes zinc status is not reduced to detrimental levels by the base diet.

The organic form of zinc was 39.0% more digestible than the zinc from the inorganic salt. Similarly Apines et al. (2001) reported 52-57% absorption of zinc from an amino acid chelate compared to 38% absorption from the sulphate form when fed to rainbow trout and a second study by Apines-Amar et al. (2004) reported an elevated % absorption and retention of zinc when rainbow trout were fed an amino acid chelated zinc compared with an inorganic sulphate, again in rainbow trout. The same study reported equal % absorption when half the

amount of zinc was supplemented as the amino acid chelate compared with the sulphate salt. It has also been reported in channel catfish *(Ictalurus punctatus)* that a zinc proteinate was 37.5% more digestible than the sulphate form in a purified diet and 74.2% more digestible in a soyabean based diet (Paripatananont and Lovell, 1997). It has been postulated that the chelation of the zinc to an amino acid increases the rate of absorption by preventing the metal ion from forming insoluble complexes and aiding its transport into the mucosal cells (Ashmead, 1992).

As dietary zinc increased the serum levels increased equally for both forms of zinc. This indicates that the zinc from either form is being transported around the animal at equal levels despite the differences in digestibility suggesting that the extra zinc absorbed from the organic source is being used somewhere within the fish and is not in general circulation with in the vascular system. Wekell et al. (1986) also reported blood zinc levels in rainbow trout increased with dietary levels. Likewise a study using an egg white-based diet to achieve a zinc-deficient base diet with 4 mg/kg zinc reported an increase in blood, liver and gill zinc levels as the dietary zinc level was increased over a 55 day period with $ZnSO_4$ (Wekell et al., 1983). That trial was a toxicity trial and the next lowest level of supplementation took the dietary zinc level to 90 mg/kg and continued up to 1700 mg/kg, hence the rapid increase in blood zinc concentration as the zinc was transported around the fish and also in the gill, a probable excretory mechanism for the removal of excess zinc. Lorentzen and Maage (1999) also reported an increase in plasma zinc and whole body zinc concentrations as the dietary zinc increased from 67 mg.kg to 141 mg/kg with $ZnSO_4$ supplementation. The plasma levels increased from 12.8 mg/L to 26.3 mg/L, comparable with the results of this present study. Conversely, Maage et al. (2001) compared zinc gluconate to $ZnSO_4$ in Atlantic salmon (*Salmo salar)* and concluded that there was no difference between the two forms based on serum, vertebrate and whole body zinc levels after 172 days on the diets. The authors report

serum levels ranging from 17.8 mg/L in the lowest supplemented diet to 26.5 mg/L in the highest supplemented diet, which is again comparable to the levels reported in this present study, however their low and high supplementation resulted in dietary levels of 139 mg/kg and 239 mg/kg respectively, both well above the stated requirements of Atlantic salmon.

Satoh et al. (1987) reported cataract formation in trout fed a white fishmeal based diet. This required a supplementation of more than 40 mg/kg zinc, additional to the endogenous zinc in the white fishmeal, in order to prevent cataract formation. In this current study more zinc from the organic supplementation is being deposited in the eyes when the fish are being fed less than 40 mg/kg zinc in total. A higher level of zinc in the eye may provide more adequate antioxidant protection and help prevent cataract formation (Ketola, 1979) especially at time of rapid growth (Bjerkaas et al. 1995). There is some debate about the importance of the role of zinc in cataract formation when using practical diets (Breck et al., 2003) but this current trial suggests that the use of an organic source of zinc makes the diet better able to maintain ocular zinc levels when the requirement for zinc is only just met by the diet. Once the dietary zinc concentration reaches 55 mg/kg the difference between the two forms of zinc disappeared. This coincides with the increase in serum levels which may indicate either a threshold level of zinc in the serum, above which the zinc is able to be deposited in the eye or that the zinc pool in the eye is of lower importance than another tissue who's requirement may only be reached when the zinc in free transport reaches a certain level.

Zinc deposition in the caudal fin increases as dietary zinc and subsequently serum zinc levels increase but only after the dietary level reaches 40 mg/kg. Again Wekell et al. (1986) reported similar results indicating that caudal fin zinc levels in rainbow trout increased with dietary levels. The results from this section of the study, as with the eyes suggests the presence of a threshold level of zinc in the serum which once reached enables zinc to be deposited in other tissues. The high level of zinc in the caudal fin of the fish on the highest

level of organic supplementation compared with the same level of inorganic supplementation may also indicate that a tissue of 'higher importance' has reached its requirement level earlier or more easily when the organic form is used allowing for greater deposition of zinc into other tissues earlier.

The hepatic level of zinc remained the same throughout all the different levels and forms of supplementation indicating that the hepatic level of zinc was maintained at a level of around 100 µg/g. Whether this remains constant no matter how much zinc is available or whether this will increase at higher levels of dietary zinc supplementation is unclear from this experiment but when combined with the zinc concentration in the serum, eyes and caudal fin suggests that the liver is the first organ in the hierarchy where zinc deposition is concerned. This could be expected due to the metabolic importance of the liver and the liver is also likely to be the first place nutrients are transported to after absorption from the gut, and it may be this organ that regulated the amount of zinc free to be transported to other tissues. A similar result was seen previously by Wekell et al. (1986) who reported that liver zinc levels in rainbow trout remained constant as dietary levels increased. Lorentzen and Maage (1999) also reported the liver of Atlantic salmon maintained its zinc concentration as the dietary zinc levels increased from 67 mg.kg to 141 mg/kg, however the concentrations recorded in their study were much lower, approximately 30-35 µg/g and it should be noted that their experiment was carried out on 0.2 g fingerlings with a final weight of only 30 g. Slightly contradictory, an amino acid zinc chelate did increase the hepatic zinc level, as well as the bone zinc level, of rainbow trout more than a sulphate form was able to achieve (Apines et al., 2003).

The negligible amount of zinc in the bile indicates that no zinc is being excreted back into to gut lumen for removal from the organism. This has two important implications: 1) the animal is trying to maintain its overall zinc pool and has no excess zinc to excrete; and 2) the results

of the digestibility measurement have not been confounded by excess zinc being excreted back into the digesta which would them be analysed as undigested zinc, artificially lowering the ADC for a particular diet.

Of the two zinc dependant proteins analysed only metallothionein was affected by the dietary zinc. Superoxide dismutase was not affected by either the level or supplementation form, nor was it affected by the imposition of the stress regime. Superoxide dismutase is found in several isoforms and may be either Cu/Zn dependent or Mn dependent. The fact that the dietary concentration of zinc had no effect indicates that the manganese dependant form is either the dominant form in rainbow trout or that the cells can compensate for the lack of zinc by increasing the manganese form. Apines-Amar at al. (2004) reported an increase in Cu/Zn SOD activity when rainbow trout were fed an amino acid chelated zinc however these authors also used a chelated form of copper which may have been responsible for the up regulation of the enzyme. Also a study by Hidalgo et al. (2002) into the oxidative stress generated by a dietary zinc deficiency identified a possible compensatory effect whereby the induction of the Mn-SOD isoform of the enzyme is stimulated when the Cu/Zn forms activity is reduced. Similar results have been observed in rats fed a Cu deficient diet (Lai et al., 1994; Taylor et al., 1988). The analysis performed on the superoxide dismutase activity in the present study was unable to distinguish between the two forms; therefore the ratio of one to the other could not be established. As the simulated husbandry stressor did not increase the activity of the enzyme this would suggest that prolonged netting and reduced water level did not induce superoxide free radical formation. However the level of activity in the unstressed fish was already high.

The molecular mechanisms for the metal induction of the metallothionein gene expression is dependent on the interaction between a *cis*-acting element in the gene (Metal Responsive Element, MRE) and a *trans*-acting factor (MRE binding transcription factor 1, MTF-1) which

reversibly binds to the MRE and mediates the induction of the metallothionein gene (Westin and Schaffner, 1988; Andrews, 2001). The binding of the MTF-1 is dependent on the availability of free zinc ions in the cytoplasm (Radtke et al., 1993; Dalton et al., 1997; Laity and Andrews, 2007). The zinc required for this activation has been shown to be able to enter the nucleus of the cells from a dietary source in rats (Cousins and Lee-Ambrose, 1992). Zinc has been shown to cause a dose-dependent increase in metallothionein induction in the immortalised liver cell line of zebra fish (Cheuk et al., 2008)

In this present study stress increased the metallothionein activity in the fish on all the supplemented diets as well as in the fish on the unsupplemented diet. This was supported by an increase in the mRNA levels for both isoforms of metallothionein in the livers of the fish that had been stressed. Simulated husbandry stress has also been shown to increase metallothionein gene expression in both the brain and kidney of rainbow trout using microarray technology (Krasnov et al., 2005). The increase in mRNA production was not affected by the dietary zinc level or form, indicating the increase in expression was solely due to the stress. There is good evidence that glucocorticoids can induce metallothionein expression in humans and mice independently of metal regulatory transcription factors (Dalton et al., 2000; Kelly et al., 2000) and various studies have shown that different types of stress induce the production of metallothionein in the livers of goldfish (Carpene et al, 1992), the crucian carp (Muto et al., 1999) and the buffalo sculpin (Sabourin et al., 1985). Cortisol has also been shown to increase metallothionein production in the primary cultures rainbow trout hepatocytes but not in the immortalised rainbow trout hepatocyte cell line RTH-149 (Olsson et al., 1990). It has since been hypothesised and subsequently shown that the cortisol produced by the stressors may indirectly cause the metallothionein production by interacting with the zinc signalling pathway and increasing the amount of free zinc in the cytoplasm and set off the MTF-1/MRE cascade (Bury et al., 2008). In the current study the stress also

increased the gene expression uniformly across the groups of fish on different dietary treatments. With the increase in expression being uniform, it would be expected to observe a uniform increase in the metallothionein production, however, in the fish on the organic supplemented diets the level of increase was not uniform and was positively correlated with the level of supplementation. As the fish on the inorganically supplemented diets showed a much lower and uniform increase across the supplementation levels this would suggest a deficiency in these fish of an integral component of the protein. There must have been a sufficient supply of all the components required to synthesis the required level of metallothionein in the unstressed fish fed the unsupplemented diet as there was extra capacity to increase the production of the protein to some extent in this group when they were subjected to the stressor. The same level of capacity was also possessed by the fish on all the diets supplemented with the inorganic zinc which implies that the inorganic supplementation did not increase the supply of the limiting component and the small increase observed was due to the zinc endogenous to the casein, fishmeal and maize gluten meal in the diet. The ability of the fish fed the organically supplemented diet to increase their metallothionein production in a dose-dependent manner points strongly towards the zinc being the limiting component and it was only bioavailable when presented in the organic form. It is significant to note that coupled with this observation is the fact that the hepatic zinc levels were equal in all the fish across the range of supplementation form and level, indicating that the zinc is present when the fish are supplemented with the inorganic form but it evidently cannot be utilized. This could be evidence that the element is bound differently or to a different chaperone depending on the form of zinc supplied by the diet. In other studies investigating different zinc-dependant enzymes, Apines et al. (2001) showed a higher activity of the zinc dependant enzyme alkaline phosphatase in rainbow trout fed an amino acid chelate compared with those fed the sulphate form indicating a greater utilization of the element from the

chelate for the production of this enzyme. A similar result has been shown in abalone-fed organic zinc (Tan and Mai, 2001).

Normal metabolism produces reactive oxygen species (ROS), a fraction of which are used for cell signalling. The remaining ROS are detoxified by antioxidants which convert them into non-toxic metabolites, preventing them causing oxidative damage to cellular macromolecules such as DNA. It is essential that this balance is maintained to avoid the onset of oxidative stress (Haliwell and Gutteridge, 1999). Physiological stress causes the reallocation of energy to increase activities such as locomotion and respiration, an evolutionary trait relating to the fight or flight response, which in captivity i.e.in aquaculture is of little or no benefit. Under stress conditions fish switch from an anabolic to a catatonic state, which is a primary stress response and involves the release of numerous hormones including catecholamine and glucocorticoids such as cortisol (Wendelaar-Bogna, 1997). A secondary effect of the stress response, especially when the stress is chronic as opposed to acute is the loss of DNA stability due to the action of the excess ROS species, created by the increase in metabolism, causing strand breaks and the oxidation of nitrogenous bases. The stress regime that the fish in this experiment were subjected to, was chronic and induced physiological responses similar to those associated with exercise that is exhaustive in nature. The damage to DNA caused by such exertions has been studied in humans (Hartman et al., 1994) and rats (Leeuwenburgh and Heinecke, 2001; Packer et al., 2008; Sachdev and Davies, 2008). The increase in % tail DNA in the stressed fish is evidence that chronic stress does cause damage to te genetic material in the erythrocytes, indicating that the ROS are increased to a level exceeding the capacity of the fish to safely detoxify the free radicals produced. The lack of a significant decrease in the level of damage, as more dietary zinc is added, suggests that this antioxidant capacity is not totally zinc dependant. The observed increase in % tail DNA does provide validation of this technique as a way of assessing the stress status of fish in a non-

terminal way, which may be preferable to measuring cortisol levels which is notorious for being confounded by the acute stress caused when sampling.

The increased digestibility of the organic zinc compared with the inorganic zinc reported in this present study should be considered an accurate indication of the uptake of the zinc from the gut due to the low levels of zinc used and the absence of zinc in the bile. This should not be confused with its availability, nor should the increase in tissue concentrations be used to assume a greater bioavailability. The true indicator of the availability of a nutrient must come from the activity and levels of its dependant proteins within the animal's cells, not just its digestibility or the tissue concentration (Swinkels et al., 1994); a fact often overlooked in the literature. The metallothionein results of this study clearly provide evidence that the zinc from the organic form of supplementation is not only more digestible but also more bioavailable and utilizable by rainbow trout than the inorganic sulphate salt, especially at times of stress characteristic of culture conditions.

CHAPTER 6. The use of mixed isotope diets to assess the distribution of dietary zinc from inorganic and organic sources

6.1 Introduction

It is common practice to assess mineral absorption, digestibility and bioavailability by the use of an element balance technique, namely the difference between the dietary intake and the faecal content or tissue levels of the mineral. However, minerals with low digestibility or when studying animals with high endogenous mineral stores this balance technique is unable to distinguish the dietary supplied minerals from the endogenous, which may have been either excreted back into the digesta or mobilised from storage pools for use in tissues with a metabolic demand (Sandstrom et al., 1993). The studies reported in previous chapters have tried to eliminate this confounding effect by using diets bordering on deficient and checking that the bile concentration of the mineral is negligible. However, the use of a stable but enriched isotope to label a fraction of the supplemented mineral should allow the endogenous minerals and dietary minerals to be distinguished.

The natural abundance of each isotope of zinc is:

^{64}Zn - 48.89%
^{66}Zn - 27.81%
^{67}Zn - 4.11%
^{68}Zn - 18.56%
^{70}Zn - 0.62%

The use of a stable enriched isotope in a component of the diet, to alter the natural abundances above, can allow that dietary component to be tracked though the body. Having a known, unnatural, ratio of isotopes in the diet (a fixed percentage coming from an inorganic sulphate source enriched with ^{70}Zn and the remaining supplementation from the organic

source with the natural ratio) the biological 'preference' of the tissues can potentially be observed by measuring the changes in the tissue specific isotope ratios.

The use of stable enriched isotopes to assess mineral kinetics in fish is, to the best of the authors knowledge at the time of writing, completely novel. However their use is a relatively common technique in other species such as rats (usually for medical use), dogs and pigs (Popov and Besel, 1977; House and Wastney, 1997; Dunn and Cousins, 1989; Lowe et al., 1995; Serfass et al., 1996).

The study in rats by House and Wastney (1997) identified the existence of slow and fast pools in muscle and bone where the turnover of zinc in these tissues was dramatically different. Turnover rates were reported to be 4hrs, 1.3 days and 50 days in the kidney, spleen and testes respectively; rates consistent with the kinetics in humans. It may be possible to make similar observations in rainbow trout and investigate whether the form of zinc affects the turnover rates. In this current study, the findings in Chapter 3 have indicated that the tissues throughout the fish can be divided into groups containing zinc pools termed either functional or exchangeable (table 6.1). The functional pools are sites where zinc is of

Table 6.1 The division of body tissues into those containing either functional or exchangeable zinc pools

Functional	Exchangeable
Liver	Bone
Fins	Blood (serum)
Eyes	Muscle
Gills	Intestine
Skin	Bile

metabolic importance and the zinc levels remain constant for as long as possible. The exchangeable pools are pools in which the zinc levels fluctuate rapidly, either storing excess zinc or supplying surplus zinc when required in other areas of the body.

Serfass et al. (1996) showed that weaned piglets fed a deficient diet compensated by increasing the absorption of zinc from the diet and the authors were able to track the zinc throughout the body tissues. It was reported that despite the increasing fractional absorption the hepatic zinc levels remained lower in the deficient piglets compared with the control animals. In the Serfass study, no investigations into the functional ability of the liver was made, failing to answer questions about the true status of the organ i.e. was it deficient or was it simply not storing excess zinc. It may be possible to identify similar trends in rainbow trout, however from the previous chapters findings it has been demonstrated that the functionality of the liver is different in trout fed the organic zinc compared with the trout fed the inorganic zinc at these levels. Tracking the uptake rates of each form may provide insight as to why those effects are seen.

Dunn (1991) investigated the role of metallothionein and its involvement in the redistribution of zinc among the body tissues of rats. The authors reported that the induction of metallothionein caused an increase in the redistribution of zinc among body tissues. The previous chapter identified a superior ability of the organic form of dietary supplementation to stimulate metallothionein production. If the increased ability of metallothionein production can be correlated with the redistribution of zinc around the rainbow trout tissues that are of zinc metabolic importance it may be possible to identify the role of this increased metallothionein production which could not be attributed to its role in oxidative protection.

This study aimed to 1) quantify the rate of uptake of dietary zinc from the digesta into the bloodstream; 2) measure the effect of feeding low zinc diets on the metabolically important

tissue levels each week of a 12 week period; 3) assess the ‘preference’ of the metabolically important tissues for the inorganic or organic forms of zinc; and 4) draw some initial conclusions about the suitability of using mixed isotope diets and the experimental design for future work in this area. For this study only the eyes, liver and fins where analysed as they are tissues known to be affected by this feeding regime. The time and cost of analysis became prohibitive for speculative analysis on all tissues to be conducted at the time.

6.2 Methods

6.2.1 Experimental Design

Two separate trials were conducted, both using the same base diet as used in chapter 5 and supplementing the diet to the same extent to achieve a set of low, medium and high zinc diets containing 30, 40 and 50mg Zn/kg diet respectively (table 6.2). The supplemented zinc was 1:1 mix of an organic source, with a natural isotope ratio giving an average atomic mass of 65.37, and an inorganic zinc salt, $ZnSO_4$, with a 95.43% enriched ^{70}Zn isotope (with an average atomic mass of approximately 69.4). All diets were analyzed for protein, lipid and total zinc content as well as isotopic ratios of ^{66}Zn, ^{67}Zn or ^{68}Zn: ^{70}Zn (table 6.3).

Trial 1: Post Prandial uptake

A total of 160, pre-acclimated (Chapter 2, section 2.1.2) rainbow trout (*O.mykiss*), weighing 103.4 ± 2.5 g were divided into 6 x 126 liter tanks (30 fish per tank) and fed the unsupplemented control diet (table 6.2) for 26 days at 1% body weight per day and food was withheld on days 27 and 28. On day 29, three fish were removed and sampled for blood and each tank was allocated either the high zinc level diet supplemented with only the organic form of zinc or the high zinc level diet supplemented with only the inorganic form of zinc (n=3). The remaining fish were fed the allocated diet to 4% body weight, resulting in a feed ration of 258 µg zinc. Blood samples were taken at 2, 4, 6, 8, 12, 16, 20, 24 and 30hrs post feed from three fish in each tank. These were allowed to clot and their serum separated. Serum was analyzed for total zinc using ICP OES and the uptake and subsequent decrease in serum zinc levels tracked.

Table 6.2 Diet Formulation; % inclusion of base diet ingredients and mg/kg of zinc supplementation.

	Control Diet	Low Zn Diet	Medium Zn Diet	High Zn Diet
Casein	14.0	14.0	14.0	14.0
LT 94 Fishmeal	15.0	15.0	15.0	15.0
MGM	37.0	37.0	37.0	37.0
Corn Starch	13.9	13.9	13.9	13.9
Fish Oil	19.0	19.0	19.0	19.0
Vitamin/Mineral Premix	1.0	1.0	1.0	1.0
L-Lysine	0.1	0.1	0.1	0.1
$^{70}ZnSO_4$ (mg/kg)	0	2.5	7.5	12.5
BioPlex Zn (mg/kg)	0	2.5	7.5	12.5

Trial 2: Tissue assimilation

A total of 600, pre-acclimated (Chapter 2, section 2.1.2) rainbow trout (*O.mykiss*), weighing 10.1 ± 0.5 g were divided into 12 x 126 liter tanks (50 fish per tank). Each of the four diets was randomly allocated to three tanks and the fish were fed a constant 3% body weight per day, adjusted daily using a predicted FCR of 1.0 for 10 weeks. Five fish from each tank were sampled each week for the four weeks and then every two weeks until week 10. The liver, eye and caudal fin were sampled, then analyzed at each time point for both the total zinc content and the ratio of ^{66}Zn:^{70}Zn, as the tissue turned over its zinc pool.

Table 6.3 Proximate composition of diets and ratios of zinc isotopes (mean ± S.D. n=3)

	Control Diet	Low Zn Diet	Medium Zn Diet	High Zn Diet
Protein (%)	46.7 ± 0.2	47.1 ± 0.2	46.5 ± 0.4	46.7 ± 0.2
Lipid (%)	21.9 ± 0.1	21.9 ± 0.5	21.8 ± 0.4	22.1 ± 0.3
Total Zn (mg/kg)	24.8 ± 0.5	30.2 ± 0.2	41.0 ± 0.6	50.5 ± 0.8
^{66}Zn: ^{70}Zn	45.0 ± 0.37	2.9 ± 0.28	1.24 ± 0.32	0.86 ± 0.14
^{67}Zn: ^{70}Zn	6.61 ±0.25	0.44 ±0.13	0.18 ±0.03	0.13 ±0.02
^{68}Zn: ^{70}Zn	30.24 ±0.27	2.06 ±0.12	0.87 ±0.05	0.62 ±0.02

6.2.2 General analytical procedures

The diets were analyzed for protein (Chapter 2, section 2.2.4), lipid (Chapter 2, section 2.2.3) and trace element profile (Chapter 2, section 2.3). The livers, eyes and caudal fins were sampled, freeze dried and then measured for their total zinc content (ICP OES) and their isotopic ratios (ICP-MS). All isotope counts were blank subtracted and standardized per mg of tissue assayed before ratios were calculated. Bracket standards (a TraceCERT® Zinc Standard with a known isotopic ratio used either side of an unknown in order to standardize the samples for drift) were used every other sample and the mass bias was calculated. Serum was also processed in the same way as the tissues, with an acid reflux digestion (Chapter 2, section 2.3.1) prior to dilution and analysis for total zinc which was was conducted using ICP OES.

6.2.3 Calculations

Table 6.4 The theoretical natural abundance ratios of Zn

^{64}Zn : ^{70}Zn	78.4
^{66}Zn : ^{70}Zn	45.0
^{67}Zn : ^{70}Zn	6.6
^{68}Zn : ^{70}Zn	30.2

The process in the differentiation of two isotopes using ICP MS incurs a mass bias, derived from the differential transmission of ions of different masses from the point at which they enter the sampling device until they are finally detected. This bias results in fewer of the lighter isotopes being detected and a lower ratio of one isotope to the other than is representative of the sample i.e. less ^{66}Zn is detected than ^{70}Zn. A modified method of standard-sample bracketing (Albarede and Beard, 2004) with internal normalization for mass bias correction was employed using natural zinc ratios as the bracketing standard. Each $^{i}Zn/^{70}Zn$ ratio ($^{64}Zn/^{70}Zn$, $^{66}Zn/^{70}Zn$, $^{67}Zn/^{70}Zn$ and $^{68}Zn/^{70}Zn$) in the bracketing standard, in conjunction with the IUPAC ^{65}Zn ratio (table 6.4), was used to separately calibrate an individual $^{i}Zn/^{70}Zr$ ratio for each 'unknown' sample. The interference from residual ^{70}Ge on ^{70}Zn was corrected for by simultaneous measurement of ^{72}Ge and the use of the IUPAC $^{72}Ge/^{70}Ge$ ratio of 1.297, which was itself mass bias corrected.

6.2.4 Statistics

Statistical analysis was performed using analysis of variance and a Fishers test of least significant difference was applied to rank significantly different means. Computer software, SPSS v18 (IBM) was used to perform the statistical calculations; $p<0.05$ was considered statistically significant for all analyses.

6.3 Results

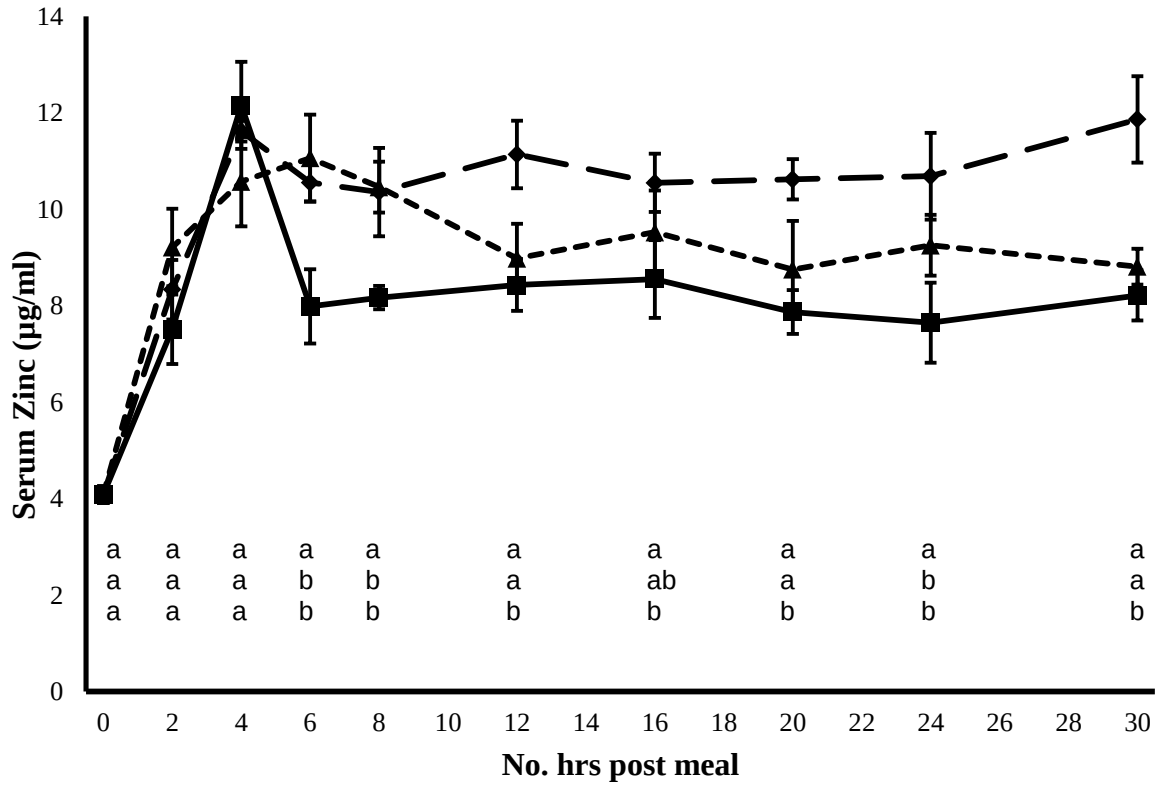

Figure 6.1 Post prandial serum zinc levels. (mean ± S.D. n=3). Solid line = unsupplemented; Long broken line + diamonds = organic zinc; Short broken line + triangles = inorganic zinc. Different letters in the same column indicates significance between diets, top row = unsupplemented; middle row = inorganic zinc; bottom row = organic zinc.

6.3.1 Post prandial serum levels

The results of the post prandial serum zinc levels are displayed in figure 6.1. The initial serum zinc level in all the fish, before the presentation of feed was 4.03 ± 0.17 µg/ml. The serum zinc levels in the fish on the control diet peaked at 12.16 ± 0.90 µg/ml 4 hrs post feed and then decreased to a level of 7.99 ± 0.77 µg/ml after 6 hrs, which remained relatively constant for the next 24 hrs. The serum zinc level in the fish on the organic zinc supplemented diet, which followed a similar trend, decreased significantly less, maintaining a zinc concentration of approximately 11 µg/ml. The fish fed the inorganic zinc supplemented diet took an extra 2 hrs for the serum zinc level to reach its peak at a concentration of 11.06 ±

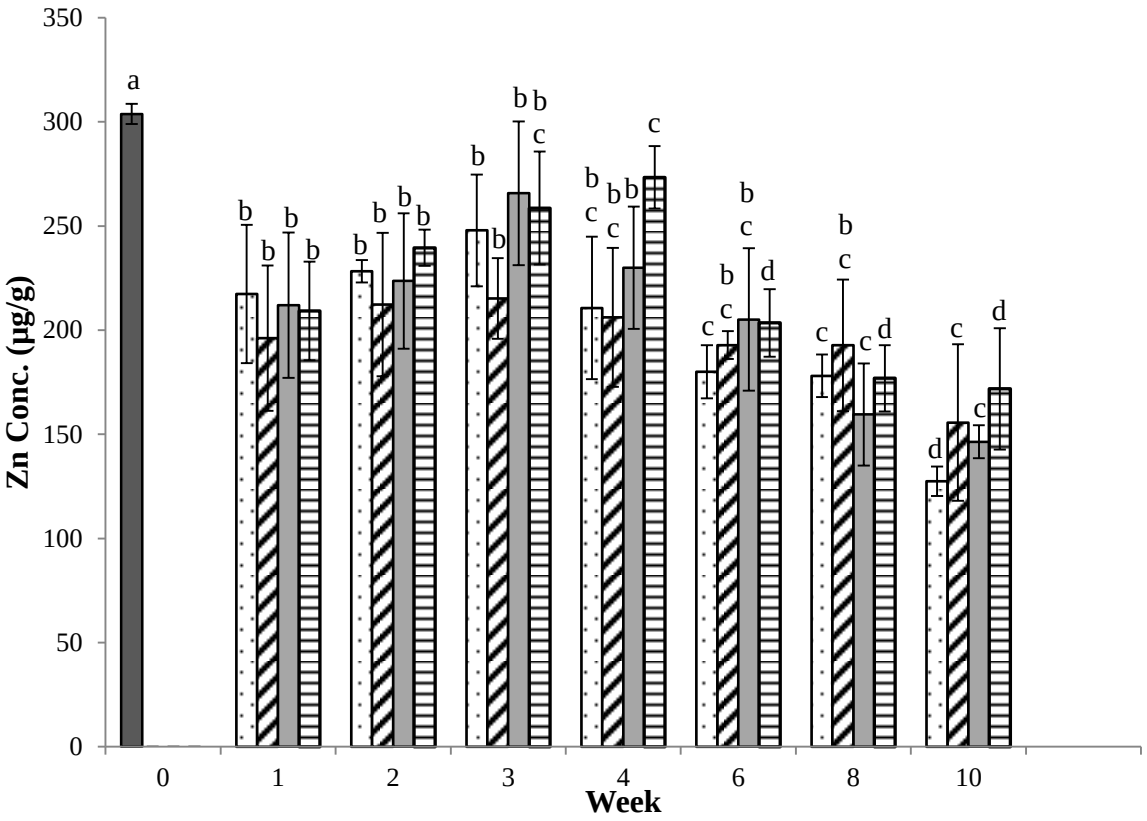

Figure 6.2 Ocular total zinc concentration (mean ± S.D. n = 5). Dark grey bar = initial fish; Dotted bar = unsupplemented; Diagonal stripe bar = low zinc; Light grey bar = medium zinc; Horizontal stripe bar = high zinc. Bars with different suffix with in the same dietary group denotes significant difference ($p<0.05$)

0.90 µg/ml at 6 hrs, which then decreased at a slower rate than the fish on the control diet dropping to a higher, but not significantly so, level than was observed in the fish on the unsupplemented control diet. After the initial spike in serum zinc levels all groups of fish maintained a serum zinc concentration above the initial concentration for the next 18-24 hrs. The fish on the organic supplemented diet maintained this serum zinc level significantly higher than the other groups.

6.3.2 Tissue total zinc levels

The initial zinc level in the eyes of the fish fed a commercial EWOS diet was 300 µg/g. After one week on the experimental diets this decreased significantly to approximately 200-210 µg/g in all diets. The ocular zinc levels in the fish on different dietary zinc levels at each

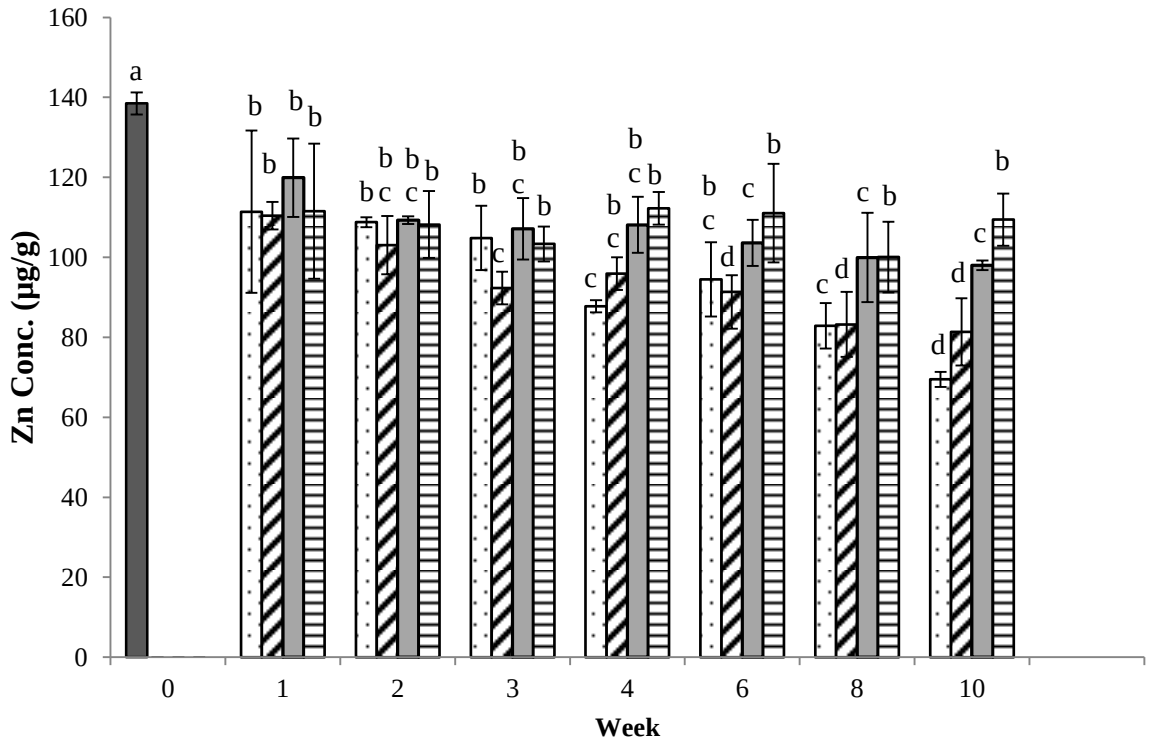

Figure 6.3 Caudal fin total zinc concentration (mean ± S.D. n = 5). Dark grey bar = initial fish; Dotted bar = unsupplemented; Diagonal stripe bar = low zinc; Light grey bar = medium zinc; Horizontal stripe bar = high zinc. Bars with different suffix with in the same dietary group denotes significant difference ($p<0.05$)

time point were significantly different at weeks 4 and 10 when the fish on the high supplemented diet had a significantly higher level of zinc in the eye compared with the fish on the control and low zinc diets in week 4 and the control diet at week 10 (fig 6.2). In the fish on the unsupplemented control diet there was a non-significant trend showing an increase over weeks 2 and 3, after which the ocular zinc levels decreased continually over the next seven weeks, becoming significantly different at week 6. The eyes of the fish on the low supplemented diet followed a similar trend and at no point were significantly different to the eyes of the fish on the unsupplemented control, however it was week 10 before this decrease was significantly different to the levels seen in week 3. The fish on the medium level supplemented diet followed the same trend, decreasing in week 1, increasing again until week

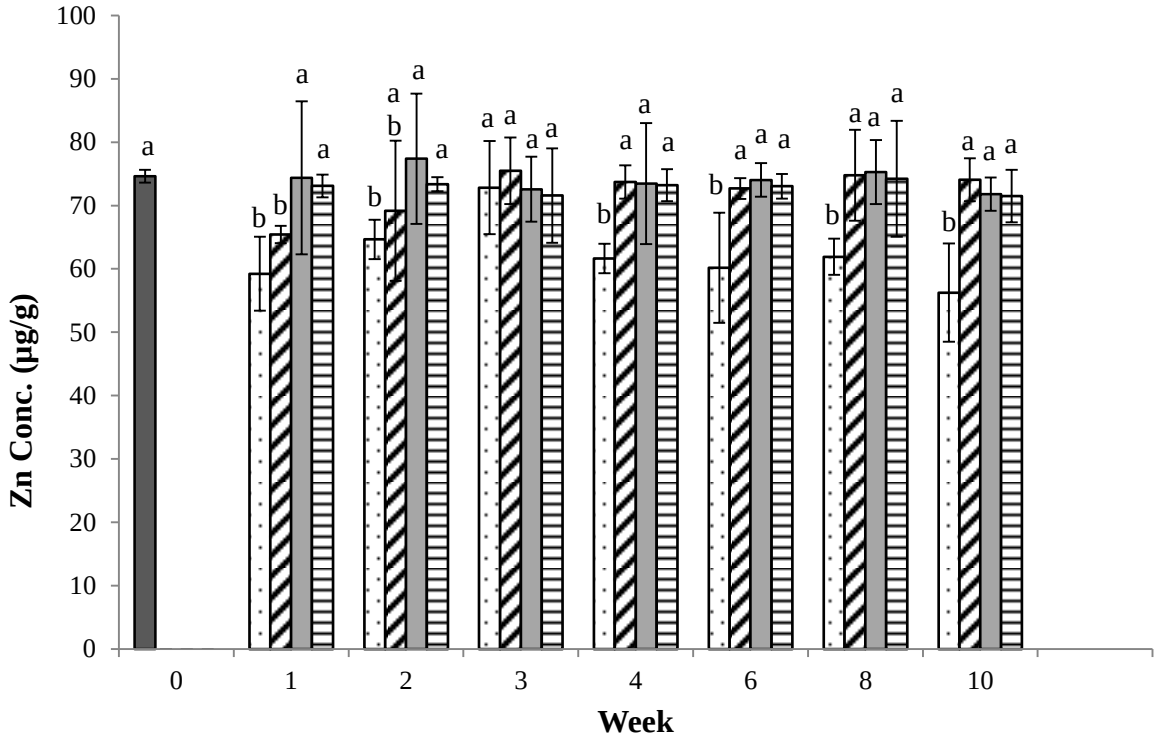

Figure 6.4 Hepatic total zinc concentration (mean ± S.D. n = 5). Dark grey bar = initial fish; Dotted bar = unsupplemented; Diagonal stripe bar = low zinc; Light grey bar = medium zinc; Horizontal stripe bar = high zinc. Bars with different suffix with in the same dietary group denotes significant difference ($p<0.05$)

3 and then continually decreasing until week 10, but in this group the difference from week 3 became significant at week 8. The zinc in the eyes of the fish on the high supplemented diet continued to rise after the initial drop in week 1 to a significantly higher level in week 4 which then dropped, again significantly, in week 6 and then continued to drop until week 10.

The initial zinc concentration in the caudal fins of fish fed the commercial EWOS diet was 140 µg/g. As with the ocular zinc levels this decreased significantly in all the groups of fish after one week on the respective experimental diets. The groups of fish on both the unsupplemented control diet and the low supplemented diet both showed a continual decrease in caudal fin levels of zinc over the 10 week period. The caudal fins of the fish on the unsupplemented diet dropped from 110 µg/g in week 1 to 70 µg/g in week 10 and the fish on

the low supplemented dropped from 110 µg/g to 80 µg/g. The zinc in the caudal fins of the fish on the medium level supplemented diet also decreased over the 10 week period but to a lesser amount, decreasing from 120 µg/g in week 1 to 100 µg/g in week 10, becoming significantly different from week 1 at week 6 having dropped to 107 µg/g. The level of zinc in the caudal fin of fish on the high supplemented diet did not decrease further after the initial decrease in week 1; maintaining a zinc concentration fluctuating around 115 µg/g.

The initial hepatic zinc level in the fish was 74 µg/g. This level was maintained throughout the 10 weeks in the groups of fish on both the medium and high zinc supplemented diets. In the fish on the low zinc supplemented diet there was an initial drop in hepatic zinc concentration in week 1, as observed in both the eyes and caudal fin, but by week 3 this had increased back to a level equal to the initial concentration and this level was maintained for the remainder of the trial. The fish on the unsupplemented control diet also showed a drop in week 1 followed by an increase back to 'normal' levels in week 3 but subsequently decreased continually from weeks 4 to 10, resulting in a final hepatic zinc concentration of 59 µg/g.

6.3.3 Tissue isotope ratios

The isotope ratios for the diets are displayed in table 6.3 and the ^{66}Zn:^{70}Zn ratio of each diet is represented by the broken lines in figures 6.5-6.7. The ^{66}Zn:^{70}Zn ratio in the control diet is 45.0 ± 0.3. At time 0, before any experimental diets had been fed, all the tissues analysed across all the groups of fish had a ^{66}Zn:^{70}Zn ratio of 45.2 ± 0.2, the natural IUPAC ratio in zinc. In the fish fed the control diet the natural ^{66}Zn:^{70}Zn ratio was maintained in all the tissues for the whole duration of the trial.

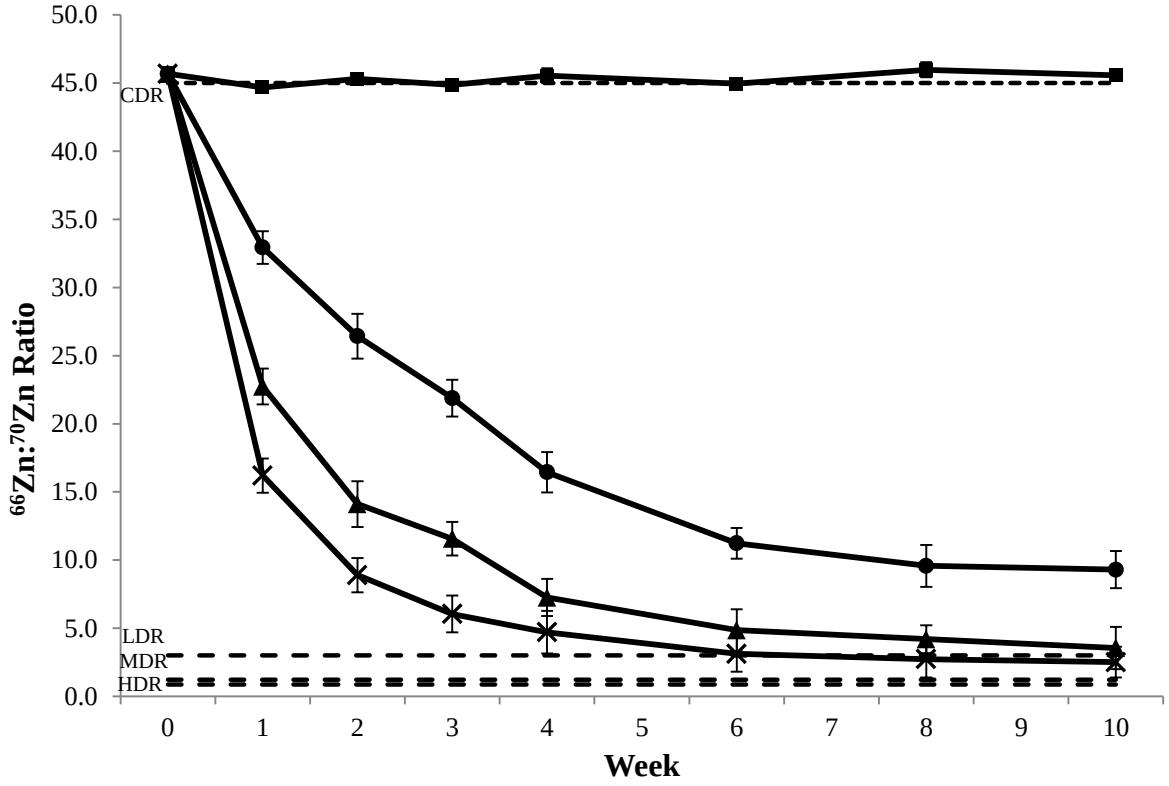

Figure 6.5 Ocular 66Zn:70 Zn ratio (mean ± S.D. n=5). Square marker = unsupplemented diet; Circular marker = low zinc; Triangle marker = medium zinc; Cross marker = high zinc. * denotes no significant difference from the dietary ratio. CDR = control diet ratio, LDR = low diet ratio, MDR = medium diet ratio, HDR = high diet ratio.

The isotope ratio in the eyes (fig 6.5) of fish on the lowest level of supplementation changed significantly each week until week 6, after which the ratio remained stable at 10.03 ± 1.04. In the fish on the medium diet the ^{66}Zn:^{70}Zn ratio stabilised at 4.96 ± 1.61after four weeks and in the eyes of fish on the high level of supplementation the ratios stabilised at 3.27 ± 0.99, also after four weeks. The rate of decrease of the ^{66}Zn:^{70}Zn ratio was positively correlated with the level of supplementation, and the ^{66}Zn:^{70}Zn ratio of the eye never dropped low as the ratio in the corresponding diet

The ^{66}Zn:^{70}Zn ratios in the caudal fins (fig 6.6) followed similar trends as the ratios in the eye, however the rate at which the ratios decreased was increased in the caudal fin. In the first week, the ^{66}Zn:^{70}Zn ratios in the fins decreased from 45.51± 0.22 to 22.21 ± 1.24, 12.40 ±

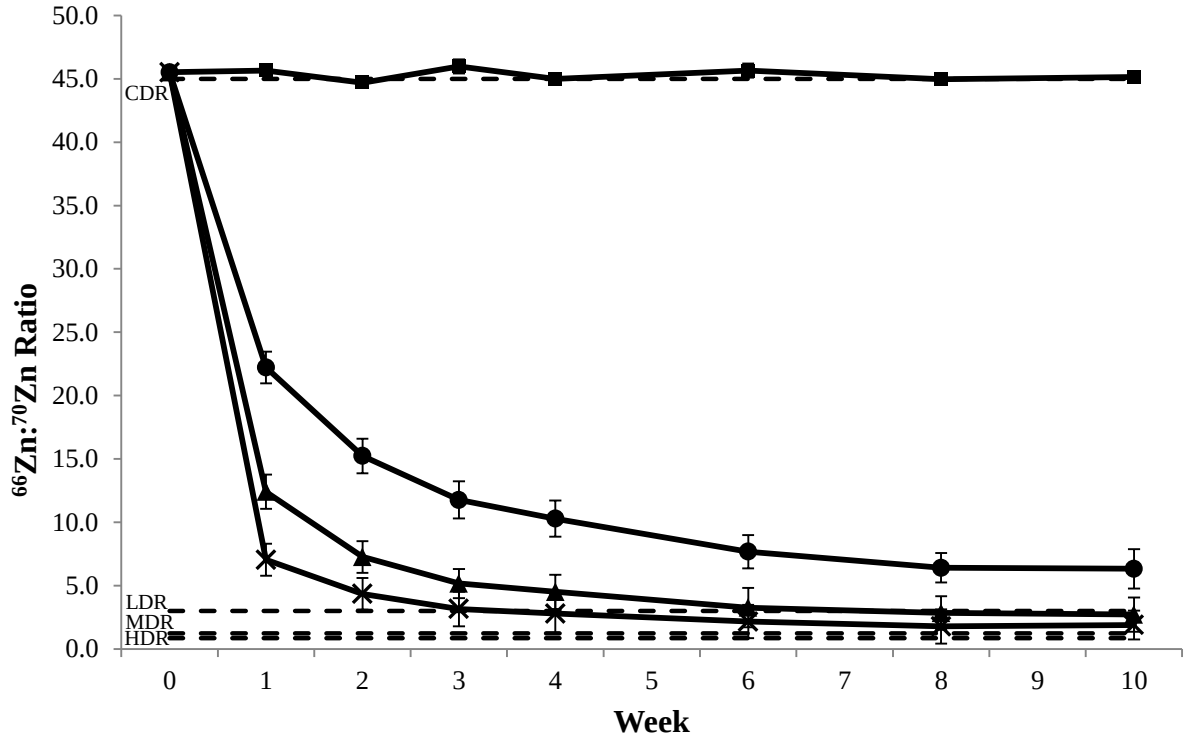

Figure 6.6 Caudal fin ^{66}Zn:^{70}Zn ratio (mean ± S.D. n=5). Square marker = unsupplemented diet; Circular marker = low zinc; Triangle marker = medium zinc; Cross marker = high zinc. * denotes no significant difference from the dietary ratio. CDR = control diet ratio, LDR = low diet ratio, MDR = medium diet ratio, HDR = high diet ratio.

1.19 and 7.10 ± 1.31 in the low medium and high supplemented fish respectively. This corresponds to a decrease from 45.68 ± 0.47 to 32.93 ± 1.20, 22.74 ± 1.31 and 16.20 ± 1.26 in the eyes of fish on the same diets over the same period. As in the eye, the decrease in the ^{66}Zn:^{70}Zn ratios stopped being significant after week 6 in the fish on the low diet but it only took until week 3 in the high diet and week 4 in the medium diet. Unlike in the eye, the caudal fins of the fish on the highest supplemented diet did reach an isotopic equilibrium with the diet when the ^{66}Zn:^{70}Zn ratio was no longer significantly different to the ratio in the respective diet. This was not achieved in either low or medium level supplemented fish.

In the liver (fig 6.7) equilibrium between the tissue and the diet was reached in fish on both the medium and high supplemented diets. Equilibrium was reached in week 4 in the high

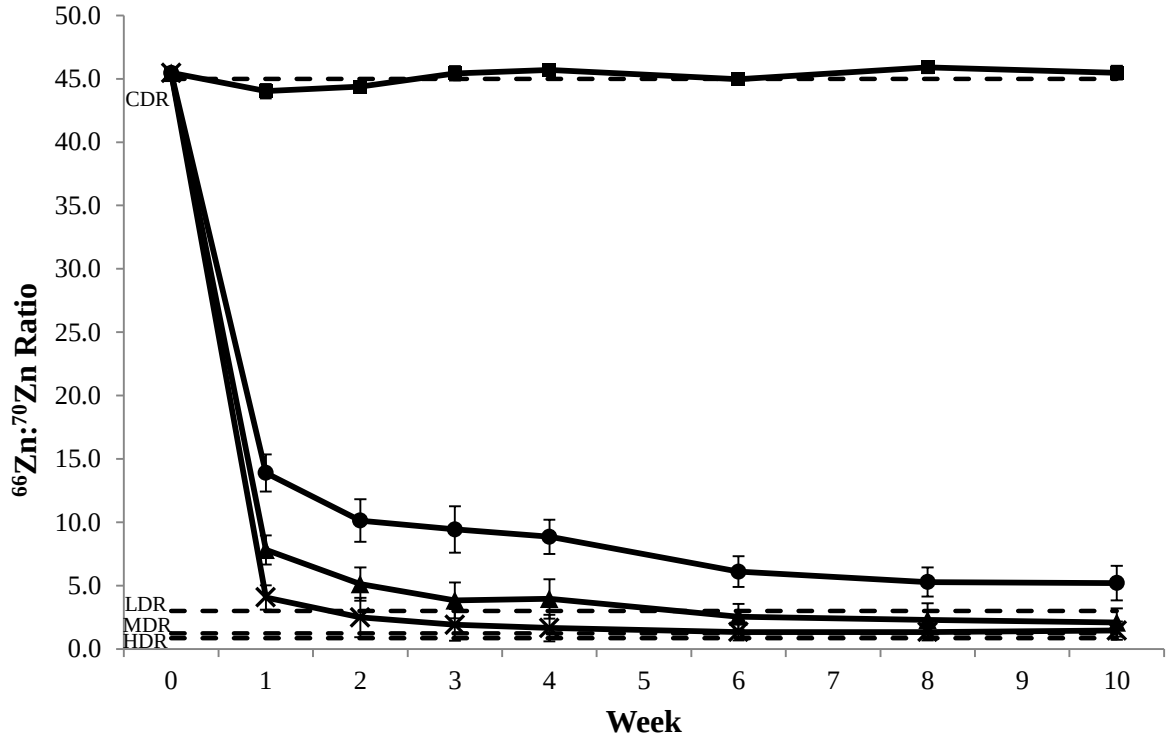

Figure 6.7 Hepatic $^{66}Zn:^{70}Zn$ ratio (mean ± S.D. n=5). Square marker = unsupplemented diet; Circular marker = low zinc; Triangle marker = medium zinc; Cross marker = high zinc. * denotes no significant difference from the dietary ratio. CDR = control diet ratio, LDR = low diet ratio, MDR = medium diet ratio, HDR = high diet ratio

supplemented group and week 8 in the medium supplemented group. The $^{66}Zn:^{70}Zn$ ratios decreased from 45.46 ± 0.31 to 13.89 ± 1.46, 7.82 ± 1.15 and 4.07 ± 0.96 in the low, medium and high supplemented fish respectively; a higher rate of decrease than observed in both the eyes and the caudal fins. In the fish on the low supplemented diet, the rate of decrease stabilized between weeks 2 and 4 at 9.2 ± 0.4 but dropped again between weeks 4 and 6 before stabilizing at 5.7 ± 0.5 between weeks 6 and 10. A similar pattern was seen in the fish on the medium diet with $^{66}Zn:^{70}Zn$ ratio stabilizing at 4.4 ± 0.5 between weeks 2 and 4, then decreasing to 2.3 ± 0.2 between weeks 6 and 10. The $^{66}Zn:^{70}Zn$ ratio in the fish on the high zinc diet stabilized at 1.91 0.6 in week 3 and maintained this ratio until week 10.

6.4 Discussion

The post prandial serum zinc levels, indicate that the zinc in both the unsupplemented control diet (the zinc endogenous to the proteins) and the zinc from the organically supplemented diet are taken up into the serum at the same level. However the zinc in the additionally supplemented diet either stays in the serum longer or is being continually supplied from the gut at the same rate it is being deposited around the fishes tissues. The delayed peak in the fish on the inorganically supplemented diet suggests a slower uptake from the diet and the subsequent decrease after six hours results in less zinc being taken up in total, as indicated by the lower total area under the graph. In a similar investigation by Savolainen and Gatlin (2010) the post prandial serum levels of hybrid striped bass were measured. The results indicated that a dietary concentration of 20 mg/kg resulted in a serum zinc peak of approximately 11 µg/ml after four hours, a level and time period highly comparable to the results obtained from this current study. Differences in experimental design allow little further comparison between this and their study. This is because in the current study the fish were effectively administered a spike of zinc on day 29 and in all treatments that spike remained evident in the serum for at least 30 hours following meal ingestion, whereas in the Savolainen and Gatlin study samples were only taken for eight hours and a stable base line serum concentration was not established. A longer time course in both studies would have allowed for further extrapolations to be made with regards to how long a single ration of food continues to contribute to the circulating zinc pool. The same study by Savolainen and Gatlin, like this study, used two forms of zinc, one organic (methionine hydroxyl analog) and one inorganic ($ZnSO_4$) and the authors concluded there was no difference between the two; a contradictory result to the outcome of this study. The literature is full of contradictory results regarding different forms of zinc supplementation, not only in fish but also in pigs, poultry

and humans. Many have reported positive effects (Paripatananont and Lovell, 1996; Leeson and Summers, 2001; Dibner, 2005; Chien et al., 2006) and many negative (Gomes and Kaushik, 1993; Li and Robinson, 1996; Maage et al, 2001; Satoh et al, 1987). Further investigation into these studies possibly explains why. For example, Li and Robinson (1996) reported negative effects of organic supplementation in channel catfish while Paripatananont and Lovell (1996) reported positive effects in the same species, as one group used zinc-methionine and the other an unspecified zinc-proteinate, of which there are many, all with different physical properties which will affect their assimilation. Comparisons between species, especially fish species, are also difficult to make as the conditions and duration spent in the different sections of the GI tract differ greatly from species to species, both of which will determine to what extent the compound will dissociate during digestion.

The second half of this study has proved very difficult to relate back to the literature as both the use of isotopes in fish studies and the use of isotope ratios to determine uptake and origin of nutrients not only in fish but in other species too, seems to be relatively novel. Isotope half-lives, combinations of oral and intravenous administrations, and balance studies have all been used to measure turnover rates and differentiate between minerals of endogenous and dietary origins in the faeces (Lowe et al., 1995; Turnlund et al., 1986; Griffin, 2002; Fairweather-Tait et al., 1993; Dunn and Cousins, 1989; House and Wastney, 1997; Serfass et al., 1996) but not to test, in direct competition the uptake of two dietary supplements simultaneously. With that taken into account, the following discussion tries to relate the results from this study with the observations made in previous chapters, to add further understanding to these conclusions and to pose further questions for future work.

There is a difference between the zinc levels measured in the livers and eyes of the fish in this trial compared to the levels measured in the same tissues in the previous chapter. This trial reports a range of 71 µg/g to 56 µg/g in the livers at the end of the trial in the fish fed the high

and low zinc diets respectively and from 171 µg/g to 127 µg/g in the eyes. This compares with a range of 100 µg/g to 90 µg/g in the livers of the fish at the end of the previous study and 140 µg/g to 100 µg/g in the eyes. The length of the trials were the same (10 weeks) and the concentrations of zinc in the diets were also the same, the only appreciable difference was the size of the fish. This trial started with 10 g fish while the previous trial started with 65 g fish, this 55 g difference is the equivalent of 3-4 weeks growth during which the tissue concentrations seem to have changed. Unfortunately the tissues were not sampled at the start of the previous study so a starting comparison cannot be made but this difference highlights the importance of knowing what the concentrations in the tissues should be for fish of a certain size if using tissue levels as a biomarker for either deficiency or good health. Interestingly, the caudal fin zinc concentrations did not seem to differ greatly between trials.

The aim of this study was to provide insight into the dynamics of the zinc in the tissues during the 10 week growth period. It was assumed from the previous trial that there would be a linear decrease in the concentration of zinc in the tissues as the trial progressed but the evidence from the current study implies that when the dietary zinc level is either low or deficient there is a compensatory effort made to increase the zinc levels in the metabolically important tissues i.e. the eyes and liver. Garretts and Molokhia (1977) showed that in humans with severe zinc deficiency, muscle catabolism occurs, releasing zinc for systemic redistribution, explaining why severely zinc deficient infants maintain normal plasma zinc concentrations when tested, in the context of this current study a similar affect may be being observed. This compensatory response was clearly evident in the eye, where all groups showed an increase between weeks 1 and 3, and in the liver, where the same trend was observed in the fish on the unsupplemented control diet. The unsupplemented control diet was the only diet with insufficient zinc to maintain the hepatic zinc content. The low supplemented diet was able to maintain the hepatic zinc content, after the initial

compensatory effort was made, for the duration of the trial. The compensatory effect seen implies that zinc is being sequestered from other tissues and when there is at least an extra 5 mg/kg being supplied by the diet this compensatory effort is sufficient to maintain the hepatic zinc pool; how long this compensatory effort could continue for could not be established in this trial but it would be interesting in future studies. Another investigation for future studies would be to monitor the ratios in the tissues after the fish were returned to a non-enriched diet. This would enable the clearance rates of the isotopes to be measured, giving an idea of turnover rates and possibly identifying a difference in longevity with in the tissues for the different zinc sources

The compensatory hypothesis is supported by the isotope ratios measured. Using the ^{66}Zn:^{70}Zn ratio provides insight into the source of the zinc found in each tissue. If there is a higher value it means there is less ^{70}Zn in the tissue and vice versa, if the tissue ratio is equal to that of the diet it means that the zinc in the tissue is solely from the diet and if it is lower than the diets it means that more ^{70}Zn is available than the organically supplied zinc. The fact that in the tissues of the fish on the low supplemented diet the ^{66}Zn:^{70}Zn ratio never dropped as low as the isotopic ratio of the diet implies that either the ^{70}Zn is unavailable or there is some non-dietary zinc, with the natural isotope ratio being incorporated into those tissues.

Taking into account the compensatory effect observed, the latter is most probable and this extra zinc can only come from the reserves in the fish. In the livers of the fish on the diets with high supplementation no compensatory effort is seen and the isotope ratios do reach equilibrium with diet. Also, the plateau seen in the ^{66}Zn:^{70}Zn ratio in the fish on the low supplemented diet, between weeks 2 and 4, coincides with the apparent compensatory effort observed from the total zinc results at the same time point.

The ^{66}Zn:^{70}Zn ratio in the eye, where a decrease in total zinc content is observed (indicating that there is insufficient dietary zinc to maintain its zinc pool) does not reach equilibrium with the diet, even in fish on the highest supplemented diet. This could be an indication that the endogenous zinc from the fishes stores is being incorporated into this tissue or it could be evidence that less of the inorganic ^{70}Zn is been taken up. Further investigations into the total zinc and isotope ratios of other tissues i.e. the muscle and bone would allow further insight into this question but was not within the scope of this preliminary study. However, when this result is combined with the evidence of an initial compensatory effect, seen in the fish on the highest supplemented diet, which subsequently failed after week 4 it is unlikely that the endogenous stores were contributing to the eye zinc pool in the latter half of the trial, where the isotope ratio still does not equilibrate with the diet but this still needs to be proven.

Unlike the eyes and liver, the caudal fin showed no evidence of a compensatory effect by utilising the fish's stores. After the initial decrease in total zinc content in week 1, the fish on the highest supplementation maintained the same concentration throughout. The zinc in the caudal fin of the fish on the medium level of supplementation decreased slowly after week 6 and the fish on the low and unsupplemented diets decreased more rapidly with further significant decreases observed in weeks 3 and 6. When combined with the isotope ratio data there is good evidence that in the medium and high supplemented fish the dietary zinc was the only source of zinc being used and by week 6 all the zinc in the fin had originated in the experimental diets. This also suggests that the two forms of zinc were equally available. In the fish on the low supplemented diets equilibrium between the diet ratio and the tissue ratio was never achieved, and when combined with the evidence that the total zinc in the caudal fin was deceasing and there is no evidence of a compensatory effect suggests that the elevated ratio must be due to an increased utilisation of the organic form of zinc over the inorganic ^{70}Zn when there is an insufficient supply.

In conclusion, it appears that this isotope ratio method of comparing two mineral sources against each other does have the potential to be an effective tool to distinguish the ‘preference’ of a tissue for zinc from different sources (the term ‘preference’ is used in this instance to describe the ability of the tissue to utilise the zinc when ligated to different chaperones in the serum). This trial also provides evidence that a complete study of the isotopic ratios in every tissue in the fish would be extremely worthwhile, however the expense, both in analysis costs and labour, may be prohibitive. A compromise may be to investigate how the ratio changes in the whole body but this would not be a detailed enough approach to use to understand how the reallocation of zinc between the body tissues occurs, it would realistically allow a comparison of uptake from the feed, a result obtainable from digestibility studies. The analysis of the muscle, blood and possibly bone isotope ratios may be a better compromise as these would include a transport pool in the case of blood, a storage pool in the case if bone and the muscle would give a good indicator of what the whole body zinc status would be as this tissue primarily dictates the outcome of whole body analyses. The hypothesis that there is a compensatory effort made by catabolism in other tissues would be to some extent confirmed by the reciprocal drop in total zinc as well as an isotopic ratio much closer to the natural ratio than the dietary ratio being maintained in these tissues; a hypothesis to be investigated in future studies.

CHAPTER 7. General discussion

7.1 Overview of programme of investigation

The focus of this thesis was to assess different methods of improving the availability of zinc from modern salmonid diets. Due to the increasing dependency on plant-based proteins the required level of zinc supplementation has increased to sometimes five times the actual requirement of the intended species. This has been necessary because of the combined effect of the poor digestibility of the supplements used and the increased level of the anti-nutritional factors (ANF's) associated with the higher inclusion of plant based proteins. The consequences of these elevated mineral supplementation levels are an increased cost and a higher environmental impact. The present study examined the actual zinc availability from several commercially used raw materials, using traditional techniques that were optimised to produce more accurate data than is currently available. It also examined the role of new processing technologies can play in increasing the availability of the raw materials endogenous minerals with the aim of reducing the need to further supplement the diets so heavily. Finally the study directly compared two supplements, one from an organic compound and the other a commonly used inorganic sulphate salt. The findings showed that mineral availability varied greatly from different protein sources, that further processing of the raw materials could significantly increase the availability of a products endogenous minerals and that if assessed correctly there is strong evidence that the use of organic supplements not only increases the digestibility but also the bioavailability of supplemented minerals. It has become evident that it is not sufficient to treat all organic compounds equally, nor is it appropriate to expect one form of supplement to perform in the same manner in different species of fish. Further work must be conducted on an individual compound, on a

specific species basis, taking into account the physico-chemical properties of the supplement and the internal conditions (temperature, pH and transit duration) of the animal's gastrointestinal tract.

7.1.1 Availability of protein endogenous zinc

The essential first step in assessing the level of supplementation required in a diet is to obtain a clear understanding of the availability of the nutrients already present in the unsupplemented diet. The first trial (chapter 3) aimed to do this by using the hypothesis that only when a fish is bordering on deficient of a certain nutrient will it utilise all the nutrients available to it in the diets. If in a deficient fish the nutrient is not utilised then that nutrient, or part of it, is not available for assimilation. This method has the advantage of reducing the excretion of already ingested nutrients back into the digesta which would confound the results of a digestibility study. To the best of the authors knowledge no other digestibility study has used this approach in the refinement of a nutrient assessment. The design of the diets used in the digestibility studies also reduces the effect that differing feed intakes may have on the digestibility of a specific nutrient. It would seem logical that if the transport pathway from the gut lumen into the body can be saturated, and the fish ingest more of a nutrient by actually eating a greater quantity of food, then this may lead to a reduced apparent digestibility being calculated. As the dietary zinc levels were maintained bordering on deficiency this should have reduced the potential for this to have an effect in this study, however, feed intake in salmonids is believed to be regulated by the energy levels in the feed. In rainbow trout this is most closely correlated to the amount of digestible energy (Boujard and Medale, 1994). The majority of digestible energy comes from the oil fraction of the diet

as carbohydrates are considered indigestible in fish; in this study all the diets were formulated to be isolipidic so the feed intake should have been similar for all groups.

The results indicated that the zinc from the soyabean meal was the most available (49.4%) which was a surprising result given the expected levels of phytate usually present in soyabean meal however the soyabean meal used was a particularly high grade meal and the result is comparable with other results in the literature (Storbaken et al., 1998; Yamamoto et al., 1997) in studies using refined, pre-treated soyabean products. The digestibilities of the animal based protein used in this current study, 15.1% for the LT94 fish meal and 15.8% for the poultry meatmeal, are also comparable with the values reported in other studies (Sigiura et al,. 1998). It seems contradictory to report higher zinc digestibilities from plant based proteins than from a high quality fishmeal but all the results prove comparable with published data from other research groups and it highlights the importance of collecting data for individual ingredients and not assuming all soya bean meals will be the same and all fishmeals will be the same. It was on the basis of these results and by measuring the effect these diets had on the tissue levels that the diets for the subsequent supplementation trials, which were intended to produce zinc deficient fish with decreased metabolically important zinc pools which could then be observed increasing as more zinc was re-supplemented, were formulated. From the tested protein sources, maize gluten meal (31.9% digestible zinc) was chosen as the base of the diet because its low zinc content, its relatively low zinc digestibility and its high protein digestibility (85.3%) allowed a high protein/low zinc diet, proven to cause the significant decrease in tissue zinc levels required for the supplementation trial, to be produce.

7.1.2 Pre-treatment of raw materials

A possible alternative to increasing the total levels of zinc in the diet with supplements in order to meet the requirements of the fish is to improve the digestibility of the zinc from the dietary components. This can be achieved by milling (Bohn et al., 2008), heat treatment of the ingredients (Pontoppidan et al., 2007) and by using genetically modified crops (Raboy, 1997). The most successful method used to date is the use of exogenous enzymes to break down anti-nutritional factors and predigest some of the raw materials. Both single enzymes and 'cocktails' of multiple enzymes are used and the method of treatment may play an important role in the efficacy of each. The pre-treatment of the raw materials during the processing method seems to prove the most effective approach (Vielma et al., 2002; Storebaken et al., 2000). When these results are compared with studies incorporating the enzymes into the pellet along with the processed raw materials it is evident that either top-spraying or using the enzyme as an additive while extruding the pellet (Forster et al., 1999; Oliva-Teles et al., 1998) is very inefficient. However, a recent study by Dalsgaard et al (2012) showed moderate improvements in nutrient digestibility in Rainbow trout from sunflower meal and rapeseed meal top-coated with β-glucanase, xylanase and proteinase while the β-glucanase and proteinase significantly improved the digestibility of the nutrients in soyabean meal. It is proposed that the ineffectiveness of the whole-feed additive technique is due to a combination of insufficient contact between the enzyme and the ingredient and suboptimal conditions for the enzymatic activity but the results from Dalsgaard et al (2012) highlight that each enzyme : protein : species combination needs to be examined separately. The results of this current study confirm that by pre-treating a soyabean meal with phytase, both the phosphorus and the zinc availability is increased in Rainbow trout.

7.1.3 The form of supplementation

It is undoubtedly the most common practice to supplement salmonid diets with zinc very highly by means of its inorganic sulphate salt. As discussed in the introduction, this practice results in large losses of zinc to the environment (Russell et al., 2011) and as the levels of plant based proteins and their associated anti-nutritional factors increase this loss is set to increase. There is much debate in the literature about whether the use of organically chelated minerals can reduce this loss by improving the digestibility. Studies by Apines et al. (2001; 2003), Apines-Amar et al. (2004) and Paripatananont and Lovell (1997) all report an increased digestibility when an organic form of zinc is supplemented but studies by Maage et al. (2001), Kjoss et al. (2006), Overnell et al (1988) and Li and Robinson (1996) state that there is either no effect or a negative effect of using organic sources. In the studies reporting no effect the dietary zinc intake was often high (>120 mg/kg). As discussed in chapter 3, it has been observed by Clearwater et al. (2002) and shown by Sappal et al. (2009), that the zinc status of the fish is highly regulated when excess zinc is present in order to prevent toxicity. Studies using levels exceeding the dietary requirements are unlikely to show differences because the zinc is simply not required. If anything these studies provide evidence that over supplementation is a waste of the mineral and the excess zinc will pass out into the local environment. The results of this present study clearly support the fact that an organic form of zinc is more digestible.

Concerns about the effect of high supplementation on the environment can be eased by increasing the digestibility, however concerns about meeting the requirements of the fish also need to take into account the bioavailability of the mineral once inside the animal. Swinkels et al. (1994) stated that the true indicator of the availability a nutrient must come from the activity and levels of it dependant proteins with in the animals cells, not just its digestibility

or the tissue concentration. The results presented in chapter 5 clearly illustrate this point; an examination of the hepatic zinc levels alone would suggest that both the organic and the inorganic zinc were equally bioavailable to the liver, however, the analysis of the zinc dependant protein, metallothionein, clearly shows that under stress conditions only the organic supplemented fish were able to synthesis the protein. Furthermore, the analysis of the mRNA levels indicate the fish on the inorganically supplemented diet up regulated the production of the mRNA to synthesise the protein to the same extent as the fish on the organic supplement, so the liver was trying to produce more metallothionein but was unable to do so.

The use of a stable isotope in identifying different 'preferences' for one form over the other in different tissues (Chapter 6) was partly successful. The approach taken for fish studies was completely novel and illustrated a tissue dependant difference with regards to how the fish attempts physiologically to compensate for zinc deficiency by seemingly reallocating its endogenous stores. Without the use of the isotope ratios it would not have been possible to identify that this increase must have been from the endogenous zinc stores and not the diet as the isotope ratios levelled out at the same time point as the total zinc in the tissue increased. The study also illustrated the rate of turnover was fastest in the liver, then the caudal fin and then the eye, and also showed that when the diet was more deficient there was a seemingly increased ability for the tissues to take up the organic form. Further studies need to be conducted in order to confirm these observations. The use of a second isotope to label the organically supplemented zinc would have been preferable as it would then allow for some firm conclusions to be made about the source of all the zinc in the tissues. However the expense of enriching the organic supplement with one isotope was prohibitive.

7.2 Future work

7.2.1 Application for other species and life stages

The focus of this thesis has been how to improve the zinc nutrition of farmed salmonids, particularly the Rainbow trout (*Oncorhynchus mykiss*) during the juvenile, fast growing stage of their life cycle. The findings and the implications of this study may also be relevant to the nutrition of other minerals, especially divalent cations, the different life stages of a production cycle and also to other intensively farmed species such as sea bream, sea bass, and tilapia.

The interactions between minerals within the gastrointestinal tract are a well-documented and complicated process (Goyer, 1997). The competition between divalent cations like, but not limited to, zinc and copper for uptake mechanisms means care should be taken when supplementing extra of one not to cause a potential deficiency of another (Hall et al, 1979). The use of organically chelated minerals may avoid this issue due to the use of different uptake mechanisms but these interactions should always be considered when increasing the supplementation in diets. For some minerals the competition for uptake may be a regulatory mechanism to avoid toxicity and the use of chelates to by-pass this competition may result in toxicity of either the supplemented or a previously out competed mineral.

The maize gluten diet in the third chapter of this thesis highlighted the need for zinc of a juvenile Rainbow trout to grow, but it is also known that zinc is required for health gonad development and gamete production in humans (Croxford et al, 2011; Tian and Diaz, 2013) and has also been shown to improve the total number of spawns, total seed production, mean fecundity, hatching rate, sperm motility and sperm viability of Nile tilapia, *O. niloticus*

(Gammanpila et al, 2007). Future studies into the effect of organic zinc supplementation or the use of pre-treated proteins in diets of brood stock feeds on egg quality may be worthwhile especially as these diets already command a premium and the increased cost associated with these products may be better accepted.

The use of both enzyme pre-treated proteins and organically chelated minerals in other intensively farmed species also need to be further investigated. Warm water species such as carp, tilapia, sea bass and sea bream may all respond differently to both attempts at increasing zinc availability. The warmer body temperatures resulting from the warmer water temperatures may produce more favourable conditions for the enzymatic activity of the proteinases, glucanases and phytases used in both top coated diets and pre-treated protein diets, increasing their efficacy. The differences in gut morphology may also play a role increasing their efficacy, both for pre-treated proteins and organically supplemented minerals. Although not technically a different species the increasing interest in the use of triploid salmon may benefit from both these attempts at improving mineral availability. Triploid salmon are prone to post smoltification bone deformities in both the jaw and vertebrae (Fjelldal and Hansen, 2010). Several minerals are required for proper bone development including both phosphorus and zinc. The faster growth associated with the freshwater pre-smolt stage of triploid development would probably result in a higher mineral requirement but with phosphorus and zinc in particular, one being a highly regulated effluent of farm sites and the other having very low digestibility, it is not feasible to simply add more to the diets. Pre-treatment of the proteins and the use of more efficient supplements may provide a solution to this problem. The decreased cost of production due to the reduced production period may provide the increased margin required for more expensive feed.

7.2.2 Commercial relevance and applications

The current EU regulations restrict the level of zinc in a salmonid diet to 200 mg/kg; while this is more than three times the requirement for Atlantic salmon and more than six times the requirement for rainbow trout, zincs poor digestibility combined with the increased use of protein sources high in ANFs that further reduce the digestibility of zinc may be resulting in diets incapable of supplying sufficient zinc. This can be exaggerated during times of stress. The easy solution is to lobby and try to decrease the restrictions on the allowable levels of zinc inclusion however the environmental impact this will have and the sustainability of this approach is questionable and it looks more likely that the restrictions are going to be tightened; reducing the maximum permitted levels to 150 mg/kg. If this does happen the importance of being able to accurately monitor the zinc status and to target biomarkers of deficiency will become very evident. The results of this study imply that whole body zinc levels could start to decrease without there being clinical signs of a deficiency, but for how long a period of time this decline could continue before the health and eventually growth would be affected needs long term studies to be conducted to investigate. Targeting tissues such as the caudal fin, liver and eye would give a strong indication of the severity of the deficiency and if found to be low would be a warning that the heath of the fish is being affected.

If the maximum permitted level of zinc is reduced to 150 mg/kg the results of this study indicate that the use of either pre-treated raw materials or organic forms of supplementation could help to mitigate the impact this may have of the fish. The degree to which these options will increase the cost of feed production and hence the final product cost is unknown to me but further, more targeted research and an increased demand from the global feed market should reduce the deficit between the standard products and the more refined options.

7.3 Overall conclusions

The results of these trials indicate that it is necessary to re-evaluate the current methods used in aquaculture, particularly salmonid aquaculture, with regards to mineral supplementation and increasing the availability of minerals from the raw materials. It has shown that both the pre-treatment of the raw materials with enzymes and the use of inorganic minerals improves both the digestibility and the true bioavailability (as described by Swinkels et al., 1994) in a salmonid species. It has identified the eyes, liver and caudal fin as metabolically important zinc pools which are maintained for as long as possible during times of deficiency and provided support to the hypothesis that a compensatory effort is made by the systemic redistribution of endogenous zinc from other zinc pools (this occurred after 3-4 weeks on a deficient diet), an effect needing to be taken into account when assessing the zinc status of fish by tissue zinc levels. Finally this study has also provided preliminary results indicating the usefulness of stable isotope enrichment ratios in studies investigating supplements in fish.

CHAPTER 8. Appendix

8.1 Mineral composition of protein sources

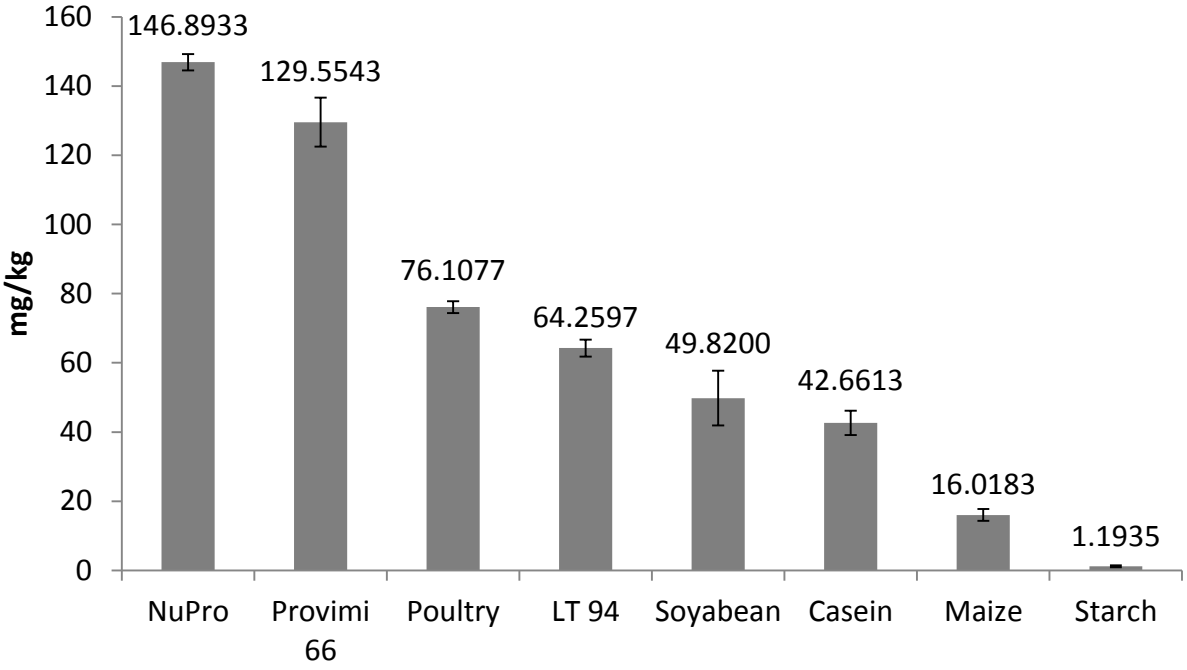

Figure 8.1 Zinc content of raw materials

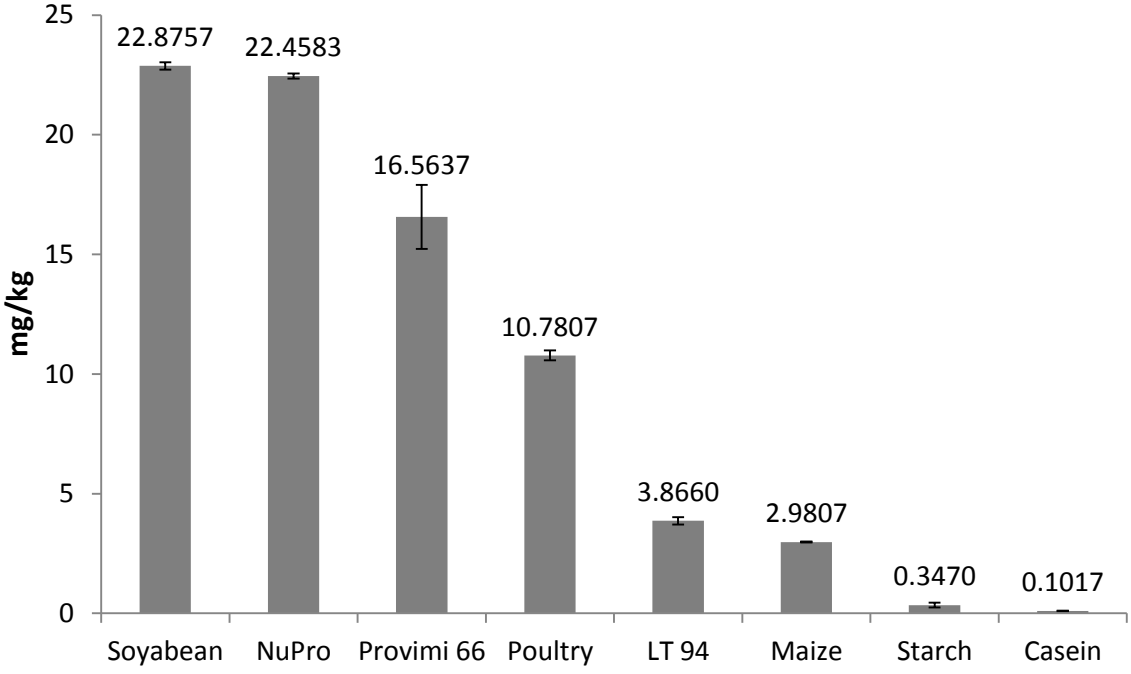

Figure 8.2 Manganese content of raw materials

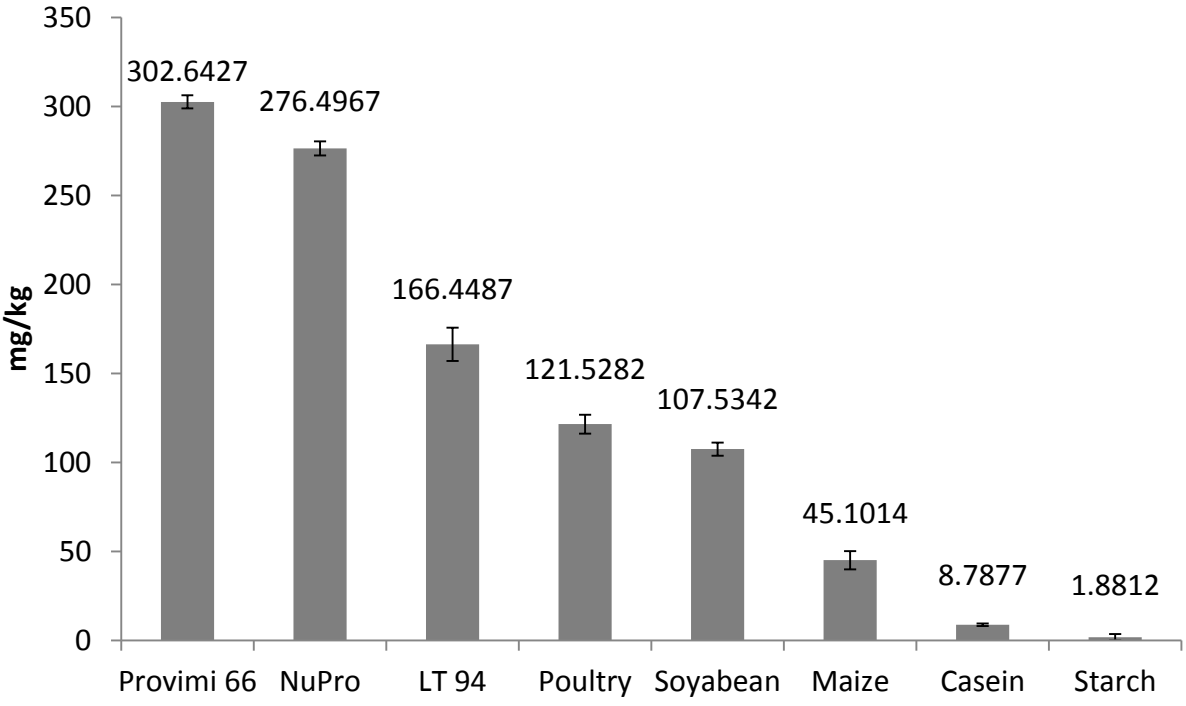

Figure 8.3 Iron content of Raw materials

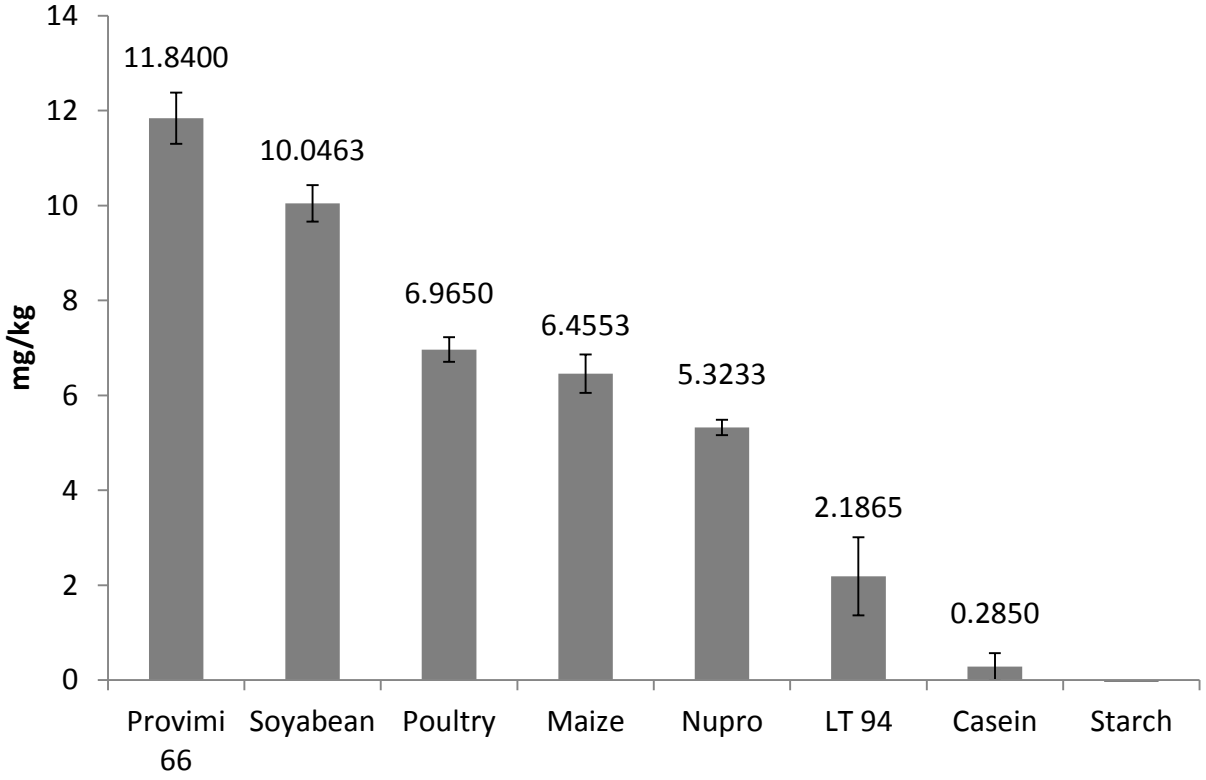

Figure 8.4 Copper content of raw materials

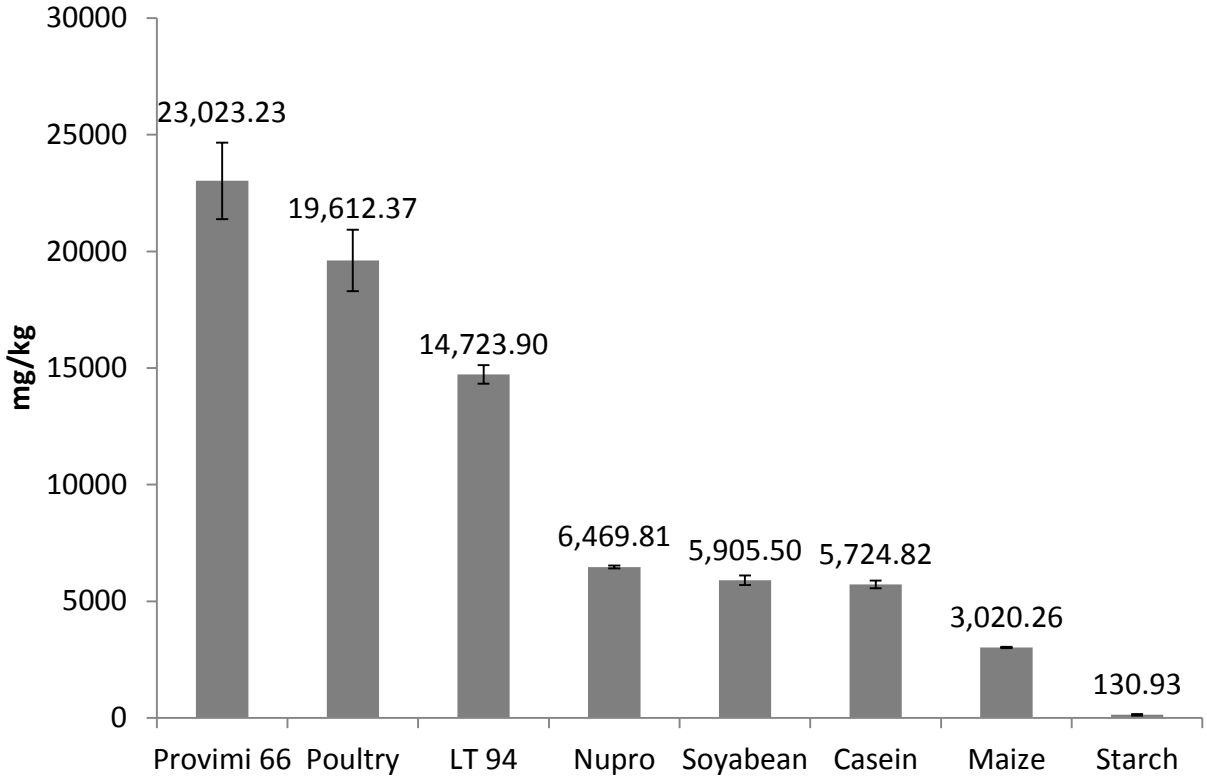

Figure 8.5 Phosphorus content of raw material

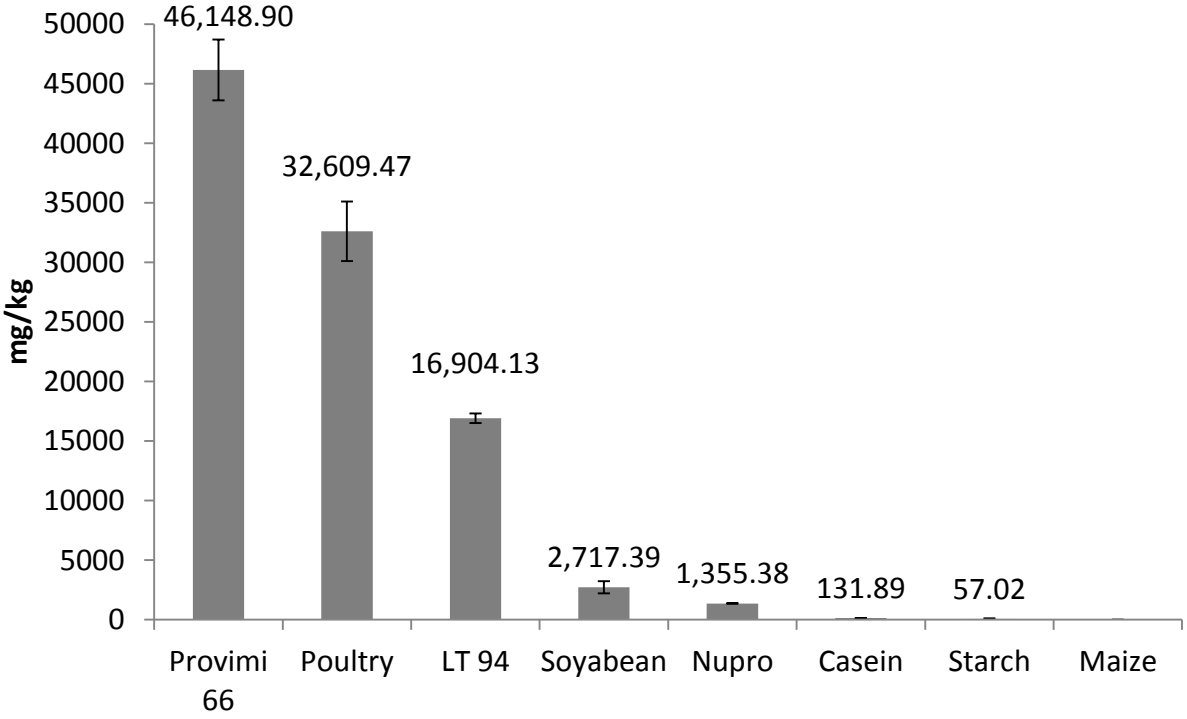

Figure 8.6 Calcium content of the raw materials

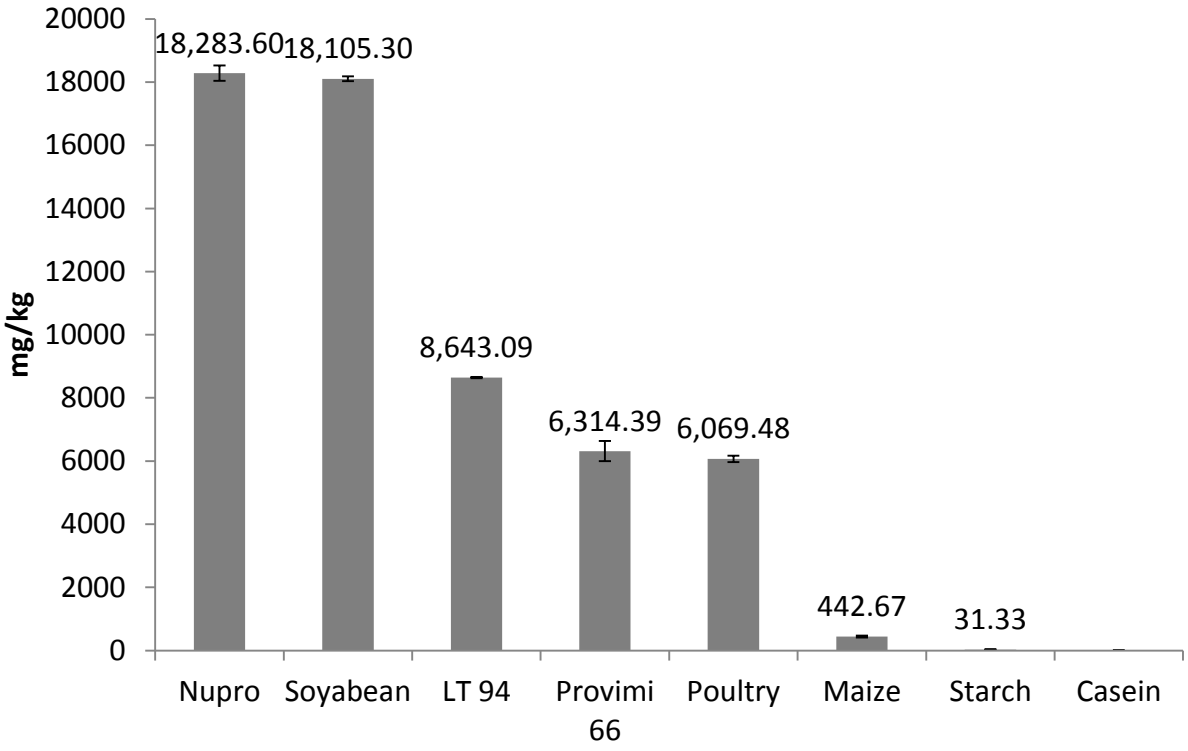

Figure 8.7 Potassium content of raw materials

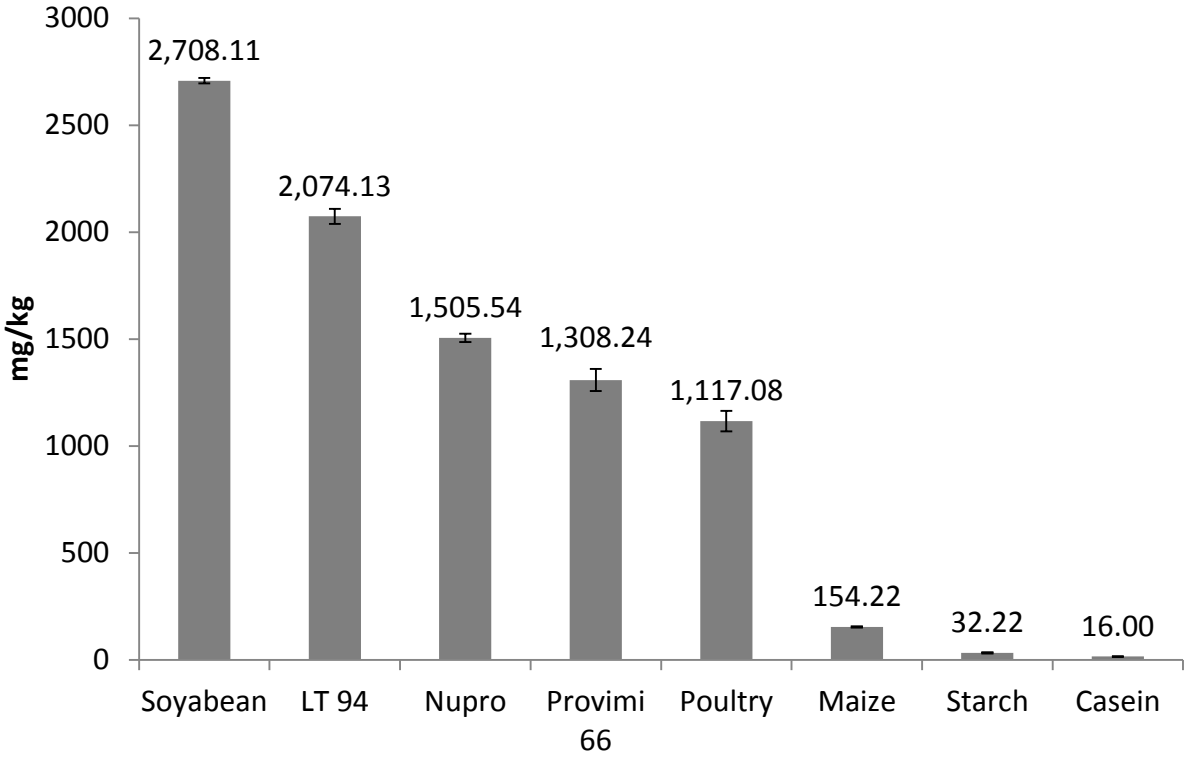

Figure 8.8 Magnesium content of raw materials

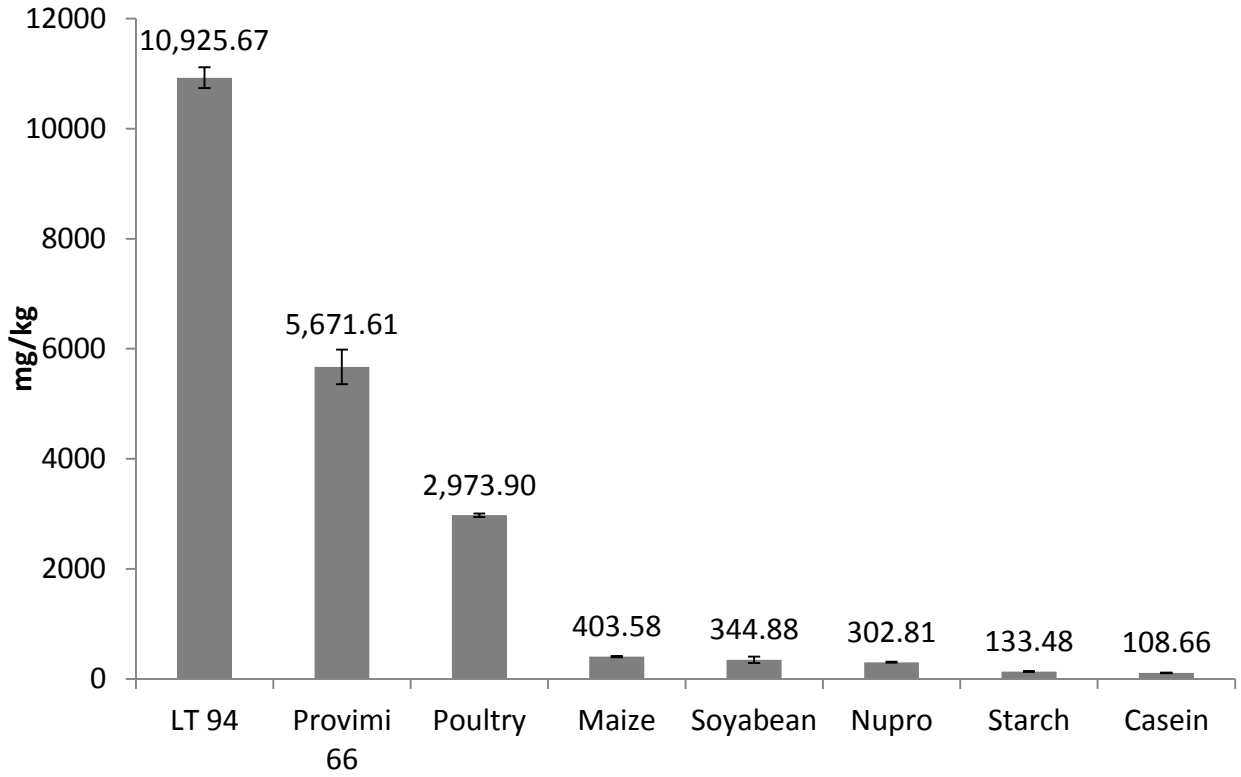

Figure 8.9 Sodium content of raw materials

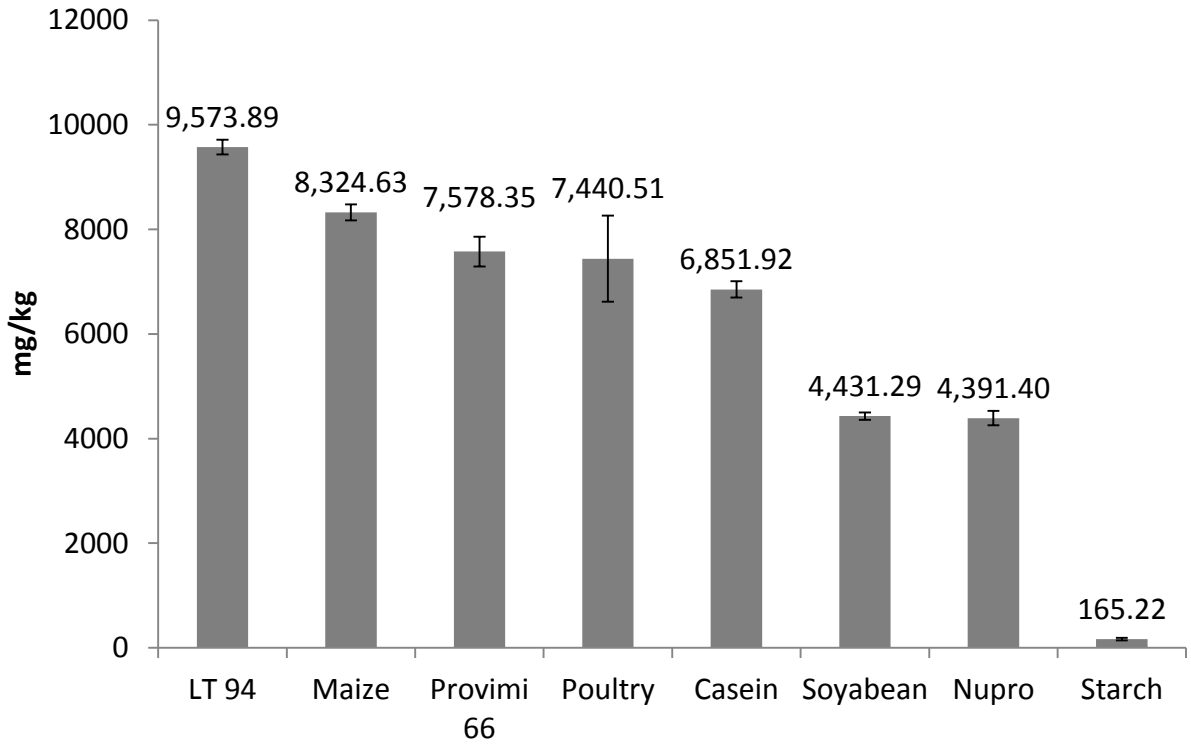

Figure 8.10 Sulphur content of raw materials

8.2 Proximate composition of protein sources

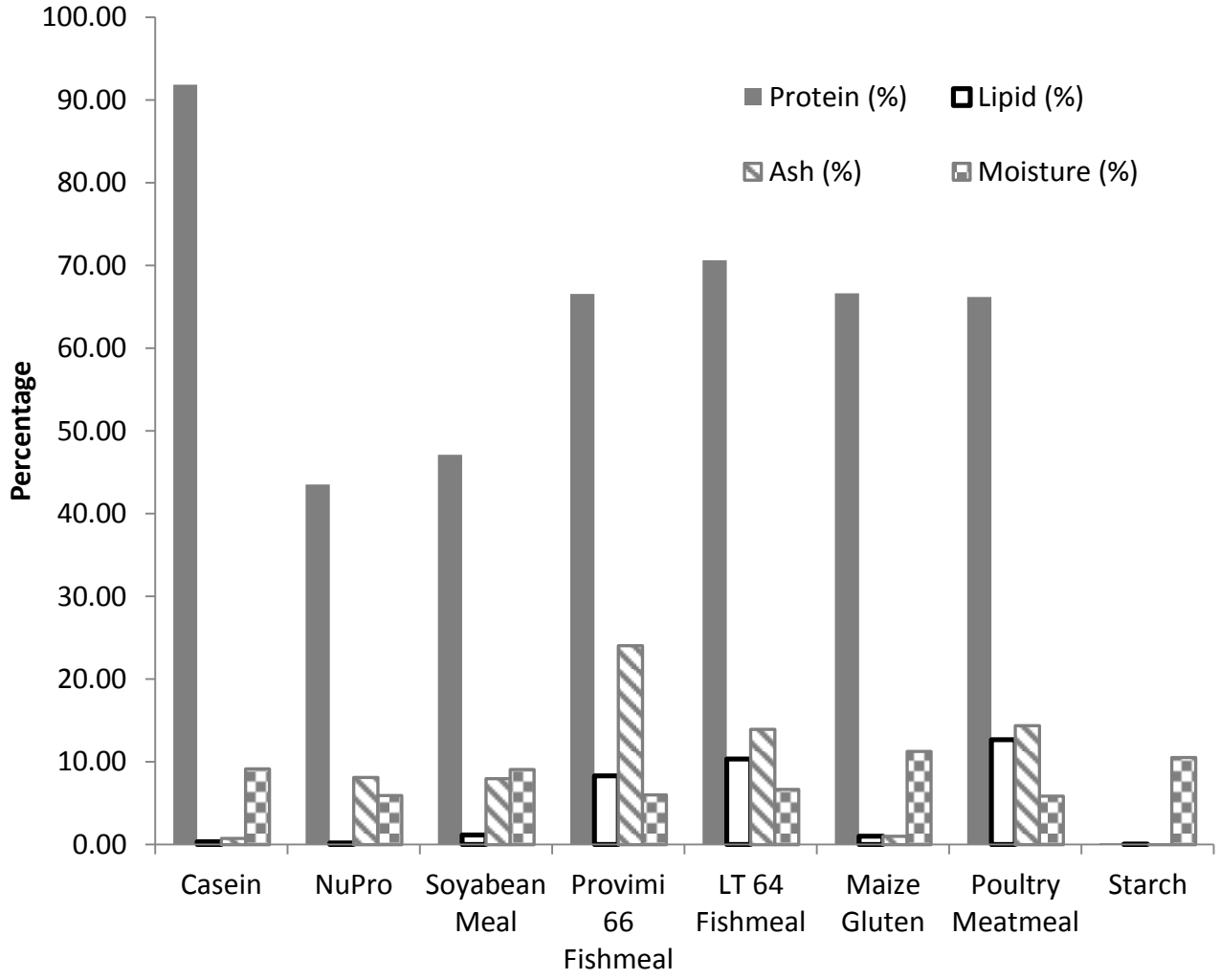

Figure 8.11 Proximate Analysis (MOPA) of raw materials

8.3 Gel electrophoresis for primer specificity

In order to test the specificity of the primers used to replicate the mRNA of MTA and MTB the PCR product was run on an Agarose gel (see 8.4 for method)

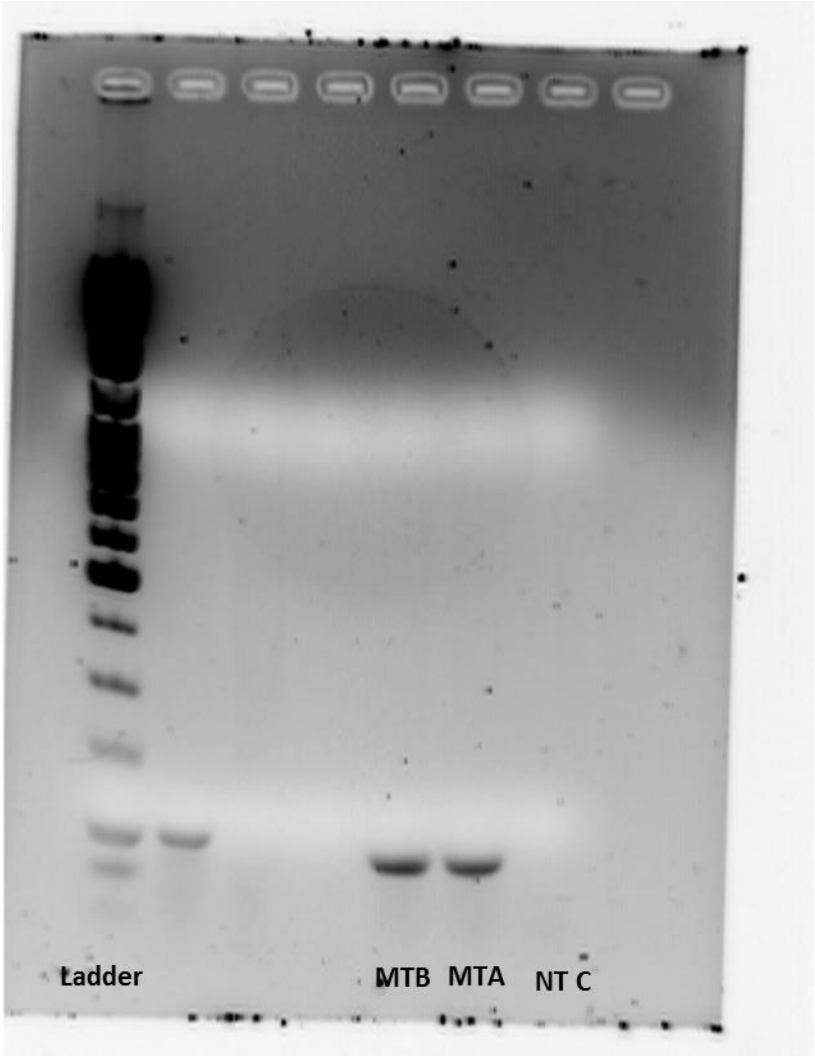

Figure 8.12 Agarose gel with 0-100bp lader, metallothionein A (MTA), metallothionein B (MTB) and no template control (NTC)

8.4 Standard operating procedures

8.4.1 Gel electrophoresis.

Objective: To establish a protocol for identification of the sizes and relative quantities of the products of RT-PCR from zebrafish (*Danio rerio*) mRNA.

Reference: This SOP was developed from the procedure used by Michelle Kiernan (July 2006).

Notable Hazards: Take care when handling hot solutions. Risk of superheating solution, causing it to boil over without warning. Swirl regularly when heating and hold at arm's length. Ethidium bromide is toxic and carcinogenic. Wear gloves when handling and dispose of all contaminated materials in the ethidium bromide waste bin. Do not allow any contaminated gloves or tools outside the area marked with biohazard tape. Store ethidium bromide at 4°C in the dark, marked with a TOXIC label. UV radiation is carcinogenic. Avoid direct exposure to eyes and skin.

Procedure:

This technique was based on one used to identify bacterial plasmids, and has been modified for use on zebrafish eggs and larvae at the age of 0-7 days post-fertilisation, though should be applicable to any RT-PCR product.

Reagent Preparation

Prepare 10xTAE **or** 10xTBE buffer as per the manufacturer's instructions, or from the following recipes.

10xTAE buffer (1l)

- 1.86g EDTA (disodium salt)
- 48.4g Tris Base
- 1.1ml glacial acetic acid
- Add 900ml deionised water, adjust pH to 8.5 with NaOH, then make up to 1l with water.

10xTBE buffer (1l)

- 109g Tris base
- 55g Boric acid
- 4.8g EDTA (disodium salt)
- Dissolve in 900ml deionised water, adjust pH to 8.3 with NaOH, then make up to 1l with water.

Preparing Agarose Gel

1. For a 2% agarose minigel (8x10cm), add to a conical flask:

- 1.0g agarose
- 5ml 10xTAE buffer (or 10xTBE)
- 45ml deionised water

2. Heat on full power in a microwave oven for 1 minute, swirling after 45s. Check that all the agarose is dissolved.
3. Leave to cool for 5min at room temperature.
4. Prepare the gel mould by taping up both ends. press along the edges to ensure a good seal. Set on a level surface.
5. Once the gel has cooled to about 60°C (enough to hold comfortably), add enough ethidium bromide to give a final concentration of 0.5µg/ml (say, 2.5µl of a 10mg/ml stock). Swirl to mix.
6. Slowly pour the molten gel into the mould. When finished, use a disposable pipette tip to sweep any air bubbles to the sides of the gel.
7. Carefully add the gel comb, making sure that no air bubbles form on the teeth.
8. Leave the gel to set for 30 minutes to 1 hour.

Preparing Electrophoresis Tank

1. Prepare 0.5xTAE (or TBE) buffer by diluting 1 part of the 10x stock with 19 parts deionised water. This is the running buffer. There should be enough to cover the gel in the tank.
2. Remove the tape from the ends of the gel mould and place on the white platform in the tank, ensuring that the comb is above the red lines at the **end** of the platform.
3. Pour on the running buffer, ensuring the gel is well covered.
4. Carefully remove the gel comb, making sure that the wells aren't torn, and do not contain air bubbles.
5. Check before loading any samples that the leads from the power pack are correctly connected and that the power pack is working properly.

Loading Samples

1. Prepare a clean 0.2ml eppendorf tube for each sample. Add 8µl of PCR product to each tube, then add 2µl of gel loading buffer to each tube.
2. Transfer the entire contents of the tube to one of the wells in the agarose gel. Take care not to pierce the gel with the pipette tip and **do not** depress the plunger completely, to prevent ejecting air into the gel.
3. When all the samples are loaded, load 10µl of DNA ladder solution another well (this should already contain loading buffer. If not, use 8µl DNA / 2µl loading buffer again).
4. Record each well's contents for reference after the gel has run.

8.4.2 PCR

Objective: Establish a protocol for routine PCR with samples obtained from fish tissue

Reference: This SOP was developed in part from a kit provided by Sigma: ReadyMix^{TM} Taq PCR Reaction Mix with $MgCl_2$ (Catalogue number P4600)

Materials: For kit see attached sheet from Sigma.

Notes:

- The PCR reaction on the thermocycler takes ~2 hours. Be sure you have enough time to run the reaction because the machine cannot be left to run overnight.
- Forward and reverse primers obtained from the manufacturer are ordered separately and based on specific design criteria. They are shipped as lyophilized vials and must be reconstituted with nuclease-free water. The amount of primer is given on the literature sent with the primer. Following the direction for reconstituting primers, make a 100 µmole/L stock solution and also prepare at least four vials of primer working solution (this is to prevent too much freezing and thawing of primers). Working solution is prepared by taking 5 µL of stock solution and diluting to 500 µL with nuclease-free water. This results in a primer working solution concentration of 1 µmole/L.
- The concentration of primers in the final reaction volume is important for the PCR reaction. If PCR does not work well, primer concentrations may need to be optimized to improve the success.
- The procedure below is designed for a final primer concentration of 300 nM. Thus, 10 µL of primer working solution will be added for each 50 µL reaction (each sample requires 10 µL).
- The DNA template concentration is important. If the sample is cDNA obtained from reverse transcription of RNA see the procedure for the reverse transcription of RNA. The reverse transcription reaction of RNA is performed on 800 ng (obtained from total RNA extracted from sample and diluted to 100 ng/µL; and final volume of RT reaction of 24 µL) of total RNA and the final concentration of DNA is ~33.3 ng/µL.
- The procedure below is designed for a final DNA template concentration of ~165 ng in the 50 µL reaction volume. Thus, 5 µL of the cDNA template (concentration 33.3 ng/ µL) will be added for each reaction.

Procedure:

1. Obtain a new 0.5 mL nuclease-free tube and label as PCR Master Mix. Keep tube on ice and add the following (where S = the number of samples + 1):
    a. S×(25 µL) ReadyMix (from kit)
    b. S×(10 µL) Forward primer
    c. S×(10 µL) Reverse primer
2. Obtain new 0.2 mL PCR tubes and label for each of your samples and for the no-template control
    a. Keep all tubes on ice.
    b. Add 45 µL of PCR Master Mix (prepared in step 1) to each tube
    c. Add 5 µL (~165 ng) of the sample cDNA (DNA template) to the correct tube. Add 5 µL of water to the tube labeled “no template control”
    d. Total final volume is 50 µL.
3. Common cycling parameters (use regular PCR machine in room 428):

a. Denature the template at 94 °C for 1 minute
b. Anneal primers at 55 °C for 2 minutes
c. Extension at 72 °C for 3 minutes
d. 25-30 cycles of amplification is recommended.
e. Remove samples when PCR machine has cooled to 4 °C and store at -80 °C or proceed directly to evaluate product on gel.
f. Do not leave the PCR machine to remain at 4 °C overnight.

CHAPTER 9. References

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