Minerals in fish: does the source matter?

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Thesis

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Abstract

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Minerals are a group of micro-nutrients essential to fish. Meta-analysis of literature data was performed to identify the appropriate response criterion to determine the mineral requirement of fish. The meta-analysis revealed that, vertebral mineral concentrations or specific enzyme activities provide stringent requirement estimates compared to weight gain. Dietary intake forms the major route of mineral supply to fish; however fish are also capable of acquiring dissolved minerals from the rearing water. Changes in the dietary composition or mineral concentration of rearing water could have an impact on the mineral balance in fish. In this thesis, high fat diets, diets devoid, or low in fish meal and rearing systems with high water mineral concentrations were studied for their impact on the mineral balance in rainbow trout or common carp. Increased available phosphorus levels were needed (0.4% vs. 0.8%) in high fat diets to improve whole body and vertebral mineralisation as indicated by ash, P and Ca concentrations. However, supplementing phosphorus to complete plant ingredient based diets negatively affected the absorption and utilisation of micro-minerals namely Zn, Cu and Se. In rainbow trout that received complete plant ingredient based diets, the endogenous loss of Zn was higher and of Cu was lower resulting in Zn depletion and Cu accumulation in the body. Further, the hepatic metabolism of Fe and Cu was affected in rainbow trout fed the plant ingredient based diets, possibly due to the alterations in bile or cholesterol metabolism. With regard to the minimal dietary levels required, supplementation of Zn and Se were required beyond the levels recommended by NRC (2011) to maintain body balance in rainbow trout and common carp when fed complete plant ingredient based diets. Common carp reared in recirculation aquaculture systems (RAS) with high concentration of minerals in the water was able to acquire and retain minerals from water. However, only in the case of Se, they were able to compensate for a part of the dietary requirement. On the whole, higher dietary levels of P and Ca are required in the diet of fast growing rainbow trout; dietary levels of Zn and Se have to be increased beyond present recommended levels in plant ingredient based diets for rainbow trout and common carp. Low water exchange RAS has multiple effects on the whole animal physiology of fish and requires further research for better understanding.

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CHAPTER 1

General Introduction

Fish in human diet and its significance in aquaculture growth

Fish have been a regular part of the diet of human even 40 millennia ago. According to certain theories, eating fish helped the human brain grow in size, and develop more complex social behaviour, which ultimately led to our ancestors rising as a dominant species (Crawford *et al.*, 1999; Broadhurst *et al.*, 2002; Cunnane and Stewart, 2010). The practice of harvesting fish stocks from natural waters which started thousands of years ago has never ceased but in turn intensified. It has now been more than twenty years since we have attained the maximal exploitable limits of our oceans and only very limited scope is available to increase wild fish catch from the oceans (Pauly *et al.*, 2002). On the other hand, the increasing demand for animal protein and the necessity to feed the billions stands as a great challenge for the nutritional security. In this scenario, aquaculture presents the best, if not the only possible avenue to cater the need for food fish resulting from the expanding global population, according to 'Agricultural Outlook 2012-2021', OECD/FAO (2012).

Sustainable intensification of aquaculture

The growing demand for seafood has led to increasing intensification of fish production (FAO, 2014). Such intensification should be sustainable with minimal impact on the environment and natural resources (Klinger and Naylor, 2012). Feed and water are among the primarily important resources required for aquaculture. Over the years, awareness of the importance to include sustainable raw ingredient resources in fish feeds has increased. Reduction in the level of inclusion of fish meal (FM) and of fish oil (FM) originating from wild fisheries has become a standard practice over the past decade (Tacon and Metian, 2008; Tacon *et al.*, 2010; Tacon and Metian, 2013) in order to ensure that aquaculture is an environment-friendly and sustainable food production sector. In a resource (nutrients, energy, water) constrained world, maximising resource utilisation efficiency of aquaculture practices is essential. Farmed finfish are considered the most efficient in converting feed proteins into edible proteins for humans (Waite *et al.*, 2014) but there can be big differences between fish production systems.

With regard to water use efficiency, re-circulating aquaculture systems (RAS) are increasingly becoming viable alternatives to conventional flow-through aquaculture systems. Besides water use efficiency, the application of RAS technology offers advantages in terms of improved prospects for waste management and nutrient

recycling; better disease management and biological pollution control (no escapees). A number of studies have shown that the environmental performance of RAS fairs well above other aquaculture production systems (d'Orbcastel *et al.*, 2009; Martins *et al.*, 2010; Wilfart *et al.*, 2013). RAS also enables the production in close proximity to markets (Masser *et al.*, 1999; Schneider *et al.*, 2010), thereby reducing carbon dioxide (CO2) emissions associated with food transport and the negative trade deficits related to imports of fish. It is also recognised that further research is warranted to understand and manage different trade-offs in these systems such as performance of fish in terms of growth and nutrient utilisation especially with regard to micronutrients (Martins *et al.*, 2009; Martins *et al.*, 2011).

Aquaculture feeds - present scenario

Feed is a vital determinant of the productivity in aquaculture operations. The common feed evaluation indices of fish feeds are 'feed efficiency' and 'protein retention efficiency' which refer to the fish or protein produced per unit of feed or protein input, respectively. The primary objective of feed formulation and management practices in aquaculture is to improve the feed or protein retention efficiency. These are largely determined by the nutritional profile of the feeds. Protein (amino acids) is the primary source of energy for fish, and the most expensive nutrient component of the feed. Therefore, improving the protein retention efficiency through increased inclusion of digestible non-protein energy sources (fat, carbohydrates) is an effective strategy to reduce feed costs, improve growth rates and reduce nitrogenous waste output (Cho and Kaushik, 1990). Besides protein economy, sustainability of aquaculture is often questioned by the use of feeds rich in fish meal (FM) and fish oil (FO) derived from capture fisheries. Current estimates are that at a global level, aquaculture feeds utilise close to 70 and 90% respectively of the available FM and FO (FAO, 2012). Thanks to progress made in the area of FM and FO replacement by alternative protein and oil sources, the total use of such capture fishery-derived ingredients has decreased significantly and will continue to do so (Tacon and Metian, 2015). This decline will possibly influence the performance of the fish feeds due to the inherent changes in the quality of the nutrients supplied and other non-nutritional components present in the alternate feed ingredients. Among the four different nutrient groups (amino acids, fatty

acids, vitamins and minerals), the latter two collectively termed as micro-nutrients have received much less attention with regard to the aforesaid changes in fish feeds.

Minerals as essential nutrients and their requirement to fish

Of all 90 naturally occurring inorganic elements, 29 are considered to be essential for all farmed animals including fish (Lall and Milley, 2008), but only few of them have been studied in fish. Dietary requirements are established for macro-minerals such as Ca, K, Mg, Na, P and micro-minerals (trace elements) such as Cu, Fe, I, Mn, Se, Zn for one or more fish species (NRC, 2011). Studies have dealt with the functions, deficiency, availability, utilisation, toxicity and the interaction with other nutrients and environment on the aforesaid minerals to several fish species. Information on mineral requirements of fish has been compiled by the National Research Council (NRC, 1993; NRC, 2011) and in reviews on mineral nutrition of fish, e.g. (Schwarz, 1995; Davis and Gatlin, 1996; Watanabe *et al.*, 1997a; Kaushik, 2002; Lall, 2002). Shearer (1995) used a factorial modeling approach to predict the dietary requirements of minerals to rainbow trout. With regard to the optimal dietary inclusion levels in particular of micro-minerals such as Fe, Cu, Mn, Zn and Se in fish feeds, our knowledge remains limited (Lall and Milley, 2008). With the changing scenarios in aquaculture systems and feeds, there is a need to strengthen our knowledge on mineral requirements of fish in a systematic and comprehensive manner and to effectively utlise the information for optimising mineral supply and utilisation by fish reared with feeds having different ingredient profiles or under different rearing systems.

Meta-analysis, the science of data based research

Systematic reviews especially with meta-analytic components have gained popularity in the field of biology, including livestock and fish nutrition as a tool to gain more knowledge out of the existing published data (Borenstein *et al.*, 2011). Meta-analysis of data from the published literature is a relevant solution for inter-study comparisons and summarising data (Sauvant *et al.*, 2008). Careful selection and standardisation of data and the use of appropriate mathematical models for data analysis are essential to make the meta-analysis to be biologically relevant. The meta-analytic methods reported thus far in nutrient requirements in animals are primarily an extension of modeling approaches. Methodological limitations concerned with data structure had prevented

the use of conventional tools of meta-analysis in studies on nutrient requirements. Meta-analytic approach has been used to analyse data on dietary requirements for selected amino acids in farm animals (Simongiovanni *et al.*, 2011) as well as in fish (Kaushik and Seiliez, 2010; Hua, 2013), to investigate nutrient balance in growing animals (Schulin-Zeuthen *et al.*, 2007), to study the effects of fish meal and fish oil replacement in fish feeds (Drakeford and Pascoe, 2008; Sales, 2009; Sales and Glencross, 2011; Hua and Bureau, 2012). Although there is a significant amount of data available in the literature on response to a particular mineral based on a wide range of response variables, it is not clear if the requirement of a micro-nutrient should be based on growth or other specific response criterion needs to be used (NRC, 2011). Meta-analysis can be used effectively to identify the best criterion for determining mineral requirement as well as to identify knowledge gaps for further research.

Impact of changing feeds on optimal dietary supply of minerals to fish

Ensuring adequate dietary supply of minerals to farmed fish is essential for proper somatic and skeletal growth, health and final flesh quality. Mineral composition of edible as well as non-edible portions in farmed fish is linked to the dietary mineral composition of the feeds (Lall, 1995; Carpene *et al.*, 1998; Fuentes *et al.*, 2010; Fallah *et al.*, 2011). Besides the mineral composition, other dietary factors such as ingredient composition of the diet, macro-nutrient levels, nutrient interactions, anti-nutritional factors affecting mineral availability and utilisation etc., can also affect mineral supply to fish.

Impact of diet composition on mineral needs for basal metabolism

The nutrient requirement of fish can be split into three parts as, (i) requirement for basal metabolism or maintenance; (ii) requirement for growth, and (iii) requirement for reproduction (Dabrowski, 1985; Cho *et al.*, 1982). Maintenance requirement for a nutrient is the level of intake required to compensate for obligatory losses and for maintaining nutrient balance, i.e. no gain-no loss situation (Mitchell, 1962; Pfeffer and Potthast, 1977; Cho and Kaushik, 1990). Maintenance requirement can be determined by regressing gain against intake obtained by feeding diets of semi-purified or practical ingredients, with graded levels of the target nutrient (Pfeffer and Pieper, 1979; NRC, 2011). This method is considered as not applicable for studies with minerals, since low levels of certain minerals in semi-purified diets, for instance, can result in biased mineral

balances, whereas the response at higher inclusion levels can be due to differences in feed intake rather than the utilisation of the mineral (Baker, 1984). Secondly, with conventional feed ingredients, it is difficult to formulate a basal diet with sufficiently low levels of minerals. Instead, the factorial method (Fig. 1.1) involves feeding graded ration levels of a diet with known nutrient concentration (Baker, 1984; Cowey, 1992).

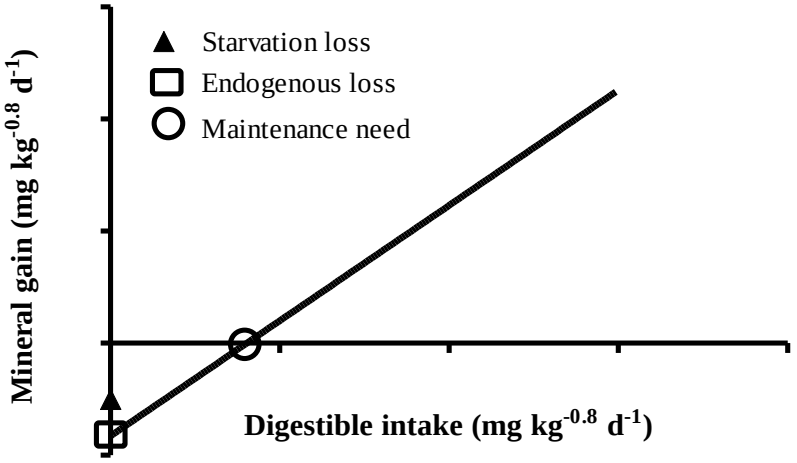

Fig. 1.1: Factorial method for estimating mineral needs for basal metabolism in animals. Upon regression of mineral gain over intake, (i) intercept on Y-axis (when, x=0; open circle) gives endogenous loss of fed fish; (ii) intercept of X-axis (when, y=0; open square) gives point of intake for zero-balance, no loss-no gain state; and (iii) observed mineral loss from body during starvation (filled diamond).

Using this method, maintenance requirements have been determined in fish for many nutrients and digestible energy (Gatlin *et al.*, 1986; Shearer, 1995; Lupatsch *et al.*, 1998; Rodehutscord and Pfeffer, 1999; Fournier *et al.*, 2002; Bureau and Encarnação, 2006; Glencross, 2008; Helland *et al.*, 2010). Such an approach has not been applied for studies with minerals in fish. In animals, starvation loss, endogenous loss of fed animals or level of intake for zero balance have been used to estimate mineral requirements for maintenance, the latter two can be affected by changes in diet composition (Dilger and Adeola, 2006b; Dilger and Adeola, 2006a).

Cellular and molecular regulation of mineral balance in fish

The level and form of supply of minerals through the diet or from the ambient water is known to affect the associated biochemical and physiological responses in fish (Bury and Grosell, 2003). In humans, vegetarian diets are reported to be limiting in the bio-available supply of micro-minerals such as Fe, Zn and Se; but are generally good sources

of Cu and Mn, present at higher concentrations in plant ingredients, in contrast with Se which is higher in animal than in plant protein sources (Hunt, 2003). The intrinsic form of the minerals in the respective dietary ingredients thus bears great significance. For instance, heme-bound Fe in fish or meat based diet is relatively more bio-available than non-heme Fe of plant derived ingredients (Standal *et al.*, 1999). Besides the difference in the form or level of minerals, secondary metabolites (anti-nutritional factors, ANFs such as phytic acid, protease inhibitors, lectins or alkaloids) in plant ingredients can directly or indirectly affect micro-mineral metabolism (Francis *et al.*, 2001) with the entero-hepato-pancreatic system is the target of major ANFs. Phytic acid can directly reduce the availability of phosphorus but can also indirectly interfere in mineral metabolism, as uptake and body status of Fe, Cu, Mn and Zn are primarily regulated at the level of gastrointestinal tract or liver (Hambidge, 2003). Despite the recognition of such links between dietary plant ingredients and micro mineral metabolism, the underlying cellular and molecular responses that could alter the mineral metabolism in fish remains little explored. Over the years, various functional proteins and their encoding genes regulating cellular transport of micro-minerals have been characterised in mammals, a few of which have also been identified in different teleost species (Bakke *et al.*, 2010). Besides the transporters, activity and expression of metaloenzymes have been used as markers for micro-mineral status (Hunt, 2003). Most of these markers are shown to respond to exogenous supply of respective micro-minerals.

High fat diets and phosphorus requirement in rainbow trout

Over the years, high energy diets (mainly with high lipid levels) are commonly used in salmonid farming and are known to improve feed and protein efficiencies, with a consequent reduction in waste output in terms of suspended matter, nitrogen (N) or phosphorus (P). Given the improved feed efficiencies with such high energy diets, if the dietary P level is maintained at a fixed level, a situation arises where less dietary P is available to the fish to support a higher (than normal) growth rate per unit quantity of feed consumed. The faster growth rates achieved with such diets can affect growth allometry resulting in vertebral deformities especially under dietary phosphorus (P) insufficiency (Hansen *et al.*, 2010). Improving the feed efficiency may require increased available phosphorus supply to meet the phosphorus requirement of fast growing fish (Shearer, 1995), as hypothesized in Fig. 1.2.

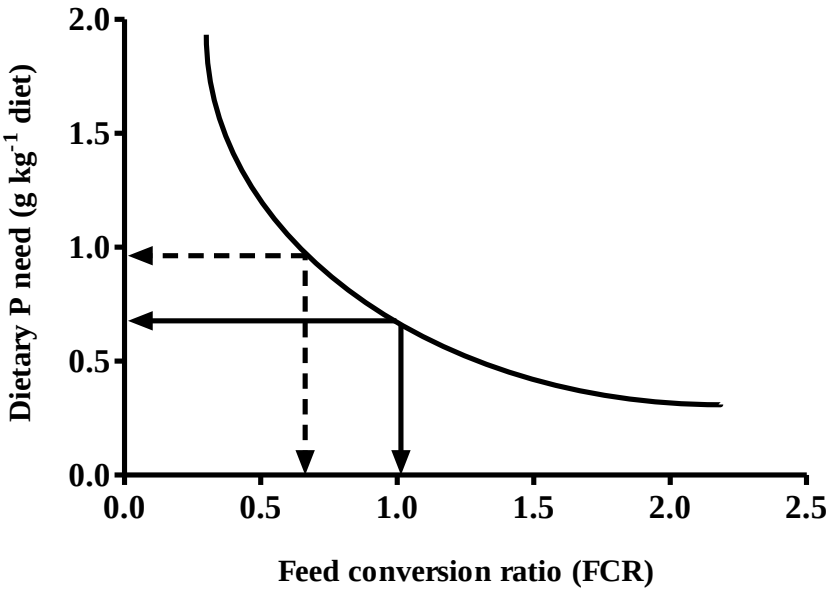

Fig. 1.2: Impact of improving feed conversion ratio in fish feeds on the dietary phosphorus needs. Hypothetical response curve, adapted from Shearer (1995).

Phosphorus supplements and micro-mineral availability

Fish meal (FM) is a good source of minerals (Julshamn *et al.*, 1978) and especially of phosphorus to farmed fish (Kaushik, 2002; Lall, 2002). The substitution of FM by plant protein sources inevitably calls for measures to ensure an adequate supply of minerals in an available form to meet the physiological demands of fish. In diets containing high levels of plant-protein ingredients and low levels of FM, supplementation with mono- or di-basic inorganic P salts has been found to be an efficient strategy to increase the levels of available P supply (Ketola, 1975; Watanabe *et al.*, 1997b; Kaushik *et al.*, 2004). However, very little is known on the impact of adding calcium-phosphate salts on the availability of other micro-minerals.

Micro-mineral supplementation in plant ingredient based diets

The dietary inclusion level of micro-minerals needed to meet the requirement can vary based on a number of physiological and environmental factors (Hilton, 1989; Sugiura *et al.*, 2000), the change in ingredient composition of practical fish feeds being one such factor (Watanabe *et al.*, 1997a). The latter authors suggested that micro-minerals such as Fe, Cu, Mn, Se and Zn are important to be supplemented in fish feeds due to the low levels in practical feed ingredients and interactions with other dietary components. The

magnitude of supplementation depends on the intrinsic contribution from the basal ingredients and interactions affecting the availability of the micro-minerals. Theoretically, levels of the essential minerals in plant derived ingredients used (Table 1) in fish feeds are comparable or even higher compared to those found in fish meals, except for phosphorus, zinc and selenium. Considering the supply of micro-minerals from the different ingredients themselves (Sugiura *et al.*, 1998; Lall and Milley, 2008), it is necessary to evaluate the need for and level of supplementing one or more micro-minerals to diets devoid of or with very low levels of FM.

Table 1: Concentrations of minerals in fish meals compared to those from different plant protein sources (drawn from iO7, INRA)

	Fishmeals		Plant protein sources	
Minerals	Min	Max	Min	Max
Ca (g/kg)	24.1	55.4	0.4	33.8
P (g/kg)	20.6	31	2.6	11.4
Phytic P (% of P)	0	0	5	85
Mg (g/kg)	1.9	2.6	0.4	5.5
K (g/kg)	7.4	12.2	0.9	21.2
Na (g/kg)	9.5	11.3	0.04	0.9
Cl (g/kg)	15.1	17.7	0.4	1.8
S (g/kg)	6.7	7.4	1.1	5.9
Mn (mg/kg)	6	23	8	1802
Zn (mg/kg)	85	89	15	69
Cu (mg/kg)	7	9	2	62
Fe (mg/kg)	252	469	24	283
Se (mg/kg)	0.4	0.41	0.06	1.1
I (mg/kg)	2	3	0.06	0.28

Re-circulating aquaculture systems (RAS) and mineral metabolism in fish

Fish farming involves diverse production systems, ranging from extensive or semi-extensive pond culture systems to totally feed-based highly intensive raceways or cage culture systems. Over the years, aquaculture systems have evolved continuously to cope up with the ever growing need for a more efficient and sustainable culture practice. The most recently evolved and steadily expanding systems are the re-circulating aquaculture systems (RAS). In RAS, the particulate faecal waste and unfed feed pellets are mechanically removed periodically, whereas the dissolved metabolic wastes and minerals leached from feed pellets and faecal matter tend to accumulate in the water. The magnitude of accumulation is based on the water exchange rate of the RAS.

Generally, water exchange rate and magnitude of mineral accumulation are inversely related, the lower the water exchange, the higher the accumulation (Martins *et al.*, 2009). Following the report of Martins *et al.* (2009), few other studies reported data on minerals accumulating in RAS water (Davidson *et al.*, 2009; Davidson *et al.*, 2011). The minerals reported to accumulate are P, Mg, K, Na, Fe, Cu, Zn, Mn, Co, Cd and Ni; the most profound accumulation has been reported for phosphorus and reports show that dissolved ortho-phosphate concentrations at 25 mg/l in RAS have beneficial effects on P-gain and growth of Nile tilapia grown in freshwater (Janssen 2010). In this scenario, studying the interaction of the accumulating aqueous mineral levels on the mineral utilisation and requirement will be useful in better managing the dietary mineral levels as well as nutrient discharge from the RAS.

Aim and Outline of the thesis

The thesis starts with the investigation of published literature data on mineral requirements of fish. Meta-analysis of available data on dose response to dietary phosphorus in fish from over 64 published studies covering over 40 species of fish was performed to identify the most appropriate response criterion in estimating phosphorus requirement in fish and also to identify factors altering dietary need for phosphorus to fish (Chapter 2). In the subsequent chapter, the approach was extended for other essential macro- and micro-minerals and a systematic review on the literature data on mineral requirements of fish and factors affecting the dietary need was made with the generation of comprehensive database on mineral nutrition in fish (Chapter 3).

During the course of the study in Chapter 2, it was identified that dietary phosphorus requirement was proportional to the growth rate of the fishes. Improving the growth rate and feed efficiency may require increased available phosphorus supply to meet the phosphorus requirement of fast growing fish (Shearer, 1995). This hypothetical prediction was tested by altering the levels of fat and phosphorus in the diet of rainbow trout (Chapter 4).

Given the recent changes in the diet composition of fish feeds with significant reductions in fish meal levels, it would be worth studying the effect of this dietary change on bio-availability, basal needs and metabolism of minerals. Phosphorus supplementation to a complete plant based diet can affect the bio-availability of other macro- and micro-

minerals to fish. In this regard, post-prandial changes in plasma mineral levels and utilisation of minerals in rainbow trout fed complete plant ingredient based diets with or without supplemental di-calcium phosphate were studied and compared with the responses of a fish meal based diet (Chapter 5). The effect of changing diet composition on basal metabolic needs such as endogenous loss and maintenance requirement was tested by regressing mineral gain over intake in rainbow trout upon feeding a fish meal or plant ingredient based diet at four different feeding levels (Chapter 6).

Subsequently, the effect of transition from a fish meal based feed to a feed based on plant ingredients on micro mineral metabolism in fish was studied. This was assessed by a study with a 2 x 2 factorial arrangement with rainbow trout, fed a fish meal based diet (M) or a totally plant ingredient based vegetable (V) diet, with or without micro-mineral (Fe, Cu, Mn, Zn and Se) premix inclusion, and responses related to absorption, transport or metabolism of micro minerals were investigated (Chapter 7).

Further trials were conducted to evaluate the need for and level of supplementing one or more micro-minerals to diets devoid of fish meal in rainbow trout and common carp. In rainbow trout it was studied by supplementing graded levels of a micro-mineral premix comprising of Fe, Cu, Mn, Zn and Se to a complete plant ingredient based diet and analysing the body mineral balances (Chapter 8). The effect of aqueous mineral availability on mineral balance and minimal dietary inclusion levels required for common carp reared in RAS with high or low aqueous mineral concentrations generated through differing water exchange rates (Chapter 9).

In the final chapter (Chapter 10) of this thesis, the findings and observations of the preceding chapters are discussed within the context of the effect of on-going and foreseen dietary and rearing system changes on the mineral supply, utilisation and metabolism in fish.

CHAPTER 2

Quantifying dietary phosphorus requirement of fish: a meta-analytic approach

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Abstract

A meta-analysis of available data on dose response to dietary phosphorus (P) in fish from over 70 feeding trials reported in 64 published studies covering over 40 species of fish was performed. Broken-line regression was used to model the datasets. The meta-analysis showed that estimated minimal dietary P level varies with the response criterion and that estimates should preferably be expressed in terms of available P than in terms of total P. Estimates based on whole body P concentration (4.7 g available P kg^{-1} dry matter, DM) or vertebral P (5.2 g available P kg^{-1} DM) were greater than that for maximising somatic weight gain (3.5 g available P kg^{-1} DM) or plasma P concentration (2.8 g available P kg^{-1} DM). P content of fish varies linearly with body mass (3.6 g kg^{-1} live weight). Use of ingredients rich in P or of diets with high basal P content or high levels of water P concentration can affect the estimations. Among the different response criteria tested, weight gain was found to be the most reliable and whole body P concentration to be the most stringent criterion to estimate P requirement of a given fish species. Expressing available P requirement as g P per unit DM or digestible energy (DE) in the diet were equally effective, but expressing in terms of g P intake per kg $BW^{0.8}$ per day would be more precise.

Introduction

Available data on mineral requirements of fish and crustaceans has been updated and summarised recently by the National Research Council (NRC, 2011). Of all minerals considered essential for fish, requirement for phosphorus (P) is the most extensively studied. Although there is a significant amount of data available in the literature on response to dietary P and P requirement based on a wide range of response variables, direct comparison of results is often difficult due to inter-study differences. Meta-analysis of data from the available literature is a relevant solution for inter-study comparisons and for summarising data (Sauvant *et al.*, 2008). Meta-analytic approach has been used to analyse data on dietary requirements for selected amino acids in animals (Simongiovanni *et al.*, 2011) as well as in fish (Kaushik & Seiliez, 2010), to investigate nutrient balance in growing animals (Schulin-Zeuthen *et al.*, 2007), to study the effects of fish meal and fish oil replacement in fish diets (Drakeford & Pascoe, 2008; Sales, 2009; Sales & Glencross, 2011; Hua & Bureau, 2012). Careful selection and standardisation of data and the use of appropriate mathematical model for data analysis are essential to make the meta-analysis to be biologically relevant. Till date, more than 70 studies on P requirement or P utilisation have been reported for over 40 different species of fish. About 90% of the studies were undertaken in the past two decades (1991-2011) and over 60% in the past decade alone (2001-2011). This reflects the recognition of the importance of P not only in growth and skeletal development but also the environmental implications of P discharge into water. P requirement of fish has been estimated using a wide range of response criteria: (i) production traits such as weight gain, growth rate or feed efficiency, (ii) levels of ash/P in whole body, plasma/serum, vertebrae, scale or skin, (iii) mineral balance indices such as P gain, retention and, (iv) to a limited extent, expression of genes involved in absorption of P from the gastro-intestinal tract. The major and widely used response criteria are in the order of weight gain, whole body P, vertebral P, plasma P and whole body P balance. Urinary P excretion has also been occasionally used, especially for large fishes. The present work aims at re-analysing available data on P requirement of fish for estimating the minimal dietary P level required based on different response criteria and to identify the most stringent criterion and mode of expression through meta-analysis. These were performed by delineating the inter-study differences through standardisation of

dependent and independent variables following selection, segregation and grouping of data.

Methodology

Construction of database

Data from published literature on P requirement or P dose response studies in fish with at least three dietary P levels were collected. Data on different factors such as fish species, initial body weight, final body weight, duration of feeding trial, diet type, P concentration in basal diet, number of dietary P levels tested and corresponding dietary P concentrations, P availability, feed efficiency, major protein source, source of supplemental P, time of sampling after the last meal, type of rearing system, water temperature, salinity, P concentration of the rearing water, response variables tested, mathematical model used, if any, response criterion based on which optimal dietary P level was estimated and the corresponding estimate values were registered. A database was created with the foresaid information with the corresponding data from a total of over 70 feeding trials reported in 64 published articles covering not less than 40 fish species. In each study, data from treatment groups, in which dietary P concentration alone was the only quantitative variable were selected and grouped for inclusion in the meta-analysis.

Selection of data for meta-analysis

Every study reported data on one or more of the different response criteria employed. The widely used four criteria (weight gain, whole body P, vertebral P and plasma P) were chosen for a comparative analysis across studies. Initially, meta-analysis was performed on the complete dataset including all fish species (All). Further to assess if P requirement differs between fish species, the total dataset was split into two, a dataset of only rainbow trout (Rbt) and a dataset with all species excluding rainbow trout (All – Rbt). Meta-analysis was performed on these sub-datasets for the four response criteria in relation to both dietary total P as well as available P concentration. Detailed description of the different datasets is reported in Table 1.

Standardisation of selected data

The selected data were standardised to a common response unit of expression prior to meta-analysis. For example, growth or weight gain data were normalised by taking the

maximum body weight gain in a given study as 100 and calculating the weight gain of the other groups in the study as a relative percentage to the maximum weight gain (Simongiovanni *et al.*, 2011). This helped in direct inter-study comparison of the growth data which would as such be difficult due to differences in fish size, duration of trial and magnitude of somatic weight gain in absolute terms. Dietary P concentration expressed as fed or on a dry matter (DM) basis in the studies were converted to g P per unit DM, or g P MJ^{-1} DE or absolute P intake (g P kg^{-1} $BW^{0.8}$ d^{-1}) on total and available P basis, wherever possible. Response data expressed in different units in different studies for a given criteria such as whole body P, vertebral P and plasma P were normalised and expressed in terms of g P kg^{-1} wet weight, percentage on dry weight basis and mmol L^{-1} respectively.

Calculations

$$\text{Relative weight gain (RWG, \%)} = \frac{\text{actual weight gain of the group}}{\text{maximum weight gain in the given study}} \times 100$$

Thermal growth coefficient (TGC, %)

$$= \frac{\text{Final body weight}^{1/3} - \text{Initial body weight}^{1/3}}{\text{Temperature} \times \text{days}} \times 100$$

$$\text{P content in fish (g fish}^{-1}) = \text{weight of fish (g)} \times \text{whole body P concentration (\%)}$$

$$\text{Metabolic body weight (MBW)} = (\text{actual body weight})^{0.8}$$

$$\text{Average metabolic body weight (Av MBW)} = (\text{Initial MBW} \times \text{Final MBW})^{0.5}$$

$$\text{Metabolic weight gain } \left(\text{g kg}_{\text{MBW}}{}^{-1}\text{day}^{-1}\right) = \frac{\text{weight gain (g)}}{\text{Av MBW (kg)} \times \text{days}}$$

$$\text{Feed intake (g)} = \text{weight gain (g)} \times \text{feed gain ratio}$$

$$\text{P intake (g kg}_{\text{MBW}}{}^{-1}\text{day}^{-1}) = \frac{\text{Feed intake (g)} \times \text{diet P concentration (g kg}^{-1})}{\text{Av MBW (kg)} \times \text{days} \times 1000}$$

Water P concentration

The response criterion weight gain in relation to dietary available P level was used to assess if this relationship (growth versus available P) was dependent on rearing system (flow through versus recirculation system). This was done for the 'Rbt' sub-dataset, which contained two of experiments done in recirculation system (Rodehutscord, 1996;

Rodehutscord *et al*. 2000). Meta-analysis was done on this dataset including and excluding these studies. Moreover, a comparison of growth data (calculated as TGC) was made between two studies in rainbow trout (Ketola & Richmond, 1994; Rodehutscord, 1996) of relatively similar initial body weights (35g and 50g) but differing in the concentration of P in the rearing water rendered by the nature of the rearing system (P concentration in water not determined, flow-through system and < 5 mg L^{-1} P in water, recirculation system).

Diet type and P concentration of the basal diet

There were large differences between studies in the basal diet composition. In order to study if the relationship between response criteria and dietary P level was affected by the basal diet concentration of available P, the total dataset for the response variable weight gain was spilt into two sub-datasets: one with having a concentration of available P in the basal diet below 3 g available P kg^{-1} DM and the other sub-dataset above 3 g available P kg^{-1} DM. Meta-analyses were performed to examine if estimated minimal dietary P levels for optimal weight gain was affected by basal diet available P concentration.

Unit of expression

Minimal (optimal) dietary P levels required for growth can be expressed in different ways such as g kg^{-1} DM and g MJ^{-1} DE (Digestible energy). In NRC (2011) it is expressed in g kg^{-1} DM, whereas it can be argued if requirements are not related to dietary nutrient concentration (e.g. DE content). In order to assess if this unit of expression (per unit of DM versus per unit of DE) improved the prediction of the response criteria, a dataset comprising of studies from which data on weight gain based on DM basis as well as DE basis could be calculated. This comparison was made based on the R^2 values of the regression.

Model selection

Two models namely, simple linear broken line regression (Robbins *et al.*, 1979) and Mercer's four parameter nutrient saturation kinetic model (Mercer, 1982) were tested to model the different data sets with a fixed effect approach. Preliminary analysis

showed that, the linear broken line regression model had a better fit and was preferred over Mercer's nutrient kinetic model. The description of the model used is as follows,

$Y_1 = a_1 + b_1.X$; Y at $X_{bp} = b_1.X_{bp} + a_1$

Y_2 = Y at $X_{bp} + b_2.(X - X_{bp})$; Y = if ($X<X_{bp}$, Y_1, Y_2)

With, Y = Response criteria such as RWG (%), WG ($g.kg^{-1}$ $BW^{0.8}.d^{-1}$), Whole body P (g/kg wet BW), Vertebral P (% DM) or Plasma P (mmol/L)

X = dietary P concentration (g kg^{-1} DM or g MJ^{-1} DE) or intake (P kg^{-1} $BW^{0.8}$ d^{-1}) on total or available basis

a_1 = intercept on y-axis for X = 0

b_1 = slope of the first line (Y_1, ascending segment)

b_2 = slope of second line (Y_2, plateau segment; < 0.1)

X_{bp} = breakpoint X value (P requirement value)

Results

Of all the 64 studies (Table 1) that report a quantitative dietary P requirement or a dose response relationship to graded dietary P levels for a given fish species, only 40% (25 studies) provide data on the dietary available P levels in the diets used. Of all the studies that report dietary P requirement in fish (Table 2), the majority (59%) have used primarily somatic growth as the response criterion. Other response variables such as vertebral ash or phosphorus content (39.7%), whole body phosphorus content (24.4%), P retention (10.3%), Scale P (10.3%), Non-faecal P excretion (6.4%), plasma P (7.7%) and gene expression of P-transporters (1.5%) have also been used to estimate the dietary P requirement of fish.

Background information on the studies used in this meta-analysis of data on P requirement is presented in Table 2. Estimation of the minimal dietary P levels based on different response criteria on different datasets, using the broken line regression is shown in Table 1. Within the different datasets (complete and sub-datasets) the minimal dietary P level was lower for available P compared with total P content and in general the R^2 of the broken line analysis was higher for available P compared to total P.

Table 1: Summary of minimal dietary P level (g kg^{-1} DM, g MJ^{-1} DE, and g kg^{-1} BW$^{0.8}$ d^{-1}) as estimated through the meta-analysis using broken line regression analysis*.

Variable	Dietary form	Data set	Sp.	St.	a_1	b_1	X_{bp}	95% CL	Unit	R^2
Weight gain[1]	Total	All	41	64	43.3	9.5	**4.7**	4.1 – 5.3	g kg^{-1} DM	0.3
Weight gain	Total	Rbt	1	10	34.4	18.7	**3.2**	2.7 – 3.7	g kg^{-1} DM	0.71
Weight gain	Total	All - Rbt	40	54	50.7	6.1	**6.4**	5.6 - 7.3	g kg^{-1} DM	0.33
Weight gain[2]	Available	All	19	25	23.5	19.5	**3.5**	3.2 – 3.8	g kg^{-1} DM	0.66
Weight gain	Available	Rbt	1	7	57.1	12.3	**2.7**	2.2 – 3.6	g kg^{-1} DM	0.42
Weight gain	Available	Rbt-RAS	1	5	49.1	9.1	**4.4**	3.5 – 5.5	g kg^{-1} DM	0.38
Weight gain	Available	All - Rbt	13	15	37.3	11.3	**4.7**	4.1 – 5.3	g kg^{-1} DM	0.6
Weight gain[3]	Total	All	17	21	37.6	176	**0.3**	0.25 -. 0.34	g MJ^{-1} DE	0.4
Weight gain	Total	Rbt	1	6	3.5	532	**0.17**	0.13 - 0.21	g MJ^{-1} DE	0.51
Weight gain	Total	All - Rbt	16	15	23.52	214	**0.31**	0.28 - 0.35	g MJ^{-1} DE	0.53
Weight gain[4]	Available	All	9	13	17.1	427	**0.17**	0.15 - 0.2	g MJ^{-1} DE	0.6
Weight gain	Available	Rbt	1	3	45.8	258	**0.16**	0.1 - 0.23	g MJ^{-1} DE	0.53
Weight gain	Available	All - Rbt	8	10	34.5	230	**0.25**	0.19 - 0.3	g MJ^{-1} DE	0.46
Weight gain[5]	Total	All	34	42	4.14	45.6	**0.16**	0.12 - 0.19	g kg^{-1} BW$^{0.8}$ d^{-1}	0.3
Weight gain	Total	Rbt	1	6	5.1	61.8	**0.19**	very wide	g kg^{-1} BW$^{0.8}$ d^{-1}	0.37
Weight gain	Total	All - Rbt	33	36	3.5	41.2	**0.19**	0.15 - 0.23	g kg^{-1} BW$^{0.8}$ d^{-1}	0.41
Weight gain[6]	Available	All	15	19	3.19	136	**0.06**	0.04 - 0.08	g kg^{-1} BW$^{0.8}$ d^{-1}	0.3
Weight gain	Available	Rbt	1	3	7.2	132	**0.07**	0.04 - 0.1	g kg^{-1} BW$^{0.8}$ d^{-1}	0.51
Weight gain	Available	All - Rbt	14	16	2.6	105	**0.08**	0.06 - 0.1	g kg^{-1} BW$^{0.8}$ d^{-1}	0.49
Whole body P[7]	Total	All	18	27	1	0.54	**6.5**	5.3 – 7.6	g kg^{-1} DM	0.25
Whole body P	Total	Rbt	1	7	1.23	0.47	**7.4**	5.9 – 8.8	g kg^{-1} DM	0.7
Whole body P	Total	All - Rbt	17	20	2.5	0.27	**7.8**	4.7 - 10.9	g kg^{-1} DM	0.13
Whole body P[8]	Available	All	14	18	1	0.72	**4.7**	3.8 – 5.7	g kg^{-1} DM	0.22
Whole body P	Available	Rbt	1	5	1	0.65	**5.8**	4.4 – 7.3	g kg^{-1} DM	0.73
Whole body P	Available	All - Rbt	8	13	2.5	0.4	**6.4**	3.4 – 9.4	g kg^{-1} DM	0.2
Vertebral P[9]	Total	All	17	23	5.9	0.41	**10.5**	8.2 – 12.8	g kg^{-1} DM	0.44
Vertebral P[10]	Available	All	9	9	4.9	0.87	**5.8**	3.1 – 7.3	g kg^{-1} DM	0.26
Plasma P[11]	Total	All	5	7	2	0.25	**7.3**	2.1 – 12.6	g kg^{-1} DM	0.16
Plasma P	Total	Rbt	1	3	1.8	0.37	**6.9**	2.6 – 11.3	g kg^{-1} DM	0.47
Plasma P[12]	Available	All	6	8	1.2	0.81	**2.8**	1.1 – 4.4	g kg^{-1} DM	0.11
Plasma P	Available	Rbt	1	3	0.43	1.27	**3**	2.7 – 3.7	g kg^{-1} DM	0.74

*Slope of the second line Y_2 (b_2) ranged from 0.011 – 0.018; All – dataset comprising all species; Rbt – dataset comprising only rainbow trout; All – Rbt: dataset comprising of all species excluding rainbow trout; Sp – Number of species; St – Number of studies.

[1] Andrews (1973); Asgard & Shearer (1997); Borlongan & Satoh (2001); Brown *et al*. (1992); Chavez-Sanchez *et al*. (2000); Chen *et al*. (2007); Choi *et al*. (2005); Coloso *et al*. (2003); Davis & Robinson. (1987); Dougall *et al*. (1996); Elangovan & Shim (1998); Fontagne *et al* (2009); Furuya *et al*. (2008); Ketola & Richmond (1994); Ketola (1975); Kim *et al*. (1998); Kousoulaki *et al*. (2010); Liang at al. (2011); Lovell (1978); Luo *et al*. (2009); Luo *et al*. (2010); Mai *et al*. (2006); Nwanna *et al*. (2009); Nwanna *et al*. (2010); Ogino & Takeda (1976); Ogino & Takeda (1978); Oliva-telas & Ana (2004); Paul *et al*. (2004); Phromkunthong & Udom. (2008); Qiu-shan *et al*. (2009); Robinson *et al*. (1987); Rodehutscord (1996);Rodehutscord *et al*. (2000); Rodriues & O-teles (2001); Roy & Lall (2003); Roy *et al*. (2004); Satoh *et al*. (2003); Schafer *et al*. (1995); Shao *et al*. (2008); Shim & Ho (1989); Skonberg *et al*. (1997); Sugiura *et al*. (2000a); Sugiura *et al*. (2000b); Sugiura *et al*. (2007); Sukumaran *et al*. (2009); Vielma & Lall (1998); Vielma *et al*. (2002); Wang *et al*. (2005); Wen *et al*. (2008); Xie *et al*. (2011); Xu *et al*. (2011); Yang *et al*. (2006); You *et al*. (1987); Youli *et al*. (2008); Yuan *et al*. (2011); Zhang *et al*. (2006); Zhang *et al*. (2006); Zhao *et al*. (2008a); Zhao *et al*. (2008b); Zheng *et al*. (2007) & Zhou *et al*. (2004).

[2] Brown *et al*. (1992); Choi *et al*. (2005); Coloso *et al*. (2003); Dougall *et al*. (1996); Fontagne *et al*. (2009); Furuya *et al*. (2008); Ketola & Richmond (1994); Kim *et al*. (1998); Kousoulaki *et al*. (2010); Liang at al. (2011); Mai *et al*. (2006); Nwanna *et al*. (2009); Phromkunthong & Udom (2008); Rodehutscord (1996);Rodehutscord *et al*. (2000); Schafer *et al*. (1995); Shao *et al*. (2008); Sugiura

et al. (2007); Sukumaran *et al*. (2009); Vielma & Lall (1998); Vielma *et al*. (2002); Xie *et al*. (2011); Yuan *et al*. (2011) & Zhang *et al*. (2006).

[3] Borlongan & Satoh (2001); Coloso *et al*. (2003); Davis & Robinson (1987); Dougall *et al*. (1996); Fontagne *et al*. (2009); Kousoulaki *et al*. (2010); Nwanna *et al*. (2010); Oliva-telas & Ana (2004); Phromkunthong & Udom (2008); Robinson *et al*. (1987); Rodehutscord (1996);Rodehutscord *et al*. (2000); Rodriues & O-teles (2001); Roy & Lall (2003); Skonberg *et al*. (1997); Sugiura *et al*. (2007); Vielma & Lall (1998); Vielma *et al*. (2002); Wang *et al*. (2005); Xu *et al*. (2011) &Yang *et al*. (2006).

[4] Brown *et al*. (1992); Coloso *et al*. (2003); Dougall *et al*. (1996); Fontagne *et al*. (2009); Furuya *et al*. (2008); Kousoulaki *et al*. (2010); Nwanna *et al*. (2010); Phromkunthong & Udom (2008);Rodehutscord (1996); Sugiura *et al*. (2007); Vielma & Lall (1998) & Vielma *et al*. (2002).

[5] Andrews (1973); Asgard & Shearer (1997); Borlongan & Satoh (2001); Choi *et al*. (2005); Coloso *et al*. (2003); Davis & Robinson. (1987); Dougall *et al*. (1996); Dougall *et al*. (1996); Elangovan & Shim (1998); Ketola & Richmond (1994); Ketola (1975); Kim *et al*. (1998); Kousoulaki *et al*. (2010); Liang at al. (2011); Luo *et al*. (2010); Nwanna *et al*. (2009); Nwanna *et al*. (2010); Ogino & Takeda (1976); Ogino & Takeda (1978); Oliva-telas & Ana (2004); Paul *et al*. (2004); Phromkunthong & Udom (2008); Robinson *et al*. (1987); Rodehutscord (1996);Rodehutscord *et al*. (2000); Rodriues & Oliva-teles (2001); Roy & Lall (2003); Roy *et al*. (2004); Schafer *et al*. (1995); Shao *et al*. (2008); Shim & Ho (1989); Skonberg *et al*. (1997); Sukumaran *et al*. (2009); Vielma & Lall (1998); Wang *et al*. (2005); Xie *et al*. (2011); Xu *et al*. (2011); Yang *et al*. (2006); Yuan *et al*. (2011); Zhao *et al*. (2008b); Zheng *et al*. (2007) & Zhi *et al*. (2009).

[6] Brown *et al*. (1992); Choi *et al*. (2005); Coloso *et al*. (2003); Dougall *et al*. (1996); Furuya *et al*. (2008); Ketola & Richmond (1994); Kim *et al*. (1998); Kousoulaki *et al*. (2010); Liang at al. (2011); Nwanna *et al*. (2010); Phromkunthong & Udom (2008); Rodehutscord (1996);Rodehutscord *et al*. (2000); Schafer *et al*. (1995); Shao *et al*. (2008); Sukumaran *et al*. (2009); Vielma & Lall (1998); Xie *et al*. (2011) & Yuan *et al*. (2011).

[7] Asgard & Shearer (1997); Borlongan & Satoh (2001); Brown *et al*. (1992); Coloso *et al*. (2003); Elangovan & Shim (1998); Ketola & Richmond (1994); Kousoulaki *et al*. (2010); Liang at al. (2011); Luo *et al*. (2009); Luo *et al*. (2010); Mai *et al*. (2006); Nwanna *et al*. (2009); Ogino & Takeda (1976); Ogino & Takeda (1978); Oliva-telas & Ana (2004); Phromkunthong & Udom (2008); Rodehutscord (1996);Rodehutscord *et al*. (2000); Rodriues & O-teles (2001); Schafer *et al*. (1995); Shao *et al*. (2008); Skonberg *et al*. (1997); Sukumaran *et al*. (2009); Xie *et al*. (2011); Xu *et al*. (2011); Yang *et al*. (2006); Yuan *et al*. (2011) & Zhang *et al*. (2006).

[8] Brown *et al*. (1992); Coloso *et al*. (2003); Fontagne *et al*. (2009); Ketola & Richmond (1994); Kim *et al*. (1998); Kousoulaki *et al*. (2010); Liang at al. (2011); Mai *et al*. (2006); Phromkunthong & Udom (2008); Rodehutscord (1996);Rodehutscord *et al*. (2000); Schafer *et al*. (1995); Shao *et al*. (2008); Sukumaran *et al*. (2009); Vielma *et al*. (2002); Xie *et al*. (2011); Yuan *et al*. (2011) & Zhang *et al*. (2006).

[9] Borlongan & Satoh (2001); Brown *et al*. (1992); Chen *et al*. (2007); Davis & Robinson (1987); Furuya *et al*. (2008); Lovell (1978); Luo *et al*. (2009); Nwanna *et al*. (2009); Ogino & Takeda (1976); Phromkunthong & Udom (2008); Robinson *et al*. (1987); Roy & Lall (2003); Schafer *et al*. (1995);Vielma & Lall (1998); Wang *et al*. (2005); Yang *et al*. (2006); Yuan *et al*. (2011) & Zheng *et al*. (2007).

[10] Brown *et al*. (1992); Furuya *et al*. (2008); Mai *et al*. (2006); Nwanna *et al*. (2009); Phromkunthong & Udom (2008); Schafer *et al*. (1995); Vielma & Lall (1998); Yuan *et al*. (2011) & Zhang *et al*. (2006).

[11] Coloso *et al*. (2003); Liang at al. (2011); Vielma & Lall (1998); Vielma *et al*. (2002); Rodehutscord *et al*. (2000); Rodehutscord (1996) & Xie *et al*. (2011)

[12] Coloso *et al*. (2003); Liang at al. (2011); Phromkunthong & Udom (2008); Rodehutscord (1996); Rodehutscord *et al*. (2000); Vielma & Lall (1998); Vielma *et al*. (2002) & Xie *et al*. (2011).

Body weight gain

Meta-analysis of the complete dataset on all species (All) showed that, minimal dietary P in terms of dietary concentration (g kg^{-1} DM) to attain maximum weight gain was estimated at 3.5 g available P kg^{-1} DM (4.7 g total P kg^{-1} DM). In terms of dietary P per unit of DE in the diet the requirement was estimated to be around 0.17 g available P MJ^{-1} DE (0.29 g total P MJ^{-1} DE) and in terms of absolute intake, the requirement level was estimated at 0.062 g available P kg^{-1} $BW^{0.8}$ day^{-1} (0.152 g total P kg^{-1} $BW^{0.8}$ day^{-1}).

Meta-analysis of the different sub-datasets based on weight gain, showed minimal dietary P levels for attaining maximal growth were lower in trout compared to the non-

trout fish species, having non-overlapping confidence intervals (Table 1). The estimated minimal dietary P concentration to attain maximum weight gain (Fig. 1a; expressed per unit DM), was 2.7 g available P kg^{-1} DM (3.2 g total P kg^{-1} DM) for data on rainbow trout and 4.7 g available P kg^{-1} DM (6.4 g total P kg^{-1} DM) for data from all species excluding rainbow trout. However, the minimal dietary P level estimate for rainbow trout showed no difference with other species when studies done in re-circulatory systems were excluded from the dataset. Minimal dietary P concentration for maximal weight gain expressed in relation to DE content was estimated as 0.16 g available P MJ^{-1} DE (0.17 g total P MJ^{-1} DE) for rainbow trout and as 0.25 g available P MJ^{-1} DE (0.31 g total P MJ^{-1} DE) for the sub-dataset on all species except rainbow trout (Fig. 1b). When P requirement was estimated from the relationship between absolute P intake (in g kg $BW^{-0.8}$ day^{-1}) and metabolic growth rate (in g kg $BW^{-0.8}$ day^{-1}), the difference in the requirement for rainbow trout versus all species excluding rainbow trout was marginal (Fig 1c and Table 1). This was indicated by the overlapping confidence interval of the break points; being 0.07 g available P intake kg $BW^{-0.8}$ day^{-1} (0.19 g total P intake kg $BW^{-0.8}$ day^{-1}) for rainbow trout and 0.09 g available P intake kg $BW^{-0.8}$ day^{-1} (0.19 g total P intake kg $BW^{-0.8}$ day^{-1}) all species excluding rainbow trout (Fig. 1c).

Whole body P

In comparison to weight gain, using whole body P concentration as the response criterion led to higher estimates for the minimal dietary P levels for all datasets (Table 1). Estimated minimal dietary P level for attaining maximal whole body P concentration (in g kg^{-1} live weight) was 4.7 g available P kg^{-1} DM (6.5 g total P kg^{-1} DM) complete dataset (All). For the sub-datasets on 'Rbt' and 'All-Rbt' it was 5.8 g available P kg^{-1} DM (7.4 g total P kg^{-1} DM) and 6.4 g available P kg^{-1} DM (7.8 g total P kg^{-1} DM), respectively (Fig. 2).

Vertebral P

Due to a limited number of studies on rainbow trout using vertebral P content as the response criterion, minimal dietary P levels based on this criterion was estimated only on the complete dataset (All). The estimated minimal dietary P levels expressed as total P was higher when vertebral P content was used as the response criterion compared to whole body P content, but when expressed as available P the estimated minimal dietary P levels were almost similar between vertebral and whole body P concentration as the

response criteria (Table 1). The estimated minimal dietary P level was 5.2 g available P kg^{-1} DM (10.5 g total P kg^{-1} DM) for all species combined (Fig. 3).

Plasma P

Due to the low number of studies providing data on plasma P concentration, the estimated minimal dietary P levels had a relatively large 95% confidence interval and overlapped with most other response criteria. Moreover, no break point could be observed on the sub-dataset on all species excluding rainbow trout (Fig 4). Based on plasma P concentration the minimal dietary P level was estimated as 3 g available P kg^{-1} DM (6.9 g total P kg^{-1} DM) for rainbow trout (Fig. 4).

Water P concentration and rearing system

Besides inter-species differences, variations in experimental conditions were also encountered among studies on P requirements of fish. In order to rule out species differences, the impact of rearing system was assessed within the rainbow trout sub-dataset. In this dataset, out of the 7 studies, two were done in recirculation systems while five in flow through systems. Using weight gain as the response criterion, the estimated minimal dietary available P level was found to be high when the two studies in recirculation systems were excluded from the sub-dataset. The minimal dietary available P level for maximal weight gain was estimated as 2.7 g kg^{-1} DM (95% confidence interval: 2.2 – 3.6) for all trout studies combined (Table 1 & Fig. 1a) and 4.4 g kg^{-1} DM (95% confidence interval 3.5 – 5.5) for rainbow trout reared in flow through systems (Table 1). Moreover, as an example for the difference between flow through and recirculation system on the estimated minimal dietary available P level, Fig. 5 gives a comparison of the relation between dietary available P and growth (calculated as thermal growth coefficient) of two rainbow trout studies with a similar initial body weight but differing in rearing system (Ketola & Richmond, 1994; Rodehutscord, 1996).

Fig. 1a

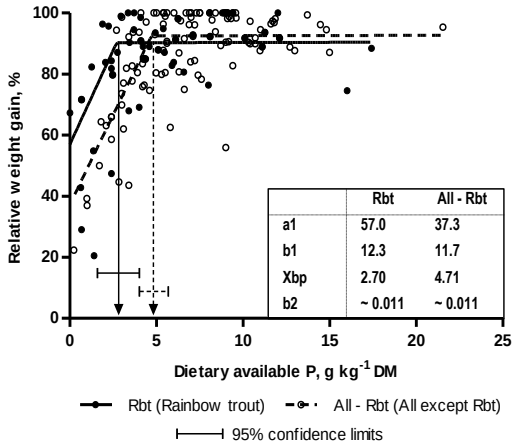

Fig. 1b

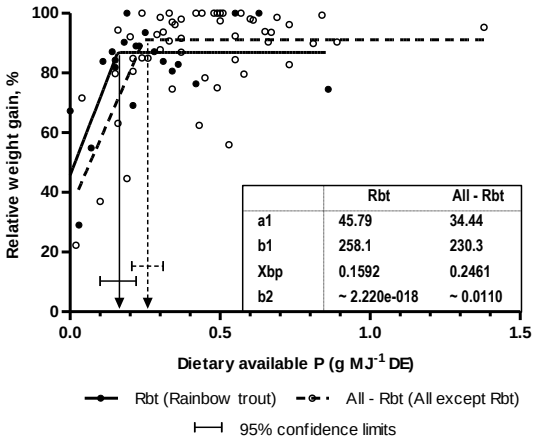

Fig. 1c

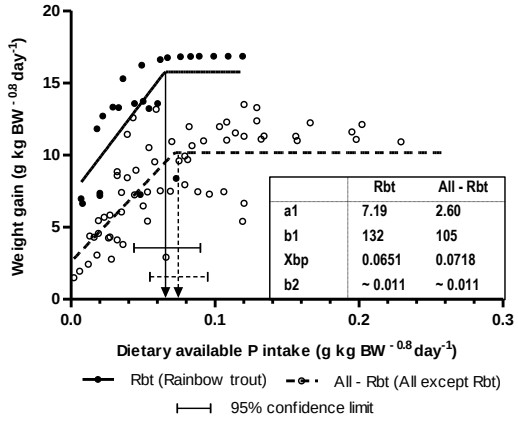

Fig. 1: Minimal dietary available P levels estimated through meta-analysis, based on weight gain for 'Rbt' (filled circles, solid line) and 'All-Rbt' sub-datasets (open circles, dotted line) expressed as (a) g P kg^{-1} DM (b) g P MJ^{-1} DE and (c) g P intake kg^{-1} $BW^{0.8}$ day^{-1} .

Fig. 2

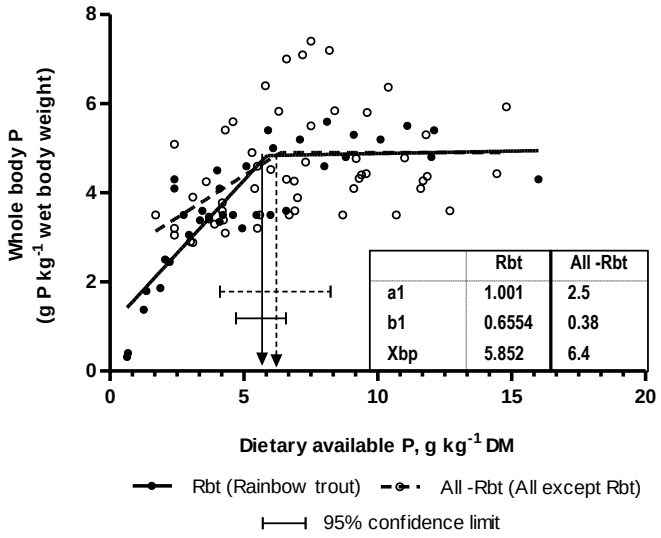

Fig. 2: Minimal dietary available P levels (g kg^{-1} DM) estimated through meta-analysis based on whole body P content (g P kg^{-1} live weight) for 'Rbt' (filled circles, solid line) and 'All-Rbt' sub-datasets (open circles, dotted line).

Fig. 3

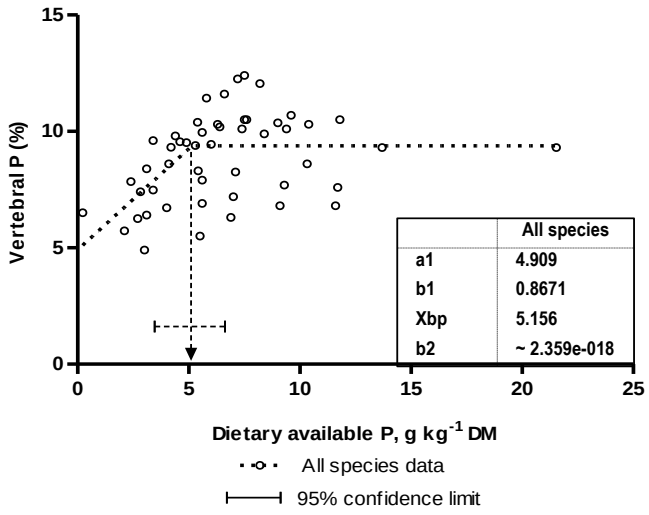

Fig. 3: Minimal dietary available P levels (g kg^{-1} DM) based on vertebral P content (% of dry weight) estimated through meta-analysis for 'All' dataset (open circles, dotted line).

Fig. 4

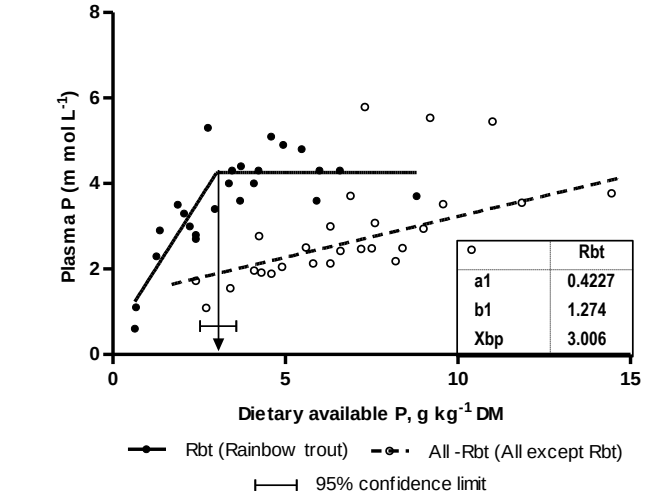

Fig. 4: Minimal dietary available P levels (g kg^{-1} DM) estimated through meta-analysis based on plasma P concentration (mmol L^{-1}) for 'Rbt' (filled circles, solid line) and 'All-Rbt' (open circles, dotted line).

Fig. 5

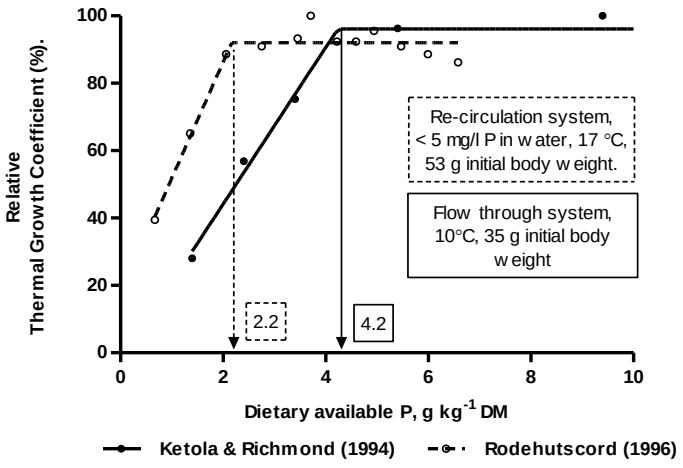

Fig. 5: Possible impact of rearing system and high water P concentration on the available P requirement estimate in rainbow trout. Growth data (TGC) from Ketola & Richmond (1994), initial body weight 35g, water P concentration not reported, flow through system, water temperature 10ºC (filled circles, solid line) and Rodehutscord (1996), initial body weight 53g, water P concentration < 5 mg L^{-1}, recirculation system, water temperature 17 ºC (open circles, dotted line).

Broken line analysis of the data resulted in a requirement estimate of 4.2 g available P kg^{-1} DM for trout reared in flow through system, while with the recirculation system

having high P concentration in water it was estimated to be lower (2.2 g available P kg^{-1} DM; Fig. 5).

Diet type and basal dietary P level

When constructing the dataset (and various sub-datasets) we noticed that there was a large variability in dietary P content, both in range of dietary P levels as well as the lowest level of P content (basal diet P concentration) tested.

Fig. 6

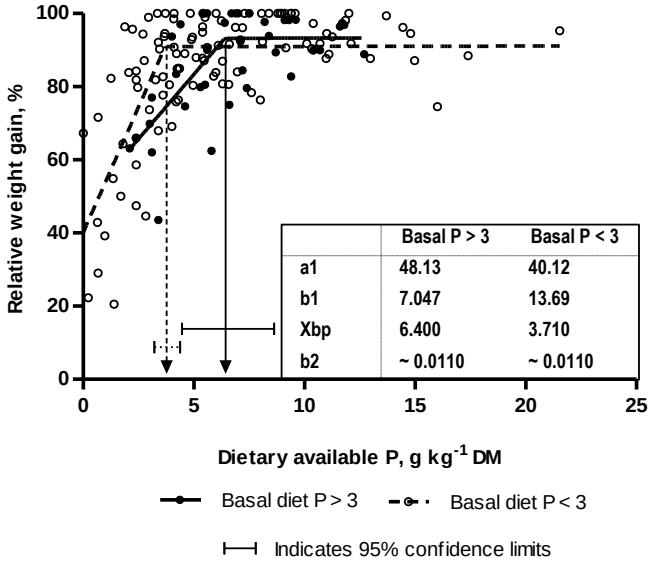

Fig. 6: Effect of high available P concentration in the basal diet on the estimate of minimal dietary P level for maximal weight gain. Sub-datasets with studies more than 3 g available P kg^{-1} DM (filled circles, solid line) and less than 3 g available P kg^{-1} DM (open circles, dotted line) in the basal diet.

Using weight gain as response criterion, minimal dietary available P content was estimated as 3.5 g kg^{-1} DM in the dataset using all species (25 studies; Table 1). Out of the 25 studies, 12 studies used diets made of non-refined (practical) ingredients for quantifying P requirements and 14 studies (56%) had an available P concentration in the basal diet above 3 g P kg^{-1} DM. Also, the type of diets used varied between studies. The mean basal dietary P concentration of the 14 studies was 3.1 g available P kg^{-1} DM (7.2 g total P kg^{-1} DM). Analysis of data from these studies showed that, the minimal dietary available P level (i.e., break point) was 2 to 2.5 times higher compared with the value estimated from the other studies having an available P content in the basal diet

below 3 g kg^{-1} DM (Fig. 6). Moreover, the 95% confidence limits of the estimated minimal dietary P levels did not overlap between these two sub-datasets.

Discussion

It is usual practice to express data on P requirement of fish and/or minimal dietary P levels in terms of total P in the diet (as fed or as on dry matter basis), despite the large variability in the availability of dietary P to fish. Minimal dietary P level expressed in terms of available P gives more reliable quantification of the requirement than total P, as indicated by the wider confidence intervals (Table 1). The availability of P from different ingredients and inorganic forms of mineral supplements used in fish diets is known to vary (Kaushik, 2001; Lall, 2003), depending upon the fish species and type of inorganic source (Ogino *et al.*, 1979; Satoh *et al.*, 1997), feed processing conditions (Satoh *et al.*, 2002; Cheng & Hardy, 2003), dietary phosphorus concentration (Riche & Brown, 1996; Sugiura *et al.*, 2000b). Hence, it is considered essential to measure the availability of P from the diets in studies aiming at determining a quantitative P requirement of fish.

In almost all P requirement data on weight gain is reported and most often used as a response criterion to estimate the P requirement. However, the use of weight gain as the response criterion to determine minimal dietary levels of the micronutrients such as vitamins, minerals and trace elements for fish is questionable (NRC, 2011). Hardy *et al*. (1993) reported that growth was not affected in rainbow trout when fed a P-deficient diet, until the body P reserves depleted to below 80% of the initial body P levels. This implies that, the estimated minimum dietary P level using weight gain as the response can be lower compared with the estimate with whole body P concentration as the response criterion, depending on the duration of the trial. In the current study, the estimated minimum dietary available P level for achieving maximum weight gain was approximately 25-30% lower than for the requirement estimate using whole body P concentration as the response criterion from datasets on all species and all species excluding rainbow trout (Table 1 & 2), which, in the case of rainbow trout alone, was even 50% lower. This might be due to an effect of rearing system in the rainbow trout sub-dataset where two of the seven studies were undertaken in re-circulating aquaculture systems (RAS) with levels of up to 5 mg P L^{-1} (normally it is less than 0.01

mg P L^{-1}) in the rearing water. When these two RAS studies were excluded (Rbt-RAS), minimal dietary P level for maximum weight gain as the response was about 25% lower (4.4 g available P kg^{-1} DM) when compared to the estimate based on whole body P concentration. Also, the difference between minimal dietary P levels for 'Rbt' and 'All-Rbt' sub-datasets was reduced to marginal (4.4 versus 4.7 g available P kg^{-1} DM). The ability of fish to absorb phosphorus from water is still debated. Though few studies (Coffin *et al.*, 1949; Winpenny *et al.*, 1998) have denied this, few others have confirmed (Mullins, 1950; Al-kholy *et al.*, 1970) the uptake of P by fish from water through radioisotope studies. Moreover, some studies looking into a P budget in fish have suspected the possibility of P uptake by fish from water when fed P-deficient diets (Cowey, 1995; Oliva-Teles, 2000; Dias *et al.*, 2005). In line with the suggestion of P uptake from water, re-analysis of weight gain data (TGC) from Ketola & Richmond, (1994) on rainbow trout (35g initial body weight, 10ºC) in a flow through system using broken line regression (fig. 5) showed that weight gain plateaued at a dietary concentration of 4.2 g available P kg^{-1} DM. Similar analysis of the data on weight gain (TGC) from Rodehutscord (1996) showed that the plateau was reached at 2.2 g available P kg^{-1} in rainbow trout of 50 g initial body weight reared in a RAS with levels up to 5 mg P L^{-1} at 17ºC. Further, analysis of growth data from another P dose response study undertaken in a RAS (Rodehutscord *et al.*, 2000, wherein the data on P concentration in the rearing water was not presented), plateaued at 1.7 g available P kg^{-1} DM. These give an indication for P uptake from water by rainbow trout and whereby the estimate of minimal dietary P level is affected. This however requires clear confirmation through more specifically designed studies.

Comparing the minimal dietary P levels as estimated through meta-analysis (Table 1) and mean of the values of P requirements reported in different studies using different response criteria (Table 2) reveals that, the values from meta-analysis are lower (25-43% for weight gain; 13-25% for whole body P and 14% for vertebral P) to the values from table 2. The large difference with weight gain can be possibly due to methodological as well as physiological and environmental factors. Methodologically, with weight gain, as the maximum response was restricted to a value of 100 and restricting the slope of the line Y_2 ($b_2 < 0.1$) might have influenced the slope (b_1) of the line Y_1 to be steeper and the plateau to be lower, forcing a lower breakpoint value than it would be if actual weight gain data were used as such (Table 1; Fig. 1a & 1b) and

difference in models used in different studies (Table 2). So, an attempt made to observe the relation between dietary P and weight gain in absolute terms estimated an requirement of 0.062 g available P kg^{-1} $BW^{0.8}day^{-1}$ (0.152 g total P kg^{-1} $BW^{0.8}$ day^{-1}) based on data from all species (Table 1). But, a low R^2 value and wide confidence limits when expressed in absolute terms indicates that the differences might not solely be due to the methodological approach but may have also been influenced by physiological and environmental factors like fish size, growth rate, feeding method, water temperature and trophic level of the species. Similar data analysis focusing on the effect of these variables on the minimal dietary P level would improve precision of our estimation of P requirement of fish.

Another widely used approach to determine P requirement of fish is the use of whole body P as the response variable. Whole body P concentration is the commonly used criterion, based on which the minimal dietary P levels are estimated to be 20-30% higher than those estimated with weight gain. The possible reasons underlying this have been discussed earlier. However, P retention (% of dietary intake) or daily P gain (g P kg^{-1} day^{-1}) can be more reliable response criteria for estimating the P requirement of fish, provided the fish are not in a status of P-deficiency (Suguira *et al*., 2000a) and the duration of the trial is long enough to nullify any effects due to the P-status of the fish. Available data is limited to verify whether there is a relation between P retention and P requirement and if daily P gain per unit body weight can change with increasing body mass or growth rate. Analysis of data (Fig. 7) however shows that the absolute phosphorus content in fish varies linearly with body weight (0.36% of live weight). This is in agreement with the previous findings that report P content in salmonids to be fairly constant at around 0.4 to 0.5% live weight (Lall, 1991; Shearer, 1994; Shearer *et al.*, 1994), and 0.37% live weight for rainbow trout through different growing stages (Bureau *et al.*, 2003).

Estimation of minimal dietary P level based on vertebral ash or P content analysis was also found to be higher than that required for maximal body weight gain (Table 1). Since data on dietary available P was provided in only 9 out of 23 studies using vertebral P content as a response criterion, it is difficult to make a conclusive statement as regards vertebral P content as the most stringent response criterion.

Fig. 7

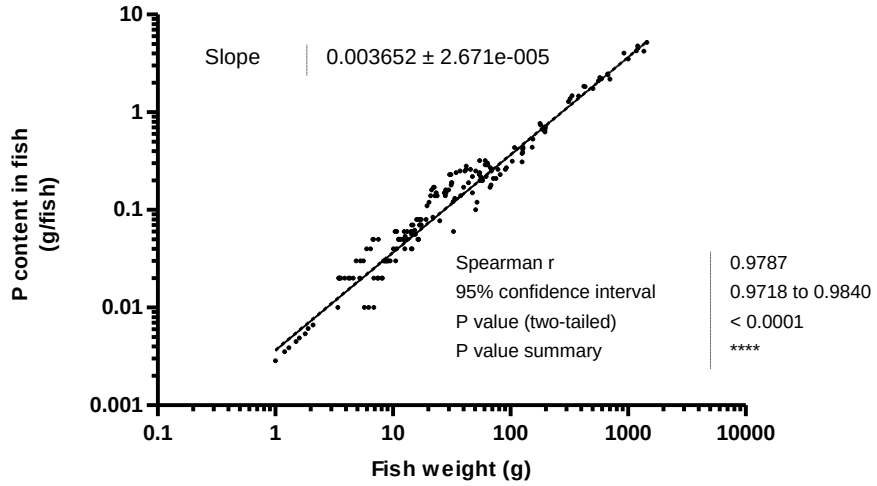

Fig. 7: Whole body P content of well growing fish (g P/fish) with varying body weight (g) from multi species data.

During P deficiency, plasma and urinary P levels are reduced (Rodehutscord, 1996; Lall, 2003), suggesting that plasma P can be used as a response criterion for estimating P requirement of fish. Asplasma P concentration can vary in relation to both dietary input as well as mobilisation from tissues, time of sampling. Thus, the use of this criterion as an index of P requirement is less reliable and especially so for cross study comparisons. From the results of the meta-analysis (Table 1 & 2), it was found that plasma P levels as an index holds well for inter-study comparisons in a single species dataset (Rbt), only when the data are expressed in terms of available P in the diet and the post-prandial time of sampling do not vary drastically across studies.

This meta-analysis shows that the P requirement estimates as minimal dietary P levels tend to vary depending on the response criterion and the levels are higher when we use whole body or vertebral P concentration as the criterion, than when we use weight gain as the response criterion. Weight gain gave a more reliable estimate (R^2 = 0.5-0.7; narrow confidence limits) followed by whole body P concentration and plasma P level which was the most variable (R^2 = 0.11; wide confidence limits) for the datasets 'All' and 'All-Rbt'. However, all response criteria analysed were found to fit better (R^2 = 0.4 - 0.7) for the single species dataset (Rbt), with whole body P and plasma P having the maximum R^2 values. Thus, under a given set of physiological and environmental conditions, in terms of precision and variance, it can be deduced that criteria based on

whole body P concentration to be the most stringent of the four different response variables analysed.

Apart from these four widely used response criteria, few other potentially effective response variables have also been investigated, as summarised in Table 2. Scales are a major site of mineral metabolism, and resorption of scales takes place during food deprivation (Lall, 2003). Based on this, scale ash or P content has been used as a non-invasive response for determining P requirement of fish in few studies, but with limited success. On the other hand, urinary P excretion has been proved to be an effective and relatively quick method for studying the P requirement and P status of fish, especially in large fish (Sugiura *et al.*, 2000a; Rodehutscord *et al.*, 2000). Lately, gene expression analyses of P transporters in pyloric caeca, intestine and kidney have also been employed to analyse the P status and estimate minimal dietary P levels with rainbow trout (Sugiura *et al.*, 2007). However, it is not clear whether such response criteria are consistent and reliable to be useful in determining P requirement of fish.

In studies aiming at quantifying the requirement of any nutrient, the use of diets made of refined ingredients is ideal. Diets made of non-refined practical ingredients may be used provided the basal diet contains very low levels of the nutrient under investigation (NRC, 2011). Therefore, under conditions where the P concentration in the basal diet itself is high enough to almost meet the P requirement of the fish, the probability of over estimating the minimal dietary P level required is high (Fig. 6). Hence, it is better to avoid diets containing ingredients which are inherently rich in available phosphorus for quantifying the dietary P requirement of fish.

The unit or mode of expressing minimal dietary P levels required to fish is very crucial and needs close attention. It was suggested that, as the feed gain ratio and hence the P intake per unit weight gain is determined by the digestible energy content of the diet, expressing minimal dietary P levels as g P MJ^{-1} DE would be more appropriate (Rodehutscord, 1996). Analysis of data to find the best way of expressing P requirement of fish however shows that g available P kg^{-1} DM (R^2 = 0.49) and g available P MJ^{-1} DE (R^2 = 0.44) are equally good based on the R^2 values of the regression. However, though a direct comparison is not possible, between expressing P requirement of fish as g available P kg^{-1} $BW^{0.8}$ day^{-1} (Fig.1c, R^2 = 0.5) would be more appropriate as it takes into

account the variations that may arise due to difference in fish size, P intake and growth rate thus providing an estimate in absolute terms.

From this meta-analysis it can be concluded that there exists considerable homogeneity of data on the P requirement of rainbow trout in flow through system and all other species when dealt with in terms of available P level in the diets. However, this needs confirmation through a mixed effect approach.; Among the response criteria analysed, weight gain was more reliable and whole body P was the most stringent, thereby using whole body P as a variable (whole body P balance, retention, deposition or daily P gain) could be more efficient in determining the P requirement of fish. Environmental factors such as, water P concentration and available P concentration of basal diet can affect the estimation of the minimal dietary P level. The results of this meta-analysis could have been more robust if more data on dietary available P were available, especially for species other than rainbow trout. Further studies on P requirement of fish should necessarily provide data on available P levels in the diets and basic information on feed intake, macro-nutrient composition, DE and levels of other minerals in the diets as well as water P levels. Similar work on the analysis of available data on requirements of fish for other essential macro and micro minerals is warranted.

Acknowledgment

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Table 2: Data on minimal dietary Phosphorus (P) level estimates by various studies on several fish species using different response criteria

Species	IW (g)	Form	Diet	Diet P level (g kg^{-1} DM)			Inorganic source	Major protein source ((g kg^{-1})	Rearing system	Temperature (ºC)	Salinity (g L^{-1})	Water P concentration (mg L-1)	Response variables tested	Minimal dietary P level reported (g kg^{-1} DM) based on,							FE	Reference
				Levels	Min	Max								WG	WB-P	Ver P	Ver ash	Pl-P	Sc-P	Ur-P		
African catfish	11	T	Pr	11	10.2	15.4	CaHPO4	SBM (430)	RAS	26	FW		1, 3, 6	12.3							0.68	Nwanna *et al*. (2009)
Amur Sturgeon	4.7	T	Pu	6	1.8	1.6.6	$Ca(H_2PO_4)_2$			26	FW	0.098	1, 3, 6	8.8		10					1	Wen *et al*. (2008)
American cichlid	0.4	T	SP	4	5	25	KH_2PO_4	Casein (485)	RAS	28	FW	0.003		15							0.65	Chavez-Sanchez *et al*. (2000)
Asian seabass	1.28	T	Pr	4	15.6	27	Na_2HPO_4	FM (630)	FL	26.5	29.5		1, 3, 5, 6	R							1	Chaimongkol & Boonyaratpalin (2001)
Asian seabass	1.28	T	Pr	4	40	52.6	Na_2HPO_4	FM (750)	FL	26.5	29.5		1, 3, 5, 6	R							1	Chaimongkol & Boonyaratpalin (2001)
Atlantic cod	154	T	SP	3	5.4	10.4	NaH2PO4 :KH 2PO4 (1:1)	FMM (290); SPC (100)	FL	11	SW		1, 3, 4, 5, 6, 7	R							1.1	Kousoulaki *et al*. (2010)
Atlantic salmon	56	T	Pu	2	0	9	KH_2PO_4		FL	15.5	32		1,3, 5	9								Lall & Bishop (1977)
Atlantic salmon	6.5	A	Pr (P)	7	7	16	Na_2HPO_4	Soybean meal (700)	FL	14	FW	< 0.5	1, 3, 6	6			6				0.54	Ketola, (1975)
Atlantic salmon	1.4	A	SP	5	4.4	25.3	$Ca(H_2PO_4)_2$	Casein (450)	FL				7, 3		9						1.6	Asgard & Shearer (1997)
Atlantic salmon	15	A	SP	8	4	13.3	$Ca(H_2PO_4)_2$	Casein (300), WGM (100)	FL	15	FW		4, 6				5.6				0.95	Vielma & Lall (1998)
Barbel chub	4	T	SP	3	6	18				25	FW		1, 3, 6	R								Zheng *et al*. (2007)
Black sea bream	11.5	A	SP	6	1.8	10.7	Na_2HPO_4	Casein (470)	FL	28	FW		1, 7, 6	5.5	8.1	8.8			8.7		0.51	Shao *et al*. (2008)
Blue Tilapia	1.5	T	SP	8	2	10	Na_2HPO_4	Casein (330)	FL	31	FW	0.52	1, 3, 6, 7	3	5	5					0.38	Robinson *et al*. (1987)
Catla	4.2	T	SP	8	1	15	KH_2PO_4	EA (430)			FW		1, 3, 7, 9	6.4	7.1					6	0.77	Sukumaran *et al*. (2009)
Channel catfish	6 & 24	A	SP	3	0.5	1	$Ca_2(H_2PO_4)_2$	SMB (200), CGM (200), YC (300)	FL	27	FW		1, 6, 7	8							0.77	Andrews (1973)
Channel catfish	1.5	T	SP	8	0.7	5.7	Na_2HPO_4	Casein (330)	FL	26	FW	0.03	1, 6	4.2			4.7					Lovell (1978)
Chinese Sucker	1.8	T	SP	5	3.1	11.8	$Ca(H_2PO_4)_2$	FM (320); SBM (360)	FL	29	FW	0.037	1, 3, 6, 7, 8	7.4	8.3	8.8			8.6		0.72	Yuan *et al*. (2011)
Chum Salmon	1.5	A	SP	8	0.7	8.4	NaH_2PO_4	EA (40)	FL	13	FW	0.002	1, 3, 6, 7	4.6	5.5	5.5	4.5					Watanabe et al. (1980)
Common carp	4.8	T	SP	4	0.6	7	NaH_2PO_4 :KH $_2PO_4$ (1:3)	EA (400)		22.5	FW	0.002	1, 6, 7	6.0 - 7.0							0.75	Ogino & Takeda (1976)
Common carp	80	A	SP	4	0.6	7	Na_2HPO_4	EA (160), PP (150), WG (150)	RAS	23	FW		1, 6,7	5.55	13.2		7.6		13.2		0.55	Nwanna *et al*. (2010)

Species	IW (g)	Form	Diet	Diet P level (g kg^{-1} DM)			Inorganic source	Major protein source ((g kg^{-1})	Rearing system	Temperature (ºC)	Salinity (g L^{-1})	Water P concentration (mg L-1)	Response variables tested	Minimal dietary P level reported (g kg^{-1} DM) based on,							FE	Reference
				Levels	Min	Max								WG	WB-P	Ver P	Ver ash	Pl-P	Sc-P	Ur-P		
Common Carp (Jian)	7.2	A	SP	6	1.7	11	Na_2HPO_4	Rice GM (315); FM (200)	RAS	25	FW		1, 3, 4, 7, 8	5.2							0.58	Xie *et al*. (2011)
Common Carp (Mirror)	18	A	SP	6	2.4	12.7	$Ca(H_2PO_4)_2$	FM (300); SBM (300); WF (250)	RAS	27	FW	0.004	1, 2, 3, 5, 7	7						7	0.98	Kim *et al*. (1998)
Common Carp (Mirror)	4	A	Pr (P)	3	2.4	6	$Ca(H_2PO_4)_2$	SMB;	RAS		FW		1, 3, 5, 6, 7	6								Schaefer *et al.* (1995)
Culter alburnus	3.8	T	SP	7	5.3	16.1	Na_2HPO_4	Casein (360)	FL	27	FW	0.02	1, 3, 6, 7	8.8	8.8						0.74	Chen *et al*. (2007)
Eueopean Seabass	10	T	SP	6	4.8	12.5	CaHPO4	Casein (470)	RAS	21	34	0.02	5		6.5						0.91	Oliva-telas & Ana (2004)
European white fish	5.2	A	Pu	5	4.4	14.9	KH_2PO_4	Casein (460)	FL	15	FW	0.14	1, 4, 6, 7	6.2			6.5					Vielma *et al*. (2002)
Gilthead seabream	5	T	SP	7	3.7	15	CaHPO4	Casein (400); Gelatin (100)	RAS	24	32	0.08	1		7.5						0.57	Rodrigues & Oliva-teles (2001)
Goby	15.8	T	Pr	5	6.3	13.6	Na_2HPO_4	SBM (320); FM (280)	FL	24	20	0.02	1, 2, 3, 5, 6, 7		8.8; 10.6	10.6					0.6	Luo *et al*. (2009)
Grass carp	5.6	A	SP	6	2.4	14.8	Na_2HPO_4	Casein (420)	RAS		FW	0.02 - 2.27	1, 3, 4, 6, 7	8.5	8.5	8.5	8.5				0.85	Liang *et al*. (2011)
Grouper	9.9	T	SP	5	6.8	12.1	$Ca(H_2PO_4)_2$	FM (350); Casein (200); WG (100)	CAGE	25	20		1, 6	8.6								Zhou *et al*. (2004)
Guppy	0.24	T	SP	3	0.5	12.3	KH2PO4	Casein (430)	RAS	84	FW	0.6	1, 3, 6	5.3 - 12.3		5.3 - 12.3	5.3 - 12.3				0.3	Shim and Ho (1989)
Haddock	4.2	A	SP	5	4.2	12.2	$Ca(H_2PO_4)_2$	Casein (210); WGM (100); FM (100); CGM (80)	FL	12	32		1,3, 6, 9				7.2				1.4	Roy & Lall (2003)
Haddock	11.2	T	SP	3	4.2	13.2	$Ca(H_2PO_4)_2$	Casein (210); WGM (100); FM (100); CGM (80)	FL	12	32		1, 3, 4, 5, 6								0.8	Roy *et al*. (2002)
Hybrid sunshinebass	2.2	A	SP	8	3.4	10.4	KH_2PO_4	Red drum muscle (480)	RAS	25	FW		1, 3, 4, 6, 7	4.1		5.4			5.5		0.59	Brown *et al*. (1992)
Japanese Flounder	2	T	SP	6	3.3	21.2	Na_2HPO_4	Casein (350); SqM (100); Flounder muscle (78)	RAS	19	31	0.63	1, 3, 6	4.5 - 5.1							0.93	Wang *et al*. (2005)
Japanese seabass	6.3	A	Pr	5	3.1	11.7	$Ca(H_2PO_4)_2$	SBM (360); FM (280)	CAGE	28	34		1, 4, 6, 7, 8	6.8								Zhang *et al*. (2006)
MilkFish	2.5	T	SP	7	3	11.9	KH_2PO_4	Casein (300)	FL	29	31,5	0.03	1, 3, 6, 7	8.5			8.5		8.5		0.48	Borlongan & Satoh, (2001)
Mrigal carp	6	T	Pu	5	0	7.5	Na_2HPO_4	Casein (360)	FL	27	FW		1, 3	7.5							0.45	Paul *et al*. (2004)
Nile Tilapia	0.4	A	SP	4	2.1	7.1	$Ca_2(H_2PO_4)_2$	SBM (500)	RAS	29	FW		1, 3, 6	5.5		6.4					0.84	Furuya *et al*. (2008)

Species	IW (g)	Form	Diet	Diet P level (g kg^{-1} DM)			Inorganic source	Major protein source ((g kg^{-1})	Rearing system	Temperature (ºC)	Salinity (g L^{-1})	Water P concentration (mg L-1)	Response variables tested	Minimal dietary P level reported (g kg^{-1} DM) based on,							FE	Reference
				Levels	Min	Max								WG	WB-P	Ver P	Ver ash	Pl-P	Sc-P	Ur-P		
Olive flounder	4	T		4	3.3	11.6	Na_2HPO_4		FL	22	31		1, 5	4.5-5.7							1	Choi *et al.* (2005)
Rainbow trout	1.2	T	SP	4	0.7	10.9	NaH_2PO_4 :KH_2PO_4 (1:3)	EA (500)		16.5	FW	0.002	1,6,7	7.0 - 8.0							1.1	Ogino & Takeda (1978)
Rainbow trout	9	A	SP	9	4.1	12.1	Na_2HPO_4	Casein (280), BM (210)	FL	9	FW		1,3,6	4.1	5.1 - 6.1		5.1				0.86	Ketola & Richmond (1994)
Rainbow trout	35	A	SP	6	1.4	17.4	Na_2HPO_4	CG (670)	FL	9	FW		6, 7, 9	3.4 - 5.4			5.4				0.87	Ketola & Richmond (1994)
Rainbow trout	50	A	SP	12	1	11	Na_2HPO_4	WG (270)	RAS	17	FW	< 5	1, 2, 4, 5, 7, 9	3.7	5.6			4.3 (36h)			0.92	Rodehutscord (1996)
Rainbow trout	278	A	SP	6	0.7	11.3	Na_2HPO_4	WG (370); BM (100)	FL		FW		1, 4, 6, 10	5.4		4.5		4.5 (6h)				Sugiura *et al*, (2007)
Rainbow trout	2	A	SP	5	2.3	11.6	Na_2HPO_4	WG (200), Casein (170)	FL	18	FW		8, 7		7.0 - 9.0						0.8	Skonberg *et al*. (1997)
Rainbow trout	203	A	SP	9	1.4	12.1	KH_2PO_4	WG (300)	FL	15	FW	0.011	4, 7, 9		7.5			7.3 (24h)			0.95	Sugiura *et al*. (1999)
Rainbow trout	400	A	SP	9	1.4	12.1	Na_2HPO_4	WG (350)	FL	15	FW	0.011	4, 7, 9		7						0.95	Sugiura *et al*. (1999)
Rainbow trout	400	A	SP	9	1.4	12.1	KH_2PO_4	WG (350)	FL	15	FW	0.011	8							5.2		Sugiura *et al*. (2000a)
Rainbow trout	203	A	SP	9	1.4	12.1	KH_2PO_4	WG (350)	FL	15	FW	0.011	9							6.1		Sugiura *et al*. (2000a)
Rainbow trout	258	T	SP	5	1	10.7	KH_2PO_4	EA (350)	FL	15	FW	0.0325	9					5.2 (12h)		5.2		Sugiura *et al*. (2000b)
Rainbow trout	400	A	SP	9	1.4	12.1	KH_2PO_4	WG (350)	FL	15	FW	0.011	9							5.3		Sugiura *et al.* (2000a)
Rainbow trout	78	A	Pr	3	2.4	8.8	Na_2HPO_4	CG (180); SBM (180)	FL	12	FW		1, 5, 4, 10, 9	R							0.87	Coloso *et al.* (2003)
Rainbow trout	0.1	A	SP	5	1	16	NaH2PO4 :KH2PO4 (1:1)	Casein (520)	FL	17	FW		7	R								Fontagne *et al.* (2009)
Rainbow trout	51	A	SP	8	1	6.2	Na_2HPO_4	WG (270)	RAS	16	FW		4, 5, 9	R							0.9	Rodehutscord *et al*. (2000)
Red drum	1.2	T	SP	8	2.6	13.1			RAS		5.5		1, 3, 6				8.6					Davis & Robinson (1987)
Red sea bream		T		4	2	13.6			FL				6			6.8	6..8				Sakamoto & Yone (1978)
Red tilapia	25.2	A	Pr (P)	6	4.6	8.2	$Ca_2(H_2PO_4)_2$	SBM (670)			FW		1, 3, 5, 6,		7.9	7.6					0.85	Phromkunthong & Udom, (2008)
Rohu carp	0.7	T	Pu	5	3.5	7.5	Na_2HPO_4	Casein (360)	FL	30	FW		1, 7	7.5	7.5						0.5	Paul *et al.* (2006)
Siberian sturgeon	14.5	T	Pu	11	1.2	13.8	Na_2HPO_4	EA (380); WGM (150)	FL	20	FW	0.04	1, 3, 5, 6, 7, 8	5	8.7	4.9					1.23	Xu *et al*. (2011)
Silver perch	2.3	T	SP	8	2.4	10.8	Na_2HPO_4	Casein (330)	RAS	25	FW	0.25	1, 3, 4, 5, 6	7.1	6.5	6	5.6	6.2 (24h)			0.51	Yang *et al*. (2006)
Spotted steed	8	T	SP	6	3.2	15.9	KH_2PO_4	Casein (360)					1, 3, 5, 6, 7	8.3	8.3		11.7				0.63	Zhao *et al*. (2008b)
Spotted steed	8	T	SP	6	3.2	15.9	KH_2PO_4	Casein (360)					1, 3, 4, 5, 6	9.1	9.1		11.7				0.63	Zhao *et al*. (2008a)

Species	IW (g)	Form	Diet	Diet P level (g kg^{-1} DM)			Inorganic source	Major protein source ((g kg^{-1})	Rearing system	Temperature (ºC)	Salinity (g L^{-1})	Water P concentration (mg L-1)	Response variables tested	Minimal dietary P level reported (g kg^{-1} DM) based on,							FE	Reference
				Levels	Min	Max								WG	WB-P	Ver P	Ver ash	Pl-P	Sc-P	Ur-P		
Striped bass	7.9	T	SP	5	1.5	9.5	KH_2PO_4	EA (150); SBM (150)	FL	24			1, 3, 4, 6	3.1		4		2.9	4		0.62	Dougall *et al*. (1996)
Striped bass	48	T	SP	5	3	6.2	KH_2PO_4	EA (150); SBM (150)	FL	24			1, 3, 4, 6	3.1		5.8	5.4	2.9	5.8		0.57	Dougall *et al*. (1996)
Striped bass	321	T	SP	3	2	6	KH_2PO_4	EA (280)	FL	24			1, 3, 4, 6	2.0 - 4.0		4.6	5.4	2.9	4.6		0.44	Dougall *et al*. (1996)
Tiger barb	0.33	T	Pu	8	1.7	15.7	KH2PO4	Casein (430)	RAS	56	FW		1, 3, 6, 7	5.2							0.52	Elangovan & Shim (1998)
Yellow catfish	2.7	T	SP	6	4.3	11.8	Na_2HPO_4	Casein (360)		27	FW	0.013 - 0.017	1, 3, 6	8.5		7.6					0.67	Luo *et al*. (2010)
Yellow catfish	4.4	T	SP	4	13	22					FW		1, 3	17								Li *et al*. (2008)
Yellow croaker	1.9	A	Pr	5	3	11.6	$Ca(H_2PO_4)_2$	SBM (360); FM (280)	CAGE	28	34		1, 4, 6, 7, 8	7	9.1	8.9						Mai *et al*. (2006)
Yellow tail	920	A	SP	6	0.2	10	Na_2HPO_4	EA (600)	FL	28	SW	0.007	9							4.4		Sarker *et al*. (2009)

Mean of the above listed P requirement estimates reported by individual studies (g available P kg^{-1} DM), Mean (SD)

		WG	WB-P	Ver-P	Ver-ash	Plasma-P	Scale-P	Ur-P	
1.	**Dataset on all species**	*6 (1.5)*	*7.6 (1.3)*	*6.5 (1.2)*	*6.5 (1.2)*	*5.4 (1.7)*	*9.1 (3.9)*	*5.6 (1)*	
2.	**Dataset on Rainbow trout alone,**	*4.4 (1.1)*	*6.7 (1.1)*	*4.5 (1)*	*6.9 (1.1)*	*5.4 (1.7)*	-	*7*	
3.	**Dataset on all species excluding rainbow trout**	*6.4 (1.4)*	*8.5 (0.5)*	*7.3 (1.6)*	*5.3 (1.3)*	*4 (1.6)*	-	*5.5 (0.5)*	

IW – Initial weight of fish; WG – weight gain; WB-P – whole body P; Ver-P – vertebral P; Ver ash - vertebral ash; Pl-P – Plasma P; Sc-P – Scale P; Ur-P – Urinary P excretion;

Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum P levels; 5 - P retention; 6 - Bone/scale mineralization; 7 - whole body P /ash; 8 - Plasma enzyme activity; 9 - Urinary P excretion; 10 - Molecular markers (transport proteins).

T – Total phosphorus; A – Available phosphorus

SP – semi-purified diet; Pu – purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet

EA – Egg albumin; BM - Blood meal; CGM – Corn gluten meal; CG – Corn gluten; FM – Fish meal; FMM – Fish muscle meal; GM – Gluten meal; PP – Potato proteins ; SBM – Soy bean meal; SqM – Squid meal; WGM – wheat gluten meal; WG – wheat gluten; WF – wheat flour; YC – Yellow corn.

FL – Flow through system; RAC – Re-circulatory aquaculture system; FW – freshwater; SW – salt water

WG – weight gain; WB-P – whole body phosphorus; Ver P – vertebral phosphorus; Ver ash – vertebral ash; Plasma P – plasma inorganic phosphorus (figures in parenthesis indicate the time after the last meal during which samples were taken)

FE – feed efficiencyR – Reported to be required but quantitative requirement not determined

CHAPTER 3

Mineral requirements of fish: a systematic review

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Abstract

Meta-analysis of literature data on mineral and trace element requirements of fish was performed with the major objectives of identifying appropriate response criteria and the factors affecting the minimal dietary inclusion levels. The primary dataset included 25 studies on available P, 20 on Ca, 24 on Mg, 5 on K, 37 for Zn, 23 for Se, 19 for Mn, 16 for Fe and 13 for Cu. Broken line regression analysis with linear plateau model (P, Ca, Mg and K) or two-linear line model (Zn, Cu, Fe, Mn and Se) were used for determining the minimal dietary inclusion levels. Vertebral mineral concentration (P, Ca, Zn and Mn), whole body mineral levels (Mg, K and Fe) and hepatic enzyme activity (Se) were found to be the most appropriate criteria for the respective minerals analysed. In general, weight gain as the criterion resulted in a lower estimate (by 18-42%) than those obtained using whole body or vertebral mineral concentrations as response criteria. The analysis also showed that different fish species do not show large variations in the mineral and trace element concentrations in the whole body and tissues. Factors such as species group and digestive physiology, type of experimental diet used and dietary interactions, type of mineral source, mineral concentration of water were found to affect the minimal dietary inclusion levels of certain minerals. Besides the meta-analysis, research needs in mineral nutrition of fish with reference to growing changes in dietary strategies and rearing systems are discussed.

Key words: fish, meta-analysis, mineral requirements, response criteria, trace elements.

Introduction

Research in fish nutrition gained momentum by the early-1950, while attention towards essentiality and requirements of minerals in the diets of fish were recognised only by mid-1970. Despite the recognition of the essential roles of minerals for various life processes, research on mineral and trace element nutrition of fish has progressed rather slowly. Although about 29 of the 90 naturally occurring inorganic elements are considered to be essential for all farmed animals including fish (Lall & Milley 2008), only few of them have been studied in detail in fish. Dietary requirements are established for macro-minerals such as Ca, K, Mg, Na, P and micro-minerals such as Cu, Fe, I, Mn, Se, Zn for one or more fish species (NRC 2011). Studies have dealt with the functions, deficiency, availability, utilisation, toxicity, interaction with other nutrients and environment on the foresaid minerals to several fish species. Available data on the requirements of different minerals is limited and poorly defined when compared to data for other nutrients in fish or to data on minerals in terrestrial animals. It is recognised that nutrient requirement of an animal should be determined in terms of a specific response criterion at a given age, sex, weight gain and body composition (Baker, 1986). This applies for studies on mineral and trace element requirements of fish as well. However, it is much more complicated in fish due to the close interaction with the aquatic environment unlike in terrestrial animals (Kaushik 2002; Lall 2002). The factors that may affect the minimal dietary levels of mineral and trace element to fish can be one or a combination of the following: biological factors such as species, life stage, sex, trophic level, feeding habits and the nutritional status of the fish; dietary factors such as diet composition, availability and nutrient interactions; and environmental factors such as water mineral concentration, salinity and temperature of the rearing system.

Reviews by different authors and compilations by the National Research Council (NRC, 1993; 2011) have updated and summarised the information on mineral and trace element requirements of fish, from time to time. Other periodic reviews on minerals and trace element nutrition in fish by Schwarz (1995), Davis and Gatlin (1996), Watanabe *et al.* (1997), Kaushik (2002), Lall (2002) are also worth

mentioning. These works have reviewed one or more aspects of the mineral and trace element nutrition in fish, using a narrative approach. Shearer (1995) predicted the dietary requirements of minerals to rainbow trout through factorial modeling. In recent years, systematic reviews have established themselves especially in the field of biology and medicine, as a tool to gain more knowledge out of the existing published data (Borenstein *et al.* 2011). Though a diverse array of definitions exists, a systematic review in a nut shell is a critical evaluation through structured analysis of data from all research studies which address a similar research objective or question. Thus, systematic reviews especially with meta-analytic components have gained popularity in the field of livestock and fish nutrition, as well. Meta-analysis in fish nutrition, especially dealing with quantifying dietary nutrient requirements are reported for amino acids (Kaushik & Seiliez 2010; Hua 2013) and phosphorus (Antony Jesu Prabhu *et al.* 2013). The meta-analytic methods reported thus far in animal nutrition are primarily an extension of modeling approaches. Methodological limitations had prevented the use of conventional tools of meta-analysis to be used for studies on nutrient requirements. This review provides a new methodology for performing systematic review of nutrient requirement data in animal nutrition using the conventional approach of a meta-analysis.

Several response criteria have been used to determine the requirement of minerals in different fish species. Besides growth, feed efficiency and gross deficiency signs, whole body mineral concentration, mineral retention or balance, tissue mineral concentrations, plasma mineral levels, activities of related enzymes, urinary excretion and even gene expression of specific mineral transporters have been used as criteria. Data based on different response criteria often show variations in the estimates of values on quantitative requirements, within and across studies. This recommends the need for identifying the most appropriate criterion for each mineral and trace element in order to determine the minimal dietary level ensuring maximal weight gain, with no compromise on the physiological wellbeing of fish. The recent edition of NRC (2011) stated that, micro-nutrient requirements for farmed fish and shrimp are another area where knowledge is lacking; whether

dietary micronutrient requirements should be based on minimal levels required to prevent clinical deficiency signs or impaired growth or based on other criteria is an open question. Studies on determination of nutrient requirement should rely on more than one response criterion in order to test the effect of increasing levels of a nutrient and to eventually estimate minimal dietary inclusion levels for each criterion tested, whenever possible. We have tried to apply this in the present analysis of data on mineral and trace element requirements in fish. Almost every study analysed has used more than one response criterion, but not all of them leading to a valid estimate of the minimal dietary level required. In studies where valid estimates were obtained using more than one response criterion, differences can exist between the estimates. The variations in the estimate based on different response criteria can be either little and negligible to large and significant. The variations observed in the minimal dietary level estimates (between response criteria within a study) do not necessarily imply variations in the requirement of the fish. However, this stresses the need for a clear understanding on how a nutrient requirement (mineral or trace element, in this case) is defined and more importantly the need to identify the most appropriate criterion which would provide the most robust estimate. If the published data on minerals and trace element requirements in fish are analysed effectively, identifying the most appropriate response criterion for each mineral and trace element would be possible. Besides these variations caused by response criteria, it is also recognised that other biotic or abiotic factors may have an impact on data on the minimal dietary levels of specific minerals required by fish. The objectives of this systematic review are to (i) introduce a simple and new method for performing meta-analysis of nutrient requirement data, (ii) identify the most appropriate response criterion for quantifying mineral and trace element requirements in fish, (iii) analyse the factors that have an effect on the minimal dietary levels required and (iv) provide insights on the research needs for the future in terms of mineral and trace element nutrition of fish with particular reference to ongoing changes in dietary strategies and rearing systems.

Table 1: Literature data on calcium requirement studies reported in finfish

Ref No	Species	IW (g)	Days (d)	Diet	Diet Ca level (g/kg DM)			Ca source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Ca (mg/l)	Response variable	Ca Req (g/kg DM)	Estimate based on	Reference
					Level	Min	Max										
1	Blue Tilapia	0.8	84	SP	8	1.7	10	$CaSO_4$	CS (33)	FL	31	FW	Ca-free	1, 3, 6, 7	7	WG	Robinson *et al.* (1986)
2	Black seabream	8	70	SP	4	0.2	25	$C_6H_{10}CaO_6$, $Ca_3(PO_4)_2$	CS (50)	FL	22	35	400	1, 6	NR	WG & Ver. min	Hossain and Furuichi (1999b)
3	Tiger puffer	1	56	SP	5	0.3	4	$C_6H_{10}CaO_6$	CS (45)	FL	24	35		1, 3, 6	1.3 - 2.4	WG & Ver. min	Hossain and Furuichi (1998)
4	Redlip mullet	0.6	70	SP	4	0.2	24.5	$C_6H_{10}CaO_6$, $Ca_3(PO_4)_2$	CS (45)	FL	28	33	400	1, 6	R	WG	Hossain and Furuichi (2000c)
5	Jap. flounder	0.7	70	SP	4	0.2	25.5	$C_6H_{10}CaO_6$, $Ca_3(PO_4)_2$	CS (45)	FL	28	35		1, 6	R	WG	Hossain and Furuichi (2000d)
6	Scorpian fish	0.8	84	SP	4	0.2	25	$C_6H_{10}CaO_6$, $Ca_3(PO_4)_2$	CS (50)	FL	24	33	400	1, 6, 7	R	WG, FE & Ver. min	Hossain and Furuichi (2000b)
7	Hybrid tilapia	0.5	56	SP	8	0.6	10.7	$C_6H_{10}CaO_6$	CS (38)	RAC	26	FW	30	1, 3, 4, 6, 9	3.5 - 4.3	WG, Ver. & scale Ca	Shiau and Tseng (2007)
8	Channel catfish	6	150	Pr	4	5	20		SBM (20)	FL	27	FW	56	1, 3, 6, 14	15	WG	Andrews *et al.* (1973)
9	Channel catfish	24	150	Pr	6	7.5	20		SBM (20)	FL	27	FW	56	1, 3, 14	15	WG	Andrews et al. (1973)
10	American cichlid	0.4	63	SP	8	0.5	4	$CaCO_3$	CS (50)	RAC	28	FW	84	1, 3, 6, 7	1.8	WG, WB-Ca	Chavez-Sanchez *et al.* (2000)
11	Guppy	0.3	84	SP	3	0.3	12.8	$CaCO_3$	CS (43)	RAC	26	FW	40	1, 3, 6	NR	WG, Ver. min	Shim and Ho (1989)
12	Atlantic cod	150	91	Pr	5	4.3	11.9	$CaSO_4$	FM (30)	FL	10.6	35		1, 3, 4, 6, 7	R	WG, WB & Ver. min	Kousoulaki *et al.* (2010)
13	Rainbow trout	1.2	42	SP	3	0.3	3.4	$C_6H_{10}CaO_6$	EA (50)	FL	16.5	FW	22	1, 3, 6, 7	NR	WG, WB & Ver. min	Ogino and Takeda (1978)
14	Common carp	4.5	42	SP	8	0.3	9.7	$C_6H_{10}CaO_6$	EA (40)	FL	22.5	FW	20	1, 3, 6, 7,	NR	WG, WB & Ver. min	Ogino and Takeda (1976)
15	Rainbow trout	0.1	84	SP	3	1	8	$CaCO_3$	CS (52)	FT	17	FW	?	1, 3, 6	NR	Ver. min	Fontagné *et al.* (2009)
16	Grouper	30	70	SP	4	2.8	23.8	$C_6H_{10}CaO_6$	CS (40)	FL	28	30	340	1, 2, 3, 6, 7	6	Scale min	Ye *et al.* (2006)
17	Channel catfish		84	SP	7	1.7	8.5	$CaSO_4$	CS (33)	FL	25	FW	Ca free	1, 3, 4, 6, 7	4.5	WG	Robinson *et al.* (1986)
18	Mrigal	6	90	SP	4	1.9	3.5		CS (36)	FL	27	FW		1, 3	1.9	WG	Paul *et al.* (2004)
19	Barbell chub	4	45		3	6	18					FW		1, 3, 6	R	Ver. min	Zheng *et al.* (2007)
20	Grass carp	5	54	SP	6	2.75	11	$C_6H_{10}CaO_6$	CS (42)	RAS	25	FW	35	1, 4, 5, 6, 7, 9	10	WG; WB-Ca; Ver-Ca	Liang *et al.* (2012b)

IW – Initial weight of fish; WG – weight gain; WB – whole body Ca; Ver-Ca – Vertebral Ca content; Ver min - vertebral mineral content; Sc-min – Scale mineral content;;

Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum Ca levels; 5 - retention; 6 - Bone/scale mineralization; 7 - whole body Ca /ash; 8 - enzyme activity; 9 - Urinary excretion

Calcium lactate ($C_6H_{10}CaO_6$). SP – semi-purified diet; Pu – purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet

CS - Casein; EA – Egg albumin; FM – Fish meal; SBM – Soy bean meal;

FL – Flow through system; RAC – Re-circulatory aquaculture system; FW – freshwater; SW – salt water

Table 2: Literature data on magnesium requirement studies reported in finfish

Ref No	Species	IW (g)	Days (d)	Diet	Diet Mg level (g/kg DM)			Mg source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Mg (mg/l	Response variable	Mg Req (g/kg DM)	Estimate based on	Reference
					Level	Min	Max										
1	Atlantic salmon	12	84	SP	6	0.2	0.7	MgSO4	CS (45)	FL	10	0.5	54	1, 3, 4, 5, 6, 7	0.33	WB-Mg, Pl-Mg, Ver-Mg	El-Mowafi and Maage (1998)
2	Blue tilapia	0.5	126	SP	6	0.03	0.65	MgSO4	CS (33)	FL			0.1	1, 3, 6, 7	0.5	WG & Tissue min	Reigh *et al.* (1991)
3	Channel catfish	167	70	SP	8	0.04	1.04	MgSO4	CS (31)	FL	27	FW	1.6	1, 4, 6	0.4	WG, Ver-Mg, Pl-Mg	Gatlin *et al.* (1982)
4	Common carp	26	63	SP	4	0.08	3.22	$Mg(C_2H_3O_2)_2$	CS (26)	FL	28	FW	9	1, 6	0.6	WG; Hep & Kidney-Mg	Dabrowska and Dabrowski (1990)
5	Common carp	26	63	SP	4	0.08	3.2	$Mg(C_2H_3O_2)_2$	CS (26)	FL	24	FW	9.4	4, 6	0.6	Ver-Mg & Plasma-Mg	Dabrowska *et al.* (1991)
6	Common carp	8.5	28	SP	6	0.08	0.99	MgSO4		FL	21	FW	3.5	1, 3, 6, 7	0.4 -0.5	WG; Ver-Mg	Ogino and Chiou (1976)
7	Grass carp	8	76	SP	6	0.07	2.48	MgSO4	CS (32)	RAS	25	FW	5.6	1, 2, 3, 4, 5, 6	0.5-0.7	WG; Mg Ret, Ver-Mg	Wang *et al.* (2011)
8	Grouper	12	70	SP	7	0.24	2.8	MgSO4	CS (40)	FL	28	30	930	1, 3, 6, 7	0.24	WG, Ver-Mg, Sc-Mg	Ye *et al.* (2010)
9	Guppy	0.1	105	SP	6		0.9	MgSO4	CS (43)	RAS	26	FW	2.07	1, 3, 6	0.54	WG	Shim and Ng (1988)
10	Japanese eel														0.4	WG	Nose & Arai (1979)
11	Milk fish	2.6	154	SP	2	0.27	1	MgSO4	CS (30)		28	33		1, 3, 5, 6, 7	R	WG, Tissue min	Minoso *et al.* (1999)
12	Nile tilapia	10	70	SP	4	0.07	3.2							1, 2, 4, 5, 6, 7	0.6 - 0.77	Growth & Tissue min	Dabrowska *et al.* (1989a)
13	Rainbow trout	16	140	SP	6	0.06	2.1	MgSO4		RAS	15	FW	1.2	1, 2, 4	0.5	WG & Plasma-Mg	Knox *et al.* (1981)
14	Rainbow trout	12	42	SP	8	0.04	2.1	MgSO4	CS (43)	FL	13.5	FW		1, 2, 3, 6	0.6; 1.35	WG & WB-Mg	Shearer (1989)
15	Rainbow trout	5	170	Pr	4	1.2	2	MgSO4	FM (55)	FL	18	FW		1, 3, 6	0.5	WG, Ver.-Mg	Satoh *et al.* (1991)
16	Rainbow trout											FW	3.1		0.6 - 0.7	WG; Ver-Mg	Ogino *et al.* (1978)
17	Redlip mullet	5	91	SP	2	0.1	0.4	MgSO4	CS (50)		22	30		1, 3, 4, 6	NR	WG, Ver-mg, Pl-Mg	El-Zibdeh *et al.* (1996b)
18	Yellow croaker	29	98	SP	2	0.1	0.4	MgSO4	CS (50)		22	33		1, 3, 4, 6	NR	WG, Ver-mg, Pl-Mg	El-Zibdeh *et al.* (1996a)
19	Gibel carp	3	90	SP	6	0.04	2.9	MgSO4	CS (37)	FL	24	FW	12	1, 3, 4, 5, 6, 7, 8	0.75	Mg Ret	Han *et al.* (2012)
20	Grass carp	6	56	SP	6	0.19	0.94	MgSO4	CS (42)	RAS	27	FW	3	1, 3, 4, 5, 6, 7, 8	0.6-0.7	WG, WB-Mg, Ver-Mg	Liang *et al.* (2012c)
21	Rainbow trout	0.8	28	SP	4	0.08	0.75	MgSO4	CS (43)	FL	14	FW	1.4; 5	1, 2, 3, 5, 6	0.33	WG, WB-Mg	Shearer and Åsgård (1992)
22	Rainbow trout	0.8	28	SP	4	0.08	0.75	MgSO4	CS (43)	FL	14	FW	150	1, 2, 3, 5, 6	0.01	WG, WB-Mg	Shearer and Åsgård (1992)
23	Hybrid tilapia	0.9	54	SP	8	0.025	0.57	MgSO4	CS (38)	RAS	28	FW	4	1, 3, 4, 5, 6, 7, 8	0.2	WG, Mg Ret	Lin *et al.* (2013)
24	Hybrid tilapia	0.9	54	SP	8	0.025	0.57	MgSO4	CS (38)	RAS	28	32	1400	1, 3, 4, 5, 6, 7, 8	0.02	WG, Mg Ret	Lin *et al.* (2013)

IW – Initial weight of fish; WG – weight gain; WB-Mg – whole body Mg; Ver-Mg – vertebral Mg; Ver ash - vertebral ash; Pl-Mg – Plasma Mg; Sc-Mg – Scale Mg; Mg ret - retention;

Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum P levels; 5 - P retention; 6 - Bone/scale mineralization; 7 - whole body P /ash; 8 - enzyme activity.

SP – semi-purified diet; Pu – purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet

CS – Casein; FM – Fish meal; FL – Flow through system; RAC – Re-circulatory aquaculture system; FW – freshwater; SW – salt water.

Table 3: Literature data on potassium requirement studies reported in finfish

RefNo	Species	IW (g)	Day (d)	Diet	Diet K level (g/kg DM)			K source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water K (mg/l)	Response variable	K Req (g/kg DM)	Estimate based on	
					Level	Min	Max										
1	Channel catfish	17	56	SP	6	0.01	0.49	$KHCO_3$	CS (26)	FL	27	FW	4	1, 3, 5	2.6	WB balance	Wilson & El Nagger (1992)
2	Chinook salmon	1.1	70	SP	5	0.01	1.2		CS (41)	FL	14	FW		1, 3, 5	8	WG	Shearer (1988)
3	Redlip mullet	5	91	SP	2	0.1		$KHCO_3$	CS (50)		22	30		1, 3, 8, H	NR	WG	El-Zibdeh et al. (1996)
4	Red sea bream											SW					Sakamoto & Yone (1978b)
5	Hybrid Tilapia	0.8	56	SP	8	0.5	9.7	KCl	CS (38)	RAS	26	FW		1, 2, 3, 5, 8	2-3	WG, WB, ATPase	Shiau & Hsieh (2001)
6	Grass carp	4	56	SP	8	0.9	12.4	KCl	CS (42)	RAS	26.3	FW	2-8	1, 2, 3, 5, 8	8-10	WG, WB, Ver, ATPase	Liang *et al.* (2012a)

IW – Initial weight of fish; WG – weight gain; WB-K – whole body potassium balance.

Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum P levels; 5 - P retention; 6 - Bone/scale mineralization; 7 - whole body P /ash; 8 - enzyme activity; H – hematological parameters.

SP – semi-purified diet; CS – Casein; FL – Flow through system; RAC – Re-circulatory aquaculture system; FW – freshwater; SW – salt water.

Table 4: Literature data on zinc requirement studies reported in finfish

Ref. No	Species	IW (g)	Day (d)	Diet	Diet Zn (mg/kg DM)			Zn source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Zn mg/l	Response variable	Zn Req. (mg/kg DM)	Estimate based on	Reference
					Level	Min.	Max.										
1	Atlantic salmon	40	56	SP	5	0.02	80	$ZnSO_4.H_2O$		FL	12	2	3	1, 4, 6,7, 8, 10	37 - 67	WB-Zn; Sr-Zn	Maage and Julshamn (1993)
2	Blue tilapia		80	SP					EA	FL		FW			20	Scale & Ver Zn	McClain and Gatlin (1988)
3	Cobia		56	SP	5	5.65	56	$ZnSO_4.H_2O$	EA	FL		30		1, 3, 4, 6, 7, 8	42.9	WG, Ver-Zn	Xu *et al.* (2007)
4	Common carp	2	54	SP	4	1	30	$ZnSO_4.H_2O$	EA (40)	FL		FW	11	1, 3, 6, 7	15	WG, Ver-Zn	Ogino and Yang (1979)
5	Common carp	3	98	SP	4	2.6	41.2	$ZnSO_4.H_2O$		FL	20	FW		1, 3, 6	15 - 30	WG	Satoh *et al.* (1992)
6	Jian carp	15.7	42	SP	6	15	93	$Zn(C_3H_5O_3)_2 \cdot {}_2H_2O$		FL		FW		1, 3, 4, 8, 12	43-49	Sr-Zn; WG	Tan *et al.* (2011a)
7	Channel catfish	5.5	84	SP	3	20	200	$ZnSO_4.H_2O$	EA (34)	FL		FW	7	1, 3, 4, 6, 8, H	R	Ver-Zn	Gatlin III *et al.* (1989)
8	Channel catfish	4.5	84	SP	2	1	28	$ZnSO_4.H_2O$	EA (34)	FL	27	FW	3	1, 2, 3, 4, 6, 8	R	WG, Ver-Zn	Gatlin and Wilson (1983)
9	Channel catfish	4	98	SP	9	1	100	$ZnSO_4.H_2O$	EA (34)	FL	27	FW	3	1, 3, 4, 6, 7, 8	20	Sr-Zn, Ver-Zn	Gatlin and Wilson (1983)
10a	Channel catfish	0.02	42	SP	4	1.3	80	$ZnSO_4.H_2O$	EA (40)	FL	28	FW	4	1, 3, 7	20	WG; WB-Zn	Scarpa and Gatlin (1992)
10b	Channel catfish	0.02	42	SP	4	1.3	80	$ZnSO_4.H_2O$	EA (40)	FL	28	FW	4	1, 3, 7	80	WG; WB-Zn	Scarpa and Gatlin (1992)
10c	Channel catfish	0.02	54	SP	4	1.3	80	$ZnSO_4.H_2O$	EA (40)	FL	28	FW	4	1, 3, 7	20	WG; WB-Zn	Scarpa and Gatlin (1992)
11	Milk fish	3	154	SP	2	11	58	$ZnSO_4.H_2O$	CS (30)	FL	28	33		1, 3, 6	R	WG	Minoso *et al.* (1999)
12	Nile tilapia	8.5	70	SP	10	1	100	$ZnSO_4.H_2O$	EA (34)		25	FW		1, 3, 4, 6, 7	30	WG, Sr, Ver-Zn	Eid and Ghonim (1994)
13	Nile tilapia	13	70	Pr(P)	8	50	400	$ZnSO_4.H_2O$	SBM (65)	RA	25	FW	8	1, 4, 6, 7, 8,H	45; 80	WG; Ver-Zn	Sa *et al.* (2004)
14	Rainbow trout	2	54	SP	4	1	30	$ZnSO_4.H_2O$	EA (40)	FL		FW	11	1, 3, 6, 7	15	WG, Ver-Zn	Ogino and Yang (1978)
15	Rainbow trout	0.35	84	SP	4	0.1	42	$ZnSO_4.H_2O$	EA (45)	FL	16.5	FW		1, 3, 7, 11	20	WG	Satoh *et al.* (1987d)
16	Rainbow trout	0.35	84	SP	4	0.1	43	$ZnSO_4.H_2O$	EA (45)	FL	16.5	FW		1, 3, 7, 11	40	WG	Satoh *et al.* (1987c)
17	Rainbow trout	0.35	84	SP	4	0.1	41	$ZnSO_4.H_2O$	EA (45)	FL	16.5	FW		1, 3, 7, 11	40	WG	Satoh *et al.* (1987c)
18	Rainbow trout	1.2	84	SP	4	0.1	85	$ZnSO_4.H_2O$	EA (45)	FL	16.5	FW		1, 3, 7	80	WG	Satoh et al. (1987c)
19	Rainbow trout	0.3	280	Pr	5	39.5	117	$ZnSO_4.H_2O$	FM (55)	FL	12.5	FW		1, 6, 11	80	WG	Satoh *et al.* (1987a)
20	Red drum	2	54	Pr	5	5	40	$ZnSO_4.H_2O$	FM (20)	FL	28	6	9	1, 3, 6	20	WG	Gatlin III *et al.* (1991)
21	Yellow catfish	6	56	SP	6	7.6	76	$ZnSO_4.H_2O$	CS (36)		26	FW	2	1, 3, 4, 6, 7, 9	17 - 21	WG	Luo *et al.* (2011)
22	GH Seabream	42	90	Pr	3	61	989	$ZnSO_4.H_2O$	FM	FL	20	16		1, 9	R	WG, Tissue Zn	Serra *et al.* (1996)
23	Starry flounder	63	66	SP	6	20	411	$ZnSO_4.H_2O$	CS					1, 3, 4, 6, 7, 8	168	WG	
24	Olive flounder	33	31		5	37.5	117	$ZnSO_4.H_2O$						1, 3, 4, 6, 8	> 120	WG; Hep-Zn	
25	Japanese seabass	10	56		5			$ZnSO_4.H_2O$			28	29		1, 3, 6, 8	103	WG; Hep-Zn	Zhou *et al*. (2009b)
26	Cobia	17	56	SP	6	13	328	Zn-Met	CS					1, 3, 6, 8	47-54	WG; Sr-ALP	
27	Europeanseabass	10.6	90	Pr	5	91	253	Zn-Met	FM (50)		20	38		1, 3, 6	240	Skin-Zn	Fountoulaki *et al.* (2010)
28	Hy Stripped bass	1	70	SP	6	7	80	$ZnSO_4.H_2O$		RA	26	2	10	1, 3, 4, 6, H	17	Ver-Zn; Se-Zn	Buentello *et al.* (2009)
29a	Hy Stripped bass	5.5	54	Pr(P)	4	45.5	66	$ZnSO_4.H_2O$	SBM (56)	RA				6			Savolainen & Gatlin (2010)
29b	Hy Stripped bass	5.5	54	Pr(P)	4	45.5	66	ZnMet	SBM (56)	RA				6			Savolainen & Gatlin (2010)
30	Channel catfish	4	70	Pr(P)		54	450	ZnO	SBM (43)	FL	26	FW	30	9	150-200	Sr-Zn; Ver-Zn	Gatlin III and Wilson (1984b)
31	Grass carp	4	54	SP	6	13	135	$ZnSO_4.H_2O$	CS (42)	RA	26		20	1, 3, 6, 7, 8	55	WB-Zn; Ver-Zn	Liang *et al.* (2012d)

Ref. No	Species	IW (g)	Day (d)	Diet	Diet Zn (mg/kg DM)			Zn source	Protein source (%)	Syst em	Temp (˚C)	Salinity (ppt)	water Zn mg/l	Response variable	Zn Req. (mg/kg DM)	Estimate based on	Reference
					Level	Min.	Max.										
32a	Asian Seabass	0.24	54	Pr	4	80	125	ZnCl2	FM; GOC		28			1	45	WG	Sapkale and Singh (2011)
32b	Magur catfish	0.5	54	Pr	4	80	125	ZnCl2	FM; GOC		29	FW		1	30	WG	Sapkale and Singh (2011)
33	Rainbow trout	50	55	Pr	3	48	108	ZnPic	FM (45)	FL	11	FW		1, 3, 4, 7, 8			Kucukbay *et al.* (2006)
34a	Atlantic salmon	2	111	Pr	2	120	223	$ZnSO_4.H_2O$	FM	FL		2		1, 4, 7		WG, WB-Zn	Maage *et al.* (2001)
34b	Atlantic salmon	2	111	Pr	2	140	239	ZnGlu	FM	FL		2		1, 4, 7		WG, WB-Zn	Maage et al. (2001)
35	Rainbow trout	12	180	Pr(P)	2	51	115	$ZnSO_4.H_2O$	SBM (31)	FL	12	FW	1	1, 3, 6, 7, 8			Ramseyer *et al.* (1999)
36a	Channel catfish	2	84	Pr(P)	4	59	149	$ZnSO_4.H_2O$	SBM (38)	FL	30	FW		1, 3, 6	50	WG	Li and Robinson (1996)
36b	Channel catfish	2	84	Pr(P)	4	59	149	Zn-Met	SBM (38)	FL	30	FW		1, 3, 6	50	WG	Li and Robinson (1996)
37a	Channel catfish	2	70	SP	5	2	32	$ZnSO_4.H_2O$	EA (40)	FL	28	FW	2	1, 3, 6	22	WG; Ver-Zn	
37b	Channel catfish	2	70	SP	5	2	32	Zn-Met	EA (40)	FL	28	FW	2	1, 3, 6	8.6	WG; Ver-Zn	Paripatananont and Lovell
37c	Channel catfish	2	70	Pr(P)	5	46	126	$ZnSO_4.H_2O$	SBM (55)	FL	28	FW	2	1, 3, 6	76	WG; Ver-Zn	(1995)
37d	Channel catfish	2	70	Pr(P)	5	46	126	Zn-Met	SBM (55)	FL	28	FW	2	1, 3, 6	52	WG; Ver-Zn	

IW – Initial weight of fish; Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum Zn levels; 5 - Zn retention; 6 - Bone/scale mineralization; 7 - whole body Zn /ash; 8 - enzyme activity.

SP – semi-purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet.

CS – casein; EA – Egg albumin; FM – Fish meal; SBM – Soy bean meal; GOC – Groundnut oil cake.

FL – Flow through system; RA – Re-circulatory aquaculture system; FW – freshwater; SW – salt water

WG – weight gain; WB-Zn – whole body zinc; Ver Zn – vertebral zinc content; Ver ash – vertebral ash; Plasma Zn – plasma Zinc concentration; Sr-Zn – Serum zinc concentration; Sr-ALP – Serum Alkaline Phosphatase activity.

Table 5: Literature data on selenium requirement studies reported in finfish

Ref. No	Species	IW (g)	Day (d)	Diet	Diet Se (Mg/kg DM) Level	Min.	Max.	Se source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Se mg/l	Response variable	Se Req. (mg/kg DM)	Estimate based on	Reference
1a	Atlantic salmon	5	54	Pr	3	1.2	3.4	Na_2SeO_3	FM (70)	FL	14	1		1, 6, 7, 8	< 1.2	WG & H-GPx	Lorentzen *et al.* (1994)
1b	Atlantic salmon	5	54	Pr	3	1.2	3.1	Se-Met	FM (70)	FL	14	1		1, 6, 7, 8	< 1.2	WG & H-GPx	Lorentzen et al. (1994)
2	Atlantic salmon	1	112	SP	2	0.04	0.14	Na_2SeO_3	CS (22)	FL	14	FW	ND	1, 8	0.14	H-GPx	Poston *et al.* (1976)
3	Atlantic salmon	6	196	SP	2	0.02	0.95	Na_2SeO_3	Yeast(35	RAS	15	FW	0.4	1, 3, 6, 7, 8	R	WG, Tissue Se, Enz.	Bell *et al.* (1987)
4a	Channel catfish	2.8	105	SP	7	0.06	15	Na_2SeO_3	CS (25)	FL	27	FW	2.5	1, 3, 7, 8	0.1 - 0.5	H-GPx, Pl-GPx	Gatlin and Wilson (1984)
4b	Channel catfish	3.2	98	SP	8	0.06	5	Na_2SeO_3	CS (25)	FL	27	FW	2.5	1, 3, 8	0.25	WG, H-GPx, Pl-GPx	Gatlin and Wilson (1984)
5	Cobia	6.3	70	SP	6	0.21	1.4	Se-Met	CS (41)	FL	29	25	ND	1, 3, 6, 7, 8	0.8	WG, WB-Se, Ver-Se	Liu *et al*. (2010a)
6	Rainbow trout	1.3	140	SP	6	0.07	13.1	Na_2SeO_3	Yeast(35	FL	15	FW	0.4	1, 3, 7, 8	0.15-0.38	Pl- GPx	Hilton *et al.* (1980)
7a	Crucian carp	15	30	SP	2	0.05	0.6	Se-nano particle	CS (32)	FL	25	FW		1, 6, 8	R	WG	Zhou *et al*. (2009c)
7b	Crucian carp	15	30	SP	2	0.05	0.6	Se-Met	CS (32)	FL	25	FW		1, 6, 8	R	WG	Zhou et al. (2009c)
8a	Grouper	12	54	SP	6	0.21	4	Se-Met	CS (51)	RAS		31	ND	1, 3, 6, 7, 8	0.77	WG and WB-ret	Lin and Shiau (2005)
8b	Grouper	12	54	SP	7	0.17	2.1	Se-Met	CS (51)	RAS		31	ND	1, 3, 6, 7, 8	0.7	WG, WB-Se-ret	Lin and Shiau. (2005)
9	Gibel carp	3	100	SP	7	0.34	5.1	Se-Met	CS (37)	FL	24	FW	0.1	1, 3, 6, 7, 8	1.18	WG, GPx, tissue Se	Han *et al.* (2011)
10	Largemouth bass	5	54	Pr	6	0.97	2.1	Na_2SeO_3	FM (45)	RAS	27		ND	1, 3, 6, 7, 8	1.6 - 1.85	WG, H-GPx, GR	Zhu *et al.* (2011)
11a	Channel catfish	2	63	SP	5	0.02	0.4	Na_2SeO_3	CS (40)	FL	28	FW	0.4	1, 3, 6, 8	0.2 - 0.3	WG; H-GPx; H-Se	Wang and Lovell (1997)
11b	Channel catfish	2	63	SP	5	0.02	0.4	Se-Met	CS (40)	FL	28	FW	0.4	1, 3, 6, 8	0.12	WG; H-GPx	Wang and Lovell (1997)
11c	Channel catfish	2	63	SP	5	0.02	0.4	Se-Yeast	CS (40)	FL	28	FW	0.4	1, 3, 6, 8	0.12	WG; H-GPx	Wang and Lovell (1997)
12a	Rainbow trout	20	84	Pr	3	0.8	1.2	Na_2SeO_3	FM (48)	FL	11	FW		1, 4, 8, IR	R	WG; IR	Küçükbay *et al.* (2009)
12b	Rainbow trout	20	84	Pr	3	0.8	1.2	Se-Met	FM (48)	FL	11	FW		1, 4, 8, IR	R	WG; IR	Küçükbay *et al.* (2009)
14a	Rainbow trout	26	70	Pr	4	0.73	7	Na_2SeO_3	FM (50)	RAS	16	FW		1, 4, 6, 7, 8,	R	WG, WB, tissue Se	Rider *et al.* (2009)
14b	Rainbow trout	26	70	Pr	4	0.73	7.4	Se-Yeast	FM (50)	RAS	16	FW		1, 4, 6, 7, 8,	R	WG, WB, tissue Se	Rider *et al.* (2009)
15a	Channel catfish	2	63	SP	5	0.02	0.4	Na_2SeO_3	CS (40)	FL	28	FW	0.4	1, IR	0.4	WG, IR	Wang *et al.* (1997)
15b	Channel catfish	2	63	SP	5	0.02	0.4	Se-Met	CS (40)	FL	28	FW	0.4	1, IR	0.2	WG, IR	Wang *et al.* (1997)
15c	Channel catfish	2	63	SP	5	0.02	0.4	Se-Yeast	CS (40)	FL	28	FW	0.4	1, IR	0.2 - 0.4	WG, IR	Wang *et al.* (1997)
16a	Hyb. striped bass	3	42	SP	2	0.03	0.23	Na_2SeO_3	CS	RAS	24	2		1, IR	R	WG, IR	Jaramillo & Gatlin (2004)
16b	Hyb. striped bass	3	42	Pr	2	1.03	1.2	Na_2SeO_3	FM (51)	RAS	24	2		1, IR	R	WG, IR	Jaramillo & Gatlin (2004)
17a	Crucian carp	15	30	SP	2	0.05	0.6	Na_2SeO_3	CS (32)	FL	25	FW		1, 6, 8	R	WG, WB-Se, Enz	Wang *et al.* (2007)
17b	Crucian carp	15	30	SP	2	0.05	0.6	Se-Met	CS (32)	FL	25	FW		1, 6, 8	R	WG, WB-Se, Enz	Wang et al. (2007)
18a	Hyb. striped bass	150	42	Pr	7	1.22	3.9	Se-Yeast	FM (59)	RAS	27	FW		1, 6, 8	0.2	WG	Cotter *et al.* (2008)
18b	Hyb. striped bass	150	42	Pr	3	1.22	2.2	Na_2SeO_3	FM (59)	RAS	27	FW		1, 6, 8	0.2	WG	Cotter et al. (2008)
19a	Hyb. striped bass	3	84	SP	4	0.11	21.2	Na_2SeO_3	CS	RAS	25	2		1, 3, 7	1.2	WG	Jaramillo Jr *et al.* (2009)
19b	Hyb. striped bass	3	84	SP	4	0.11	2.6	Se-Met	CS	RAS	25	2		1, 3, 7	0.9	WG, WB-Se	Jaramillo Jr *et al.* (2009)
20a	Grouper	10	54	SP	3	0.39	1.6	Se-Met	CS (51)	RAS		32		1, 3, 6, 8	1.6	WG	Lin and Shiau (2009)
20b	Grouper	10	54	SP	3	0.38	1.7	Se-Met	CS (51)	RAS		32		1, 3, 6, 8	0.4	WG	Lin and Shiau. (2009)

Ref. No	Species	IW (g)	Day (d)	Diet	Diet Se (Mg/kg DM) Level	Min.	Max.	Se source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Se mg/l	Response variable	Se Req. (mg/kg DM)	Estimate based on	Reference
20c	Grouper	10	54	SP	3	0.36	1.8	Se-Met	CS (51)	RAS		32		1, 3, 6, 8	0.4	WG	Lin and Shiau. (2009)
21	Beluga sturgeon	13	54	Pr	6	1.26	20.2	Se-Met	FM	FL	28	FW		1, 3, 6, 7	12 (Max)	WG, WB, tissue Se	Arshad *et al.* (2011)
22a	Coho salmon		270	Pr	4	1.1	13.6	Na_2SeO_3	FM	FL		FW		1, 8, IR	8.6 (Max)	WG, WB-Se, Enz.	Felton *et al.* (1996)
22b	Coho salmon		180	Pr	4	1.1	13.6	Na_2SeO_3	FM	FL		30		1, 8, IR	8.6 (Max)	WG, WB-Se, Enz.	Felton et al. (1996)
23	Cut throat trout	10	870	Pr	6	1.2	11.2	Se-Met	FM	FL	15	FW		1, 5, 6, 7	11.2 (Max)	WG, WB-Se, Egg Se, fry survival	Hardy *et al.* (2010)

IW – Initial weight of fish; Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum Se levels; 5 - Se retention; 6 – Tissue Se content; 7 - whole body Se; 8 - enzyme activity; IR – Immune response; H – hematological parameters.

SP – semi-purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet

CS – casein; EA – Egg albumin; FM – Fish meal; SBM – Soy bean meal; GOC – Groundnut oil cake.

Se-Met – Selenomethionine; Na-Se – Sodium selenite; Se-Y – Selenium yeast; Se-nan – Selenium nanoparticle.

FL – Flow through system; RAC – Re-circulatory aquaculture system; FW – freshwater; SW – salt water

WG – weight gain; WB-Se – whole body Se; Tissue Se – Tissue Se content; Plasma Se – plasma Se concentration; H-GPx - .Hepatic Gluthothione peroxidase activity.

Table 6: Literature data on manganese requirement studies reported in finfish

RefNo	Species	IW (g)	Day (d)	Diet	Diet Mn (Mg/kg DM)			Mn source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Mn (mg/l	Response variable	Mn Req (mg/kg DM)	Estimate based on	Reference
					Level	Min	Max										
1	Atlantic salmon	0.18	84	SP	6	1.1	25.5	$MnSO_4.H_2O$	CS (45)	FL	10	FW	0.003	1, 7, 9	7.5 - 10.5	WB-Mn	Maage *et al.* (2000)
2	Atlantic salmon	4.7	84	Pr	5	5	106	$MnSO_4.H_2O$	FM (68)	FL	11	2.5		1, 6, 7, 8	15	WB-Mn, Ver-Mn	Lorentzen *et al.* (1996)
3	Channel catfish	4	84	SP	7	2.4	63	$MnSO_4.H_2O$	CS (25)	FL		FW	0.002	1, 3, 6, 7, 8	2.4	WG	Gatlin III and Wilson (1984a)
4	Common carp	1.9	84	SP	2	4	12	$MnSO_4.H_2O$	EA (40)	FL	24	FW		1, 3, 6,7, 8	12.0 - 13.0	WG & deformity	Ogino and Yang (1980)
5	Common carp	4	595	Pr	6	3	25	$MnSO_4.H_2O$	FM (55)	FL	15	FW		1, 3, 6, 8	12.0 - 13.0	WG	Satoh *et al.* (1987c)
6	Common carp	4.3	77	Pr	3	5	30	$MnSO_4.H_2O$	FM (55)	FL	20	FW		1, 3, 6	R	WG, Ver-Mn	Satoh *et al.* (1989a)
7	Common carp	2	105	SP	5	1	20	$MnSO_4.H_2O$	EA (45)	FL	20	FW		1, 3, 6	13 - 15	WG	Satoh *et al.* (1992)
8	Milk fish	3	154	SP	2	1.8	23	$MnSO_4.H_2O$	CS (30)	FL	28	33		1, 3, 6	R	Ver-Mn	Minoso *et al.* (1999)
9	Tilapia	0.04	70	SP	2	2.8	34	$MnSO_4.H_2O$	CS (54)	FL	25	FW	0.003	1	R	WG	Ishac and Dollar (1968)
10	Grouper	13	56	SP	6	4	1350	$MnSO_4.H_2O$	CS (40)	FL	28	30	0.012	1, 3, 6, 7	19	WB-Mn, Ver-Mn	Ye *et al.* (2009)
11	Rainbow trout	15	168	SP	2	1.3	33	$MnSO_4.H_2O$	CS (50)			FW		1, 4, 6, 8, H	R	Ver-Mn	Knox *et al.* (1981)
12	Rainbow trout	2.4	182	Pr	5	5	24	$MnSO_4.H_2O$	FM (55)	FL	18	FW		1, 3, 6	19	Ver-Mn	Satoh *et al.* (1991)
13	Rainbow trout	1.5	84	SP	2	4	13	$MnSO_4.H_2O$	EA (40)	FL	18	FW		1, 3, 6,7, 8	12.0 - 13.0	WG & deformity	Ogino and Yang (1980)
14	Gibel carp	3.2	68	SP	7	7.2	22	$MnSO_4.H_2O$	CS (42)	FL	24	FW	0.011	1, 6, 7, 8	12.6 – 13.7	WG; Ver-Mn; WBMn	Pan *et al.* (2008)
15	Hybrid tilapia	0.64	54	SP	8	2.9	64	$MnSO_4.H_2O$	CS (38)	RAS		FW	0.001	1, 5, 6, 7, 8	7	Hep-MnSOD; WBMn	Lin *et al.* (2008b)
16	Yellow catfish	4	54	SP	6	3.1	20	$MnSO_4.H_2O$	CS (36)		25	FW	0.014	1, 3, 7, 8	5.5 - 6.4	WG; Hep-MnSOD	Tan *et al.* (2012)
17	Grass carp	3.8	30		4	3	20	$MnSO_4.H_2O$			26	FW		1, 3, 6, H	15	Ver-Mn	
18	Cobia	6.3	70	SP	6	6	41	$MnSO_4.H_2O$	CS (42)	FL	29	25	0.01	1, 3, 6, 7, 8	22 - 25	WG; WB-Mn; VerMn	Liu *et al.* (2012)
19	Rainbow trout	16	420	Pr	2	4.4	23	$MnSO_4.H_2O$	FM (55)	FL	21	FW		1, 6, 7	R	WG; Ver-Mg	Yamamoto *et al.* (1983)

IW – Initial weight of fish; Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum Mn levels; 5 - Mn retention; 6 – Tissue Mn content; 7 - whole body Mn; 8 - enzyme activity; H – hematological parameters.

SP – semi-purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet

CS – Casein; EA – Egg albumin; FM – Fish meal.

FL – Flow through system; RAC – Re-circulatory aquaculture system; FW – freshwater; SW – salt water

WG – weight gain; WB-Mn – whole body Mn; Ver Mn – Vertebral Mn content; Plasma Mn – plasma Mn concentration; Hep-MnSOD - .Hepatic Mn Superoxide dismutase activity..

Table 7: Literature data on iron requirement studies reported in finfish

Ref. No	Species	IW (g)	Day (d)	Diet	Diet Fe (mg/kg DM)			Fe source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Fe (mg/l	Response variable	Fe Req (mg/kg DM)	Estimate based on	Reference
					Level	Min	Max										
1	Atlantic salmon	5.6	84	SP	9	11	409	$FeSO4.7H_2O$	CS (48)	FL	11	2.5	0.003	1, 3, 4, 7, H	60 - 100	H & Hep-Fe	Andersen *et al.* (1996)
2	Channel catfish	107	70	SP	6	9.6	60	$FeSO4.7H_2O$	EA (40)	FL		FW	0.43	1, 3, 4, H	30	WG & H	Gatlin III and Wilson (1986a)
3	Common carp														150		Takeuchi et al. (2002)
4	Common carp	11.4	60	SP	7	54	266	$C_4H_2FeO_4$	CS (18)	RAS	25	FW	0.005	1, 3, 4, 8	147.4	Serum Fe	Ling *et al.* (2010)
5	Gilthead seabream	2	84	Pr	5	22	309	Org-Fe	FM (35)	RAS	20	38			R	WG, H	Rigos *et al.* (2010)
6	Japanese eel														170		Nose & Arai (1976)
7	Orange grouper	21	56	SP	6	145	395	$FeSO4.7H_2O$	CS (40)	FL	24	35	0.005	1, 3, 4, 6, 7	245	Hep-Fe	Ye *et al.* (2007)
8	Rainbow trout	80	48	SP	3		1975	$FeSO4.7H_2O$		FL	15	FW		1, 5, 7, 8, 9, 10	R		Carriquiriborde *et al.* (2004)
9	Red sea bream														150		Sakamoto and Yone (1978b)
10	Yellow tail	163	42	Pr	6	58	148	Fe-Protnate	FM (45)	Cage	26	35		1, 4, 6, H	101	H & Hep-Fe	Ukawa *et al.* (1994)
11	Grass carp	7.1	78	SP	6	0	500	$FeSO4.7H_2O$						1, 3, H	300	WG	Su *et al.* (2007)
12	Red drum	173	56	SP	5	285	444	$FeSO4.7H_2O$	CS (50)	Cage	28	28		1, 3, 4, 6, 8	330	WG	Zhou *et al.* (2009a)
13	Rainbow trout	3	84	SP	6	25	5934	$FeSO4.7H_2O$	CS (40)	FL	15	FW	0.03	1, 3, 4, H, 6, 7			Desjardins *et al.* (1987)
14	Gibel carp	2	83	SP	6	138	273	$FeSO4.7H_2O$	CS (53)	FL	25	FW	0.01	1, 3, H, 6,	202	Hep-Fe; HCT	Pan *et al.* (2009)
15	Atlantic salmon	5	140	SP	6	16.4	252	$FeSO4.7H_2O$	CS (40)	FL		FW		1, 3, 4, 6, H	60	WG, Hep-Fe, H	Naser (2000)
16a	Tilapia	0.63	56	SP	7	24.7	200	$FeSO4.7H_2O$	CS (38)	RAS	26	FW	1.1	1, 3, 4, H	85	WG, Hep-Fe, H	Shiau and Su (2003)
16b	Tilapia	0.64	56	SP	7	23.6	209	$C_6H_5FeO_7$	CS (38)	RAS	26	FW	1.1	1, 3, 4, H	150-160	WG, Hep-Fe, H	

IW – Initial weight of fish; Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum Fe levels; 5 - Fe retention; 6 – Tissue Fe content; 7 - whole body Fe; 8 - enzyme activity; H – hematological parameters.

SP – semi-purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet

CS – Casein; EA – Egg albumin; FM – Fish meal.

FL – Flow through system; RAC – Re-circulatory aquaculture system; FW – freshwater; SW – salt water

WG – weight gain; WB-Fe – whole body Fe; Hep Fe – Hepatic Fe concentration; Plasma Fe – plasma Fe concentration; HCT – Hematocrit; H – hematological parameters.

Table 8: Literature data on copper requirement studies reported in finfish

Ref No	Species	IW (g)	Day (d)	Diet	Diet Cu (Mg/kg DM)			Cu source	Protein source (%)	System	Temp (˚C)	Salinity (ppt)	water Cu (mg/l	Response variable	Cu Req (mg/kg DM)	Estimate based on	Reference
					Level	Min	Max										
1	Atlantic salmon	7.5	84	SP	5	3.5	98	$CuSO_4.5H_2O$		FL	11	2.5		1, 7, 8	8.5 - 13.7	liver Cu	Lorentzen *et al.* (1998)
2	Channel catfish	5.5	91	SP	6	0.9	4.9	$CuSO_4.5H_2O$	EA (35)	FL		FW	0.11	1, 3, 4, 6, 8, H	5	Hepatic SOD	Gatlin III and Wilson (1986b)
3	Channel catfish	5.5	84	SP	2	1	20	$CuSO_4.5H_2O$	EA (35)	FL		FW	0.06	1, 3, 4, 6, 8, H	R	Hepatic SOD	Gatlin III *et al.* (1989)
4	Common carp	1.9	84	Pu	2	0.7	3	$CuCl_2$		FL	24	FW		1, 3, 6,7, 8	3	WB-Cu, Ver-Cu and Liver-Cu	Ogino and Yang (1980)
5	Malabar Grouper	14	56	SP	8	0.1	20.1	$CuSO_4.5H_2O$	CS (51)	RA		31	0.001	1, 3, 4, 5, 6, 7	4.0 - 6.0	WG, H-SOD, WB-Cu	Lin *et al.* (2008a)
6	Malabar Grouper	22	56	SP	7	0.2	12.8	CuPep	CS (51)	RA		31	0.001	1, 3, 4, 5, 6, 7	2.0 - 3.0	WG, H-SOD, TBARS; WB-Cu	Lin *et al.* (2010)
7	Rainbow trout	20	60	SP	4	22	424	$CuSO_4.5H_2O$		FL		FW		1, 2, 3, 5, 7, 8	R		Gundogdu *et al.* (2009)
8	Rainbow trout	1.5	84	SP	2	0.7	3	$CuSO_4.5H_2O$	EA (40)	FL	18	FW		1, 3, 6,7, 8	3	WB-Cu, Ver-Cu and Liver-Cu	Ogino and Yang (1980)
9	Rockfish	26	60		5		500	$CuSO_4.5H_2O$		FL	18	33		1, 4, 6, 8, H	R	WG	Kim and Kang (2004)
10	Yellow catfish	3	49	SP	6	2.1	22.25	$CuSO_4.5H_2O$	CS (36)		26	FW	0.001	1, 3, 7	3.1; 4.2	WG, Cu Ret	Tan *et al.* (2011b)
11	Atlantic salmon	0.9	84	Pr	6	2.2	1780	$CuSO_4.5H_2O$	FM (57)	FL	13	5	0.006	1, 5, 6, 7	37	WG	Berntssen *et al.* (1999)
12a	Crusian carp	18	55	Pr	4	3.5	12.5	Cu-AA	FM (58)		26	FW	0.004	1, 3, 4, 6, 7	6.5 - 12.5	WG; WB-Cu	
12b	Crusian carp	18	55	Pr	4	3.5	12.6	$CuCl_2$	FM (58)		26	FW	0.004	1, 3, 4, 6, 7	6.5 - 12.5	WG; WB-Cu	Shao *et al.* (2010)
12c	Crusian carp	18	55	Pr	4	3.5	12.5	$CuSO_4.5H_2O$	FM (58)		26	FW	0.004	1, 3, 4, 6, 7	6.5 - 12.5	WG; WB-Cu	
13	Blunt snout bream	31	56	Pr	8	8.5	148	$CuCl_2$	FM (50)		28	FW	0.004	1, 3, 4, 6, 7, 8	12-18	WG; plasma ALP and ACP	Shao *et al.* (2012)

IW – Initial weight of fish; Response variable tested: 1 – Growth; 2 - feed Intake; 3 - Feed efficiency; 4 - plasma/blood/serum Cu levels; 5 - Cu retention; 6 – Tissue Cu content; 7 - whole body Cu; 8 - enzyme activity; H – hematological parameters.

SP – semi-purified diet; Pr – practical diet; Pr (P) – Plant ingredient based practical diet; CuPep – Copper peptide; Cu-AA – Copper amino-acid chelate.

CS – Casein; EA – Egg albumin; FM – Fish meal.

FL – Flow through system; RA – Re-circulatory aquaculture system; FW – freshwater; SW – salt water

WG – weight gain; WB-Cu – whole body Cu; Hep Cu – Hepatic Mn concentration; Plasma Cu – plasma Cu concentration; Hep-enzyme activities and . H – Hematological parameters.

Meta-analysis of literature data

Collection, screening and selection of studies

Collection of data and clearly defining the eligibility criteria for a study to be included in the meta-analysis are vital to the quality of meta-analysis (Egger *et al.* 1997). Published reports on the effect of essential macro-minerals such as P, Mg or Ca and micro-minerals such as Cu, Fe, Mn, Se or Zn in fish were collected from various sources. A primary dataset was prepared comprising of reports with at least three dietary levels of the mineral studied. The primary dataset thus included 25 studies on available phosphorus (refer Antony Jesu Prabhu *et al.* 2013), 20 studies on calcium (Table 1), 24 studies on magnesium (Table 2), 7 studies on potassium (Table 3), 37 studies on zinc (Table 4), 23 in the case of selenium (Table 5), 19 for manganese (Table 6), 16 for iron (Table 7) and 13 for copper (Table 8). Information on factors such as fish species, fish size, duration of the trial, diet type, dietary mineral levels, source of the mineral, water salinity, temperature, response criteria tested, mathematical model used and corresponding requirement estimates were incorporated into the primary dataset. Primary data on dietary mineral levels and the corresponding values for different response criteria tested were retrieved from each study. The primary data thus obtained were standardised to a common response unit following the method of Antony Jesu Prabhu *et al.* (2013). Rigorous screening of the studies included in the primary dataset was performed in order to select only those studies appropriate for further meta-analytic procedure. All the studies included in the primary dataset grouped under each mineral were subjected to broken line regression analysis (Robbins *et al.* 2006). Linear plateau model and two-linear line model were used for each study and the estimates from the best fit model were chosen to be used in the meta-analysis. In general, linear plateau model was chosen for the macro-minerals (P, Ca, Mg and K) whereas two-linear line model was selected for the micro-minerals (Zn, Cu, Fe, Mn and Se). The choice was based on the regression coefficient of the curve-fit and as well as considering the biological property of the element, where most of the micro-minerals tend to have a negative effect on growth and accumulate in different tissues at higher dietary inclusion levels. The broken line

regression was used to estimate the break-point (X_{bp}), plateau (L) and slope (b in case of plateau model; b1 and b2 for two-linear line model) value along with their corresponding error terms. The studies where definite values for all the three parameters along with their error terms could be estimated were then used in the meta-analysis and the rest were rejected. The number of studies thus selected for further analysis varied depending on the response criterion within each mineral. In all cases, the most number of studies were recorded for weight gain as the response criterion. It included 20 studies for available phosphorus, 11 for magnesium, 8 for calcium, 4 for potassium, 21 for zinc, 25 for selenium, 5 for copper, 6 for iron and 10 in case of manganese. Detailed account of studies selected and used under different response criteria is provided in Table 9.

Response criteria and mineral requirement estimates

Among the different response criteria examined for each mineral, analysis of variance (ANOVA) was used to identify the most reliable criterion. The estimates obtained for each study were subjected to ANOVA in order to test the significance of different response criteria over each other. The most common and widely used response criteria were weight gain, whole body mineral levels, vertebral mineral concentration, plasma or serum mineral levels, specific enzyme activities or haematological parameters. As weight gain was observed to be the most common and widely used response criterion, a comparison of the requirement estimates from other criteria with reference to weight gain as 100 was also used to quantify the magnitude of the variation on a relative scale.

Factors affecting minimal dietary inclusion levels

The studies in which a valid estimate of requirement along with the variations could be obtained were categorised into different class groups based on one or more of the following factors, (i.) Species group or digestive physiology: based on the finfish species examined in the studies, they were classified into one of the following groups, Salmonids, Cyprinids, Silurids or others); Further, species with a gastric acid secretion in the stomach were classified into gastric and those which

lack gastric glands for acid secretion in the stomach were classified as agastric or stomach-less (Smith, 1989) (ii.) Diet type, as semi-purified or practical: experimental diets primarily based on refined ingredients (casein, gluten, egg white etc.) were classified as semi-purified and those based on unrefined ingredients such as fish meal, soybean meal etc. were considered as practical; (iii.) Mineral source, sources of inorganic mineral salts or chelated sources used in different studies were categorised into respective classes; (iv.) From the information provided in the individual studies, the possible effects of environmental factors such as salinity or mineral concentrations of the rearing water were also tested: studies with 0 to 0.5 ppt of salinity were classified as freshwater and those above them were collectively classified into seawater category. The estimates on the minimal dietary inclusion level obtained from each study were subjected to ANOVA for testing the significance of one or more of the above mentioned factors in influencing the minimal dietary inclusion level of minerals.

Statistical and analytical tools

The break-point (effect size) analysis of the individual studies from the primary dataset using linear plateau or two-linear line model was performed through generalised linear model (GLM) protocol of SAS version 9.2 following the method of Robbins *et al.* (2006). The computation of weighted effect size, overall mean effect size and selection of random effects based on Q and I^2 statistics for the meta-analysis was performed in Microsoft Excel, 2010 based on the method modified from Neyeloff *et al.* (2012). In the aforementioned study, the size effect was calculated as a proportion of an event to the total number of subjects. Calculating effect size (step 1) and the standard error of the effect size (step 2) require adjustments according to study type and outcome (Neyeloff et al. 2012). In the present study, the break-point estimate and its standard error were used in steps 1 and 2 respectively. Analysis of response criteria for identifying the most appropriate criterion was performed through one-way ANOVA. In order to test the factors influencing minimal dietary inclusion levels of minerals (P, Mg, Zn and Se),

ANOVA based on least square means (LS means) was performed and accordingly type-III F-test was used to evaluate the effects.

Table 9: Studies selected for inclusion in the meta-analysis for each mineral with respect to different response criteria tested

Mineral	Response criteria	Studies	Reference number	Table no.
Phosphorus	Weight gain	20	All studies, 1-20 listed in table A1.	Table A1
	Whole body P	11	1, 2, 4, 7, 8, 11, 12, 15, 19, Luo *et al.* (2010), Phromkunthong and Udom (2008).	
	Vertebral P	9	6, 12, 15, 17, 19, Vielma and Lall (1998a), Furuya *et al.* (2008), Luo *et al.* (2010), Phromkunthong and Udom (2008).	
	Plasma P	6	1, 2, 7, 8, Vielma and Lall (1998a), Phromkunthong and Udom (2008).	
Calcium	Weight gain	8	1, 3, 7, 9, 14, 17, 18, 20	Table 1
	Vertebral ash or Ca	8	1, 3, 7, 9, 14, 17, 18, 20	
Magnesium	Weight gain	12	2, 3, 6, 9, 13a, 14, 19, 20, 21a, 21b, 23, 24	Table 2
	Whole body Mg	11	1, 5a, 5b, 6, 7, 14, 19, 20, 21a, 21b, 23	
	Vertebral Mg	7	1, 2, 3, 6, 7, 9, 20	
	Plasma Mg	5	3, 13a, 13b, 20, 23	
Potassium	Weight gain	4	1, 2, 4, 5	Table 3
	Whole body K	4	1, 2, 4, 5	
	Na-K ATPase	2	4, 5	
Zinc	Weight gain	24	2, 5, 6, 9, 12, 13, 15, 16, 17, 28, 19, 20, 21, 23, 24, 25, 28, 31, 32b, 37a, 37b, 37c, 37d, 38	Table 4
	Whole body Zn	10	1, 2, 10c, 12, 14, 17, 18, 21, 31, 38	
	Vertebral Zn	21	2, 6, 9, 13, 15, 16, 17, 18, 19, 20, 21, 23, 24, 25, 26, 27, 32b, 37a, 37c, 37b, 38	
	Serum Zn	5	1, 9, 12, 30, 31	
	Serum-ALP	4	1, 9, 13, 31	
Selenium	Weight gain	25	2, 3, 4a, 4b, 5, 6, 8a, 8b, 9, 10, 11a, 11b, 11c, 15a, 15b, 15c, 16a, 16b, 18a, 18b, 19a, 19b, 20a, 21a, 21b, 22	Table 5
	Hepatic-GPx	14	4a, 4b, 5, 8a, 8b, 9, 10, 11a, 11b, 11c, 14b, 17a, 17b, 18a	
	Serum-GPx	8	4a, 4b, 5, 6, 11a, 11b, 11c, 17a	
	Hepatic-GR	4	11a, 11b, 11c, 17a	
Manganese	Weight gain	10	1, 3, 5, 7a, 7b, 10, 14, 15, 16, 18	Table 6
	Whole body Mn	6	1, 2, 10, 14, 16, 18	
	Vertebral Mn	5	2, 3, 5, 7a, 12, 14, 18	
Iron	Weight gain	6	1, 2, 7, 13, 14, 16a	Table 7
	Whole body Fe	3	1, 13, 16a	
	Plasma Fe	4	2, 4, 13, 15	
	Haemoglobin	6	1, 2, 5, 14, 15, 16a	
	Haematology	5	1, 2, 14, 15, 16a	
Copper	Weight gain	5	5, 6, 10, 12c, 13	Table 8
	Hepatic Cu	6	1, 2, 5, 6, 10, 12	
	Hepatic CuZn SOD	4	2, 5, 6, 13	

Results and Discussion

Response criteria and mineral requirement estimates

The results of the meta-analysis used to study the impact of the response criteria on the estimated minimal dietary inclusion levels of P, Mg, Ca, Zn, Se, Mn, Fe and Cu are reported in Table 10. Minerals have structural as well as functional roles as essential nutrients. Macro-minerals such as P, Ca and Mg are major constituents of the hard structures such as scales and bones of the body; Na and K as the major intra- and extra-cellular cations are involved in ionic balance (Lall, 2002). The response criteria to determine the dietary deficiency, adequacy or excess of a mineral therefore should be based on the vital role for which the respective mineral is utmost indispensable. Therefore, weight gain, the most commonly used response criterion may not necessarily be the most appropriate (Baker 1986), particularly so for micro-nutrients. In our meta-analysis, vertebral concentration for P and Ca; vertebral and whole body concentration for Mg; whole body concentration and gill Na-K ATPase activity for K were found as relevant criteria to assess the quantitative dietary supply of respective macro-minerals. On the other hand, micro-minerals are important for their catalytic functions in the metalloenzymatic processes. The functional role of Zn is known to be associated with the activities of alkaline phosphatase (ALP), Cu-Zn super-oxide-dismutase (SOD) and carbonic anhydrase; while Mn is linked to Mn-SOD activity. However, vertebral concentration remained the defining criterion for both Zn and Mn, as vertebrae are known to be the major reservoir for Zn and Mn storage in fish (Satoh et al. 1987a, b, c, d and 1991). Next to vertebral Zn content, biochemical criteria such as serum Zn concentration and ALP activity in the serum were also responsive to dietary Zn levels. Selenium is essential for the functioning of numerous selenoproteins, particularly of Se-dependent glutathione peroxidase (GPx). Se deficiency reduces the activity whereas Se inclusion increases the activity of Se-GPx (Bell et al. 1986). In the meta-analysis, hepatic-GPx activity as the criterion resulted in the highest estimate for Se, compared to serum GPx or glutathione reductase activities. In the case of Fe, although not statistically significant, the use of haematological indices such as haemoglobin, haematocrit,

plasma Fe, mean corpuscular volume (MCV) and haemoglobin (MCH) resulted in lower estimates than required for body Fe homeostasis. The reason for this could be that dietary Fe requirements for haematopoiesis are lower than needed to maintain body Fe stores. In mammals, Fe required for haematopoiesis is mainly supplied from recycling of Fe from senescent erythrocytes (Andrews and Schmidt, 2007). From the limited published data available for Cu, requirement estimates based on weight gain, hepatic Cu concentration and Cu-Zn-SOD activity were not statistically different. It could therefore be stated that, estimates based on criteria which utilises the mineral concentration of the whole body or vertebrae were found to result in significantly higher estimates than those obtained using weight gain as the criterion for P, Mg, Ca, Zn and Mn. The use of specific enzyme activities (hepatic GPx for Se and serum ALP for Zn) led to significantly higher estimates than weight gain. Although statistical significance was not observed, differences were observed between weight gain and other functional biochemical criteria used in the case of K, Fe, and Cu. In summary, a clear difference can be observed between the requirement estimates based on weight gain and a specific response criterion for each mineral based on its functional significance. Only a limited number of response criteria were compared in this meta-analysis due to lack of sufficient data for many other response criteria. Moreover, exploring the effectiveness of specific criteria especially focusing on non-invasive samples of hard and soft tissues such as scales, blood, plasma, serum, mucus and even fin-rays could provide pertinent biomarkers to assess the micro-mineral status of fish.

Comparison of meta-analytic estimates with published data

We took into account data from individual studies in the published literature on macro- and micro-mineral requirements of fish, most of which have been compiled in the recent revision of the NRC (2011). Factorial modeling was used by Shearer (1995) to predict values on the minimal dietary inclusion level of minerals based on whole body mineral concentration as the criterion. The meta-analytic estimates obtained in the present study were compared with these two datasets from literature and the data are also reported in Table 10.

Table 10: Mineral dietary inclusion levels of minerals based on different response criteria tested through meta-analysis

Criteria	Nb. of studies	Meta-analytic estimate[†]	95% confidence limit[‡]		Relative %[¶]	P-value[¶]	From literature[§]	
		Mean ± SE	Lower	Upper	Mean ± SE		Shearer (1995)[1]	NRC (2011) range[2]
Phosphorus (g available P/kg DM)								
Weight gain	20	5.31 ± 0.51	4.31	6.31	100 ± 4.5 [a]	< 0.001		
Whole body-P	11	6.34 ± 0.81	4.75	7.93	118.1 ± 4.4 [ab]		5	3.3 to 8
Vertebral P	9	7.16 ± 0.5	6.19	8.14	133.3 ± 6.5 [b]			
Plasma P	6	5.33 ± 1.35	2.69	7.98	99.3 ± 6.6 [a]			
Calcium (g/kg DM)								
Weight gain (Ca free water)	3	4.7 ± 0.23	4.25	5.14	100 ± 4.9 [a]	< 0.001		
Vertebral ash or Ca (Ca free water)	3	5.31 ± 0.75	3.83	6.79	113.2 ± 14.2 [a]		5.5	NR to 7
Weight gain (normal water)	5	2.6 ± 0.11	2.39	2.82	55.5 ± 4.2 [b]			
Vertebral ash or Ca (normal water)	5	4.32 ±0.81	2.73	5.92	92.1 ± 18.8 [a]			
Magnesium (g/kg DM)								
Weight gain	11	0.34 ± 0.05	0.25	0.42	100 ± 6.1 [a]	< 0.001		
Whole body-Mg	11	0.49 ± 0.02	0.45	0.53	146.3 ± 5.3 [b]		0.4	0.4 to 0.6
Vertebral Mg	7	0.42 ± 0.04	0.35	0.50	126.4 ± 4.9 [b]			
Plasma Mg	5	0.42 ± 0.09	0.24	0.59	124 ± 10.6 [b]			
Potassium								
Wight gain	4	4.2 ± 1.6	1.07	7.41	100 ± 38	NS		
Whole body-K	4	6.9 ± 1.8	3.36	10.36	162 ± 26		2.6	2 to 8
Na-K ATPase activity	2	6 ± 3.8	1.52	13.44	141 ± 64			
Zinc (mg/kg DM)								
Weight gain	21	36.05 ± 6.98	22.38	49.73	100 ± 4 [a]	0.003		
Whole body-Zn	7	33.47 ± 8.04	17.70	49.23	93 ± 5 [a]		20.6	
Vertebral-Zn	19	64.61 ± 11.39	42.29	86.92	179.1 ± 14 [b]			15 to 37
Serum-Zn	5	53.4 ± 14.35	25.27	81.53	148.9 ± 16 [b]			
Serum ALP activity	4	46.96 ± 20.85	14.04	81.76	130 ± 11 [ab]			

Criteria	Nb. of studies	Meta-analytic estimate[†]	95% confidence limit[‡]		Relative %[¶]	P-value[¶]	From literature[§]	
		Mean ± SE	Lower	Upper	Mean ± SE		Shearer (1995)[1]	NRC (2011) range[2]
Selenium (mg/kg DM)								
Weight gain	25	0.35 ± 0.04	0.27	0.43	100 ± 12 [a]	0.001		
Hepatic GPx activity	14	0.78 ± 0.09	0.61	0.95	221.2 ± 11 [b]			0.15 to 0.7
Serum GPx activity	8	0.43 ± 0.07	0.29	0.57	122.4 ± 17 [ab]			
Hepatic-GR activity	4	0.41 ± 0.11	0.19	0.63	116.8 ± 27 [ab]			
Manganese (mg/kg DM)								
Weight gain	10	10.7 ± 1.7	7.4	14.1	100 ± 16	0.08		
Whole body-Mn	6	13.4 ± 2.01	9.5	17.4	124.7 ± 15		3	2.4 to 12
Vertebral-Mn	5	18.4 ± 4.1	10.3	26.5	171.6 ± 22			
Iron (mg/kg DM)								
Weight gain	6	125.8 ± 34.6	58.0	193.7	100 ± 27	NS		
Whole body-Fe	3	166.7 ± 48	72.5	260.8	132.5 ± 29		21	
Plasma -Fe	4	67.4 ± 15.4	37.3	97.5	53.6 ± 22			30 to 150
Haemoglobin (Hb)	6	58.8 ± 12.6	34.0	83.6	46.7 ± 21			
Haematocrit (Hct)	4	93.1 ± 25.2	43.7	142.5	73.9 ± 27			
Mean corpuscular volume (MCV)	3	93.9 ± 35.4	24.6	163.4	74.7 ± 37			
Mean corpuscular Hb (MCH)	3	75.5 ± 9.2	57.5	93.5	60.1 ± 17			
Copper (mg/kg DM)								
Weight gain	5	6.5 ± 1.7	3.09	9.88	100 ± 27	NS	1.1	3 to 5
Hepatic Cu	5	5.7 ± 2	1.54	9.51	88 ± 24			
Hepatic CuZn-SOD	4	5.7 ± 2.2	1.49	9.90	87.9 ± 27			

[†] Estimate of minimal dietary inclusion level (mean ± SE) based on each response criteria obtained through meta-analysis of literature data from selected studies listed in table 9.

[‡] 95% confidence intervals of the mean estimate for each response criteria.

[¶] Relative comparison and statistical analysis of each response criteria with estimate based on weight gain as the reference. Estimate based on criteria with different superscripts within a single mineral are statistically different at $P < 0.05$; NS – not significant.

[§1] Estimate values for each mineral reported by Shearer (1995) based on his factorial model with whole body mineral balance as the response criterion.

[§2] Range of minimal dietary inclusion level estimates reported by NRC (2011) for different fish species.

i. Comparison with model predicted estimates

The minimal dietary inclusion level estimates (based on whole body mineral concentration as the response criterion) obtained from meta-analysis were in close agreement with the model predicted values reported by Shearer (1995) for macro-minerals P, Ca, Mg and K. In the case of micro-minerals such as Zn, Mn, Cu and Fe, the meta-analytic estimates of the present study were higher compared to values reported by Shearer (1995). The possible reasons appear to be the different assumptions made in the model such as, 100% dietary availability of the minerals, feed efficiency of 1, no endogenous loss or uptake from water. In the present study, the dataset on P, for instance, was based on data on available P levels and all the studies included under Ca, Mg and K in the meta-analysis were based on diets made of refined ingredients (close to 100% availability, according to NRC). Therefore no large differences were found between the estimates obtained from meta-analysis and values predicted by Shearer (1995). This was not the case for micro-minerals such as Zn, Mn, Cu and Fe where most of the studies undertaken using practical ingredients were also included in the meta-analysis. Therefore, the mean values were higher as it corresponds to the total dietary levels and not available levels as assumed by Shearer (1995). However, the lower limit of the 95% confidence intervals of the meta-analytic estimates (Table 10) were closer to the model predicted estimates of Shearer (1995) indicating the influence of dietary mineral availability in the differences observed between the values from the afore-mentioned work and the meta-analytic estimates of the present study. This was also clearly seen from the results of analysis on factors affecting minimal dietary inclusion levels, wherein diet type as a factor significantly affected minimal dietary inclusion estimates of selected minerals such as Zn and Se.

ii. Comparison with NRC recommended minimal dietary inclusion levels

NRC (2011) presented mineral requirement data on 8 freshwater and 4 marine finfish species; the data on seawater reared fish being restricted to one or two minerals at the maximum for a given species. The meta-analytic estimates of the present study are in good agreement with the range of minimal dietary inclusion

levels for macro-minerals (P, Ca, Mg or K) obtained from NRC (2011). However, similar to the observation made in the comparison with Shearer (1995), the upper limit of the NRC (2011) range for the micro-minerals (Zn, Se, Mn, Fe and Cu) were lower compared to the meta-analytic estimated range (Table 10). The possible reasons are, (i) similar to the model predicted values on minimal dietary inclusion levels reported by Shearer (1995), the recommendations of NRC (2011) were also based on data representing near 100% availability of the dietary minerals and (ii) the values presented by NRC (2011) were not criteria specific unlike the meta-analytic estimates of the present study. The need to identify a more appropriate criterion than whole body weight gain, to assess the nutrient requirements and minimal dietary inclusion levels of micronutrients such as minerals and vitamins is well recognised (NRC, 2011). In this context, the estimates obtained in the present study could serve as a source of reference for minerals, supplementing the information provided in the NRC (2011). Moreover, the minimal dietary inclusion level based on different response criteria expressed as a relative percentage to the estimate based on weight gain (Table 10) will provide a relative scale to quantify the magnitude of difference that can be expected with changes in response criteria.

Normal range of whole body and tissue mineral concentrations in fish

The concentration of minerals in the whole body and specific tissues are considered better indicators of mineral status, as clinical deficiencies are preceded by disturbance in normal tissue or whole body mineral levels in terrestrial animals (Underwood 1981) as well as in fish (Shearer 1984). Rainbow trout, Atlantic salmon and common carp are the three most studied fish species with regard to mineral nutrition, till date. Of these, mineral composition of whole body and different tissues in rainbow trout (Hardy *et al.* 1984; Shearer 1984; Satoh *et al.* 1987e) and Atlantic salmon (Shearer *et al.* 1994) are available for the entire life cycle. In common carp, an extensive database on changes in mineral composition of vertebrae and gonads as affected by various dietary factors is available from reports of Ogino & Takeda (1976) and of Satoh *et al*. (1987e, f). Taking advantage of the database generated, an attempt was made to analyse the data on mineral and trace element composition of whole body and different tissues in order to

Table 11: Mineral and trace element concentration reported in literature on different tissues of fish

	Min	Max	Median	Studies	Species	observations	Plateau values from meta-analysis		Rainbow trout ¶ (mean ± SD)	Atlantic salmon † (mean ± SD)	Common carp ‡ (mean ± SD)
							Mean ± SD	Studies (n)			
Whole fish (wet weight basis)											
Ash (%)	1.1	8.7	3.3	62	37	328				1.5 to 3	2.8 ± 0.3
Ca (g kg^{-1})	0.6	16.4	**6.5**	40	27	203	**6.2 ± 1.5**	8	5.2 ± 1.2	3 to 5.5	7.1 ± 0.8
Mg (g kg^{-1})	0.12	0.63	**0.33**	26	17	131	**0.32 ± 0.1**	11	0.33 ± 0.2	0.3 to 0.5	0.25 ± 0.05
P (g kg^{-1})	1.06	11.9	**4.9**	52	42	275	**4.6 ± 1.1**	16	4.8 ± 1	4 to 5	4.9 ± 0.7
K (g kg^{-1})	0.96	4.5	3.85	8	6	37	**2.8 ± 0.3**	4	3.2 ± 0.5	2.3 to 4	1.9 ± 0.1
Na (g kg^{-1})	0.98	4.1	**1.5**	7	5	33			1.3 ± 0.3	1.5 to 2	1.1 ± 0.1
Cu (mg kg^{-1})	0.35	2.58	**1**	24	12	122	**1 ± 0.4**	11	1.2 ± 0.5	1 to 3	1.2 ± 0.1
Fe (mg kg^{-1})	4.3	115	**13.6**	21	9	122	**14.5 ± 4.3**	4	12 ± 3.8	10 to 20	31.5 ± 5
Mn (mg kg^{-1})	0.37	5.9	**2.5**	22	12	114	**3.6 ± 1.8**	6	1.8 ± 0.9	1.5 to 3	1.2 ± 0.1
Se (mg kg^{-1})	0.08	1.55	0.38	7	5	34			0.3 ± 0.05*	0.2 to 0.4*	
Zn (mg kg^{-1})	7.36	52.4	**20.6**	31	12	153	**26.1 ± 13.2**	17	25 ± 1.6	25 to 60	
Vertebrae (Dry weight basis)											
Ash (%)	12.5	66	49.9	43	32	227			52.8 ± 0.6	51.2 ± 3.4	49.7 ± 0.7
Ca (%)	4.7	33	**18.8**	49	25	255	**20.3 ± 2.6**	8	17.6 ± 0.1	21.2 ± 2.2	19.8 ± 0.6
Mg (%)	0.12	0.85	**0.32**	28	22	142	**0.32 ± 0.07**	7	0.3 ± 0.02	0.2 ± 0.1	0.35 ± 0.1
P (%)	2.57	14.1	**9.5**	54	34	284	**8.58 ± 2.23**	10	9.2 ± 0.3	10.1 ± 1.1	7.9 ± 0.02
K (%)	0.01	0.25	0.03	10	10	47			0.2 ± 0.1		0.6 ± 0.02
Na (%)	0.13	1	0.47	8	8	42			0.3 ± 0.02	0.5 ± 0	0.3 ± 0.1
Cu (mg kg^{-1})	0.9	14	3.8	9	9	40			5.2 ± 2.3		8 ± 2.3
Fe (mg kg^{-1})	8	323	59	18	15	95			24.4 ± 6.1	30 ± 3.7	10.6 ± 1.7
Mn (mg kg^{-1})	6.1	135	**15.4**	15	14	65	**18.1 ±10.8**	20	9.4 ± 1.2	11.2 ± 2.9	9.8 ± 0.4
Se (mg kg^{-1})	0.98	4.16	2.58	2	2	10					
Zn (mg kg^{-1})	56.7	650	**151**	30	19	157	**168.9 ± 30**	29	153.2 ± 28.8	168.3 ± 14.9	138.2 ± 13.3
Liver (wet weight basis)											
Ash (%)	4.97	10.6	9.5	3	3	14					
Ca (g kg^{-1})	0.02	0.11	0.04	9	7	44			0.08 ± 0.03	0.1 ± 0.03	0.04 ± 0.01
Mg (g kg^{-1})	0.07	0.44	0.19	9	9	45			0.2 ± 0.03	0.4 ± 0.02	0.3 ± 0.04
P (g kg^{-1})	1.36	5.5	2.32	8	8	39			3.3 ± 0.4	3.8 ± 0.3	
K (g kg^{-1})	1.07	3.12	2.04	4	4	16			3.2 ± 0.5	2.4 ± 0.5	
Na (g kg^{-1})	0.67	0.8	0.75	1	1	4			1.4 ± 0.3		
Cu (mg kg^{-1})	3.5	191	**15**	15	9	75	**9.8 ± 4.9**	10	101 ± 41 (21.5±12)	67 ± 44	11.2 ± 1.6
Fe (mg kg^{-1})	13.2	214	64.2	21	11	105			89 ± 55	96 ± 45	70 ± 10
Mn (mg kg^{-1})	0.58	3.02	1.18	9	7	42			1.5 ± 0.3	0.85 ± 0.2	

	Min	Max	Median	Studies	Species	observations	Plateau values from meta-analysis		Rainbow trout ¶ (mean ± SD)	Atlantic salmon † (mean ± SD)	Common carp ‡ (mean ± SD)
							Mean ± SD	Studies (n)			
Se (mg kg^{-1})	0.55	3.75	1.75	8	6	41			3 ± 0.7*	2 ± 0.6	
Zn (mg kg^{-1})	7.99	101	24.3	17	10	82			24 ± 4	27.6 ± 7	
Plasma											
Ca (mmol L^{-1})	1.61	5.4	2.93	20	14	104			3.6 ± 0.1	3 ± 0.4	2.1 ± 0.1
Mg (mmol L^{-1})	0.15	1.79	**0.9**	13	9	61	**1.08 ± 0.3**	5	0.85 ± 0.04	1 ± 0.2	0.9 ± 0.06
P (mmol L^{-1})	1.4	8.04	**4.1**	17	14	89	**3.31 ± 1.3**	10	4.2 ± 0.6	3.5 ± 1.7	
K (mmol L^{-1})	2.8	4	3.6	2	2	8			3.8 ± 0.2		
Cu (µmol L^{-1})	9.6	29.9	15.1	8	4	34			14.6 ± 3.7	25.6 ± 3.3	
Fe (µmol L^{-1})	1.79	16.1	**8.1**	8	6	39	**13.97 ± 4.5**	4	12.9 ± 1.4	10.9 ± 0.5	
Mn (µmol L^{-1})	0.53	2.37	1.46	3	3	13				1.3 ± 0.1	
Se (µmol L^{-1})	1.45	4.19	2.3	5	2	23			2.3 ± 0.6	3.3 ± 0.7	
Zn (µmol L^{-1})	50	600	270	20	10	96			268 ± 82	319 ± 86	
Scales (Dry weight basis)											
Ash (%)	13	51	33.4	17	12	91				46 ± 2	
Ca (%)	5.98	19.8	13.7	20	14	109				19 ± 0.3	
Mg (%)	0.15	0.46	0.29	11	7	60				0.2 ± 0.03	
P (%)	2.44	11.4	6.5	22	17	121				9.4 ± 0.5	
Na (%)	0.15	0.55	0.34	3	3	12					
Fe (mg kg^{-1})	30.2	87.5	66	2	1	12					
Zn (mg kg^{-1})	34.8	172	123	5	4	27					
Operculum (Dry weight basis)											
Ash (%)	28.19	62.1	47.7	4	4	20					
P (%)	5.05	12.6	8.2	4	4	19					
Ca (%)	15.5	26.8	20.3	2	2	10					
Mg (%)	0.25	0.43	0.41	1	1	6					
Skin (Wet weight basis)											
Ash (%)	1.18	5.1	3.6	4	3	25			3.5 ± 0.7		
P (%)	0.43	1.02	0.72	4	3	22			1 ± 0.6		
Ca (%)	0.62	1.71	1.05	4	3	21			0.7 ± 0.5		
Mg (%)	0.03	0.08	0.05	3	2	16			0.06 ± 0.02		
Fe (mg kg^{-1})	15.6	31	20	1	1	7			13 ± 11		
Zn (mg kg^{-1})	30	57.4	51	2	2	13			23 ± 9		

¶ Rainbow trout: Shearer (1984) except for vertebral mineral composition from Satoh *et al.* (1987d).

† Atlantic salmon: Whole body, Shearer *et al.* (1994) and vertebral, Vielma and Lall (1998a), El-Mowafi *et al.* (1997) and Lorentzen *et al.* (1996).

‡ Common carp: Whole body, Ogino and Kamizono (1975), Dabrowska *et al.* (1991) and Vertebral, Satoh *et al.* (1983); Satoh *et al.* (1987c).

Note: Data on muscle tissue was not included in this study as an extensive database is already available in the literature on the mineral and trace element concentrations in the edible flesh of different fish species (Lall 1995).

ascertain a normal range for mineral concentration in the whole body and different tissues of fish. The minimal, maximal and median values of mineral concentrations reported in the studies included in Tables 1 to 8 and plateau value from the regression analysis were compared with the data from rainbow trout, Atlantic salmon and common carp (Table 11). Although the minimal and maximal mineral concentration showed extreme values in certain tissues, the median and the plateau range were found to be in close agreement with each other and also with data on rainbow trout, Atlantic salmon and common carp for most of the minerals in all the tissues for which there was sufficient data available for a valid comparison. In general, the analysis showed that different fish species do not show drastic intrinsic variations with regard to mineral and trace element concentrations in the whole body and tissues. Thus the values presented in Table 11 can serve as reference for the range of concentrations useful to assess the mineral status for many finfish species.

Factors influencing minimal dietary inclusion level of minerals

A delicate balance is believed to exist between deficiency and surplus with regard to mineral utilisation by fish and excess intake through dietary or aqueous route can cause toxicity (Lall & Milley 2008). The status and distribution of elements in the body of fish may vary with age, sex, health and physiological conditions such as smoltification, gonadal maturation etc. (Lall 2002). Therefore, any change in the physiology and/or environment of the animal may alter the dietary need for the element under question. Dietary inclusion level which might be adequate under a specific physiological or environmental condition may in turn become deficient or excess subject to any change in aforesaid conditions. Thus it is important to understand the various factors that can influence the minimal dietary mineral level required by fish.

i. Species group and digestive physiology

The major groups of finfish species of aquaculture importance and well-studied in the context of mineral nutrition included in this study were classified into four

groups: salmonids (trout and salmon), cyprinids (carps), silurids (catfishes) and all other species classified as others. The species group as a factor of variance did not significantly affect the minimal dietary inclusion level of P. Although not significant, the mean estimate for salmonids was lower when compared to those for cyprinids or other species (Figure 1a). The variation observed was mainly due to the methodological limitation arising from weighing of studies based on the precision of the estimates. In the salmonid dataset, two major studies on rainbow trout (Rodehutscord 1996; Rodehutscord *et al.* 2000) were found to be more precise in terms of the minimal dietary inclusion level estimate obtained, compared to all other studies. However, the estimated value (1.8 ± 0.04 g kg^{-1}) from Rodehutscord *et al*. (2000) was significantly lower in comparison to other studies in rainbow trout itself (3.7 to 6.3 g kg^{-1}), probably due to the observed differences in water P concentrations, as already mentioned in our earlier study (Antony Jesu Prabhu *et al.* 2013). In the case of magnesium, the requirement estimate for cyprinids was significantly higher compared to that for salmonids and others. The differences thus observed could be possibly due to the inherent physiological differences or even influenced by other extrinsic factors. According to Flik *et al.* (1993) and Bucking and Wood (2007), diet is the primary source of Mg accounting for about 60-80% of the total Mg requirement, in freshwater teleost. Moreover, Bucking and Wood (2007) proposed that stomach may play a major role in the absorption of dietary magnesium by facilitating free Mg^{2+} ion formation through acid secretion and further functioning as the major site of Mg^{2+} ion absorption in the gut of rainbow trout. If so, absence of acid secretion in the stomach of Cyprinid species could result in reduced availability of dietary Mg and hence may require higher dietary levels to meet the requirement when compared to Salmonid and Silurid fishes. If this hypothesis holds true, variation in the digestive physiology and gut anatomy between major species groups might play a role in determining the minimal dietary Mg level required by the species. Analysis on dataset split into species with a true stomach (Salmonids, Silurids)' and those which lack a true stomach '(cyprinids)' showed a significant difference in the estimates of minimal dietary level of Mg required. Cyprinid species were found to

have a higher requirement estimate than salmonids and silurids, with weight gain as the response criterion (Figure 1b).

Fig. 1a

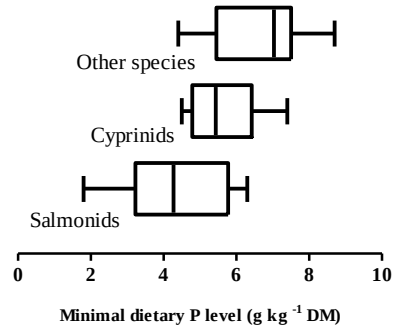

Fig. 1b

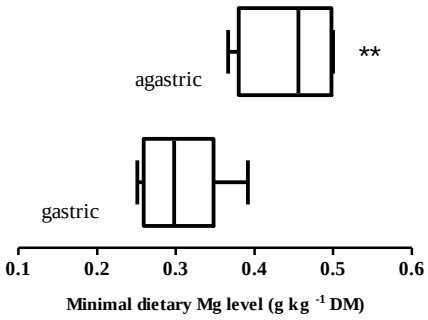

Fig. 1c

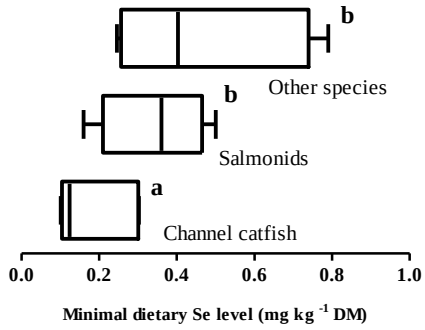

Figure 1: Impact of species group or digestive physiology of the species on the minimal dietary inclusion level of phosphorus, magnesium and selenium. Fig. 1a: Available phosphorus. Fig. 1b: Magnesium, star (**) indicates that the groups were significantly different at $P<0.001$. Fig. 1c: Selenium, different superscripts represent significant difference between groups at $P<0.05$.

Selenium is the other element for which we observed a significant difference between species groups in the minimal dietary inclusion level. Data for channel catfish showed significantly lower estimates than those for all other fish species based on weight gain as the response criterion (Figure 1c). The possibility of this being a species effect cannot be overruled, because such differences between species have been reported for maximum tolerable toxicity limits of dietary Se. Cutthroat trout showed no signs of growth retardation or toxicity when fed up to 12.5 mg Se kg^{-1} DM as seleno-methionine throughout the life cycle (Hardy *et al.* 2010), whereas in other freshwater species, such high levels are toxic especially when fed for long periods (Hilton *et al.* 1980, 1982; Gatlin & Wilson 1984; Hamilton *et al.* 1990; Felton *et al.* 1996). However, a possibility of interaction with other nutrients leading to this difference cannot be excluded and is discussed in the following section on the effect of nutrient interactions.

Analysis of data on zinc based on weight gain and vertebral Zn concentration showed that, there was no significant difference between the gastric and agastric species in the minimal dietary level of zinc. This is in accordance with the findings in rainbow trout (Ogino & Yang 1978) and common carp (Ogino & Yang 1979), where similar estimates waer obtained between the two species; one with stomach and the other stomach less.

ii. Mineral interactions

The existence of many nutrient and non-nutrient interactions are known in fish (Lall & Milley 2008). In studies aimed at determining mineral requirements, a target mineral source is incorporated into a basal feed mixture, made of refined or non-refined ingredients. Depending on the type of ingredients used, the level and magnitude of interaction with the added mineral source can vary. The effect of this factor termed as 'type of experimental diet' was tested for Zn and Se.

Data on response of fish to dietary zinc is the most abundant of all the micro-minerals considered to be essential for fish. NRC (2011) reported data on zinc requirement for 8 species, (7 freshwater; and 1 seawater) to range from 15 to 40 mg kg^{-1} DM of the diet. However, studies on response to dietary zinc are more

numerous (13 freshwater and 12 seawater) with substantial variations (15 – 240 mg kg^{-1} DM) in the reported minimal dietary inclusion levels. Among the different factors analysed, diet type was found to have the most significant impact on the minimal dietary inclusion level of zinc. Meta-analytic estimates from studies using practical diets were about three folds higher than those derived from studies using semi-purified diets, based on both weight gain as well as vertebral Zn as the response criteria (Figure 2a). Practical diets may contain phytic acid or hydroxyapatite, both shown to inhibit gastro-intestinal Zn absorption and hence reduce the availability of dietary Zn to fish (Gatlin & Wilson 1984b; Satoh *et al.* 1987b; Satoh *et al.* 1987d; McClain & Gatlin 1988; Gatlin & Phillips 1989; Satoh *et al.* 1989b; Satoh *et al.* 1992; Porn-Ngam *et al.* 1993; Satoh *et al.* 1993).

With selenium, when weight gain is used as the response criterion, the type of diet did not have a significant effect on the minimal dietary inclusion level, whereas it was significant when hepatic-GPx activity is used as the response criterion (Figure 2b). Even with weight gain, although the estimates derived from studies using practical diets were higher than those from studies using semi-purified diets, it was not significant due to large variations and overlapping confidence intervals. The magnitude of difference between the estimates resulting from studies based on semi-purified diets and from those based on practical diets was much higher with hepatic-GPx activity as the response criterion, than with weight gain (Figure 2b), showing that the hepatic-GPx activity is a more pertinent indicator of the availability and utilisation of dietary selenium in fish. Early studies have shown that fish meal based practical diets lacking additional Se do not affect growth (Poston *et al.* 1976; Hilton *et al.* 1980), whereas the use of semi-purified diets required additional Se supplementation to avoid growth retardation induced by dietary Se inadequacy. Hybrid striped bass juveniles fed soybean meal or casein based diets without Se supplementation showed reduced growth and GPx activity compared to those fed fish meal based diets (Cotter 2006). Soybean meal based diets lacking Se supplementation were sufficient enough to support growth but the need for additional selenium was recommended to maintain optimal GPx activity in rainbow trout fry (Fontagne et al. 2013). Apart from these, knowledge on the effect of plant ingredient based practical diets on the minimal dietary Se

requirement to fish are almost absent. This lacuna needs to be filled, especially with the decreasing incorporation of fish meal and concomitant increase in plant based ingredients to fish diets.

Apart from the above mentioned non-nutrient interaction of basal ingredient composition with minerals, specific nutrients present in the diet can also interact with minerals. We observed that the estimates of Se requirement of channel catfish were lower than that of all other species; this effect was significant with weight gain but not when based on hepatic-GPx activity as the response criterion (refer to Figure 1c). Further analysis of data indicated that interaction with a specific micro-nutrient (vitamin-E) might have had an influence on the minimal dietary inclusion level of selenium. Dietary vitamin E is known to interact with the physiological functions of Se in fish as in higher animals (Poston *et al.* 1976; Bell *et al.* 1985; Jaramillo Jr *et al.* 2009; Lin & Shiau 2009). The vitamin E levels in the diets of the Se requirement studies used in the meta-analysis was found to range from 22-400 mg kg^{-1} DM whereas the NRC (2011) recommendations for different fish species range from 25-115 mg kg^{-1} DM. Depending on the dietary vitamin-E levels, the anti-oxidant status of the fish would be different. Consequently, the vitamin-E level in the diets, hepatic-GPx activity and the estimated minimal dietary inclusion level for Se were found to be inter-related (Figure 2c and 2d). Besides vitamin-E, vitamin-C is also a part of the anti-oxidant defense system in fish along with the activity of metalloenzymes such as Se-GPx, Cu-Zn-SOD, Mn-SOD and Fe-CAT (Martínez-Álvare et al. 2005). These molecules help in scavenging the oxygen free radicals thereby protecting the cells from oxidative damage. The higher the dietary vitamin-E supply, lower will be the free radical formation with a reduced physiological need for maintenance of a high hepatic GPx activity. The activity of hepatic-GPx (expressed as μ moles NADPH min^{-1} mg^{-1} protein) reported in channel catfish was lower (0.05-0.22 units min^{-1} mg^{-1} protein) when compared to the activity reported in other species of fish (0.5-2.3 units min^{-1} mg^{-1} protein). The anti-oxidant status of the animal which in turn depends on the dietary anti-oxidant levels can significantly affect the estimated minimal dietary inclusion level of Se to fish (Küçükbay *et al.* 2009; Lin & Shiau 2009). The mutual sparing effects of Se and vitamin-E can have practical implication in fish feed formulation (Lin &

Shiau 2009). The total selenium concentration and use of selenium additives in animal feeds has been strictly regulated by the European Food Safety Authority, EFSA (2014). With ESFA (2014a) recommending a total selenium content of animal feeds to be not more than 0.5 mg/kg, it is essential to consider the dietary vitamin-E or other anti-oxidant nutrient levels in diets as a counter measure to keep the dietary Se levels within the allowable limits and at the same time maintain the anti-oxidant status of the fish.

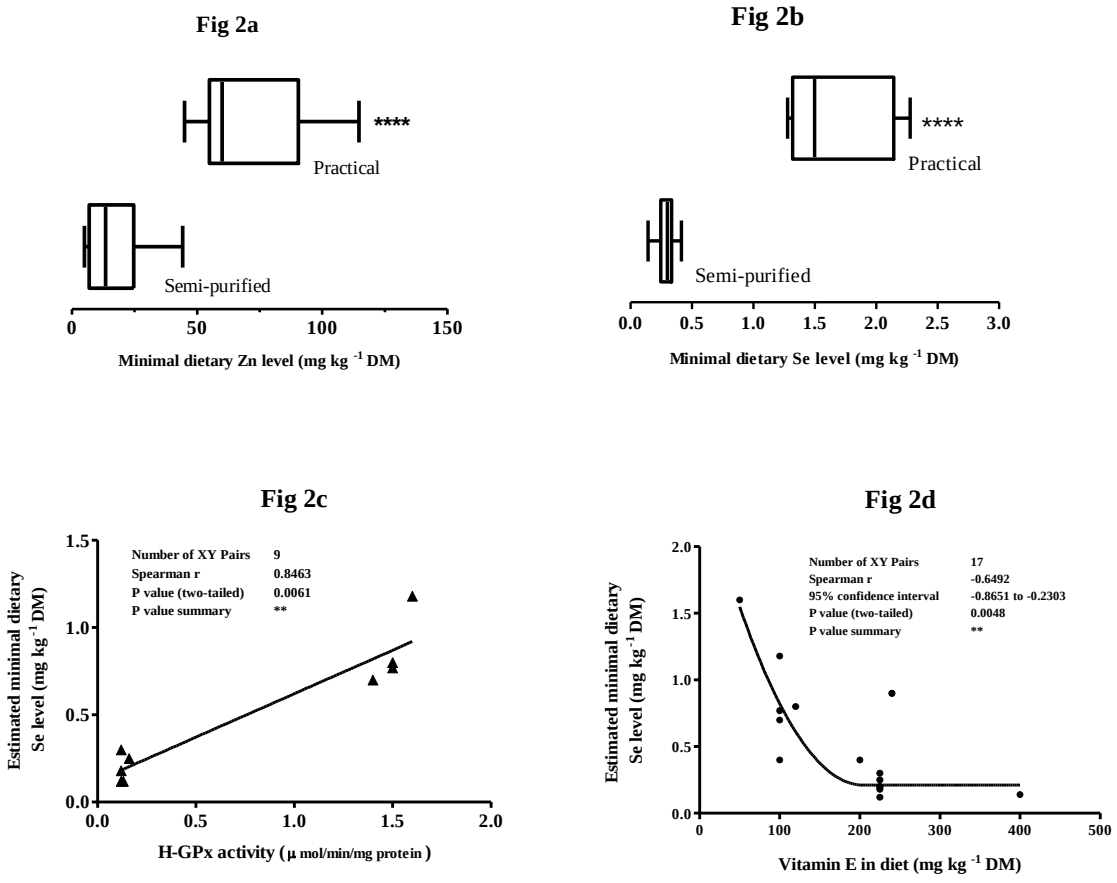

Figure 2: Impact of diet type and other dietary interaction on the minimal dietary inclusion level of zinc and selenium. Fig. 2a (Zn) and 2b (Se): Estimates based on practical diets were significantly higher compared to estimates based on semi-purified diets (P<0.0001). Fig. 2c: Correlation between basal level of hepatic-GPx activity on the minimal dietary inclusion level of selenium (spearman r, 0.85); higher the activity, greater the dietary requirement. Fig. 2d: Correlation between dietary vitamin-E levels on the minimal dietary inclusion level of selenium (spearman r, -0.65); higher the level of vitamin-E, lower the dietary requirement of selenium.

Meta-analysis of available data on other minerals to analyse their interactions was not possible, due to insufficient data. Published reports on the possible interactions with other nutrients are discussed in short. Dietary protein level has been reported to influence the minimal dietary magnesium level required for freshwater tilapia (Dabrowska *et al.* 1989b) and common carp (Dabrowska *et al.* 1991), an increase in dietary protein level required increased dietary Mg inclusion (Dabrowska & Dabrowski 1990). Excess tri-calcium phosphate in egg albumin based semi-purified diets drastically reduced the vertebral Mn concentration though growth was unaffected in common carp juveniles (Satoh *et al.* 1992), suggesting that with practical diets, a higher dietary level of Mn may be required to compensate for the loss due to poor availability and further confirms that vertebral manganese concentration is a more pertinent criterion than weight gain. Reciprocal interactions between Zn and Cu were observed for gastro-intestinal uptake in rainbow trout, examined through in vitro gut-sac technique (Ojo et al. 2009), where high concentration of Zn reduced the absorption of Cu and vice-versa. However, no antagonistic effects of high dietary supply of Zn on Cu or vice-versa was observed in the utilisation of Cu and Zn by rainbow trout from chelated as well as inorganic Cu sources (Read et al. 2014).

iii.Type of dietary mineral source: chelated and inorganic

Availability of the essential micro-minerals such as iron, zinc, manganese, copper and selenium had been studied to a small extent in fish. Iron sulphate was found to be the best inorganic source of iron to Atlantic salmon while metallic iron and iron (III) oxide were completely unavailable (Andersen *et al.* 1997; Maage & Sveier 1998). However, Naser (2000) showed that iron from ferric chloride to be as available as from ferrous sulphate to Atlantic salmon. Among different inorganic sources of zinc, zinc sulphate is the most available to rainbow trout (Satoh *et al.* 1987d) and channel catfish (Paripatananont & Lovell 1995, 1997); zinc gluconate is as efficient as zinc sulphate to Atlantic salmon (Maage *et al.* 2001). Availability of manganese to carp from sulphate and chloride forms was superior to the corresponding oxide and carbonate forms (Satoh *et al.* 1987c). Although literature data on availability of dietary copper to fish is limited, Shao *et al.* (2010) showed

that tribasic copper chloride was a better source of available copper than copper sulphate to carp. Sodium selenite is the widely used and regarded as the best inorganic source of selenium to fish (Bell & Cowey 1989).

There is increasing interest for the use of micro-minerals chelated to ligands such as amino acids or microorganisms such as yeast enriched with specific micro-minerals, although precise quantitative data on their availability to fish is scarce. Selenium is the only element for which there is evidence on the advantage of chelated sources over inorganic forms to fish. Isotope dilution study in fathead minnow showed that after oral administration, the whole body half-life, rate of uptake and magnitude of accumulation to be greater for seleno-methionine (Se-Met) than for the inorganic forms (Kleinow & Brooks 1986). Bell and Cowey (1989) found Se-Met to be the best available form of dietary selenium to rainbow trout. Later studies have demonstrated that chelated sources such as Se-Met or Se-yeast but not Se-Cys to be more available than sodium selenite to fish (Lorentzen et al. 1994; Wang & Lovell 1997; Wang et al. 2007; Jaramillo Jr et al. 2009; Küçükbay et al. 2009; Rider et al. 2009; Rider et al. 2010). At high dietary intakes, non-specific incorporation of Se-Met into skeletal muscle tissue proteins and other organs may eventually reduce excretion. The literature data on selenium in fish is found to be in agreement with this, with the muscle selenium content significantly increasing with increased dietary intake of Se-Met (Wang *et al.* 2007; Küçükbay *et al.* 2009; Rider *et al.* 2009; Zhou *et al.* 2009c; Rider *et al.* 2010) and the additionally retained selenium being effectively utilised under stress conditions caused by handling (Rider et al. 2009) or crowding (Küçükbay et al. 2009). Thus, the improved retention and efficient utilisation better explains the advantage of chelated selenium sources over inorganic forms. Among other sources, results of a recent study show that selenium nano-particle to be a better source than Se-Met to crucian carp in improving the retention of dietary selenium in the skeletal muscle (Zhou *et al.* 2009c).

The availability to fish of chelated sources of zinc, manganese and copper has also been studied to some extent. Compared to the inorganic salts, better availability of chelated forms of zinc (Paripatananont & Lovell 1995, 1997; Apines et al. 2001;

Tan & Mai 2001; Apines et al. 2003a; Apines et al. 2003b; Apines-Amar et al. 2004a; Buentello et al. 2009; Rider et al. 2010), manganese (Paripatananont & Lovell 1997; Satoh et al. 2001; Apines-Amar et al. 2004a; Apines-Amar et al. 2004b) and copper (Paripatananont & Lovell 1997; Lin et al. 2010; Shao et al. 2010) has been reported. On the contrary, few studies indicate that inorganic sources are equally efficient or even better than chelated sources of zinc (Gomes & Kaushik 1992; Li & Robinson 1996; Apines et al. 2001; Maage et al. 2001; Apines et al. 2003b; Do Carmo e Sa et al. 2005; Savolainen & Gatlin 2010; Ma et al. 2014) or of manganese and copper (Apines et al. 2003b). Despite limited data, the chelated or organic sources of iron were found to be more available than ferrous sulphate (Figure 3c). The availability of iron to channel catfish was reported to be better from iron-methionine than ferrous sulphate (Paripatananont & Lovell 1997), whereas Lim et al. (1996) found no difference between the two sources. Another organic form of iron, the heame-bound iron from spray dried blood cells was found to be more efficient than inorganic forms of iron to Atlantic salmon (Andersen *et al.* 1997; Standal *et al.* 1999).

Meta-analysis on data from requirement studies on zinc and selenium were performed to analyse the effect of the type of mineral source used, namely chelated or inorganic sources, on the minimal dietary inclusion levels of zinc or selenium. In the case of selenium, sodium selenite, seleno-methionine and selenium yeast were analysed on the basis of weight gain and hepatic GPx activity as the response criteria. Of the three selenium sources compared, minimal dietary inclusion level estimated from selenium yeast was significantly lower compared to selenite (selenium yeast < seleno-methionine < selenite), but only based on weight gain (Figure 3a). Estimates based on hepatic-GPx activity were statistically similar between the three groups, due to large variations in selenite and yeast groups. Availability of zinc from sulphate and amino acid chelated sources was compared, based on weight gain and vertebral Zn content (Figure 3b). The comparison was confounded by the fact that, out of the six studies included in the dataset on Zn-chelate, four were performed using practical diets (ref. no. 28, 29b, 37b and 38d in Table 4), thereby resulting in higher estimates as discussed in the earlier section.

Fig 3a

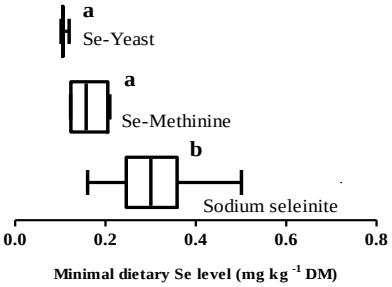

Fig 3b

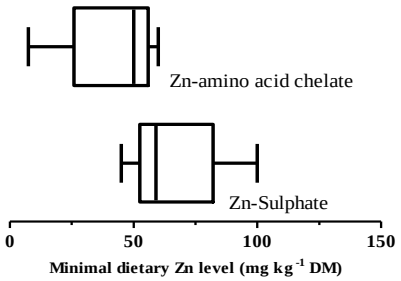

Fig. 3c

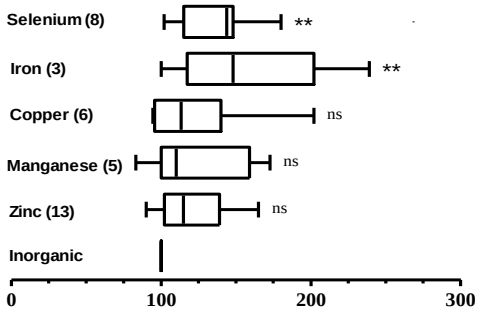

Figure 3: Impact of type of mineral source on the minimal dietary inclusion level of micro-minerals. Fig. 3a: Selenium, estimates based on sodium selenite, seleno-methionine and selenium yeast; different superscripts represent significant difference between groups at $P<0.05$. Fig. 3b: Zinc, estimates based on zinc sulphate and zinc amino acid chelates. Fig. 3c: Relative efficiency of chelated organic sources of micro-minerals such as Zn, Mn, Cu, Fe and Se in comparison with the respective inorganic forms (sulphate for all, except for Se); star (**) indicates that the groups were significantly different at $P<0.001$; ns – not significant.

As it was not possible to compare the requirement estimates from inorganic and chelated forms of minerals other than for Se and Zn, and because analysis of data on Zn was inconclusive, a secondary analysis on the relative efficiency of amino acid chelates of Zn, Se, Mn, Cu and Fe was performed. Whole body or tissue mineral retention was used as the criteria and in addition blood haemoglobin levels were also used for Fe.. The response to chelated or organic source was calculated as a relative percentage to the response of the corresponding inorganic form taken as 100%. The commonly used inorganic forms (sulphates) were compared against their respective Zn, Mn, Cu and Fe chelates, whereas for Se, selenite was used as the reference inorganic form. The results (Figure 3c) showed that, the chelated sources were significantly ($p<0.05$) more efficient than their corresponding inorganic forms, by 47% for selenium and by 57% for iron. The relative efficiency of chelated zinc (by 21%), manganese (by 23%) and copper (by 27%) were also higher, but not statistically significant compared to their corresponding inorganic forms. It should be noted that these results correspond to the relative efficiency of organic chelated sources over inorganic forms and not apparent availability or absorption coefficients. Any differences in the metabolism of the two forms of minerals resulting in preferential retention of one over the other is likely to result in higher efficiency, as observed for all the chelated organic sources analysed.

Although a large number of studies and the results of the meta-analysis (Figure 3) show that chelated mineral sources are more efficient over the respective inorganic forms, there are large variations, considerable inconsistencies and even lack of statistical significance in the case of certain minerals (Zn, Mn and Cu). The variations observed can be attributed to the quality of chelated sources influenced by (i) proportion of total mineral concentration that is chelated to the ligand and (ii) presence of any antagonistic impurities which in turn are linked to the production method and properties of the ligand used. The proportion of different chemical forms of the element also influences the bioavailability of the chelated source (Rayman 2004). Caution is warranted since the quality of the commercial products can vary depending on the manufacturer and at times, the specifications may be ambiguous and misleading (Rosen 2010). Moreover, EFSA (2013; 2014a, b

& c) regulates the supplementation of mineral sources such as for Se (total, 0.5 mg/kg) and Se-Met as an additive (max. 0.2 mg/kg); Zn (total, max. 200 mg/kg); Cu (total, max. 25 mg/kg) and Mn (total, max. 100 mg/kg). Based on the regulatory limits and the requirement of fish for the aforementioned minerals, the major advantage of reducing dietary inclusion levels by improving availability as stated in many studies may not of practical interest, except for Se. Therefore, understanding the metabolic pathways would help in exploring the physiological benefits of chelated mineral sources as opposed to inorganic forms to fish, for example incorporation of metaloproteins.

iv. Mineral concentration of rearing water

Fish are capable of acquiring dissolved minerals from water. The ability of fish to uptake minerals from the aquatic environment has been studied to some extent with emphasis on physiological and molecular mechanisms underlying ion transport, salt and water balance, metal toxicity and bio-accumulation. Branchial and cutaneous uptakes in freshwater environment and active oral uptake through drinking in seawater conditions are the major uptake routes for minerals in fish. Osmotic differences of freshwater (0-20 mOsm/L) and seawater (1050 mOsm/L) when compared to the tissue fluids (≈300 mOsm/L) of the fish drives these ion uptake processes (Kaushik 2002; Grosell, 2010; Wood and Bucking, 2010). An inter-related regulation between metal uptake at gills and uptake in the gastrointestinal tract is known to exist (Bury *et al.* 2003). Knowledge on water-borne uptake affecting dietary needs of the fish is known for calcium (Robinson *et al.* 1986; Robinson *et al.* 1987), magnesium (Shearer & Åsgård 1992) and potassium (Wilson & Naggar 1992).

Calcium uptake studies (Lovelace & Podoliak 1952; Perry & Wood 1985) estimated that, almost 50-80% of the body calcium is obtained through absorption from water. Moreover, calcium requirement studies in few freshwater teleosts under calcium-free water conditions (Robinson *et al.* 1984; 1986; 1987) show that dietary essentiality of this mineral is felt only under severe dearth of calcium in the rearing water. Consequent to the above findings, it is far more complicated to

assess the minimal dietary inclusion level of certain minerals, to which fish have unlimited access through the rearing water. As described earlier, NRC (2011) recommends that calcium supplementation may not be required under practical conditions, as calcium from water is available to the fish and a dietary supplementation of calcium is needed only when reared under Ca-free environmental conditions. However, meta-analysis of the limited literature data (Table 10), and a thorough review of the literature indicates that this needs to be re-considered. The belief that aqueous calcium supply was sufficient to meet the calcium requirement of fish was in most studies based on weight gain as the response criterion (Table 1). This may not hold true if the mineral or calcium status of the fish were to be used as the criterion. The estimated minimal dietary Ca level based on weight gain was lower by 45% in fish reared in water containing Ca when compared to fish reared in Ca free water; whereas with vertebral ash or Ca as the response criterion, it was only lower by 18% between the two groups (Table 10). Literature data from channel catfish (Andrews *et al.* 1973), tilapia (O'connell & Gatlin 1994), rainbow trout (Vielma *et al.* 1998), Atlantic salmon (Vielma & Lall 1998b) and grass carp (Liang *et al.* 2012b) indicate the need for dietary calcium at levels of 9-15 g Ca kg^{-1} DM, even when reared in water containing appreciable levels of dissolved calcium (35-60 mg Ca L^{-1}). Likewise, Hossain and Furuichi (1999a, 2000a, c, d, b) recommend the need for a minimal dietary supplementation of calcium (1-2 g kg^{-1} DM) to casein based semi-purified diets (0.2 g Ca kg^{-1}) to several species of seawater finfish and Ye *et al.* (2006) reported the need for an optimal supplementation of 6 g Ca kg^{-1} DM to casein based basal diets (3.3 g Ca kg^{-1}) for juvenile grouper. In these studies, although growth was not affected, the whole body ash or calcium levels were reduced when fed diets without calcium supplementation. The normal Ca:P ratio in rainbow trout is 0.9-1 (Rodehutscord 1996) whereas when fed with diets low in available calcium it can be reduced up to 0.5 to 0.6 even at non-limiting water calcium levels (Vielma *et al.* 1999; Antony Jesu Prabhu *et al.* 2014). Based on the published data available (Hossain & Yoshimatsu 2014) and the results of the meta-analysis, dietary supply of available Ca to fish needs to be taken into account irrespective of

the Ca levels in the rearing water, especially with the changing diet composition (Vielma & Lall 1998b).

Response to dietary magnesium has been investigated in about 16 finfish species (8 freshwater, 4 marine and 4 euryhaline). The ability of fish to utilise water borne Mg and its effect on the dietary supply has been studied in freshwater fish. It was demonstrated that rainbow trout (Shearer & Åsgård 1992) and tilapia (Van Der Velden *et al.* 1991) were able to utilise water borne magnesium to fulfil the requirement for growth when fed low-Mg diets for periods up to 4 weeks. Mg-uptake from water is dependent on the Mg-concentration of water (Van Der Velden *et al.* 1991) and may not be sufficient to meet the need of a fast growing fish on a long term (Dabrowska *et al.* 1991). Moreover, a recent study in gibel carp (Han *et al.* 2012) conducted in water containing 10-12 mg Mg L^{-1} reports a lack of response in growth to increasing levels of dietary magnesium. These show that freshwater fish are capable of utilising water borne magnesium. As discussed above, if water Mg concentration has an impact in freshwater finfish species, salinity of the rearing environment can be expected to impart a similar phenomenon in marine finfish species as well. As stated earlier, marine fish species drink water continuously to maintain osmotic balance of body fluids. Given the natural abundance of magnesium in seawater (3.7%, second most abundant cation only next to sodium) and the basic differences in osmoregulatory mechanisms between marine and freshwater fishes, available data were analysed to look for differences, if any between the two groups. Although the Mg requirement of eight finfish species reared in seawater have been studied, clear dose response was observed only in Atlantic salmon (El-Mowafi & Maage 1998), grouper (Ye *et al.* 2010) and recently in hybrid tilapia, a euryhaline species studied under both freshwater and seawater condition (Lin *et al.* 2013). Even with the meta-analysis, the results showed that it was not possible to determine a break point for the seawater species, using weight gain as the response criterion. This would imply that, the minimal inclusion levels of magnesium in the diet for fish reared in seawater conditions would be low, at least for ensuring normal growth. The lack of sufficient data on the marine finfish especially on whole body Mg as

response criterion makes it difficult to arrive at a definitive conclusion from the meta-analysis. However, Lin *et al.* (2013) clearly showed the effect of salinity on the minimal dietary Mg levels required in an euryhaline species. Hybrid tilapia reared in freshwater required 0.2 g Mg kg^{-1} DM, while those in seawater (32 ppt) required not more than 0.02 g Mg kg^{-1} DM based on whole body Mg balance. Thus the impact of seawater on minimal dietary magnesium levels seems evident. However, more studies in marine species on the response to dietary magnesium is required to further confirm, if the fish are able to maintain growth as well as whole body Mg balance irrespective of the dietary Mg supply.

In the case of phosphorus, there is evidence for a branchial uptake from water through radioactive tracer studies (Al-Kholy *et al.* 1970; Winpenny *et al.* 1998). However, unlike Ca and Mg no direct and conclusive evidence is available to support a possible interaction with dietary supply. Certain studies have speculated the possibility of P absorption from water when the dietary available P supply was low (Pimentel Rodrigues & Oliva Teles 2001; Dias *et al.* 2005). A more direct evidence for uptake of phosphorus from water under dietary P limitation was demonstrated in Nile tilapia (Janssen 2010).

Among the micro-minerals, uptake from water has been well studied for copper and zinc mainly in freshwater fishes. At normal dietary concentrations, the relative contribution of water-borne copper to whole body copper content accounts for about 10% and increases up to 60% at low dietary levels (Kamunde *et al.* 2002); fairly similar pattern of regulation was observed for zinc in rainbow trout (Spry *et al.* 1988). Iron uptake through gills has been studied in both freshwater and seawater fish suggesting water-borne iron can be utilised by fish (Bury & Grosell 2003). The uptake of iodine from water is documented in marine (Moren *et al.* 2008) as well as freshwater fish (Hunn & Fromm 1966). In the meta-analysis, seawater as a factor of variance tested for zinc was not found to have any significant effect due to large variations in the salt water group. However, the analysis indicated that the minimal dietary inclusion level of zinc could be affected by seawater and in general, seawater seemed to increase the minimal dietary inclusion level of zinc. Hypothetically this could be the case for all trace-metal

cations, as the continuous drinking of saline water could reduce the dietary availability by forming ion complexes with chloride, the major anion in sea water. Testing this hypothesis by comparative studies with euryhaline fish species as studied for Mg in hybrid tilapia by Lin et al. (2013), would be informative. From the results of the meta-analysis and the information available in the published reports discussed in this section, the effect of water mineral concentrations and flow through or recirculating aquaculture system on minimal dietary inclusion level of minerals to fish cannot be ignored and needs further investigation.

Research needs for the future

i. Knowledge gap in mineral requirements and their functional importance

Despite the significant progress made in mineral nutrition of fish, our current knowledge on the mineral requirements and their versatile functions is limited, more so for marine fish species. The macro-minerals of importance such as P, Ca and Mg have been well studied as structurally important components, but information on endogenous losses, requirements for basal metabolic or maintenance needs, specific requirements during different developmental stages, physiological conditions or changing diet composition are lacking. Information on micro-minerals is rather scarce as compared to macro-minerals; among them, micro-minerals such as Fe, Cu and Mn are relatively less studied compared to Zn or Se. It was quite astonishing to note that quantitative requirement for Fe is not established even for the most widely studied fish species, the rainbow trout (NRC, 2011). Other than the five well recognised essential micro-minerals, the role of other elements such as boron (B), cobalt (Co), and chromium (Cr) have also been studied in fish, but knowledge on their dietary essentiality and requirement are lacking for the most part (Lall & Milley 2008). Boron (B) is essential for normal embryonic development of zebra fish and rainbow trout (Rowe *et al.* 1998; Rowe & Eckhert 1999) and is known to be involved in bone metabolism and immune functions in higher vertebrates (Armstrong *et al.* 2000; Armstrong & Spears 2001; Fry *et al.* 2007). Cobalt supplementation to practical diets improved growth in Asian sea bass and Asian catfish (Sapkale and Singh, 2011). The dietary essentiality

of chromium is not clear; however the role of dietary chromium as a modulator of carbohydrate metabolism in a warm water fish species, hybrid tilapia (O. missanbicus x O. niloticus) has been proposed (Shiau & Chen 1993; Shiau & Liang 1995; Shiau 1997; Shiau & Shy 1998). It is necessary to assess the quantitative requirements for these micro-minerals considering their roles in developmental and physiological functions.

Apart from dietary essentiality and quantitative requirements, knowledge on the dietary supply of the aforementioned minerals affecting health and immune response is also limited (reviewed by Kiron, 2012). The use of cellular and molecular markers would help in understanding the mechanisms behind the overt phenotypic changes observed in body composition or enzyme activities. Existence of common regulatory pathways between (i) mineral metabolism and immune modulatory mechanisms for example, properties of hepcidin and other associated molecules such as ferroportin and transferrin receptor in regulating iron metabolism as well as anti-microbial responses has been known in higher vertebrates (Andrews and Schmidt, 2007) and even in fish species (Hsieh et al. 2010; Yang et al. 2013) (ii) mineral metabolism and hepatic intermediary metabolism, for example link between cholesterol or bile metabolism and micro-minerals such as Fe, Cu, Zn and Mn, as hepato-biliary system plays a vital role in regulating their metabolism (Hambidge, 2003).

ii. Fish meal replacement - mineral nutrition perspective

The reduction of fishmeal inclusion levels in fish feeds might change the supply of minerals to fish. Theoretically, dietary levels of the essential minerals in plant derived ingredients used in fish feeds are higher compared to fishmeal, except for phosphorus, zinc and selenium. Nevertheless, the availability of these minerals to fish is the major point of concern due to the presence of various anti-nutritional factors. Improving the availability of intrinsic minerals in feed ingredients to fish should be given due consideration to reduce the dependence on exogenously supplemented mineral sources to fulfill the mineral requirements of fish. In recent years, dietary supplementation of mineral availability enhancers has been the focus of research in terms of improving the availability of phosphorus and other

minerals to fish (Cheng & Hardy 2002; 2003; 2004; Baruah *et al.* 2005; Debnath *et al.* 2005). Apart from phytic acid, other plant derived anti-nutritional factors (ANFs) such as gossypol, oxalates, glucosinolates, saponin, lectin, tanin and even non-starch polysaccharides can affect mineral absorption and utilisation in fish (Francis et al. 2001). Entero-hepato-pancreatic system is the main route of action for the ANFs listed above. Phytic acid directly interferes with the absorption of minerals while others can indirectly disrupt mineral metabolism, as uptake and homeostatic balance of Fe, Cu, Mn and Zn are regulated at the level of gastrointestinal tract or liver (Hambidge, 2003). Therefore, the possible effect of the above listed anti-nutritional factors from alternate plant ingredient sources on the underlying cellular and molecular mechanisms in mineral metabolism requires better understanding.

As mentioned earlier, some trace minerals are present in higher concentrations in plant derived ingredients when compared to fish meal. Tacon & De Silva (1983) documented exceedingly high concentrations above recommended levels and 2 to 11 fold variations for trace minerals such as Fe (80-540 mg/kg), Cu (5-40 mg/kg), Mn (35-100 mg/kg) and Zn (50-260 mg/kg) within similar feed categories of commercial salmonid feeds that were available in Europe. Three decades down the line, the situation has not changed much, according to the recent report based on a survey on different Norwegian fish feeds over a decade from 2000-10' (Sissener et al. 2012). The reported minimal and maximal dietary levels are as follows, Fe (65-493 mg/kg); Cu (2.5-21 mg/kg); Mn (4.4-226 mg/kg); Zn (36-330 mg/kg) and Se (0.39 to 4.1 mg/kg). With trace minerals, a bell shaped or quadratic pattern is expected in the associated responses as higher dietary concentrations can be toxic (Mertz, 1981). As the source and origin of ingredients used in fish feed formulation is bound to change dynamically with time, it is of utmost importance to proportionately balance the inclusion of various trace minerals in a premix, considering the trace mineral supply from the feed ingredients to be used. Availability of essential minerals from different fish feed ingredients have been studied in salmonids (Storebakken et al. 1998; Sugiura et al. 1998b; Sugiura et al. 1999; Sugiura et al. 2000a; Cheng & Hardy 2002; Cheng & Hardy 2004), but such

knowledge on other species of farmed fish is limited. Therefore it becomes necessary not only to ensure the minimal dietary levels meet the requirement but also make sure that the levels do not exceed the maximal limit which can affect the growth and other responses in fish.

iii. Re-circulating aquaculture systems (RASs): possible impact of accumulated minerals

Over the years, aquaculture systems have evolved continuously to cope up with the ever growing need for a more efficient and sustainable culture practice. The most recently evolved and steadily expanding systems are the re-circulating aquaculture systems (RAS). In RAS, the particulate faecal waste and unfed feed pellets are mechanically removed periodically, whereas the dissolved metabolic wastes and minerals leached from feed pellets and faecal matter tend to accumulate in the water. The magnitude of accumulation is based on the water exchange rate of the RAS. Generally, water exchange rate and magnitude of mineral accumulation are inversely related, lower the water exchange higher the accumulation (Martins et al. 2009). Following the report of Martins et al. (2009), studies that have reported data on minerals accumulating in RAS water have increased (Davidson et al. 2009; 2011; 2014 van Bussel et al. 2014). The minerals reported to accumulate are P, Mg, K, Na, Fe, Cu, Zn, Mn, Co, Cd and Ni. The most profound accumulation has been reported for phosphorus and reports show that dissolved ortho-phosphate concentrations at 25 mg/l in re-circulating aquaculture systems have beneficial effect on P-gain growth of a freshwater fish, Nile tilapia (Janssen 2010) and a seawater fish, turbot (van Bussel et al., 2013), respectively. With regard to micro-minerals, a positive correlation was observed for the body concentration and water micro-mineral concentrations, of fish reared in RAS with high mineral accumulated water (van Bussel et al. 2014). In this scenario, studying the interaction of these aqueous mineral levels on the dietary mineral utilisation and requirement will be useful in better managing the dietary mineral levels as well as nutrient discharge from the RAS.

iv. Other approaches

Given the growing concerns on the global availability and sustainable use especially of phosphorus in food production systems, the following approaches may also play a role in the years to come, (i) *Gut micro-flora:* although fish are believed to lack endogenous synthesis of phytase, some studies have reported the possible existence and activity of a phytate digesting enzyme in fish (LaVorgna 1998; Ellestad *et al.* 2002). Other studies dealing with the characterisation of gut micro-flora in fish have brought to light the existence of phytase producing bacterial strains in the digestive tract of some teleost fishes (Ray *et al.* 2012). (ii) *Genetic selection* as a tool can be used to improve mineral and trace element absorption and utilisation in fish through direct as well as indirect approach. Selective breeding of fish for genetically superior traits with regard to mineral absorption and utilisation can be considered a direct approach, while the use of genetically selected low phytate varieties of plant ingredient sources in the diets to be an indirect approach. Although the former has not gained much attention, report exists on the genetic variation in apparent mineral absorption in Atlantic salmon (Thodesen *et al.* 2001). With regard to the latter, the use of low phytate varieties of barley and corn lead to improved absorption and reduced faecal excretion of phosphorus but not for other minerals (Sugiura *et al.* 1999; Overturf *et al.* 2003). (iii) *Genetic engineering (GE):* genetically engineered pig "Enviropig" capable of producing phytase in the salivary glands and secrete active enzyme in the saliva was developed (Golovan *et al.* 2001), enabling a better utilisation of phosphorus form cereal grains and soybean meal thereby reducing faecal P loss up to 60% less than non-transgenic pigs fed the same diet lacking supplemental phosphate (Forsberg *et al.* 2003), but failed to pass through USFDA regulations. The possibility of a genetically improved fish for better utilisation of phytate rich plant ingredients should be seriously taken into account. It is also not clear whether the fast growing genetically engineered Atlantic salmon "AquAdvantage Salmon" (USFDA 2010) is efficient in terms of mineral utilisation and if the fast growth rate alters the dietary mineral needs when compared to non-transgenic fish.

Conclusion

This meta-analysis of literature data on mineral and trace element requirements of fish demonstrates that large amount of information can be generated from the published data by systematic approach and the use of appropriate analytical tools. Information such as (i) impact of response criteria on minimal dietary inclusion levels, (ii) comparison of the meta-analytic estimates with that of model predicted estimates and NRC values available in literature, (iii) compilation of a large array of data on mineral and trace element concentrations of whole fish and different tissues (iv) analysis of published data for factors that might affect the minimal dietary inclusion level estimates and (v) prospective discussion on ideas to propel research in mineral and trace element nutrition of fish have been provided in this review. A serious lack of sufficient data on micro-minerals such as Cu, Fe and Mn has to be noted and more emphasis needs to be laid in such areas. Regulation of nutrient requirement studies by a set of guidelines and re-validation of the estimates with time needs to be practiced in order to improve the quality of the data generated on nutrient requirements of fish, adapting to the changing dietary and rearing conditions. Furthermore, the possible mechanisms by which dietary mineral levels or sources interact with hepatic intermediary metabolism, affecting the utilisation of macro-nutrients needs better understanding.

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Appendices

Appendix 1: Meta-analytic elements - effect size, variance and weighted effect size

After having carefully selected the studies to be included in the meta-analysis the next step involves the computation of the effect size. Effect size is the central player of a conventional meta-analysis which quantifies the effect of a treatment group against a control group, in most cases. Effect size based on means such as hedges's g, cohen's d, glass's Δ or root-mean-square standardised effect (Ψ) are commonly used. According to McGaw and Glass (1980), it is difficult to use one of these effect sizes under certain conditions wherein a control group does not exist or effect size has to be a measure of multiple group differences or when factorial arrangements are used in the experimental design. These are few specific issues to have been confronting the use of conventional meta-analysis in animal nutrition studies especially to investigate the nutrient requirements of farm animals. As per Borenstein *et al.* (2009) any outcome can be used as an effect size, provided (i) if it is a meaningful estimate of the parameter under study (ii) Outcomes from different studies are comparable (iii) the variance and confidence intervals can be computed. Based on these characteristics, the most meaningful outcome that would be possible from a nutrient requirement study is the estimate of the requirement itself i.e. the breakpoint value (X_{bp}). This is comparable across studies and the variance along with the confidence intervals can be computed if the variance (SE^2) of X_{bp} is available from the model. Once the effect size (X_{bp}) and its corresponding variance (SE^2) are computed for each individual study, then it is essential to weigh each study to compute the weighted effect size ($\overline{X}_{bp}$). Weighing the studies included in a meta-analysis can be based on size of the study or precision of the study (Borenstein *et al.* 2011). In animal nutrition studies, size of the study pertains to aspects such as, number of replicates used, number of animals used per replicate, duration of the trial or number of nutrient levels used to determine the requirement. The larger the size, more weightage would be given. In terms of precision, magnitude of weight gain during the experimental period or variance of the response criteria or variance of the estimated requirement value can be considered. Smaller the variance, large is the weightage for that particular study. In this review, as large variations were not observed in the size of the studies, the precision parameter namely the variance of the breakpoint (SE^2) was used to weigh the individual studies. This is computed as the inverse of variance ($w=1/SE^2$). Now, the

product of the study effect size (X_{bp}) and the corresponding weight of the study (w) will give the weighted effect size ($\bar{X}_{bp}$).

Appendix 2: Testing heterogeneity of variance, fixed or random effects model selection

Having computed the weighted effect size for each study, the next step is to determine the heterogeneity of variance between the studies that are to be used in the meta-analysis. The test for heterogeneity can be done through Q statistics or chi-square statistics. The formula for which is,

$$Q = \sum(w * (Xbp)^2) - \frac{[\sum(w * Xbp)]^2}{\sum w} \quad \text{---------- eq. 1}$$

If the calculated Q value is lower than the table value for the corresponding degrees of freedom (df), the heterogeneity between studies is low and a fixed effect approach can be used. Whereas, if the heterogeneity is high (calculated Q > table value) a random effect approach must be taken. Furthermore, computation of I^2statistics provides an approach to quantify heterogeneity in dataset. The formula used to calculate I^2 statistics is as follows,

$$I^2 = \frac{(Q-df)}{Q} * 100 \quad \text{---------- eq. 2}$$

This presents the true heterogeneity in a dataset as a percentage of the total variance observed in the dataset. The maximal threshold I^2 value for lack of heterogeneity is considered to be 40% (Deeks *et al.* 2008). Studies in a dataset are said to heterogeneous if I^2 > 40% and above it the degree of heterogeneity increases with the percentage. Therefore, the individual study variance (SE^2) needs to be corrected by a common factor (v) which accounts for the inter-study variation observed in the dataset. This factor is computed using the formula,

$$v = \frac{Q-(n-1)}{\sum w - \frac{\sum w^2}{\sum w}} \quad \text{---------- eq. 3}$$

Once this constant factor is computed, the new weight for each study to be used in the random effect model needs to be calculated by the following formula,

$$w_v = \frac{1}{(SE^2+v)} \quad \text{---------- eq. 4}$$

This new individual study weight (w_v) was used to calculate the new mean effect size based on the random effect model.

The final estimates of weighted mean effect size was calculated as,

Estimate based on fixed effect model: $mean\ \overline{X}bp = \frac{\sum (w * X_{bp})}{\sum w}$ ---------- eq. 5

Estimate based on random effect model: $mean\ \overline{X}bp = \frac{\sum (w_v * X_{bp})}{\sum w_v}$ ---------- eq. 6

The corresponding standard errors were calculated as $\sqrt{(1/w)}$ and $\sqrt{(1/w_v)}$, respectively.

Appendix 3: Meta-analysis, a step by step procedure

A step by step approach in performing the meta-analysis is presented below taking the example of phosphorus with weight gain as the response criterion. The results are tabulated in Table 10 along with a graphic presentation of the results in the form of a forest plot (Figure 1A). The estimation of breakpoint values (X_{bp}) along with the corresponding standard error (SE) were obtained for 20 studies using the linear plateau model of regression analysis. The breakpoint value (X_{bp}) is taken to be the effect size or outcome. The variance of the effect size was calculated as the square of the standard error (SE^2). After computing the variance, individual study weights (w) were computed as the inverse of variance ($1/SE^2$). This individual study weight is assigned to the corresponding study for computing the weighted effect size ($\bar{X}_{bp} = w * X_{bp}$) as the product of effect size (X_{bp}) and individual study weight (w). The product of individual study weight and square of effect size ($w * X_{bp}{}^2$) was also obtained for testing the heterogeneity of variance among the studies in a Q statistic or chi square statistic.

The sum of each of these variables for all the 20 studies provided the following values in the case of phosphorus (Table 1A),

Sum of individual study weights, $\sum w = 704.99$

Sum of weighted effect size, $\sum (w * X_{bp}) = 1483.37$

Sum of the product of w and $X_{bp}{}^2$, $\sum (w * X_{bp}{}^2) = 3711.7$

Using the above three values, the heterogeneity of variance was tested with chi-squared statistic using equation (1) presented in the methodology. The chi-squared statistic for

breakpoint estimates based on weight gain data of phosphorus was 590.56. This value is higher than the chi-squared table value for 19 degrees of freedom even at a probability level of $p<0.0005$ (45.97). Using this observed value for chi-squared statistic, I^2 value calculated with 19 degrees of freedom (n-1) following equation (2) presented in the methodology, resulted in a value of 96.8, which was also used for testing the heterogeneity of variance. This is higher than the threshold of 40% to indicate heterogeneity in a dataset as described by Deeks *et al.* (2008). Both Chi-squared and I^2 statistic showed that, the variance observed between studies is heterogeneous and therefore a fixed effect model is not ideal.

As described in the methodology, the constant factor (v) to correct for the inter-study variations was calculated using the equation (3) provided in the methodology (v = 3.837). This factor was used to calculate a new set of individual study weights (w_v) calculated based on the equation (4) provided in the methodology in order to use the random effect model. Thereafter, the variables such as $\sum w_v$, $\sum (w_v * X_{bp})$ and $\sum (w_v * X_{bp}{}^2)$ were calculated to be 3.83, 20.82 and 118.85. These values were again tested for heterogeneity using chi-squared and I^2 statistic. The calculated values thus obtained for chi-squared test with the random effect model was 10.9, which is lower than the chi-square table values for 19 degrees of freedom even at a probability level of $p>0.25$ and the corresponding I^2 value was -73%, indicating that the inter-study variation has been accounted in the model . The overall mean effect size (mean $\bar{X}_{bp}$) for all the 20 studies, calculated using the equations (5) and (6) resulted in estimates of 2.1±0.4 (calculated as 1483.9/704.99 from eq. 5) in case of fixed effect model and 5.3±0.5 (calculated as 20.82/3.83 from eq. 6) with random effect model from the 20 studies. In this study, the chi-squared and I^2 values of all the datasets (Table 2A) were more than the table values for chi-square or 40% in case of I^2, therefore random effects model was used all the datasets analysed.

Table A1: Step-by-step procedure on computing different parameters involved in the meta-analysis: Example of requirement data on available phosphorus based on weight gain as response criteria.

Study	Xbp (es)	SE	Var ($1/SE^2$)	Fixed effect model					Random effect model				
				w	w*es	$w*(es^2)$	w^2	w,%	w_v	w_v*es	$w_v*(es^2)$	w_v^2	w_v,%
1. Rainbow trout, Rodehutscord (1996)	3.7	0.18	0.032	30.86	114.198	422.531	952.59	4.378	0.258	0.956	3.538	0.067	6.75
2. Rainbow trout, Rodehutscord *et al.* (2000)	1.8	0.04	0.002	625.0	1125.0	2025.0	390625.0	88.65	0.261	0.469	0.844	0.068	6.81
3. Rainbow trout, Ketola & Richmond (1994)	4.5	0.5	0.250	4.000	18.000	81.000	16.000	0.567	0.245	1.101	4.955	0.060	6.39
4. Rainbow trout, Ketola & Richmond (1994)	8.8	6.8	46.240	0.022	0.190	1.675	0.000	0.003	0.020	0.176	1.546	0.000	0.52
5. Rainbow trout, Sugiura *et al.* (2007)	6.3	1.6	2.560	0.391	2.461	15.504	0.153	0.055	0.156	0.985	6.205	0.024	4.09
6. Atlantic salmon, Ketola (1975)	5.6	0.3	0.090	11.11	62.222	348.444	123.457	1.576	0.255	1.426	7.986	0.065	6.65
7. Chum salmon, Watanabe *et al.* (1980)	4.036	0.2787	0.078	12.87	51.961	209.714	165.749	1.826	0.255	1.031	4.161	0.065	6.68
8. Common carp, Xie *et al.* (2011)	5.456	1.138	1.295	0.772	4.213	22.986	0.596	0.110	0.195	1.063	5.801	0.038	5.09
9. Jian carp, Kim *et al.* (1998)	4.496	0.3218	0.104	9.657	43.416	195.200	93.252	1.370	0.254	1.141	5.130	0.064	6.63
10. Grass carp, Liang et al. (2011)	8.4	1.364	1.860	0.537	4.515	37.925	0.289	0.076	0.176	1.474	12.385	0.031	4.59
11. Catla, Sukumaran *et al.* (2009)	5.433	0.9871	0.974	1.026	5.576	30.294	1.053	0.146	0.208	1.129	6.135	0.043	5.43
12. Black sea bream, Shao *et al.* (2008)	5.457	0.8749	0.765	1.306	7.129	38.904	1.707	0.185	0.217	1.186	6.471	0.047	5.68
13. European white fish, Vielma et al. (2002)	7.398	0.9571	0.916	1.092	8.076	59.747	1.192	0.155	0.210	1.557	11.515	0.044	5.5
14. Japanese seabass, Zhang *et al.* (2006)	7	0.9658	0.933	1.072	7.505	52.532	1.149	0.152	0.210	1.468	10.274	0.044	5.48
15. Yellow croaker, Mai *et al.* (2006)	7.03	2.132	4.545	0.220	1.547	10.873	0.048	0.031	0.119	0.839	5.896	0.014	3.12
16. Mirror carp, Schafer *et al.* (1995)	5.085	1.304	1.700	0.588	2.990	15.206	0.346	0.083	0.181	0.918	4.670	0.033	4.72
17. Chinese sucker, Yuan *et al.* (2011)	7.5	0.8066	0.651	1.537	11.528	86.458	2.362	0.218	0.223	1.671	12.535	0.050	5.82
18. Atlantic cod, Kousoulaki *et al.* (2010)	8.7	4.4	19.360	0.052	0.449	3.910	0.003	0.007	0.043	0.375	3.263	0.002	1.13
19. Striped bass, Dougall *et al.* (1996)	3	2.4	5.760	0.174	0.521	1.563	0.030	0.025	0.104	0.313	0.938	0.011	2.72
20. Hybrid striped bass, Brown *et al.* (1992)	4.4	0.6089	0.371	2.697	11.868	52.217	7.275	0.383	0.238	1.046	4.601	0.056	6.21
			Sums:	**704.99**	**1483.36**	**3711.68**	**391992.2**	**100.0**	**3.827**	**20.32**	**118.85**	**0.83**	**100.0**

n	**20**	
df	**19**	
Q	**590.5**	
I^2	**96.7**	
es (fixed)	**2.1**	
SEes (fixed)	**0.04**	
CI (fixed)	**2.03**	**2.18**

v	**3.837**	
Q_v	**10.92**	
I^2_v	**-73.93**	
es (random)	**5.31**	
SEes (random)	**0.51**	
CI (random)	**4.31**	**6.31**

Table A2: Chi-square and I square statistic values from heterogeneity of variance test based on fixed and random effects model.

	Response criteria	df	Q_f	I^2_f	v	Q_r	I^2_r
P	Weight gain	19	590.6	96.8	3.84	10.9	-74
	Whole body P	10	1347	99.3	6.8	6.4	-55.5
	Vertebral P	8	93.6	91.5	1.7	6.3	-26.7
	Plasma P	5	276.2	98.2	9.4	3.6	-38.9
Ca	Weight gain	7	475.9	98.5	7.2	4.3	-62.9
	Vertebral ash or Ca	7	25.8	72.9	1.7	7.6	8
Mg	Weight gain	11	19.8	49.6	0.01	9.9	-1
	Whole body Mg	11	248.7	95.9	0.3	76.3	66.9
	Vertebral Mg	6	39.1	84.6	0.01	9.4	36.3
	Plasma Mg	4	99.9	96	0.4	3.1	-30
K	Weight gain	3	22.3	86.6	8.5	4.7	35.8
	Whole body Mg	3	217.2	98.6	10.7	3.3	9.8
	Na-K ATPase	1	219.5	99.5	29	1	0.1
Zn	Weight gain	24	17469	99.9	630.4	41.7	52.1
	Whole body Zn	7	473.8	99.7	495.2	6.4	5.9
	Vertebral Zn	19	9136	99.8	552.3	46.1	60.1
	Serum Zn	5	337.4	98.8	959.5	6.7	40.4
	Serum-ALP	4	29.5	89.8	817	2.3	-28.7
Se	Weight gain	24	433.1	94.5	0.02	54.4	55.9
	Hepatic-GPx	14	329.7	96.1	0.08	69.6	61.3
	Serum-GPx	8	46.2	84.8	0.03	13	46.1
	Hepatic-GR	4	16.8	82.1	0.04	3.8	21.9
Mn	Weight gain	10	48.2	81.3	15.3	10.7	15.6
	Whole body Mn	6	57.2	93	13.2	3.4	-18.1
	Vertebral Mn	7	132.8	95.5	93.2	10.5	42.6
Fe	Weight gain	6	566	99.1	6447.8	8.6	41.8
	Whole body Fe	3	23.5	91.5	4948.4	7.9	54.9
	Haemoglobin	6	29.7	83.2	684.3	6.1	17.9
Cu	Weight gain	5	112	96	14.2	4.1	1.1
	Hepatic Cu	5	89.2	92	11.7	8.1	21.5
	Hepatic CuZn SOD	4	74.6	95.8	16.8	1.58	-89.9

df – degrees of freedom; Q_f – Observed Q statistics for fixed effect model; I^2_f – Observed I^2 statistics value for fixed effect model; Q_r – Observed Q statistics for random effect model; I^2_f – Observed I^2 statistics value for random effect model; v – correction factor for converting from fixed effect to random effect model.

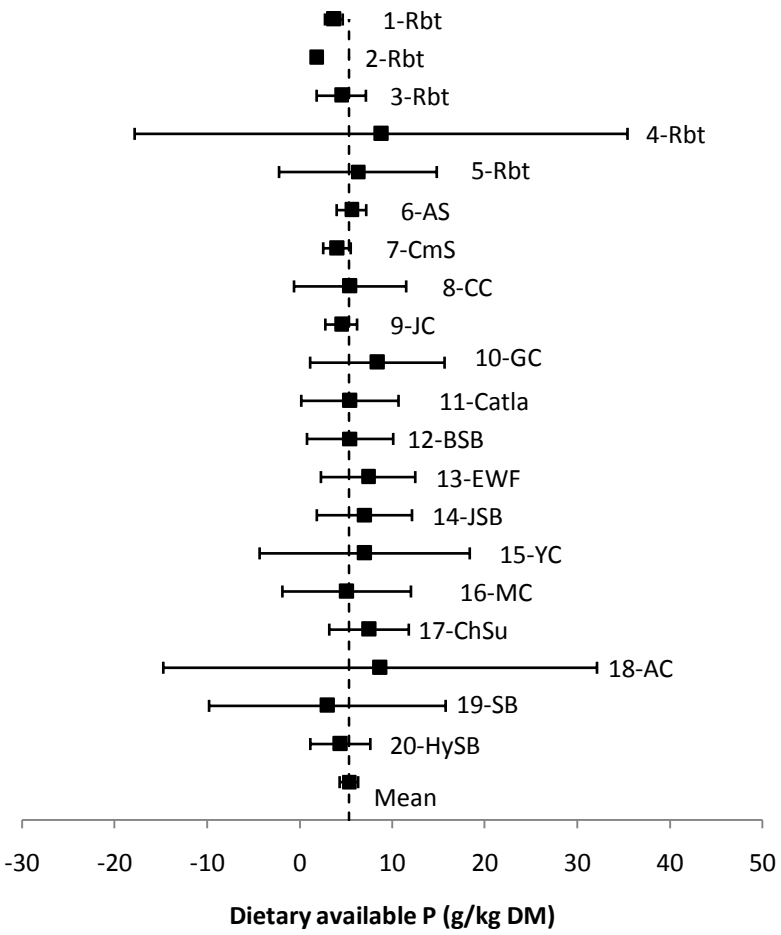

Figure A1: Forest plot presentation of the meta-analytic estimates on the minimal dietary inclusion levels of available P, data presented in Table A1 of appendix.

CHAPTER 4

Dietary fat (F) and phosphorus (P) interactions: Effects on mineralization and dietary P needs in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Nutrient dense high-energy diets (with high fat levels) have improved feed efficiency, growth rate and protein retention but also leads to increased fat deposition and vertebral deformities in salmonids. In this scenario, it was hypothesized that high fat salmonid diets lacking higher available P levels will affect mineralization by depleting vertebral Ca and P levels. To test this, two levels of dietary fat (12 and 22%, LF and HF) and two levels of available P namely adequate and high (0.4 and 0.8%, AP and HP) were studied in a 2x2 factorial design. Groups (n=30 fish) of rainbow trout juveniles (IBW: 49±1g) were distributed into 12 experimental units and each unit was randomly assigned one of the four dietary treatments (in triplicates). All treatments were fed equal restricted rations, twice a day for 12 weeks at 15°C. Weight gain, daily growth index (DGI), feed efficiency (FE) and protein efficiency ratio (PER) were significantly ($P<0.05$) higher in fish fed high fat diets compared to low fat diets. No effect of phosphorus was observed on weight gain or any other growth indices analysed ($P>0.05$). Inclusion of high fat significantly ($P<0.01$) improved the availability of dietary P at both AP and HP conditions. HP diet fed fish had higher ash, P and Ca in their body compared with LP fed fish; within the AP fed groups, fish fed AP-HF diets had the lowest levels of ash, Ca and P. HF diets resulted in high fat deposition in the vertebrae; while the HP diets resulted in higher ash, Ca and P content in the vertebrae at both fat levels. To conclude, higher weight gain and faster growth rates induced by high dietary fat requires increased available P supply to assure proper mineralization of whole body and vertebrae in rainbow trout.

Introduction

Bones form a major reserve of minerals especially of phosphorus (P) in fish. Mineral homeostasis in vertebrates is largely maintained through constant formation and resorption of bones by the process of remodelling (Lall and Lewis-McCrea, 2007). Among the dietary or nutritional factors that affect bone mineral metabolism, dietary lipids and phosphorus are important components. Dietary lipids play an important role in the production and regulation of active modulators of bone metabolism (such as eicosanoids, cytokines, leucotriens, lipoxygenase, PGE2). Both dietary lipid levels and sources are known to affect bone formation and resorption in higher animals and humans (Watkins *et al.*, 2001). It is also often observed that P deficiency leads to increased fat deposition in the fish body (Lall, 2002). However, less is known on the relation between dietary lipids and bone or phosphorus metabolism in fish (Lall and Lewis-McCrea, 2007).

Over the years nutrient dense, high energy diets (mainly with high lipid levels) are commonly used in salmonid farming and are known to improve feed and protein efficiencies, with a consequent reduction in dissolved waste output (N and P). High-energy diets also lead to increased fat deposition (Sargent *et al.*, 2002), which is at times associated with P-deficiency (Lall, 2002). The reduction in dissolved N output is achieved through the protein sparing effect by the higher dietary lipids. With phosphorus, it is achieved through reduced intake of P per unit weight gain due to reduced intake and higher conversion efficiency of high-energy diets. This imposes a situation where less dietary P is available for the fish to support rather a higher (than normal) growth rate per unit amount of feed consumed. Moreover, the faster growth rates achieved with such diets can affect growth allometry resulting in vertebral deformities especially under dietary phosphorus (P) insufficiency. It was predicted by Shearer (1995) that improving the feed efficiency will require increased dietary phosphorus supply to meet the phosphorus requirement of fast growing fish.

Based on the literature data, the dietary requirement of phosphorus to rainbow trout for maximal weight gain was found to be 0.35% and for maintaining vertebral P concentration the required level was 0.52% (Antony Jesu Prabhu et al., 2013). This showed that the requirement for maintaining vertebral P concentration was 50% higher than the level required for maximal weight gain. Therefore it was hypothesised that,

higher growth rates induced by higher dietary energy intake will require additional dietary P supply to maintain normal phosphorus and calcium balance in the body.

Materials and Methods

Two levels of dietary fat, low fat (LF, 12%) and high fat (HF, 22%) and two levels of available P, namely adequate (AP, 0.4%) and high (HP, 0.8%), were tested in a 2x2 factorial design. Table 1 provides the detailed ingredient and nutrient composition of the experimental diets. Groups (n=30 fish) of rainbow trout juveniles (IBW: 49±1g) were distributed into 12 experimental units and each unit was randomly assigned one of the four dietary treatments (in triplicates). The fish were housed in the metabolic research unit with 12 tanks each of 200L capacities, which are all connected to a RAS system equipped with a nitrification and a de-nitrification unit. All treatments were fed equal restricted rations (14 g $kg^{-0.8}$ d^{-1}), twice a day for 12 weeks at 15°C.

At the end of the 12-week experimental period, individual weight (W), standard length (SL) and caudal peduncle length (CL) were measured (n=20 fish per tank) for analysis of allometric length-weight relations (LWR) expressed as $W=aL^b$ and tail ratio (TR = CL/SL). Whole fish samples were taken for body composition and vertebral composition analysis (each n=10 fish per tank) and immediately frozen until analysis. Blood samples were withdrawn from anesthetised fish (0.25ml L^{-1}, 2-phenoxyethanol) in heparinised syringes 24h after the last meal at the end of the 12 weeks. The samples were immediately frozen at -20 °C until analysis. All animal handling and sampling protocols were approved by the animal ethics and welfare committee of Wageningen University. Apparent availability coefficients (AAC) of Phosphorus and Calcium were determined using yttrium oxide as the inert marker (Table 1). Faecal samples were collected by stripping, 9h after the last meal, at the end of 12 weeks. The stripped faeces were used for AAC measurements of calcium and phosphorus.

Table 1: Ingredient and nutrient composition of the experimental diets

	AP		HP	
Ingredients (%)	LF	HF	LF	HF
Fish meal, 70	18	18	18	18
Maize gluten	11	7	7	7
Wheat gluten	7	11	8	10
Soybean meal, 48	7	7	7	7
Soybean concentrate	10	10	10	10
Rapeseed meal, colza00	8	8	8	8
Whole wheat	27.7	16.8	26.8	13.2
Soy lecithine	1	1	1	1
L-Lysine	0.4	0.4	0.4	0.4
Monocalcium phosphate	-	0.5	3.8	5
Min. premix INRA	1	1	1	1
Vit. Premix	1	1	1	1
Tracer, Yttrium oxide	0.01	0.01	0.01	0.01
Fish oil	7.9	18.3	8.0	18.4
Analysed nutrient content on DM basis (g kg^{-1})				
Dry Matter (DM g kg^{-1} diet)	952,4	970,3	956,8	957,7
Crude protein (N x 6.25)	450	431	433	436
Crude Fat	138	224	129	231
Ash	61	62	90	96
Gross Energy (GE kJ g^{-1})	22,4	24,4	21,6	23,6
Phosphorus	8.6	9.3	17.5	19.6
Calcium	8.6	9.3	15.5	17.3
Yttrium	0.084	0.08	0.085	0.083
Digestible Energy (DE kJ g^{-1})	18.5	20.8	17.3	20.1
DP/DE (mg kJ^{-1})	22.6	19.5	22.9	20.3
Nitrogen free extract (NFE)	303	253	305	195

AP: adequate phosphorus; LF: low fat; HP: high phosphorus; HF: high fat; Nitrogen Free extract calculated as: DM – (ash + crude protein + crude fat).

Mineral premix (g or mg/kg diet): calcium carbonate (40% Ca), 2.15 g; magnesium oxide (60% Mg), 1.24g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; copper sulphate (25%Cu), 0.3 g; manganese sulphate (33% Mn), 0.3 g; dibasic calcium phosphate (20% Ca, 18%P), 5g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; KCl, 0.9 g; NaCl, 0.4 g (UPAE, INRA).

Vitamin premix (IU or mg/kg diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (UPAE, INRA).

The chemical composition of experimental diets, whole fish and vertebral samples were analysed for moisture (dry heat; 105 °C for 24h), crude protein (Kjeldhal nitrogen), crude fat (Soxtherm), ash (combustion at 550 °C for 8h) and gross energy (adiabatic bomb calorimeter, IKA, Germany). The analysis of P and Ca in the diets, whole fish and faecal samples were made by ICP-OES at CBLB, Wageningen University, Netherlands. The fish meant for vertebral analysis were microwave treated for 2-3 min., upon which the flesh was removed and the vertebrae were collected. The collected vertebrae were rinsed with hexane and dried at room temperature for 24h. The analysis of P and Ca were made on the ash samples by ICP-OES at USRAVE, INRA-Bordeaux, France.

Concentrations of plasma metabolites such as P (Sobioda, France), free fatty acids (NEFA C kit, Wako Chemicals, Neuss, Germany), triglycerides (PAP 150, BioMérieux, France), cholesterol (RTU, bioMérieux, France) and phospholipids (PAP 150, Biomérieux, France) were analysed with commercial colorimetric kits adapted to a microplate format, according to the recommendations of the manufacturer.

Tanks were used as experimental units for growth, body composition and other performance indicators. For parameters on plasma metabolites individual fish was used as the experimental unit. Two-way ANOVA was used to test the effects of fat and phosphorus.

Results

Data on growth and body condition indices are presented in Table 2. Weight gain and final body weight were significantly ($P<0.05$) higher in fish fed high fat diets compared to low fat diets. Similar results were obtained for other growth indices such as daily growth index (DGI), feed efficiency (FE) and protein efficiency ratio (PER).

Table 2: Growth performance indices of rainbow trout fed the experimental diets varying in dietary fat and phosphorus levels for 12 weeks

	AP		HP		Pooled SEM	Fat (F)	Phos (P)	F x P
	LF	HF	LF	HF				
IBW (g)	49.2	49.5	49.2	50.1	0.9	0.89	0.91	0.93
FBW (g)	222.5	246.3	221.8	246.2	14.6	0.001	0.23	0.19
WG (g)	173.4	196.8	172.6	196.1	14.1	0.001	0.24	0.21
FI (g)	179.9	177.2	180.2	177.9	1.9	0.98	0.41	0.18
Survival (%)	96.7	98.9	96.7	96.7	1.57	0.63	0.63	0.63
DGI	2.9	3.1	2.8	3.1	0.11	0.0003	0.32	0.14
FE	0.9	1	0.9	1.0	0.08	0.0002	0.26	0.13
PER	2.0	2.3	2	2.3	0.21	0.0001	0.56	0.58
HSI	1.05	0.99	0.93	0.98	0.2	0.04	0.001	0.02
VSI	7.9	7.4	9.3	9.3	1.3	0.42	0.01	0.02
TR	0.221	0.220	0.221	0.224	0.009	0.44	0.09	0.09
LWR-b	2.4	3.4	2.5	2.7	0.2	0.09	0.01	0.03
K-factor	1.77	1.72	1.7	1.77	0.2	0.59	0.59	0.001

AP, adequate phosphorus; HP, high phosphorus; LF, low fat; HF, high fat. IBW, initial body weight; FBW, final body weight; WG, weight gain; FI, feed intake; DGI, daily growth index; FE, feed efficiency; PER, Protein efficiency ratio; HSI, hepato-somatic index; VSI, viscera-somatic index; TR, tail ratio; LWR-b, coefficient of length weight relationship; K-factor, condition factor. P-values presented from 2-way ANOVA indicate statistical significance at $p<0.05$.

No effect of phosphorus was observed on weight gain or any other growth indices analysed ($P>0.05$). Significant effect ($P<0.05$) of phosphorus and interaction with fat

levels were observed for body condition parameters such as hepato and viscera-somatic index (HIS and VSI) and allometric coefficient of length-weight relationship (b). A tendency towards significance (P=0.09) was also observed on the effect phosphorus and interaction on tail ratio (TR).

The apparent availability coefficients (AAC) of phosphorus and calcium are presented in Table 3. High fat (HF) diets had a higher AAC of phosphorus (P<0.01) than the low fat (LF) diet; the dietary phosphorous level did not affect AAC of phosphorus. The AAC of Ca was significantly (P=0.01) increased by fat inclusion at both dietary P levels; AAC of Ca was reduced (P=0.01) in the HP diets compared to the LP diets at both fat levels.

Table 3: Apparent availability coefficients (%) of P and Ca in experimental diets to rainbow trout measured at the end of 12 weeks.

	AP		HP		Pooled SEM	Fat (F)	Phos (P)	F x P
	LF	HF	LF	HF				
Phosphorus	43.4^{a}	52.5^{b}	46.1^{ab}	50.7^{b}	2.06	0.003	0.69	0.16
Calcium	8.6^{ab}	14.9^{a}	0.1^{b}	5.2^{ab}	1.64	0.01	0.01	0.97

AP, adequate phosphorus; HP, high phosphorus; LF, low fat; HF, high fat. P-values presented from 2-way ANOVA indicate statistical significance at p<0.05. Values having different superscript within the same row are statistically different.

Analysis of circulating levels of plasma phosphorus and lipid metabolites (Table 4) showed that plasma phosphorus, free fatty acids and triglycerides were significantly higher in HF fed fish; interaction effect was also significant for triglycerides and phospholipids with AP-HF being higher than other groups. Plasma cholesterol levels were the lowest in AP-LF fed fish while no significant difference was observed between the other dietary treatments.

Table 4: Plasma phosphorus and lipid metabolites in rainbow trout fed the experimental diets of varying fat and phosphorus levels for 12 weeks

	AP		HP		Pooled SEM	Fat (F)	Phos (P)	F x P
	LF	HF	LF	HF				
Phosphorus	3.12	3.88	3.49	3.78	0.4	0.001	0.3	0.1
Free fatty acids	0.39	0.49	0.41	0.52	0.1	0.01	0.7	0.8
Triacylglycerides	2.43^{a}	3.43^{b}	2.61^{ab}	2.68^{ab}	0.6	0.01	0.15	0.03
Cholesterol	3.08^{a}	3.55^{b}	3.51^{b}	3.23^{ab}	0.3	0.49	0.73	0.01
Phosholipids	6.71^{a}	7.66^{b}	7.17^{ab}	6.95^{a}	0.6	0.09	0.57	0.01

AP, adequate phosphorus; HP, high phosphorus; LF, low fat; HF, high fat. P-values presented from 2-way ANOVA indicate statistical significance at p<0.05. Values having different superscript within the same row are statistically different.

Body composition data (Table 5) showed that fish fed high fat diets had more dry matter, energy and lower protein due to higher fat deposited in the body. With regard to the mineral composition, HP diet fed fish had higher ash, P and Ca in their body compared with AP fed fish; within the AP fed groups, fish fed AP-HF diets had the lowest levels of ash, Ca and P.

Table 5: Initial and final body composition of rainbow trout fed the experimental diets for 12 weeks

	Initial	AP		HP		pSD	Fat (F)	Phos (P)	F x P
		LF	HF	LF	HF				
Dry matter	267	291	310	292	303	8.2	0.02	0.09	0.04
Protein	162	174	165	175	168	4.6	0.003	0.29	0.6
Lipid	85	102	127	100	118	12.5	0.01	0.04	0.14
Energy	20	8.5	8.9	8.1	8.6	0.4	0.02	0.04	0.67
Ash	6.9	19.5^{ab}	18.4^{a}	20.9^{ab}	22.1^{b}	1.4	0.9	0.001	0.01
Phosphorus	3.5	3.7^{ab}	3.3^{a}	3.9^{b}	4.1^{b}	0.3	0.5	0.001	0.01
Calcium	3	3.4^{ab}	2.9^{a}	3.7^{ab}	4.0^{b}	0.5	0.3	0.001	0.001

AP, adequate phosphorus; HP, high phosphorus; LF, low fat; HF, high fat. P-values presented from 2-way ANOVA indicate statistical significance at $p<0.05$. Values having different superscript within the same row are statistically different.

The vertebral composition analysis (Table 6) revealed that HF diets resulted in high fat deposition in the vertebrae; while the HP diets resulted in higher ash, Ca and P content in the vertebrae. No effect of fat or an interaction was observed in the mineral composition of the vertebrae.

Table 6: Vertebral proximate and mineral composition of rainbow trout fed the experimental diets varying in fat and phosphorus levels for 12 weeks

	AP		HP		pSD	Fat (F)	Phos (P)	F x P
	LF	HF	LF	HF				
Dry matter	51.9	49.5	52	51.7	0.4	0.12	0.36	0.17
Protein	36.9^{c}	33.2^{b}	33.3^{b}	31.7^{a}	0.6	0.001	0.001	0.02
Lipid	35.1	37.5	34.5	38	1.2	0.04	0.98	0.72
Ash	24.8	24.2	30.2	28.5	0.8	0.17	0.001	0.49
Phosphorus	4.6	4.5	5.7	5.4	0.2	0.19	0.001	0.58
Calcium	8.3	8.2	10.1	9.3	0.3	0.22	0.003	0.35

AP, adequate phosphorus; HP, high phosphorus; LF, low fat; HF, high fat. P-values presented from 2-way ANOVA indicate statistical significance at $p<0.05$. Values having different superscript within the same row are statistically different.

Discussion

High energy diets with additional P supplementation have been influential in reducing the nutrient load (N and P) in aquaculture effluents by improving growth rates, nitrogen retention and dietary P availability (Green *et al.*, 2002a; Green *et al.*, 2002b; Sarker and Satoh, 2013). However, many physiological changes that occur along with the increased growth rates have not been studied. Mineralisation processes and the physiological need for phosphorus and calcium are a few to mention. The faster growth rates achieved with energy dense, high fat diets can possibly affect the mineral balance in the body resulting in vertebral deformities (Hansen *et al.*, 2010). In this case, high dietary fat induced faster growth rates and will require higher dietary supply of minerals especially P and Ca for proper mineralisation. It was recently found that, increased dietary phosphorous prevented vertebral deformities in triploid Atlantic salmon (Fjelldal et al. 2015). Although NRC (2011) does not recommend a quantitative requirement for Ca in the diets for rainbow trout, many studies have shown that adequate dietary supply of calcium is required for proper mineralisation in salmonids and other fish species (Antony Jesu Prabhu et al., 2014; Hossain and Yoshimatsu, 2014). In the present study, apparent availability coefficient of phosphorus and calcium were significantly higher in the high fat groups compared to the low fat groups. The magnitude of increase was more prominent under adequate dietary phosphorus rather than at high dietary phosphorus. These findings suggest that, fish fed high fat diets would attempt to maximise the dietary availability of P and Ca, especially when the later are limiting in the diet.

The effect of dietary P deficiency resulting in increased lipid deposition in fish is known for years with previous studies involving common carp (Takeuchi and Nakazoe, 1981), channel catfish (Eya and Lovell 1997), Japanese sea bass (Zhang et al., 2006), black sea bream (Shao et al., 2008) and tilapia (Yao et al., 2014). However, the physiological mechanism has not been uncovered. In the present study, increase in circulating levels of plasma lipid metabolites (cholesterol, phospholipids, TG) due to high dietary lipid intake was attenuated by a higher inclusion of dietary phosphorus. Exogenous dietary lipid supply as well as mobilisation of endogenous lipid from body stores may serve as sources of circulating levels of plasma lipid metabolites (Tocher, 2003). However the question that remains is, whether the observed differences are brought about by changes in lipid absorption and re-synthesis at the intestinal level or by differential

mobilisation of lipid reserves from adipocytes, myocytes or hepatocytes? The possible role of P in the former is unknown, whereas phosphorus has been clearly associated to processes involved in peripheral lipid mobilisation. Phosphorylation of 'perilipin' and 'hormone activated lipase' are the rate limiting reactions in the mobilisation of lipid reserves from the adipose tissue (Sheridan, 1994) and might well be responsible for the increased lipid deposition in tissues during dietary P deficiency. Further studies focusing on molecular and cellular responses might shed light on the mechanism underlying the dietary lipid and P interactions on lipid metabolism and mobilisation in fish.

Based on the meta-analysis of Antony Jesu Prabhu *et al.* (2013), minimal dietary phosphorus inclusion required for maximal weight gain for rainbow trout was estimated to be 3.5 g available P kg^{-1}. Confirming this, the AP (4 g available P kg^{-1}) diets which were formulated based on the aforementioned finding resulted in similar growth in par with the HP (8-9 g kg^{-1}) diet fed groups. However, this may not be sufficient to maintain proper mineralization of hard tissues especially bones and vertebrae. Many studies have investigated the impact of dietary P levels on the incidence of vertebral deformities in salmonids. Although many factors are responsible for the occurrence of vertebral deformities, sufficiency of dietary P supply attained most interest (Fjelldal *et al.*, 2009; Fjelldal *et al.*, 2012; Fjelldal *et al.*, 2007; Gil Martens *et al.*, 2012; Deschamps *et al.*, 2014b; Deschamps *et al.*, 2014a; Le Luyer *et al.*, 2014). Very recently, Fjelldal *et al.* (2015) reported that higher inclusion of dietary P (similar to the levels present in the HP diets of present study; 16 g kg^{-1}) prevents vertebral deformities in triploid Atlantic salmon. Although vertebral deformities were not studied in the present study, mineral content of the vertebra as indicated by ash, P and Ca were reduced by high fat diet fed with 4 g available P kg^{-1} available P. Inclusion of higher dietary P levels (8-9 g available P kg^{-1}) increased the mineral content of the whole body and vertebrae even at high fat conditions. According to the report of Malfotruite (2007), caudal peduncle and neck are the two most susceptible regions of the vertebrae to be affected by compressions. In the present study, data on the body condition indices suggest a compression in the tail region as indicated by a tendency for low TR in AP-HF group. Moreover, the drastic change in the value of allometric growth coefficient (LWR-b) of trout fed the AP-HF vs HP-HF diet is an indicator of the disturbance in the normal growth allometry. The

increase in the allometric coefficient, as seen in the AP-HF group (b > 3) signifies that the fish is too heavy for its length (Froese, 2006).

To conclude, higher weight gain and faster growth rates induced by high dietary fat requires increased available P supply to assure proper mineralisation of whole body and vertebrae in rainbow trout. Long term feeding of high fat diets can reduce vertebral mineralisation in rainbow trout. Further investigations are required to better understand the interactions between phosphorus and lipid metabolism in fish.

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CHAPTER 5

Post-prandial changes in plasma mineral levels in rainbow trout fed a complete plant ingredient based diet and the effect of supplemental di-calcium phosphate

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Abstract

Post-prandial changes in plasma mineral levels and utilisation of minerals in rainbow trout fed complete plant ingredient based diets with or without supplemental di-calcium phosphate (DCP) were studied over an 8 week period. Three diets were used: diet M was FM and fish oil (FO) based diet (control); diets VP0 and VP+ (V diets) were completely based on plant derived protein and lipid sources. One of the V diets (VP+) was supplemented with DCP to supply 5 g kg^{-1} dry matter available phosphorus (P); while the other diet (VP0) was not supplemented with DCP. Change in dietary protein source significantly affected the post-prandial pattern in plasma levels of P ($p<0.05$), Ca ($p<0.007$), Mg ($p<0.001$) and Zn ($p<0.03$). Area under the curve analysis indicated that compared to VP0, DCP supplementation in VP+ improved plasma levels of P ($p<0.01$) and K ($p<0.05$); Cu ($p<0.002$), Se ($p<0.009$) and Zn ($p<0.001$) levels were reduced while Ca, Mg and Fe levels were unaffected ($p>0.05$). Based on measurement of apparent digestibility, growth and whole body composition analyses, mineral balances were established showing that supplementation of DCP led to significant increase in whole body P concentration and P retention in VP+, comparable to fish fed diet M with significantly ($p<0.05$) reduced faecal and non-faecal P losses. There was improved post-absorptive retention (as % of available intake) of Ca ($p<0.05$), Mg ($p<0.05$) and K ($p<0.05$) in VP+ compared to VP0. Utilisation of Cu ($p<0.05$) and Zn ($p<0.01$) was negatively affected. DCP supplementation to complete plant ingredient based diet increased the post-prandial plasma levels, whole body concentration and utilisation of macro-minerals (P, Ca, Mg and K) whereas that of micro-minerals especially Zn and Cu were negatively affected.

Key words: Rainbow trout; fish meal replacement; minerals; post-prandial absorption; area under the curve; utilisation.

Introduction

Ensuring an adequate dietary supply of minerals to farmed fish is essential for proper somatic and skeletal growth, health and final flesh quality. Mineral composition of edible as well as non-edible portions in farmed fish is linked to the dietary mineral composition of the feeds (Lall, 1995; Carpene et al., 1998; Fuentes et al., 2010; Fallah et al., 2011). Fish meal (FM) is a major source of minerals and trace elements (Julshamm et al. 1978) and especially of phosphorus (P) to farmed fish (Kaushik, 2001; Lall, 2002). The substitution of FM by plant protein sources which is well under way for farmed fish (Kaushik and Hemre, 2008; Tacon and Metian, 2008) inevitably calls for measures to ensure an adequate supply of minerals in an available form to meet the physiological demands of fish. The bioavailability of minerals supplied by the diets to meet such physiological demands is markedly influenced by efficiency with which the body uptakes and utilises the dietary minerals (Watanabe et al. 1997a; Lall, 2002).

Post-prandial plasma levels of minerals can serve as an indicator of absorption through dietary intake and bio-availability (Navarro and Wood, 2003). As already pointed out by Rodehutscoerd (1996) with regard to phosphorus, in order to assess the mineral status using circulating levels of minerals as indicators, knowledge on post-prandial changes as affected by dietary factors is necessary. However, data on time course of changes in the post-prandial plasma mineral levels are scarce in fish. Secondly, in diets containing high levels of plant-protein ingredients and low levels of FM, supplementation with mono- or di-basic inorganic P salts has been found to be an efficient strategy to increase the levels of available P supply (Ketola, 1975; Watanabe et al., 1997b; Kaushik et al., 2004). Many studies have focused on the adverse effects of tri-calcium phosphate and calcium phytate on the utilisation of other minerals (Hardy and Shearer, 1985; Satoh et al., 1987; Satoh et al., 1989; Gatlin and Phillips, 1989a; Satoh et al., 1992a; Satoh et al., 1993). But, only a few (Vielma and Lall, 1998; Kousoulaki et al., 2010) have studied the effect of mono- or di-basic P salts, which are the commonly used supplements under both experimental and practical conditions. The objective of this study was to test the effects of change in dietary protein sources and of DCP supplementation on the post-prandial patterns of plasma mineral levels and mineral balance in rainbow trout.

Materials and methods

Diets

Three diets (M, VP0 and VP+) were formulated with two different basal ingredient compositions (Table 1).

Table 1: Ingredient and analytical composition of the experimental diets

Ingredients (%)	M	VP0	VP+
Norwegian Herring meal, (CP 70; Sopropêche, France)	62.6	.	.
Corn Gluten meal (CP 60; Inzo, France)	.	18.0	18.0
Wheat Gluten (CP 70; Roquette, France)	.	20.0	20.0
Soybean mea(CP 48; Inzo, France)	.	6.1	6.1
Soy protein concentrate (Estrilvo ; CP 70; Sopropêche, France)	.	15.0	15.0
White lupin meal (Terrena, France)	.	5.0	5.0
Extruded peas (Aquatex, Sotexpro, France)	.	3.8	3.8
Rapeseed meal (Primor 00; Sud Ouest Alimants, France)	.	5.2	5.2
Whole wheat	24.6	4.2	2.0
Soy lecithin (Louis François, France)	.	2.0	2.0
L-Lysine (Eurolysine)	.	1.3	1.3
L-methionine (Evonik, Germany)	.	0.3	0.3
CaHPO4.2H20 (18%P; 22% Ca)	.	.	2.2
Attractant Mix§	.	1.5	1.5
Mineral premix¶	.	1.0	1.0
Vitamin premix†	1.0	1.0	1.0
Fish Oil (Southern Hemisphere, Sopropêche, France)	11.9	.	.
Rapeseed Oil (Daudruy, France)	.	6.2	6.2
Linseed Oil (Daudruy, France)	.	6.2	6.2
Palm Oil (Daudruy, France)	.	3.1	3.1
Analysed proximate composition			
Dry matter (DM), %	91.8	91.4	93.1
Crude Protein, % DM	49.2	51.1	49.9
Crude Lipid, % DM	19.6	21.7	22.2
Crude Ash, % DM	9.0	3.1	4.9
Energy, kJ/g DM	22.9	24.7	24.4
Analysed mineral composition			
P, g/kg DM	14.1	6.1	10.3
Ca, g/kg DM	16.0	4.5	10.8
Ca/P ratio	1.1	0.7	1.0
Mg, g/kg DM	2.1	1.7	1.8
K, g/kg DM	11.0	5.4	5.1
Fe, mg/kg DM	176.3	244.3	246.6
Mn, mg/kg DM	10.5	77.8	79.7
Cu, mg/kg DM	4.3	16.1	17.4
Se, mg/kg DM	1.3	0.4	0.4
Zn, mg/kg DM	58.9	85.7	84.7

§ *Attractant mix (g/kg mixture):* glucosamine, 5g; taurine, 3g; betaine, 3g; glycine, 2g and alanine, 2g.
¶ *Mineral premix (g or mg/kg diet):* calcium carbonate (40% Ca), 2.15 g; magnesium oxide (60% Mg), 1.24g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; copper sulphate (25%Cu), 0.3 g; manganese sulphate (33% Mn), 0.3 g; dibasic calcium phosphate (20% Ca, 18%P), 5g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; KCl, 0.9 g; NaCl, 0.4 g (UPAE, INRA).
† *Vitamin premix (IU or mg/kg diet):* DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (UPAE, INRA).

Diet M was formulated to contain fish meal (FM) and fish oil (FO) as protein and lipid sources and served as a control. Diets VP0 and VP+ (collectively addressed as V diets) were based on plant ingredients (with no FM or FO). The V diets were supplemented with a mineral pre-mixture at 10 g kg^{-1} diet to meet all the essential mineral requirements of rainbow trout (NRC 2011), except for available P. To one of the V diets (VP+), additional supplementation of di-calcium phosphate, DCP at 22 g kg^{-1} diet was made to supply adequate levels of available P as per NRC (2011). For measurement of the apparent digestibility of minerals, the same diets were prepared with the incorporation of 10 g kg^{-1} chromic oxide (Cr_2O_3) as the inert marker by replacing an equivalent amount of whole wheat.

Fish, feeding and rearing condition

Rainbow trout juveniles (78.1 ± 0.6 g, IBW) were randomly distributed into nine experimental units, each of 500 L (35 fish $unit^{-1}$) and acclimatised to the rearing conditions two weeks prior to the start of the experiment. Triplicate groups of fish were hand fed twice a day to visual satiation for a period of 8 weeks (6 days a week). The experiment was carried out in a flow through rearing system at the INRA experimental fish farm at Donzacq (Landes, France). Water temperature was 17.5 ± 0.5°C and flow rate was maintained at 50 L min^{-1} during the course of the study. The mineral concentrations in the water (mg L^{-1}) were analysed to be P, < 0.2; Ca, 41.7 ± 6.3; Mg, 19.2 ± 0.8; K, 1.8 ± 0.1; Fe, < 0.02; Mn, < 0.02; Cu, < 0.008 and Zn, < 0.007.

Weight and tissue sampling

A total of 15 fish were sampled at the start of the study for whole body composition analysis. Fish from each experimental unit were bulk weighed at the start, after 4 weeks and at the end of the 8 week growth trial. Feed was withheld for 24h before every weighing. At the end of the trial, six fish from each replicate were withdrawn, anaesthetised (benzocaine, 30 mg L^{-1}) and euthanized subsequently by a sharp blow to the head; liver and viscera were dissected and weighed for calculating hepato- and viscero-somatic indices (HSI and VSI). The carcass along with liver and viscera were immediately frozen and kept at -20°C awaiting analyses.

Post-prandial mineral absorption study

After the end of the growth trial and samplings, the remaining fish (n=25 per replicate) were used for the post-prandial mineral absorption study. The fish were not fed for 24h

and were subsequently fed a single ration (55g per tank of 25 fish, equal amount to all groups) at 09:00 h in the morning. Following this single meal, blood samples were collected from the caudal vein into heparinized syringes at 8 time points (0, 1, 2, 4, 6, 9, 12 and 24h). At each time point, a total of 18 fish (6 per treatment) were anesthetized in a solution of benzocaine (30 mg L^{-1}) prior to sampling of blood. A sample size of 6 fish per time point was used to reduce variation arising from possible differences in feed intake, as this was not monitored for individual fish within each tank. All the fish used for blood collection were independent samples and no repeated sampling was made on the same fish. Plasma was recovered from centrifuged (3000g for 5 min) blood samples, immediately frozen and stored at -20 °C until mineral analysis.

Apparent digestibility study

After the end of the post-prandial blood sampling, the remaining fish of each treatment group were pooled together into two replicates (n=25 fish per replicate) for the determination of apparent digestibility coefficients (ADC) of minerals. Fish were fed twice a day with the chromic oxide (Cr_2O_3) incorporated diets to visual satiation for 2 weeks. At the end of these two weeks and 16h after the last meal , faecal samples were collected by the method of stripping. The samples were collected over ice, frozen immediately and stored at -20°C until analysis. The analysis of minerals in the faeces was done on pooled samples from duplicate groups for each dietary treatment. Contamination of faeces with residual volume of urine was unavoidable while stripping.
Animal experiments and sampling procedures followed the guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree no. 2001-464, May 29, 2001) and the animal ethics committee of INRA (INRA 2002-36, April 14, 2002).

Chemical composition analysis

Frozen samples of fish were homogenously ground, freeze-dried and stored at 4°C until further analyses. The moisture content of the fish was estimated by drying freshly ground samples at 105°C for 24h. The chemical composition of the diets and of freeze-dried whole fish samples were analysed by the following methods: dry matter after drying at 105°C for 24h, ash by combustion at 600°C for four hours in a muffle furnace, crude protein (Nx6.25) by Kjeldahl method in acid digested samples, crude lipid by petroleum ether extraction using Soxhlet method (Soxtherm) and gross energy content

in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany). The concentration of P in plasma was analysed using a plasma P calorimetric kit (Sobioda, France). The concentrations of P, Ca, Mg, K, Fe, Cu and Zn in the diets, whole fish, faeces and plasma (except P) were analysed using inductively coupled plasma-mass spectrometry (ICP-MS) at USRAVE, INRA, Bordeaux, France. The Se content in diet, whole fish and plasma were analysed by the method of Bierla et al. (2008) using ICP-MS at LCABIE, UPPA, Pau, France. Chromic oxide in the diet and faeces were analysed by the method of Bolin et al. (1952).

Data analysis

Tanks were used as experimental units (group of 30 fish) for growth, body composition and mineral retention. The data were subjected to one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests and the treatment effects were considered significant at $p<0.05$ significance level. Individual fish (6 independent fish per time point per diet) was the experimental unit for data on post-prandial plasma mineral levels. The data on post-prandial plasma mineral levels were subjected to both one-way (to identify significant peak within each dietary group) and two-way ANOVA (to test the main effects of diet, time and diet x time interactions). Area-under-the-curve (AUC) was calculated based on the principle of trapezoidal rule. All the data analysis was performed using a GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California, USA).

Results

Apparent digestibility of minerals

Apparent digestibility coefficients (ADC, %) of minerals in diet VP0 were higher than in diet VP+ for almost all the analysed minerals and higher than those of diet M in the case of micro-minerals such as Fe, Cu, Mn and Zn (Table 2).

Table 2: Apparent digestibility coefficients (ADC, %) of minerals in the diets fed to rainbow trout

	P	Ca	Mg	K	Fe	Cu	Mn	Zn
M	59.1	15.3	52.9	96.7	-8.6	2.6	17.6	32.4
VP0	69.4	-3.2	52.5	85.3	33.4	29.7	42.4	39.3
VP+	54.5	-0.3	45.1	79.7	-25.1	9.8	14.8	28

$$\text{ADC}, \% = 100 - \left\{100 \text{ x} \left(\frac{\% \text{ mineral in faeces}}{\% \text{ mineral in diet}} \text{ x } \frac{\% \text{ marker in diet}}{\% \text{ marker in faeces}}\right)\right\}$$

ADC values presented here were calculated from minerals analysed in pooled faecal samples from duplicate tanks of each treatment. Therefore no statistical tests were perfomed on this data.

Growth performance

Rainbow trout from all three treatment groups more than doubled their body weight at the end of the 8 week feeding trial and attained an individual final body weight ranging from 200-230 g (Table 3). Weight gain was significantly higher ($p<0.01$) in trout fed diet M (151.7 ± 6.2 g fish^{-1}), than in the other two groups (125-130 g fish^{-1}). Voluntary feed intake (VFI) was significantly higher ($p<0.05$) in fish fed VP0 (162 ± 2.4 g fish^{-1}) than in those fed M or VP+ diets (≈153 g fish^{-1}). Daily growth increment (DGI), feed and protein utilisation efficiencies (FE and PER) were significantly higher ($p<0.01$) and VSI the lowest in fish fed diet M; HSI was not significantly ($P>0.05$) affected by dietary treatments.

Table 3: Weight gain and growth indices of rainbow trout after the 8 weeks feeding trial

	M	VP0	VP+
IBW (g)	78.3 ± 1	78 ± 0.3	77.9 ± 0.3
FBW (g)	230 ± 7.1^{a}	207.8 ± 7.4^{b}	203.1 ± 4.5^{b}
FI (g/fish)	153.4 ± 1.2^{a}	161.7 ± 2.3^{b}	153.8 ± 4.7^{a}
WG (g)	151.7 ± 6.2^{a}	129.8 ± 7.4^{b}	125.2 ± 4.2^{b}
WG (%)	193.6 ± 5.8^{a}	166.5 ± 9.3^{b}	160.7 ± 4.7^{b}
DGI	3.3 ± 0.08^{a}	2.95 ± 0.12^{b}	2.87 ± 0.07^{b}
FCR	0.96 ± 0.03^{a}	1.21 ± 0.04^{b}	1.18 ± 0.03^{b}
PER	2.3 ± 0.08^{a}	1.77 ± 0.05^{b}	1.83 ± 0.05^{b}
HSI	1.58 ± 0.24	1.41 ± 0.17	1.58 ± 0.3
VSI	8.8 ± 0.5^{a}	10.9 ± 1.5^{b}	10.6 ± 1.1^{b}

Data represented as mean ± SD. Means with different superscript within a row differ significantly ($p < 0.05$). Absence of superscript denotes lack of significance between treatment groups.
DGI = 100*(FBW$^{1/3}$ - IBW$^{1/3}$)/duration (54 d); FCR = Dry feed intake (g)/wet weight gain (g); PER = wet weight gain (g)/crude protein intake (g); HSI = 100*(wet liver weight (g)/wet body weight (g)); VSI = 100*(wet visceral weight (g)/wet body weight (g)).

Post-prandial plasma mineral levels

The post-prandial plasma profiles of the eight minerals in rainbow trout for all three diets are shown in Figure 1 and data on the time of post-prandial peak, mean 24h baseline and the 24h area under the curve (AUC) levels are presented in Table 4. The post-prandial time point of peak in plasma levels of minerals P, Ca, Mg, K, Zn, Fe, Cu and Mn showed significant variation with change in diet. Significant diet x time interaction was observed for P, Ca, Mg and Zn indicating that plasma mineral levels at a given time point can be affected by the dietary changes.

Post-prandial pattern in plasma mineral levels: The post-prandial changes in plasma concentrations of P, Ca and Mg showed similar patterns in the time of their respective peaks, at 4h in M and VP0 groups whereas there was no significant positive peak in the VP+ group. There was a significant drop in the post-prandial profile of Ca and Mg (at 6h) in all three groups. A similar drop below the basal line was also observed for potassium (K) in the M and VP+ groups (at 9h) with no change in the VP0 group. The decline started after 4h for Ca and Mg or 6h for K, reaching minimum levels at 6h and 9h, respectively. Subsequently, a steady increase was observed up to 12h to regain the basal level. The peak in plasma Fe level appeared 4-6h after the meal in all three groups: a significant ($p<0.05$) peak at 4h in the M group and at 6h in the V groups although not significant. Concentration of plasma Cu in the M group exhibited a small peak (not statistically significant) during 2-4h, whereas in the V diet groups, there were two peaks during 2-4h and later at 9h after feeding. However, the peaks for plasma Cu were significant ($p<0.05$) only in VP0. No clear rhythm in the pattern of plasma Mn levels was found although there was a significant peak at 1h in the VP+ group. In the case of plasma Zn, a significant ($p<0.05$) peak appeared at 2h in the M group but not in the V groups. The plasma Se concentrations did not present a clear post-prandial pattern.

Based on 2-way ANOVA of post-prandial plasma mineral concentrations (Fig.1), significant effect of diet and time was observed with all the analysed minerals except for Fe with diet and Cu with time, taking all the groups together. Comparing VP0 and VP+, plasma concentrations of Ca ($p<0.004$), Cu ($p<0.001$), Se ($p<0.009$) and Zn ($p<0.001$) were found to be decreased in fish fed VP+ with additional DCP supplementation. Plasma levels of P ($p<0.05$) and K ($p<0.05$) increased by DCP supplementation in VP+. Significant diet-time interactions were observed only in the case of Ca ($p<0.02$) and Zn ($p<0.01$) between VP+ and VP0. Although postprandial time interval had a significant effect in the case of Mg ($p<0.001$) and Fe ($p<0.03$), neither a significant effect of diet, nor a diet x time interaction was observed when data from VP+ and VP0 were compared.

Baseline and area under the curve: The baseline concentration of P was significantly higher in the control (diet M) and VP+ groups than in VP0, and the reverse was true for Ca and Zn. The groups fed V diets had significantly higher baseline levels of K and Mn than fish fed diet M, whereas the contrary was observed for baseline Se levels. Baseline values of Mg and Fe were similar between the dietary treatments. The mean 24h AUC

values were significantly different between the three dietary groups for P in the order of M > VP+ > VP0; for K as VP+ > VP0 > M; for Se (12h) in the order of M > VP0 > VP+ and as VP0 > VP+ > M in case of Zn. There was no effect of dietary treatment on the AUC of Ca, Mg and Fe.

Table 4: Data on 24h post-prandial plasma minerals in rainbow trout following a single meal

	Baseline concentration† (mmol or µmol L^{-1})			Area under the curve* (mmol or µmol L^{-1} 24 hr^{-1})			Time of peak§ (hours)		
	M	**VP0**	**VP+**	**M**	**VP0**	**VP+**	**M**	**VP0**	**VP+**
P[1]	3.3 ± 0.4[a]	1.7 ± 0.2[b]	3.2 ± 0.3[a]	85.4 ± 5.5[a]	46 ± 5[c]	76.7 ± 3.9[b]	4	4	NS
Ca[1]	2.5 ± 0.1[b]	2.7 ± 0.1[a]	2.5 ± 0.1[b]	60.8 ± 1.6	64 ± 3	59.3 ± 1.8	4	4	6 (-)
Mg[1]	0.78 ± 0.02	0.74 ± 0.04	0.74 ± 0.04	18.2 ± 0.4	17.7 ± 1.1	17.8 ± 0.8	4	4	6 (-)
K[1]	1.7 ± 0.3[b]	2.5 ± 0.2[a]	2.8 ± 0.3[a]	41.3 ± 4[c]	56 ± 7.3[b]	67.6 ± 3.8[a]	9 (-)	NS	9 (-)
Fe[2]	12.2 ± 3.2	13.1 ± 1.9	10.5 ± 2.1	349 ± 54	330.5 ± 47	367 ± 74	4	NS	NS
Cu[2]	9.9 ± 1.1	9.9 ± 1	9.8 ± 0.5	240 ± 16.2[b]	266.3 ± 11[a]	237 ± 16[b]	NS	9	NS
Mn[2]	< 0.36	0.53 ± 0.12	0.51 ± 0.1	NA	11.6 ± 0.9	9.5 ± 4.4	NA	NS	1
Se[2]	2.2 ± 0.11[a]	1.61 ± 0.12[b]	1.63 ± 0.1[b]	28.9 ± 1.2[a]	22.3 ± 1.1[b]	19.9 ± 1.1[c]	NS	NS	NS
Zn[2]	128 ± 26[b]	257 ± 35[a]	153 ± 11[b]	3513 ± 373[c]	6245 ± 401[a]	4198 ± 479[b]	2	NS	NS

Data represented as mean ± SD. Means with different superscript within a row differ significantly ($p < 0.05$). Absence of superscript denotes lack of significance between treatment groups. [1] mmol units; [2] µmol units.
†Baseline concentration calculated as mean of 0h and 24h values; in case of selenium it was 0h and 12h.
*12h area under the curve for selenium.
§ Time point of significant peak as indicated by ANOVA. NS – not significant; NA – data not available; Negative sign in parenthesis (-) indicates lack of a significant positive peak and the given value pertains to a negative peak (post-prandial dip).

Body composition and mineral balance

The moisture content of the fish reduced from 80% at the start to 68-70% at the end of the trial, which was effectively displaced by lipid increasing up to a level of 11-14% from an initial level of 1.1% (Table 5). The ash content of fish at the end of the trial (1.7-2.2%) was lower compared to the initial level of 2.7%. At the end of the growth trial, moisture, crude protein (CP), crude lipid (CL) and gross energy contents of fish were not significantly affected by the dietary treatments, whereas the total ash content and levels of individual minerals were altered by the dietary treatments. VP0 fed fish had significantly lower whole body ash, P, Ca, Mg and Ca:P ratio than the groups fed M and VP+ diet, with no significant difference between the two latter groups. Potassium and iron levels in the body were not affected by the dietary treatments. Copper content was significantly higher in the V diet fed groups than in fish fed diet M. Whole body Se level was higher in fish fed diet M than in those fed the V diets. Mn level was below detectable limits in M and VP0 fed fish.

Table 5: Initial and final whole body composition (fresh weight basis) of rainbow trout

	Initial	M	VP0	VP+
Moisture (%)	79.3	69.5 ± 1.4	67.5 ± 0.2	68.8 ± 2
Crude protein (%)	16.8	16.9 ± 0.2	17 ± 0.5	16.6 ± 0.3
Crude lipid (%)	1.1	11.2 ± 1.1	13.7 ± 0.4	12.2 ± 1.9
Energy, kJ/g	4.2	8 ± 0.4	9.1 ± 0.2	8.4 ± 0.7
Crude ash (%)	2.7	2.2 ± 0.1^{a}	1.7 ± 0.1^{b}	2.2 ± 0.1^{a}
Phosphorus (g/kg)	5.0	4.1 ± 0.1^{a}	2.9 ± 0.1^{b}	3.7 ± 0.3^{a}
Calcium (g/kg)	6.1	2.6 ± 0.3^{a}	1.1 ± 0.1^{b}	2.3 ± 0.4^{a}
Ca/P ratio	1.2	0.63 ± 0.1^{a}	0.39 ± 0.04^{b}	0.62 ± 0.1^{a}
Magnesium (g/kg)	0.3	0.29 ± 0.01^{a}	0.25 ± 0.01^{b}	0.28 ± 0.01^{a}
Potassium (g/kg)	4.0	3.8 ± 0.1	3.7 ± 0.1	3.8 ± 0.1
Iron (mg/kg)	17.0	12.7 ± 3.1	11.4 ± 0.7	12 ± 0.8
Copper (mg/kg)	3.8	1.4 ± 0.1^{b}	2.7 ± 0.5^{a}	2.4 ± 0.5^{a}
Manganese (mg/kg)	1.5	< 0.65	< 0.65	0.9 ± 0.2
Selenium (mg/kg)	0.2	0.34 ± 0.02^{a}	0.26 ± 0.01^{b}	0.24 ± 0.01^{b}
Zinc (mg/kg)	30.7	16.5 ± 0.8^{b}	19.9 ± 0.6^{a}	17.6 ± 0.5^{b}

Data represented as mean ± SD. Means with different superscript within a row differ significantly ($p < 0.05$). Absence of superscript denotes lack of significance between treatment groups.

Based on measurement of digestibility, growth and comparitive carcass analyses, mineral balances were established to analyse the effects of dietary protein sources and of DCP supplementation of the V diet (Table 6). Absolute daily gains (expressed per unit metabolic body weight, MBW) of P, Ca, Mg, K and Se were significantly higher in trout fed the fish meal based control diet (M) than in the other groups. DCP supplementation to the V diet led to increased daily gain and total retention of P, Ca and Mg, and to a decreased deposition of Zn compared to VP0. Significant decrease ($p<0.05$) in faecal and non-faecal P losses were also observed in VP+ compared to M. Post-absorptive retention (% of available intake) of P, Mg and K were significantly increased by dietary supplementation of DCP, with no effects on post-absorptive retention of Zn and a small but insignificant increase on retention of Cu. Daily gain, total and post-absorptive retention of Fe and Se were not affected by DCP supplementation when comparing VP+ and VP0. Fish fed VP+ diet were statistically similar or better ($P<0.05$) to their counterparts fed diet M for crude ash and all minerals except Se in terms of whole body levels, 24h plasma baseline and 24h AUC values.

Table 6: Mineral budget in rainbow trout fed the experimental diets for 8 weeks

	Intake[1]	Gain[1]§	Faecal loss[1]¶	Non-faecal loss[1]‡	Retention¥ (% TI)[2]	Retention† (% AI)[3]
Phosphorus (mg)						
M	327.6 ± 3.1 [a]	101.1 ± 1.1 [a]	134 ± 1.3 [a]	92.5 ± 2.5 [a]	30.9 ± 0.5 [a]	52.2 ± 0.9 [a]
VP0	155.5 ± 1.1 [c]	37.2 ± 4.8 [c]	47.6 ± 0.3 [c]	70.8 ± 4.1 [b]	23.9 ± 2.9 [b]	34.4 ± 4.2 [b]
VP+	260 ± 5.1 [b]	69.3 ± 7.1 [b]	118.3 ± 2.3 [b]	72.4 ± 9.7 [b]	26.7 ± 3.3 [ab]	49 ± 6 [a]
Calcium (mg)						
M	370.8 ± 3.5 [a]	22 ± 13.1 [a]	314.1 ± 2.4	34.7 ± 11.1	6 ± 3.6 [a]	39 ± 23.4
VP0	113.7 ± 0.8 [c]	-46.4 ± 3 [b]	NA	NA	-40.8 ± 2.6 [b]	NA
VP+	271.1 ± 5.4 [b]	-1.5 ± 15.1 [a]	NA	NA	-0.6 ± 5.5 [a]	NA
Magnesium (mg)						
M	49.3 ± 0.5 [a]	7.2 ± 0.4 [a]	23.2 ± 0.2 [b]	18.9 ± 0.6 [a]	14.6 ± 0.8 [a]	27.6 ± 1.6 [a]
VP0	43.1 ± 0.3 [b]	4.9 ± 0.2 [c]	20.5 ± 0.1 [c]	17.7 ± 0.2 [b]	11.4 ± 0.5 [b]	21.7 ± 0.9 [b]
VP+	44.1 ± 0.9 [b]	5.8 ± 0.3 [b]	24.2 ± 0.5 [a]	14.1 ± 0.1 [c]	13.2 ± 0.4 [a]	29.3 ± 0.8 [a]
Potassium (mg)						
M	254 ± 2.4 [a]	101.1 ± 2.4 [a]	8.5 ± 0.1 [a]	144.4 ± 1.8 [a]	39.8 ± 0.7 [a]	41.2 ± 0.7 [c]
VP0	137.8 ± 0.9 [b]	84.9 ± 3.9 [b]	20.2 ± 0.1 [c]	32.7 ± 4.3 [b]	61.6 ± 3.1 [b]	72.2 ± 3.6 [b]
VP+	129.4 ± 2.6 [c]	84.6 ± 3 [b]	26.2 ± 0.5 [b]	18.6 ± 1.6 [c]	65.4 ± 1.4 [b]	82 ± 1.7 [a]
Iron (µg)						
M	4088.6 ± 38.4 [b]	287.4 ± 122.6	NA	NA	7 ± 2.9	NA
VP0	6219.4 ± 42.4 [a]	192.1 ± 35	4138.9 ± 23	1888.4 ± 20	3.1 ± 0.6	9.2 ± 1.6
VP+	6212.8 ± 122.8 [a]	208.3 ± 41.2	NA	NA	3.3 ± 0.6	NA
Copper (µg)						
M	99.5 ± 0.9 [c]	3.6 ± 6.1 [b]	96.9 ± 0.9 [c]	2.4 ± 2.8 [b]	3.6 ± 6.2	137.9 ± 234.4
VP0	409 ± 2.8 [b]	49.9 ± 18.6 [a]	287.5 ± 2 [b]	71.7 ± 19.4 [a]	12.2 ± 4.6	41.1 ± 15.5
VP+	438.7 ± 8.7 [a]	36.5 ± 20.3 [ab]	395.9 ± 7.8 [a]	17.9 ± 2.1 [b]	8.3 ± 4.6	85.2 ± 47.3
Zinc (µg)						
M	1366.2 ± 12.8 [b]	252.8 ± 41.5 [ab]	923.2 ± 8.7 [c]	190.3 ± 45.1 [c]	18.5 ± 3.2 [a]	57.1 ± 9.9 [a]
VP0	2182.4 ± 14.9 [a]	318.8 ± 22 [a]	1325 ± 9 [b]	538.7 ± 26.9 [a]	14.6 ± 1.1 [ab]	37.2 ± 2.8 [b]
VP+	2133.6 ± 42.2 [a]	220.2 ± 34.5 [b]	1536.1 ± 30.4 [a]	377.3 ± 24 [b]	10.3 ± 1.4 [b]	36.8 ± 5.2 [b]
Selenium (µg)*						
M	34.1 ± 0.3 [a]	11.5 ± 0.9 [a]	22.6 ± 1.2 [a]		33.8 ± 2.9 [a]	NA
VP0	11.6 ± 0.1 [b]	7.1 ± 0.6 [b]	4.5 ± 0.6 [b]		61 ± 5.2 [b]	NA
VP+	11.3 ± 0.2 [b]	6.2 ± 0.3 [b]	5 ± 0.4 [b]		55.3 ± 2.8 [b]	NA

Data represented as mean ± SD. Means with different superscript within a column differ significantly ($p < 0.05$). Absence of superscript denotes lack of significance between treatment groups. NA – data not available

[1] (mg or µg kg^{-1} MBW^{-1} day^{-1}); [2] TI: Total intake; [3] AI: Available intake

§ Whole body gain = [(FBW*FWB content)-(IBW*IWB content)]/[((IBW$^{0.8}$*FBW$^{0.8}$)$^{0.5}$)*days]

¶ Faecal loss = [(daily intake $kg^{-0.8}$ d^{-1})*ADC,%]

‡ Non-faecal loss = [(daily intake – daily gain) – (daily faecal loss)]

¥ Total retention, % = (gain/intake)*100

† Post-absorptive retention, % = (gain/available intake)*100

*Se was not analysed in faeces. The loss data pertains to total loss (faecal + non-faecal).

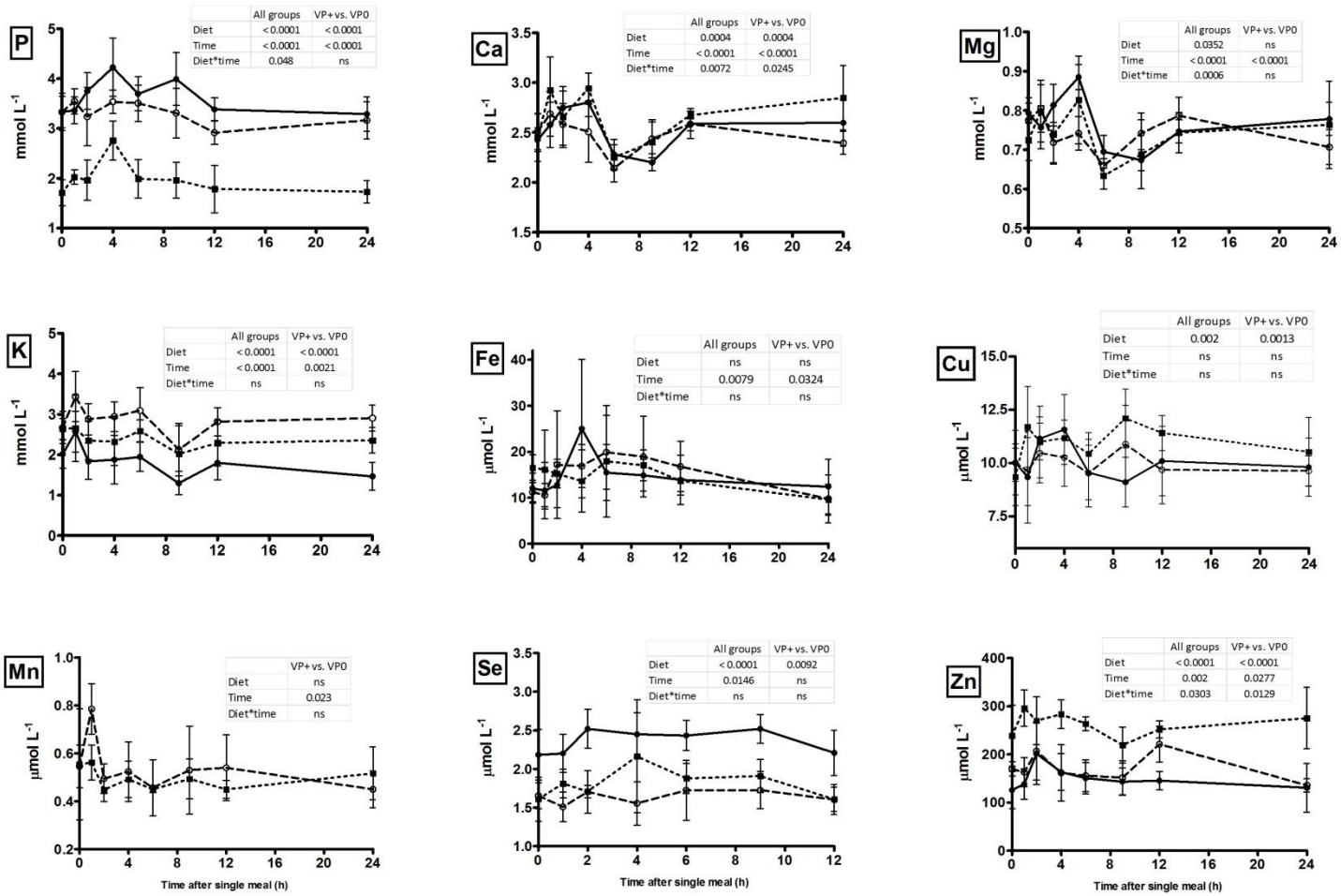

Figure 1: Post-prandial pattern of plasma minerals such as P, Ca, Mg, K, Fe, Cu, Mn, Se and Zn in rainbow trout fed diet M (dark circles and solid line), VP0 (dark square and dotted line) and VP+ (white circles and dashed line) diets. Results (p values) from 2-way ANOVA on all groups together or comparing VP+ and VP0 are provided in insets. Each point represents the mean ± SD (n=6).

Discussion

Post-prandial pattern of plasma mineral levels

Information available on post-prandial changes in plasma mineral levels in teleosts is limited to those on blood P in brook trout (8 and 10-13 °C) (Phillips, 1962); Ca, Mg, Na and K (Bucking and Wood, 2006; 2007) in rainbow trout (10-13 °C); Zn, Cu and Mn measured at 6-hr intervals after meal in rainbow trout (18 °C) (Apines et al. 2003); and Zn in hybrid striped bass (25-26°C) (Savolainen and Gatlin, 2010). Plasma levels of P and other minerals are considered as useful index of mineral status in terrestrial animals (Plus, 1990; Sands et al., 2001) and have been used in salmonids (Rodehutscord, 1996; Sugiura et al. 2000b; and in some other finfish species (Lall, 2002; Antony Jesu Prabhu et al. 2013). Data on post-prandial profile of plasma P over a full diel cycle (24h) is lacking in rainbow trout or in any fish species with data being limited to one or two time points after a meal. A decrease of 20-30% in plasma P concentrations was observed between 4h and 24h after meal in Atlantic salmon (15 °C) (Vielma and Lall, 1998) indicating possible effects of the time of feeding. A post-prandial pattern of urinary P excretion has also been described in rainbow trout (Sugiura et al., 2000b; Coloso et al., 2003; McDaniel et al., 2005), milkfish (Sumagaysay-Chavoso, 2003), haddock and Atlantic salmon (Roy and Lall, 2004) or yellow tail (Sarker et al., 2009). Coloso et al. (2003) reported two peaks in urinary P excretion in rainbow trout (12 °C), a short sharp first peak during 0-1h and a more prominent peak 4-6h after feeding. At the digestive level, inorganic P sources are reported to be better available than ingredient bound organic forms in salmonids (Lall, 1991; 2002; Rodehutscord et al. 2000b) and in European sea bass (Pimentel-Rodrigues and Oliva-Teles, 2007). The small but significant interaction observed in the post-prandial profile of plasma P in the present study would suggest that a fraction of dietary P supply from inorganic P sources are absorbed rapidly (within the first hour after feeding) while the rest is absorbed relatively later (3-5 h after feeding). An additional early sampling point before 1h in the present study would have provided further insights on this observation. However, existence of a diffusive and saturable component for P transport in intestine of rainbow trout as reported by Avila et al. (2000) possibly explains this pattern.

Analogies between gastro-intestinal absorption of Ca and Mg have been reported in higher vertebrates (Ferment and Touitou, 1985) as well as in rainbow trout (Bucking and Wood, 2007, 2009). Bucking and Wood (2006; 2007) showed that the ionic concentration of Ca and Mg in the chyme and plasma of rainbow trout (10-13 °C) attains a peak 8h post-meal in the plasma. Acidic pH of stomach facilitated absorption whereas the alkaline intestinal environment resulted in precipitation of these divalent cations, thereby decreasing absorption (Bucking and Wood, 2009). Lower levels of plasma Ca and Mg concentrations at 6h compared to 24h observed here is similar to the difference reported in Atlantic salmon (15 °C) between 4h and 24h (Vielma and Lall, 1998). As the diet used in the aforementioned study was a semi-purified diet, the peak could have preceded the 4h time point and started to decline thereafter. The significant decline in plasma concentrations of Ca and Mg 4h to 8h post-feeding (as the chyme passes through the alkaline intestinal environment) can be a combined effect of precipitation and influx of Ca and Mg ions through bile secretions.

While the bioavailability of Fe from different dietary sources has been studied in fish, no information is available on the plasma Fe levels at more than one time point. Standal et al. (1999) suggested the factors affecting Fe bioavailability to be similar between Atlantic salmon and terrestrial vertebrates. Conway et al. (2006) reported that serum Fe levels in humans fed Fe supplemented diet attained a peak during 3-4h post-meal. High levels of dietary Zn and Cu are known to inhibit the intestinal Fe uptake in rainbow trout (Kwong and Niyogi, 2009) and reduce hepatic Fe concentrations in channel catfish (Gatlin and Phillips, 1989b). The differences in the plasma Fe peaks between M and V groups might be related to the higher Zn and Cu content in the V diets. Post-prandial plasma Zn levels measured at 6h intervals in rainbow trout showed that the plasma Zn levels peaked 0-1h after feeding, with no significant increase up to 36h after which it declined (Apines et al., 2003). Savolainen and Gatlin (2010) reported a peak in plasma Zn 2-4h post-meal in striped bass (25-26 °C) fed a soybean meal based diet supplemented with $ZnSO_4$. Similar to Vielma et al. (1998) in Atlantic salmon (15 °C), our data show no difference in plasma Zn levels between 4h and 24h.

As regards Cu, Apines et al. (2003) observed no difference in plasma Cu concentrations in rainbow trout (18 °C) between 0h and 6h, with a peak only at 12h after-meal. We also observed a peak between 9h-12h but it was secondary to a small peak (statistically

insignificant) at 2h after feeding, subsequent to which the plasma Cu rapidly returned to the basal level (at 6h). The lack of adequately spaced early sampling points might have masked the observation of such an initial peak in the study of Apines et al. (2003). Stomach as an effective absorption site for Cu in addition to mid and posterior intestine as suggested by Nadella et al. (2006) possibly explains the two peaks, at 0-2h and 9h. In the case of Mn, a significant peak observed at 1h in VP+ was similar to the peak obtained by Apines et al. (2003) in rainbow trout (18 °C) at 0.5h after feeding with a practical diet supplemented with $MnSO_4$. For Se, although an effect of time was significant, no significant peak could be obtained over the 12h period tested.

The changes in plasma mineral levels observed in the present study show that distinct patterns exist in the post-prandial levels of plasma minerals. This is of significance as plasma mineral levels are often used to access the mineral status and requirements in fish wherein considerable differences exist in the time point of sampling as suggested by Rodehutscord (1996) and Sugiura et al. (2004).. Moreover, significant diet X time interactions for Ca and Zn along with alterations in time point of peak for P, Ca, Mg, K, Cu and Mn among VP+ and VP0 showed that, DCP supplementation of a V diet can alter the absorption pattern of these minerals. This can serve as base-line information for the study of bio-markers on regulation of gastro-intestinal mineral absorption through changes in dietary sources.

Mineral availability, utilisation and balance

The bioavailability of micronutrients from dietary sources is influenced by the form of supply as well as the possible interactions between micronutrients. One way of estimating the bioavailability of minerals is based on their post-prandial plasma concentrations, using the area-under-the-curve (AUC) approach in plasma or serum for a given mineral. In humans, Navarro and Wood (2003) estimated the bioavailability of seven different micronutrients including Fe, Zn and Cu based on their plasma 12h AUC concentrations. The 24h AUC of P clearly reflected the dietary P supply with M and VP+ showing statistically similar values, higher than in VP0. However, improved available P supply to rainbow trout from a complete plant ingredient based diet by di-calcium phosphate (DCP) supplementation had concomitant effects on the 24h plasma AUC of other minerals. In fish fed VP+ diet, AUC of plasma K levels increased whereas Cu, Se and

Zn decreased compared to VP0. Although no reports are available on the measure of mineral availability to fish by the AUC method, these observations agree with the results obtained by apparent digestibility measurements and also with earlier findings that Ca or P supplementation can adversely affect absorption of other micro-minerals in different fish species (Hardy and Shearer, 1985; Satoh et al., 1987; Satoh et al., 1989; Satoh et al., 1992a; Satoh et al., 1993; Vielma and Lall, 1998; Kousoulaki et al., 2010). The ADCs of P, Ca, K, Mg and Mn of diet M were in the range of values reported for rainbow trout (Sugiura et al., 1998b; Sugiura et al., 1999), while those of Cu and Zn were lower by 40-45% and 15-20%, respectively. Compared to VP0, DCP supplementation of VP+ reduced the ADC and/or 24h plasma AUC concentrations of Ca, Cu, Se and Zn. Similar observations were made for ADC of Ca, Mn, Fe and Se in the Atlantic cod (Kousoulaki et al., 2010) and for plasma Mg and Zn in the Atlantic salmon (Vielma and Lall, 1998).

Feeding in the present study was sufficiently long to assess mineral balance by means of a comparative carcass analysis. Requirement for P in rainbow trout to maintain normal whole body P concentration is higher than that required to maximise weight gain, as growth is not affected until the whole body P concentration declines below critical limits (Hardy et al. 1993; Antony Jesu Prabhu et al., 2013). Despite differences in dietary total as well as available P levels between VP0 and VP+, growth was not affected but there was a reduction in the final whole body P content, 24h baseline and AUC of plasma P concentrations were significant in VP0. The relatively lower level of dietary P in VP0 resulted in higher ADC value for P from VP0 compared to VP+ and M, as observed in other studies (Satoh et al., 1992b; Riche and Brown, 1996; Rodehutscord et al., 2000a). The significantly lower retention and final whole body P concentration with VP0 compared to the fish meal control (diet M) and VP+, confirms the insufficiency and poor utilisation of endogenous P from the plant protein ingredients. Various studies have shown that solid and dissolved P losses can be significantly reduced by low FM diets with adequate inorganic P supplementation (Jahan et al., 2001; Coloso et al., 2003; Jahan et al., 2003; Hernandez et al., 2004, 2005; Kaushik et al. 2004). Our data also shows that DCP supplementation to a complete plant ingredient based diet (VP+) could maintain P balance and reduce P waste outputs (faecal and non-faecal) in rainbow trout compared to the control fishmeal diet (M) fed group.

It is generally recognised that rainbow trout and fish in general except for a few marine species may not have a specific requirement for dietary Ca when reared in water containing more than 20 mg Ca L^{-1} (NRC, 1993, 2011). According to Lall (2002), resorption of skeletal tissues and uptake from rearing water can serve as Ca sources to support growth at times of dietary Ca deprivation. Fjelldal et al. (2006) found that high growth rates in post-smolts of Atlantic salmon corresponded with elevated plasma Ca concentrations and high incidence of vertebral deformities. In the present study despite the low Ca supply in VP0, higher plasma Ca concentration (significant for 24h baseline, non-significant for 24h AUC) and significantly low whole body Ca:P ratio (0.4), indicate mobilisation of endogenous Ca to support growth comparable to VP+. It could be argued that this is primarily due to lack of proper bone mineralisation resulting from low available P supply in the VP0 fed trout. However, despite an adequate supply of available P, as in the control and the VP+ fed fish, a low Ca:P ratio (0.6) as was also observed in these groups as compared to the normal range of 0.9-1 reported in rainbow trout (Rodehutscord, 1996). Similar low range (0.5-0.8) was reported by Vielma et al. (1998) when the dietary Ca level was only 1.5 g kg^{-1}DM in rainbow trout reared in low-Ca water (4 mg L^{-1}). Under conditions of adequate available P supply, supplemented Ca (7.5 g kg^{-1} DM) improved bone mineralisation in blue tilapia reared in low-calcium water (O'Connell and Gatlin, 1994) and also in juvenile Atlantic salmon reared in freshwater with 15 mg L^{-1} dissolved Ca (Vielma and Lall 1998). Vielma and Lall (1998) suggested that, improved bone mineralisation in Atlantic salmon by Ca supplementation cannot be excluded especially with increasing incorporation of plant protein ingredients having low Ca:P ratios compared to diets with FM. Our data also show that even under adequate available P supply and appreciable levels of water-borne Ca (40 mg L^{-1}), a low dietary supply of available Ca can drastically reduce the whole body Ca reserves. Although according to (NRC, 2011), a dietary Ca supply may not be required for salmonids under practical conditions, more investigation is needed especially in fast growing fish to analyse the possible role of dietary Ca availability on whole body Ca balance as emphasised recently (Hossain and Yoshimatsu, 2014).

The gastro-intestinal absorption of Mg is regulated by dietary intake in rainbow trout (Shearer and Åsgård, 1992; Bucking and Wood, 2007). Reflecting this, both 24h baseline and AUC did not differ significantly between the groups as the dietary Mg concentrations

were similar. The close interrelation in the regulation of Mg metabolism with P and Ca is well recognised in terrestrial vertebrates (Hardwick et al., 1991). Magnesium balance as affected by changes in dietary Ca (Robinson et al., 1987; Oconnell and Gatlin, 1994) or P (Skonberg et al., 1997; Vielma and Lall, 1998) has also been reported in fish. Similar to our observation, a reduction in whole body Mg was reported in Atlantic cod (Kousoulaki et al., 2010), rainbow trout (Skonberg et al., 1997) and in the exoskeleton of pacific white shrimp (Cheng et al., 2006) when fed diets low in Ca or P and the magnitude of reduction was more with P deficiency. Despite a higher ADC of Mg in VP0 than in VP+, significantly lower whole body Mg, daily gain and retention (total and post-absorptive) values in trout fed diet VP0 than in the other groups suggest an increased non-faecal loss of absorbed dietary Mg. Enhanced renal reabsorption of Ca induced by low dietary supply to maintain plasma Ca levels (Quamme, 1997) could have reduced Mg reabsorption resulting in excess Mg loss in the urine as suggested by Bijvelds et al. (1998).

The plasma K levels (measured as AUC) in M group were significantly lower than in V groups despite the dietary K level in M being twice that in the V diets. Significantly high 24h AUC in plasma K suggest that additional DCP might have enhanced the post-absorptive retention of K by reducing the non-faecal K excretion. The interaction between K and P or Ca has not been reported in fish as the dietary essentiality of K to fish has often been largely overlooked. Shearer (1988) reported a dietary K requirement of 6 to 12 g kg^{-1} DM in chinook salmon. Wilson and Naggar (1992) observed no dose-response relationship between dietary and whole body K concentration in Channel catfish, but based on whole body K balance, they could demonstrate a requirement for K which could be met by either a dietary supply of K or the uptake of K from the rearing water.

Dietary iron absorption depends on the chemical form of iron (Standal et al. 1999; Andersen et al. 2003); other nutrients such as ascorbic acid, fructose or amino acids can enhance the absorption of iron, whereas phytates, oxalates and phosphates reduce Fe absorption (Hurrell and Elgi, 2010). The ADC of Fe in the diet VP0 was about 33% whereas no reliable estimate could be made for the other diets. Sugiura et al. (1998b; 2000a) in rainbow trout and Leenhouwers et al. (2007) in African catfish have similarly reported low or negative ADC for Fe in different fish meals and other ingredients.

Kousoulaki et al. (2010) reported that supplementation of Ca and P salts reduced the ADC of Fe to Atlantic cod. Studies in terrestrial vertebrates also show that excess calcium taken along with the diet containing Fe may adversely affect Fe absorption (Cook et al., 1991; Whiting, 1995; see Hurrell and Egli, 2010). The higher ADC of Fe in VP0 than in the other two groups also suggests a similar phenomenon. However, no significant differences were observed in plasma Fe levels, daily gain, retention and final whole body Fe concentration among the groups. The high levels of Fe in the diets, almost 3-4 times the recommended levels (60 mg/kg DM) for salmonids (NRC, 2011), might have reduced the effect of Ca as suggested by Cook et al. (1991) in humans. The high dietary Fe may be a possible reason for the very low retention (3-7%) of dietary Fe in this study. Thus studying the effect Ca or P on absorption and utilisation of Fe at dietary Fe concentration close to the requirement of the species could be more informative.

Fishmeal-based diets often contain levels of Se and Zn well above the requirements, but their availability appears to be lower than that from other sources (Bell and Cowey, 1989; Lorentzen and Maage, 1999). Supplemental Zn as $ZnSO_4$ improved the whole body and tissue zinc status in rainbow trout fed a fish meal based diet (Rider et al., 2010). However, in plant ingredient based diets, the bioavailability of endogenous as well as supplemented Zn is reduced by phytates and supplemented inorganic phosphate salts, leading to reduction in ADC, plasma, whole body and tissue Zn concentrations (Hardy and Shearer, 1985; Satoh et al., 1987; Satoh et al., 1989; Satoh et al., 1992a; Satoh et al., 1993; Vielma and Lall, 1998; Kousoulaki et al., 2010). Data from the present study confirms this as shown by reduction in the ADC, 24h plasma Zn baseline and AUC, daily gain and final whole body Zn levels in VP+ compared to VP0. Reports on the effect of P or Ca supplementation on other micro-minerals such as Mn, Cu and Se are scarce. A significant effect of diet on the post-prandial plasma mineral concentrations and AUC of Cu and Se between VP+ and VP0 show that DCP reduced Cu and Se absorption as observed for ADC in Atlantic cod (Kousoulaki et al., 2010). Whole body Cu gain was also affected but final retention showed no significant difference due to high variations.

Conclusion

The data on the 24h post-prandial profiles of plasma minerals reported here show that plasma concentrations of P, Ca, Mg, K, Cu, Fe, Mn and Zn exhibit a clear pattern with at

least one significant peak whereas no clear peak is observed for Se. Plasma levels, whole body concentration or utilisation of macro-minerals (P, Ca, Mg and K) were improved and micro-minerals especially Zn and Cu were adversely affected by DCP supplementation of a complete plant ingredient based diet.

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CHAPTER 6

Comparison of endogenous loss and maintenance need for minerals in rainbow trout (*Oncorhynchus mykiss*) fed fishmeal or plant ingredient-based diets

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Abstract

Mineral needs as affected by changes in dietary protein and oil sources were studied in rainbow trout. Duplicate groups (n=30 fish per replicate) of rainbow trout (initial BW: 37g) were fed either a fish meal / fish oil based (M) or a complete plant ingredient (V) based diet at four graded ration (R) levels (apparent satiation (AS), R75, R50 and R25% of AS); one treatment group was maintained under starvation. The feeding trial lasted 12 weeks at a water temperature of 17°C. Dietary intake, apparent digestibility, initial and final whole body composition data were used to calculate mineral gain which were regressed against digestible mineral intake (both expressed as mg or µg $kg^{-0.8}$ d^{-1}). Starvation loss (SL), endogenous loss of fed fish (ELF, y-intercept at x=0) and point of intake for zero balance (PZB, x-intercept at y=0) were used as estimates of maintenance requirements. SL provided the lowest estimate, ELF provided the net requirement of a mineral for maintenance and PZB provided the digestible dietary intake required to meet maintenance (SL<ELF<PZB). Dietary ingredient composition did not significantly affect the digestible mineral supply required for maintenance (PZB) for any of the minerals (P, Mg, K, Cu, Zn) studied. However, ELF of micro-minerals such as Cu and Zn were significantly affected. The ELF of Cu was significantly lower and that of Zn was significantly higher in V group compared to M fed fish. Further studies on the effects of such changes in dietary formulations on micro-mineral metabolism are warranted.

Keywords: rainbow trout; dietary changes; minerals; endogenous loss; maintenance.

Introduction

Maintenance requirement for a nutrient is the level of intake required to compensate for obligatory losses and for maintaining nutrient balance, i.e. no gain-no loss situation (Mitchell, 1962; Pfeffer and Potthast, 1977; Cho and Kaushik, 1990). Maintenance requirement can be determined by analysing the relation between nutrient gain and nutreient intake obtained by feeding diets composed of semi-purified or practical ingredients, containing graded levels of the target nutrient (Pfeffer and Pieper, 1979; NRC, 2011). This method is not favourable for minerals, low levels of certain minerals in semi-purified diets result in biased mineral balances, whereas the response at higher inclusion levels can be due to differences in feed intake or in the utilisation of the mineral (Baker, 1984). Further, with practical ingredients, it is difficult to formulate a basal diet with sufficiently low mineral levels. Instead, the factorial method involves feeding graded ration levels of a diet with known nutrient concentration (Baker, 1984; Cowey, 1992). Using this method, maintenance requirements have been determined in fish for many nutrients and digestible energy (Gatlin et al. 1986; Shearer, 1995; Lupatsch et al. 1998; Rodehutscord and Pfeffer, 1999; Fournier et al. 2002; Bureau et al. 2006; Glencross, 2008; Helland et al. 2010). Such knowledge on minerals is lacking in fish.

In animals, starvation loss, endogenous loss of fed animals or level of intake for zero balance have been used to estimate mineral requirements for maintenance, the latter two factors can be affected by changes in the ingredient composition of the diets (Dilger and Adeola, 2006a; 2006b). Given the recent changes in the composition of fish feeds with significant reductions in fish meal levels, it is considered worth studying the effect of this dietary change on basal needs and utilisation of minerals. Hence, the objective of this study was to estimate and compare the mineral (P, Mg, K, Cu and Zn) maintenance needs in rainbow trout when fed diets containing either fish meal and fish oil (diet M) or plant protein sources and vegetable oils (diet V).

Material and methods

Experimental diets

Two practical diets of varying ingredient compositions, based either on fish meal and fish meal (diet M) or on proteins and oils of plant origin (diet V) were used (Table 1).

They were formulated to have similar digestible protein contents. Both the diets were supplemented with vitamin (1%) and mineral (1%) blends. Diet V was further supplemented with 2.7% dicalcium phosphate in order to meet the digestible P requirement of rainbow trout juveniles (NRC, 2011).

Table 1: Ingredient composition of the experimental diets

Ingredients (%, as fed basis)	**M (Fish meal based)**	**V (Plant based)**
Norwegian herring meal, (CP 70; Sopropêche, France)	60	-
Corn gluten meal (CP 60; Inzo, France)	-	18
Wheat gluten (CP 70; Roquette, France)	-	20
Soybean meal (CP 48; Inzo, France)	-	8.3
Soy protein concentrate (Estrilvo; Sopropêche, France)	-	15
White lupin meal (Terrena, France)	-	7.2
Extruded peas (Aquatex, Sotexpro, France)	-	4.9
Whole wheat	24	-
Soy lecithin (Louis François, France)	-	2
L-lysine (Eurolysine)	-	1.4
L-methionine (Evonik, Germany)	-	0.3
CaHPO4.2H20 (18%P; 22% Ca)	-	2.9
Attractant mix†	-	1.5
Mineral premix§	1	1
Vitamin premix¶	1	1
Fish oil (Southern hemisphere, Sopropêche, France)	14	-
Rapeseed oil (Daudruy, France)	-	5
Linseed oil (Daudruy, France)	-	6.5
Palm oil (Daudruy, France)	-	5
Analysed macro-nutrient composition		
Dry matter (DM), %	91.6	93.6
Crude protein (Nx6.25), % DM	44.3	50.5
Crude lipid, % DM	23.7	19.1
Crude ash, % DM	10.3	6.3
Gross energy, kJ g^{-1} DM	23.1	23.5
Analysed mineral composition		
P, g kg^{-1} DM	15	12
Mg, g kg^{-1} DM	2.9	2.5
K, g kg^{-1} DM	11	9.5
Ca, g kg^{-1} DM	18.7	13.9
Na, g kg^{-1} DM	12.0	6.3
Cu, mg kg^{-1} DM	18	18
Zn, mg kg^{-1} DM	122	83

† Attractant mix: glucosamine, 0.5g; taurine, 0.3g; betaine, 0.3g; glycine, 0.2g and alanine, 0.2g.

§ Mineral premix (g or mg kg^{-1} diet): calcium carbonate (40% Ca), 2.15 g; magnesium oxide (60% Mg), 1.24 g; ferric citrate, 0.2 g; potassium iodide (75% I), 0.4 mg; zinc sulphate (36% Zn), 0.4 g; copper sulphate (25% Cu), 0.3 g; manganese sulphate (33% Mn), 0.3 g; dibasic calcium phosphate (20% Ca, 18%P), 5 g; cobalt sulphate, 2 mg; sodium selenite (30% Se), 3 mg; KCl, 0.9 g; NaCl, 0.4 g (UPAE, INRA).

¶ Vitamin premix (IU or mg kg^{-1} diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg (UPAE, INRA).

Two similar diets (M^c and V^c), prepared with incorporation of an inert marker, (chromic oxideat1%) by replacing equivalent proportion of whole wheat, were used for apparent digestibility measurements.

Fish, feeding and rearing conditions

The experimental animals (rainbow trout, *Oncorhynchus mykiss*) used were produced by the unit of Génétique Animale and Biologie Intégrative (GABI), INRA, Jouy-en-Josas, France. The fish were maintained at the experimental fish farm of INRA at Donzacq in a flow through rearing system. Prior to the experimental period, the fish were randomly allotted into 18 experimental units of 30 fish each and fed with a commercial diet. During the study, duplicate groups (30 fish $tank^{-1}$) of rainbow trout (initial BW: 36.5±0.9g) were fed one of the two experimental diets at four ration (R) levels, twice a day, 6 days a week for 12 weeks. The ration levels were 'apparent satiation' (AS), 75% (R75), 50% (R50) and 25% (R25) of apparent satiation, respectively. Two other groups of fish were kept starved during the entire length of the study period. The water temperature was 17.0±0.5° C and the dissolved oxygen concentration of the water was always maintained at or above 6 mg L^{-1}. The analysed mineral concentrations of the rearing water (mg L^{-1}) were P, < 0.2; Ca, 41.7 ± 6.3; Mg, 19.2 ± 0.8; K, 1.8 ± 0.1; Fe, < 0.02; Mn, < 0.02; Cu, < 0.008 and Zn, < 0.007.

Sampling

At the beginning of the feeding trial, 12 fish were sampled for analysis of whole body composition. During the study, the fish were group weighed every two weeks. At the end of the 12 week feeding trial, 6 fish from each experimental unit were sampled for body composition analysis. The fish were sedated in a solution of benzocaine (30 mg L^{-1}), prior to each weight sampling. Similarly, prior to sampling for whole body analysis, the fish were completely anaesthetised in benzocaine (30 mg L^{-1}), euthanized subsequently by a sharp blow to the head and frozen. The frozen fish were homogenously ground, freeze dried and stored at 4°C until further analysis.

Apparent digestibility trial

Rainbow trout (*Oncorhynchus mykiss*) juveniles (initial BW: 35g) belonging to the same population were held (12 fish per unit) in six cylindro-conical fibre-glass tanks (each 60 L) connected to a recirculation system with a flow rate of 4 L min^{-1} at St-Pee-sur-Nivelle,

INRA, France. The fish were fed one of the two diets (M^c or V^c), in triplicate groups. The water was well aerated and thermostatically maintained at 17.0±0.5 °C. The fish were allowed to adapt to the rearing conditions for 2 weeks, after which collection of faeces was performed by the method of Choubert et al. (1982). The faeces were collected on a daily basis for a period of two weeks and stored at -20°C. The faeces collected over two weeks from each tank were pooled, freeze dried and used for further mineral analysis. The apparent digestibility coefficients (ADC, %) of minerals were calculated as follows, $\text{ADC}, \% = 100 - \left\{100 \text{ x} \left(\frac{\% \text{ mineral in faeces}}{\% \text{ mineral in diet}} \text{ x } \frac{\% \text{ marker in diet}}{\% \text{ marker in faeces}}\right)\right\}$.

All the experimental conditions and sampling procedures were in agreement with the guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree no. 2001-464, May 29, 2001) and the animal ethics committee of INRA (INRA 2002-36, April 14, 2002).

Chemical and mineral analysis

The moisture content of the fish was estimated by drying the freshly ground samples at 105°C for 24h. The chemical composition of the diets and freeze-dried whole fish samples were analysed as follows: dry matter as mentioned above, gross energy content using an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany), crude protein (Nx6.25) by Kjeldahl method in acid digested samples, crude lipid by petroleum ether extraction using Soxhlet method (Soxtherm, Gerhardt, Germany) and ash content by combustion at 600°C for four hours in a muffle furnace. The concentrations of P, Mg, K, Cu, and Zn in the diets, whole fish and faeces were analysed using inductively coupled plasma-radial spectrometry (ICP-RS) at USRAVE, INRA, Bordeaux, France. Chromic oxide in the diet and faeces were analysed by the method of Bolin et al. (1952).

Data analysis

Students' t-test was used to analyse data on apparent digestibility of minerals (Table 2). Daily mineral intake and gain were calculated as unit per metabolic body weight (mg or µg $kg^{-0.8}$ d^{-1}). Mean metabolic body weight (MBW) was calculated as $[(\text{initial BW})^{0.8} \text{ x } (\text{final BW})^{0.8}]^{0.5}$. Apparent digestibility coefficient (ADC, %) was used to calculate the mineral intake on digestible basis. Starvation loss (SL) was calculated through mass balance from the initial and final body mineral composition data of the starved group. Endogenous loss of fed fish (ELF), utilisation efficiency (b) and point of intake for zero

balance (PZB) were estimated by regressing whole body mineral gain as the dependent variable (y) and digestible mineral intake as the independent variable (x) for P (Fig.1a), Mg (Fig.1b), K (Fig.1c), expressed as mg $kg^{-0.8}$ d^{-1} and Cu (Fig.2a), Zn (Fig.2b), expressed as µg $kg^{-0.8}$ d^{-1}. Simple linear regression (y = a + bx) was used to estimate parameters such as slope (b), y-intercept (a, ELF) and x-intercept (-a/b, PZB) for specific minerals. Logistic regression of Gahl et al. (1991) was used for the analysis on re-calculated data from literature on phosphorus (Fig.3a) and potassium (Fig.3b). Logistic regression was preferred over simple linear regression for literature data as the data did not fit the model of simple linear regression. One way ANOVA was used to compare SL, ELF of M and V groups. Regression analyses along with comparison of slope and intercepts between M and V groups were performed using unpaired t-test in GraphPad Prism version5 for windows (GraphPad software, San Diego, CA, USA). The standard error along with the 95% confidence interval was used to compare the x-intercepts (-a/b, PZB) of P and Cu, wherein the slopes were significantly different between M and V groups.

Results

Apparent digestibility coefficients (ADC, %) for the minerals from the present study are presented in Table 2. ADC for P, Mg and Cu were significantly higher in V diet fed groups while for K, groups fed M diet showed higher ADC ($p<0.01$). ADC of Zn was not different between the two dietary treatments ($p>0.05$).

Table 2: Apparent digestibility coefficients (ADC, %) of minerals in rainbow trout juveniles fed the M or V diet.

	P	Mg	K	Cu	Zn
Diet M	40.7	48.3	99.6	37.3	22.9
Diet V	47.9	57.1	98.3	74.8	21.2
pSE†	1.8	3.0	0.1	1.3	1.8
P-value§	0.005	0.007	0.001	0.0001	0.227

† pSE, pooled standard error.

§ Test of statistics was performed using an unpaired t-test in GraphPad Prism version5 for MS Windows (GraphPad software, San Diego, CA, USA).

Table 3: Feed intake, growth and feed efficiency in rainbow trout juveniles starved or fed diet M or V at different ration levels for 12 weeks.

		Initial BW, g	Final BW, g	Feed intake, g fish^{-1}	Wt. gain, g fish^{-1}	Wt. gain, % ‡	FE $
Starved		37.8	31.1	-	-6.7	-17.7	-
Diet M	**R25**†	36.3	48.9	12.8	12.6	34.7	1
	R50†	36.5	63.1	24.4	26.5	72.7	1.1
	R75†	37.2	99	48.1	61.8	165.9	1.3
	AS§	35.1	136.2	77.7	101	287.6	1.3
Diet V	**R25**†	36.2	47.8	12.4	11.6	32	0.9
	R50†	36.4	63.2	24.2	26.9	73.9	1.1
	R75†	36.5	88.8	45.5	52.3	143.2	1.1
	AS§	35.8	123.3	79.3	87.6	244.9	1.1
	pSE¶		1.8	1.2	1.7	2.4	0.2

†R, ration level; §AS, apparent satiation; ¶pSE, pooled standard error; ‡Weight gain, (WG, %) = 100 x [(final – initial BW) / initial BW]; $Feed efficiency (FE) = weight gain (g) / feed intake (g)

Table 4: Initial and final body mineral composition of rainbow trout starved or fed diet M or V at different ration levels for 12 weeks (wet weight basis).

		P, g kg^{-1}	Mg, g kg^{-1}	K, g kg^{-1}	Cu, mg kg^{-1}	Zn, mg kg^{-1}
	Initial	3.85	0.27	3.55	1.43	17.9
	Starved	5.08	0.29	3.49	1.29	20.1
Diet M	**R25**†	2.83	0.23	3.18	2.12	13.4
	R50†	3.94	0.30	4.13	1.22	19.4
	R75†	4.02	0.29	3.99	1.60	17.9
	AS§	3.68	0.27	3.74	2.05	16.1
Diet V	**R25**†	2.22	0.19	2.70	1.84	11.4
	R50†	3.48	0.26	3.76	2.60	14.8
	R75†	3.11	0.27	3.98	2.69	14.9
	AS§	2.80	0.26	3.93	2.95	14.1
	pSE¶	0.54	0.11	0.28	0.81	0.92

†R, ration level; §AS, apparent satiation; ¶pSE, pooled standard error.

Data on feed intake, growth and body mineral composition provided in Table 3 and 4 were used for calculating daily mineral gain per unit metabolic body weight to be further used in regression analysis, the results of which are presented in Table 5; Fig. 1 and 2.

The observed mineral loss during starvation (SL) and parameter estimates of regression analysis i.e. such as y-intercept (a, ELF), slope (b) and x-intercept (-a/b, PZB) are

presented in Table 5. Analysis of variance showed no significant difference between starvation loss (SL) and endogenous loss of fed fish (ELF, y-intercept) obtained through regression from M or V diet groups for macro-minerals P ($p=0.66$), Mg ($p=0.15$) and K ($p=0.10$). Also the estimated ELF for P (3.4 mg $kg^{-0.8}$ d^{-1}), Mg (0.85 mg $kg^{-0.8}$ d^{-1}) and K (10.5 mg $kg^{-0.8}$ d^{-1}) were not significantly different between M and V diet groups. For micro-minerals, namely Cu ($p=0.004$) and Zn ($p=0.025$), the differences between SL and ELF were significant. Moreover, the estimate of ELF of Zn from M diet fed group (36.1 µg $kg^{-0.8}$ d^{-1}) was lower than the 57.5 µg $kg^{-0.8}$ d^{-1} obtained with V diet group. A similar but opposite trend was observed for Cu wherein ELF estimate from M fed group (6 µg $kg^{-0.8}$ d^{-1}) was higher than from V group (4.1 µg $kg^{-0.8}$ d^{-1}). Utilisation efficiency of digestible P ($p<0.0001$) and Cu ($p<0.001$) for gain were significantly higher in diet M fed group and no significant difference was observed for the other minerals between the two diet groups. Estimated digestible intake to meet maintenance requirement (PZB, x-intercept) was not significantly different between the two diet groups for all the five analysed minerals ($p>0.5$).

Discussion

Starvation loss (SL) and endogenous loss of fed fish (ELF)

Mineral requirements for maintenance in fish have been subjected to limited investigation. Starvation loss (SL) was used as a measure of maintenance requirement of essential minerals such as P, Ca, Na, K and Mg in rainbow trout (Pfeffer and Potthast, 1977). The SL of Mg (0.42 mg $kg^{-0.8}$ d^{-1}) obtained in the present study was similar to SL of 0.44 and 0.60 mg $kg^{-0.8}$ d^{-1} calculated from Pfeffer and Potthast (1977) in rainbow trout and from El-Mowafi et al. (1997) in Atlantic salmon, respectively. In case of K, data from Pfeffer and Potthast (1977) provide a calculated SL in rainbow trout of 17 mg K $kg^{-0.8}$ d^{-1}, almost twice the 9 mg K $kg^{-0.8}$ d^{-1} SL value from the present study. Also for P, calculated SL values from the above studies (3.2 and 8 mg P $kg^{-0.8}$ d^{-1}) were higher than the 2.0 mg P $kg^{-0.8}$ d^{-1} SL value in the present study.

Table 5: Mineral loss observed during starvation (SL), endogenous loss of fed fish (ELF) and point of intake for zero balance (PZB) in rainbow trout fed diet M or V estimated through regression of daily mineral gain over intake.

Mineral	SL†	ELF § (-a)		pSE¥	P-value¥	Slope ¶ (b)		pSE	P-value£	PZB ‡ (-a/b)		pSE	P-value£
		M	V			M	V			M	V		
P$	2.01	3.7	2.9	3	0.66	0.95	0.55	0.07	0.001	3.89	5.37	6.16	0.81
Mg$	0.42	0.74	1.1	0.55	0.15	0.36	0.33	0.05	0.53	2.08	3.28	1.36	0.17
K$	9	7.6	13.6	7.8	0.10	0.59	0.71	0.09	0.22	12.87	19.23	9.56	0.41
Cu*	3.2 ^{a}	6 ^{b}	4.1 ^{a}	1.5	0.004	0.32	0.23	0.02	0.001	18.72	18.19	6.68	0.98
Zn*	18.4 ^{a}	36.1 ^{ab}	57.5 ^{b}	17.5	0.025	0.85	0.96	0.13	0.49	42.58	59.69	22.27	0.92

† SL, observed loss during starvation;

§ ELF (-a, y-intercept), endogenous loss of fed fish estimated through regression analysis;

¶ Slope, (b), utilisation efficiency of absorbed mineral intake for gain;

‡ PZB (-a/b, x-intercept), digestible intake to meet maintenance requirement.

$ SL, ELF and PZB expressed as milligram per unit metabolic body weight per day (mg $kg^{-0.8}$ d^{-1})

* SL, ELF and PZB expressed as microgram per unit metabolic body weight per day (µg $kg^{-0.8}$ d^{-1}).

¥ One-way ANOVA was used to test the statistical difference between SL, ELF (M) and ELF (V). Values with different superscript within a row are statistically different ($P<0.05$). pSE, pooled standard error.

£ Regression analyses along with comparison of slope and intercepts between M and V groups were performed using unpaired t-test in GraphPad Prism version5 for windows (GraphPad software, San Diego, CA, USA). The standard error along with the 95% confidence interval was used to compare the x-intercepts (-a/b, PZB) of P and Cu, wherein the slopes were significantly different between M and V groups.

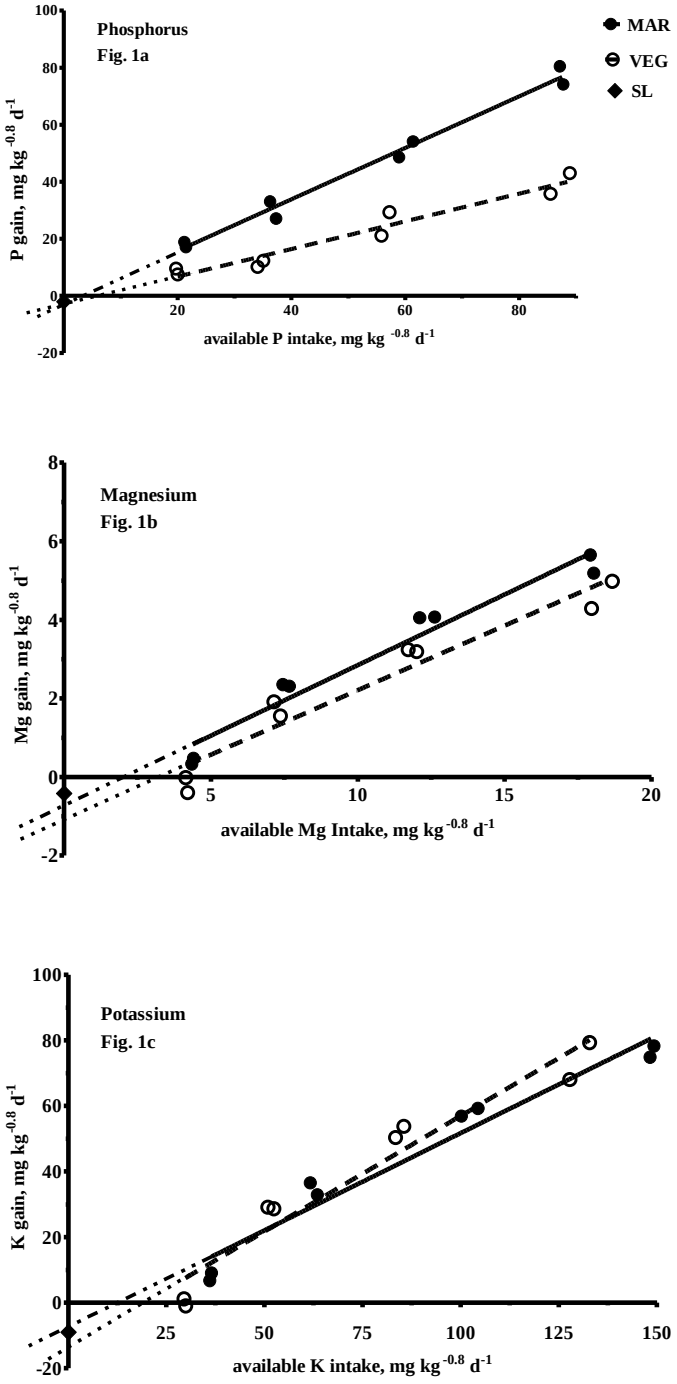

Figure 1: The daily digestible intake of macro-minerals namely P (Fig.1a), Mg (Fig.1b) and K (Fig.1c) plotted against daily whole body gain (both expressed as mg $kg^{-0.8}$ d^{-1}) in rainbow trout fed M (filled circles and solid line) or V (open circles and dashed line) diets. The observed starvation loss (SL) is plotted on y-axis (filled diamond). The regression lines of M and V groups are extrapolated by 'dotted lines' to intersect the y-axis and x-axis at corresponding estimates of endogenous loss of fed fish (ELF) and point of intake for zero balance (PZB), respectively.

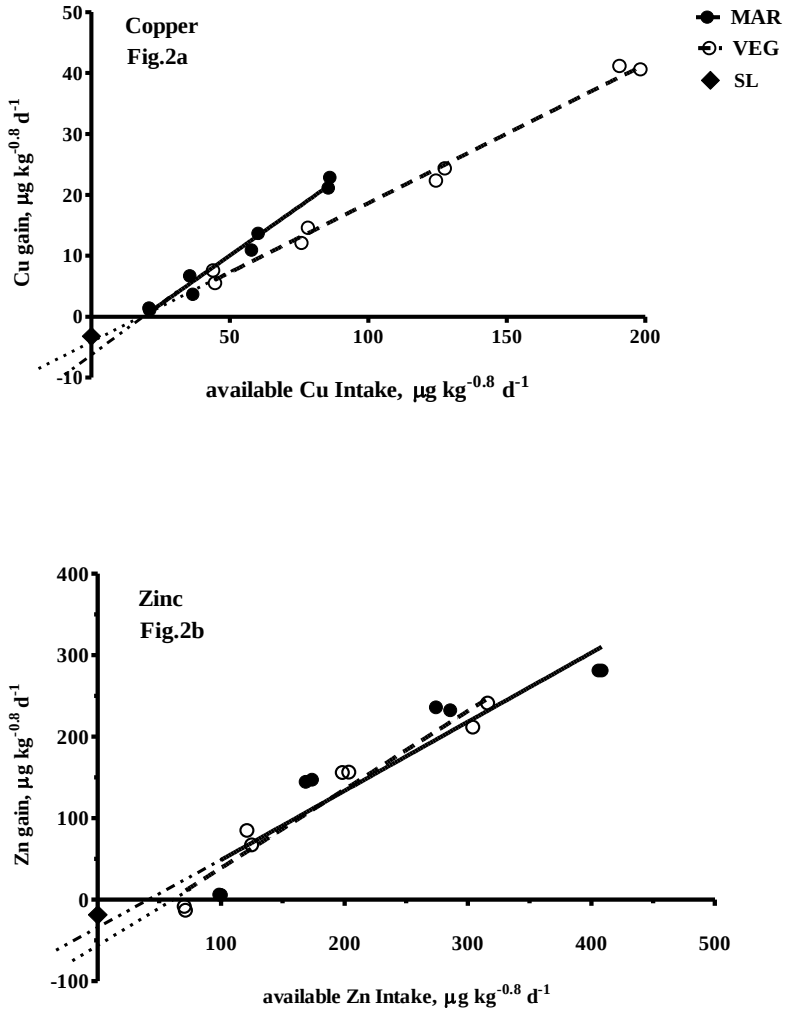

Figure 2: The daily digestible intake of micro-minerals namely Cu (Fig.2a) and Zn (Fig.2b) plotted against daily whole body gain (both expressed as µg $kg^{-0.8}$ d^{-1}) in rainbow trout fed M (filled circles and solid line) or V (open circles and dashed line) diets. The observed starvation loss (SL) is plotted on y-axis (filled diamond). The regression lines of M and V groups are extrapolated by 'dotted lines' to intersect the y-axis and x-axis at corresponding estimates of endogenous loss of fed fish (ELF) and point of intake for zero balance (PZB), respectively.

Calculated SL values for the microminerals Zn and Cu (El-Mowafi et al. (1997) in Atlantic salmon were 34.4 and 1.8 µg $kg^{-0.8}$ d^{-1} respectively, being again higher in case of Zn but lower for Cu as compared to the present SL values. The discrepancy in daily SL rates observed between the present study and published values for the studied minerals (except for Mg) may be related to the length of the starvation period. In Atlantic salmon starved for 6 weeks, the rate of daily loss of minerals was higher during the initial days

of starvation compared to later stages (El-Mowafi et al. 1997). The lower daily SL rate observed in the present study as discussed above could be due to the longer period of starvation (12 weeks) compared to that in the studies of Pfeffer and Potthast (1977) and El-Mowafi et al. (1997) (4 to 6 weeks). This indicates that SL rates do not provide a realistic estimate of the maintenance requirements for minerals in fish. In addition, SL may underestimate the true maintenance requirement of a fed fish since the digestion and processing of food in the alimentary tract may lead to increased loss of certain nutrients especially minerals (Pfeffer and Pieper, 1979). This is confirmed here by the numerically lower values obtained following starvation than by extrapolating endogenous loss of fed fish (ELF) in all cases, except for K in M diet group.

Point of intake for zero balance (PZB)

Due to limited data on maintenance requirements of minerals in fish, we re-calculated estimates from literature on mineral balances. The point of intake for zero balance (PZB), as calculated here, provides the digestible mineral intake required to compensate for the ELF. In other words, PZB for a given mineral equals its endogenous loss (ELF) only if its utilisation efficiency is close to 1 PZB with PZB increasing at low utilisation efficiency levels and decreasing at high efficiency levels. Estimates of digestible P intake level to reach PZB (no gain-no loss equilibrium), obtained by logistic regression (Gahl et al. 1991) of re-calculated P balance data from fish literature (expressed as mg P $kg^{-0.8}$ d^{-1}) were as follows: rainbow trout, 1.7 (Ketola and Richmond, 1994), 1.9 and 2.5 (Ogino and Takeda, 1978), 3.6 (Rodehutscord, 1996); 5.3 (Skonberg et al. 1997); 4 (Bureau and Cho, 1999) Atlantic salmon, 1.2 (Åsgård and Shearer, 1997). The overall regression (Fig.3a) estimate of digestible P requirement for maintenance in salmonids from the above cited literature was 2.7 mg P $kg^{-0.8}$ d^{-1}. This value agrees with present observations, being intermediate between the observed loss due to starvation (SL, 2 mg $kg^{-0.8}$ d^{-1}) and PZB (3.9 mg P $kg^{-0.8}$ d^{-1}) estimates for the M group. It was much lower than the PZB estimate of 5.4 mg P $kg^{-0.8}$ d^{-1} for fish fed diet V which displayed a poor P utilisation efficiency (0.55, Table 5) compared to that in the M group. This leads to higher PZB estimates of maintenance requirement in V group (but not statistically significant), as also seen for amino acids in Atlantic salmon (Grisdale-Helland et al. 2011). In porcine nutrition, Schulin-Zeuthen et al. (2007) using a meta-analytic approach of literature data on P balance from more than 350 pigs, estimated endogenous P loss (ELF as defined in

present study) and the corresponding dietary P required for maintenance (PZB as defined in present study) to be 17 and 37 mg P $kg^{-0.75}$ on total P basis; 14 and 15 mg P $kg^{-0.75}$ on digestible basis. These findings highlight that the difference between endogenous loss of fed animals (ELF) and point of zero balance (PZB) is caused by availability and corresponding changes in efficiency of utilisation. A point worth mentioning here is that we did not specifically measure urinary losses of minerals. It has been shown that nonfecal excretion of P can be high and affected by dietary factors in both freshwater and marine species (Vielma and Lall, 1998; Bureau and Cho, 1999; Roy and Lall, 2004) the measurement of which has indeed been found to be a valid approach to estimate the minimum dietary requirement for P (Sugiura et al. 2000). Data on concentrations of minerals in the urine in farmed fish is indeed limited.

The overall regression estimate of PZB for K obtained from literature data on fish (Fig.3b) was 9.71 mg K $kg^{-0.8}$ d^{-1} (Shearer, 1988; Wilson and Naggar, 1992; Shiau and Hsieh, 2001; Liang et al. 2012a). This is in range with the SL, ELF and PZB of the present study (Table 5). It is well documented that ADC of potassium is between 96-100% in salmonids (Sugiura et al. 1998), similar to the results of the present study (99 ± 0.7, Table 2). Therefore, the value of 9.7 mg K $kg^{-0.8}$ d^{-1} was considered to be digestible intake required for maintenance (PZB), as the correction factor is negligible. However, the higher PZB of the present study (16.1 mg K $kg^{-0.8}$ d^{-1}, Table 5) is due to the low utilisation efficiency of 60-70 % compared to the 90% of the literature data (Shearer, 1988). If corrected for utilisation efficiency, the estimates would be 8.7 and 10.5 mg K $kg^{-0.8}$ d^{-1} from literature data and present study, respectively. In case of Mg, PZB estimates calculated from literature data were highly variable, ranging between 2.4 and 8.2 mg $kg^{-0.8}$ d^{-1} (Shearer, 1989; Shearer and Asgard, 1990; Shearer and Åsgård, 1992; El-Mowafi and Maage, 1998) for salmonids and between 3.9 and 7.3 mg $kg^{-0.8}$ d^{-1} (Dabrowska et al. 1991; Han et al. 2012; Liang et al. 2012b) for cyprinids.

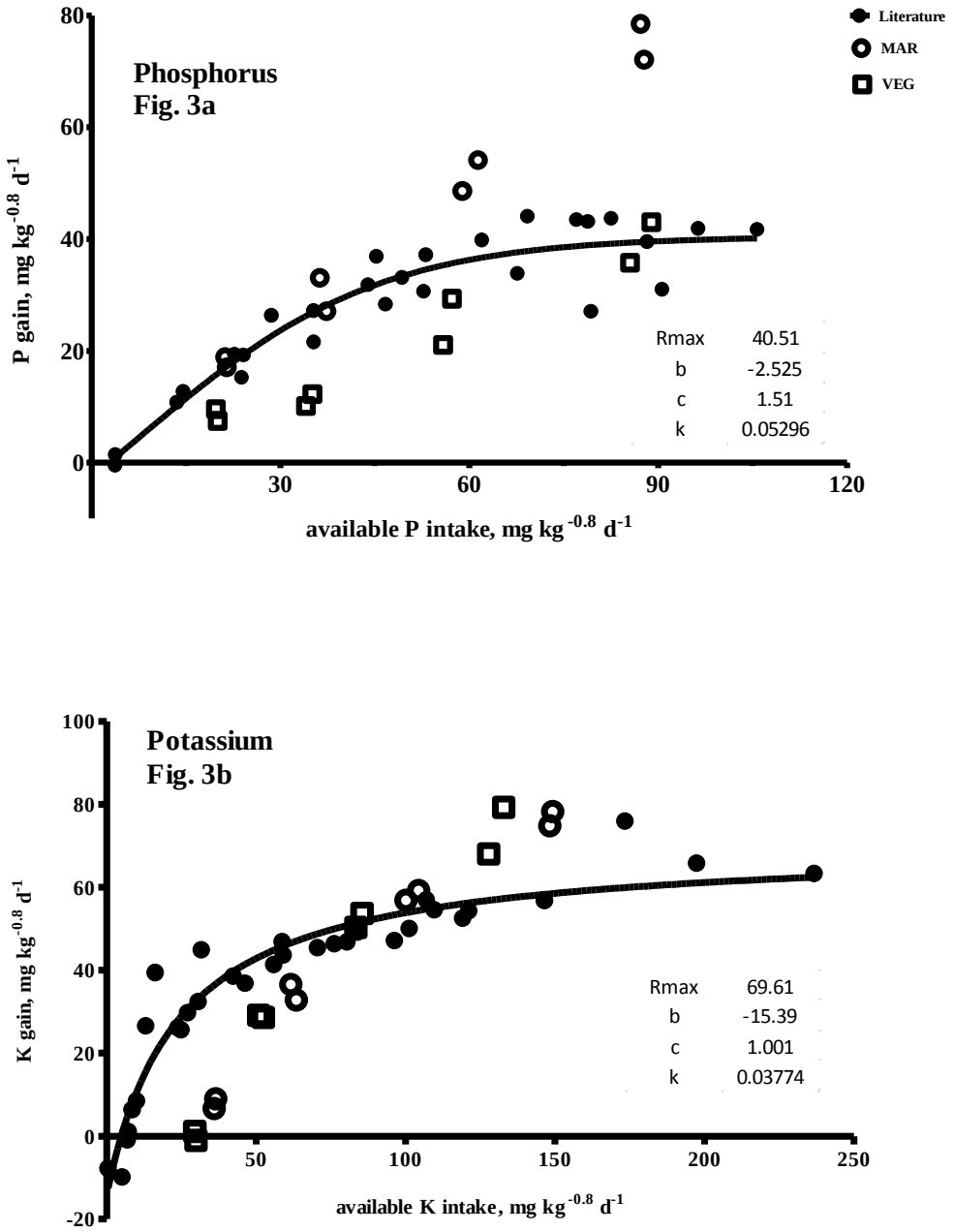

Figure 3: Calculated daily digestible intake and whole body gain (both expressed in mg $kg^{-0.8}$ d^{-1}) data from published literature in salmonids for P, Fig.3a (Ogino and Takeda, 1978; Ketola and Richmond, 1994; Rodehutscord, 1996; Åsgård and Shearer, 1997) and fish in general for K, Fig.3b (Shearer, 1988; Wilson and Naggar, 1992; Shiau and Hsieh, 2001; Liang et al. 2012a). The data of the present study from M (open circles) and V (open squares) groups are also plotted along with the literature data.

Unlike for phosphorus, the lack of data on Mg availability limited the estimation of digestible Mg requirement for maintenance and thus comparison with the current estimates. Taking the estimate of 2.4 mg $kg^{-0.8}$ d^{-1} obtained for rainbow trout (Shearer and Asgard, 1990) and a theoretical assumption of 60% apparent digestibility based on the literature data for Mg from $MgSO_4$ (Shearer and Asgard, 1990; Satoh et al. 1991), the PZB estimate would be 1.4 mg $kg^{-0.8}$ d^{-1} which is lower than the PZB observed in our study (2.7 ± 0.8 mg $kg^{-0.8}$ d^{-1}, Table 5). As seen for potassium, this is due to the low utilisation efficiency of Mg observed in the present study (33-36%) relative to that observed in literature (70-80%). This in turn can be explained by the high digestible Mg content in our diets (1.4 mg kg^{-1} diet) compared to the Mg requirement of rainbow trout (0.5 mg kg^{-1} diet). Subsequent correction for utilisation efficiency would however result in similar estimates of 1 to 1.1 mg $kg^{-0.8}$ d^{-1} in both M and V groups, which is also close to the ELF estimate of 0.7 to 1.1 (Table 5) obtained in this study.

Effect of change in diet composition on ELF and PZB

As stated earlier, it has been shown that ELF and PZB of minerals can be affected by dietary changes in pigs (Dilger and Adeola, 2006b) and poultry (Dilger and Adeola, 2006a). In our study, both ELF and PZB of macro-minerals (P, K and Mg) were not significantly affected by the change in diet composition. Even with micro-minerals, PZB did not significantly differ between the M and V groups. However, our data showed significant differences in extrapolated endogenous loss values (ELF) for both micronutrients (Zn and Cu) between the two dietary groups, higher for Zn and lower for Cu in fish fed diet V compared to those fed diet M. Anti-nutritional factors such as phytic acid, present in plant-derived feed ingredients have been shown to increase the endogenous loss of minerals such as Fe and Na in broiler chickens (Cowieson et al. 2004); Mg, K and Na in piglets (Woyengo et al. 2009). Although such direct evidence is yet to be reported in fish, there are ample indirect indications. We did not measure phytic acid content, but based on ingredient composition of the two feeds, we estimated diet M to contain no phytic acid and diet V to have about 0.27% of phytic acid. It has been often observed that apparent digestibility of dietary Zn is reduced by phytic acid or calcium phytate or even by supplementation of inorganic calcium and phosphorus salts (Richardson et al. 1985; Hardy and Shearer, 1985; McClain and Gatlin, 1988; Gatlin and Phillips, 1989; Satoh et al. 1989; Ramseyer et al. 1999; Kousoulaki et al. 2010; Antony

Jesu Prabhu et al. 2014). This may explain the increased loss of dietary as well as endogenous Zn in fish fed diet V compared to diet M. Moreover, ELF of K ($p=0.11$) and Mg ($p=0.15$) also tended to be slightly higher in trout fed diet V, as reported in piglets when fed diets containing phytic acid (Woyengo et al. 2009). A similar yet opposite phenomenon was observed in case of Cu. The estimated endogenous Cu loss (ELF) was higher with diet M than with diet V, as also reflected by a lower apparent digestibility of Cu in fish fed diet M. Along with renal pathways, hepato-biliary regulation of Cu homeostasis forms a major route of Cu excretion in mammals (Cousins, 1985; Gross Jr et al. 1989; Roelofsen et al. 2000) and also in rainbow trout (Lanno et al. 1987). As such, daily hepatic Cu excretion can be affected by changes in bile synthesis or secretion. In rat, fish oil, rich in polyunsaturated fatty acids, has been reported to increase bile secretion (Levy and Herzberg, 1995, 1996; Du et al. 2004). A similar phenomenon possibly contributed to the higher endogenous Cu loss in trout fed diet M, containing fish oil. On the other hand, soybean meal, present in diet V, has also been reported to adversely affect bile synthesis pathways (Vilhelmsson et al. 2004) and decrease the quantity of bile secreted into the gallbladder (Yamamoto et al. 2007) in rainbow trout and Atlantic salmon (Kortner et al. 2013; Gu et al. 2014). The lack of significant difference between Cu loss in starved group (SL of Cu, Table 5) and the fed groups (ELF for Cu from V diet group, Table 5) suggests that normal homeostatic regulation of Cu through hepato-bilary secretions may be impaired in rainbow trout fed the plant ingredient based diet V. This may lead to accumulation of Cu, possibly explaining the significantly high whole body Cu in fish fed the V diet (Table 4). In mammals, impaired hepato-biliary copper homeostasis is a characteristic feature of Menkes (resulting in Cu deficiency) and Wilson's (resulting in Cu accumulation) diseases. The study of Cu transporting P-type ATPase namely ATP7A and ATP7B homologues and other hepatic Cu transporters in trout might provide basic insights into the effect of changes in dietary ingredients on hepato-biliary Cu metabolism in rainbow trout. Interaction of Cu with Zn has also been reported in rainbow trout (Ojo et al. 2009). Although the existence of mineral interactions is recognised in all animals, there is limited data available today in fish (Antony Jesu Prabhu et al. 2014).

In summary, changes in ingredient composition did not affect the dietary mineral supply required for maintenance (PZB) for any of the minerals studied. However, ELF of micro-minerals such as Cu and Zn were significantly affected. These findings offer interesting

insights for further investigations on the effect of changing dietary composition of fish feeds on micro-mineral metabolism especially that of Zn and Cu in rainbow trout.

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CHAPTER 7

Responses in micro mineral metabolism in rainbow trout (*Oncorhynchus mykiss*) to change in dietary ingredient composition and inclusion of a micro-mineral premix

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Abstract

Responses in micro mineral metabolism to changes in dietary ingredient composition and inclusion of a micro mineral premix (Fe, Cu, Mn, Zn and Se) were studied in rainbow trout. In a 2 x 2 factorial design, triplicate groups of rainbow trout (initial weight: 20g) were fed over 12 weeks at 17°C a fishmeal-based diet (M) or a plant-ingredient based diet (V), with or without inclusion of a mineral premix,. Trout fed the V vs M diet, had lower feed intake, growth, hepato-somatic index, apparent availability coefficient (AAC) of Fe, Cu, Mn and Zn and also lower whole body Se and Zn concentration, whereas whole body Fe and Cu and plasma Fe concentrations were higher. Feeding the V diet increased intestinal ferric reductase activity and transcription of hepatic hemeoxygenase1 and ferroportin1 and decreased that of hepcidin in liver. Transcription of intestinal Cu-transporting ATPases and hepatic copper transporter1 were higher in V compared to M groups. Among the hepatic metaloenzyme activities assayed, only Se-dependent glutathione peroxidase was affected, being lower in V fed fish. Premix inclusion reduced the AAC of Fe, Cu and Zn; increased the whole body concentration of all trace minerals; increased hepatic hepcidin and decreased intestinal ferroportin1 transcription; reduced the transcription of Cu-transporting ATPases in the intestine. An interaction of premix with basal diet was observed for some parameters such as AAC of Fe, Cu and Zn; whole body Se and Mn concentrations; and hepatic transcription of hepcidin and Cu-transporting ATPases. Overall, the regulation of trace mineral metabolism in rainbow trout, especially Fe and Cu was affected both by a change in ingredient composition and premix inclusion.

Keywords: ATP7a; ATP7b; Bile metabolism; Ferroportin; Fishmeal; Hemeoxygenase1; Hepcidin; Plant protein sources; Trace metals; Transporters.

Abbreviations

AAC – Apparent availability coefficient; CR – Cupric reductase; FPN1 – Ferroportin1; FR – Ferric reductase; HAMP – Hepcidin anti-microbial peptide; HO1 – Hemeoxygenase1; M – Marine ingredients; M0 – Marine ingredient basal diet; M1 – Micro-mineral supplemented marine ingredient diet; V – Vegetable ingredients; V0 – Micro-mineral Vegetable ingredient basal diet; V1 – Supplemented vegetable ingredient diet.

Introduction

Trace minerals such as Fe, Cu, Mn, Zn and Se are essential to fish (Ogino and Kamizono, 1975; Kaushik, 2002; Lall, 2002). A low or a high supply of dietary or aqueous trace minerals affects the associated biochemical and physiological responses in fish (Bury *et al.*, 2003). Fish meal, rich in trace minerals (Julshamn *et al.*, 1978), has been the major protein source in feed of farmed fish over the years. However, due to the limited supply, the use of fishery-derived ingredients such as fish meal (FM) in fish feeds, particularly in salmonid feeds, has seen a significant reduction, being replaced to a large extent by plant ingredient sources (Tacon and Metian, 2008). In humans, vegetarian diets are reported to be limiting in the supply of bioavailable trace minerals such as Fe, Zn and Se, but are generally good sources of Cu and Mn. The latter are present at high concentrations in plant ingredients, in contrast with Se which is higher in animal than in plant protein sources (Hunt, 2003). The intrinsic form of the dietary trace minerals is also important. For instance, heme-bound Fe present in fish or meat based diets is relatively more bioavailable than non-heme Fe present in plant-derived ingredients (Standal *et al.*, 1999). It is generally recommended to supplement Fe, Cu, Mn, Zn and Se to fish feeds due to their low and variable levels in practical feed ingredients and also due to possible interactions with other dietary components which may affect their availability (Watanabe *et al.*, 1997). Indeed, secondary metabolites (anti-nutritional factors, ANFs) in plant ingredients may directly or indirectly affect trace mineral metabolism (Francis *et al.*, 2001). The entero-hepato-pancreatic system is the target of major plant ingredient ANFs such as phytic acid, protease inhibitors, lectins, alkaloids (Francis *et al.*, 2001). Phytic acid can directly reduce availability of trace minerals whereas other ANFs may modify uptake and body trace mineral status by interfering in trace mineral metabolism at the level of gastrointestinal tract or liver (Hambidge, 2003). The underlying cellular and molecular mechanisms by which plant ingredients interact with trace mineral metabolism in fish remain however little explored.

Over the years, various functional proteins and their encoding genes regulating cellular transport of trace minerals have been characterised in mammals, a few of which have also been identified in teleost species (Bakke *et al.*, 2010). These include brush border metal reductases and divalent metal transporters (DMTs) for apical uptake of Fe, Cu, Mn or Zn (Bury *et al.*, 2003); ferroportin (FPN1) for basolateral extrusion of Fe; hepcidin

(HAMP) the iron regulatory peptide; and heme-oxygenase (HO) for heme degradation to release Fe (Fraenkel *et al.*, 2005; Rodrigues *et al.*, 2006); Cu-chaperons (ATOX1 and CCS) and Cu-transporting-ATPases, ATP7a and ATP7b for intra-cellular trafficking of Cu (Minghetti *et al.*, 2008; Minghetti *et al.*, 2010); family of ten SLC30A (Zn transporters, ZnT) and fourteen SLC39A (Zn importers, ZIP) genes for cellular import and export of zinc (Zheng *et al.*, 2010; Feeney *et al.*, 2005). Besides the transporters, activity and expression of metaloenzymes have been used as markers for trace mineral status (Hunt, 2003). Most of these markers are shown to respond to exogenous supply of respective trace minerals. In this scenario, the intriguing questions are, (i) does the transition from fish meal based diets to predominantly plant ingredient based diets affect trace mineral metabolism in fish?, and (ii) does the supplementation of a trace mineral premix affect or interact with the ingredient composition of the diet in trace mineral absorption or metabolism? In order to address these questions, we undertook a 2 x 2 factorial design study with rainbow trout fed either a FM-FO based diet (M) or a totally plant ingredient based (V) diet, with or without trace mineral (Fe, Cu, Mn, Zn and Se) premix inclusion, and analysed the responses related to absorption, transport or metabolism of trace minerals.

Material and Methods

Animal experiments and sampling procedures followed the guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree no. 2001-464, May 29, 2001) and the animal ethics committee of INRA (INRA 2002-36, April 14, 2002).The INRA experimental facility is certified under the permit number A402281 for animal services and permit number FR40090951-40090002 for animal feed production by the French veterinary services. The scientist in charge of the experimentation received training and personal authorization (N°B64 11 001).

Experimental diets

Two basal diets namely M0 (FM-FO based) and V0 (plant-ingredient based) were formulated to meet the protein, energy and macro-mineral requirements of rainbow trout (Table 1). The M0 diet was based on fishmeal (FM) and fish oil (FO), while the V0 diet was made entirely of plant-derived ingredients. Essential trace minerals (Fe, Cu, Mn, Zn and Se) were supplemented to the basal diets, as a premix at 1% inclusion level, to

provide diets M1 and V1, respectively. The premix was formulated to provide Fe, 52.5 mg; Cu, 7.5 mg; Mn, 12 mg; Zn, 14 mg and Se, 0.15 mg per kg diet, at 1% inclusion, on as fed basis. These concentrations are based on data on trace mineral requirements of rainbow trout (National Research Council, NRC (NRC, 2011; NRC, 1993). The levels of analysed trace mineral concentrations of the basal and the premix supplemented diets are provided in Table 2.

Table 1: Ingredient (g/kg) and chemical composition of the basal diets

Ingredients	**M**	**V**
Norwegian herring meal, (CP 70; Sopropêche, France)	625.7	-
Corn Gluten meal (CP 60; Inzo, France)	-	180.0
Wheat Gluten (CP 70; Roquette, France)	-	200.0
Soybean meal (CP 48; Inzo, France)	-	50.0
Soy protein concentrate (Estrilvo ; CP 70; Sopropêche, France)	-	170.0
White lupin meal (Terrena, France)	-	50.0
Extruded peas (Aquatex, Sotexpro, France)	-	30.0
Rapeseed meal (Primor 00; Sud Ouest Alimants, France)	-	40.0
Whole wheat	245.6	31.8
Soy lecithin (Louis François, France)	-	20.0
L-Lysine (Eurolysine)	-	13.4
L-methionine (Evonik, Germany)	-	3.0
CaHPO4.2H20 (18%P; 22% Ca)	-	21.7
Attractant Mix§	-	15.0
Trace mineral premix¶	0 or 10	0 or 10
Vitamin premix†	10	10.0
Yttrium oxide (Sigma-Aldrich, USA)	0.1	0.1
Fish oil (southern hemisphere, Sopropêche, France)	118.6	-
Rapeseed Oil (Daudruy, France)	-	66.0
Linseed Oil (Daudruy, France)	-	66.0
Palm Oil (Daudruy, France)	-	33.0
Analytical composition		
Dry matter (DM), g/kg	948	949
Crude protein, g/kg DM	494	500
Crude lipid, g/kg DM	221	227
Crude ash, g/kg DM	82	40
Energy, kJ/g DM	23.8	25.1
Analysed concentration of major minerals (g/kg DM)		
Phosphorus, g/kg DM	12.9	9.4
Calcium, g/kg DM	11.3	8.0
Ca/P ratio	0.9	0.9
Magnesium, g/kg DM	1.76	0.95
Potassium, g/kg DM	12.2	4.2
Sodium, g/kg DM	9.4	1.4

§Attractant premix: glucosamine, 5g; taurine, 3g; betaine, 3g; glycine, 2g and alanine, 2g.
† Vitamin premix (IU or mg/kg diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg. (UPAE, INRA).
¶ Trace mineral premix (g/kg premix): FeSO4.7H2O (21% Fe; 11.5% S), 25 g; CuSO4.5H2O (25.45% Cu; 12.8% S), 3 g; MnSO4.H2O (33% Mn; 19% S), 3 g; ZnSO4.H2O (36% Zn; 18% S), 4 g; Na2SeO3 (46% Se; 27% Na), 0.03 g and α-cellulose, 964.93 g.

Table 2: Analysed dietary concentration of supplemented micro-minerals (mg/kg DM)

Diet code	M0	M1	V0	V1
Iron	161.6	212.9	153.8	205.4
Copper	6.9	14	7.0	12.9
Manganese	9.4	22.7	88.6	100.9
Zinc	62.8	72.5	42.9	52.5
Selenium	1.09	1.27	0.24	0.40

Experimental animals, design and rearing condition

Rainbow trout juveniles (19.8 ± 0.8 g, initial weight) were distributed into 12 experimental units, each of 500 L (40 fish unit^{-1}). Each unit was randomly assigned to one of the four dietary treatments in triplicates following a 2x2 factorial design. The fish were hand fed twice a day to apparent visual satiation for a period of 12 weeks (6 days a week). The fish were reared in flow-through systems at the experimental fish farm (INRA, Donzacq, Landes, France). Water temperature was 17 ± 0.5°C and the water flow rate was set at 50 L min^{-1} during the experimental period.

Fish, tissue and faecal sampling

At the end of the 12 week growth trial and 24h after the last meal, a pooled sample of 6 fish from each experimental unit was taken for final body composition analysis. Further, 3 more fish from each experimental unit were randomly withdrawn, anaesthetised (benzocaine, 30 mg L^{-1}) and sampled for blood from the caudal vein using heparinised syringe. The blood was then centrifuged (3000g for 5 min) and the recovered plasma were immediately stored at -20 °C until mineral analysis. Subsequent to blood sampling, the fish were euthanized by a sharp blow to the head. Liver and anterior intestine (without caeca) were dissected, rinsed in 0.9 % NaCl to remove any blood (in liver) or food and faecal remains (in intestine) and immediately frozen in liquid nitrogen and stored at -80 °C until further analysis. After the tissue sampling, 25 fish of each experimental unit were used for the determination of apparent availability coefficient (AAC) of minerals. The fish were fed a single meal (86 g tank^{-1}) and 9h after the meal, faecal samples were collected by the method of stripping (Antony Jesu Prabhu *et al.*, 2014). The samples were collected over ice, frozen immediately and stored at -20°C until mineral analysis.

Analytical methods

Chemical composition: The chemical composition of the diets was analysed by the following methods: dry matter after drying at 105°C for 24h, ash by combustion at 600°C for four hours in a muffle furnace, crude protein (N x 6.25) by Kjeldahl method in acid digested samples, crude lipid by petroleum ether extraction using Soxhlet method (Soxtherm) and gross energy content in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany). The concentrations of Fe, Cu, Mn and Zn in the diets, whole fish, faeces and plasma were analysed using inductively coupled plasma-mass spectrometry (ICP-OES) at USRAVE-INRA, Bordeaux, France. The Se and yttrium content in the samples (except plasma) were analysed using ICP-MS at LCABIE-UPPA, Pau, France.

Selection of trace mineral responsive bio-markers for molecular analysis: Bio-markers known to be involved in absorption, transport and metabolism of Fe and Cu were studied in detail, based upon the similar levels of Fe and Cu in diets M and V (Table 2), upon the inherent differences in the form of Fe between diets M and V and based upon previous observations of lower endogenous Cu loss when fed diet V (our own unpublished data). The study on the molecular responses of other trace minerals Mn, Zn and Se was restricted to the activity or expression of their respective metalloenzymes such as superoxide dismutase (Mn-SOD and CuZn-SOD); alkaline phosphatase (for Zn) and glutathione peroxidase (for Se), respectively. Details of the primer sequences used for the amplification of the target genes are presented in Table 3.

Enzyme assays: Samples of liver and anterior intestine were ground respectively in 8 and 4 volumes of ice cold buffer (TRIS HCl, 50mM; NaCl, 150mM; pH, 4). After homogenization, the samples were subjected to sonic disruption for one minute. The samples were kept on ice during sonication. Homogenates were then centrifuged for 10 min at 3500 rpm at 4°C and the supernatants were immediately used for enzyme assays. Activities of ferric reductase (FR, EC 1.16.1.7) and cupric reductase (CR, EC 1.16.1) were determined from the same extract. FR activity was measured as described by Mazoch et al. (2004), omitting flavin mononucleotide in the mixture. The reduction of iron was followed by the formation of the coloured Fe(II)-ferrozine complex and monitoring the change in absorbance at 562 nm. CR activity was assayed according to Wyman et al. (2008), the Cu(I)-bathocuprionedisulfonate complex was monitored by the change in

absorbance at 482 nm. ALP activity was measured using a commercial kit (Enzyme ALP, bioMérieux, ref 63509). Antioxidant enzyme activities CAT (EC 1.11.1.6) and GPX (EC 1.11.1.9) were assayed in the liver homogenates following the method of Fontagne et al. (2008). Total SOD (EC 1.15.1.1) activity was measured using a commercial kit (Sigma, St Louis, MO, USA ref 19160-1KT-F); MnSOD (SOD2) was measured by inhibiting Cu-ZnSOD using 5 mM KCN (Sigma) as specific inhibitor and Cu-ZnSOD (SOD1) activity was calculated by subtracting MnSOD activity from total SOD activity (Knox et al., 1981). In all the enzyme assays, the reaction was initiated by the addition of a specific substrate; a blank with water instead of the substrate was run for each sample. One unit of enzyme activity was defined as the amount of enzyme that catalysed the transformation of 1 µmol of substrate per min at 30 °C. Protein concentration of all the samples were measured in triplicate by the method of Bradford (1976), using a protein assay kit (Bio Rad, Munich, Germany) with bovine serum albumin as the standard. In all cases, a Power Wave X (BioTek Instrument, Inc.) was used as the plate reader. The enzyme activities were expressed specific to per mg of protein.

Gene expression analysis: Total RNA was extracted from liver and anterior intestine samples (n=9 per treatment) using Trizol reagent (Invitrogen, Cergy-Pontoise, France) as previously described (Fontagne et al., 2008). For quantitative RT-PCR, complementary DNA was generated from 1 µg total RNA using SuperScript® III reverse transcriptase (Invitrogen) with a mix of oligo$(dT)_{15}$ and random primers (Promega, Charbonnières, France). RT was performed in duplicate for each sample and the quantitative PCR analyses were performed in LightCycler 480 II thermocycler (Roche) using LightCycler 480 SYBR Green I Master mix (Roche Diagnosis, Indianapolis, IN, USA). Total reaction volume was 6µL, with 2µL of cDNA (RT product) and 4 µL of master mix added with 0.4mM of each primer. Relative quantification of target gene transcripts were normalized using Elongation Factor 1α (EF1α) as the reference gene and M0 as the reference group following the method of Pfaffl (2001).

Data analysis

Tanks (n=3) were used as experimental unit for data on body mineral composition and mineral balance. Individual fish (n=9; 3 from each tank) was the experimental unit for data on enzyme assays and gene expression analysis. Two-way ANOVA was used to analyse the main effects of the basal diet, trace mineral premix inclusion and their

interactions. In the case of a significant interaction, one-way ANOVA with Tukeys' multiple comparison test was performed and the significant difference at $P<0.05$ were indicated by different superscript letters. All the data analyses was performed using SPSS version 20, IBM Statistics Inc., USA.

Table 3 : Primers used for gene expression analysis by real-time quantitative RT-PCR

Gene	Accession no. (GenBank* or INRA-SIGENAE)	Primer sequence (5′ → 3′)	Product size (bp)	Annealing temp. (°C)
EF1α	AF498320.1*	F: TCCTCTTGGTCGTTTCGCTG R: ACCCGAGGGACATCCTGTG	159	59
HAMP	BX088223.s.om.10	F: GGAGGAGGTTGGAAGCATTG R: GATGGTTTTAGTGCAGGCAGG	196	59
HO1	CA387878.s.om.10	F: ACTCTTCCGCAGTACAAGCT R: CTGTGTGTTGCAGCAGGAAT	212	59
FPN1	CA351776.s.om.10	F: GTCCTCTTACTGGGCGCTAT R: GCCAGGTTAGCGATGTTAGC	224	59
Nramp-β	AF048761.1*	F: CACCTCCCCTCCGGCTT R: CCTGGGTCAAGATAGGCGAT	156	60
Nramp-γ	EF495162.1*	F: GCCATCCTCAACAGTGTCT R: CTTTAGCTCCAGACTGTAGATCA	200	57
CTR1	GU723513.1*	F: GTTGTTTCCTGCTGGCTGTG R: GTAACACCGTCTGCAGCAAG	192	59
ATP7a	BX295327.s.om.10	F: CATGCCGGTGACTAAGAAGC R: AATGAGGATCCAGGCGAACA	244	59
ATP7b	FYV3OTN01C7RN9.s.om.10	F: CTGAGATGACTGGGGTGTGT R: GTCTTTGAAGGGGAGGGGTT	183	59
ATOX1	BX300064.s.om.10	F: ATGTGAGGGATGCTCTGGTG R: AGCCTCCTTTCCAGTCTTCT	154	59
SOD1	AF469663.1*	F: TGGTCCTGTGAAGCTGATTG R: TTGTCAGCTCCTGCAGTCAC	201	56
SOD2	CA352127.1*	F: TCCCTGACCTGACCTACGAC R: GGCCTCCTCCATTAAACCTC	201	57
CAT	BX087110.3*	F: TGATGTCACACAGGTGCGTA R: GTGGGCTCAGTGTTGTTGAG	195	55
GPX1	CA357669.1*	F: CGAGCTCCATGAACGGTACG R: TGCTTCCCGTTCACATCCAC	183	59
GPX4	CA344428.1*	F: TTGGAGGTCAGGAGCCAGGT R: ACCCTTTCCCTTGGGCTGTT	152	59

F, forward primer; R, reverse primer; EF1α, elongation factor 1α; HAMP, Hepcidin anti-microbial peptide; HO1, heme oxygenase 1; FPN1, ferroportin1; Nramp-β, Natural resistance associated macrophage protein beta polypeptide; Nramp-γ, Natural resistance associated macrophage protein gama polypeptide ; CTR1, copper transporter I; ATP7a, Cu^{++} transporting ATPase-alpha polypeptide; ATP7b, Cu^{++} transporting ATPase-beta polypeptide; ATOX1, copper transporter protein ATOX1; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; CAT, catalase; GPX1, glutathione peroxidase 1; GPX4, glutathione peroxidase 4.

Results

Feed intake and growth-related parameters (Table 4) were significantly higher in groups fed the M vs V diets. No effect of premix supplementation was observed on growth or feed intake. The hepato-somatic index (HSI) was significantly higher in M diet groups. Premix inclusion had an impact on HSI only in the M groups, with M1 fed fish having a higher HSI than M0 fed fish (Table 4).

Table 4: Growth performance of rainbow trout fed the experimental diets for 12 weeks

	M0	M1	V0	V1	Basal diet	Premix	Diet x Premix
FI (g/fish)	127.6 ± 1.7	131.2 ± 2	113.7 ± 6	115.9 ± 4	< 0.001	0.12	0.76
FBW (g)	187.9 ± 6	190.8 ± 6	143.7 ± 13	145.3 ± 6	< 0.001	0.5	0.89
WG (g)	168.3 ± 5.3	171.1 ± 4	123.8 ± 12	126 ± 5.4	< 0.001	0.46	0.76
DGI	3.6 ± 0.1	3.6 ± 0.04	3 ± 0.15	3 ± 0.07	< 0.001	0.57	0.98
FE	1.4 ± 0.03	1.4 ± 0.03	1.2 ± 0.04	1.1 ± 0.03	< 0.001	0.54	0.73
HSI	1.21 ± 0.2^{b}	1.43 ± 0.28^{c}	1.05 ± 0.11^{a}	1.07 ± 0.13^{a}	< 0.001	0.01	0.03

Initial body weight (IBW): 19.8 ± 0.8 g; FBW, final body weight; FI, feed intake; WG, weight gain; Daily growth index, DGI = 100*(FBW^1/3 - IBW^1/3)/duration (84 d); FE = Wet weight gain (g)/dry feed intake (g); Hepato-somatic index, HSI = (wet liver weight, g/weight of fish, g)*100. Data are expressed as mean ± SD of n=3 observations. P-value indicates statistical significance as obtained through two-way ANOVA. Values in the same row with different superscripts are statistically different (P<0.05) as obtained through Tukeys' multiple comparison test.

Apparent availability coefficients (AAC, Table 5) of Fe, Cu and Zn were significantly affected by change in basal diet and premix supplementation. AAC of Mn was affected only by change in basal diet, being higher in groups fed diet M. AAC of Se was not different between diets. A significant interaction of premix inclusion with basal diet was observed for AAC of Fe and Cu, in both cases, premix inclusion decreased the AAC but to a larger extent in the M diet compared with V diet groups.

Table 5: Apparent availability coefficient (AAC, %) of trace mineral concentration of rainbow trout

	M0	M1	V0	V1	Basal diet	Premix	Diet x Premix
Fe	39.2 ± 3.1^{d}	3.7 ± 0.9^{a}	13.4 ± 1.9^{c}	10.1 ± 1.2^{b}	0.027	0.007	0.012
Cu	74.8 ± 0.2^{d}	35.1 ± 3.7^{c}	39.8 ± 5.5^{b}	28.3 ± 4.4^{a}	0.014	0.012	0.04
Mn	31 ± 4.8^{b}	25.7 ± 6.8^{b}	7.3 ± 0.4^{a}	10 ± 2.6^{a}	0.01	0.975	0.349
Zn	56.1 ± 7.4^{b}	45.8 ± 3^{b}	40.2 ± 3^{a}	39.4 ± 2^{a}	0.011	0.043	0.024
Se	80.5 ± 0.7^{b}	74.4 ± 1.5^{a}	81.4 ± 1.4^{b}	79.8 ± 1.7^{b}	0.272	0.199	0.238

AAC,% =100-(100 x((% mineral in faeces)/(% mineral in diet) x (% marker in diet)/(% marker in faeces))). Data are expressed as mean ± SD of n=3 observations. P-value indicates statistical significance as obtained through two-way ANOVA. Values in the same row with different superscripts are statistically different (P<0.05) as obtained through Tukeys' multiple comparison test.

The effect of basal diet on the circulating levels of plasma trace minerals (Table 6) was significant only for Fe, being higher in V-fed compared to M-fed fish. Premix inclusion did not increase the plasma levels of any of the analysed trace minerals, except for Mn in M1 vs M0 fed fish. The final whole body concentration of trace minerals (Table 7) such as Cu, Zn and Se were higher in diet M than in diet V fed fish. Premix inclusion increased the final body concentration of all the analysed trace minerals, except for Se. Significant interactions between both dietary factors were observed for Mn and Se: premix inclusion increased body Mn concentration in M-fed fish but not V-fed fish. The reverse was observed for Se, where premix inclusion to diet V increased the body Se level, but not in trout fed diet M.

Table 6: Trace mineral concentration in the plasma of rainbow trout (µmol L^{-1})

	M0	M1	V0	V1	Basal diet	Premix	Diet x Premix
Fe	11.1 ± 2	8.5 ± 2.8	13.6 ± 4.1	14.3 ± 2.9	0.006	0.52	0.23
Cu	7.5 ± 2.5	9.5 ± 1.3	10 ± 1.3	8.8 ± 2.2	0.25	0.56	0.72
Mn	0.4 ± 0.4^{b}	1 ± 0.2^{a}	0.9 ± 0.3^{a}	0.6 ± 0.1^{a}	0.85	0.28	0.01
Zn	143.6 ± 27.9	162.3 ± 34	140.4 ± 45.5	150.9 ± 58.4	0.2	0.42	0.4

Data are expressed as mean ± SD of n=6 observations. P-value indicates statistical significance as obtained through two-way ANOVA. Values in the same row with different superscripts are statistically different (P<0.05) as obtained through Tukeys' multiple comparison test.

Table 7: Initial and final whole body trace mineral concentration of rainbow trout (mg kg^{-1} fresh weight)

	Initial	M0	M1	V0	V1	Basal diet	Pre mix	Diet x Premix
Fe	26	14. ± 0.23	22.5 ± 5.58	17.9 ± 0.6	21.4 ± 2.5	0.06	0.02	0.38
Cu	0.9	0.91 ± 0.12	1.4 ± 0.35	1.9 ± 0.1	2.8 ± 0.2	< 0.01	0.001	0.12
Mn	0.9	0.73 ± 0.04^{a}	1.2 ± 0.09^{b}	1 ± 0.1^{b}	0.9 ± 0.1^{b}	0.61	0.004	0.002
Zn	24.9	15.4 ± 0.51	17.28 ± 0.4	12.1 ± 0.5	13.8 ± 0.6	< 0.001	0.01	0.23
Se	0.25	0.3 ± 0.01^{b}	0.3 ± 0.005^{b}	0.13 ± 0.007^{a}	0.16 ± 0.002^{a}	< 0.001	0.1	0.03

Data are expressed as mean ± SD of n=3 observations. P-value indicates statistical significance as obtained through two-way ANOVA. Values in the same row with different superscripts are statistically different (P<0.05) as obtained through Tukeys' multiple comparison test.

Data on the activity of the analysed enzymes is presented in Table 8. Of the two apical metal reductases assayed in intestine and liver, only ferric reductase activity in the intestine was significantly affected by the change in basal diet, being 2-fold higher in fish fed the V diet. Among the different metaloenzymes analysed, only the activity of hepatic Se-dependent GPX was differentially regulated, being higher in liver of fish fed M diet. Premix inclusion did not affect the activity of any of the analysed enzymes.

Table 8: Analysed activities of selected enzymes involved trace mineral absorption, transport or metabolism (unit per mg protein)

	Tissue	M0	M1	V0	V1	Basal diet	Premix	Diet x Premix
Ferric reductase	Intestine	0.71 ± 0.33	0.8 ± 0.36	1.87 ± 0.66	1.89 ± 0.89	0.00	0.81	0.88
	Liver	3.52 ± 0.94	4.17 ± 3.25	6.4 ± 4.73	3.59 ± 1.31	0.32	0.35	0.14
Cupric reductase	Intestine	189.2 ± 43	199.6 ± 66	180.1 ± 39	189.6 ± 46	0.61	0.59	0.98
	Liver	94 ± 23	91 ± 5.9	76.8 ± 10.6	94.1 ± 12.1	0.20	0.19	0.07
ALP	Intestine	455.3 ± 149	444 ± 246	383.1 ± 119	409.8 ± 89.9	0.38	0.89	0.76
	Liver	221.9 ± 40.7	221 ± 101	182.4 ± 53.5	208.3 ± 66.4	0.32	0.63	0.61
Total SOD	Liver	101.6 ± 15.1	98.6 ± 8.2	108.7 ± 13.1	97 ± 12.4	0.68	0.35	0.38
CuZnSOD	Liver	40 ± 12.9	41.2 ± 22.3	43.7 ± 14.5	40.1 ± 15.2	0.88	0.89	0.78
Mn SOD	Liver	57.3 ± 15.8	61.6 ± 4.2	56.9 ± 5.4	64.9 ± 10.1	0.41	0.21	0.99
Catalase	Liver	969 ± 156	975 ± 297	1023 ± 190	1064.3 ± 219	0.39	0.78	0.83
Se-GPX	Liver	37 ± 17.5	49.4 ± 15.7	31.7 ± 3.6	31.4 ± 7.3	0.02	0.20	0.17

ALP, Alkaline phosphatase; SOD, Super-oxide-dismutase; GPX, Glutathione peroxidase; Data presented as mean ± SD of n=9 samples for ferric reductase, cupric reductase, alkaline phosphatase, catalase and glutathione peroxidase; for the three SOD enzymes namely totalSOD, Cu-ZnSOD and MnSOD, n=6. P-value indicates statistical significance as obtained through two-way ANOVA.

Data on relative mRNA expression of genes involved in the transport and metabolism of the studied trace minerals are presented in Fig. 1 to 3: Fe (Fig.1), Cu (Fig.2), and Mn, Zn, Se together (Fig.3). (i) Iron: The expression of HO1 (Fig.1a) and FPN1 (Fig.1b) was higher in fish fed the V vs M diet without effect of premix inclusion. In the liver of trout, HAMP expression was increased by feeding diet M and by premix inclusion, in particular in diet M groups (Fig.1c). In the intestine, the expression of DMT1 isoforms namely, Nramp-β (Fig.1d) and Nramp-γ (Fig.1e), was not significantly different among the groups. Feeding diet M and the inclusion of premix reduced the expression of intestinal FPN1 (Fig.1f). (ii) Copper: Intestinal expression of CTR1 was not different between the groups (Fig.2a). The intestinal expression of Cu-transporting P-type ATPase, ATP7a was slightly higher in V0 fed fish (Fig.2b). That of ATP7b was upregulated by feeding the V diet and reduced by premix inclusion in both M and V groups (Fig.2c). In liver, the basal V diet V increased CTR1 expression (Fig.2d). As in intestine, hepatic gene expression of ATP7a was higher in V0 fed fish (Fig.2e); whereas that of ATP7b was not affected by the dietary treatments (Fig.2f). ATOX1 expression in both intestine and liver was not significantly different between the groups (data not presented). (iii) Metaloenzymes: Hepatic gene expression of catalase (Fig.3a) and Cu-Zn super-oxide-dismutase (SOD1) (Fig.3b) in liver was not different between the treatments. Hepatic expression of Mn-SOD (SOD2) strongly decreased by premix inclusion to basal V diet, being the lowest in liver of the V1 fed group (Fig.3c). Expression of GPX1b1 (Fig.3d) and GPX1b2 (Fig.3e) was higher in liver of fish fed the V than the M diet, whereas the expression of GPX4a1 (Fig.3f) was lower in liver of V vs M fed fish.

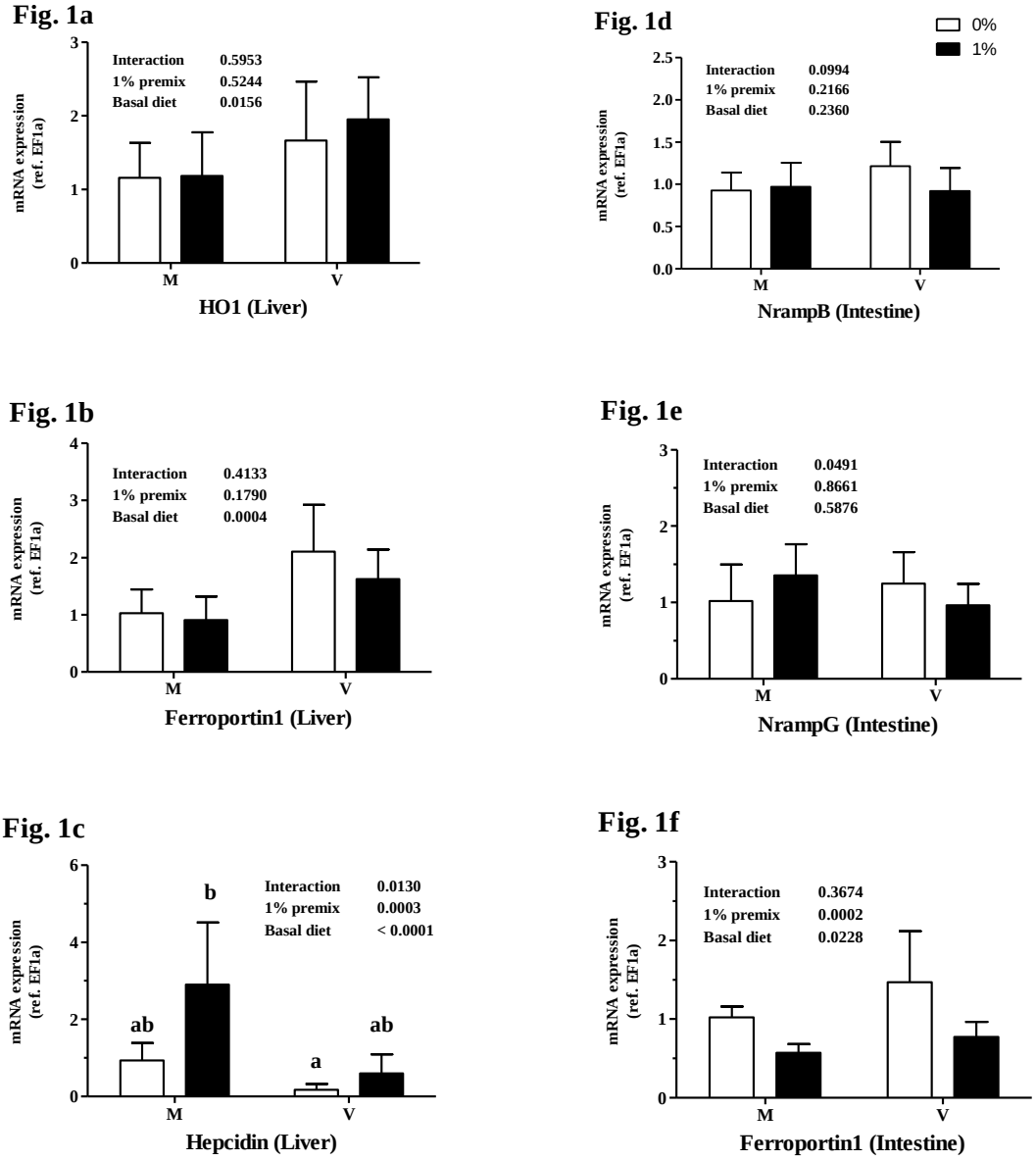

Figure 1: Transcription of selected transporters and regulators of iron metabolism in the liver and intestine of rainbow trout expressed relative to elongation factor 1alpha (EF1a). (i) Liver: hemeoxygenase1 (Fig.1a); ferroportin1 (Fig.1b); hepcidin (Fig.1c). (ii) Intestine: Nramp-β, (Fig.1d) and Nramp-γ (Fig.1e); ferroportin (Fig.1f). M, marine ingredient based diet and V, vegetable ingredient based diet; white bars, un-supplemented diet (0%); black bars, and premix supplemented diet (1%). Each bar represents mean ± SD of n=9 samples. P-values obtained from two-way ANOVA on the main effects of basal diet, premix inclusion and interaction, if any are provided in insets. In the case of the interaction being significant, one-way ANOAV with Tukeys' multiple comparison test was performed and different super scripts indicate significant difference at $P<0.05$.

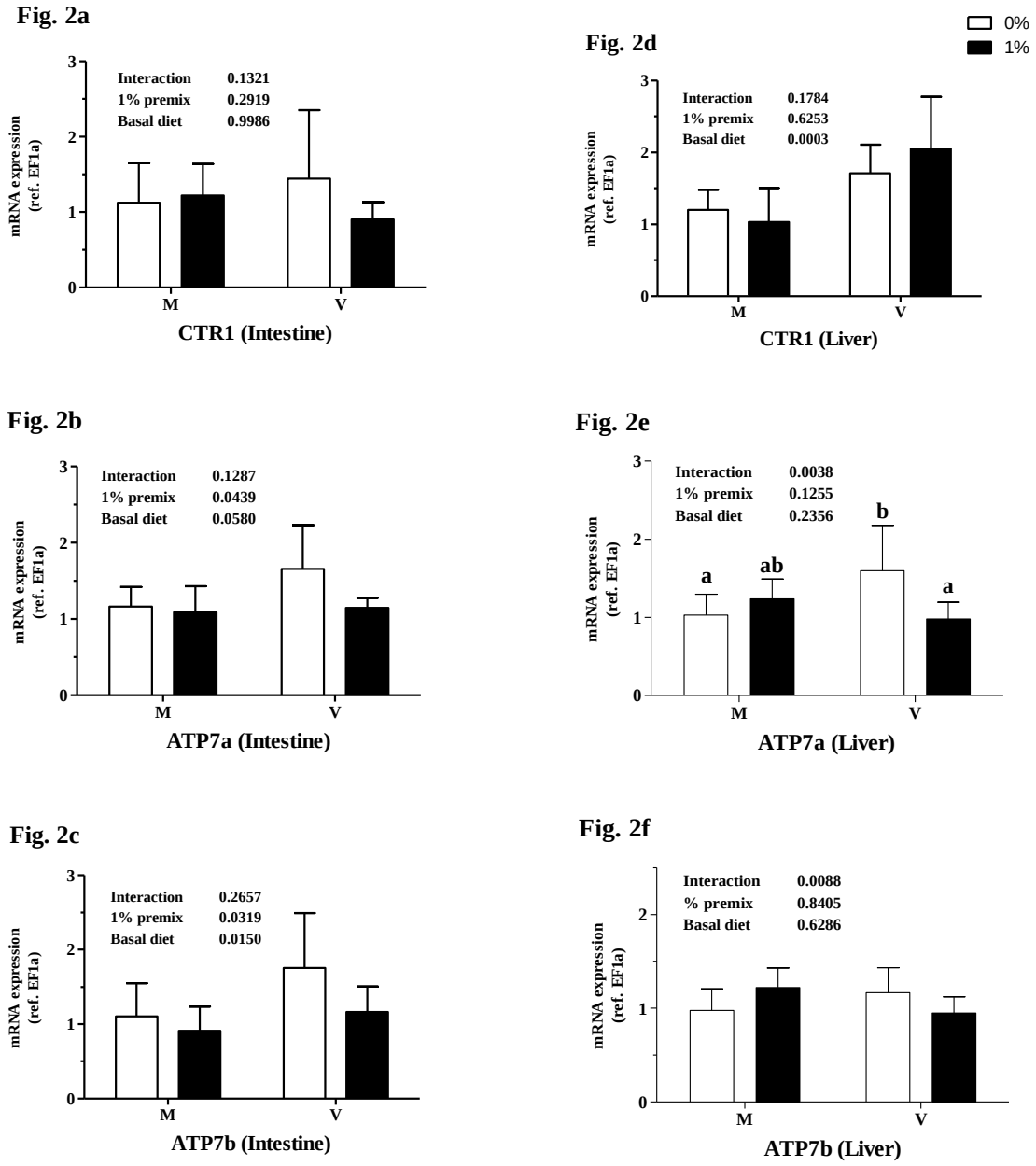

Figure 2: Expression of intestinal and hepatic copper transporters expressed relative to elongation factor 1alpha (EF1a). Intestine: copper transporter1, CTR1 (Fig.2a) ATP7a (Fig.2b) and ATP7b (Fig.2c). Liver: copper transporter1, CTR1 (Fig.2d) ATP7a (Fig.2e) and ATP7b (Fig.2f). M, marine ingredient based diet and V, vegetable ingredient based diet; white bars, un-supplemented diet (0%); black bars, premix supplemented diet (1%). Each bar represents mean ± SD of n=9 samples. P-values obtained from two-way ANOVA on the main effects of basal diet, premix inclusion and interaction, if any are provided in insets. In the case of the interaction being significant, one-way ANOAV with Tukeys' multiple comparison test was performed and different super scripts indicate significant difference at $P<0.05$.

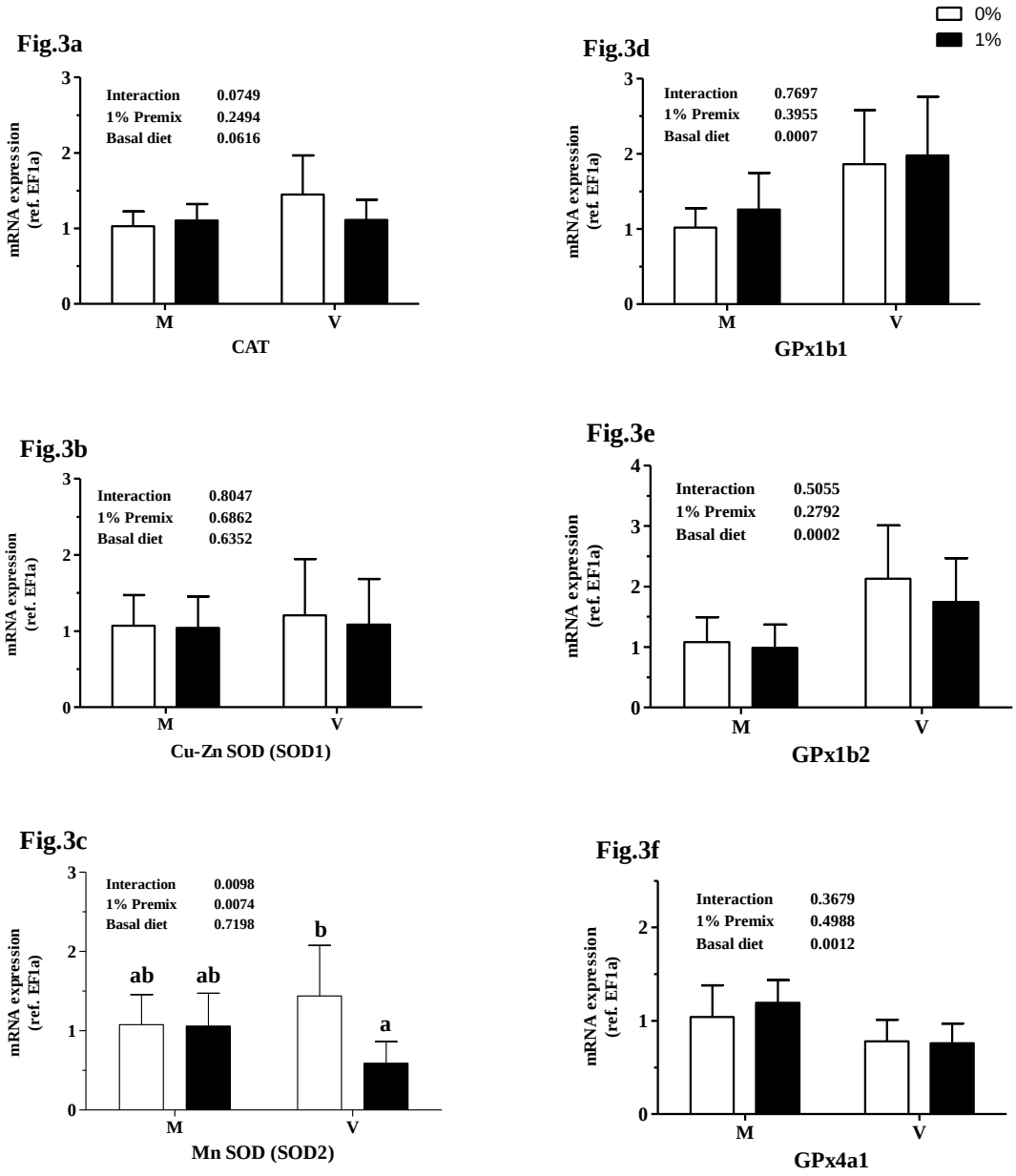

Figure 3: Transcription of metaloenzyme genes in liver expressed relative to elongation factor 1alpha (EF1a). Catalase (Fig.3a); Cu-Zn superoxide dismutase, Cu-Zn SOD (Fig.3b); Mn superoxide dismutase, Mn-SOD (Fig.3c); Glutathione peroxidase, GPx1b1 (Fig.3d); GPX1b2 (Fig.3e) and GPX4a1 (Fig.3f). M, marine ingredient based diet and V, vegetable ingredient based diet; white bars, un-supplemented diet (0%); black bars, premix supplemented diet (1%). Each bar represents mean ± SD of n=9 samples. P-values obtained from two-way ANOVA on the main effects of basal diet, premix inclusion and interaction, if any are provided in insets. In the case of the interaction being significant, one-way ANOAV with Tukeys' multiple comparison test was performed and different super scripts indicate significant difference at $P<0.05$.

Discussion

The growth performance of the trout fed the all plant based diets in this study was considerably lower than when fed the fish meal based diets, as observed in our earlier study (Antony Jesu Prabhu *et al.*, 2014). The lack of a positive effect of adding the trace mineral premix on growth performance indicates that the trace mineral supply from the un-supplemented basal diets was sufficient to support good growth of the trout in both diet groups. High dietary Fe has been shown to increase HSI of rainbow trout (Kwong *et al.*, 2011; Kwong *et al.*, 2013), but the Fe levels in the present study were much lower than in the studies cited above. Nevertheless, the higher hepato-somatic index in the M1 compared with M0 group might be due to the long duration of feeding trial or imply that even low levels of dietary Fe supplementation (50 mg/kg DM, as in this study) above the requirement can impact hepatic metabolism in rainbow trout. Effects of the change in basal diet, the premix inclusion and the interaction between the two variables on apparent availability, plasma and whole body mineral levels, activity of brush border apical reductases and expression of genes involved in Fe and Cu metabolism and hepatic activity and expression of metaloenzymes containing Fe, Cu, Mn, Zn or Se are discussed in the sections to follow.

Changes related to hepatic iron metabolism

Change in basal diet had a significant effect on the analysed markers of hepatic Fe metabolism. Diets based on plant protein ingredients lack heme, present in fish meal based diets. Apart from dietary heme being a good source of Fe, degradation of heme supplies biliviridin, the precursor of bile pigment bilirubin. Activity of heme-oxygenase (HO) in splenic or hepatic macrophages is essential for catabolizing heme to produce biliverdin and free iron (Poss and Tonegawa, 1997), whereas hepatic ferroportin1 (FPN1) expression is considered vital for the cellular export of Fe recycled from heme (Donovan *et al.*, 2005). In vertebrates, the export of Fe from macrophage or enterocyte to plasma by the FPN1 transporter is transcriptionally regulated by cellular Fe levels and by hepcidin, which exert an inhibitory action on Fe export by FPN1 (Ganz and Nemeth, 2012). Hepcidin is encoded by HAMP gene in liver of mammals as also documented in some teleosts (Douglas *et al.*, 2003), including rainbow trout (Alvarez *et al.*, 2013). In our study, we noted a down-regulation of hepcidin together with increased expression of HO1 and FPN1 in the liver of fish fed the V diet, suggestive of increased

degradation of endogenous heme in the hepatic macrophages by diet V feeding. The premix inclusion also up-regulated the expression of HAMP expression in liver, but did not modify that of HO1 and FPN1. This suggests that factors other than dietary Fe supply may regulate the hepatic expressions of HO1 and FPN1, possibly in relation with cholesterol metabolism. Indeed, an over-expression of HO1 in rat astrocytes has been found to be associated with enhanced cholesterol biosynthesis and intra-cellular Fe levels (Vaya *et al.*, 2007; Hascalovici *et al.*, 2009). In rainbow trout, it is well known that plant ingredients such as soybean meal may negatively affect cholesterol metabolism and bile status (Kaushik *et al.*, 1995; Yamamoto *et al.*, 2007; Iwashita *et al.*, 2009). A recent study with Atlantic salmon fed soybean indicated increased transcriptional capacity for bile salts and cholesterol biosynthesis to compensate for defective reabsorption of bile due to inflammation in the distal intestine (Kortner *et al.*, 2013). As such, increasing the degradation of the endogenous heme-pool, as suggested by the enhanced HO/FPN1 expression, could be an adaptive strategy to meet the physiological demand for bile salts essential in cholesterol biosynthesis in the V-fish fed plant-based diets devoid of heme. On the other hand, such higher endogenous heme-degradation may increase the supply of Fe to the plasma, saturating the Fe binding capacity of plasma transferrin and hence increase the non-transferrin bound Fe which is stored in the liver. Like in mammals, body iron stores in fish are known to regulate intestinal Fe absorption and homeostasis (Standal *et al.*, 1999). Accordingly, the heme-degradation induced systemic Fe overload in V-fish, as indicated by higher plasma and whole body Fe levels, may explain the lower AAC of Fe in the V0 vs M0 fed fish, despite similar dietary Fe concentrations. These findings suggest that disturbances in hepatic metabolism when fed plant ingredient based diets have an impact on mechanisms regulating Fe homeostasis in rainbow trout.

Regulation of intestinal iron absorption

The proportion of Fe absorbed from the diet, which is the major source of Fe to fish (Bury *et al.*, 2001; Bury and Grosell, 2003), has been found to decrease with increasing dietary levels (Standal *et al.*, 1999), as reflected here by the lowered Fe AAC values following premix addition. Divalent metal transporters (DMT1 or Nramp) are known to facilitate cellular transport of Fe^{2+} across intestinal apical membranes (Dorschner and Phillips, 1999) and a brush border ferric reductase (FR) activity exists for the reduction

of non-heme Fe^{3+} to Fe^{2+}, the substrate for DMT1 (Carriquiriborde *et al.*, 2004; Cooper *et al.*, 2006). In line with observations by Carriquiriborde et al. (Carriquiriborde *et al.*, 2004), the mineral premix did not stimulate intestinal FR activity in our study probably since the added Fe was already supplied in the Fe^{2+} form as ferrous sulphate ($FeSO_4.7H_2O$). The activity of intestinal FR also indicated that the available Fe supply wah higher from M than from V diets and that, as in mammals, FR independent mechanism might exist for heme-Fe uptake in rainbow trout when fed fish meal based diets. *In vitro,* iron uptake in the anterior intestine of rainbow trout is considered to occur via simple diffusion while carrier mediated transport occurs in the mid- and posterior intestinal segments (Kwong and Niyogi, 2008). In trout, gastro-intestinal expression of Nramp (DMT) genes were initially induced at day 7 on exposure to high dietary Fe but decreased at day 14 (Kwong *et al.*, 2013). Such a phenomenon may explain the lack of differential expression in intestinal DMT isoforms (Nramp-β and Nramp-γ) after 12 weeks of feeding as in the present study. Moreover, DMTs are also transporters of several other divalent metal ions, and interactions with Cu^{++} or Zn^{++} were shown to exist in the apical uptake of Fe by DMTs in trout intestine (Carriquiriborde *et al.*, 2004; Kwong and Niyogi, 2009). Given this non-specific nature of DMTs, it is likely that a more specific and critical step exists in regulating intestinal Fe absorption. In this respect, Kwong et al. (Kwong *et al.*, 2013) emphasized the need for better understanding the role and physiological importance of ferroportin and hepcidin in systemic Fe regulation in fish. In mammals and also in zebrafish, ferroportin assisted basolateral extrusion of Fe was identified as the rate limiting step of intestinal iron absorption (Andrews, 2000; Fraenkel *et al.*, 2005), which, as described earlier, is regulated by hepatic hepcidin or intracellular Fe levels. *In vitro*, experimental addition of exogenous hepcidin was foud to reduce Fe export from enterocytes due to internalisation and degradation of ferroportin by hepcidin (Nemeth *et al.*, 2004). *In vivo*, experimental Fe overload increased liver HAMP expression in mammals (Pigeon *et al.*, 2001) and in fish (Rodrigues *et al.*, 2006). In the same line, Yeh et al. (Yeh *et al.*, 2004) reported that a suppression of intestinal FPN1 transcription by Fe overload induced hepcidin over-expression in rats *in vivo*. Although dietary Fe levels were similar between M and V diets and although we only moderate increased dietary Fe supply through premix supplementation, such opposite expression pattern and regulation intestinal FPN1 and of hepatic HAMP was also seen in our study in response to changes

in basal diet and premix inclusion, This might possibly be the effect of excess Fe supplemented to M diet in the form of Fe SO_4 when Fe in M0 diet was sufficient to meet the requirement of rainbow trout. In contrast, the suppression of liver HAMP might be have been to avoid systemic Fe overload in plasma derived from the degradation of heme in the hepatic macrophages, as explained previously. Cooper et al. (Cooper *et al.*, 2006) reported a biphasic pattern of Fe accumulation in the intestinal epithelia of gulf toad fish, suggestive of the periodic partial loss of dietary Fe due to epithelial sloughing. Rainbow trout also showed accumulation of dietary Fe in the intestine (Kwong *et al.*, 2013). The enterocytes of the intestinal epithelium are sloughed off and replenished regularly, at 2-3 day intervals in mammals and probably few weeks in the case of fish (Buddington and Kuz'mina, 2000). Consequently, the Fe accumulated in the intestinal epithelium will not be absorbed and end up in the faeces, resulting in low AAC. The significantly low intestinal FPN1 expression related with the high liver HAMP possibly explains the very low AAC in M1 fed fish. Our results suggest that systemic Fe homeostasis in rainbow trout could be transcriptionally regulated by hepcidin and ferroportin, but as suggested by Kwong et al. (Kwong *et al.*, 2013), further studies are required to better understand the mechanism.

Intestinal and hepatic changes related to copper transport

Copper uptake, transport and homeostasis in mammals are mediated by a high affinity copper transporter (CTR1), Cu chaperons (ATOX1 and CCS) and P-type Cu-transporting-ATPases (ATP7a and ATP7b) (Lutsenko, 2010). The existence of a high-affinity Cu transporter in the gastrointestinal tract with conserved regions for cellular Cu transport has been characterised in zebrafish (Mackenzie *et al.*, 2004), gilthead seabream (Minghetti *et al.*, 2008) and rainbow trout (Nadella *et al.*, 2011). In the intestine, ATP7a is believed to be involved in the basolateral transfer of Cu (Boyle *et al.*, 2009). Gastrointestinal expressions of CTR1 in rainbow trout (Nadella *et al.*, 2011), and of CTR1 and ATP7a in gilthead sea bream (Minghetti *et al.*, 2008; Minghetti *et al.*, 2010) were down-regulated by high dietary Cu supply. In the present study, AAC of Cu and intestinal expression of ATP7a was down-regulated by inclusion of premix.

According to Bertinato and L'Abbé (Bertinato and L'Abbé, 2004), elevated hepatic cellular Cu concentrations stimulate hepatic Cu transport (by ATP7a) to the secretory pathways for Cu to be incorporated into cuproenzymes or to be excreted into the bile

(by ATP7b). Expression of hepatic CTR1 and ATP7a were down-regulated and hepatic ATP7b was up-regulated in gilthead seabream exposed to high dietary Cu for 30 days (Minghetti *et al.*, 2008; Minghetti *et al.*, 2010). Of interest, and in contrast to the above observations(Minghetti *et al.*, 2008; Minghetti *et al.*, 2010), liver of fish fed the premix supplemented plant-based diet (V1 vs V0) showed a persistently elevated CTR1 expression and no decrease in expression of hepatic ATP7b in response to the premix inclusion even after 12 weeks. The V1-fed fish indeed had high body Cu, in line with observations on Cu accumulation in the liver due to the lack of a functional homologue of ATP7b in mammals, commonly known as Wilson's disease (Terada *et al.*, 1998). In stickleback, transcription of the rate limiting enzyme in cholesterol biosynthesis (HMGCR) increased during acute exposure to Cu (10 µg/L), but decresaed at higher concentrations (128 µg/L) (Santos *et al.*, 2009). Similarly, changes in HMGCR enzyme transcription were also reported in Atlantic salmon fed soybean meal, showing that its transcriptional up-regulation was not able to restore the plasma cholesterol and bile acid levels (Kortner *et al.*, 2013), indicating defective cholesterol biosynthesis. As such, the high whole body Cu in the fish fed the plant-based V diet (in the present and in previous study (Antony Jesu Prabhu *et al.*, 2014)), may result from impaired hepato-biliary Cu excretion leading to Cu accumulation in the body.

Activity and expression of metaloenzymes

Activity and expression of metaloenzymes have been used as biomarkers of trace mineral status in mammals and fish. Catalase activity and expression decreases with a dietary deficiency in Fe supply in rabbits (Zhang *et al.*, 2012). Alkaline phosphatase has been used as a marker for Zn status in several fish species including Atlantic salmon (Maage and Julshamn, 1993) and rainbow trout (Kucukbay *et al.*, 2006), where the activity is reduced during Zn deficiency. No such effect was observed in the present study indicating dietary Fe and Zn supply was not deficient even in the un-supplemented diets. Low dietary supply of Mn results in low whole body Mn-status in rainbow trout (Satoh *et al.*, 1991) and Atlantic salmon (Lorentzen and Maage, 1999), as observed for whole body-Mn and hepatic expression of Mn-SOD in the present study, especially with M fed fish. In fish, hepatic MnSOD (SOD2) activity is reduced at high dietary supply of Mn (Lin *et al.*, 2008; Tan *et al.*, 2012). In the present study, the inherently high level of Mn in V0 diet resulted in higher MnSOD expression but was

reduced by premix inclusion, while the activity was unaffected. The results might indicate insufficient supply of Mn in M0 diet and an excess Mn supply with V1 diet. With regard to Se, the lower activity of Se-dependent glutathione peroxidase in V diet groups might be due to the low Se content of V diets compared to M diets. At the transcriptional level, we found a reduction in the expression of GP4a1 and increased expression of GPX1b1 and GPX1b2 in V diet fed groups. According to Pacitti et al. (Pacitti *et al.*, 2013), among the different transcripts of GPX described in rainbow trout, GPX1 isoforms are the most sensitive and even for GPX1, transcriptional changes do not coincide with enzyme activity. Similarly, in the present study, the response in expression of GPX1 was not in line with the measured GPX activity. Moreover, as no effect of premix supplementation was observed, it is possible that the changes in the expression GPX isoforms are influenced by other unknown factors. On the whole, except for Mn in M diet and Se in V diets, the dietary supply of other trace minerals was sufficient to meet the normal physiological needs of rainbow trout.

Conclusion

The results of the present study suggest that regulation of trace mineral absorption and metabolism in rainbow trout depends on the dietary ingredient composition (fish- versus plant-ingredient based diets) and on the inclusion of mineral premix. We also found that the dietary ingredient composition interacts with premix supplementation, especially for Fe and Cu. It is suggested that the disturbances in Fe and Cu metabolism are secondary effects of hepato-biliary disfunction in relation to cholesterol and bile metabolism when fed plant-ingredient based diets. Further detailed investigations are required to better understand such interactions and the cellular and molecular regulation of trace mineral status in rainbow trout as affected by dietary factors.

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CHAPTER 8

Effect of change in ingredient composition on micro-mineral supplementation to the diets of juvenile rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Two basal diets M0 and V0 were formulated with marine and plant ingredient based ingredient composition. Seven experimental diets were prepared from the two basal diets namely M0, M100, V0, V30, V60, V100 and V150 by incorporating different levels of a micro-mineral premix. Triplicate groups of rainbow trout (initial weight: 20g) reared at 17°C were fed one of each diet to apparent visual satiation over 12 weeks. Among the V diet fed fish, growth and feed intake exhibited maximal response at V60 level of premix inclusion. Among V diet fed fish, apparent availability coefficient of Fe, Cu and Zn decreased linearly with increasing level of premix whereas apparent availability coefficient of Mn and Se were unaffected. The available dietary concentration in basal V0 diet was for Fe, 20.6; Cu, 2.8; Mn, 6.5; Zn, 17.3 and Se, 0.195 (in mg/kg DM) and in the M0 diet for Fe, 63.3; Cu, 5.2; Mn, 2.9; Zn, 35.2 and Se, 0.87 (in mg/kg DM). In reference to NRC (2011) recommendations, the V0 basal diet accounted for 34.3, 92.9, 53.9, 115 and 130.2%; and the contribution from M0 diet for 105.5, 173.3, 24.2, 234.7 and 580% of the minimal dietary inclusion levels of Fe, Cu, Mn, Zn and Se to rainbow trout, respectively. However, Cu and Mn supply from basal V0 diet, Fe from V30 diet were sufficient to maintain the normal whole body concentrations. Additional supplementation of Zn and Se were required beyond the levels found in V150 to maintain normal body levels in rainbow trout fed complete plant ingredient based diets. To conclude, optimal dietary inclusion levels of micro-minerals to rainbow trout are altered when fed fish-meal free diets.

Keywords: fishmeal replacement; premix; rainbow trout; requirement; supplement; trace minerals

Introduction

Mineral requirements of fish and optimal dietary inclusion level in particular of trace minerals such as Fe, Cu, Mn, Zn and Se in fish feeds are not well understood (Lall and Milley, 2008). As regards rainbow trout, estimates on requirements for all trace minerals, except for iron (Fe) are available (NRC, 2011). The recommendations of National Research Council (NRC) are based on *"requirement estimates determined with highly purified ingredients in which the nutrients are highly digestible and therefore the values represent near 100% bioavailability".* Under practical conditions, the dietary inclusion level of trace minerals needed to meet the requirement can vary based on a number of physiological and environmental factors (Hilton, 1989, Sugiura et al., 2000). The ingredient composition of practical fish feeds is one such factor (Watanabe et al., 1997). The latter authors found that trace minerals such as Fe, Cu, Mn, Se and Zn are important to be supplemented in fish feeds due to the low levels in practical feed ingredients and interactions with other dietary components. The magnitude of supplementation depends on the intrinsic contribution from the basal ingredients and interactions affecting the availability of the trace minerals. Over the years, the use of marine ingredients such as fish meal and fish oil in fish feeds has seen a significant reduction and are, replaced to a large extent by plant-derived protein and lipid sources (Tacon and Metian, 2008). Fish meal is generally rich in trace minerals (Julshamn et al., 1978) but appreciable levels of certain trace minerals are also found in plant-derived ingredients, at times even many folds higher than in fish meal, as seen for the Mn content of white lupin (NRC, 2011). Considering the supply of trace minerals from the different ingredients themselves (Sugiura et al., 1998, Lall and Milley, 2008), it is necessary to evaluate the need for and level of supplementing one or more trace minerals to plant ingredient based diets which are completely devoid of fish meal. Based on the foresaid considerations, a study with juvenile rainbow trout was undertaken to evaluate the NRC (2011) recommendations for trace minerals and determine the optimal range of trace mineral supplementation required to a complete plant ingredient based diet.

Material and Methods

Experimental diets

Two basal diets (M0 and V0) were formulated with the basal ingredient composition provided in Table 1.

Table 1: Ingredient and chemical composition of the basal diet

Ingredients	V0	M0
Norwegian herring meal, (CP 70; Sopropêche, France)	-	62.57
Corn Gluten meal (CP 60; Inzo, France)	18.00	-
Wheat Gluten (CP 70; Roquette, France)	20.00	-
Soybean meal (CP 48; Inzo, France)	5.00	-
Soyproteinconcentrate (Estrilvo ; CP 70; Sopropêche, France)	17.00	-
White lupin meal (Terrena, France)	5.00	-
Extruded peas (Aquatex, Sotexpro, France)	3.00	-
Rapeseed meal (Primor 00; SudOuestAlimants, France)	4.00	-
Whole wheat	3.18	24.56
Soylecithin (Louis François, France)	2.00	-
L-Lysine (Eurolysine)	1.34	-
L-methionine (Evonik, Germany)	0.30	-
CaHPO4.2H20 (18%P; 22% Ca)	2.17	-
Attractant Mix§	1.50	-
Trace mineral premix¶	0.00	0.00
Vitamin premix†	1.00	1.00
Yttrium oxide (Sigma-Aldrich, USA)	0.01	0.01
Fish oil (southern hemisphere, Sopropêche, France)	-	11.86
Rapeseed Oil (Daudruy, France)	6.60	-
Linseed Oil (Daudruy, France)	6.60	-
Palm Oil (Daudruy, France)	3.30	-
Analysed proximate composition		
Dry matter (DM), %	94.9	94.8
Crude Protein, % DM	50.0	49.4
Crude Lipid, % DM	22.7	22.1
Crude Ash, % DM	4.0	8.2
Energy, kJ/g DM	25.1	23.8
Analysed concentration of major minerals (g/kg DM)		
Phosphorus	9.4	12.9
Calcium	8.0	11.3
Ca/P ratio (no unit)	0.9	0.9
Magnesium	0.95	1.76
Potassium	4.2	12.2
Sodium	1.4	9.4

§Attractant premix: glucosamine, 5g; taurine, 3g; betaine, 3g; glycine, 2g and alanine, 2g.

† Vitamin premix (IU or mg/kg diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg; B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 2000 mg. (UPAE, INRA).

¶ Trace mineral premix (g/kg premix): $FeSO_4$.7H2O (21% Fe; 11.5% S), 25 g; $CuSO_4.5H_2O$ (25.45% Cu; 12.8% S), 3 g; $MnSO_4.H_2O$ (33% Mn; 19% S), 3 g; $ZnSO_4.H_2O$ (36% Zn; 18% S), 4 g; KI (76% I; 24% K), 0.04 g; Na_2SeO_3 (46% Se; 27% Na), 0.03 g; α-cellulose (carrier) 964.93 g.

The diets were made either with fish meal as the major protein source (M) or entirely of plant-derived ingredients (V) and supplemented with di-calcium phosphate to meet the available phosphorus requirement of rainbow trout (Antony Jesu Prabhu et al., 2014; NRC 2011). A micro mineral (Fe, Cu, Mn, Zn and Se) pre-mixture was designed to meet the theoretical requirements of rainbow trout (NRC, 2011) at an inclusion level of 10 g/kg diet contributing Fe, 52.5 mg; Cu, 7.5 mg; Mn, 12 mg; Zn, 14 mg; and Se, 0.15 mg per kg diet, on as fed basis. This premix was included at incremental levels of 0, 3, 6, 10 and 15 g kg^{-1} diet to the basal V diet to provide diets V0, V30, V60, V100 and V150, respectively; whereas, in the M diet, the premix was included at 0 and 10g. kg^{-1} diet to give M0 and M100, respectively. For the measurement of the apparent availability of minerals, the diets were prepared with the incorporation of 1 g kg^{-1} yttrium oxide (Y_2O_3) as the inert marker.

Fish, feeding and rearing condition

Rainbow trout juveniles (19.8 ± 0.8 g, IBW) were randomly distributed into 21 experimental units, each of 500 L (40 fish $unit^{-1}$). Triplicate groups of fish were hand fed twice a day to apparent visual satiation for a period of 12 weeks (6 days a week). The fish were reared in flow-through systems at the INRA experimental fish farm at Donzacq (Landes, France). Water temperature was 17 ± 0.5°C and the flow rate of water was maintained at 50 L min^{-1} during the experimental period. The analysed concentrations of minerals in the rearing water (mg L^{-1}) were P, < 0.2; Ca, 41.7 ± 6.3; Mg, 19.2 ± 0.8; K, 1.8 ± 0.1; Fe, < 0.02; Mn, < 0.02; Cu, < 0.008, Zn, < 0.007; and Se, 0.002.

Fish, tissue and faecal sampling

Fish samples for initial body composition analysis (n=20) were collected at the start of the experiment. Fish from each experimental unit were bulk weighed at the start, every 3 weeks and at the end of the 12 week growth trial. Feed was withheld for 24h before every weighing. At the end of the growth trial and 24h after the last meal, 6 fish were randomly selected from each experimental unit and pooled for final body composition analysis. Out of the remaining fish, 25 fish per tank were used for the determination of apparent availability coefficient (AAC) of minerals. The fish were fed a single meal (86 g $tank^{-1}$) and 9h after the meal, faecal samples were collected by the method of stripping (Antony Jesu Prabhu et al., 2014). The samples were collected over ice, frozen immediately and stored at -20°C until mineral analysis. Animal experiments and

sampling procedures followed the guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree no. 2001-464, May 29, 2001) and the animal ethics committee of INRA (INRA 2002-36, April 14, 2002).

Analytical methods

Moisture content of the pooled fish samples were determined by drying freshly ground samples at 105°C for 24h. The chemical composition of the diets and of fish samples were analysed by the following methods: dry matter after drying at 105°C for 24h, ash by combustion at 600°C for four hours in a muffle furnace, crude protein (Nx6.25) by Kjeldahl method in acid digested samples, crude lipid by petroleum ether extraction using Soxhlet method (Soxtherm) and gross energy content in an adiabatic bomb calorimeter (IKA, Heitersheim Gribheimer, Germany). The concentrations of P, Ca, Mg, K, Fe, Cu and Zn in the diets, whole fish and faeces were analysed using inductively coupled plasma-mass spectrometry (ICP-OES) at USRAVE-INRA, Bordeaux, France. The Se and yttrium content in the samples were analysed using ICP-MS at LCABIE-UPPA, Pau, France. The analysed chemical composition and macro-mineral concentration of the basal V0 and M0 diets are presented in Table 1 and the micro-mineral concentration of the diets is provided in Table 2.

Table 2: Analysed dietary concentration of supplemented micro-minerals (mg/kg DM)

Diet code	V0	V30	V60	V100	V150	M0	M100
Iron	153.8	169.1	185.2	205.4	234.8	161.6	212.9
Copper	7.0	8.8	10.0	12.9	14.2	6.9	14.0
Manganese	88.6	92.2	94.1	100.9	102.1	9.4	22.7
Zinc	42.9	44.1	46.8	52.5	58.1	62.8	72.5
Selenium	0.24	0.28	0.33	0.40	0.54	1.09	1.27

V0 – Basal plant ingredient based diet; M0 – Basal fish meal based diet;
V30, V60, V100 and V150 – plant ingredient based diets with respective percentage inclusion of micro-mineral premix; M100 – Fishmeal based diet with 1% inclusion of micro-mineral premix.

Data analysis

Tanks (n=3) were used as experimental units for data on growth, body composition and mineral balance. All data were subjected to one-way ANOVA followed by Tukeys' multiple comparison test at a probability of $P<0.05$. All the data analysis was performed using SPSS version 20, IBM Statistics Inc., USA.

Results

Feed intake and growth performance

Data on feed intake (FI), weight gain (WG) and other indices of growth performance such as feed efficiency (FE) and daily growth index (DGI) are provided in Table 3. During the 12 week feeding trial, fish gained more than 6 folds their initial weight. Fish fed M diets had significantly higher feed intake, weight gain and feed efficiencies compared with V diet fed fish. Inclusion of the micro-mineral premix did not have an impact on the growth of the fish, irrespective of the type of basal diet. However, among the 5 groups fed the V diet, a tendency for higher weight gain was observed in the fish fed with the V60 diet.

Table 3: Growth performance of rainbow trout fed the experimental diets for 12 weeks

	IBW (g/fish)	FBW (g/fish)	Feed intake (g/fish)	Weight gain (g/fish)	FE	DGI
V0	19.8 ± 0.6	144 ± 12.8 ^{a}	113.7 ± 5.7 ^{a}	123.8 ± 12 ^{a}	1.2 ± 0.04 ^{a}	3 ± 0.15 ^{a}
V30	19.8 ± 0.5	145.8 ± 4.5 ^{a}	116.9 ± 1.3 ^{a}	126 ± 4.8 ^{a}	1.1 ± 0.02 ^{a}	3 ± 0.08 ^{a}
V60	19.9 ± 0.8	155.1 ± 2.2 ^{a}	121.6 ± 1.4 ^{ab}	135.2 ± 1.5 ^{a}	1.2 ± 0.02 ^{a}	3.2 ± 0.02 ^{b}
V100	19.7 ± 0.5	145.3 ± 5.7 ^{a}	115.9 ± 3.5 ^{a}	125.5 ± 5.4 ^{a}	1.1 ± 0.03 ^{a}	3 ± 0.07 ^{a}
V150	20 ± 0.3	146.8 ± 4.5 ^{a}	120.4 ± 4.5 ^{ab}	126.8 ± 4.8 ^{a}	1.1 ± 0.07 ^{a}	3.1 ± 0.1 ^{ab}
M0	19.7 ± 0.5	187.9 ± 6 ^{b}	127.6 ± 1.7 ^{b}	168.3 ± 5.3 ^{b}	1.4 ± 0.03 ^{b}	3.6 ± 0.1 ^{c}
M100	19.7 ± 0.5	190.8 ± 5.5 ^{b}	131.2 ± 2.4 ^{b}	171.1 ± 4.1 ^{b}	1.4 ± 0.03 ^{b}	3.6 ± 0.04 ^{c}
p-value	0.52	0.01	0.03	0.01	0.02	0.02

V0 – Basal plant ingredient based diet; M0 – Basal fish meal based diet; V30, V60, V100 and V150 – plant ingredient based diets with respective percentage inclusion of micro-mineral premix; M100 – Fishmeal based diet with 1% inclusion of micro-mineral premix. IBW – Initial body weight; FBW – final body weight; FE – feed efficiency; DGI – Daily growth index. DGI = 100*(FBW^1/3 - IBW^1/3)/duration (84 d); FE = Wet weight gain (g)/Dry feed intake (g). Values within the same column with different superscript are statistically significant.

Apparent availability of minerals

Data on apparent availability coefficient (AAC, %) of minerals in the experimental diets to rainbow trout are presented in Table 4. The AAC of all the analysed macro-minerals and micro-minerals were higher in fish fed the M diet. Supplementation of micro-mineral premix to the M0 diet, resulted in reduction of AAC of the micro-minerals, most prominent for Fe. Among the V diet fed fish, the AAC of micro-minerals such as Fe, Cu and Zn decreased with increasing level of premix and attained the lowest with V150 fed group. Premix supplementation to basal V diet did not have an impact on the AAC of Mn, Se and also on the AAC of the macro-minerals such as P, Ca, Mg and K, for which the dietary levels were kept constant.

Table 4: Apparent availability (%) of minerals in experimental diets fed to rainbow trout

	P	Mg	Ca	K	Fe	Cu	Mn	Zn	Se
V0	58.5 ± 7.9 [a]	68.6 ± 5.2 [b]	7.4 ± 1.1	88.4 ± 1.1 [a]	13.4 ± 1.9 [d]	39.8 ± 5.5 [c]	7.3 ± 0.4 [a]	40.2 ± 3 [ba]	81.4 ± 1.4 [b]
V30	60.8 ± 0.8 [a]	69.4 ± 4.5 [b]	8.4 ± 0.9	88.9 ± 0.6 [a]	12.9 ± 0.1 [d]	35.9 ± 3.1 [c]	8.7 ± 1 [a]	38.6 ± 2.2 [ba]	80.7 ± 1.8 [b]
V60	60.9 ± 6.2 [a]	68.9 ± 4.8 [b]	9.2 ± 7.1	88.9 ± 1.2 [a]	13.1 ± 1.1 [d]	27.7 ± 2.1 [b]	9.9 ± 2.6 [a]	40.4 ± 2.3 [ba]	80.4 ± 2.4 [b]
V100	59.6 ± 5.9 [a]	70 ± 3.9 [b]	11.8 ± 1.9	89.2 ± 0.4 [a]	10.1 ± 1.2 [bc]	28.3 ± 4.4 [b]	10 ± 2.6 [a]	39.4 ± 2 [ba]	79.8 ± 1.7 [b]
V150	58.7 ± 2.3 [a]	68.1 ± 4.9 [b]	7.9 ± 5.6	88.7 ± 0.4 [a]	7.5 ± 0.5 [b]	19.1 ± 2.3 [a]	6.6 ± 2.1 [a]	34.5 ± 1.8 [a]	80.8 ± 2.1 [b]
M0	70.5 ± 1.8 [b]	65.4 ± 3.3 [ab]	13.4 ± 3.1	97.4 ± 0.2 [b]	39.2 ± 3.1 [e]	74.8 ± 0.2 [e]	31 ± 4.8 [b]	56.1 ± 7.4 [c]	80.5 ± 0.7 [b]
M100	67.5 ± 1.2 [ab]	58.5 ± 3.8 [a]	8.3 ± 0.1	97.1 ± 0.1 [b]	3.7 ± 0.9 [a]	35.1 ± 3.7 [d]	25.7 ± 6.8 [b]	45.8 ± 3 [b]	74.4 ± 1.5 [a]
p-value	0.051	0.069	0.707	0.000	0.000	0.000	0.000	0.000	0.004

V0 – Basal plant ingredient based diet; M0 – Basal fish meal based diet; V30, V60, V100 and V150 – plant ingredient based diets with respective percentage inclusion of micro-mineral premix; M100 – Fishmeal based diet with 1% inclusion of micro-mineral premix. Data presented as mean ± SD; values within the same column having different superscripts are statistically different.

Table 5: Comparison between the NRC (2011) recommendation on micro-mineral requirements to rainbow trout and available supply of micro-minerals from the basal M0 and V0 diets in the present study

	Requirement of Rainbow trout NRC (2011)	Available supply from basal diet (mg/kg DM)§		Percentage contribution of M0 diet to NRC (2011) recommendation	Percentage contribution of V0 diet to NRC (2011) recommendation
		M0-diet	V0-diet		
Iron	NT (21†)	63.3	20.61	301.4	98.1
Copper	3	5.2	2.79	173.3	92.9
Manganese	12	2.9	6.47	24.2	53.9
Selenium	0.15	0.87	0.195	580.0	130.2
Zinc	15	35.2	17.25	234.7	115.0

NT, not tested in rainbow trout as per NRC (2011). †Fe requirement provided within parenthesis is based on data from Shearer (1995).
§Calculated from data presented in table 2 (dietary trace mineral concentration) and table 4 (AAC of trace minerals) for V0 diet.
¶Calculated as relative percentage of available supply from basal diet to the respective available requirement estimates.

Table 6: Mineral composition of rainbow trout fed the experimental diets for 12 weeks (fresh weight basis)

	Ash (%)	P (g/kg)	Ca (g/kg)	Mg (g/kg)	K (g/kg)	Na (g/kg)	Fe (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Zn (mg/kg)	Se (mg/kg)
Initial	2.3	4	4.2	0.3	3.7	1.1	26	0.9	0.9	24.9	0.25
V0	1.8 ± 0.04	3.3 ± 0.1	2.4 ± 0.1	0.3 ± 0.02	3.6 ± 0.02	0.8 ± 0.02	21.4 ± 2.5	1.9 ± 0.1 ^{c}	1 ± 0.1^{b}	12.1 ± 0.5 ^{c}	0.13 ± 0.007^{e}
V30	1.7 ± 0.03	3.1 ± 0.2	2 ± 0.1	0.3 ± 0.01	3.7 ± 0.04	0.8 ± 0.03	26.5 ± 9.8	2.4 ± 0.3 ^{cb}	0.9 ± 0.1^{b}	13.1 ± 0.1 ^{cb}	0.13 ± 0.004^{e}
V60	1.8 ± 0.1	3.1 ± 0.1	2.2 ± 0.3	0.3 ± 0.02	3.6 ± 0.06	0.8 ± 0.02	26 ± 5.5	2.5 ± 0.2 ^{cb}	1 ± 0.2^{b}	13.8 ± 0.2 ^{b}	0.14 ± 0.005^{d}
V100	1.7 ± 0.1	3.1 ± 0.2	2.2 ± 0.4	0.3 ± 0.07	3.5 ± 0.07	0.8 ± 0.03	17.9 ± 0.6	2.8 ± 0.2 ^{b}	0.9 ± 0.1^{b}	13.8 ± 0.6 ^{b}	0.16 ± 0.002^{c}
V150	1.7 ± 0.1	3.2 ± 0.1	2.2 ± 0.3	0.3 ± 0.06	3.6 ± 0.06	0.9 ± 0.02	18.5 ± 2.4	4 ± 0.4 ^{a}	0.9 ± 0.1^{b}	16.4 ± 0.5 ^{a}	0.18 ± 0.006^{b}
M0	1.8 ± 0.07	3.3 ± 0.16	2.4 ± 0.28	0.3 ± 0.01	3.7 ± 0.04	0.9 ± 0.02	14.4 ± 0.23	0.9 ± 0.12^{e}	0.7 ± 0.04^{a}	15.4 ± 0.51^{ab}	0.3 ± 0.01^{a}
M100	1.8 ± 0.08	3.4 ± 0.14	2.6 ± 0.18	0.3 ± 0.01	3.7 ± 0.1	0.9 ± 0.04	22.5 ± 5.58	1.4 ± 0.35^{d}	1.2 ± 0.09^{c}	17.3 ± 0.4	0.3 ± 0.01^{a}
p-value	0.1	0.09	0.24	0.17	0.03	0.2	0.67	0.00	0.01	0.01	0.01

V0 – Basal plant ingredient based diet; M0 – Basal fish meal based diet; V30, V60, V100 and V150 – plant ingredient based diets with respective percentage inclusion of micro-mineral premix; M100 – Fishmeal based diet with 1% inclusion of micro-mineral premix. Data are expressed as mean ± SD. P-value indicates statistical significance as obtained through ANOVA. Values within the same column with different superscripts are statistically significant.

Contribution of basal diet in meeting available micro mineral requirements

A comparison between the NRC recommendations (2011) and the contribution of available micro mineral supply from the basal V0 and M0 diet to the requirements of corresponding micro-minerals for rainbow trout are presented in Table 5. In reference to NRC (2011) recommendations for rainbow trout,, the V0 basal diet covered 34.3, 92.9, 53.9, 115 and 130.2%; and the M0 diet for 105.5, 173.3, 24.2, 234.7 and 580% of the minimal dietary inclusion levels of Fe, Cu, Mn, Zn and Se, respectively.

Chemical composition of fish

The major chemical composition of the body such as moisture (674 ± 5 g/kg), crude protein (155 ± 2 g/kg), crude fat (166 ± 6 g/kg), ash (17 ± 1 g/kg) and energy (9.2 ± 0.2 kJ/g) were not different between the treatments (data not shown). The initial and final mineral concentrations of fish fed the experimental diets are presented in Table 6. Micro mineral premix supplementation did not affect the concentration of macro-minerals such as P, Ca, Mg, K and Na. The final body concentration of Cu ($P=0.04$), Zn ($p<0.01$) and Se ($p<0.01$) increased with premix inclusion level, while Fe and Mn were not significantly different between the V diet fed groups. In comparison with M diet fed groups, it was found that Zn and Se levels even in the V150 diet were insufficient to maintain the normal body mineral levels in rainbow trout.

Discussion

Excessive use of inorganic minerals in practical diets can be avoided by utilising the intrinsic potential of minerals in feed ingredients (Sugiura et al., 1998). Tacon and De Silva (1983) documented exceedingly high concentrations above recommended levels and 2 to 11 fold variations for micro minerals such as Fe (80-540 mg/kg), Cu (5-40 mg/kg), Mn (35-100 mg/kg) and Zn (50-260 mg/kg) within similar feed categories of commercial salmonid feeds that were available in Europe. Three decades down the line, the situation has not changed much, according to the recent report based on a survey on different Norwegian fish feeds over a decade from 2000-10' (Sissener et al., 2012). The reported minimal and maximal dietary levels are as follows, Fe (65-493 mg/kg); Cu (2.5-21 mg/kg); Mn (4.4-226 mg/kg); Zn (36-330 mg/kg) and Se (0.39 to 4.1 mg/kg). These data suggest that, (i) high variations could be related to the differences in micro mineral composition of the major feed ingredients; due to a diverse array of source and origin

(ii) commercial fish feed formulations are more often negligent to the negative effects of excess micro mineral supply. Given the diverse array of alternate feed ingredients of varying mineral concentrations, necessary strategies need to be devised to provide optimal levels, avoiding deficient or excess of micro minerals in fish feeds.

The available micro-mineral levels from the basal diets (both M0 and V0) fulfilled more than 90% of the respective micro-mineral requirements to rainbow trout except for Mn with reference to NRC (2011) recommendations. However, the final whole body concentrations indicated deviations from the earlier statement. Final body composition is a robust response to quantify macro-mineral requirement (Shearer, 1989, Rodehutscord, 1996, Antony Jesu Prabhu et al., 2013) and can also be applied for micro minerals (Shearer, 1995). Iron is the only essential micro mineral for which the requirement is yet to be tested in rainbow trout (NRC, 2011). Shearer (1995) predicted an estimate of 21 mg/kg diet for normal Fe homeostasis in 10g rainbow trout. Dietary Fe requirement of Atlantic salmon is reported to be 60-100 mg/kg diet (Andersen et al., 1996, Naser, 2000), when fed $FeSO_4$ supplemented casein-based diets. It can be assumed that total Fe supply of 154 mg/kg DM (from V0 diet) almost satisfied the minimal dietary Fe needs of rainbow trout. The AAC of Fe was constant at 13% until V60 and reduced significantly thereafter to 10% and 7.5% in V100 and V150, suggestive of an excess dietary supply only beyond V60. This is more likely because iron homeostasis is primarily regulated at intestinal absorption stage by body iron stores and dietary Fe levels (Standal et al., 1999). Judging from the AAC and the higher but insignificant values of whole body Fe in V30 and V60, it could be proposed that supplementation of 30 mg Fe /kg (V60, 185 mg Fe /kg) would be required to the basal V0 diet.

The NRC (2011) recommended and the only available report on dietary Cu requirement of rainbow trout (3 mg/kg DM) was based on growth and body Cu concentration, obtained by testing only two dietary Cu levels (Ogino and Yang, 1980). Reports on Cu requirement for several other fish species ranges from 3.1 to 12 mg/kg DM (Gatlin and Wilson, 1986, Lorentzen et al., 1998, Shiau and Ning, 2003, Lin et al., 2008, Shao et al., 2010, Tan et al., 2011, Shao et al., 2012, Cao et al., 2014). In our study, the minimal dietary Cu concentration of 8.7 mg/kg DM (3.2 mg/kg DM on available basis) was sufficient to maintain normal body Cu concentration. The present estimate on available basis (3.2 mg/kg DM) was higher by 7% compared to the estimate of Ogino and Yang (1980) in rainbow trout, but actually this difference could be larger as the latter

estimate of 3 mg/kg DM is on total basis. Although the diet used in the study of Ogino and Yang (1980) was based on purified ingredients, 100% availability may not be realistic, as assumed by NRC. Certain micro minerals, especially Cu is very often reported to accumulate in the body when exposed to high dietary or aqueous supply (Kamunde et al., 2002). Even in the present study, it was observed that, accumulation rate of Cu in the body increased steeply when dietary Cu was in excess of 13.2 mg/kg DM (> 4 mg/kg DM on available basis). This sharp increase in whole body Cu was concurrent with the significant reduction in AAC of Cu in V150 possibly indicating a regulatory mechanism in response to the dietary Cu overload, as observed in Atlantic salmon (Berntssen et al., 1999). In view of this, an optimal range of 8.7 to 13.2 mg/kg DM total dietary Cu (3.2 to 4 mg/kg DM on available basis) could be considered for rainbow trout fed the V diets to avoid excess accumulation of Cu in the body.

The Mn requirement of rainbow trout ranges from 12-13 mg/kg DM in purified diets on total basis, with growth and tissue Mn levels as response criteria (Ogino and Yang, 1980). Similar to Cu, this estimate was obtained from testing only two dietary Mn levels. In the present study, fish fed the basal V0 diet with a total Mn content of 89 mg/kg DM (6.5 mg/kg DM on available basis) were able to maintain normal whole body Mn level, presumably due to the contribution from lupin meal. Lupin meal is reported to contain very high levels of Mn, up to 1390 mg/kg, as fed basis (NRC, 2011). Nevertheless, the estimate of 6.5 mg/kg on available basis which was sufficient to maintain body Mn level in the present study, was lower by 46% than the estimate (12 mg/kg DM) recommended by NRC (2011) based on data from Ogino and Yang (1980). This could possibly be due to (i) over estimation of Mn requirement by Ogino and yang (1980), as only two dietary levels were tested; (ii) present estimate is on available basis while the earlier was on total basis as explained for Cu; (iii) possibility of an underestimated Mn availability due to high total dietary Mn concentration in the basal V0 diet and bilary excretion of absorbed Mn making a significant contribution to faecal Mn load (Sugiura et al., 1998). However, vertebral Mn concentration would have provided a more robust indicator to evaluate if the Mn supplied by diet V0 was sufficient to rainbow trout, as shown by Satoh et al. (1991); in view of this, supplementation of 6-10 mg/kg inorganic Mn to the V diet may be beneficial.

Zinc and selenium concentrations are naturally low in plant-derived ingredients as compared with fish meal (Lall, 2002). Moreover, the availability of both Zn and Se can be

reduced by phytic acid (Cheng and Hardy, 2002) and inclusion of inorganic P or Ca salts (Kousoulaki et al., 2010, Antony Jesu Prabhu et al., 2014). Zinc is by far the most studied among the micro minerals essential to fish, and rainbow trout in particular. NRC (2011) recommends the Zn requirement of rainbow trout as 15 mg/kg DM; while NRC (1993) recommended 30 mg/kg DM, both based on the range of 15-30 mg/kg DM reported for maximal growth and body Zn levels when fed purified diets (Ogino and Yang, 1978). In the present study, the whole body levels of Zn in both M and V diet fed fish were lower compared to the initial levels. Tri-calcium phosphate (TCP) at 7% (equivalent to levels present in fish meal) resulted in complete unavailability of Zn to rainbow trout even with inclusion of 20 mg/kg $ZnSO_4$ (Satoh et al., 1987a). Thereby, supplementation of 40 mg/kg Zn (80 mg/kg total dietary Zn) was required to maintain whole body or vertebral Zn content when fed white fish meal based practical diets (Satoh et al., 1987a, Satoh et al., 1987b). In diets containing 4% TCP, dietary available Zn level of 15.7 mg/kg DM satisfied the growth and body Zn requirements of rainbow trout (Satoh et al., 1987a). Thereby, Zn supplementation higher than by NRC (2011) recommendation is required in rainbow trout feeds to maintain normal Zn balance in the body, as recently suggested by Read et al. (2014).

As described earlier, the Se levels in plant ingredients are low compared to fish meal. The basal V0 diet had a total Se concentration of 0.24 mg/kg DM which is about 3-4 folds lower than observed with a fishmeal based diet. NRC (2011) recommends a Se requirement of 0.15 mg/kg DM based on weight gain as response criterion; while NRC (1993) had recommended 0.35 mg/kg DM which was based on maximal plasma GPx activity in rainbow trout reported by Hilton et al. (1980). Maximal weight gain at 0.2 mg/kg DM and Se accumulation in tissues beyond this level was reported in hybrid striped bass (Cotter et al., 2008). With increasing dietary Se levels, while a fixed proportion was defecated, excess Se was excreted by the kidneys (Hilton et al., 1982). Data on AAC and Se balance in the present study are in line with afore-mentioned study (Hilton et al., 1982) with a constant proportion (18-20%) of ingested Se being eliminated through faeces and the non-faecal loss increasing from 30% in V0 fed fish to 50% in fish fed diet V150. Regarding the optimal inclusion level of dietary Se to fish, some of the recent reports advocate high levels of dietary Se (in mg/kg DM): 0.9-1.2 for hybrid striped bass (Jaramillo Jr et al., 2009); 0.8 for cobia (Liu et al., 2010); 1.18 for gibel carp (Han et al., 2011); 1.6 for largemouth bass (Zhu et al., 2011); 5.56 for

yellowtail kingfish (Le and Fotedar, 2013) and 1.6 for grouper (Lin, 2014) under normal health conditions. The findings of the present study suggested that dietary supply of 0.54 mg/kg DM (0.4 mg/kg DM on available basis) was not sufficient to maintain body Se-balance. Fontagné-Dicharry et al. (2015) also reported similar results in whole body Se content of rainbow trout fry fed similar V-based diets, and also on GPx activity. Therefore higher inclusion of Se in plant ingredient based diets is recommended to maintain normal Se balance and GPx levels in the body. High levels of Se fortification in the form of Se-met or Se-yeast are reported to be beneficial in improving resistance to abiotic stress (Rider et al., 2009, Ribeiro et al., 2012, Saleh et al., 2014) and pathogen challenge (Le and Fotedar, 2013, Le and Fotedar, 2014), particularly in early life stages.

To conclude, with reference to NRC (2011) recommendations, the mineral levels in the V0 (basal) diet covered 98.1, 92.9, 53.9, 115 and 130.2% of the minimal dietary inclusion levels of Fe, Cu, Mn, Zn and Se to rainbow trout, respectively. However, data on whole body mineral content showed that Cu and Mn supply from basal V0 diet and, Fe supply from V30 diet were sufficient to maintain the normal whole body concentrations. Supplementation of Zn and Se were required beyond the levels supplemented in V150 to maintain body balance in rainbow trout fed complete plant ingredient based diets. On the whole, optimal dietary inclusion levels of microminerals are altered while using fishmeal free diets for rainbow trout.

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CHAPTER 9

Effects of water exchange rates in RAS and dietary micro-mineral supplementation on growth, body composition and mineral metabolism in common carp (*Cyprinus carpio*)

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Abstract

The effect of water refreshment rate in recirculation aquaculture systems (RAS) and of the consequent accumulation of minerals in RAS water on the mineral balance and minimal dietary inclusion levels of micro-minerals for common carp were studied. The system with low-water exchange rate (70 L/kg feed) accumulated more minerals in the water and was termed high accumulation system (HAS), while the mineral concentration in high water exchange rate system (2000 L/kg feed) was less and accordingly termed as low accumulation system (LAS). The experiment had a 2 by 5 factorial arrangement: 2 RAS systems namely HAS and LAS; and 5 dietary treatments differing in the premix inclusion level (0, 0.3, 0.6, 1 or 1.5%). The diets were made completely of plant derived ingredients. Each treatment was done in triplicates and the experiment lasted for 8 weeks. A tendency for higher growth was observed in the HAS compared to the LAS ($P=0.10$). With increasing dietary premix inclusion levels, the growth and feed efficiency decreased ($P<0.05$). Fish reared in HAS systems had higher whole body levels for all minerals ($P<0.05$), except Cu. With increasing inclusion levels of the premix, the whole body concentration of Cu, Se and Zn increased ($P<0.001$), while Fe and Mn content were unaffected ($P>0.05$). The minimal dietary mineral level (in mg/kg DM) over both systems, as estimated by broken line analysis was 10.2-11.3 for Cu, 273-278 for Fe and 49.2-50.2 for Zn. The minimal dietary Se level appeared to be lower ($P<0.05$) in carp reared in the HAS (0.28 mg/kg) compared to LAS (0.32 mg/kg) system. The estimated minimal dietary mineral levels based on vertebral mineral concentration for Fe, Cu, Mn and Zn was 305, 16.5, 39.5 and 55 mg/kg DM respectively. Activities of enzymes and expression of genes involved in mineral metabolism and transport were studied in gills, liver and intestine. Of all enzyme activities studied, only glutathione peroxidase showed significant and consistent results (2 to 4 fold higher in supplemented groups) across all three tissues. GPX expression was also increased in the tissues of fish fed the premix supplemented diets in both systems. In general, significant interactions between system and diet in the expression of target genes were observed the most in the gills. To conclude, common carps reared in HAS were able to acquire and retain minerals, and were only able to compensate for a part of the dietary requirement in the case of selenium.

Keywords: Recirculation aquaculture system; plant based diet; minerals; requirement.

Introduction

Space availability, water utilisation efficiency and nutrient discharge in wastewater are major challenges facing the sustainable development of aquaculture. Recirculation aquaculture system (RAS) offer potential ways to handle the foresaid issues (Martins *et al.*, 2010). In RAS, either complete or a part of the used water is re-used after treatment to remove the toxic nitrogenous metabolites through bio-reactors. Removal of nitrogenous waste metabolites forms the core activity in ensuring the successful functioning of the RAS. Although, the toxic nitrogenous metabolites are removed or transformed to non-toxic forms in RAS, many particulate and dissolved substances tend to accumulate in the re-circulated water, for example minerals and trace elements (Martins *et al.*, 2009b). Increasing the re-use of water is often observed to be associated with increased accumulation of the essential mineral ions in RAS water, such as P, K, Mg, Cu, Fe, Mn, Zn, and heavy metals such as arsenic (As), barium (Ba), boron (B), lithium (Li), Nickel (Ni), strontium (Sr) (Martins *et al.*, 2009b; Davidson *et al.*, 2011; Davidson *et al.*, 2009; van Bussel *et al.*, 2014).

Uptake of micro-minerals from water has been studied for iron, copper, zinc and selenium in fishes (Bury et al. 2003; Hodson and Hilton, 1983). Although diet is the preferred source for most of the essential micro-minerals to fish, absorption from branchial and cutaneous pathways can fulfil part of the requirements when dietary supply is sub-optimal. Recently, there is increasing evidence that the utilisation or accumulation of minerals in fish tissues is dependent on water exchange management of RAS (low vs high exchange rate). Tilapia and common carp fry were able to acquire water-borne minerals when reared in water from a low-water exchange RAS (Martins *et al.*, 2011; Martins *et al.*, 2009b). Van Bussel *et al.* (2014) observed increasing accumulation of minerals in turbot reared in RAS with decreasing water exchange rates.

In this scenario, the question arises whether fish can utilise water-borne minerals in RAS and compensate for a part of their dietary supply? It is also suggested that an inter-relation exists between the acquisition of dissolved minerals from the water at the gills and absorption of dietary minerals at the intestine (Bakke *et al.*, 2010). In rainbow trout, the relative contribution of water-borne Cu to whole body copper load amounts for 10% and increases up to 60% at low dietary Cu levels (Kamunde *et al.*, 2002). A similar pattern of regulation was observed for zinc in rainbow trout (Spry *et al.*, 1988). Given

the accumulation of minerals in RAS and the ability of fish to acquire dissolved minerals, the objective of the study was to examine the effect of aqueous mineral availability on mineral balance and minimal dietary inclusion levels required for common carp reared in RAS with high or low aqueous mineral concentrations generated through differing water exchange rates.

Material and Methods

RAS design and pre-conditioning of the system

The experiment consisted of two identical recirculating aquaculture systems (RAS; Fig.1), which were operated differently regarding water quality. The purpose was to create a contrast in water mineral contents, by applying different water-exchange rates. The high mineral accumulation system (HAS) had low-water exchange rate and the low mineral accumulation system (LAS) had a high water exchange rates. Water exchange levels of 70 and 2000 L/kg feed were used for HAS and LAS, respectively. Each RAS system consisted of 15 experimental tanks (i.e., 70-L glass aquaria) and 4 donor tanks (450-L), a trickling filter, drum filter and two sumps.

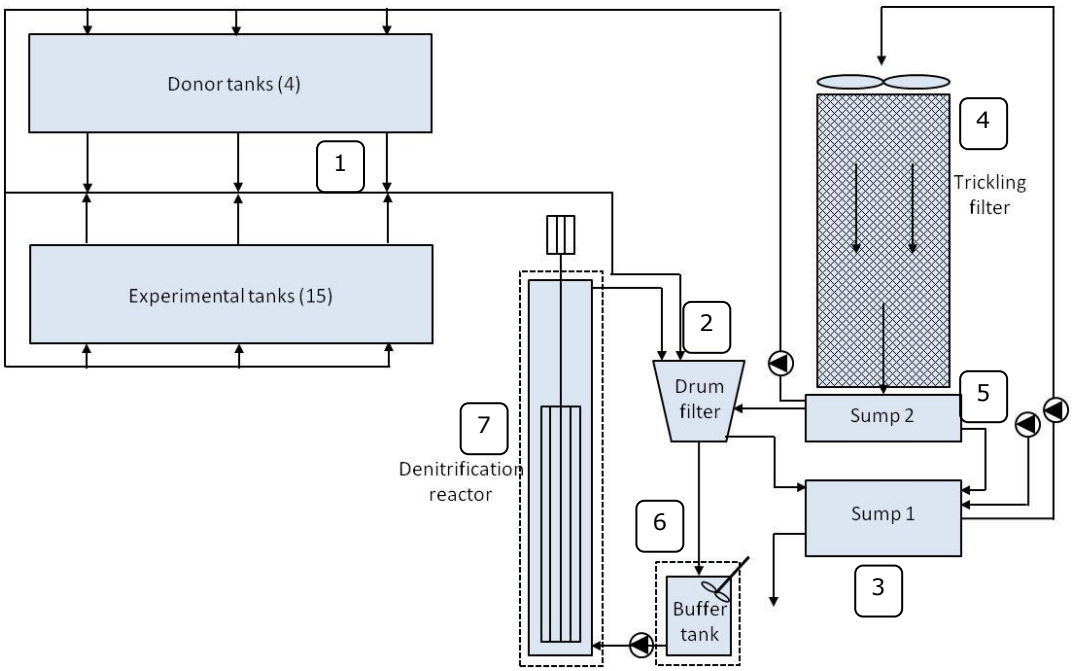


Fig. 1: Recirculating aquaculture system setup. The dotted lined boxes indicate additional components of the high accumulation system. Main path water: 1. Experimental (15 x 66L) and donor tanks (4 x 450L), 2. Drumfilter (Hydrotech HDF 501-1P , 90 µm meshsize, 20L), 3. Sump 1 (600L), 4. Tricklingfilter (70L), 5. Sump 2 (300L). The high accumulation system has an additional 6. Buffertank (20L) and 7.Denitrification reactor (480L) with faeces as a carbon source. Water refreshment occurs via sump 1, which is controlled manually or is set at a certain refreshment rate, for the high and low accumulation systems respectively. A fan (Mini-air® IV)on top of the trickling filter allows for degassing. Adapted from (Martins et al. 2009).

The daily water exchange from each system was done via the sump. This was performed manually for HAS to prevent accidental water loss.The HAS differed from the LAS in two ways, e.g. by the addition of a denitrification reactor and a buffer tank. A denitrification reactor was placed in HAS system to ensure nitrate and ammonia were maintained below toxic limits. Faecal carbon originating from the drum filter was the only carbon source for the denitrification reactor. The system start-up phase of the experiment lasted two months, which enabled the denitrification unit and biofilter to stabilize and to obtain the aimed water exchange levels combined with a stable accumulation of minerals (measured as conductivity in µS/m). The basal mineral accumulation level was established in the 2-month pre-period and maintained during the experimental period by feeding a commercial diet to the carp kept in the 'donor tanks'. The carp in the donor tank, which were not the experimental fish, were fed a commercial carp feed (Skretting Carpe F) at 300g/d per tank, thus 1.2 kg/d per system. This feeding load per system was aimed to load both systems equally with faecal and non-faecal waste. This, in combination with the daily water exchange per system, created a difference in dissolved minerals between HAS and LAS. The water flow rate for the experimental and donor tanks was set at respectively 7 and 16L/min per tank. pH (7.0-7.5) and temperature (24°C) were aimed to be similar for both systems. Photoperiod was set at 12 hours light and 12 hours dark.

Experimental design and set-up

The experiment had a 2 by 5 factorial design: 2 water quality management strategies, one RAS system (HAS) having a high water mineral accumulation level and the other RAS system (LAS) a low accumulation level; and 5 dietary treatments differing in the premix inclusion level (0, 0.3, 0.6, 1 or 1.5%). Accumulation of substances in RAS water and dietary mineral premix inclusion, and their interaction, were the factors of investigation in the study. Each treatment combination was done in triplicate (tanks). The experiment lasted for 8 weeks.

Experimental animals

A standard line R8R3 (F11) of common carp (*Cyprinuscarpio*) was bred at the Aquatic Research Facility (Carus-ARF) of Wageningen University. At the start of the experiment, average fish weight was 8.5g, and 25 fish were stocked per experimental tank. The 15

experimental tanks within each RAS were randomly assigned to one of the 5 dietary treatments. Moreover, 30 fish was sampled for initial body composition analysis.

Table 1: Ingredient and analysed nutrient composition of the basal diet

Ingredients (g/kg)	*Basal 'V' diet*
Corn gluten meal (CP 60; Inzo, France)	100
Wheat gluten (CP 70; Roquette, France)	100
Soybean meal (CP 48; Inzo, France)	100
Soyproteinconcentrate (Estrilvo ; CP 70; Sopropêche, France)	100
Extruded peas (Aquatex, Sotexpro, France)	100
Rapeseed meal (Primor 00; SudOuestAlemants, France)	100
Whole wheat	219.4
Soylecithin (Louis François, France)	20.0
L-Lysine (Eurolysine)	31.9
$CaHPO_4.2H_20$ (18%P; 22% Ca)	16.0
Attractant Mix §	15.0
Mineral premix ¶	**0, 3, 6, 10 or 15**
Vitamin premix †	10.00
Rapeseed oil (Daudruy, France)	24.00
Linseed oil (Daudruy, France)	31.80
Palm oil (Daudruy, France)	31.80
Analysed nutrient composition	
Dry matter (DM), %	93.8
Crude protein, % DM	38.8
Crude lipid, % DM	8.2
Crude ash, % DM	4.3
Energy, kJ/g DM	21.0
Phosphorus, g/kg DM	8.6
Calcium, g/kg DM	6.4
Ca/P ratio	0.74
Magnesium, g/kg DM	1.5
Potassium, g/kg DM	6.8
Sodium, g/kg DM	1.1

§Attractant premix: glucosamine, 5g; taurine, 3g; betaine, 3g; glycine, 2g and alanine, 2g.
† Vitamin premix (IU or mg/kg diet): DL-a tocopherol acetate, 60 IU; sodium menadione bisulphate, 5 mg; retinyl acetate, 15,000 IU; DL-cholecalciferol, 3000 IU; thiamin, 15 mg; riboflavin, 30 mg; pyridoxine, 15 mg;B12, 0.05 mg; nicotinic acid, 175 mg; folic acid, 500 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate,50 mg; choline chloride, 2000 mg. (UPAE, INRA)
¶ Trace mineral premix (g/kg premix): $FeSO_4.7H_2O$ (21% Fe; 11.5% S), 25 g; $CuSO_4.5H_2O$ (25.45% Cu; 12.8% S), 3 g; $MnSO_4.H_2O$ (33% Mn; 19% S), 3 g; $ZnSO_4.H_2O$ (36% Zn; 18% S), 4 g; Na_2SeO_3 (46% Se; 27% Na), 0.03 g; KI (76% I; 24% K), 0.04 g and α-cellulose, 964.93 g.

Experimental diet and feeding

The basal diet was a complete plant-ingredient based diet and was formulated to meet the nutritional requirements of common carp as given by NRC (2011), except for the micro minerals, Fe, Cu, Mn, Zn and Se. The micro-mineral pre-mixture contained Fe, Cu, Mn, Zn and Se and was formulated to meet the theoretical requirements of these mineral for common carp as given by NRC (2011) at an inclusion level of 10 g/kg diet (Table 1). The premix formulation was based on the assumption that the minerals in the basal diet were not available for the fish. The five different experimental diets were then

formulated by incorporating 0.0, 0.3, 0.6, 1.0 and 1.5% of this micro-mineral premix to the basal diet. Feeds were produced by extrusion into a 1.5mm floating pellet at the INRA experimental fish farm, Donzaq, France. The analysed micro-mineral concentrations of the experimental diets are shown in Table 2. The amount of feed given per fish was kept equal at all treatments in order to avoid any bias in mineral balances arising due to differences in feed intake between treatments. Therefore, the fish were fed restrictively at a feeding rate of 18 g $kg^{-0.8}$ d^{-1} throughout the experiment. Daily feed ratio was increased based on an expected feed conversion ratio of 0.9. This feeding level was calculated to attain 80%of the quantitative feed intake at apparent satiation feeding. Fish were fed twice daily at 9:30 and 15:30 for 57 days.

Table 2: Analysed trace mineral concentration of the experimental diets with graded levels of mineral premix inclusion (mg/kg DM)

	Fe	Cu	Mn	Zn	Se
V0	259.3	8.4	31.3	40.5	0.14
V0.3	276.4	10.4	33.1	44.0	0.19
V0.6	286.3	12.9	37.8	48.1	0.23
V1	304.6	15.7	41.0	51.9	0.29
V1.5	331.5	19.9	44.7	57.2	0.36

V0, V0.3, V0.6, V1 and V1.5 represent experimental diets with micro-mineral premix inclusion levels of 0%, 0.3%, 0.6%, 1% and 1.5% , respectively.

System and water quality management

Daily water quality measurements were performed on both systems. Measurements were made from samples taken at the combined outlet of the experimental tanks, but also at the denitrification unit outlet before feeding. This included testing for pH, temperature, conductivity and nitrate levels, water outflow of the denitrification reactor and the water exchange flow of the LAS and HAS. The denitrification reactor was checked for sludge removal once every second day. Nitrite and ammonia were checked weekly by Merck tests and also by using an auto-analyzer (SKALAR). Water exchange, denitrification reactor management and pH were daily monitored. The accumulation of minerals were controlled by water exchange of 70 L kg^{-1} and 2000 L kg^{-1} for the HAS and LAS respectively. System water samples for analysing the mineral concentration were taken on week 0, week 4 and week 8. Water refreshment rates were daily calculated via the amount of feed given and measured water losses after feeding and correction of evaporation. Water exchange in the LAS was set at a constant flow, which was adjusted every few days. The use of a denitrification reactor contributes to water losses. Sludge

was removed and weighed daily. In the start-up phase, the sludge removal was set at a standard rate of 7.4 kg a day (about 10% of the start-up phase stable sludge bed). Efficiency of the sludge-bed increased during the experimental period and therefore more sludge needed to be removed. This occurred by performing multiple sludge removals. The occurrence of multiple sludge removals was highly dependent on the amount of nitrogen in the outlet of the denitrification reactor, to prevent the formation of hydrogen sulphide. The stable height of the sludge-bed decreased from 65cm to 35cm. At this point a daily amount of 7.4 kg sludge was taken, resulting in a sludge removal of about 20% daily. The pH in the HAS was regulated by increasing denitrification reactor inflow or by adding sodium bicarbonate. In the LAS, pH control activities was not required as it was mainly regulated by the water exchange and therefore in balance with alkalinity losses caused by the amount of feed.

Sampling and analytical methods

Water samples for mineral composition were taken at the combined experimental tank outflow within each system (Week 0, 4 and 8). Fish from each experimental unit were bulk-weighed at the start (Week 0), Week 4 and at end of the experiment (Week 8).The fish were anaesthetised during each weighing (0.25ml L^{-1}, 2-phenoxyethanol). During the final weighing, a random sample of 5 fish per unit was taken for blood sampling. After sampling for blood while being anaesthetised, the fish were then euthanized by a sharp cut on the head and sampled for gill, intestine and hepatic tissue. Ten other fish from each experimental unit were sampled for body composition analysis. Another 10 fish were sampled from each experimental unit, for separation of vertebrae for mineral analysis following the method described by Vielma and Lall (1998).

Moisture content of the pooled fish samples were determined by drying freshly ground samples at 105°C for 24h. The chemical composition of the diets and of fish samples were analysed by the following methods: dry matter after drying at 105°C for 24h, ash by combustion at 550°C for four hours in a muffle furnace, crude protein (Nx6.25) by Kjeldahl method in acid digested samples, crude lipid by petroleum ether extraction using Soxhlet method (Soxtherm) and gross energy content in an adiabatic bomb calorimeter (IKA, HeitersheimGribheimer, Germany). The mineral analyses (P, Ca, Mg, K, Fe, Cu, Se and Zn) in feed, fish, vertebrae and water samples were performed in ICP-MS at Chemical Biological Laboratory (CBLB, Wageningen).The concentrations of Fe, Cu, Mn

and Zn in the plasma samples were analysed using inductively coupled plasma-mass spectrometry (ICP-OES) at USRAVE-INRA, Bordeaux, France. The Se content in the plasma samples were analysed using ICP-MS at LCABIE-UPPA, Pau, France.

Enzyme assays: Samples of gill, liver and anterior intestine were ground in 4 volumes of ice cold buffer (TRIS HCl, 50mM; NaCl, 150mM; pH, 4). After homogenization, the samples were subjected to sonic disruption for one minute. The samples were kept on ice during sonication. Homogenates were then centrifuged for 10 min at 3500 rpm at 4°C and the supernatants were immediately used for enzyme assays. Activities of ferric reductase (FR, EC 1.16.1.7) and cupric reductase (CR, EC 1.16.1) were determined from the same extract. FR activity was measured as described by Mazoch et al. (2004), omitting flavin mononucleotide in the mixture. The reduction of iron was followed by the formation of the coloured Fe(II)-ferrozine complex and monitoring the change in absorbance at 562 nm. CR activity was assayed according to Wyman et al. (2008), the Cu(I)-bathocuprionedisulfonate complex was monitored by the change in absorbance at 482 nm. ALP activity was measured using a commercial kit (Enzyme ALP, bioMérieux, ref 63509). Antioxidant enzyme activities CAT (EC 1.11.1.6) and GPX (EC 1.11.1.9) were assayed in the liver homogenates following the method of Fontagne et al. (2008). Total SOD (EC 1.15.1.1) activity was measured using a commercial kit (Sigma, St Louis, MO, USA ref 19160-1KT-F); MnSOD (SOD2) was measured by inhibiting Cu-ZnSOD using 5 mM KCN (Sigma) as specific inhibitor and Cu-ZnSOD (SOD1) activity was calculated by subtracting MnSOD activity from total SOD activity (Knox et al., 1981). In all the enzyme assays, the reaction was initiated by the addition of a specific substrate; a blank with water instead of the substrate was run for each sample. One unit of enzyme activity was defined as the amount of enzyme that catalysed the transformation of 1 µmol of substrate per min at 30 °C. Protein concentration of all the samples were measured in triplicate by the method of Bradford (1976), using a protein assay kit (Bio Rad, Munich, Germany) with bovine serum albumin as the standard. In all cases, a Power Wave X (BioTek Instrument, Inc.) was used as the plate reader. The enzyme activities were expressed specific to per mg of protein.

Table 3: Details of the primer sequences used for the amplification of the target and reference genes in common carp.

Gene	Accession no. from GenBank or contig from Carp genome*	Primer sequence (5′ → 3′)	Product size (bp)	Annealing temperature (°C)
18S	gi:29336521	F: CATGGCCGTTCTTAGTTGGT; R: CGGACATCTAAGGGCATCAC	180	58
40S	AB012087	F: GTTGAAGGAAGTGGCAAGGA; R: AGAATACGGCCTCTGATGGA	146	58
B-actin	Contig_52499	F: GATGGACTCTGGTGATGGTGTGAC; R: TTTCTCTTTCGGCTGTGGTGGTG	167	59
HAMP	JX855261	F: ACATGCGTCTGCTTCCTCC; R: CTGGTTCTCCTGTGGTGCTT	96	59
FRRS	Contig_25630	F: GCAAGCGGGCAGGAATAC; R: GAGGAAGATGGTGATGACAG	179	58
FPN1	Contig_43385	F: GTCCTCTTACTGGGCGCTAT; R: GCCAGGTTAGCGATGTTAGC	224	59
HO1	JX257180.1	F: AGAGAGATTGGCAAGAACAGC; R: CGACACTCCAGGAAACGAGA	162	59
HO2	Contig_10644	F: GTGCAGGACATTGGCTTTCA; R: CACATGAGGTACCAAGCAGC	248	59
CTR1	Contig_41221	F: CACAGTGACCATGTGCACC; R: GAACACAGCCAGCAAGAACA	222	59
ATP7a	Contig_14035	F: AACCGAGAGTGGATGAGGAG; R: CTGTTGTCTCCCGTCATCAG	224	58
ZnT5	Contig_3678	F: TCCTATGGGTATGGTCGTGTG; R: TGTTGATGTTCGGTGGATCG	124	59
ZnT7	Contig_44487	F: ATGTTCTTTGACTGCACCGC; R: CGCCCTCTCTACTCCTTCTG	180	59
ZIP7	Contig_7463	F: CGATCAGCACCATCATTCCC; R: CGGAGGCTTCGTCTACATCGC	128	60
ZIP10	Contig_5927	F: TGTTGCCAGAGATGCTTCATG; R: GATTCCAAACCCTGTGAGCA	101	59
GPx1	GQ376155.1	F: ACCAGTTCGGACATCAGGAG; R: CCGTTCACTTCCAGCTTCTC	121	60
GPx4a	FJ656211.1	F: TGGAAATGGCATCAAATGGA; R: ACGCTTGGATCCGATAATGG	93	62
GPx4b	FJ656212.1	F: AGCAGGAGCCTGGGACAGAG; R: TCCATTTCCAGAGCGGATGA	120	24
CAT	GQ376154.1	F: TTTCAACTGTGGCTGGTGAG; R: GGATGAAGGACGGAAACAGA	159	60
SOD1	Contig_43323	F: AAGAGAGTGACGGGTCTCCA; R: GATCTCCGACGTGTCTGTCA	186	60
SOD2	AJ492825.1	F: GAACCACAGGGTGAGCTGTC; R: GACCTGTAGTGCCCTGCAAA	193	61
GR	JN126053.1	F: GGCAGCTCATTAAACGCAAA; R: TGGACTCAATTTTGGCCTTG	90	61
GST	DQ411313.1	F: GGTCGCAAACATGGTGCATA; R: TTTGAGGTGGTTGGGCAGAT	165	63

F, forward primer; R, reverse primer; 18S, 18s rRNA; 40S, 40s rRNA; HAMP, Hepcidin anti-microbial peptide; HO1, heme oxygenase 1; HO2, Heme oxygenase 2; FPN1, ferroportin1; FRRS, Ferric reductase; CTR1, copper transporter I; ATP7a, Cu^{++} transporting ATPase-alpha polypeptide; ZnT5, Zinc transporter protein5; Zinc transporter protein7; ZIP7, Zinc importer7; ZIP10, Zinc importer10; GPX1, glutathione peroxidase 1; GPX4a, glutathione peroxidase 4a; GPX4a, glutathione peroxidase 4b; CAT, catalase; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; GR, Glutathione reductase; GST, Glutathione S-transferase.

Gene expression analysis: Total RNA was extracted from gill, liver and anterior intestine samples (n=6 per treatment) using Trizol reagent (Invitrogen, Cergy-Pontoise, France) as previously described (Fontagne et al., 2008). For quantitative RT-PCR, complementary DNA was generated from 1 µg total RNA using SuperScript® III reverse transcriptase (Invitrogen) with a mix of oligo$(dT)_{15}$ and random primers (Promega, Charbonnières, France). RT was performed in duplicate for each sample and the quantitative PCR analyses were performed in LightCycler 480 II thermocycler (Roche) using LightCycler 480 SYBR Green I Master mix (Roche Diagnosis, Indianapolis, IN, USA). Total reaction volume was 6µL, with 2µL of cDNA (RT product) and 4 µL of master mix added with 0.4mM of each primer. The primer details of the genes analysed for mRNA expression are provided in Table 3. Relative quantification of target gene transcripts were normalized using B-actin for liver, 18S for gills and 40S for intestine as reference genes and LAS-V0 as the reference group following the method of Pfaffl (2001).

Data analysis

The data on chemical and mineral concentration in the water of the two systems (LAS and HAS) were subject to Student's t-test. Tanks (n=3) were used as experimental units for data on growth, body and tissue mineral concentration. Rest of the data were subjected to two-way ANOVA followed by Tukeys' multiple comparison test at a probability of $P<0.05$ to test the effect of rearing system, dietary mineral mix and their interaction. Broken line regression (Robbins *et al.*, 2006) was used to estimate the minimal dietary inclusion levels with data on whole body and vertebral mineral content as dependent (Y) variables and analysed dietary micro-mineral content as the independent (X) variable. Individual fish (n=6; 2 from each tank) was the experimental unit for data on enzyme assays and gene expression analysis. All the statistical analyses were performed through SPSS version 20, IBM Statistics Inc., USA.

Results

The water quality parameters of the low and high accumulation system are summarised in Table 4. The major purpose of accumulation in this study was to increase the level of minerals in the water of HAS. With the exception of Ca, all other mineral levels in the HAS were higher compared to the LAS ($P<0.01$). The concentrations of P and K in the water of HAS showed about 80 and 40 fold increase respectively, compared to the levels

found in water of LAS. Among micro-minerals, Fe and Se showed about 20-fold increase in water of HAS compared to LAS. As intended, the mean conductivity, ortho-phosphate concentration and water exchange differed significantly between the two systems. Conductivity and ortho-phosphate concentration were higher in the HAS with a smaller volume of water exchange ($P<0.05$). The differences in pH and temperature between the systems were also significant ($P<0.05$), but the magnitude was minimal. The HAS also shows increased levels of nitrate compared to the LAS, being 51.5 and 16.6 mg per L respectively. As a consequence, nitrite levels are elevated in the HAS ($P<0.05$).

Table 4: Analysed water quality parameters and mineral concentrations in the water of the two types of RAS namely low and high accumulation systems used in the study.

	Low accumulation (LAS)	High accumulation (HAS)	P-value
Temperature (°C)	24.2 ± 0.3	24.0 ± 0.3	0.000
pH	7.13 ± 0.09	7.22 ± 0.23	0.005
Conductivity (µS cm^{-1})	232 ± 4	1062 ± 51	0.000
TAN (mg L^{-1})	0.25 ± 0.03	0.25 ± 0.02	0.976
NO2-N (mg L^{-1})	0.09 ± 0.01	0.13 ± 0.01	0.003
NO3-N (mg L^{-1})	16.62 ± 0.84	51.52 ± 1.57	0.000
o-Phosphate (mg L^{-1})	0.33 ± 0.09	27.66 ± 0.91	0.000
Water exchange (L kg^{-1} feed)	1959 ± 150	70 ± 0.2	0.000
Minerals (mg§ or µg† L^{-1})			
Phosphorus (P)§	0.38 ± 0.09	27.97 ± 4.70	0.001
Calcium (Ca)§	26.5 ± 0.38	20.7 ± 2.91	0.026
Magnesium (Mg)§	2.46 ± 0.14	23.83 ± 3.84	0.001
Potassium (K)§	4.4 ± 0.79	137.7 ± 24.79	0.001
Sodium (Na)§	6.8 ± 0.45	81.6 ± 12.62	0.001
Iron (Fe)†	3.3 ± 0.01	73.3 ± 1.84	0.001
Copper (Cu)†	2.3 ± 0.01	15.7 ± 0.12	0.000
Manganese (Mn)†	0.3 ± 0.01	1.7 ± 0.01	0.001
Zinc (Zn)†	10.3 ± 0.01	36.3 ± 0.27	0.001
Selenium (Se)†	0.07 ± 0.01	1.05 ± 0.29	0.004

Data presented as mean ± SD. Temperature, pH and conductivity include 58 measurements. The TAN, NO2-N, NO3-N and o-Phosphate is the mean of 4 measurements. Mineral concentrations were the mean of 3 measurements made at 0, 28 and 56 days. The P-values indicate statistical significance as tested by students' t-test.

Data on feed intake, growth and proximate body composition are given in Table 5. As intended, feed intake (g per day) of fish did not differ between the treatments ($P>0.05$). A tendency for higher growth was observed in the HAS compared to the LAS, being 0.72 and 0.68 g/d respectively ($P=0.10$). With increasing dietary premix inclusion levels, the growth and feed efficiency decreased ($P<0.05$). During the experiment, the body fat content increased and the ash content decreased (Table 5), indicated by the initial and final proximate composition.

Table 5: Growth performance, initial and final body proximate composition of common carp fingerlings fed the experimental diets for 8 weeks and reared in the low or high accumulation systems.

	Growth performance				Body composition (g kg-1 DM)				
	FBW (g fish^{-1})	FI (g fish-1)	WG (g fish^{-1})	FE	DM	CP	CL	Ash	Energy (J)
Initial	8.5 ± 0.2				244.1	148.0	70.58	22.7	6.2
System									
LAS	47.19^{a} ± 0.75	59.5 ± 0.2	38.8 ± 2.8	0.70^{a} ± 0.01	294.2^{a} ± 1.1	133.5 ± 0.5	135.7^{a} ± 0.9	16.8^{a} ± 0.1	8.62^{a} ± 0.04
HAS	49.45^{b} ± 0.48	59.5 ± 0.2	40.8 ± 1.7	0.74^{b} ± 0.01	283.0^{b} ± 1.3	133.2 ± 0.5	123.9^{b} ± 1.0	17.6^{b} ± 0.1	8.11^{b} ± 0.06
Diet									
V0	50.43^{a} ± 0.86	59.5 ± 0.2	42 ± 1.8	0.76^{a} ± 0.01	295.0^{a} ± 3.0	133.7 ± 0.9	134.7^{a} ± 2.6	17.1 ± 0.3	8.64^{a} ± 0.11
V0.3	49.58^{a} ± 0.31	59.5 ± 0.2	41.2 ± 0.7	0.74^{ac} ± 0.01	285.1^{b} ± 3.4	134.2 ± 0.9	127.6^{b} ± 3.3	17.3 ± 0.2	8.16^{b} ± 0.14
V0.6	48.2^{ab} ± 0.8	59.5 ± 0.3	39.6 ± 1.5	0.72^{abc} ± 0.01	288.5^{b} ± 1.9	133.9 ± 0.8	127.0^{b} ± 2.3	17.3 ± 0.3	8.34^{bc} ± 0.12
V1	46.26^{b} ± 1.31	59.5 ± 0.2	37.7 ± 2.9	0.68^{b} ± 0.02	286.2^{b} ± 2.9	132.6 ± 0.5	130.2^{b} ± 3.1	17.1 ± 0.2	8.31^{bc} ± 0.13
V1.5	47.2^{ab} ± 1.2	59.6 ± 0.3	38.5 ± 2.5	0.70^{bc} ± 0.02	288.2^{b} ± 2.4	132.3 ± 0.6	129.6^{b} ± 2.6	17.3 ± 0.2	8.37^{c} ± 0.12
P-values, two-way ANOVA									
System	0.02	0.301	0.09	0.001	0.001	0.55	0.001	0.001	0.001
Premix	0.002	0.387	0.01	0.001	0.001	0.02	0.001	0.323	0.001
S x P	0.144	0.281	0.149	0.196	0.2	0.3	0.498	0.921	0.820

Data presented as mean ± SD. Initial body weight, 8.5 ± 0.2 g; LAS, low accumulation system; HAS, high accumulation system; V0, V0.3, V0.6, V1and V1.5, experimental diets with graded premix inclusion levels; FBW, final body weight; FI, feed intake; WG, weight gain; FE, feed efficiency.DM, dry matter; CP, crude protein; CL, crude lipid. Data are analyzed by 2-way ANOVA. Significant differences between diets are established with the Tukey test. Means having a different superscript letter within a column within each main effect are significantly different ($P<0.05$).

System water refreshment affected the final fat content ($P<0.05$), being higher in carp from LAS compared to HAS. Also final body ash content differed between both systems, being higher in carp reared in HAS compared to LAS. No interaction effect between system and dietary treatment was present for any of the performance and proximate body composition parameters ($P>0.05$; Table 5). Dietary mineral premix inclusion levels also lead to differences in protein and fat content of fish at the end of the experiment ($P<0.05$). Body crude protein content decreased with increasing levels of mineral premix, but these differences were rather small, being < 2%. Dietary mineral premix inclusion levels did not lead to differences in final body ash content of the fish ($P>0.05$).

Data on final body mineral composition of common carp fed the different diets, reared at either low or high water exchange, are provided in Table 6. Fish reared in HAS systems (i.e., low water exchange) had higher whole body levels for all minerals ($P<0.05$), except for Cu. With increasing inclusion levels of the micro-mineral premix, the whole body concentration of Cu, Se and Zn increased ($P<0.001$), but whole body Fe and Mn content were unaffected by the premix inclusion ($P>0.05$).

The dose-response relationship between whole body mineral concentration and analysed dietary mineral content is illustrated in Fig 2, and the results of broken line analysis are provided in Table 7. A significantly higher slope (i.e. utilisation efficiency) and higher plateau value in whole body concentration were observed for Fe in fish reared in HAS compared to fish reared in LAS. However, except for Se, there was no significant effect of the system (HAS vs LAS) on the minimal dietary mineral level (i.e., breakpoint) for any of the analysed micro-minerals (Table 7). The minimal required dietary mineral level range over both systems, as estimated by broken line analysis, was 10.2-11.3 mg/kg for Cu, 273-278 mg/kg for Fe and 49.2-50.2 mg/kg for Zn. The minimal required dietary Se level appeared to be lower ($P<0.05$) in carp reared in the HAS (0.28 mg/kg) compared to LAS (0.32 mg/kg) system. In the case of Fe, although the minimal dietary Fe level required (i.e. breakpoint) was unaffected by system changes, the utilisation efficiency and plateau level were significantly higher in carp from HAS than in carp from LAS. Data on vertebral mineral composition of common carp fed the experimental diets reared in the two systems are provided in Table 8.

Table 6: Whole body macro- and micro mineral concentration of common carp fingerlings fed the experimental diets for 8 weeks and reared in the low or high accumulation systems.

	Macro-minerals (g/kg fresh weight)					Micro-minerals (mg/kg fresh weight)				
	P	**Ca**	**Mg**	**Na**	**K**	**Fe**	**Cu**	**Mn**	**Se**	**Zn**
Initial	3.35	3.95	0.24	1.67	2.75	45.87	1.8	1.23	0.14	39.02
System										
LAS	3.37 ± 0.1^{a}	3.7 ± 0.2^{a}	0.24 ± 0.01^{a}	1.04 ± 0.03^{a}	2.73 ± 0.08^{a}	21.8 ± 0.7^{a}	1.64 ± 0.18	0.86 ± 0.17^{a}	0.14 ± 0.03^{a}	33.5 ± 4.2
HAS	3.75 ± 0.2^{b}	4.18 ± 0.3^{b}	0.26 ± 0.01^{b}	1.14 ± 0.06^{b}	2.98 ± 0.12^{b}	24.7 ± 1.6^{b}	1.59 ± 0.21	0.99 ± 0.15^{b}	0.15 ± 0.03^{b}	34.9 ± 4.3
Diet										
V0	3.33 ± 0.2^{a}	3.56 ± 0.26	0.24 ± 0.02^{a}	1.02 ± 0.04^{a}	2.7 ± 0.14	21.2 ± 1	1.34 ± 0.05^{a}	0.85 ± 0.17	0.11 ± 0.002^{a}	27.6 ± 0.5^{a}
V0.3	3.67 ± 0.4^{b}	4.1 ± 0.5	0.26 ± 0.03^{b}	1.14 ± 0.09^{b}	2.98 ± 0.23	23.8 ± 2.5	1.67 ± 0.1^{b}	1 ± 0.14	0.13 ± 0.01^{b}	32.8 ± 1.7^{b}
V0.6	3.66 ± 0.3^{b}	4.11 ± 0.49	0.26 ± 0.01^{b}	1.09 ± 0.04^{b}	2.86 ± 0.15	23.9 ± 2.4	nd	0.87 ± 0.18	0.15 ± 0.011^{bc}	35.4 ± 1.2^{c}
V1	3.56 ± 0.3^{b}	3.95 ± 0.29	0.25 ± 0.01^{c}	1.11 ± 0.08^{b}	2.89 ± 0.19	23.7 ± 2.6	1.68 ± 0.03^{b}	0.94 ± 0.18	0.17 ± 0.006^{c}	36.8 ± 1^{c}
V1.5	3.57 ± 0.2^{b}	3.96 ± 0.17	0.25 ± 0.01^{c}	1.09 ± 0.07^{b}	2.84 ± 0.18	23.6 ± 1.7	1.77 ± 0.04^{c}	0.98 ± 0.14	0.18 ± 0.01^{d}	38.4 ± 0.6^{d}
P-values, two-way ANOVA										
System	< 0.01	0.004	<0.01	< 0.01	0.01	0.001	0.52	0.04	< 0.01	0.6
Premix	0.01	0.15	0.001	0.008	0. 1	0.37	0.002	0.42	< 0.01	< 0.01
Sys x Premix	0.53	0.79	0.21	0.67	0.56	0.16	0.7	0.46	0.269	0.8

Data presented as mean ± SD. LAS, low accumulation system; HAS, high accumulation system; V0, V0.3, V0.6, V1and V1.5, experimental diets with graded premix inclusion levels; Data are analyzed by 2-way ANOVA. Significant differences between diets are established with the Tukey test. Means having different superscript letters within a column and within each main effect are significantly different (P<0.05).

Table 7: Parameter estimates from broken line regression model of final whole body mineral concentration in response to the analysed dietary mineral content for carp reared the low or high accumulation system.

	R (Breakpoint, mg/kg diet)			U (Slope)			L (Plateau, mg/kg live wt)			Overall effect
	LAS	HAS	*P-value*	LAS	HAS	*P-value*	LAS	HAS	*P-value*	*P-value*
Cu	10.2	11.3	0.26	0.19	0.15	0.7	1.72	1.74	0.88	0.53
Zn	50.2	49.2	0.64	0.97	1.07	0.73	36.9	38.2	0.9	0.21
Fe	278	273	0.81	0.09	0.24	0.01	22.2	25.4	0.01	0.0001
Se	0.32	0.28	0.04	0.37	0.48	0.17	0.17	0.18	0.33	0.006

Statistical comparison of model parameters of brokenline regression between the two rearing systems: R, breakpoint (i.e., minimal required dietary mineral content), U, slope (i.e., efficiency) and L, plateau value using GraphPadprisim. LAS, low accumulation system; HAS, high accumulation system;

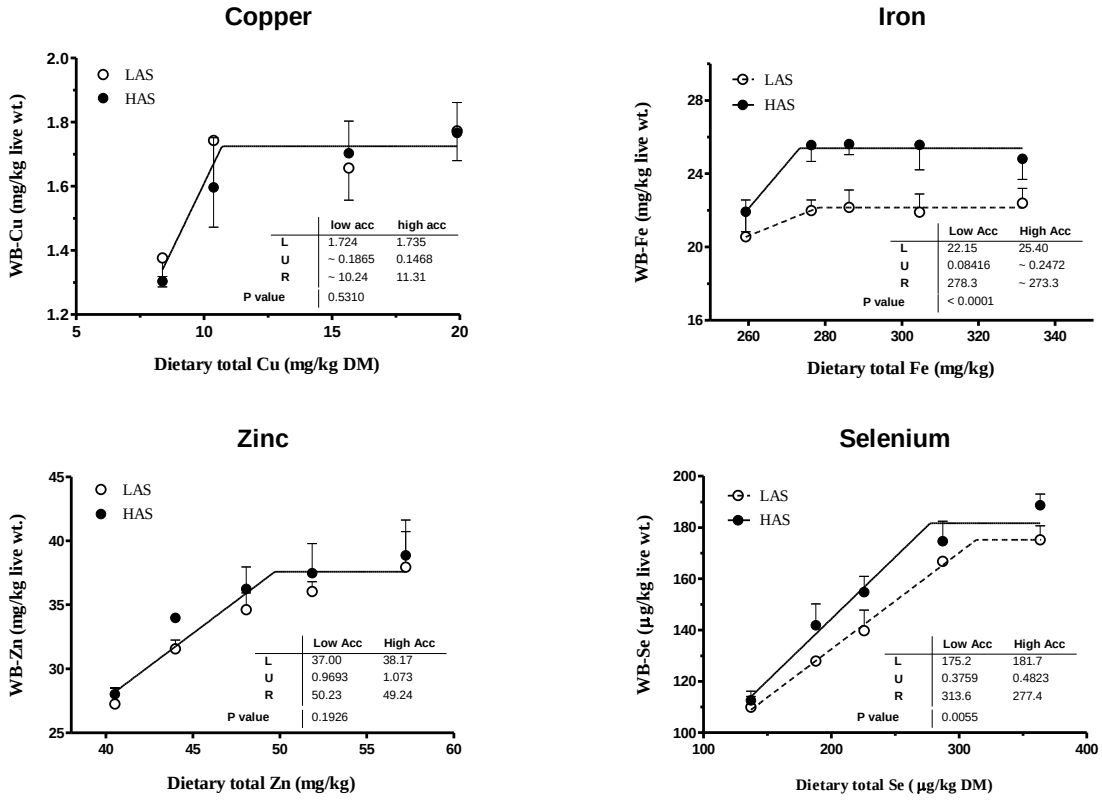


Fig. 2: Broken line regression for determining optimal dietary inclusion level of micro-minerals to common carp based on whole body (WB) mineral concentration when fed a complete plant ingredient based diet in two different RAS systems of varying water exchange rates and thus contrast in water mineral concentrations. Open circles and dotted line, LAS – Low accumulation system; Filled circles and solid line, HAS – High accumulation system. Model parameters: L, plateaue value; U, slope; and R, requirement estimate or breakpoint.

The vertebral concentration of Cu and Mn were significantly higher in fish reared in HAS; for all other minerals no effect of system was observed. Dietary micro-mineral inclusion resulted in a dose-dependent increase in all of the four analysed micro-minerals such as Fe, Cu, Mn and Zn (Se was not analysed). The dose-response relationship between vertebral mineral concentration and analysed dietary mineral content is illustrated in Fig 3 and the results of broken line analysis are provided in Table 9. The estimated minimal required dietary mineral levels (i.e., breakpoint) based on vertebral mineral levels for Fe, Cu, Mn and Zn were 305, 16.5, 39.5 and 55 mg/kg DM respectively. The effect of system was not strong enough to alter significantly the breakpoint values for any of the analysed micro-minerals. However, the estimated slope and breakpoint values based on vertebral mineral concentration (Table 9) were in general higher compared to the estimated breakpoints based on whole body mineral concentration.

Table 8: Vertebral lipid (on dry matter, DM), ash and mineral concentration (on fat free dry matter, ffdm) of common carp fed the experimental diets for 8 weeks and reared in the low or high accumulation systems.

	Proximate composition		Ash	Macro-minerals (g/kg ffdm)				Micro-minerals (mg/kg ffdm)			
	DM	CL (%DM)	(% ffdm)	P	Ca	Mg	K	Fe	Cu	Mn	Zn
Initial	63.87	24.34	66.16	11.03	20.49	0.54	1.99	10.01	7.96	8.67	229.9
System											
LAS	57.4 ± 0.8[a]	30 ± 1.7[a]	64.8 ± 1	10.1 ± 0.3	19.4 ± 0.5	0.42 ± 0.02	1.8 ± 0.1	13.4 ± 1.6	5.9 ± 0.9	9.6 ± 0.7[a]	220.8 ± 28.6
HAS	59.4 ± 1[b]	27.9 ± 1.1[b]	64.8 ± 0.9	10.2 ± 0.2	19.6 ± 0.3	0.43 ± 0.02	1.8 ± 0.1	14 ± 2.5	6.3 ± 1.1	10.4 ± 0.7[b]	222.8 ± 25.4
Diet											
V0	57.7 ± 0.9	28.8 ± 2.9	64.6 ± 0.5	10.1 ± 0.1	19.5 ± 0.2	0.42 ± 0.02	1.8 ± 0.1	11.2 ± 1.1[a]	4.9 ± 0.3[a]	9 ± 0.5[a]	200.3 ± 5[a]
V0.3	59.1 ± 1	29.1 ± 0.7	64.2 ± 1.1	10 ± 0.3	19.4 ± 0.7	0.4 ± 0.02	1.7 ± 0.1	12.7 ± 1[b]	5.4 ± 0.3[ab]	9.5 ± 0.4[a]	194.1 ± 11[a]
V0.6	58.5 ± 1.5	28.3 ± 1.1	64.3 ± 0.9	10 ± 0.2	19.3 ± 0.3	0.42 ± 0.01	1.7 ± 0.1	13.1 ± 0.6[b]	6 ± 0.5[ab]	10.3 ± 0.5[b]	214.2 ± 10[a]
V1	58 ± 1.8	29.5 ± 1.8	65.4 ± 0.7	10.3 ± 0.2	19.6 ± 0.3	0.44 ± 0.01	1.8 ± 0.1	16 ± 1.1[c]	7 ± 0.6[b]	10.5 ± 0.5[b]	242.4 ± 10.1[b]
V1.5	58.8 ± 1.5	29.1 ± 2.1	65.6 ± 0.6	10.3 ± 0.2	19.7 ± 0.3	0.43 ± 0.03	1.8 ± 0.1	15.5 ± 1.4[c]	7.2 ± 0.6[b]	10.8 ± 0.7[b]	258.1 ± 11[b]
P-values, two-way ANOVA											
System	< 0.001	0.002	0.918	0.081	0.217	0.316	0.439	0.108	0.05	< 0.001	0.590
Premix	0.02	0.753	0.031	0.067	0.468	0.042	0.198	< 0.001	0.001	< 0.001	< 0.001
S x P	0.352	0.735	0.513	0.797	0.926	0.967	0.527	0.198	0.268	0.568	0.402

DM, dry matter; CL, crude lipid; LAS, Low accumulation system; HAS, High accumulation system; Data presented as mean ± SD. LAS, low accumulation system; HAS, high accumulation system; V0, V0.3, V0.6, V1and V1.5, experimental diets with graded premix inclusion levels; Data is analyzed using 2-way ANOVA. Significant differences between diets are established with the Tukey test. Different superscript letters within a column indicate no significant difference (P<0.05).

Table 9: Parameter estimates from broken line regression model of final vertebral mineral concentration in response to graded levels of minerals in the diet.

	R (Breakpoint, mg/kg diet)			U (Slope)			L (Plateau,mg/kg live wt)			Overall effect
	LAS	HAS	*P-value*	LAS	HAS	*P-value*	LAS	HAS	*P-value*	*P-value*
Fe	304	306	*0.8*	0.08	0.12	*0.18*	14.8	16.4	*0.03*	*0.09*
Cu	16.7	16.4	*0.8*	0.23	0.34	*0.09*	6.8	7.6	*0.02*	*0.01*
Mn	39.3	39.9	*0.7*	0.17	0.19	*0.65*	10.2	11.1	*0.03*	*< 0.01*
Zn	53.4	56.9	*0.17*	7.4	7.8	*0.1*	256	259	*0.7*	*0.41*

LAS, Low accumulation system; HAS, High accumulation system. Statistical comparison of model parameters of brokenline regression namely breakpoint (R), slope (U, slope) and plateau (L) value using GraphPadprisim.

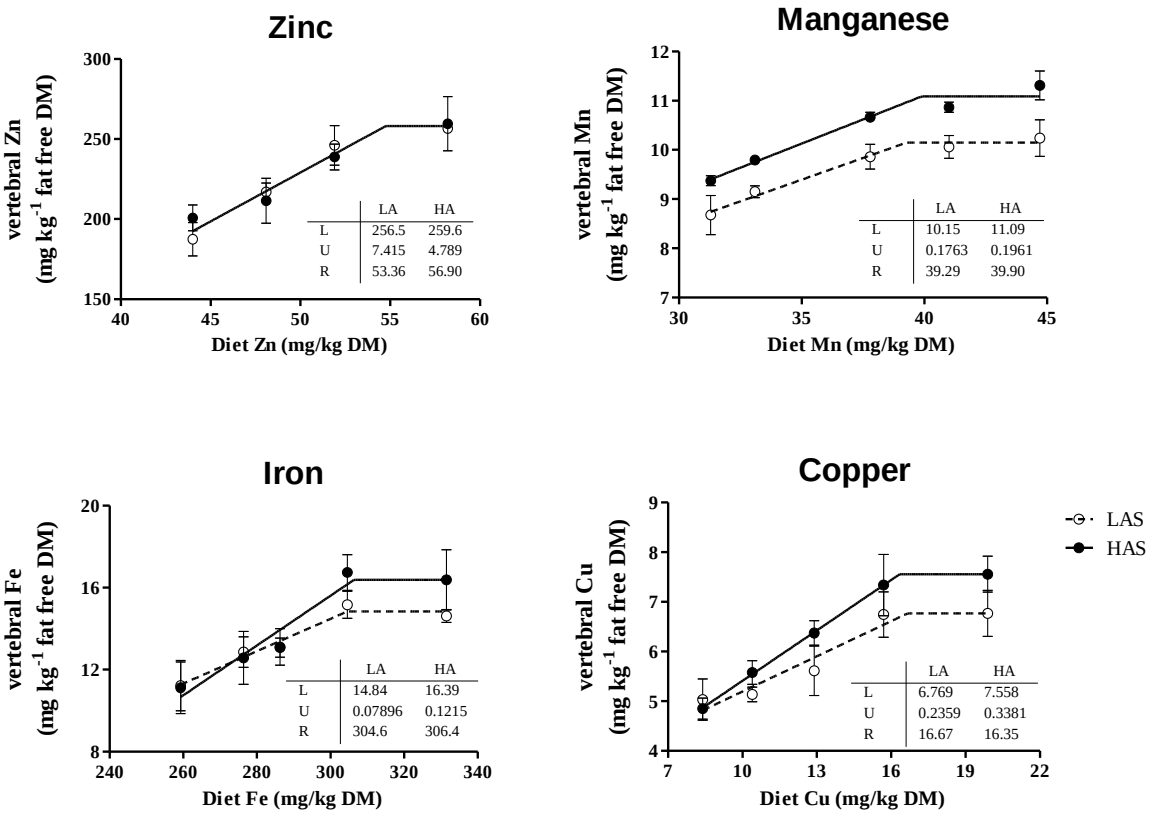


Fig. 3: Broken line regression for determining optimal dietary inclusion level of micro-minerals to common carp based on vertebral (Ver) mineral concentration when fed a complete plant ingredient based diet in two different RAS systems of varying water exchange rates and thus contrasting in water mineral concentrations. Open circles and dotted line, LAS – Low accumulation system; Filled circles and solid line, HAS – High accumulation system. Model parameters: L, plateaue value; U, slope; and R, requirement estimate or breakpoint.

Plasma mineral levels

Data on 24-h postprandial blood plasma mineral concentration are presented in Table 10. Fish reared in the HAS system had significantly higher plasma P and K levels. Among the micro-minerals, only Mn was significantly higher. With regard to the inclusion of a micro-mineral premix, no clear dose response was observed for any of the analysed micro-minerals. However, the plasma P and Mg levels increased and plasma K decreased with increasing premix inclusion levels.

Activities of specific enzymes involved in micro-mineral metabolism

Enzymes which are related to transport and metabolism of micro-minerals were analysed in three tissues: liver, gills and intestine (Table 11). Only one enzyme, GPX, showed significant and consistent results across all three tissues. GPX had increased 2 to

4 fold in these tissues of fish fed the premix supplemented diets in both systems. In the intestine, the activity of GR was significantly doubled in the HAS reared fish compared to LAS, and premix supplementation significantly reduced GR activity. However, this effect of premix supplementation tended to be present only in the HAS reared fish. The activity of other analysed enzymes showed minor differences between the treatments.

Table 10: Plasma mineral concentrations (24-h post prandial) in common carp fingerlings fed the experimental diets for 8 weeks and reared in the low or high accumulation systems.

	Macro-minerals (mmol/L)				Micro-minerals (µmol/L)			
	P	**Ca**	**Mg**	**K**	**Fe**	**Cu**	**Mn**	**Zn**
System								
LAS	3 ± 0.5[a]	2.6 ± 0.2	0.9 ± 0.1	1.7 ± 0.9[a]	43.8 ± 26.7	20.6 ± 3.5	0.5 ± 0.2[a]	80.7 ± 10.9
HAS	3.6 ± 0.7[b]	2.7 ± 0.2	0.9 ± 0.2	2.6 ± 1.5[b]	49.8 ± 27.7	19.7 ± 3	0.9 ± 0.3[b]	75.5 ± 12.6
Diet								
V0	3.1 ± 0.6	2.7 ± 0.1	0.8 ± 0.1	3.5 ± 0.8[a]	38.8 ± 21.9	20.6 ± 2.4	1 ± 0.4	80 ± 10
V0.3	3 ± 0.5	2.7 ± 0.3	0.9 ± 0.2	2.8 ± 1.1[a]	41.5 ± 31	19.9 ± 4	0.7 ± 0.4	79.4 ± 12.9
V0.6	3.2 ± 0.3	2.6 ± 0.2	0.9 ± 0.2	2.1 ± 1.5[ab]	38.6 ± 28.3	19.9 ± 3.4	0.6 ± 0.2	75.8 ± 12.4
V1	3.5 ± 0.7	2.7 ± 0.2	0.9 ± 0.1	1.2 ± 0.5[a]	61.8 ± 24	20.1 ± 2.9	0.7 ± 0.3	80.7 ± 13.7
V1.5	3.8 ± 0.8	2.6 ± 0.4	1 ± 0.2	1.1 ± 0.5[a]	53.4 ± 25.8	20.3 ± 4.1	0.6 ± 0.2	74.6 ± 11.2
P-values, two-way ANOVA								
System	0.0001	0.038	0.28	< 0.01	0.367	0.346	< 0.01	0.081
Premix	0.002	0.98	0.06	< 0.01	0.113	0.986	0. 02	0.602
Sys x Prmix	0.036	0.26	0.001	0.0001	0.113	0.408	0.085	0.070

Data presented as mean ± SD. LAS, low accumulation system; HAS, high accumulation system; V0, V0.3, V0.6, V1and V1.5, experimental diets with graded premix inclusion levels; Data is analyzed using 2-way ANOVA. Significant differences between diets are established with the Tukey test. Different superscript letters within a column indicate no significant difference.

Gene expression of metal transporters and metaloenzymes

The m-RNA expression pattern of the different analysed genes, which are related to mineral transport and metabolism, are presented in Fig. 4. Both premix inclusion and system changes had significant effects on the expression pattern of the genes involved in mineral transport and metabolism. Moreover, the m-RNA expression pattern of GPX and GR were in the direction of the results obtained through enzyme activity.

Discussion

Mineral accumulation in RAS water

The contrast in water mineral concentrations between LAS and HAS as observed in the present study were in accordance with earlier reports in RAS (Martins *et al.*, 2009b; van Bussel *et al.*, 2014; Davidson *et al.*, 2009). The magnitude of accumulation in individual minerals seemed to vary, and this could primarily be dependent on the mineral content of the feed and consequently the faecal wastes. Under similar water exchange rates, the

concentration of the accumulated minerals in the water varied based on the type of feed used. The concentration of phosphorus in the water of the system which used fish meal based diet was thrice as much to the system in which a plant-ingredient based diet was used (Davidson *et al.*, 2013). In the studies with RAS systems reporting water mineral concentrations, it is striking that the concentration of Ca has either remained stable or declined, while the concentration of all other macro-minerals have increased with decreasing water exchange rates (Martins *et al.*, 2009b; van Bussel *et al.*, 2014; Davidson *et al.*, 2009). With regard to micro-minerals, the level of accumulation is likely to be dependent on factors like dietary mineral content, mineral availability to fish, water exchange rate as well as the preferential utilisation and/or liberation of mineral by the microbial populations within the different system compartments. Hence there is need for more knowledge on the dynamics of mineral accumulation in the water of RAS as affected by the aforementioned factors.

Growth and body composition of fish reared in RASs of differing water exchange rates

Growth retardation was observed in common carp larvae and tilapia reared in water exhibiting similar metal concentrations as observed in the HAS of the present study (Martins *et al.*, 2009b; Martins *et al.*, 2009a). However, no difference in growth performance was reported in rainbow trout reared in such LAS and HAS (Davidson *et al.*, 2009). Ortho-phosphate concentrations of 25 mg PO_4-P L^{-1} in water improved growth in Nile tilapia (Eding et al., 2012). Recently, a study in marine RAS has shown that increased water ortho-phosphate concentrations improved growth in turbot (van Bussel *et al.*, 2013). Apart from improving growth, high water ortho-phosphate concentrations were also associated with reduced fat deposition in body (Eding et al., 2012; van Bussel *et al.*, 2013). Phosphorus is associated with processes involved in peripheral lipid mobilisation. Phosphorylation of 'perilipin' and 'hormone activated lipase' is the rate limiting reaction in the mobilisation of lipid reserves from the adipose tissue (Sheridan, 1994) and might well be responsible for the decreased lipid stores in fish reared in RAS containing high PO_4-P concentrations. Further studies focusing on molecular and cellular responses might shed light on the mechanism underlying the changes in body composition in fish reared in RAS with low water exchange rates (i.e. high accumulation).

Improved mineralisation in fish reared in HAS compared to LAS

Low hatching rate, higher mortality and growth retardation were observed in common carp eggs and larvae incubated in water from a similar RAS set up as in the HAS treatment in the current study (Martins *et al.*, 2009b). In contrast, the occurrence of deformities was lower in larvae reared in water from HAS (Martins *et al.*, 2009b). Van Bussel *et al.* (2014) reported an increase in body concentrations of Fe, Cu, Zn and Mn with increasing concentration of the respective minerals in water. Similar results were obtained in the present study for whole body levels of Fe, Zn and Se in fish reared in HAS compared to LAS. In fish, vertebrae form the primary storage site for most of the minerals. Hence, vertebral mineral concentration is a more pertinent and better indicator of mineralisation than whole body stores and it has been shown that vertebral mineral concentration, as the response criteria, is more stringent in estimating mineral requirement estimates in fish (Antony Jesu Prabhu et al., 2013; 2014). However, there is no data available till date to corroborate the effect of HAS in increasing mineralisation in fish, based on vertebral mineral concentration. In the present study, vertebrae of fish reared in HAS showed significantly higher mineral concentration than in LAS. Fish reared in HAS presumably retained minerals acquired from water in addition to the dietary supply, as reported earlier (Wilson and Naggar, 1992; Shearer and Åsgård, 1992).

Effect of micro-mineral premix supplementation on growth

Irrespective of the system, mineral premix inclusion of 0.6% and beyond reduced the growth performance. Highest growth and lowest FCR were observed at the lowest level of mineral premix inclusion, which suggests that mineral content of the basal diet (0% mineral premix) was sufficient. However, results of the dose-response regression analyses based on body and vertebral mineral concentration contradicts the aforesaid possibility. The exact cause of the decreased growth with increasing mineral content remains unclear. Apparently, mineral content due to premix inclusion could have affected the physiological processes resulting in increased energy expenditure for maintenance. Literature data on the effect of mineral premix on the retention of nitrogen, fat and energy is very scarce. Moreover, in the current study the use of a complete mineral premix makes it hard to substantiate the observed response in growth to an individual mineral. In Nile tilapia (*Oreochromis niloticus*), an increase in dietary

electrolyte balance (dEB) resulted in a higher amount of energy spent on maintenance (MEm) (Saravanan et al. 2013). The dEB is mainly affected by ions of Na^+, K^+, Ca^+, Mg^+, S^-, Cl^- and P^- (Patience 1990). The cations were kept constant in the current experimental diets; however, as most of the micro-mineral salts added to the premix were in the form of sulphate, it is possible that the anionic proportion increased with increasing premix inclusion, thereby increasing the dEB. If dEB increases with higher dietary mineral premix content, the acid-base homeostasis would get affected, leading to active excretion of ions out of the body and therefore a higher MEm. In the case of which, a curvi-linear or linear relation will be obtained between mineral premix inclusion against retained energy or growth. A higher MEm leads to a lower amount of energy spent on production (MEp) and therefore a decrease in growth with increasing dietary mineral premix inclusion. This, in one way, could be a reason behind the decline in the growth performance of common carp with increasing premix levels, in both systems.

Minimal dietary inclusion level of micro-minerals to common carp

NRC (2011) reports quantitative dietary requirements of micro-minerals (on available basis) for common carp to be as follows: Cu, 3 mg/kg; Fe, 150 mg/kg; Mn, 12 mg/kg and Zn, 15 mg/kg. With regard to Se, NRC (2011) reports it as not tested. In the present study, the estimate for Cu was 11.3 and 16.7 mg/kg based on whole body and vertebral mineral concentration, respectively. The requirement for Fe was estimated to be 278 and 305 mg/kg based on whole body and vertebral mineral concentration, respectively, and similarly, the requirement of Zn was estimated at 50.2 and 56.9 mg/kg, respectively. The minimal dietary inclusion level of Mn was estimated at 39.9 mg/kg based on vertebral mineral concentration. These estimates of the present study are much higher than the reported values by NRC (2011). Most likely, this is due to a difference in availability of minerals. In the current study, the breakpoints were estimated by using the analysed total dietary mineral content, which consists of both minerals originating for the basal diet as well as the premix. At the 1% premix inclusion, 48 to 85% of the supplemented micro-minerals originated from the basal diet. In contrast, most NRC estimates are based on studies using basal diets devoid from the studied mineral (thus a high availability of minerals). On the whole, the micro-mineral premix at 1% inclusion level was sufficient to supply the micro-minerals required by common carp to maintain whole body or vertebral mineral concentrations. However, it is not clear, from the

results of this study, if this level of inclusion will comply with the recommendations of NRC (2011) on available basis, as the availability of mineral was not measured in this study. Nevertheless, from the responses it can be stated that Se was the most limiting micro-mineral to common carp fed the vegetable based diet. Additional Se supplementation, beyond the recommendations of NRC (2011), may be required while using plant ingredient based diets, as shown by Fontaine-Dicharry et al. (2015) in rainbow trout.

Effect of system on the minimal dietary inclusion levels

It was hypothesised that the utilisation of dissolved minerals in water by fish would compensate for a part of the dietary need of the respective minerals. In general, for almost all parameters analysed (except for Se in whole body), the breakpoint estimates were similar in both systems. The system water management seemed to affect the relationship only in plateau value and slope, but not regarding the breakpoint. The high mineral concentration in HAS water had a positive effect on mineralization but this was not reflected back in a lower breakpoint estimate. The reason behind this remains unclear, but it suggests that most of the minerals acquired through absorption from water could be deposited in the tissues but may not be nutritionally/metabolically available to the fish. Minerals acquired from water via the gills can directly be transported to the different tissues, whereas minerals absorbed in the intestine will first pass through the liver via the portal system. The liver is the major site of intermediary-metabolism and, for most micro-elements (e.g. selenium), the site where conversion from inorganic to organic forms takes place. Hodson and Hilton (1983) suggested that the metabolism of dietary Se can differ from that of waterborne Se. This could well be the case behind the observations of the present study with regard to most micro-minerals studied.

Expression pattern of the genes and activity of enzymes involved in mineral transport and metabolism

In line with the findings of the brokenline estimate based on whole body Se concentration, wherein whole body Se levels reached a plateau at 1% inclusion level, the expression and activity of Se-dependent GPX was 2-4 fold increased in all the three tissues analysed. The increased activity and expression of glutathione reductase (GR) in the tissues, especially in the intestine of fish reared in HAS, supports the possibility of

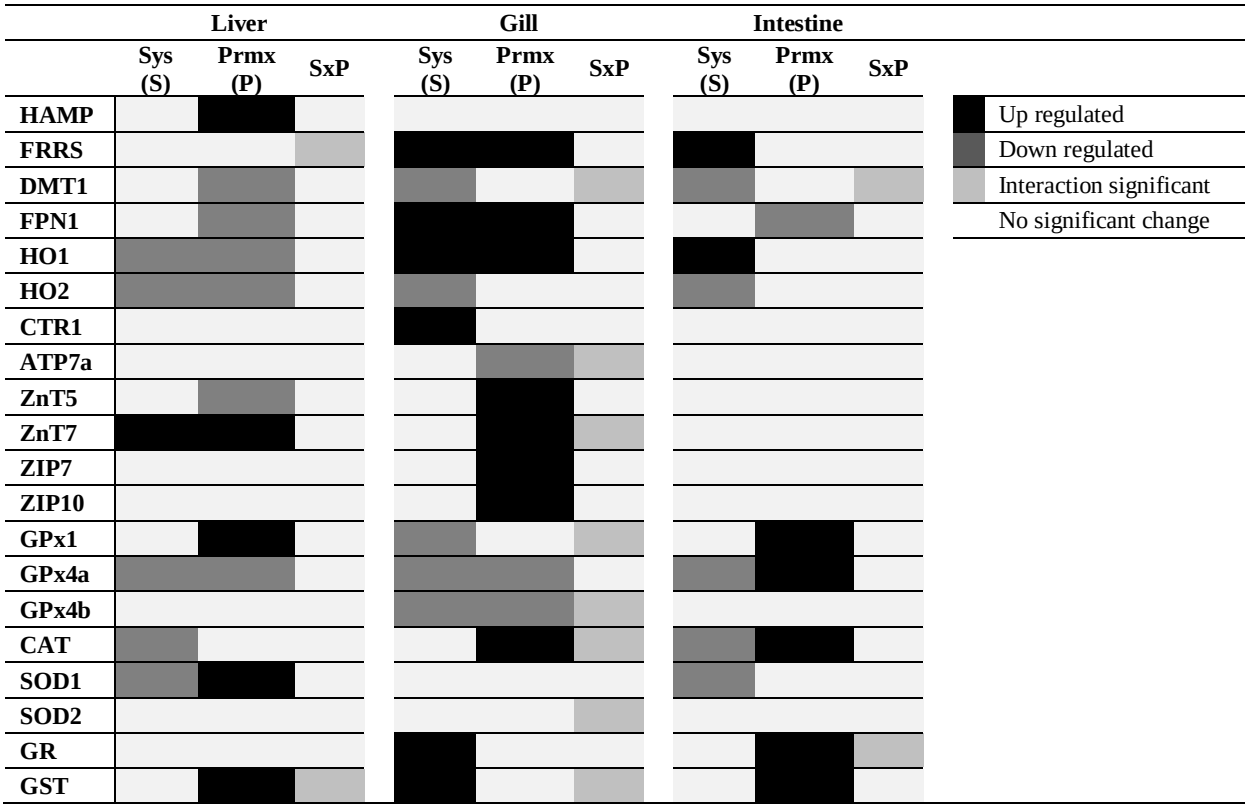
increased lipid peroxidation resulting in lower body fat content in the body. GR is reported to the best responsive anti-oxidant marker for lipid peroxidative stress (Fontagné-Dicharry *et al.*, 2014), and intestine is one of the primary sites of fatty acid bio-synthesis in rainbow trout (Kamalam *et al.*, 2013). Further studies focusing on molecular and cellular responses might shed light on the mechanism underlying low body lipid in fish reared in RAS of high mineral accumulation rates. The interesting observation for further investigation is that, of the three tissues analysed, effect of system was more pronounced in the gills with significant interactions between system and premix inclusion.

Table 11: Activities of selected enzymes involved in mineral transport or metabolism in liver, gill and intestine of common carp

	LAS-V0	LAS-V1	HAS-V0	HAS-V1	System	Premix	S x P
Liver							
FR	3.9 ± 2.3	2.9 ± 0.5	2.7 ± 0.7	2.8 ± 0.4	0.53	0.47	0.71
CR	19.1 ± 11.5	17.5 ± 8.4	17.7 ± 7.1	37.5 ± 51.2	0.31	0.35	0.34
ALP	17.5 ± 3.9	16.7 ± 3.7	16.4 ± 2.2	22.3 ± 6.1	0.17	0.23	0.08
GPX	**27 ± 12.7**	**118.2 ± 19**	**20.2 ± 8.3**	**111.1 ± 24**	**0.05**	**0.00**	**0.31**
CAT	365.9 ± 58.3	403 ± 52.5	394.8 ± 59.3	401.2 ± 44.1	0.68	0.11	0.24
GR	**9.6 ± 2.2**	**8.7 ± 1.7**	**9.8 ± 2**	**9.7 ± 2.3**	**0.82**	**0.05**	**0.71**
GST	152.9 ± 36.5	133.1 ± 33.4	155.9 ± 22.3	156.6 ± 44	0.27	0.01	0.41
Intestine							
FR	3.8 ± 0.4	4.2 ± 1.4	4 ± 1.9	3.7 ± 0.8	0.62	0.55	0.21
CR	18.1 ± 4	30.2 ± 15.1	16.3 ± 7.5	21.3 ± 6.5	0.52	0.08	0.88
ALP	2.19 ± 0.52	3.77 ± 0.89	3.24 ± 1.8	2.37 ± 0.68	0.81	0.74	0.01
GPX	**18.1 ± 4.4**	**93.4 ± 12**	**21.1 ± 7.8**	**99 ± 7.8**	**0.27**	**0.00**	**0.50**
CAT	46.9 ± 3.2	52.1 ± 4.3	52.3 ± 12.6	48.1 ± 2.9	0.95	0.45	0.15
GR	**39.3 ± 6.9**	**35.6 ± 4**	**58.7 ± 9.9**	**46.9 ± 12**	**0.00**	**0.01**	**0.08**
GST	626.1 ± 53.2	592.9 ± 72.5	658.3 ± 95.4	555.3 ± 61.3	0.76	0.09	0.41
Gills							
FR	0.6 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.2	0.74	0.03	0.00
CR	92.5 ± 32.6	111.9 ± 29.3	106 ± 17.4	137 ± 53	0.48	0.12	0.66
ALP	770.1 ± 102	775 ± 102.5	712.8 ± 96.1	675 ± 113.8	0.19	0.97	0.98
CAT	3.8 ± 0.6	4.9 ± 0.8	4.2 ± 0.4	4.7 ± 0.6	0.66	0.04	0.25
GPX	**46 ± 2.8**	**99.3 ± 13.3**	**45.6 ± 3.9**	**105.9 ± 8.5**	**0.48**	**0.00**	**0.58**
GR	15.2 ± 2.2	13.5 ± 2.2	16.7 ± 1.5	14.3 ± 2.1	0.15	0.32	0.99
GST	191.8 ± 27.7	206.4 ± 18	182.5 ± 23.3	213.5 ± 40	0.25	0.10	0.27

Units of expression: ALP, CAT as U/mg protein; FR, CR, ALP, GPX, GR, GST as mU/mg protein. Analysis of enzyme activities were performed only on the V0 and V1 diets at both systems. LAS, low accumulation system; HAS, high accumulation system; V0, 0% premix; V1, 1% premix.. Data presented as mean ± SD of n=6 samples.FR, ferric reductase; CR, cupric reductase; ALP, Alkaline phosphatase; GPX, Glutathione peroxidase; CAT, catalase; GR, Glutathione reductase; GST, Glutathione-S-transferase. Data areanalyzedby 2-way ANOVA.

Fig. 4: Effect of system (Sys), premix inclusion (Prmx) and their interaction (S x P) on the mRNA expression of genes involved in mineral transport or metabolism in liver, gill and intestine of common carp



Upregulated – significantly higher mRNA expression in HAS group (system effect) or Premix supplemented group (premix effect). Downregulated – significantly lower mRNA expression in HAS group (system effect) or Premix supplemented group (premix effect). No significant change – lack of an effect of neither system or premix inclusion. HAMP, Hepcidin anti-microbial peptide; HO1, heme oxygenase 1; HO2, Heme oxygenase 2; FPN1, ferroportin1; FRRS, Ferric reductase; CTR1, copper transporter I; ATP7a, Cu^{++} transporting ATPase-alpha polypeptide; ZnT5, Zinc transporter protein5; Zinc transporter protein7; ZIP7, Zinc importer7; ZIP10, Zinc importer10; GPX1, glutathione peroxidase 1; GPX4a, glutathione peroxidase 4a; GPX4a, glutathione peroxidase 4b; CAT, catalase; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; GR, Glutathione reductase; GST, Glutathione S-transferase.

From the results of the present study, it can be concluded that common carp are able to acquire and retain minerals which are accumulated in recirculating aquaculture systems if these systems are operated at a low water refreshment rate. However, the increased accumulation of minerals in the system water did not reduce the optimal dietary mineral supply to meet the requirement. The results also suggest that the rearing system changes could have multiple effects on the whole animal physiology of fish apart from mineral balance alone.

Acknowledgements

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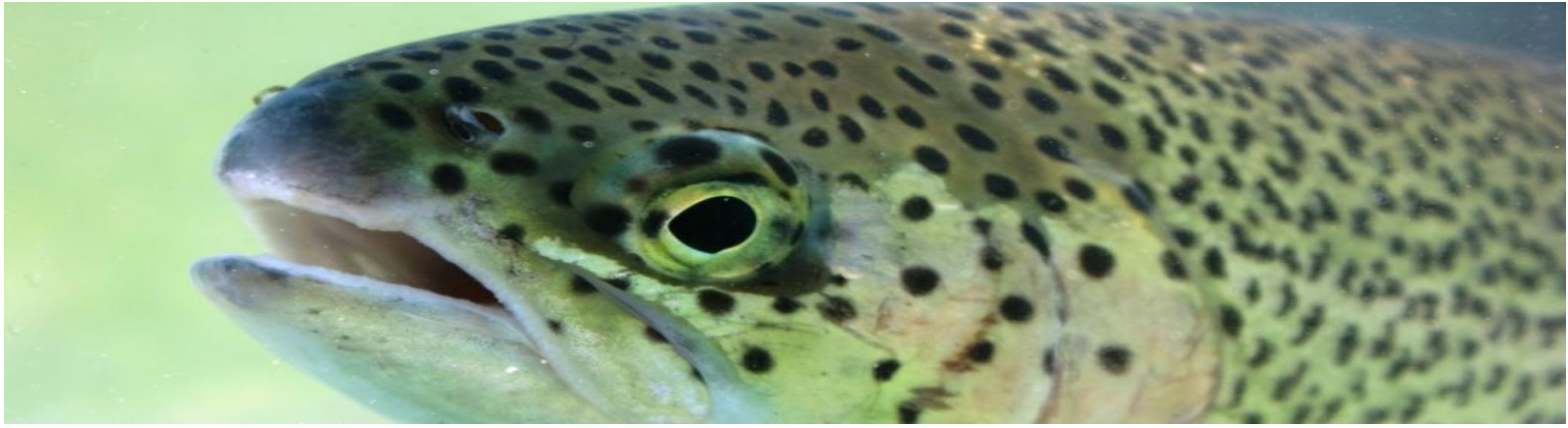

CHAPTER 10

GENERAL DISCUSSION

Introduction

After an extensive analysis of literature data on mineral requirements and utilisation in finfish (chapters 2 and 3), a series of dedicated studies was undertaken to analyse how changes in diet and in the rearing system affect the mineral balance in fish. Dietary supply, utilisation and metabolism of one or more minerals in two species of fish (Rainbow trout and Common carp) were described in the preceding chapters (chapters 4 to 9). In this chapter, an attempt is made to summarise the main conclusions of the different chapters, to integrate and corroborate the findings and to provide insights for future research in this hitherto less explored area of fish nutrition.

Impact of dietary changes on Ca-P supply and utilisation

Phosphorus, in the form of ATP, plays a vital role in nutrient and energy metabolism. In humans, obesity is linked to changes in dietary habits favouring low phosphorus intake (Obeid, 2013). The changes that have occurred in the ingredient and nutrient composition of fish feeds mirrors the scenario of human dietary habits, with increasing energy and reduced phosphorus intakes. Although, a large body of literature is available on dietary phosphorus requirements and utilisation in fish (Chapter 2), the impact of high energy diets in altering the demand for phosphorus supply has eluded the investigators. Physiologically, this is similar to the relation between dietary Ca-P with femur bone strength in laying hens (Wilson, 1991) and incidence of milk fever in dairy cows (Goff and Horst, 1993; Boda and Cole, 1953). The reason being that, egg production (shell formation) in the former and milk production in the latter, demands additional supply of Ca, possibly P as well. A similar phenomenon was hypothesised to prevail in fast growing fish fed high energy diets with dietary P supply. This, when tested during the course of the thesis In Chapter 4 we confirmed that high energy diets yield better feed efficiencies demanded additional Ca-P supplementation to meet the physiological demands of faster growth rate.

Apart from high energy diets, the second question was whether inclusion of more and more plant ingredients affects the dietary availability and utilisation of phosphorus by fish. The dietary requirement for phosphorus to rainbow trout (available basis) is reported to be 7 g kg^{-1} (NRC, 2011) and 5.8 g kg^{-1} according to the meta-analysis (Chapter 2). The available phosphorus supply from M and V diets used in Chapters 4, 6

and 8 ranged from 5.6 to 8.8 g kg^{-1} DM, and the whole body P levels ranged from 3.1 to 4.1 g kg^{-1}; and the corresponding Ca:P ratio of rainbow trout ranged from 0.61 to 0.78 (Table 10.1).

Table 10.1: Data on dietary Ca and P from the different experiments in the present thesis indicating reduction in Ca:P ratios in fish despite adequate available phosphorus supply.

	Diet	Total Ca in diet	Total P in diet	Diet Ca/P	Available P in diet	WB-P in fish	WB-Ca in fish	Fish Ca/P
Chapter 5	M	16	14.1	1.1	8.3	4.1	2.6	0.63
Trout	V	10.8	10.3	1.0	5.6	3.7	2.3	0.62
Chapter 6	M	18.7	15	1.2	6.1	3.7	2.9	0.78
Trout	V	13.9	12	1.2	5.7	3.1	1.9	0.61
Chapter 7	M	11.3	12.9	0.9	8.8	3.4	2.6	0.76
Trout	V	8	9.4	0.9	5.6	3.3	2.4	0.73

M, Fish meal based diet; V, Plant ingredient based diet. WB-P, whole body phosphorus; WB-Ca, whole body calcium; Ca/P, calcium to phosphorus ratio. Units: g/kg DM for diets and g/kg fres weight for fish.

The normal range of Ca:P ratio in rainbow trout is 0.9 to 1 (Pfeffer and Pieper, 1979; Rodehutscord, 1996). The lower Ca:P ratios observed were mainly due to decreasing Ca levels in the body. We also observed that, the whole body levels of P, Ca and Ca:P ratio, exhibited a bell shaped response with increasing feeding levels (Fig. 10.1; unpublished data). The decrease in whole body Ca:P ratio at higher feeding levels was more pronounced in V fed fish than in M fed fish, the reasons for which are unclear. In humans and higher animals, the importance of P and Ca on mineralisation of bones goes hand-in-hand; in fish, however, the importance of dietary Ca supply has been largely overlooked due to ability of fish to acquire dissolved Ca from water. Nevertheless, these results do emphasise the need to re-consider the NRC (2011) recommendation for dietary Ca as being “not required, NR” for rainbow trout, especially with more and more inclusion of plant derived ingredients.

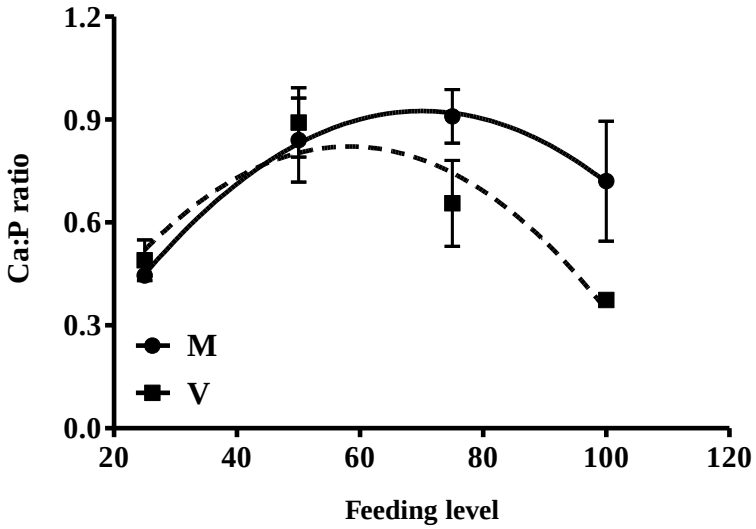

Fig. 10.1: Relation between Ca:P ratio and feeding levels (%) in rainbow trout (unpublished data). In rainbow trout fed both the fish meal as well as plant ingredient based diets, the Ca:P raito of the body declined at higher feed levels and attained the lowest at apparent satiation feeding. Apparent satiation level is indicated as 100 in the x-axis.

Impact of plant ingredients on micro-mineral supply and utilisation

With increasing inclusion of plant ingredients in fish feeds, dietary supplementationof phosphorus has become a common practice. However, the effects of supplementing phosphorus as di-calcium phosphate (DCP) to plant ingredient based diets on the availability and utilisation of other minerals are not well understood. The study on the impact of DCP supplementation to the V-diet on absorption and utilisation of other minerals (Chapter 5), showed that DCP improved phosphorus supply but also reduced the apparent absorption coefficients of micro-minerals such as Fe, Cu, Mn and Zn. The 24h postprandial profile of plasma minerals measured as area under the curve (24h-AUC) for Cu, Zn and Se were also reduced by DCP supplementation in rainbow trout fed the V-diets. This trend was again reflected in the final whole body mineral concentration for Zn, with significantly lower levels of whole body Zn in DCP supplemented group.

In order to determine the basal metabolic needs for minerals, different estimates were made based on starvation loss, endogenous loss of fed fish and maintenance need by plotting mineral gain over intake (Chapter 6). Endogenous loss and maintenance needs are shown to be affected by dietary changes in pigs (Dilger and Adeola, 2006b) and poultry (Dilger and Adeola, 2006a). However, no such data was available in fish to show

whether replacement of fish meal by plant ingredients affects the basic metabolic processes involved in the utilisation of minerals. It has been often observed that apparent digestibility of dietary Zn is reduced by dietary phytic acid, calcium phytate or even by supplementation of inorganic calcium-phosphate salts (Chapter 5). Significant differences in the estimates of endogenous loss were observed for Zn and Cu between the two dietary groups, higher for Zn and lower for Cu in fish fed diet V compared to M-diet fed fish (Chapter 6). The estimated endogenous Cu loss was higher with diet M than with diet V, as also reflected by a lower apparent digestibility of Cu in fish fed diet M (Chapter 5). Along with renal pathways, hepato-biliary regulation of Cu homeostasis forms a major route of Cu excretion in mammals (Cousins, 1985; Roelofsen *et al.*, 2000) and also in rainbow trout (Lanno *et al.*, 1987). As such, daily hepatic Cu excretion can be affected by changes in bile synthesis or secretion. Soybean meal, present in diet V, has also been reported to adversely affect bile synthesis pathways (Vilhelmsson *et al.*, 2004) and decrease the quantity of bile secreted into the gallbladder (Yamamoto *et al.*, 2007) in rainbow trout and Atlantic salmon (Kortner *et al.*, 2013; Gu *et al.*, 2014). The lack of significant difference between whole body Cu levels despite DCP lowering Cu absorption and AUC of plasma (Chapter 5), and the not significant difference between Cu loss in starved group andthe fed groups (Chapter 6), suggest that normal homeostatic regulation of Cu through hepato-bilary secretions may be impaired in rainbow trout fed the plant ingredient based diet V. This may lead to accumulation of Cu, possibly explaining the significantly high whole body Cu in fish fed the V diet (Chapter 5 and 6). These findings offer interesting insights for further investigating the effect of changing dietary composition of fish feeds on micro-mineral metabolism in rainbow trout.

Impact of plant ingredients in metabolic regulation of micro-mineral balance

The replacement of fish meal with plant ingredients in the diets triggers several changes in mineral metabolism. With the objective of better understanding the micro-mineral imbalance observed in fish fed plant ingredient based diets, molecular and cellular responses were studied in rainbow trout fed M or V diets, with or without the inclusion of a micro-mineral premix (Chapter 7). Differences in the form or level of micro-minerals, secondary metabolites in plant ingredients, can directly or indirectly affect micro-mineral metabolism in fish. Entero-hepato-pancreatic system is considered the target of ANFs known in plant ingredients (Francis *et al.*, 2001). Phytic acid can directly

reduce the availability of micro-minerals and other ANFs can indirectly interfere in trace mineral metabolism, as uptake and body status of Fe, Cu, Mn and Zn are primarily regulated at the level of gastrointestinal tract or liver (Hambidge, 2003). Diets based on plant protein ingredients lack heme, a vital component present in fish meal based diets. Apart from being a good source of Fe, degradation of heme supplies biliviridin, the precursor of bile pigment bilirubin. In rainbow trout, plant ingredients such as soybean meal may negatively affect cholesterol metabolism and bile status (Kaushik *et al.*, 1995; Yamamoto *et al.*, 2007; Iwashita *et al.*, 2009). A recent study with Atlantic salmon fed soybean (Kortner *et al.*, 2013) indicated increased transcriptional capacity for bile and cholesterol biosynthesis (up-regulation of hydroxy-3-methyl-glutaryl-CoA reductase gene expression, HMGCR) to compensate for defective re-absorption of bile due to inflammation in the distal intestine. Besides, increasing the degradation of endogenous heme-pool, as suggested by the higher HO/FPN1 (Chapter 7; Fig. 10.2), could also be an adaptive strategy to meet the physiological demand for bile salts, essential in cholesterol biosynthesis, when fed plant ingredient based diets devoid of heme.

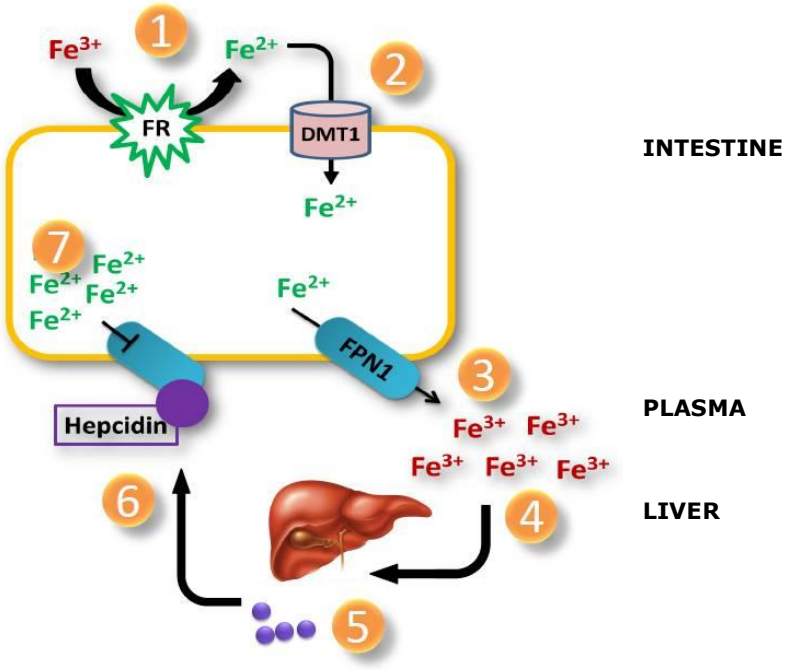

Fig. 10.2: Schematic outline on regulation of Fe metabolism: (1) Apical ferric reductase activity; (2) Apical Fe uptake; (3) Basolateral extrusion of Fe; (4) Systemic Fe overload; (5) Feedback induction of hepcidin in liver; (6) Ferroportin1 expression reduced by hepcidin; (7) Fe accumulated within enterocytes defecated by epithelial sloughing.

On the other hand, such higher endogenous heme-degradation can lead to increased Fe supply to the plasma, saturating the Fe binding capacity of plasma transferrin and hence increase the non-transferrin bound Fe which is stored in the liver. Like in mammals, body iron stores in fish are known to regulate intestinal Fe absorption and homeostasis (Standal *et al.*, 1999). Accordingly, the heme-degradation induced systemic Fe overload in V-diet fed fish, resulted in higher plasma and whole body Fe levels, followed by increased liver hepcidin (HAMP) expression and low intestinal ferroportin1 (FPN1) expression, thereby reducing the AAC of Fe in the V fed fish, despite similar dietary Fe concentrations. These findings suggest that, like in higher animals, systemic Fe homeostasis in rainbow trout could be transcriptionally regulated by HAMP and FPN1, however further studies are required to better understand the mechanism as suggested by Kwong *et al.* (2013). Nevertheless, it could be said that plant ingredient-based diets have an impact on the mechanisms regulating Fe homeostasis in rainbow trout. Moreover, as hepcidin and few other Fe regulatory genes are related to inflammatory and immune response in mammals (Ganz and Nemeth, 2012) and in fish (Hsieh *et al.*, 2010; Yang *et al.*, 2013), it will be useful to study the health status and immune response of rainbow trout fed plant ingredient based diets.

According to Bertinato and L'Abbé (2004), elevated hepatic cellular Cu concentrations stimulate hepatic Cu transport (by ATP7a) to the secretary pathways for Cu to be incorporated into cuproenzymes or to be excreted into the bile (by ATP7b) (Fig. 10.3).

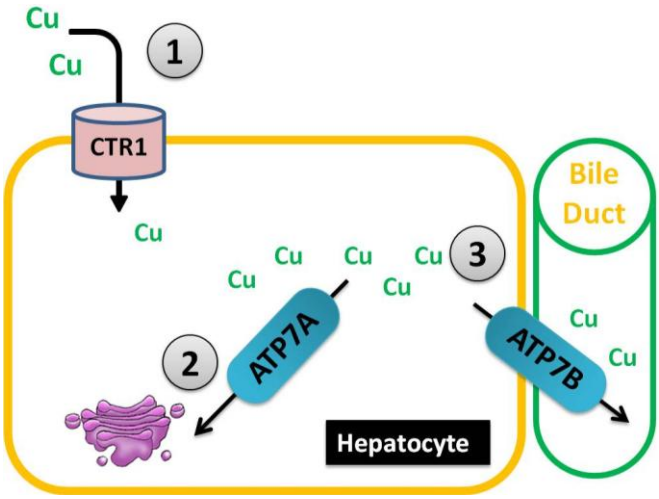

Fig. 10.3: Schematic outline of hepatic Cu metabolism: (1) Apical Cu uptake; (2) Cu to be incorporated into cuproenzymes; (3) Cu excreted into the bile.

The V-fed fish indeed showed high body Cu, in line with observations on Cu accumulation in the liver due to the lack of a functional homologue of ATP7b in mammals, commonly known as Wilson's disease (Terada *et al.*, 1998). As described earlier, defective cholesterol metabolism in fish fed plant ingredient based diets, when related with the high whole body Cu in V diet fed fish (Chapter 4, 6 and 7), indicate impaired hepato-biliary Cu excretion leading to Cu accumulation in the body, presumably in the liver.

Micro-mineral supplementation and non-specific metabolic responses

The effect of supplementing a micro-mineral premix on the metabolic responses was studied in rainbow trout (Chapter 8) and in common carp (Chapter 9). The response in growth was similar between the two species, with a bell shaped response; but the magnitude of decrease was more pronounced in common carp (Chapter 9). Energy expenditure for maintenance (Mem) was linearly affected by the mineral premix levels, which can be postulated to be a reason for the reduced growth performance with increasing mineral premix levels. In Nile Tilapia (*Oreochromis niloticus*), an increase in dietary electrolyte balance (dEB) resulted in a higher amount of energy spent on maintenance (Saravanan *et al.*, 2013). The dEB is mainly affected by ions of Na^+, K^+, Ca^+, Mg^+, S^-, Cl^- and PO_4^- (Patience 1990). All the cations were kept constant in the diets used, although most of the micro-mineral salts added to the premix were in sulphate forms. Increasing the premix inclusion reduced plasma K concentrations from 3.5 to 2.8 mmol L^{-1} in rainbow trout (Chapter 8); whereas, a linear reduction in plasma K levels from 3.5 to 1.1 mmol L^{-1} was observed in common carp (Chapter 9). This gave an indication of a possible anionic shift in the circulating body fluid thereby hinting a dietary ionic imbalance with increasing premix inclusion, with a curvi-linear or linear relation between dietary mineral premix inclusion levels and retained energy or MEm, respectively (Fig. 10.4). If dEB increases with higher dietary mineral premix content, the acid-base homeostasis would get affected, leading to active excretion of ions out of the body and therefore a higher MEm. A higher MEm leads to a lower amount of energy spent on production (MEp) and therefore a decrease in growth with increasing dietary mineral premix inclusion.

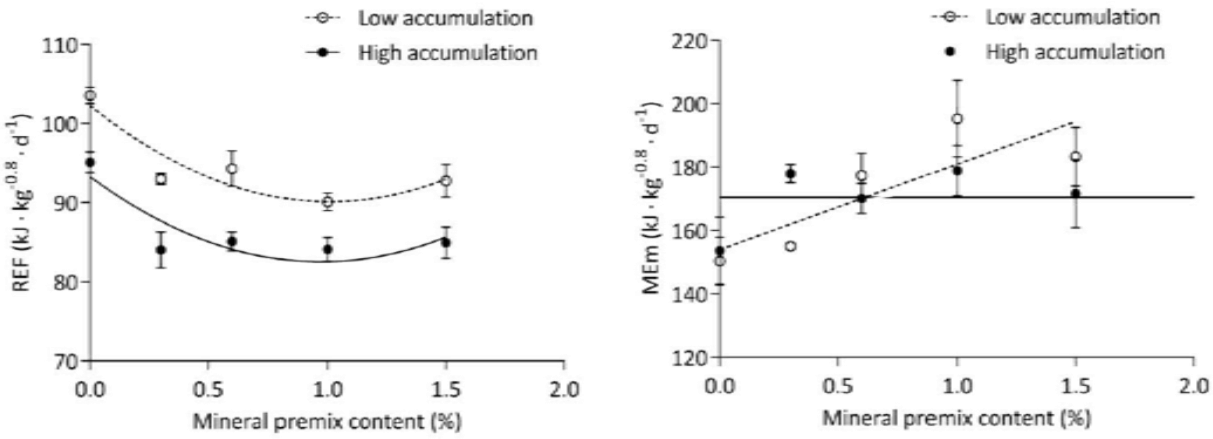

Fig. 10.4: Effect of RAS typt (low mineral accumulation system, LAS vs high mineral accumulation system, HAS) on energy metabolism in common carp: (i) The graph on the left illustrates the quadratic regression on retained energy as fat; (ii) The right graph illustrates the effect of mineral premix content on MEm.

Metabolic changes of fish reared in RASs of differing water exchange rates

Living in an aquatic habitat, fish have to maintain the osmotic balance of the body fluids with regard to the surrounding water. Freshwater fish in general, are not used to high levels of dissolved ions in the water. Thus the rearing conditions in high mineral accumulation systems (HAS) is likely to affect various processes related to ion-exchange and metabolism in different tissues. Common carp reared in low mineral accumulation systems (LAS) had significantly higher lipid levels in the whole body and in vertebrae when compared to those reared in HAS. One could consider this being an effect of higher energy as fat spent on maintenance (osmoregulation) in HAS thereby reducing the retained energy as fat. However, maintenance energy (MEm) and retained energy as fat and protein, calculated from energy balances were not different between the fish of the two system groups. Possibly, the missing link between the HAS reared fish and low fat deposition, might be the difference in the ortho-phosphate concentrations of rearing water. Phosphorus (as ATP) is associated with processes involved in peripheral lipid mobilisation through induction of lipolysis (Obeid, 2013; Obeid *et al.*, 2010). Exposure to phosphate solution in rats increased lipolysis both *invitro* and *invivo* (Lee *et al.*, 1982). Phosphate (P_i) dependent activation or phosphorylation of 'perilipin' and 'hormone activated lipase' is the rate limiting reaction in the mobilisation of lipid reserves from the adipose tissue (Sheridan, 1994). High ortho-phosphate concentrations of about 25 mg PO_4-P L^{-1}significantly reduced fat deposition in body of Nile tilapia (Eding et al.,

2002) and turbot (van Bussel *et al.*, 2013). This might well be responsible for the decreased lipid deposition in fish reared in RAS containing high PO_4-P concentrations (Chapter 9). The possibility of increased lipid peroxidation resulting in lower body fat content is another point worth taking into account. The increased activity and expression of glutathione reductase (GR) in the tissues, especially in the intestine of fish reared in HAS (Chapter 9) supports the latter possibility. GR is reported to be the best responsive anti-oxidant marker for lipid peroxidative stress (Fontagné-Dicharry *et al.*, 2014) and intestine is one of the primary sites of fatty acid bio-synthesis in rainbow trout (Kamalam *et al.*, 2013). Further studies focusing on molecular and cellular responses might shed light on the mechanism underlying low body lipids in fish reared in RAS of high mineral accumulation rates. With regard to the minerals *per se*, fish reared in HAS systems contained higher levels of all the analysed minerals in the whole body, except for Cu. This observation is in agreement with the reports of Martins *et al.* (2011) in Nile tilapia and van Bussel *et al.* (2014) in turbot. The occurrence of deformities was lower in larvae reared in water from HAS (Martins *et al.*, 2009), possibly due to better mineralisation. van Bussel *et al.* (2014) reported an increase in body concentrations of Fe, Cu, Zn and Mn with increasing concentration of respective minerals in RAS water. However, it was not clear from the aforementioned studies, if these acquired minerals improved the mineralisation of the hard tissues, primarily the vertebrae. Among the different response variables studied in the literature, mineralisation of vertebrae has been shown to best represent the mineral status of the fish (Chapters 2 and 3). In this regard, the vertebral mineral concentration of carp reared in HAS system had better mineralisation of vertebrae (Chapter 9). The overt phenotypic changes discussed above are more likely the outcome of metabolic changes at tissue and cellular levels. Blood plasma was analysed for circulating mineral levels; different tissues (gills, liver and intestine) were analysed for gene expression and enzyme activities of various markers involved in mineral transport and metabolism. Overall, the most important differences in responses were found in the gills, followed by intestine and liver. Significant interactions with dietary premix supplementation were also the most observed in gills. Thereby, it is suggested that the system changes could have multiple effects on the whole animal physiology of fish apart from mineral balance alone.

Mineral inclusion levels affected by dietary and system changes

The available P requirement of rainbow trout was estimated to be 3.5 g kg^{-1} (based on weight gain) and 5.8 g kg^{-1} (based on vertebral P content) through meta-analysis of literature data (Chapter 2). These estimates were further tested in chapter 4, where diets containing 3.5-4 g kg^{-1}available P were unable to maintain body P balance and available phosphorus of 8-9 g kg^{-1} was required to prevent vertebral de-mineralisation when rainbow trout were fed high fat, energy dense diets. In chapter 4, a complete plant ingredient based diet containing 6 g kg^{-1} total P (4.2 g kg^{-1} available P), when supplemented with 4 g P kg^{-1} in the form of di-calcium phosphate to obtain 10.3 g kg^{-1} total P (5.7 g kg^{-1} available P) whole body P balance and postprandial plasma P levels were restored in rainbow trout. As discussed earlier in this chapter and the previous chapters (Chapters 4, 5, and 6) the supply of sufficient dietary calcium needs to be ensured especially with increasing use of plant ingredients in fish feeds. It is also better to change the NRC (2011) recommendation for calcium from "not required, NR" to a quantitative value based on literature data (Chapter 3; Hossain and Yoshimatsu, 2014) awaiting further research findings.

Micro-mineral inclusion levels in complete plant ingredient-based diets were studied for rainbow trout (Chapter 8) and common carp (Chapter 9). This was done by taking a premix approach as opposed to the classical single-mineral approach. A pre-mix of Fe, Cu, Mn, Zn and Se was added in incremental proportions to a complete plant ingredient based diet and the balances were made on the body or tissue mineral concentrations of individual minerals regressed against their respective intake levels. Recently, it was reported that the NRC (2011) recommended level of 15 mg Zn kg^{-1} was not sufficient for rainbow trout and a minimal supplementation of 30 mg kg^{-1} was required when fed a plant ingredient-based diet (Welker *et al.*, 2015). Our results (in Chapter 8) also indicate that, supplementation of Zn (up to 15 mg kg^{-1}) was unable to restore whole body balance in rainbow trout fed complete plant ingredient based diets. Read *et al.* (2014) observed no adverse effects with Zn supplementation even up to 1500 mg kg^{-1} in complete plant ingredient based diets to rainbow trout. Besides, Fe and Cu levels in plant based ingredients are well in excess of the requirement of rainbow trout, whereas Zn is the most limiting micro-mineral. Among the essential micro-minerals, divalent metal ions such as Cu^{2+}, Fe^{2+} and Zn^{2+} are known to be competitive inhibitors of each

other. Copper inhibits intestinal Zn uptake in rainbow trout at equimolar luminal concentrations (Kwong and Niyogi, 2009); in humans, Fe/Zn ratio of 2:1 to 3:1 substantially inhibited zinc uptake (Solomons and Jacob, 1981). Thereby, Zn supplementation in plant based practical diets has to take into account the adverse effects of inherently high levels of Fe and Cu in the ingredients on Zn availability.

With regard to Se, supplementation of 0.3 mg kg^{-1} as sodium selenite (total Se: 0.54 mg kg^{-1})was unable to improve hepatic-GPx activity (Chapter 7) as well as maintain normal body Se levels (Chapter 8) inrainbow trout. In rainbow trout fed a similar plant-based diet, Se levels of 0.5 mg kg^{-1} resulted in low GPx activity and low body Se levels (Fontagné-Dicharry *et al.*, 2015). However, Se supplementation to the same level resulted in 4-5 fold increase in hepatic-GPx activity in common carp (Chapter 9). Among the five micro-minerals tested, Zn and Se were the limiting micro-minerals in plant ingredient-based diets. With regard to the effect of rearing system influencing the minimal dietary inclusion level of minerals, it was found that fish were able to utilise the minerals from water (indicated by difference in slope of regression lines, Chapter 9), but were however not able to compensate to meet the minimal dietary levels required during sub-optimal dietary supply. Thus, adequate dietary supply of micro-minerals needs to be ensured, irrespective of the water mineral concentrations in RASs. On the other hand, care should be taken to avoid excessive supply of inorganic minerals in practical diets and due recognition should be given to the levels of available minerals supplied by the feed ingredients (Sugiura *et al.*, 1998). Even very early, Tacon and de Silva (1983) reported exceedingly high concentrations, much above the recommended levels of the NRC, even within similar feed categories of commercial salmonid feeds. About 2 to 11 fold variations for micro-minerals such as Fe (80-540 mg/kg), Cu (5-40 mg/kg), Mn (35-100 mg/kg) and Zn (50-260 mg/kg) were observed. Three decades down the line, the situation has not changed much, according to the recent report based on a survey on different Norwegian fish feeds over the decade 2000-10' (Sissener *et al.*, 2013). The reported minimal and maximal dietary levels are as follows, Fe (65-493 mg/kg); Cu (2.5-21 mg/kg); Mn (4.4-226 mg/kg); Zn (36-330 mg/kg) and Se (0.39 to 4.1 mg/kg). These high variations might be related to the differences in micro-mineral composition of the major feed ingredients, due to a diverse array of source and origin.

Implications of the present findings

The findings of the present thesis in different chapters when integrated provide sustainable alternatives for aquaculture development.

1. Sustainable use of phosphorus

Phosphorus is the most limiting and vital nutrient in freshwater ecosystem. The ever increasing global population and the need to feed the billions have put global phosphorus availability at stake raising concerns on global phosphorus security (Cordell et al., 2009; 2011). As regards, animal feeds including aquafeeds utilise only a small proportion (7%) of the global phosphorus reserves relative to fertiliser (82%) production (GPRI 2010). Nevertheless, strict environmental regulations have been imposed on aquaculture with regard to phosphorus in the farm effluents. Feed is the primary source of phosphorus in aquaculture effluents and hence the level of phosphorus in fish feeds is strictly regulated. Considerable research had focused on reducing the phosphorus load in fish farm effluents either by reducing the total dietary P levels or by improving the availability of dietary phosphorus (Cho and Bureau, 2001). In this context, low water exchange RASs provide multiple advantages, such as (i) dietary phosphorus levels can be increased to meet the phosphorus demand of fast growing fish without affecting natural ecosystem; (ii) the excreted and unutilised phosphorus accumulated in the RAS water (up to certain limits) can improve growth (iii) the phosphorus accumulated in RAS water can be harnessed by suitable phosphorus recovery mechanisms or effectively utilised for secondary crop production through hydroponics.

2. Relation between phosphorus and lipid metabolism

Lipid accumulation in the body of fish as a symptom of dietary phosphorus deficiency has been known over the years. However, the underlying physiology in relating phosphorus and lipid metabolism is not well understood. The only study thus far in this context concluded that the inability to use dietary lipid as energy source led to lipid accumulation in phosphorus deficient fish (Sakamoto and Yone, 1981). On the other hand, lipolysis was stimulated by phosphate loading in rats (Lee at al., 1982). Recent observations in literature (Eding et al., 2012; van Bussel et al., 2013) and this thesis (Chapter 3 and 9) indicate that high phosphorus levels in diet or rearing water reduce lipid deposition in fish. Further research in this area is required to shed light on energy

metabolism and body composition of fish reared in low water exchange RAS with high phosphate concentrations.

3. Importance of the dietary supply of calcium

Owing to the appreciable level of dissolved calcium in freshwater, it was reported that dietary supply of calcium is essential only when fish are reared in calcium-free water (Robinson et al., 1984, 1986 and 1987). Almost three decades down the line, the dietary Ca recommendations in fish feeds have not changed (NRC 2011), while numerous changes have occurred in nutrient and ingredient composition of fish feeds, growth potential of cultured fish species with fast growing varieties or strains, developments in aquaculture systems etc. The importance of dietary calcium supply is described in the results of this thesis. In the context of fast growing fish and RAS taken together, dietary supply of Ca requires more attention. In low water exchange RAS, all the minerals accumulate and increase in concentration, except calcium. Thus it would be even more important to ensure adequate dietary supply of calcium for fast growing fish raised in low water exchange RAS.

4. Plant based diets and innate immunity of fish

Like in other vertebrates, iron homeostasis in rainbow trout is most likely achieved by regulation of hepcidin expression in the liver when exposed to dietary Fe overload (Chapter 7). Hepcidin, is described to be a peptide hormone at the interface of innate immunity and iron metabolism (Ganz, 2006). Cellular and systemic Fe homeostasis by hepcidin and associated Fe regulatory genes are related to inflammatory and immune response in mammals (Ganz and Nemeth 2012) and also in fish (Hsieh et al. 2010; Yang et al. 2013). We found that, hepcidin mRNA was relatively less expressed in rainbow trout fed plant based diets compared to fish fed the fish meal based diets. Thereby, further studies on innate immunity and health status of fish fed plant ingredient based diets are warranted.

Conclusion

Based on an extensive survey of literature data on mineral requirements of fish (Chapter 1 and 2), dedicated studies were undertaken to analyse the factors affecting dietary supply of minerals to fish. Dietary supply of available P and Ca needs to be adapted according to nutrient and ingredient composition of the feeds to avoid demineralization

of hard structures resulting from increased physiological demand for P and Ca in fast growing rainbow trout (Chapter 4 and 5). Supplementation of additional Ca-P reduced post-prandial levels of plasma micro-minerals especially Zn and Se (Chapter 5). Basal metabolic needs for Zn and Cu, as well as cellular and molecular processes regulating Fe and Cu homeostasis were affected in rainbow trout fed plant ingredient based diets (Chapter 6 and 7). Micro-mineral supply of Zn and Se were most limiting to rainbow trout and common carp, respectively fed plant ingredient-based diets and requires increased supplementation than recommended by NRC (2011) to meet the requirements (Chapters 8 and 9). In RAS, accumulation of minerals in re-circulating water reduced fat deposition and improved vertebral mineralisation in common carp (Chapter 9). Common carp was able to utilise dissolved minerals from water, but was however unable to compensate for sub-optimal dietary supply to meet the requirements (Chapter 9). Slight evidence for dietary mineral supply interacting with aqueous mineral uptake appeared at transcriptional level, especially in the gills (Chapter 9).

On the whole, the findings of this thesis showed that micro-nutrient (macro- and micro-minerals) metabolism is altered by changing the nutrient / ingredient composition of the feeds and the rearing conditions. Further, the existing recommendations on the minimal dietary levels of certain macro- and micro- minerals needs to be reconsidered taking into account the changing scenario in feed composition. Moreover, intriguing leads have been identified in support of the relation between minerals and hepatic intermediary metabolism of macro-nutrients. These can serve as the starting point of further research on the impact of dietary and system changes on mineral metabolism of fish. It should also be noted that, data on novel molecular markers related to mineral metabolism especially for micro minerals need to be supported with the conventional body and tissue mineral concentration, for a better understanding.

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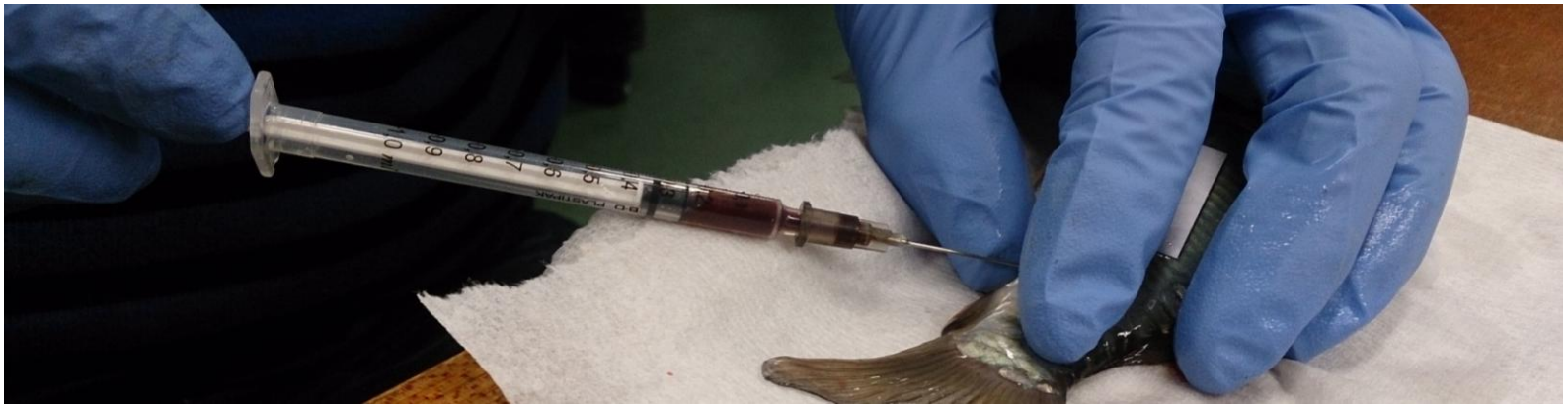

APPENDICES

Summary

Minerals are a group of essential nutrients. The requirement of minerals to fish has attained less attention when compared to other macro-nutrients. In order to understand and analyse the existing information in the literature, extensive meta-analytic reviews of literature data were made in chapter 2 and chapter 3 on the mineral requirements of fish and the factors affecting the dietary inclusion levels. The meta-analysis showed that response criteria based on vertebral mineral concentration or specific enzyme activities were more robust and appropriate in determining the mineral requirements of fish.

Any change in the diet can influence the supply of minerals to fish. Over the years, aquaculture feeds have witnessed dynamic changes in dietary composition. Nutrient dense, high fat diets are being used in commercial salmonid farming. Faster growth rates and increased feed efficiencies are achieved through such high fat diets. However, the effect of such high fat diets on dietary phosphorus needs has been overlooked. In **chapter 4**, the effect of dietary macronutrient levels (Fat) on mineral (P and Ca) balance was studied. In this study, rainbow trout were fed diets with two levels of fat (low vs high) and two levels of Ca-P (adequate vs high). It was found that high fat diets required higher P-Ca levels in the diet to maintain proper mineralisation of body and vertebre.

Research on the replacement of fish meal with plant protein sources in fish feeds has been underway since two decades. However, very few information is available on how these change in diet composition affected the mineral absorption and metabolism in fish. This was studied in chapters 5, 6 and 7. In **chapter 5**, the post-prandial changes in the plasma mineral levels over 24h was monitored in rainbow trout fed a complete plant ingredient based diet (with or without phosphorus supplementation) and a fish meal based diet. Dietary phosphorus supply in plant based diet was improved by inorganic phosphate supplementation, on par with the fish meal diet. However, supplemental phosphate reduced the bio-availability of Zn, Cu and Se to rainbow trout.

In **chapter 6** the effect of change in diet composition on the basal metabolic needs for minerals were studied in rainbow trout. A complete plant based diet and fish meal based diets were fed at 4 different feeding levels and the mineral gain was regressed over intake. Endogenous loss of microminerals, namely Zn, was increased, and Cu was reduced in rainbow trout fed plant based diet. As a consequence, lower level of Zn and higher levels of Cu were observed in the body of rainbow trout fed the plant based diet

compared to fish fed the fish meal diet. This showed that basal metabolic processes involved in micro-mineral metabolism were affected in rainbow trout fed the plant ingredient based diet.

From the lead obtained in the previous study, the cellular and molecular changes underlying micro-mineral metabolism were studied in **chapter 7**. In this study, rainbow trout were fed a fish meal or plant ingredient based diet, with or without a micro-mineral premix. Changes in body mineral composition, enzyme activities and mRNA expression of molecular markers related to micro-mineral absorption, transport and metabolism, were studied. The study revealed that rainbow trout fed plant ingredient based diets had higher levels of Cu in the body and higher circulating levels of Fe in the plasma. The results also indicated that these changes could be secondary effects of disturbances in bile salt or cholesterol metabolism.

In chapters 5, 6 and 7, the impact of plant based diets on the availability, utilisation and metabolism of micro-minerals were studied in rainbow trout. In **chapter 8**, the question was, will the metabolic changes induced by plant based diets alter the dietary levels of micro-minerals needed in rainbow trout feeds? This was studied by supplementing five graded levels of a micro-mineral premix (Fe, Cu, Mn, Se and Zn) to a plant ingredient based diet. It was found that Zn was the most limiting micro-mineral in plant based diets to rainbow trout, followed by Se. It is suggested that dietary levels higher than those recommended by NRC (2011) is required for Zn and Se in rainbow trout feeds made of plant derived ingredients.

In terrestrial animals, dietary supply forms the only source of minerals; however fish can acquire minerals from the diet as well as the rearing water. It is reported that recirculation aquaculture system with low water exchange rates accumulate dissolved minerals in the water. In **Chapter 9**, the impact of high water mineral concentrations, achieved through low water exchange rates in RAS, were studied for their impact on the mineral balance and the minimal dietary levels of micro-minerals required in common carp fed a plant based diet. The study showed that common carps were able to acquire dissolved minerals from low water exchange RAS, but were able to compensate for a part of the dietary need only for Se. Even for Se, no effect was observed on hepatic-GPX activity between the systems. With regard to minimal dietary inclusion levels of micro-

minerals in plant based diets to common carp, Zn and Se were found to be the most limiting micro-minerals.

On the whole, the following conclusions can be made from the thesis,

1. Vertebral mineral concentration and specific enzyme activities are more robust and appropriate response criteria for determining mineral requirement of fish

2. Increased supply of available phosphorus and calcium is required to maintain proper mineralization in fast growing rainbow trout fed high fat or plant ingredient based diets

3. Endogenous loss of Zn is increased and endogenous loss of Cu is reduced in fish fed by diets made of plant derived ingredients, resulting in Zn depletion and Cu accumulation in the body, respectively.

4. Among the micro-mineals essential to fish, Zn and Se were found to be the most limiting in diets made of plant derived ingredients to rainbow trout and common carp. Dietary levels higher than recommended by NRC (2011) are required to meet the requirement.

5. Fish reared in low water exchange RAS are able to acquire dissolved minerals but could not compensate for low dietary supply to meet the requirement.

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About the Author

Antony Jesu Prabhu, P., was born on 14th March 1987 in Chennai, India. Right from school days, biology was his go to subject of interest; such was the passion, he used to visit fish farms and hatcheries during his holidays in graduate school. He graduated with distinction in his bachelor program (2004-08') on Fisheries Science from Tamil Nadu Veterinary and Animal Sciences University, India. He was an all-round performer in the graduate school excelling in studies, sports and entertainment. In 2008, the Indian Profesionel Fisheries Graduates Forum ranked him 2nd in the top 5 best fisheries graduate of the country. By the end of graduation, he had his interest oriented in aquaculture and aquaculture nutrition to be specific. He was awarded with Junior Research Fellowship by ICAR for pursuing masters at CIFE, Mumbai (2008-10') where he specialized in Fish Nutrition and Biochemistry. As a part of his masters' program he made a dissertation on "Nutritional strategies to counter the effects of endosulfan and elevated temperature on the productive performance and reproductive physiology in Tilapia". He published his first full length research paper in 2010, graduated in masters' with gold medal and was determined to do Ph.D from a leading research lab in Fish Nutrition. Soon after his masters', he worked 1 year for the FAO in reviewing the feed management strategies of Indian carp farming in ten states of the country. In 2011 he was seleteced for this Ph.D position on 'micronutrient balance in fish as affected by dietary factors and rearing systems' at NuMeA, INRA St-pee-sur-Nivelle. The project was in collaboration with Aquaculture and Fisheries group, Wageningen University under the INRA-WUR platform for sustainable aquaculture. During his Ph.D, he had won two independent research grants under AquaExcel-TNA. His publication on "Mineral requirements of fish: a systematic review" was awarded as the best publication in 2014 by Wageningen Institute of Animal Sciences. He is presently employed as Assistant Professor in Aquaculture at Tamil Nadu Fisheries University, India.

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List of publications

Ph.D thesis

Antony Jesu Prabhu, P., Schrama, J. W., & Kaushik, S. J. (2013). Quantifying dietary phosphorus requirement of fish – a meta-analytic approach. Aquaculture Nutrition, 19(3), 233-249.

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Co-authored publications during Ph.D

Fontagné-Dicharry, S., Godin, S., Liu, H., **Antony Jesu Prabhu, P**., Bouyssière, B., Bueno, M., Tacon, P., Médale, F., & Kaushik, S. J. (2015). Influence of the forms and levels of dietary selenium on antioxidant status and oxidative stress-related parameters in rainbow trout (Oncorhynchus mykiss) fry. *British Journal of Nutrition*, 1-12.

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Nandeesha, M. C., Kumar, S., & **Antony Jesu Prabhu, P.** (2013). Feed management of the major carps in India with special reference to feed management practices adopted by farmers in Tamil Nadu, India. In M. R. Hasan (Ed.), *FAO Aquaculture and Fisheries, Technical Paper*, (pp. 433-463). Rome: FAO.

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Kumar, N., **Antony Jesu Prabhu, P**., Pal, A. K., Remya, S., Aklakur, M., Rana, R. S., Gupta, S., Raman, R. P., & Jadhao, S. B. (2011). Anti-oxidative and immuno-hematological status of Tilapia (Oreochromis mossambicus) during acute toxicity test of endosulfan. *Pesticide Biochemistry and Physiology, 99*(1), 45-52.

WIAS Graduate School Training and Supervision Program

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WIAS Introduction Course, 2013 Introduction to Bioethics, 2015	
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Tim Stouten (2014). Mineral Requirements of Common Carp (Cyprinus carpio) in Relation to Water Management. Wageningen University, Aquaculture and Fisheries. M.Sc Major Thesis number. T 1925. p 54.

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Mathilde Arque (2014). Molecular responses in transporters and enzymes of mineral metabolism in common carp: effect of dietary changes and water exchange rate in recirculation aquaculture systems. Internship in Biochemistry. University of Rochellee. P 36.

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