EFFECTS OF PHYTATE, PHYTASE AND DIETARY PHOSPHORUS ON FEED INTAKE, GROWTH PERFORMANCE AND PHOSPHORUS UTILISATION IN ATLANTIC SALMON (*SALMO SALAR*, L.)

by

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DECLARATION

This thesis contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis. To the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.

Mirmasoud Sajjadi

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Co-authorship

The following people and institutions contributed to the publication of the work undertaken as part of this thesis:

Paper 1 Sajjadi, M., Carter, C.G., 2004. Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar*, L.). Aquaculture Nutrition 10, 135-142.

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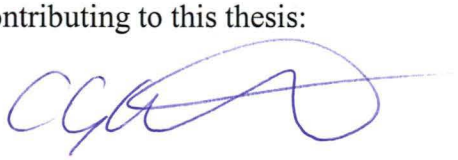
We the undersigned agree with the above stated "proportion of work undertaken" for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:



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Abstract

One of the key research areas in aquaculture nutrition is fish meal replacement. Plant derived materials such as legume and oilseed meals and protein concentrates are being investigated and used in aquafeeds. However, the use of these ingredients is limited by antinutritional factors. Phytate is an important antinutritional factor that impacts on phosphorus utilisation and excretion in monogastric animals including fish. Phytate has negative effects on growth performance, protein and mineral, especially phosphorus, digestibility and utilisation. Both purified phytic acid and natural sources of phytate were used in the present study. Different criteria such as growth performance, chemical composition and nutrient apparent digestibility were measured to assess the effect of phytate and phytase on Atlantic salmon (*Salmo salar*, L.). Furthermore, due to the importance of phosphorus in environmental loading, the effect of phosphorus intake on phosphorus utilisation was also investigated.

The first experiment established that phytate (0.8%) did not affect feed intake or growth performance, but significantly reduced protein digestibility without negative effect on trypsin activity in Atlantic salmon. Phytase *per se* enhanced the growth of fish and when phytase and phytate were added together feed efficiency ratio was higher. Phytase neutralized the effect of phytic acid on protein digestibility.

Use of phytase in plant meal based diets prevented the negative effects of phytate. Phytase had positive effects on feed intake, growth, whole-body chemical composition and nutrient digestibility. When phytase was used in a diet with sufficient dietary phosphorus, the positive effects of phytase were not observed. Efficacy of phytase strongly depends on endogenous and exogenous factors. Phytase inclusion in a fish meal based diet partially replaced with plant meal had no effect on fish performance, but when phytase was used in a soy protein concentrate (SPC) based diet, improved performance was observed. Probably, phosphorus apparent digestibility is the best criterion for determining phytase efficacy. Furthermore, phytase had a significant effect on reducing phosphorus

waste from a plant protein based diet. Phytase supplementation of diet and pre-treatment of ingredients were compared using a SPC based diet. Supplementation at levels greater than 1000 U phytase kg^{-1} diet was necessary to improve salmon performance and phosphorus utilisation, while pre-treatment of SPC with 250 U phytase kg^{-1} had similar significant effects.

The effect of phosphorus intake on apparent digestibility of phosphorus was investigated in Atlantic salmon. Phosphorus apparent digestibility was negatively correlated with dietary phosphorus concentration. Digestible phosphorus was approximately the same for salmon in all treatments despite using different levels of dietary phosphorus that ranged from suboptimal to excess amount. The effect of feed intake on nutrient and mineral especially phosphorus digestibility was also investigated. Ration affected nutrient digestibility except energy digestibility in fish and there were significant correlations between dry matter, protein and phosphorus digestibilities.

Experiments generally showed that phytase supplementation of diet or pre-treatment of ingredients has the potential to improve growth performance and phosphorus utilisation of Atlantic salmon and reduce the phosphorus load from fish farm provided proper experimental design is used. Since dietary phosphorus concentration and phosphorus intake affect apparent phosphorus digestibility these factors must be taken into account when conducting phosphorus nutrition research.

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List of abbreviations

AD	Apparent digestibility
Ag	Silver
ANF	Antinutritional factor
ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
BAPNA	*α-N-* benzoyl-D,L-arginine-*p*-nitroanilide
BW	Body weight
° C	Degree Celsius
Ca	Calcium
Co	Cobalt
Cu	Copper
CP	Crude protein
DM	Dry matter
DO	Dissolved oxygen
FAO	Food and Agriculture Organization of the United Nations
FBW	Final body weight
Fe	Iron
FCR	Feed conversion ratio
FER	Feed efficiency ratio
G	gram(s)
H	hour(s)
H	Hydrogen
I	Iodine
IBW	Initial body weight
ICP-OES	Inductively coupled plasma optical emission spectrometry
In vitro	In glass
In vivo	In animal
K	Condition factor
K	Potassium
Kg	Kilogram(s)
l	Litre(s)

L	Length
M	Molar
mg	Milligram(s)
Mg	Magnesium
MJ	Mega joule
min	Minute(s)
ml	Millilitre(s)
Mn	Manganese
Mo	Molybdenum
N	Nitrogen
N	Number(s)
Na	Sodium
NRC	National Research Council
ns	Not significant
O	Oxygen
P	Phosphorus
P	Probability
ppt	Part per thousand
PPV	Productive protein value
RPC	Rapeseed protein concentrate
S	Sulphur
SD	Standard Deviation
Se	Selenium
SEM	Standard error of the mean
SGR	Specific growth rate
Sp.	Species
SPC	Soy protein concentrate
Sr	Strontium
U	Phytase unit
µ mol	Micro mole
V	Volume
W	Weight
Zn	Zinc

CHAPTER 1

GENERAL INTRODUCTION

1.1. Protein sources for aquafeeds

Fish meal in comparison with other ingredients is the best source of essential amino acids for finfish and crustaceans (De Silva and Anderson, 1995). In aquafeeds, especially for carnivorous aquatic animals, fish meal is an expensive and important source of protein and constitutes a major part of the feed. Fish meal and fish oil constitute more than 50% of current salmon and trout feeds and expansion of the aquaculture of carnivorous species has increased the consumption of fish meal and fish oil. Fish meal production has been argued to place pressure on marine resources (Naylor et al., 2000), nutritionists are concerned about this and considerable research has been conducted in order to replace fish meal with other sources of protein (Lim and Sessa, 1995; Hertrampf & Pascul, 2000). More than one-third of the global fish meal production is currently used by the aquafeed industry and the proportion of fish meal used in the aquaculture industry tripled during 1988 – 1997 (Naylor et al., 2000). Global demand for fish meal is predicted to increase, due to both the increase in Chinese aquaculture production and aquafeed industry expansion (FAO, 2002) and a possible shift from extensive aquaculture to more intensive production that needs cost-effective formulated diets (Allan, 1997). Fish meal demand may increase to 70% by 2015 and the global demand for fish meal for aquafeed industry would exceed total fish meal supply by 2020 (FAO, 2002).

It is well established that plant meals are a promising source for fish meal replacement. They are produced in comparatively large amounts compared to fish meal production, for example soybean production was more than 25 times higher than fish meal production in 2001 (FAO, 2004a; 2004b). Plant protein sources are cheaper (Tacon, 1994; Stone, 2003), their availability throughout the year is better than that of fish meal. However, there are limitations to their use in aquafeeds, in particular, high levels of carbohydrate, amino acid deficiency and antinutritional factors (ANFs) are major disadvantages (Tacon, 1994; Jobling, 1998; Carter and Hauler, 2000).

1.2. Antinutritional factors

According to Makkar (1993), antinutritional factors (ANFs) have been defined as "substances which either by themselves, or through their metabolic products arising in living systems, interfere with food utilisation and affect the health and production of animals". ANFs are natural defensive chemicals that protect plants against moulds, bacteria and animals (Harborne, 1999; Huisman and Tolman, 2001). Protease inhibitors (in soybean, corn, barley, rapeseed, cottonseed, lupin, pea), glucosinolates (in rapeseed, soybean), saponins (in soybean), as well as more generally tannins, lectins, phytoestrogens, alkaloids, antigenic compounds, gossypols, oligosaccharides and phytates are antinutritional factors in plant protein sources (Huisman et al., 1990; Makkar, 1991; Harborne, 1999; de Lange et al., 2000; Francis et al., 2001; Huisman and Tolman, 2001). Some of these factors act as biopesticides in plants and plant seeds (Sequeira, 1978) and some of them are chemicals that are used for defence against mammalian herbivory, so when the plant is eaten, it is not generally suitable (Harborne, 1999). ANFs have negative effects on growth, feed intake, feed utilisation, histo-pathological changes in the different organs and immune responses of fish (Van der Ingh et al., 1991; Francis et al., 2001).

Based on their effects on animals, antinutritional factors can be categorized into five groups: (1) factors that affect protein digestion and utilisation (trypsin and chymotrypsin inhibitors, lectins, tannins, phenolic compounds, saponins); (2) factors that have negative effects on carbohydrate digestion (amylase inhibitors, polyphenolic compounds, flatulence factors); (3) factors that have negative effects on mineral utilisation (glucosinolates, oxalates, phytates, gossypols); (4) antivitamin factors; and (5) remaining factors (mycotoxins, mimosine, cyanogens, nitrates, alkaloids, photosensitizing agents, isoflavones) (Huisman et al.,1990; Makkar, 1991, 1993; Francis et al., 2001; Huisman and Tolman, 2001).

1.3. Phytic acid and phytate

Although phytate is classified as an ANF, it is also a nutrient, because it contains phosphorus that is there for the seedling, as well as for animals that have the phytase enzyme. Furthermore, it is a source of inositol (Cosgrove, 1980). It is an antinutritional factor for monogastric animals that do not have phytase in their gastrointestinal tract. Its phosphorus is not available (Nelson, 1967; Erdman, 1979; Reddy et al., 1982) and it binds with other minerals (multivalent cations) and affects their bioavailability (Erdman, 1979; Maga, 1982; Reddy et al., 1982).

Chemically, phytic acid is myo-inositol 1,2,3,4,5,6 hexakis dihydrogen phosphate (IUPAC-IUB, 1977) or hexaphosphoinositol ($C_6H_{18}O_{24}P_6$) with a molecular weight of 660. It contains 28.18 % phosphorus. This chemical was first discovered about 150 years ago by Hartig (1855)(cited by Reddy et al., 1982) but the current chemical structure of phytic acid was proposed by Anderson (1914). Phytate, sometimes called phytin, is the salt of phytic acid that is bound to potassium, magnesium and calcium (Pallauf and Rimbach, 1997) (Fig. 1.1). The international abbreviation of phytic acid, PA, is also used for phytate (Pallauf and Rimbach, 1997) and these two different terms, sometimes are used with the same meaning (Harland and Oberleas, 1999). Phytates are sometimes called phytin. Phytic acid phosphorus represents 50–85% of total phosphorus in plant seeds (Lolas et al., 1976; Cosgrove, 1980; Reddy et al., 1982; Pallauf and Rimbach, 1997; Hardy, 1998). Commercial purified Na phytate, Ca, Mg, Cu and Ag salt have been made (Cosgrove, 1980). Phytic acid is a highly charged molecule with six phosphate groups, therefore it is an excellent chelator of cations (Cheryan, 1980).

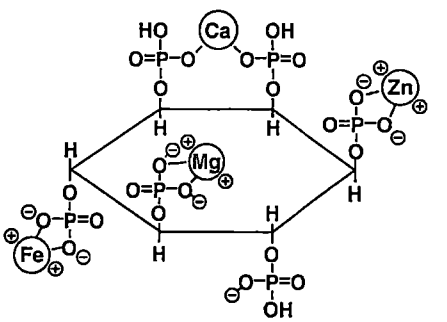

Figure 1.1. Structure of phytic acid and its chelate binding at neutral pH (from Erdman, 1979).

Phytate is found in a wide range of feed ingredients (Tables 1.1 and 1.2) including promising protein sources for fish meal replacement in aquaculture feeds such as soybean, canola, wheat, barley, cottonseed, corn, lupin, maize and their by-products (Ufodike and Matty, 1989; Olli and Krogdahl, 1994; Olli et al., 1994 [cited by Hertrampf and Piedad-Pascual, 2000]; Higgs et al., 1995; Robinson and Li, 1995; Rumsey et al., 1995). Phytate content of different plant protein sources ranges between 0.5-7% (Tables 1.1 and 1.2). The phytic acid content of some plants and their products such as wheat bran, rice bran and rapeseed protein is very high (Table 1.2). By upgrading of ingredients, the level of phytate increases, for example the level of phytate in plant protein concentrate is higher than the plant protein meal (Erdman, 1979; Higgs et al., 1995).

Table.1.1. Total phosphorus and phytate phosphorus content of common feed ingredients grown in Australia

Feed ingredient	Total P ($g\ kg^{-1}$)	Phytate-P ($g\ kg^{-1}$)	Phytate-P/P x 100 (%)	References
Barley	2.60-2.73	1.69-1.86	65.0-67.3	1,2
Maize	2.40	2.05	85.0	1, 2
Maize gluten	5.00	4.20	84.0	2
Sorghum	2.92	2.41-2.42	82.7-83.0	1, 2
Wheat	2.64-3.80	1.79-2.20	73.0-74.9	1, 2, 3
Canola meal	8.76-8.80	6.65-6.69	76.0-76.4	1, 2
Cottonseed, whole	6.05	4.25	70.2	2
Cottonseed meal	11.36-11.46	9.04-9.11	79.0-80.5	1, 2
Soybean, whole	5.55	3.08	55.5	2
Soybean meal	6.66-6.71	4.52-4.53	67.0-68.3	1, 2
Sunflower meal	9.03-9.05	7.48	82.8-83.0	1, 2
Rice bran	17.51-17.59	15.83-15.93	90.2-91.0	1, 2
Wheat by-products	7.96-8.02	6.85-7.00	86.0-87.3	1, 2
Peas	3.45	1.67	48.4	
Lupins				
L. albus, whole	4.47	2.49	55.7	2
L. angustifolius, whole	3.10	1.60	51.6	2
L. angustifolius, dehulled	3.80	1.89	49.7	2

(1) From Selle et al. (2000)(data supplied by A.R. Walker, BRI Australia Ltd, North Ryde, NSW, Australia)

(2) Selle et al. (2003)

(3) Kim et al. (2002)

Table 1.2. Total phosphorus, phytate phosphorus and phytic acid content of different ingredients

Ingredients	Total-P ($g\ kg^{-1}$)	Phytate-P ($g\ kg^{-1}$)	Phytic acid (%)	Reference
Barley	3.00-4.90	1.80-3.26	0.70-1.16	1, 4, 6, 9, 11, 14
Beans (*Phaseolus vulgaris* L.) (50 varieties)	2.59-5.56	-	0.54-1.58	8
Canola meal	-	11.3-13.2	3.00 – 5.00	2, 10, 23
Coconut (expeller)	5.3	1.8	0.64***	4
Corn	2.90-3.10	2.08-2.8	0.89-0.99	3, 7, 14, 17
Corn distillers	9.0	1.9	0.67***	4
Corn gluten meal	5.81-6.40	2.93-3.6	1.20-1.30	7, 14
Corn meal	1.01*	0.7	0.3	14
Cottonseed flour, glanded	-	-	2.90-2.94	5, 26
Cottonseed flour, glandless	-	-	4.78-4.80	5, 26
Cottonseed globoids	-	-	60.00	5
Cottonseed meal	10.71-12.96	5.40-9.20	1.92-3.30	14, 16
Field beans (heated)	5.0	2.3	0.82***	4
Linseed (expeller)	7.5	4.2	1.49***	4
Linseed (extracted)	8.2	4.7	1.67***	4
Lupins	2.5	0.5	0.18***	4
Maize	2.8	1.9	0.67***	4
Maize feed flour	2.3	1.4	0.5***	4
Maize feed flour (USA)	5.0	2.7	0.96***	4
Maize germs (extracted)	6.5	4.2	1.49***	4
Maize gluten feed	8.7	4.7	1.67***	4
Maize gluten feed (pellets)	8.9	5.2	1.84***	4
Maize (moist ensiled)	3.0	1.3	0.46***	4
Malt sprouts (pellets)	6.0	0.1	0.04***	4
Oats	3.60-4.50	2.10-2.79	0.74-1.41	4, 6, 9, 12, 13
Palm kernel meal	8.0	4.0	1.42	16
Palm-kernel (expeller)	5.9	3.9	1.38***	4
Peas	3.8	1.7	0.28-0.71	4, 24
Peanut (extracted) (pellets)	6.80	3.20	1.13***	4

Peanut meal, defatted and dehulled	-	-	1.70	5
Rapeseed	11.2	4.0	1.13-2.00	4, 18
Rapeseed meal	11.06-12.50	5.40-7.80	1.92	7, 16
Rapeseed protein concentrate	-	-	5.30-7.50	5, 18
Rice bran	14.29-17.1	10.43-14.4	3.90-5.10	4, 7, 14
Rice bran (extracted)	18.9	7.9	2.80***	4
Rice feed flour	3.2	2.3	0.82***	4
Rye	3.1-3.6	1.9-2.2	0.67-0.78***	1, 4
Sesame meal	12.72*	10.3	3.6	14
Sesame meal (defatted)	-	14.60	5.18	3
Sesame meal, defatted and dehulled	-	-	5.20	5
Sesame meal, dehulled	-	-	3.60	5
Sorghum	2.7	1.9	0.67***	4
Soybean	5.30-9.40	2.81-4.17	0.92 – 2.29	4, 6, 9, 11, 19
Soybean meal	6.07-8.7	2.4-4.00	0.85-2.98	3, 7, 14, 16, 23
Soybean meal (full-fat or defatted and hulled)	-	-	1.40 – 1.60	5
Soy flour (defatted)	6.65-7.53	4.57-5.22	-	21
Soy protein concentrate	6.50-8.99	3.50-6.12	-	21
Soybean protein isolate	8.00-8.14	3.25-4.80	1.15-2.20	5, 14, 15, 21, 25
Sunflower (extracted) (pellets)	10.0	4.4	1.56***	4
Triticale	3.7	2.5	0.89***	4
Wheat	2.8-5.2	1.75-3.75	0.62-1.35	3, 4, 7, 9, 14, 17
Wheat bran	11.60-13.71	8.84-9.7	3.40-3.44	4, 7, 14
Wheat feed flour	5.6	3.9	1.38***	4
Wheat fine bran	9.5	7.2	2.55***	4
Wheat fine bran (pellet)	10.1	7.8	2.76***	4
Wheat gluten	7.8	5.60-6.00	1.99-2.12	4, 15, 25
Wheat middlings	8.0	5.3	1.88***	4
Wheat mill bran (5 cultivars)	15.0-17.8	12.92-15.54**	4.59-5.52	9
Wheat protein concentrate	5.75-8.41	5.27-7.64	1.87-2.71	20, 21
Wheat standard middlings	4.73*	3.5	1.2	14

* In the original paper, phytate phosphorus / total phosphorus x 100 were given, these values calculated

** In the original paper, phytate phosphorus / total phosphorus x 100 were given, these values calculated

*** Calculated as Phytic acid = Phytate phosphorus (%) / 28.2%

(1) Centeno et al., 2001 (2) Cheryan, 1980 (3) de Boland et al., 1975 (4) Eeckhout and De Paepe, 1994 (5) Erdman, 1979 (6) Harland and Prosky, 1979 (7) Kirby and Nelson, 1988 (8) Lolas and Markakis, 1975 (9) Lolas et al., 1976 (10) Mc curdy and March, 1992 (11) McKenzie-Parnell and Guthrie, 1986 (12) Miller et al., 1980a (13) Miller et al., 1980b (14) Nelson et al., 1968a (15) Nelson and Potter, 1979 (16) Nwokolo and Bragg, 1977 (17) O'Dell et al., 1972 (18) Ohlson and Anjou, 1979 (19) Raboy et al., 1984 (20) Ranhotra, 1972 (21) Ranhotra et al., 1974 (22) Uppström and Svensson, 1980 (23) Vaintraub and Lapteva, 1988 (24) Vidal-Valverde et al., 2003 (25) Wallace and Satterlee, 1977 (26) Wozenski and Woodburn, 1975

Due to its special molecular structure, phytic acid can bind with various divalent and trivalent cations like Ca^{2+}, Zn^{2+}, Mg^{2+} and Fe^{3+} and also trace minerals such as Mn^{2+}, $Cu^{2+}$and Mo^{+} (Erdman, 1979; Maga, 1982; Reddy et al., 1982; Lönnerdal, 2002) leading to formation of different insoluble salts and decreased bioavailability (Gatlin and Wilson, 1984; Richardson et al., 1985; McClain and Gatlin, 1988; Gatlin and Phillips, 1989; Satoh, et al., 1989; reviewed in Francis et al., 2001). Furthermore, the phosphorus in the phytate structure is not available for monogastric animals including fish (Ketola, 1975; Ogino et al., 1979). Kumar and Chauhan (1993) showed that *in vitro* digestibility of protein in plant seed improved during germination due to a reduction in the phytic acid content of the seed. Some studies have shown that phytate can bind with starch and protein (amino acids) (Spinelli et al., 1983; Thompson, 1988; reviewed in Selle et al., 2000). Also, phytic acid can reduce gastrointestinal enzymes activities under both *in vitro* and *in vivo* conditions (Singh and Krikorian, 1982; Morz et al., 1995) and cause damage to the gastrointestinal tract (Richardson et al., 1985). Singh and Krikorian (1982) suggested that binding between phytic acid and trypsin calcium probably causes the inhibitory effect of phytic acid on trypsin, because calcium ion promotes activation of trypsinogen to form trypsin. Richardson et al. (1985) suggested that the toxic effect of phytic acid on the epithelial layer of the pyloric caeca, the negative effect of phytic acid on magnesium bioavailability, or both, might be the cause of abnormal pyloric caeca in fish fed diets with high phytic acid content.

Phytate also, reduced the growth rate of fish (Spinelli et al., 1983; Richardson et al., 1985; Satoh et al., 1989; Hossain and Jauncey, 1993; Usmani and Jafri, 2002). Impaired growth in phytate supplemented diets was attributed to a reduction in protein digestibility (Spinelli et al., 1983) and partly due to a reduction in Zn bioavailability (Richardson et al., 1985). The results of experiments indicate that the effect of phytate on growth performance of fish probably depends on fish species and level of phytate in the diet (Usmani and Jafri, 200). Generally, there are very limited studies about the effect of purified phytate on different fish species (Spinelli et al., 1983; Richardson et al., 1985; Gatlin and Phillips, 1989;

Satoh et al., 1989; Hossain and Jauncey, 1993; Usmani and Jafri, 2002) and to the best of our knowledge there is no published study in this field for Atlantic salmon.

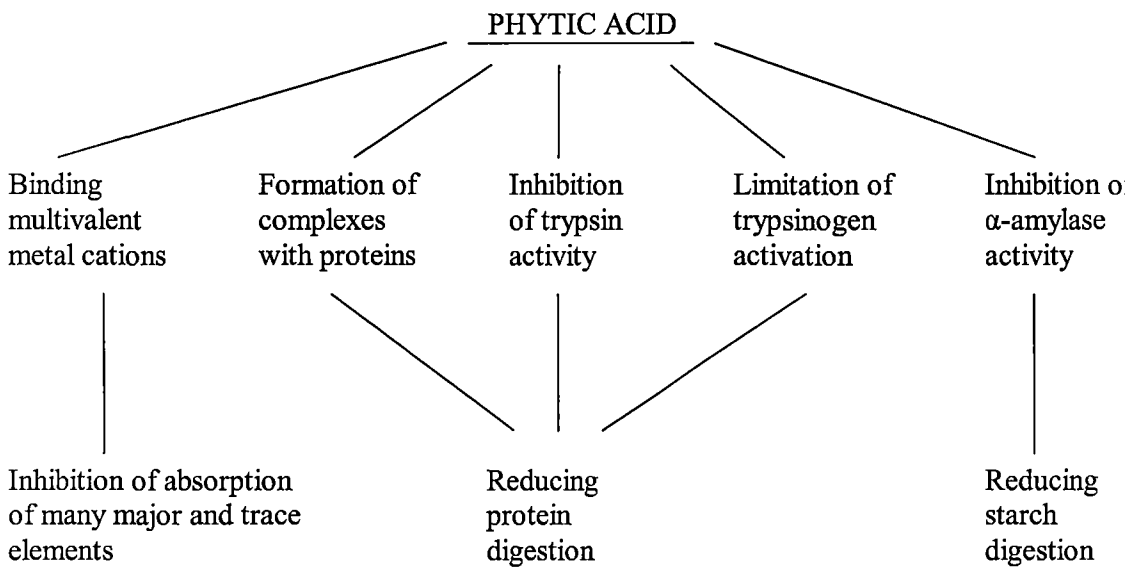

Figure 1.2. Effects of phytic acid in the digestive tract (after Szkudelski, 1997)

1.4. Phytase

Phytase (meso-inositol hexaphosphate phosphohydrolase) is the enzyme that hydrolyses phytate and is found in plants, animals and fungi (Cosgrove, 1966). It is a phosphatase that catalyses the hydrolysis of one or more phosphate groups from phytate (Kies, 1999). Phytase was discovered and extracted from rice bran by Suzuki et al. (1907) (cited by Wodzinski and Ullah, 1996), but commercially produced in 1993-1994 by Gist-Brocades (Wodzinski and Ullah, 1996). Due to lack of phytase in the gastrointestinal tract of monogastric animals, phytase is used in the plant meal based diet in these kinds of animals. Most animal feed

studies since 1994 have used commercial phytase. The first study that used phytase in animal feed was conducted by Nelson et al. (1968b), using phytase from *Aspergillus ficcum*. Commercially produced phytase has been successfully used in poultry, pig and fish feed. Phytase increased feed intake (Selle et al., 1999), growth performance (Cabahug et al., 1999), phosphorus digestibility (Denbow et al., 1995; Ravindran et al., 1995), protein digestibility (Yi et al., 1996; Selle et al., 2000), apparent metabolizable energy (AME) (Rajas and Scott, 1969; Farrell et al., 1993) and mineral digestibility (Lei et al., 1993) in monogastric terrestrial animals. The effectiveness of phytase depends on the amount of phytase that is included in the diet. Jongbloed et al. (1999) showed that 1000 U kg^{-1} diet phytase was needed to maximize the responses in pig.

Phytase has been used in some fish species and improvement in feed intake (Rodehutscord and Pfeffer, 1995; Jackson et al., 1996; Li and Robinson, 1997; Carter and Hauler, 1998b), growth performance (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Schäfer et al., 1995; Jackson et al., 1996; Li and Robinson, 1997; Carter and Hauler, 1998b; Papatryphon et al., 1999; Vielma et al., 2002), phosphorus digestibility (Brown, 1993; Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Teskeredžić et al., 1995; Riche and Brown, 1996; Carter and Hauler, 1998a, b; Lanari et al., 1998; Vielma et al., 1998; Forster et al., 1999; Sugiura et al., 2001), protein digestibility (Storebakken et al., 1998) and mineral digestibility (Storebakken et al., 1998; Sugiura et al., 2001, Carter and Hauler, 1998a) has been reported. To the best of our knowledge there is only one published paper about using phytase in Atlantic salmon diet (Storebakken et al., 1998).

1.5. Phosphorus nutrition

Phosphorus is one of the most important minerals in fish nutrition and feeds. It is a mineral with a high absolute requirement but its absorption from water is negligible and must mainly be supplied via the diet (Lall, 1991; NRC, 1993). Phosphorus is a major component of bone and has important roles in

carbohydrate, lipid and amino acid metabolism (NRC, 1993; Lall, 2002). General signs of phosphorus deficiency are poor growth, feed efficiency ratio and bone mineralization (Andrews et al., 1973; Ketola, 1975; Ogino and Takeda, 1976; Davis and Robinson, 1978; Lovell, 1978; Ogino and Takeda, 1978; Watanabe et al., 1980; Wilson et al., 1982; Robinson et al., 1987; NRC, 1993; Davis and Gatlin, 1996; Baeverfjord et al., 1998; Borlongan and Satoh, 2001; Pimentel-Rodrigues and Oliva-Teles, 2001; Lall, 2002).

Chemical composition of ingredients, their digestibility and their effect on fish growth are important factors for assessment of ingredients for aquafeeds. Both ingredient and nutrient digestibility are important for assessment of diets in aquaculture. Available information concerning factors influencing digestibility is controversial and in some cases contradictory (De Silva and Anderson, 1995). Fish size, fish age, density, feeding level, meal size, dietary composition, physical properties of the diet, temperature, salinity and the method of digestibility measurement may affect digestibility values (De Silva and Anderson, 1995). The influence of feed intake on digestibility is not clear. Some studies found feed intake had no effect on nutrient digestibility (Cho and Kaushik, 1990) whereas others showed nutrient digestibility decreased (Henken et al., 1985) or increased (Cui et al., 1996) with increasing feed intake. No research has been conducted on the effect of feed intake on phosphorus digestibility.

Phosphorus waste is lost as both faecal and urinary phosphorus and mainly originates from feeds. Phosphorus wastes pollute the surrounding waters and are directly or indirectly used by algae (Balazsi and Wikfors, 2000). In extreme situations, phosphorus pollution of waters leads to eutrophication of aquatic environment. Phosphorus excretion in fish can be minimized by dietary manipulation (Cho and Bureau, 2001). The most important factors are the amount of phosphorus and the digestibility of phosphorus. Phosphorus sources with a low digestibility will clearly result in high faecal loss. In addition, high concentration of digestible phosphorus will also increase faecal loss because phosphorus absorption is regulated in intestine (Avila et al., 2002). Previous studies have

shown that including an excess amount of dietary phosphorus leads to lower estimates of its digestibility (Vielma and Lall, 1998; Sugiura et al., 1999; Avila et al., 2000; Rodehutscord et al., 2000). Phosphorus digestibility in rainbow trout depends on the dietary phosphorus concentration, digestibility peaks at the dietary requirement and then declines with increasing dietary phosphorus (Riche and Brown, 1996). Some studies showed that when dietary phosphorus concentration exceeds requirement level non-faecal excretion also increases (Rodehutscord, 1996; Bureau and Cho, 2000).

1.6. Aims of this study

Information about the effects of phytate and phytase on Atlantic salmon is limited, this study aimed to provide information on the effects of phytate and phytase in Atlantic salmon and to take dietary phosphorus into consideration. In the present study, the effect of phytic acid and phytase on feed intake, growth performance and nutrient utilisation was studied and the following aims were investigated:

- The effect of purified phytic acid on feed intake, growth performance and digestibility in Atlantic salmon
- The effect of phytase supplementation in plant meal based diet and finding the best criterion for assessing phytase efficacy
- Efficacy of phytase supplementation and phytase pre-treatment in a plant meal based diet for Atlantic salmon
- The optimum dose of phytase supplementation in Atlantic salmon diets
- Effect of dietary phosphorus on phosphorus digestibility in Atlantic salmon

- Effect of feed intake on phosphorus, energy and protein digestibility

1.7. Notes on this study

The experimental chapters (Chapters 2-6) in this thesis are presented in the format of manuscripts for publication in peer-reviewed journals. For that reason, some text may be repeated especially in the introduction and materials and methods. Chapters 2 and 3 have been published or accepted for publication as:

Sajjadi, M., Carter, C.G., 2004. Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar*, L.). Aquaculture Nutrition 10, 135-142.

Sajjadi, M., Carter, C.G., Dietary phytase supplementation and the utilisation of phosphorus by Atlantic salmon (*Salmo salar*, L.) fed a canola meal based diet. Aquaculture (*accepted*).

Chapter 5 was originally designed to assess phosphorus requirement of Atlantic salmon smolt at a high temperature. Seawater temperature in the Southern Hemisphere during the summer (the time of salmon transfer to saltwater) reaches 21-22 °C. This situation has been observed in Chilean and Tasmanian salmon farms. In Chile during 1998-1999, ankylosis of the mandibular articulation, spinal foreshortening, fracture of vertebrae and rarefaction of osseus and cartilaginous tissues including operculum was observed in salmon smolt when they were transferred to seawater at high temperature (20 °C plus) (Roberts et al., 2001). Outbreak of this condition was up to 70-80% in some farms. They suggested that phosphorus deficiency probably caused these pathogenic symptoms in fish. Furthermore, this situation mainly was observed in fast-growing salmon. Roberts et al. (2001) suggested that most likely cause for this situation observed in Chile, was smolting of fast-growing salmon in very high seawater temperature on a diet deficient in both phosphorus and vitamin C. So, the initial aim of the present

experiment was to investigate phosphorus requirement in fast-growing salmon at high temperature fed a low FCR diet. Unfortunately the fish become infected with *Flexibacter* sp. and the experiment was terminated after 6 weeks. Therefore, it was decided to analyse the data collected from sick fish to investigate an important issue, the relationship between dietary phosphorus and phosphorus digestibility.

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CHAPTER 2

EFFECT OF PHYTIC ACID AND PHYTASE ON FEED INTAKE, GROWTH, DIGESTIBILITY AND TRYPSIN ACTIVITY IN ATLANTIC SALMON (*SALMO SALAR*, L.)

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2.1. Abstract

In the majority of experiments, the effects of phytic acid (with or without phytase) are not separated from the effects of adding plant meals containing phytic acid. A 12-week experiment was conducted with Atlantic salmon (28.9 g) to determine the separate and combined effects of phytic acid and phytase on feed intake, digestibility, trypsin activity and growth. Diets were prepared without phytic acid and phytase; with 2000 U phytase kg^{-1} diet; with 10 g sodium phytate kg^{-1} diet; with 10 g sodium phytate and 2000 U phytase kg^{-1} diet. The basal diet contained sufficient phosphorus and other minerals to meet salmonid requirements. The addition of phytic acid had no significant effect on feed intake or weight gain, it significantly ($P < 0.05$) reduced protein digestibility although there was no reduction in trypsin activity. Phytase inclusion neutralized the effect of phytic acid on protein digestibility. Phytase had no effect on feed intake but significantly enhanced growth whether included with or without phytic acid. Feed efficiency ratio was significantly improved for fish fed the diet containing both phytase and phytic acid but not separately. The significance of this experiment was to separate the direct effects of phytic acid, added in a pure form, from effects due to other components in ingredients containing phytic acid.

Keywords: Atlantic salmon, Feed intake, Phytase, Phytic acid, Protein digestibility, Trypsin activity

2.2. Introduction

Phytate is an antinutritional factor (ANF) found in many plant meals, it is not digested by fish (Rodehutscord and Pfeffer, 1995; Oliva-Teles et al., 1998) and has negative effects on protein digestibility (Spinelli et al., 1983) and availability of other minerals (Gatlin and Phillips, 1989; Hossain and Jauncey, 1993; Sugiura et al., 2001). Phytase catalyses the breakdown of phytic acid (myoinositol hexaphosphate) to sequentially produce myoinositol penta-, tetra-, tri-, di- and monophosphate. Consequently, it neutralizes the negative effects of phytic acid on protein and other nutrients in the diet of monogastric animals. Phytase supplementation may also stimulate appetite and therefore increases growth directly through increased feed intake (Jackson et al., 1996; Hauler and Carter, 1997; Li and Robinson, 1997). Phytase added to soybean meal based diets for rainbow trout (*Oncorhynchus mykiss* Walbaum) increased feed intake and weight gain (Rodehutscord and Pfeffer, 1995). In this case, higher performance was attributed to increased phosphorus digestibility in a diet with a suboptimal phosphorus content (Rodehutscord, 1996). Hauler and Carter (1997) fed Atlantic salmon (*Salmo salar* L.) with soybean meal based diet containing phytase at 2000 U kg^{-1} and showed phytase stimulated appetite. They used a diet with slightly deficient in available phosphorus.

The majority of studies have investigated the efficacy of phytase in relation to a natural source of phytate (Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Schäfer et al., 1995; Jackson et al., 1996; Hauler and Carter, 1997; Li and Robinson, 1997; Oliva-Teles et al., 1998; Bransden and Carter, 1999; Vielma et al., 2000). Consequently, purified phytic acid was used in the present study in place of plant ingredients and it was added at a level to equate to diets that use plant ingredients. In addition, the present experiment aimed to investigate the independent effects of phytic acid and of phytase on feed intake and growth. To do this, four diets with either phytase or phytic acid, with both phytic acid and phytase or with neither were compared. The level of phosphorus and other

essential minerals were included above salmonid requirements in order to assess only the effect of phytic acid and or phytase on performance. The experiment was designed to measure the effect of phytic acid and phytase *per se* on feed intake and protein digestibility and consequently growth performance.

2.3. Materials and methods

2.3.1. Experimental diets

The basal diet (diet C) was formulated to contain 480 g kg^{-1} crude protein, 22.5 MJ kg^{-1}gross energy and 7 g kg^{-1} phosphorus (Table 2.1). All nutrient requirements of Atlantic salmon were satisfied based on values for salmonids (NRC, 1993). Phosphorus concentration was set at the requirement level (7 g kg^{-1}) and concentrations of the remaining essential minerals were in excess of requirements. Protein sources were selected to contain no phytate: South American fish meal (Skretting, Cambridge, Tasmania, Australia), blood meal (Skretting, Cambridge, Tasmania, Australia), and casein (New Zealand Dairy Board, Wellington, New Zealand). Two factors were investigated in a factorial arrangement: phytase level (0 and 2000 U kg^{-1}) (where 1 U is defined as the amount of phytase that liberates inorganic phosphorus from a 0.0015 M sodium phytate solution at a rate of 1 μmol min^{-1} at pH 5.5 and 37 °C) and sodium phytate level (0 and 10 g kg^{-1}). Therefore, there were four diets including a diet that contained no sodium phytate or phytase (diet C); only sodium phytate (10 g kg^{-1}) (diet PA); only phytase (2000 U kg^{-1}) (diet PS); or both phytase (2000 U kg^{-1}) and sodium phytate (10 g kg^{-1}) (diet PAPS). Phytase 10000 U g^{-1} (Natuphos: BASF, Homebush, NSW, Australia), phytic acid [inositol hexaphosphoric acid (sodium salt)] (Sigma Chemical Co., NSW, Australia) were used. Dietary ingredients were mixed using a Hobart mixer, pelleted with a laboratory pelletizer (California Laboratory Pellet Mill) and the pellets dried at 30 °C for 24 h. The diets were refrigerated at 4 °C until use.

2.3.2. Growth experiment

Atlantic salmon were obtained from Springfield Fish Farm (Scottsdale, Tasmania, Australia) and transferred to School of Aquaculture, University of Tasmania. Fish were kept in 300-l circular tanks in a recirculation system where water quality was maintained through physical and biological filters (Bransden et al., 2001). Fish were maintained on a commercial feed (2mm Salmon, Skretting, Cambridge, Tasmania, Australia) for two weeks. Water replacement rate in the system was about 20% d^{-1} and water flow through the tanks was 8-10 l min^{-1}. Oxygen concentration was 90.0 ± 0.5% of saturation, average water temperature was 15.1 ± 0.9 °C and a natural photoperiod (approximately, 10L: 14D) was, in effect, over the 84-day experiment.

At the beginning of experiment, fish were anaesthetized in benzocaine (50 mg l^{-1}) and the wet weight and fork length of individual fish measured. One fish from each tank was sacrificed (100 mg l^{-1} benzocaine) and used for the measurement of initial whole-body chemical composition (n=12). Twenty-five fish were returned to each tank (28.9 ± 3.6 g, mean ± SD, n=300). Triplicate groups of fish were fed each experimental diet (see below) to satiation twice daily at 0900 and 1600 h, satiation was judged to have been reached when approximately 20 pellets were not eaten and lost via the outlet water. Uneaten pellets were collected by a mesh collector placed in the outlet water and feed intake calculated by subtracting the number of uneaten from supplied pellets (Helland et al., 1996). Feed intake was monitored in this way every day throughout the experiment. Fish in each tank were anaesthetized, counted and bulk-weighed every three weeks. Fish were not fed on the day of weighing to avoid the inclusion of ingested feed in the weight measurement.

Feed efficiency ratio (FER) was calculated as:

$$\text{FER (g g}^{-1}\text{ DM)} = \text{total weight gain (g) / total feed intake (g DM)} \qquad [2.1]$$

Condition factor (K) was calculated as:

$$K = (W / L^3) \times 100 \qquad [2.2]$$

Where L is fork length (cm).

At the end of experiment, the fish were fasted for 24 h and the wet weight and fork length measured for individual fish. Five fish from each tank were killed and frozen at –20 °C and then pooled by tank for analysis of final whole-body chemical composition.

2.3.3. Apparent digestibility

Apparent digestibility (AD) was measured over the last week of the growth experiment. Yttrium oxide (1 g kg^{-1} diet) was added to the diets as an inert marker (Sugiura et al., 1998). The fish were fed the diets containing yttrium oxide for the last 20 days of the experiment and on days 15, 16, 17, and 18 of this period, faecal samples were collected using Guelph-type settlement collectors (Carter and Hauler, 2000). One hour after feeding at 1600 h, the collectors were washed of uneaten food and faeces collected from that time until 1 hour before next feeding time (0900 h). Faeces were frozen and then freeze-dried and used for the analysis of yttrium oxide and nutrients (see below). The faecal samples from the four collection days were pooled in equal weights for each tank and frozen at –20 °C until analysis. AD was calculated according to Maynard and Loosli (1969):

$$AD\ (\%) = 100 - [100\ (\%\ I\ diet / \%\ I\ faeces) \times (\%\ N\ faeces / \%N\ diet)] \qquad [2.3]$$

Where I is the inert marker and N is the nutrient.

2.3.4. Trypsin activity

At the end of the experiment, five fish per tank were killed to measure trypsin activity (24 h after feeding). The fish were dissected and the whole pyloric caeca removed. The fat around the pyloric caeca was removed and the samples were weighed, labeled, wrapped in aluminium foil, frozen in liquid nitrogen and then stored at –80 °C. Trypsin activity was measured as previously described (Preiser et al., 1975; Pringle et al., 1992). Samples were taken from the same part of the caeca (anterior), homogenized in Tris-HCl buffer and then centrifuged to produce a crude enzyme extract. Trypsin activity in the enzyme extract was measured using the synthetic, chromogenic trypsin–specific substrate, *α-N-* benzoyl-D,L-arginine-*p*-nitroanilide (BAPNA) in combination with the Bratton-Marshall (BM) reaction (Bratton and Marshall, 1939). The *p*-nitroaniline (pNA) liberated by the reaction of trypsin gives a purple colour in the presence of *N*-1-naphthylethylenediamine and trypsin activity (μmol pNA min^{-1}) was expressed as the total activity per gram of caecal tissue (μmol pNA min^{-1} g^{-1}).

2.3.5. Chemical analysis

Chemical analysis of diets, fish whole-body and faeces were performed according to standard methods: dry matter (freeze dry to constant weight); ash (AOAC, 1995); crude fat (Bligh and Dyer, 1959); protein (Kjeldahl using a selenium catalyst [N × 6.25]) and energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid). For analysis of the yttrium marker in diets and faeces, 5 ml of HCl: HNO_3 2: 1 (V V^{-1}) was added to 250 mg samples in digestion tubes and boiled (Vandecasteele and Block, 1993). After cooling, the samples were diluted to 25 ml with distilled water and yttrium and phosphorus content of diets and faeces measured by inductively coupled plasma optical emission spectrometry (Thermo Jarrell-Ash IRIS Axial ICP-OES) at the central science laboratory (University of Tasmania, Hobart, Tasmania, Australia).

2.3.6. Statistical analysis

Mean values are reported ± Standard Error of the Mean (S.E.M). After confirming the normality and homogeneity of variance, data were analyzed by two-way ANOVA using phytase and phytic acid concentrations as the two factors (SPSS, version 10.0). Where two-way ANOVA showed a significant interaction between the two factors, one-way ANOVA was used. Multiple comparison was by Tukey. Differences were considered significant at $P < 0.05$.

Table 2.1. Ingredient and chemical composition of experimental diets

Ingredient composition (g kg^{-1})	Diets			
	C	PS	PA	PAPS
Casein	272	272	272	272
Fish meal	200	200	200	200
Blood meal	110	110	110	110
Pre-gelatinized starch	150	150	150	150
Fish oil	165	165	165	165
Alpha cellulose	50	50	50	50
Bentonite	32	31.8	22	21.8
Carboxymethyl cellulose	10	10	10	10
Mineral mix[1]	3	3	3	3
Vitamin mix[2]	3	3	3	3
Choline chloride	1	1	1	1
Stay C[3]	3	3	3	3
Yttrium oxide	1	1	1	1
Sodium phytate [4]	-	-	10	10
Phytase	-	0.2	-	0.2
Chemical composition (g kg^{-1} DM)				
Dry matter (g kg^{-1})	955	958	955	951
Crude protein	480	484	482	480
Crude fat	216	213	211	217
Ash	73	73	71	74
Gross energy (MJ kg^{-1} DM)	22.42	22.67	22.50	22.73
Total Phosphorus (g kg^{-1} DM)	7.8	7.8	9.1	9.9

[1]Supplied (mg kg^{-1} diet): 70, $CuSO_4$ $5H_2O$; 1089.3, $FeSO_4$ $7H_2O$; 184.5, $MnSO_4$ H_2O; 1.98, Na_2SeO_3; 395.82, $ZnSO_4$ $7H_2O$; 4.32, KI; 28.62, Co SO_4 $7H_2O$.

[2]Supplied (mg kg^{-1} diet): 15.0, Vitamin A acetate; 18.0, Vitamin D_3 powder; 300, Rovimix E50; 6.0, Menadione sodium bisulphate; 12.0, Riboflavin; 65.22, Calcium D-pantothenate; 30.0, Nicotinic acid; 0.03, Vitamin B12; 0.45, d-Biotin; 3.00, Folic acid; 3.37, Thiamin HCl; 10.98, Pyridoxine HCl; 900, Myo-inositol.

[3] L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia Ltd, Sydney, Australia).

[4] Contains 8.2 g kg^{-1} phytic acid

2.4. Results

2.4.1. Growth performance

No mortality occurred over the 12-week experiment. There were no significant differences in overall feed intake or between morning and afternoon feed intake (Table 2.2). Phytase supplementation of diets enhanced salmon growth during the trial as shown by both final weight and weight gain (Table 2.2). In contrast, phytate had no effect on growth (Table 2.2). The significantly higher FER showed that fish fed diet with the combination of phytase and phytate utilized the diet more efficiently than the others (Table 2.2). There was a slight decline in feed intake (% BW day^{-1}) with time but there were no significant differences between feed intake of fish over the different periods of trial (Fig. 2.1). Growth was linear for all diets over the period of the experiment (Fig. 2.2). Neither phytase nor phytate had a significant effect on final carcass dry matter or crude protein (Table 2.3). Both phytase and phytate had independent effects on carcass crude lipid and phytate significantly increased the carcass ash content (Table 2.3).

2.4.2. Nutrient digestibility and trypsin activity

There were no significant differences in energy digestibility but there was an interaction effect on apparent digestibility for protein and fish fed diet PA had a significantly lower value than for the other diets (Table 2.4). Phytate but not phytase had a significant effect on phosphorus digestibility (Table 2.4). There were no significant differences in trypsin activity among the fish fed different diets and the overall mean of trypsin activity was 0.33μmol pNA min^{-1} (Table 2.4).

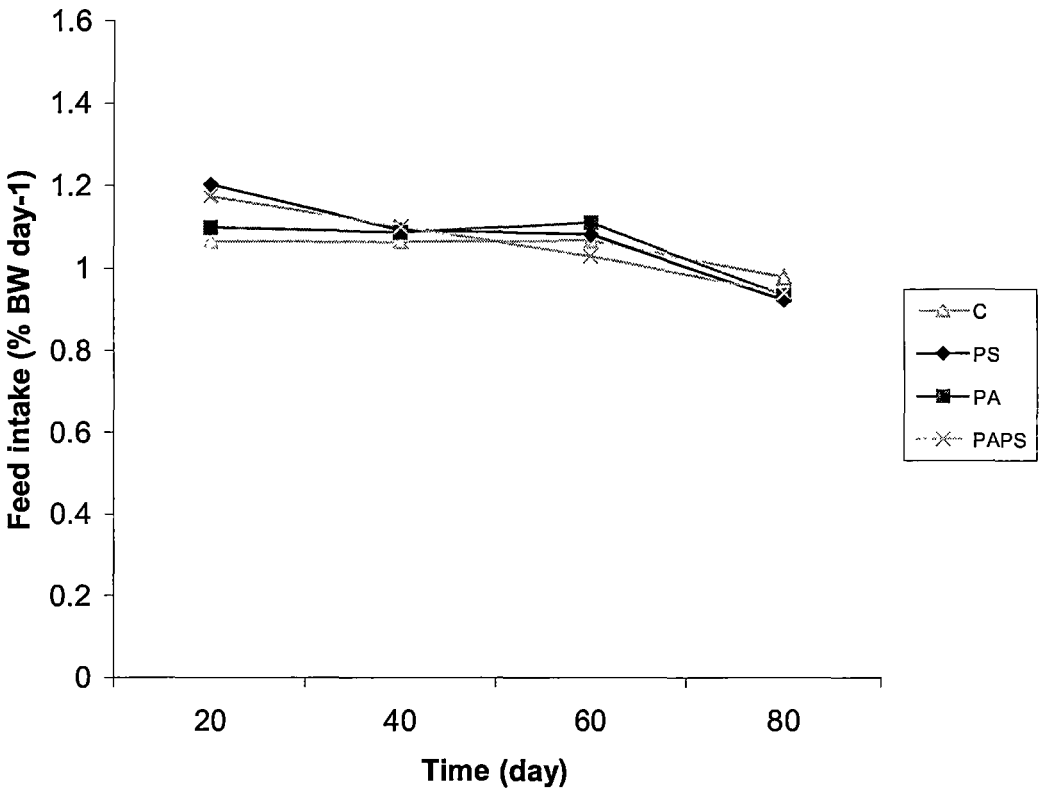

Figure 2.1. The change in mean feed intake of Atlantic salmon fed different diets (n=3). (C, no phytase or sodium phytate; PS, 2000 U kg^{-1} phytase; PA, 10 g kg^{-1} sodium phytate; PAPS, 2000 U kg^{-1} phytase and 10 g kg^{-1} sodium phytate)

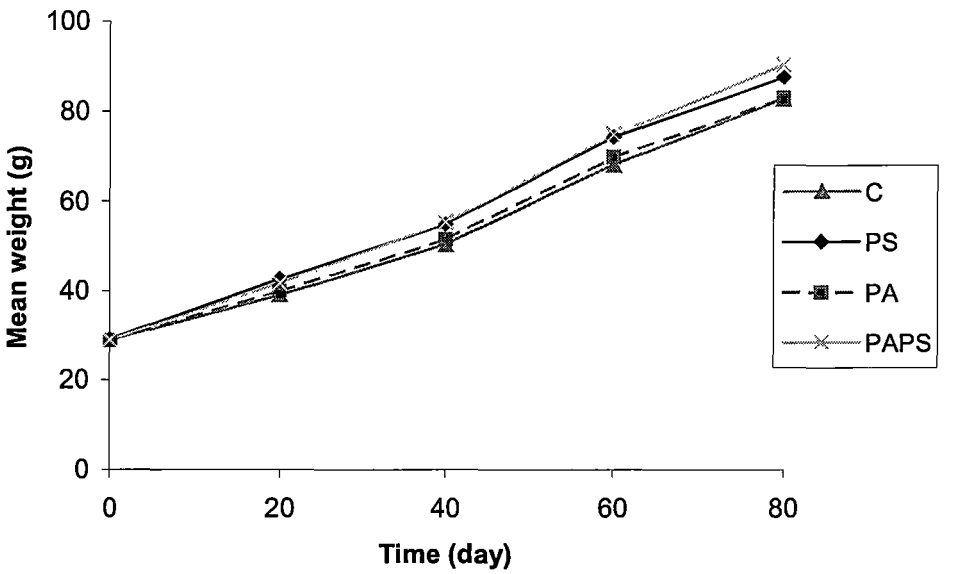

Figure 2.2. The change in mean wet weight over time of Atlantic salmon fed different diets (n=3). (C, no phytase or sodium phytate; PS, 2000 U kg^{-1} phytase; PA, 10 g kg^{-1} sodium phytate; PAPS, 2000 U kg^{-1} phytase and 10 g kg^{-1} sodium phytate)

Table 2.2. The performance of Atlantic salmon fed different diets with phytate and phytase inclusions (mean ± S.E.M, n=3)

Parameter	Unit	Diets				*P*		
		C	PS	PA	PAPS	*Phytase*	*Phytate*	*Phytase×Phytate*
Initial weight	(g)	28.9	29.1	28.8	28.9	*ns*	*ns*	*ns*
		0.2	0.2	0.2	0.1			
Final weight	(g)	82.6	88.4	82.8	90.3	*0.036*	*ns*	*ns*
		2.2	0.7	3.4	2.5			
Weight gain	(g)	53.8	58.3	54.0	61.4	*0.036*	*ns*	*ns*
		2.0	0.5	3.4	2.6			
Total feed intake	(kg DM)	1.12	1.20	1.11	1.22	*ns*	*ns*	*ns*
		0.04	0.01	0.05	0.05			
Total feed intake	(kg DM)	0.57	0.62	0.57	0.61	*ns*	*ns*	*ns*
(morning)		0.013	0.007	0.029	0.027			
Total feed intake	(kg DM)	0.55	0.58	0.54	0.61	*ns*	*ns*	*ns*
(Afternoon)		0.007	0.010	0.019	0.019			
FER	(g g^{-1} DM)	1.22^{a}	1.21^{a}	1.20^{a}	1.28^{b}	*0.036*	*0.013*	*0.005*
		0.01	0.01	0.02	0.01			
PPV	(%)	43.22	43.07	42.05	45.25	*ns*	*ns*	*ns*
		0.89	2.85	1.40	1.59			
K	-	1.35	1.35	1.35	1.36	*ns*	*ns*	*ns*
		0.006	0.011	0.013	0.018			
Survival	(%)	100	100	100	100			

(C, no phytase or sodium phytate, PS, 2000 U kg^{-1} phytase; PA, 10 g kg^{-1} sodium phytate; PAPS, 2000 U kg^{-1} phytase and 10 g kg^{-1} sodium phytate)

FER (g g^{-1} DM) = total wet weight gain (g) / total feed intake (g DM)

PPV (%) = 100 x (total protein gain (g) / total protein intake (g))

K = wet weight / fork $length^{3}$

Means with same letter are not significantly different (Tukey multiple comparison)

Table 2.3. Chemical composition (% wet weight) of Atlantic salmon fed different diets with phytate and phytase inclusions (mean ± S.E.M, n=3, five fish pooled per replicate)

Parameter	Diet				*P*		
	C	PS	PA	PAPS	*Phytase*	*Phytate*	*Phytase×Phytate*
Dry matter	32.64 0.36	32.72 0.14	31.85 0.33	32.57 0.13	*ns*	*ns*	*ns*
Crude protein	17.36 0.11	17.14 0.14	16.98 0.32	17.13 0.09	*ns*	*ns*	*ns*
Total lipid	12.62 0.20	12.89 0.13	11.85 0.21	12.42 0.06	*0.033*	*0.005*	*ns*
Ash	1.87 0.03	1.85 0.03	2.00 0.02	2.04 0.03	*ns*	*0.001*	*ns*

Initial group (mean ± S.E.M; n = 12): 29.48 ± 0.27% DM; 15.60 ± 0.30% crude protein; 12.67 ± 0.12% total lipid; 2.07 ± 0.03% ash.

(C, no phytase or sodium phytate; PS, 2000 U kg^{-1} phytase; PA, 10 g kg^{-1} sodium phytate; PAPS, 2000 U kg^{-1} phytase and 10 g kg^{-1} sodium phytate)

Table 2.4. Apparent digestibility (AD) for crude protein (N), energy (kJ) and phosphorus (P) (mean ± S.E.M, n=3) and trypsin activity in pyloric caeca in Atlantic salmon fed different diets with phytate and phytase inclusions (mean ± S.E.M, n=3, five fish pooled per replicate)

Parameter	Unit	Diet				P		
		C	PS	PA	PAPS	*Phytase*	*Phytate*	*Phytase×Phytate*
AD_N	(%)	95.98[a] 0.03	96.36[a] 0.16	94.98[b] 0.12	96.01[a] 0.08	*0.000*	*0.000*	*0.023*
AD_{KJ}	(%)	91.35 0.06	91.30 0.39	90.73 0.22	91.61 0.18	*ns*	*ns*	*ns*
AD_P	(%)	74.34 0.75	74.71 0.46	68.83 1.59	74.04 1.69	*ns*	*0.041*	*ns*
Trypsin activity	$\mu mol\ g^{-1}\ min^{-1}$	0.304 0.032	0.331 0.043	0.318 0.040	0.350 0.032	*ns*	*ns*	*ns*

(C, no phytase or sodium phytate; PS, 2000 U kg^{-1} phytase; PA, 10 g kg^{-1} sodium phytate; PAPS, 2000 U kg^{-1} phytase and 10 g kg^{-1} sodium phytate)

Means with same letter are not significantly different (Tukey multiple comparison)

2.5. Discussion

2.5.1. Feed intake and growth

In the present experiment the effect of phytic acid and the effect of phytase, independent of dietary phosphorus level, on feed intake, growth performance and digestibility were investigated. Phytase *per se* enhanced fish growth without being influenced by the presence of phytate or without having an effect on feed intake. Phytic acid reduced protein digestibility without any effect on feed intake and growth. Interestingly, FER was significantly higher in fish fed diet containing both phytase and sodium phytate. There was a slight decline in feed intake with time, as would be expected as a result of increased fish size with time.

The effect of phytic acid on growth depends primarily on the amount in the diet and on the presence or absence of a distinct stomach. Inclusion of 0.5 or 1% phytic acid in purified diets for the agastric common carp (*Cyprinus carpio*) caused a significant reduction in growth and feed efficiency (Hossain and Jauncey, 1993). Catfish (*Ictalurus punctatus*) growth was reduced only when fed diets containing 2.2% or more phytic acid (Satoh et al., 1989) but diets containing up to 1.5% phytic acid had no effect (Gatlin and Phillips, 1989; Satoh et al., 1989). In comparison with low (0.16%) or medium (0.65%) levels of phytate juvenile Chinook salmon (*Oncorhynchus tshawytscha*) showed reduced weight gain when fed at 2.6% phytate (Richardson et al., 1985). In the present study, 0.8% phytic acid was included in the diet and appeared to be below the level that would depress growth in Atlantic salmon.

There is some evidence to show phytase increases feed intake of fish fed diets with phosphorus at sub-optimal or requirement levels. Rodehutscord and Pfeffer (1995) recorded increased feed intake in rainbow trout fed a diet containing phytase at a sub-optimal phosphorus level. They concluded that increased availability of phosphorus due to phytase was the reason for increased feed intake

and resulted in higher growth performance. Higher feed intake was observed in catfish fed diets containing at least 250 U phytase kg^{-1} (Li and Robinson, 1997) and at least 500 U phytase kg^{-1} (Jackson et al., 1996). Hauler and Carter (1997) showed phytase in a plant protein based diet with a slightly deficient level of available phosphorus (5 g kg^{-1}) increased feed intake in Atlantic salmon. In the present study, the level of phosphorus in the diet was purposefully set to match salmonid requirements and there was no difference in feed intake due to the phytase.

At least part of the improved weight gain observed in some studies can be attributed to increased feed intake in fish. However phytase supplementation led to an increase in growth rate in trout (Cain and Garling, 1995) and carp (Schäfer et al., 1995) fed restricted rations. In contrast, phytase addition to reduce the phytic acid content of the diets had no significant effect on growth rate and feed efficiency of seabass (*Dicentrarchus labrax*) (Oliva-Teles et al., 1998) and rainbow trout (Vielma et al., 2000). Use of dephytinized rapeseed protein concentrate in rainbow trout diet had no significant effect on feed intake, growth rate, feed and protein utilisation, health or survival (Teskeredžić et al., 1995). They assumed that the intrinsic phytic acid in rapeseed protein concentrate (RPC) has a minor (negligible) effect on protein availability in salmonids, because the protein digestibility coefficient for RPC in trout and salmon is high (89 – 98%). In the present study, fish fed diets supplemented with phytase regardless of phytic acid level had better growth and fish fed diet supplemented with phytase and sodium phytate had better feed efficiency ratio. FER was better in fish fed phytase supplemented plant meal based diet (Li and Robinson, 1997).

Phytic acid affected the whole-body chemical composition. Fish fed diets containing phytic acid in comparison with fish fed phytase had lower lipid and higher ash contents, trends that are in agreement with result of Usmani and Jafri (2002). They observed lower lipid content in fish fed more than 0.5% phytic acid and higher ash content in fish fed more than 1.5% phytic acid. Also, Chinook

salmon fed high dietary phytic acid had higher moisture, protein and ash and lower lipid content in the body (Richardson et al., 1985).

2.5.2. Phosphorus balance

Voluntary feed intake increases when dietary phosphorus increases from severely sub-optimal to optimal levels (Rodehutscord, 1996; Pimentel-Rodrigues and Oliva-Teles, 2001). When fish are fed diets deficient in a nutrient, two opposite responses are possible: when the deficiency is mild, increasing feed intake will compensate by increasing intake of the nutrient but when the deficiency is severe, an inhibitory response is observed (de la Higuera, 2001). Decreased feed intake in fish fed severely deficient diets may prevent or delay the onset of metabolic disorder (de la Higuera, 2001). Rodehutscord and Pfeffer (1995) concluded that the inclusion of phytase in diets with sub-optimal phosphorus led to increased feed intake in rainbow trout. Total phosphorus was 4.8 g kg^{-1} but the level of available phosphorus in the control diet was 1.2 g kg^{-1}, phytase increased available phosphorus to 2.74 g kg^{-1} (recalculated from Rodehutscord and Pfeffer, 1995). According to Rodehutscord (1996), 2.5 and 4.0 g kg^{-1} dietary phosphorus stimulated 90 and 98% of the maximum feed intake, respectively, in rainbow trout. Thus, the increased available phosphorus due to phytase resulted in a large increase in feed intake and therefore growth.

However, lowered growth performance of fish fed diets with phytate may be attributed to a variety of other factors such as reductions in mineral bioavailability, protein digestibility, or nutrient absorption due to damage caused by phytate in pyloric caeca region of intestine (Francis et al., 2001) or cumulative effect of some or all of these factors. For example, reduced growth was observed due to protein digestibility reduction in rainbow trout (Spinelli et al., 1983) and partly due to reduction of Zn bioavailability in Chinook salmon (Richardson et al., 1985).

2.5.3. Digestibility

The effects of phytase on digestibility may depend on a variety of dietary factors such as source and concentration of phytate in the diet, source and concentration of protein in the diet, digestibility of protein source, mineral levels, calcium and phosphorus levels and phytase inclusion rate (Selle et al., 2000; Sugiura et al., 2001). Feedstuffs that are more poorly digested are more responsive to phytase (Selle et al., 2000). Furthermore, methods used for adding phytase during diet manufacture, such as ingredient pre-treatment or direct supplementation of the diet with phytase may also have effects.

In the present study, casein, blood meal and fish meal were used as protein sources and have high protein digestibility. The capacity of phytate to complex with protein sources varies according to the protein (Teskeredžić et al., 1995; Ravindran et al., 1999). Phytate probably complexed with casein (Spinelli et al., 1983) in the present study and the digestibility values showed protein digestibility was significantly lower for the diet containing phytate (but no phytase).

Inclusion of 1% sodium phytate reduced protein digestibility in salmon and was in agreement with previous studies (Spinelli et al., 1983). Phytic acid and protein bind at neutral to acidic pH (Cosgrove, 1966, Anderson, 1985) with the maximum interaction between phytate and protein at low pH (Okubo et al., 1976) such as the acidic environment of the salmon digestive system. Wise (1983) suggested that at low pH, phytate is mainly bound to proteins rather than minerals. Phytase pre-treatment of soy concentrate in an Atlantic salmon diet improved protein digestibility (Storebakken et al., 1998). Improvement in protein digestibility in pre-treated soybean meal with phytase and phytase-added soybean meal based diet was observed (Sugiura et al., 2001). Papatryphon and Soares (2001) showed that protein digestibility of some plant ingredients in striped bass (*Morone saxatilis*) was not affected by phytase supplementation. In the present study, the negative effect of phytic acid on protein digestibility was counter-acted by the addition of 2000 U kg^{-1} phytase. Phytase inclusion in the plant protein based diets

increases phosphorus digestibility in fish (Storebakken et al., 1998; Forster et al., 1999). In the present study, phosphorus digestibility in fish fed diets containing phytic acid was significantly lower.

2.5.4. Trypsin activity

In the current experiment the activity of trypsin was between 0.304 and 0.350 μmol pNA g^{-1} min^{-1}, and higher compared to other studies on salmon. Trypsin activity of feeding salmon ranged between 0.075 and 0.135 μmol pNA g^{-1} min^{-1} (Pringle et al., 1992; Carter et al., 1994). The final weight of salmon in the present study was between 82 and 90 g, but the weights in the previous studies were more than 200 g. The weight of fish is an important factor that influences trypsin activity in salmon, trypsin activity in 100 g salmon was five times more than for 400 g salmon (Torrissen et al., 1994).

There is some evidence that phytic acid has a negative effect on protease activity. *In vitro* trypsin activity decreased by up to 46% in the presence of phytate (Singh and Krikorian, 1982). Phytase increased the ileal nitrogen digestibility by 3.7% and trypsin activity by 10.9% in an *in vivo* study in sows (Morz et al., 1995). In the present study, there were no significant differences in trypsin activity in fish fed different diets. However, the majority of studies on terrestrial farm animals failed to show that phytic acid decreases protease activity (Reddy et al., 1988; Knuckles et al., 1989; Vaintraub and Bulmaga, 1991). As yet, the possibility that phytate inhibits trypsin and other digestive enzymes is an important but unresolved issue (Selle et al., 2000).

2.6. Conclusion

At 1% dietary inclusion, phytate had no effect on feed intake, growth or trypsin activity (*in vivo*) but reduced protein digestibility in Atlantic salmon. The use of phytase *per se* had no effect on feed intake but enhanced growth and neutralized negative effect of phytic acid on protein digestibility.

2.7. Acknowledgements

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CHAPTER 3

DIETARY PHYTASE SUPPLEMENTATION AND THE UTILISATION OF PHOSPHORUS BY ATLANTIC SALMON (*SALMO SALAR*, L.) FED A CANOLA MEAL BASED DIET

Accepted as:

Sajjadi, M., Carter, C.G., Dietary phytase supplementation and the utilisation of phosphorus by Atlantic salmon (*Salmo salar*, L.) fed a canola meal based diet. Aquaculture (*accepted*).

3.1. Abstract

The effect of phytase supplementation to a canola meal based diet on phosphorus digestibility in Atlantic salmon was studied in a 2-by-2 factorial design. Diets were prepared without phytase or inorganic phosphorus supplementation, with phytase, with supplemental inorganic phosphorus and with both phytase and supplemental inorganic phosphorus. Available phosphorus was set below requirement and total phosphorus set to meet requirements for salmonids. After 12 weeks, there were no significant differences in survival, feed intake and weight gain between diets. There was an interaction effect on bone ash, bone phosphorus and whole-body phosphorus so that adding phytase, phosphorus or both resulted in significantly ($P < 0.05$) higher values for these parameters. An interaction effect was also observed for phosphorus digestibility, phosphorus retention efficiency and phosphorus load. Phosphorus digestibility and retention efficiency were significantly ($P < 0.05$) higher and phosphorus load significantly ($P < 0.05$) lower in fish fed the phytase supplemented diet compared with diets containing supplemental inorganic phosphorus. In conclusion, phytase increased phosphorus availability, therefore reducing the need to add inorganic phosphorus and reducing phosphorus waste from plant meal based diets for Atlantic salmon.

Keywords: Atlantic salmon, Bone phosphorus, Canola meal, Inorganic phosphorus, Phosphorus digestibility, Phytase

3.2. Introduction

Canola meal is a suitable protein replacement for fish meal because it has a relatively high protein content (38%) and the protein is of high digestibility for salmonids (Cheng and Hardy, 2002). The cost of canola meal is generally lower than both fish meal and soybean meal, the major protein sources currently used in aquafeeds (Higgs et al., 1995). However, canola meal inclusion in aquafeeds is mainly limited due to the presence of glucosinolate and phytic acid (Higgs et al., 1995). Canola meal contains up to 3.7% phytic acid, representing approximately two thirds of total phosphorus (Cheryan, 1980; McCurdy and March, 1992). The digestibility and availability of phytate phosphorus for fish including salmonids, is very low (Ketola, 1975; Ogino et al., 1979; Sajjadi and Carter, 2004). Consequently, the available phosphorus of diets containing large amounts of plant protein may not meet the requirements of fish. Phytate phosphorus will be excreted via faeces and contribute to phosphorus pollution, it is either degraded by microorganisms and becomes available for algae or it can be used directly by some microalgae (Balazsi and Wikfors, 2000). In extreme situations, excreted phosphorus leads to eutrophication of the aquatic environment.

Canola meal was chosen as the main protein source in the present study, because in comparison with soybean [1.00-1.47% phytic acid (Lolas et al., 1976)] or soybean meal [1.40-1.60% phytic acid (Erdman, 1979)], it contains more phytic acid. The concentration of phytic acid in a protein concentrate is even higher than in the original plant meal. For example, phytic acid content of canola meal is between 3.1-3.7% (Cheryan, 1980; McCurdy and March, 1992) but between 5.3-7.5% in canola protein concentrate (Erdman, 1979). Results concerning the use of phytase to increase phosphorus availability from plant meals are inconsistent. It increased weight gain in rainbow trout *(Oncorhynchus mykiss)* (Rodehutscord and Pfeffer, 1995; Vielma et al., 1998) and channel catfish *(Ictalurus punctatus)* (Jackson et al., 1996; Li and Robinson, 1997) but had no effect in other studies (Lanari et al., 1998; Vielma et al., 2000; Yan et al., 2002). Several factors may

influence the efficacy of phytase including type and amount of phytase, method of inclusion, method of pre-treatment, the type of ingredient, diet formulation, fish species, and dietary concentrations of nutrients (especially protein and minerals).

In the previous experiment (Sajjadi and Carter, 2004), diets with adequate available phosphorus were used. In the present experiment, four canola meal based diets with either phytase or inorganic phosphorous, with both phytase and inorganic phosphorus or with neither were compared. Total phosphorus was set above salmonid requirement but available phosphorus was set below requirement (NRC, 1993). This was undertaken to assess the potential of phytase to enhance growth performance and phosphorus utilisation in Atlantic salmon fed a canola meal based diet. Furthermore, different criteria were measured to study the sensitivity of these criteria for assessing the effect of supplemental phytase on phosphorus utilisation.

3.3. Materials and methods

3.3.1. Experimental diets

The basal diet (diet C) was formulated to contain 470 g kg^{-1} diet crude protein, 21.5 MJ kg^{-1}gross energy (Table 3.1). All nutrient requirements of Atlantic salmon, except phosphorus, were according to values for salmonids (NRC, 1993). Total phosphorus concentration was set at 8.0 g kg^{-1} diet, with 4.7 g kg^{-1}available phosphorus (Table 3.2). Canola meal (38% protein, Skretting, Cambridge, Tasmania, Australia) was used in the diets at 350 g kg^{-1} inclusion, the maximum level of canola meal specified for rainbow trout feed (Higgs et al., 1995). Two factors were investigated in a factorial arrangement: Phytase level (0 and 2000 U kg^{-1}) [where 1 U is defined as the amount of phytase that liberates inorganic phosphorus from a 0.0015 M sodium phytate solution at a rate of 1 μmol min^{-1} at pH 5.5 and 37 °C] and diabasic sodium phosphate level (0 and 10 g kg^{-1}). Experimental diets were formulated: C, no phytase or diabasic sodium phosphate; CP, contained phytase (2000 U kg^{-1}); CS, contained diabasic sodium phosphate (10 g kg^{-1}); CSP, contained phytase (2000 U kg^{-1}) and diabasic sodium phosphate

(10 g kg^{-1}). Phytase (10000 U g^{-1}, Natuphos: BASF, Homebush, NSW, Australia) was dissolved in water and added to the diet mix. Dry ingredients were mixed in a Hobart mixer and then mixed with water for 10 min before being pelleted with a laboratory pelletizer (California Laboratory Pellet Mill) (pellet diameter: 4 mm) and dried at 30 °C for 24h. The diets were stored in a cold room (4 °C) until feeding.

3.3.2. Growth experiment

The experiment was performed at the School of Aquaculture, University of Tasmania. Atlantic salmon were obtained from Springfield Fish Farm (Scottsdale, Tasmania, Australia) and transferred to the Aquaculture Centre, School of Aquaculture. Fish were kept in 300-l circular tanks in a recirculation system where water quality was maintained through physical and biological filters (Bransden et al., 2001). Fish were maintained on a commercial feed (3mm Salmon, Skretting, Cambridge, Tasmania, Australia) for two weeks. Water replacement rate in the system was about 20% d^{-1} and water flow through the tanks was 8-10 l min^{-1}. Oxygen concentration was 90.0 ± 0.5% of saturation, average water temperature was 15.5 ± 0.8 °C and a natural photoperiod (approximately, 14L: 10D) was in effect over the 84-day experiment.

At the beginning of experiment, fish were anaesthetised in benzocaine (50 mg l^{-1}) and the wet weight and fork length of individual fish measured. One fish from each tank (n=12) was sacrificed (100 mg l^{-1} benzocaine) and used for the measurement of initial whole-body chemical composition. The remaining 20 fish (100.7 ± 0.96 g, mean ± S.E.M, n=240) were returned to each tank. Triplicate groups of fish were fed each experimental diet to satiation twice daily at 0900 and 1700 h, satiation was judged to have been reached when approximately 20 pellets were not eaten and lost via the outlet water. Uneaten pellets were collected by a mesh collector placed in the outlet water (Helland et al., 1996) and feed intake calculated by subtracting the number of uneaten from supplied pellets. Feed intake was monitored in this way every day throughout the experiment. Fish in each tank were anaesthetised, counted and bulk-weighed every three weeks. Fish were not

fed on the day of weighing to avoid the inclusion of ingested feed in the weight measurement. At the end of the 12-week experiment, the fish were fasted for 24 h and the wet weight and fork length measured for all individual fish. Five fish from each tank were killed and frozen at –20 °C and pooled by tank for final whole-body chemical composition. Another 5 fish per tank were killed and frozen at –20 °C for bone ash and phosphorus analyses.

Feed efficiency ratio (FER) was calculated as:

$$\text{FER (g g}^{-1}\text{ DM)} = \text{total weight gain (g) / total feed intake (g DM)} \quad [3.1]$$

Nutrient retention efficiency of phosphorus was calculated as:

$$NR_{\text{phosphorus}}\ (\%) = 100 \times ([FBW \times N_f] - [IBW \times N_i])/ (\text{feed intake} \times N_{diet}) \quad [3.2]$$

(Storebakken et al., 1998), where FBW is final body weight and IBW is initial body weight of fish, N is the concentration of nutrient (P) in the fish at the start (N_i) and end (N_f) of experiment. Phosphorus load was calculated as:

$$\text{Nutrient load (g P kg}^{-1}) = (\text{Nutrient fed (g)} - \text{Nutrient deposited (g)}) / \text{Weight gain (kg)} \quad [3.3]$$

(Vielma et al., 2002).

3.3.3. Apparent digestibility

Apparent digestibility (AD) was measured during the growth experiment. Yttrium oxide (1 g kg^{-1}) was added to the diets as an inert marker (Sugiura et al., 1998). The fish were fed the diets containing yttrium oxide for two weeks and then faecal samples were collected by Guelph-type settlement collectors (Carter and Hauler, 2000) for three days. One hour after feeding at 1600 h, the collectors were washed of uneaten food and faeces collected from that time until 1 h before next feeding time (0900h). Faeces were frozen, freeze-dried and used in the analysis of yttrium

oxide and nutrients (see below). The faecal samples from the three collection days were pooled in equal weights for each tank and frozen at –20 °C until analysis. AD was calculated according to:

$$AD\ (\%) = 100 - [100\ (\%\ I\ diet\ /\ \%\ I\ faeces) \times (\%\ N\ faeces\ /\ \%N\ diet)] \quad [3.4]$$

(Maynard and Loosli, 1969), where I is the inert marker and N is the nutrient.

3.3.4. Chemical analysis

Chemical analysis of diets, fish whole-body and faeces were performed according to standard methods: dry matter (freeze dry to constant weight); ash (AOAC, 1995); crude fat (Bligh and Dyer, 1959); crude protein (Kjeldahl using a selenium catalyst [N × 6.25]) and energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid). For analysis of the yttrium marker in diets and faeces, 5 ml HNO_3 were added to 250 mg samples in digestion tubes and boiled (Vandecasteele and Block, 1993). After cooling, the samples were diluted to 25 ml with distilled water and yttrium content of diets and faeces measured by inductively coupled plasma optical emission spectrophotometry (Thermo Jarrell-Ash IRIS Axial ICP-OES) at the Central Science Laboratory (University of Tasmania, Hobart, Tasmania, Australia) (Sajjadi and Carter, 2004).

To determine bone ash and phosphorus, previously frozen fish were cooked for about 6 min in a microwave until flesh and bone were easily separated. Soft tissues were carefully removed from the vertebrae. Isolated vertebrae were rinsed with distilled water and dried in an oven at 105 °C for 24 h. After drying, they were ground with a pestle and mortar and then defatted with solvent (Chloroform, Methanol 1:1), dried and ashed in a muffle furnace (Vielma and Lall, 1998 a,b). The ash was weighed and subsequently analysed for phosphorus by the molybdovanadate method (AOAC, 1995). Phosphorus in the diet, initial and final whole-body and faeces was also analysed by the molybdovanadate method. Phytic

acid was determined by David Harris (Western Australia Chemistry Centre, Perth) according to a number of published methods (Harland and Oberleas, 1977; Ellis and Morris, 1983; Harland and Oberleas, 1986). Phytate phosphorus was analysed on the ICP-OES after extraction from the diets and ingredient according to Latta and Eskin (1980).

3.3.5. Statistical analysis

Mean values are reported ± Standard Error of the Mean (S.E.M). After confirming normality and homogeneity of variance, data were analysed by two-way ANOVA using phytase and inorganic phosphorus concentrations as the two factors (SPSS, version 11.5). Where two-way ANOVA showed a significant interaction between two factors one-way ANOVA was used to identify significantly different means using Tukey multiple comparison. Differences were considered significant at $P < 0.05$.

Table 3.1. Ingredient and chemical composition of experimental diets

Ingredient composition (g kg^{-1})	Diet C	Diet CP	Diet CS	Diet CSP
Canola meal	350	350	350	350
Casein	200	200	200	200
Blood meal	100	100	100	100
Fish meal	85	85	85	85
Fish oil	142	142	142	142
Alpha cellulose	50	50	50	50
Bentonite	50	48.8	39.8	39.6
Carboxymethyl cellulose	9	9	9	9
Mineral mix[1]	5	5	5	5
Vitamin mix[2]	3	3	3	3
Choline chloride	2	2	2	2
Stay C[3]	3	3	3	3
Yttrium oxide	1	1	1	1
Dibasic Sodium Phosphate	-	-	10.2	10.2
Phytase[4]	-	0.2	-	0.2
Chemical composition (g kg^{-1} DM)				
Dry matter (g kg^{-1})	956	949	944	951
Crude protein	471	477	484	464
Crude fat	167	164	168	164
Gross energy (MJ kg^{-1} DM)	21.65	21.74	21.16	21.71
Ash	92.1	93.4	97.1	96.7

[1]Supplied (mg kg^{-1} diet): 116.67, $CuSO_4 5H_2O$; 1815.50, $FeSO_4 7H_2O$; 307.50, $MnSO_4 H_2O$; 3.30, $Na_2 SeO_3$; 659.70, $ZnSO_4 7H_2O$; 7.20, KI; 47.70, Co SO_4 $7H_2O$.

[2] Supplied (mg kg^{-1} diet): 15.00, Vitamin A acetate; 18.00, Vitamin D3 powder; 300.00, Rovimix E50; 6.00, Menadione sodium bisulphate; 12.00, Riboflavin; 65.22, Calcium D-pantothenate; 30.00, Nicotinic acid; 0.03, Vitamin B12; 0.45, d-Biotin; 3.00, Folic acid; 3.37, Thiamin HCl; 10.98, Pyridoxine HCl; 900.00, Myo-inositol.

[3] L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia Ltd, Sydney, Australia).

[4] BASF, Homebush, NSW, Australia

3.4. Results

3.4.1. Diet analysis

Comparing the phytate phosphorus in the diets confirmed that despite sub-optimal conditions for phytase activity, phytase was active during the feed making period because after making the diets, the level of phytate phosphorus in phytase-supplemented diets CP and CSP decreased by 13.2 and 10.9%, respectively (Table 3.2).

3.4.2. Growth performance

There was no mortality nor were there significant differences in feed intake, weight gain or feed efficiency ratio (Table 3.3). Total feed intake of fish was between 3.88 kg and 4.39 kg (Table 3.3) and weight gain was between 192.3 and 220.3 g $fish^{-1}$.

3.4.3. Composition of fish

There were no significant differences in whole-body dry matter, crude protein, total lipid or ash (Table 3.4). There was a dietary interaction effect on bone ash, bone phosphorus and whole-body phosphorus and the fish fed diets with phytase or inorganic phosphorus or both had significantly higher bone ash, bone phosphorus and whole-body phosphorus content (Table 3.4). Bone ash in fish fed the basal diet was 46.5% and bone phosphorus was 9.8%, but in fish fed diets with phytase or inorganic phosphorus or both, bone ash was between 50.2-50.5% and bone phosphorus was 10.6-10.7%.

3.4.4. Digestibility

There were no significant differences between protein and energy digestibility. There was a dietary interaction effect on phosphorus digestibility, it was

significantly higher for CP, intermediate for CS and CSP and significantly lower for C (Table 3.5).

3.4.5. Nutrient retention and load

There was a dietary interaction effect on phosphorus retention efficiency, which increased significantly ($P < 0.05$) when phytase was used in the basal diet but not in the inorganic phosphorus supplemented diet. There were no significant differences in phosphorus retention efficiency between diets CS and CSP (Fig. 3.1). There was also an interaction for phosphorus load, which was lower in fish fed diet CP (Fig. 3.2).

Table 3.2
Phytic acid, total P, phytate P and available P content of canola meal and experimental diets (mean; n=2) (C, no phytase or sodium phosphate; CP, 2000 U kg^{-1} phytase; CS, 10 g kg^{-1} sodium phosphate; CSP, 2000 U kg^{-1} phytase & 10 g kg^{-1} sodium phosphate)

Ingredient / Diet	pH during feed making	Phytic acid (%)	Total P	(g kg^{-1})	Phytate P	(g kg^{-1})	Available P	(g kg^{-1}) [1]
			As fed [2]	Dry	As fed	Dry	As fed	Dry
Canola meal	-	2.950	-	-	-	9.65	-	-
C	7.0	1.025	7.70	8.07	3.25	3.40	4.46	4.67
CP	7.0	1.030	7.80	8.20	2.79	2.95	4.99	5.25
CS	7.0	1.057	10.80	11.50	3.20	3.40	7.60	8.10
CSP	7.0	1.065	10.20	10.70	2.88	3.03	6.88	7.20

[1] Calculated as: Available P= Total P – Phytate P
[2] Calculated as: Nutrient (as fed) = Nutrient (in DM) × (%DM)

Table 3.3. The growth performance of Atlantic salmon fed experimental diets (C, no phytase or sodium phosphate; CP, 2000 U kg^{-1} phytase; CS, 10 g kg^{-1} sodium phosphate; CSP, 2000 U kg^{-1} phytase & 10 g kg^{-1} sodium phosphate) (mean ± S.E.M, n=3)

Parameter	Unit	Diets				*P*		
		C	CP	CS	CSP	*Phytase*	*Phosphorus*	*Interaction*
Initial weight	(g)	100.4	100.3	100.5	101.4	*ns*	*ns*	*ns*
		0.6	0.2	0.3	0.4			
Final weight	(g)	292.7	307.2	320.8	313.0	*ns*	*ns*	*ns*
		8.6	3.3	10.8	5.7			
Weight gain	(g)	192.3	206.9	220.3	211.6	*ns*	*ns*	*ns*
		8.1	3.3	10.7	6.1			
Total feed intake	(kg DM)	3.88	4.15	4.39	4.10	*ns*	*ns*	*ns*
		0.09	0.07	0.18	0.15			
FER	(g g^{-1} DM)	0.99	0.99	1.00	1.00	*ns*	*ns*	*ns*
		0.01	0.01	0.01	0.04			
Survival	(%)	100	100	100	100	*ns*	*ns*	*ns*

Table 3.4. Chemical composition (% wet weight), whole-body phosphorus content, bone ash and bone phosphorus of Atlantic salmon fed experimental diets (C, no phytase or sodium phosphate; CP, 2000 U kg^{-1} phytase; CS, 10 g kg^{-1} sodium phosphate; CSP, 2000 U kg^{-1} phytase & 10 g kg^{-1} sodium phosphate) (mean ± S.E.M, n=3, five fish per replicate)

Parameter	Unit	Diet				P		
		C	CP	CS	CSP	*Phytase*	*Phosphorus*	*Interaction*
Dry matter	(%)	31.74	31.83	31.67	31.87	*ns*	*ns*	*ns*
		0.52	0.43	0.27	0.25			
Crude protein	(%)	18.12	18.12	17.73	17.90	*ns*	*ns*	*ns*
		0.19	0.27	0.30	0.14			
Total lipid	(%)	10.39	10.58	10.89	10.66	*ns*	*ns*	*ns*
		0.12	0.23	0.21	0.16			
Ash	(%)	1.91	2.01	2.00	2.00	*ns*	*ns*	*ns*
		0.02	0.01	0.04	0.01			
Whole-body P	(%)	1.21 [b]	1.32 [a]	1.32 [a]	1.33 [a]	*0.019*	*0.031*	*0.036*
		0.02	0.02	0.02	0.02			
Bone ash	(%)	46.53 [b]	50.52 [a]	50.38 [a]	50.15 [a]	*0.024*	*0.032*	*0.014*
		0.75	0.25	0.77	0.77			
Bone P	(%)	9.78 [b]	10.71 [a]	10.59 [a]	10.62 [a]	*0.01*	*0.036*	*0.015*
		0.23	0.11	0.08	0.10			

Means with same letter are not significantly different (Tukey multiple comparison)

Initial group (mean ± S.E.M; $n = 12$): 29.17 ± 0.37% DM; 15.42 ± 0.11% crude protein; 8.92 ± 0.14% total lipid; 1.80 ± 0.03% ash.

Table 3.5
Apparent digestibility (%) of crude protein (CP), energy (KJ) and phosphorus (P) for Atlantic salmon fed experimental diets (C, no phytase or sodium phosphate; CP, 2000 U kg^{-1} phytase; CS, 10 g kg^{-1} sodium phosphate; CSP, 2000 U kg^{-1} phytase & 10 g kg^{-1} sodium phosphate) (mean ± S.E.M, n=3)

Parameter	Unit	Diet				*P*		
		C	CP	CS	CSP	*Phytase*	*Phosphorus*	*Interaction*
AD_{CP}	(%)	95.09 0.13	94.89 0.19	94.90 0.08	94.95 0.08	*ns*	*ns*	*ns*
AD_{KJ}	(%)	84.76 0.30	86.24 0.25	83.90 0.43	84.52 0.58	*ns*	*ns*	*ns*
AD_{P}	(%)	63.84^{c} 1.23	74.06^{a} 0.43	68.33^{b} 1.03	69.15^{b} 0.68	*0.001*	*ns*	*0.001*

Means with same letter are not significantly different (Tukey multiple comparison)

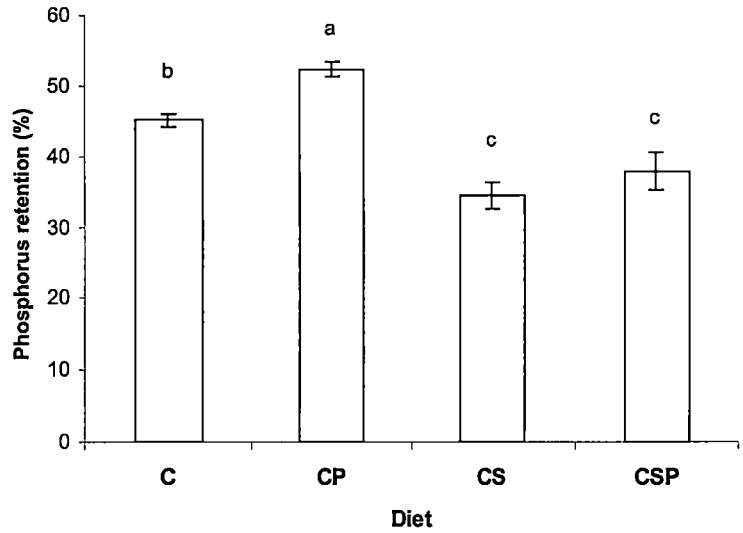

Figure 3.1. Phosphorus retention in Atlantic salmon fed experimental diets for 12 weeks (C, no phytase or sodium phosphate; CP, 2000 U kg^{-1} phytase; CS, 10 g kg^{-1} sodium phosphate; CSP, 2000 U kg^{-1} phytase & 10 g kg^{-1} sodium phosphate) (mean ± S.E.M, n=3) (one-way Anova [Tukey multiple comparison])

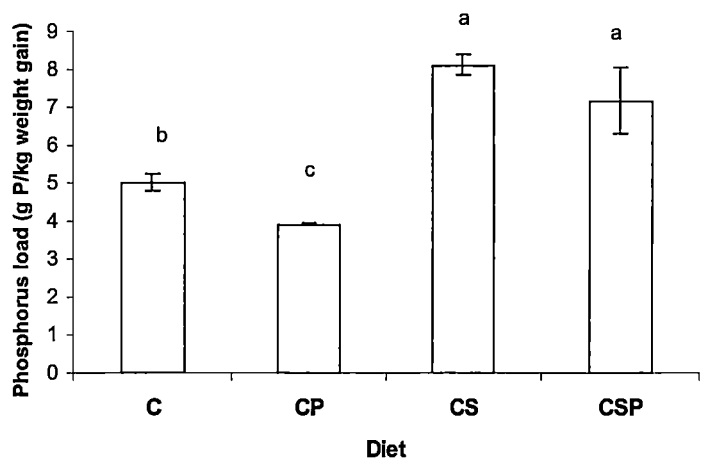

Figure 3.2. Phosphorus load in Atlantic salmon fed experimental diets for 12 weeks (C, no phytase or sodium phosphate; CP, 2000 U kg^{-1} phytase; CS, 10 g kg^{-1} sodium phosphate; CSP, 2000 U kg^{-1} phytase & 10 g kg^{-1} sodium phosphate) (mean ± S.E.M, n=3) (one-way Anova [Tukey multiple comparison])

3.5. Discussion

The present study has clearly shown that apparent phosphorus digestibility and bone mineralisation are the most sensitive criteria for assessing the influence of phytase on phosphorus utilisation in Atlantic salmon. Similarly, rib ash percentage and phosphorus digestibility in swine (Yi and Kornegay, 1999) and bone ash in poultry (Sullivan, 1999) are more sensitive criteria of phosphorus utilisation than growth performance. This is because phosphorus requirement for maximum bone mineralisation is greater than the requirement for maximum body weight gain. According to Rodehutscord (1996), different levels of dietary phosphorus are needed to fulfill phosphorus requirement for a variety of response criteria.

3.5.1. Phytase activity and efficacy

The phytic acid concentration of 3% in the canola meal used in the present study was similar to 3-4% reported previously (Cheryan, 1980; McCurdy and March, 1992). Phytase is mainly activated in the acidic stomach of salmonids and phytate is hydrolysed mainly in the stomach. However, there was an 11-13% reduction of phytate phosphorus during diet preparation. Significant dephosphorylation of phytate occurred in the phytase supplemented diet during diet preparation despite conditions being less than optimal for phytase activity (Schäfer et al., 1995; Bransden and Carter, 1999; Masumoto et al., 2001; Yan et al., 2002).

3.5.2. Feed intake and growth

In the present study, there were no significant differences in feed intake and growth performance in fish fed different diets. Similarly, inclusion of phytase or phosphorus did not improve growth in some studies (Lanari et al., 1998; Forster et al., 1999; Vielma et al., 2000). In contrast, growth improvement was observed in some studies in which fish were fed either phytase supplemented diets (Schäfer et al., 1995; Rodehutscord and Pfeffer, 1995; Papatryphon et al., 1999) or phytase

pre-treated ingredients (Cain and Garling, 1995; Vielma et al., 2002). Generally, growth improvements were observed in the studies that used diets entirely or almost entirely based on plant protein sources. When an all-plant meal diet is used, available phosphorus content is lower and amino acid profile poorer than in a fish meal based diet. Additionally, phytate binds to amino acids in the fish stomach and decreases the amino acid availability. The capacity of phytase to increase the availability of phosphorus, and perhaps amino acids explains the growth improvement when all-plant meal diets are used. In the present study the diets contained casein and blood meal as well as 35% plant meal and they were only marginally deficient in phosphorus, consequently the effect of phytase supplementation was not observed on growth performance.

3.5.3. Phosphorus digestibility

Phytase supplementation of a plant meal diet in the present study increased phosphorus digestibility in Atlantic salmon, similar to other studies with Atlantic salmon (Storebakken et al., 1998) and other salmonids (Brown, 1993; Cain and Garling, 1995; Rodehutscord and Pfeffer, 1995; Teskeredžić et al., 1995; Riche and Brown, 1996; Lanari et al., 1998; Vielma et al., 1998; Forster et al., 1999; Sugiura et al., 2001). Digestibility of phosphorus was highest in diet CP and despite inorganic phosphorus supplementation in diets CS and CSP, the digestibility was lower than diet CP. Using an excess amount of phosphorus leads to lower estimates of its digestibility (Rodehutscord et al., 2000). Phosphorus digestibility in rainbow trout peaks at approximately the dietary requirement level and then declines with increasing dietary phosphorus (Riche and Brown, 1996). Dietary phosphorus absorption is regulated by blood phosphorus level (Lall, 1991), so when the blood phosphorus level is saturated, absorption and therefore measured digestibility decreases. Avila et al. (2000) found that concentration of inorganic phosphorus in the luminal fluid was the same at different levels of dietary phosphorus when dietary inorganic phosphorus was less than requirement, but when dietary phosphorus level increased beyond requirement level, luminal fluid phosphorus increased with increasing level of dietary phosphorus.

Digestible phosphorus in the control diet was 5.15 g kg^{-1} diet, despite 4.67 g kg^{-1} available phosphorus in the diet (Table 3.2). Consequently, part of the phytate phosphorus was available for salmon. Plant phytases were isolated from different species such as wheat, corn, rice and rapeseed. Optimum pH for plant phytase activity is approximately 5.0 (Nys et al., 1999). Some activity of the natural canola phytase would be expected in the low pH of salmon stomach. The differences between digestible phosphorus and available phosphorus in diet C may have resulted from canola's intrinsic phytase activity. Despite differences in phosphorus digestibility, no differences were observed in crude protein and gross energy digestibility, which agreed with previous studies (Schäfer et al., 1995; Oliva-Teles et al., 1998; Masumoto et al., 2001).

3.5.4. Bone ash and phosphorus

Since salmon initially grow rapidly on diets that are deficient in phosphorus (Åsgård and Shearer, 1997) bone ash and phosphorus are sensitive indicators of the phosphorus status in fish (Vielma and Lall, 1998b). Insufficient phosphorus intake leads to mobilization of phosphorus from bone and transfer to soft tissues and metabolic processes (Baeverfjord et al., 1998). Whole-body growth is only affected by phosphorus deficiency when whole-body phosphorus level falls below a critical level (Nordrum et al., 1997). The effect of phosphorus deficiency on the growth rate of Atlantic salmon depends on the severity of the dietary phosphorus deficiency, both in terms of the time and the dietary phosphorus content.

Bone ash increased in fish fed diets containing phytase which was in agreement with other studies conducted with rainbow trout (Vielma et al., 2002), channel catfish (Jackson et al., 1996) and striped bass *(Morone saxatilis)* (Papatryphon et al., 1999). Bone ash increased in rainbow trout (Vielma et al., 1998; 2002), striped bass (Papatryphon et al., 1999) and channel catfish (Jackson et al., 1996; Li and Robinson, 1997; Yan et al., 2002) fed phytase containing diets. Increased bone phosphorus in fish fed phytase containing diets has been reported in catfish

(Jackson et al., 1996; Li and Robinson, 1997; Yan et al., 2002) and striped bass (Hughes and Soares, 1998).

The lower value for bone ash and phosphorus in fish fed diets without phyatse shows dietary available phosphorus content of 4.65 g kg^{-1} was deficient for bone mineralization. Phosphorus deficiency may lead to increased fat deposition in fish (Takeuchi and Nakazoe, 1981; Rodehutscord, 1996; Eya and Lovell, 1997; Skonberg et al., 1997; Watanabe et al., 1999), but this situation was not observed in the present study.

3.5.5. Phosphorus retention and load

Phosphorus retention efficiency increased in rainbow trout (Vielma et al., 2002), striped bass (Papatryphon et al., 1999), and Japanese flounder *(Paralichthys olivaceus)* (Masumoto et al., 2001) fed phytase containing diets. Phosphorus retention efficiency was significantly higher in fish fed phytase supplemented diet in the present study that showing improved phosphorus utilisation. When dietary phosphorus concentration increases above requirement the efficacy of utilisation of retention decreases (Rodehutscord, 1996; Jahan et al., 2003). The lower retention values in diets CS and CSP was due to their exceeding the phosphorus requirement.

Factors like feed conversion ratio, ingredient digestibility and dietary phosphorus content affect phosphorus load. Ketola and Richmond (1994) recorded 1.1-2.8 g kg^{-1} phosphorus load for diets with 5.1-7.1 g kg^{-1} dietary phosphorus. Using a low fish meal diet with 9.9 g kg^{-1} phosphorus led to 7.8 g kg^{-1} phosphorus load, but using a commercial diet with 18.1 g kg^{-1} phosphorus resulted in 17.3 g kg^{-1} phosphorus load (Satoh et al., 2003) that is comparable with 7.2 g kg^{-1} phosphorus load for diet CSP (10.7 g kg^{-1} dietary phosphorus) in the present study. Rainbow trout fed phytase supplemented diet had lower phosphorus load (Cain and Garling, 1995; Lanari et al., 1998; Vielma et al., 1998; Vielma et al., 2002) in comparison with the present study. Dietary phosphorus in phytase supplemented diet in the present study was 8.2 g kg^{-1} but dietary phosphorus in the other studies was less

than 6.0 g kg^{-1} so, the higher dietary phosphorus led to higher load in the present study (1-3 g kg^{-1} in the previous studies vs. 3.9 g kg^{-1} in the present study). Using phytase in plant meal based diets in comparison with inorganic phosphate reduced phosphorus load. So, inclusion of phytase in the diets can be used as a tool for aquaculture waste management (Cain and Garling, 1995; Vielma et al., 2002).

In the present study, phosphorus utilisation was higher in the fish fed either the phytase or phosphorus supplemented diets, but there was no further advantage when combination of phytase and sodium phosphate was used. Phytase incorporation in diets containing high levels of plant protein will substantially increase phosphorus digestibility and reduce phosphorus leaching from faeces. In the present study, the level of canola meal in the diet was 35% and the level of dietary phosphorus in the basal diet was marginally deficient, in comparison to diets including high level of plant protein (with consequently high level of phytate phosphorus), so the effect of phytase on increasing phosphorus digestibility and retention was not as high as previous experiments (Rodehutscord and Pfeffer, 1995; Vielma et al., 2002).

3.6. Conclusion

Phytase increased the apparent digestibility of phosphorus and enhanced the deposition of phosphorus. The results of the present study demonstrated that supplementation with phytase improved body phosphorus retention in Atlantic salmon fed a plant meal based diet deficient in available phosphorus but containing an adequate level of total phosphorus. Therefore, using phytase in plant meal based diets will reduce the need for inorganic phosphorus supplementation in diets which will lead to reductions in phosphorus discharge from fish farms.

3.7. Acknowledgements

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CHAPTER 4

COMPARISON OF PRE-TREATMENT AND DIRECT INCLUSION AT DIFFERENT PHYTASE ADDITIONS TO ATLANTIC SALMON (*SALMO SALAR*, L.) DIETS CONTAINING SOY PROTEIN CONCENTRATE

4.1. Abstract

The experiment aimed to determine the optimal dose of phytase for pre-treatment of soy protein concentrate (SPC) and for direct dietary supplementation of phytase into diets containing SPC. SPC was either pre-treated with 250, 500 or 1000 U phytase kg^{-1} SPC or phytase was included at 0, 250, 500, 1000 and 4000 U kg^{-1} diet. All diets contained 60% SPC and available phosphorus levels were set below requirement. The fish were fed for 12 weeks and there were significant ($P < 0.05$) differences in feed intake, growth, whole-body and bone ash and bone phosphorus content between the direct supplemented diets. There were no differences between the pre-treated diets but they showed better performance than the diets supplemented at less than 1000 U phytase kg^{-1}. In conclusion, to improve salmon feed intake, growth and phosphorus utilisation of SPC, phytase should be added directly at greater than 1000 U kg^{-1} diet or pre-treated with 250 U kg^{-1} diet (equivalent to 417 U kg^{-1} SPC).

Keywords: Atlantic salmon, Phytase dose-response, Phytase pre-treatment, Soy protein concentrate

4.2. Introduction

There are two approaches to using phytase in diets: direct supplementation of the diet or pre-treatment of plant ingredients. Supplementation of phytase to the diet (Rodehutscord and Pfeffer, 1995; Schäfer et al., 1995; Jackson et al., 1996; Li and Robinson, 1997; Carter and Hauler, 1998a; b; Hughes and Soares, 1998; Lanari et al., 1998; Vielma et al., 1998, 2000; Forster et al., 1999; Papatryphon et al., 1999; Papatryphon and Soares, 2001; Masumoto et al., 2001; Sugiura et al., 2001; Yan et al., 2002), or pre-treatment of ingredients (Cain and Garling, 1995; Storebakken et al., 1998; Van Weerd et al., 1998; Sugiura et al., 2001; Vielma et al., 2002), have been reported to improve phosphorus availability in fish. Pre-treatment of ingredients was more effective than direct phytase supplementation (Masumoto et al., 2001). Generally, a single and high level of phytase was used to evaluate the effect of phytase on different criteria whereas multiple levels of phytase have been used in fewer studies (Schäfer et al., 1995; Jackson et al., 1996; Li and Robinson, 1997; Forster et al., 1999; Papatryphon et al., 1999; Sugiura et al., 2001). Papatryphon et al. (1999) used 0, 500, 1000 and 2000 U kg^{-1} phytase in a high phytate diet and concluded that at least 1000 U kg^{-1} phytase was needed to improve growth, feed conversion and overall performance in striped bass (*Morone saxatilis*). Jackson et al. (1996) showed that the plateau of weight gain and phosphorus bioavailability was achieved at the lowest level of phytase (500 U kg^{-1} diet) in channel catfish (*Ictalurus punctatus*) (a warmwater fish) when they used between 0-4000 U kg^{-1} diet phytase and Li and Robinson (1997) concluded that 250 U kg^{-1} diet phytase (the lowest level in their study) was sufficient too, when they used between 0-750 U kg^{-1} diet phytase in the diet.

There are different kinds of phytases that can hydrolyse phytic acid and liberate orthophosphate, but the rate of hydrolysis of phytate strongly depends on conditions (Engelen, et al., 1994). Microbial phytases release phosphate from phytic acid across a range of pH and temperatures but optimum pH and temperature are 5.3-5.5 and 55 °C, respectively (Engelen, et al., 1994; Sugiura et

al., 2001). Phytase activity, like all enzymes, depends on temperature, thus using phytase is a concern with coldwater fish (Hardy, 1998). For example, use of 1000 U kg^{-1} phytase in rainbow trout diet at 10 °C improved phosphorus availability, without a positive effect on growth and feed efficiency, at 15 °C, growth, feed efficiency as well as phosphorous availability increased (Rodehutscord and Pfeffer, 1995).

Phytase pre-treatment and phytase supplementation have only been compared in Japanese flounder (*Paralichthys olivaceus*) (Masumoto et al., 2001) and rainbow trout (*Oncorhynchus mykiss*) (Sugiura et al., 2001). Furthermore, there is only one published study that investigated phytase in Atlantic salmon diet (Storebakken et al., 1998), using pre-treated soy protein concentrate. In the present experiment, different approaches (phytase pre-treatment and supplementation) and multiple levels of phytase were used in a soy protein concentrate (SPC) based diet, to determine the optimal level of phytase for Atlantic salmon parr.

4.3. Materials and methods

4.3.1. Experimental diets

A commercial soy protein concentrate (HP 300, Hamlet Protein A/S, Horsens, Denmark) was used as the major protein source in the trial. Phytase (Phytase 5000L, Natuphos: BASF, Homebush, NSW, Australia) was used for pre-treatment of the ingredient and direct inclusion in the diets.

To pre-treat the soy protein concentrate (SPC), 1 kg SPC was mixed with 1 L of distilled water at pH 5.5 (adjusted with HCl) at room temperature, and appropriate amounts of phytase were dissolved in the water. The mixture was incubated at 55 °C for 6 h, then dried in an oven at 30 – 40 °C for approximately 24h, to reduce the moisture to 10 - 15%. The dried, phytase-treated SPC was added to the experimental diets. A batch of SPC was treated in the same way but without addition of phytase and used in the remaining diets (sham treatment).

The SPC based diet was formulated to contain 41% crude protein and 20.0 MJ kg^{-1} energy (Table 4.1). The diet was formulated to meet the nutrient requirements of salmonids (NRC, 1993) except for available phosphorus. Diets were formulated with a low level of available phosphorus, in order to assess the effect of phytase treatment using phosphorus utilisation (Chapter 3). Other dietary mineral concentrations (Table 4.2) were equal or more than salmonid requirement (NRC, 1993). The basal diet contained no phytase. Four levels of phytase (250, 500, 1000 and 4000 U kg^{-1} diet) [where 1 U is defined as the amount of phytase that liberates inorganic phosphorus from a 0.0015 M sodium phytate solution at a rate of 1 μmol min^{-1} at pH 5.5 and 37 °C] were used for inclusion in the diet and three levels of phytase (250, 500 and 1000 U kg^{-1} diet) used for ingredient pre-treatment (the equivalent phytase for 1 kg diet [600 g SPC] was calculated for pre-treatment of diet, so 417, 833 and 1667 U phytase kg^{-1} SPC was used). For inclusion of phytase in the diet, all dry ingredients were mixed in a Hobart mixer and fish oil was added to the diet and completely mixed, the liquid phytase was then mixed with water and added to the diet before being pelleted (diameter: 3 mm) with a laboratory pelletizer (California Laboratory Pellet Mill). Diets were dried at 30 – 40 °C in an air-forced oven overnight and then stored in a cold room (4 °C) until fed.

4.3.2. Growth experiment

The experiment was conducted at the School of Aquaculture, University of Tasmania. Atlantic salmon (*Salmo salar* L.) parr were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia) and transferred to the Aquaculture Centre, School of Aquaculture. Fish were kept in 300-l tanks in a recirculation system where water quality was maintained through physical and biological filters (Sajjadi and Carter, 2004). Fish were maintained on a commercial feed (2mm Salmon, Skretting, Cambridge, Tasmania, Australia) for three weeks. Water replacement rate in the system was about 20% d^{-1} and water flow through the tanks was 8-10 l min^{-1}. Oxygen concentration was 95.0 ± 0.7%

of saturation, average water temperature was 15.5 ± 0.3 °C and a photoperiod (12L: 12D) was in effect over the 84-day experiment.

At the beginning of the experiment, fish were anaesthetised in benzocaine (50 mg l^{-1}) and the wet weight and fork length of individual fish measured. One fish from each tank (n=24) was sacrificed (100 mg l^{-1} benzocaine) and used for the measurement of initial whole-body chemical composition. The remaining 20 fish (45.5 ± 0.19 g, mean ± SD, n=24) were returned to each tank. Triplicate groups of fish were fed each experimental diet to satiation twice daily at 0900 and 1700 h, satiation was judged to have been reached when approximately 20 pellets were not eaten and lost via the outlet water. Uneaten pellets were collected by a mesh collector placed in the outlet water (Helland et al., 1996) and feed intake calculated by subtracting the number of uneaten from supplied pellets. Feed intake was monitored in this way every day throughout the experiment. Fish in each tank were anaesthetised, counted and bulk-weighed every three weeks. Fish were not fed on the day of weighing.

Feed Efficiency Ratio (FER) was calculated as:

$$\text{FER (g g}^{-1}\text{ DM)} = \text{total weight gain (g) / total feed intake (g DM)} \qquad [4.1]$$

At the end of the experiment, the fish were fasted for 24 h and the wet weight and fork length measured for individual fish. Five fish from each tank for final whole-body chemical composition and another ten fish per tank were killed for bone ash and phosphorus analysis. The fish were frozen at –20 °C until analyses.

4.3.3. Apparent digestibility

Apparent digestibility (AD) was measured during the growth experiment. Yttrium oxide (1 g kg^{-1}) was added to the diets as an inert marker (Sugiura et al., 1998). The fish were fed the diets containing yttrium oxide during the entire 12-week experiment and faecal samples were collected by Guelph-type settlement

collectors (Carter and Hauler, 2000) for three days during the last week of the growth experiment. One hour after feeding at 1600 h, the collectors were washed of uneaten food and faeces collected from that time until 1 h before next feeding time (0900h). Faeces were frozen, freeze-dried and used in the analysis of yttrium oxide and nutrients (see below). The faecal samples from the three collection days were pooled in equal weights for each tank and frozen at –20 °C until analysis. AD was calculated according to:

$$AD\ (\%) = 100 - [100\ (\%\ I\ diet\ /\ \%\ I\ faeces) \times (\%\ N\ faeces\ /\ \%N\ diet)] \quad [4.2]$$

(Maynard and Loosli, 1969), where I is the inert marker and N is the nutrient.

4.3.4. Chemical analysis

Chemical analysis of diets, fish whole-body and faeces were performed according to standard methods: dry matter (freeze dry to constant weight); ash (AOAC, 1995); crude fat (Bligh and Dyer, 1959); protein (Kjeldahl using a selenium catalyst [N × 6.25]) and energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid). For analysis of the Yttrium marker and minerals in diets and faeces, 5 ml HNO_3 were added to 100-150 mg samples in digestion tubes and boiled (Vandecasteele and Block, 1993). After cooling, the samples were diluted to 20 ml with distilled water and yttrium and minerals content of diets and faeces measured by inductively coupled plasma optical emission spectrometry (Thermo Jarrell-Ash IRIS Axial ICP-OES). Phytic acid was determined by David Harris (Western Australia Chemistry Centre, Perth) according to a number of published methods (Harland and Oberleas, 1977; Ellis and Morris, 1983; Harland and Oberleas, 1986).

To determine bone ash and phosphorus, previously frozen fish were cooked for about 6 min in a microwave until the flesh and bone were easily separated. Soft tissues were carefully removed from the vertebrae. Isolated vertebrae were rinsed

with distilled water and dried in an oven at 105 °C for 24 h. After drying, they were ground with a mortar and pestle and then de-fatted with solvent (Chloroform, Methanol 1:1) (Vielma and Lall, 1998a,b), dried and ashed in a muffle furnace at 600 °C for 4 h. The ash was weighed and subsequently analysed for phosphorus by the molybdovanadate method (AOAC, 1995). Phosphorus in the diet, initial and final whole-body and faeces were also analysed by the molybdovanadate method (Chapter 3).

4.3.5. Statistical analysis

Mean values are reported ± Standard Error of the Mean (S.E.M). Comparison between means was by one-way ANOVA after confirming the normality and homogeneity of variance (SPSS, version 11.5). Multiple comparison was by Tukey. Differences were considered significant at $P < 0.05$. Tow-way ANOVA was not used, because indirect and direct supplemented diets were not directly comparable. Correlation matrices of mineral digestibility were compared by Pearson correlation coefficient (SPSS, version 11.5). Non-linear equations were used to find the best curve ($P< 0.05$) fit for whole-body ash and phosphorus-phytase supplementation levels and bone ash and phosphorus-phytase supplementation levels (SigmaPlot, 2000).

Table 4.1. Ingredient and chemical composition of experimental diets

Ingredient composition (g kg^{-1})	Control	P 250	P 500	P 1000	P 4000	Pre 250	Pre 500	Pre 1000
Soy Protein Concentrate HP	600	600	600	600	600	600	600	600
Blood meal[2]	50	50	50	50	50	50	50	50
Fish meal[3]	45	45	45	45	45	45	45	45
Pre-gelatinized starch	64	64	64	64	64	64	64	64
Alpha cellulose	50	50	50	50	50	50	50	50
Bentonite	20.5	20.5	20.5	20.5	20.5	20.5	20.5	20.5
Carboxymethyl cellulose	10	10	10	10	10	10	10	10
Fish oil	150	150	150	150	150	150	150	150
Vitamin mix[4]	2	2	2	2	2	2	2	2
Mineral mix[5]	1	1	1	1	1	1	1	1
DL Methionine	2	2	2	2	2	2	2	2
Stay C[6]	3	3	3	3	3	3	3	3
Choline chloride	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Yttrium oxide	1	1	1	1	1	1	1	1
Phytase (U kg^{-1})[7]	0	250	500	1000	4000	250	500	1000
Chemical composition (g kg^{-1})								
Dry matter (g kg^{-1})	930	944	961	952	960	949	934	957
Crude protein	409	403	409	410	411	411	414	412
Total fat	180	181	179	182	181	180	181	184
Gross energy (MJ kg^{-1} DM)	20.03	20.03	20.02	20.04	20.01	20.08	20.06	20 05
Ash	67	68	67	67	68	67	68	69
Total phosphorus	6.10	6.10	6.10	6.10	6.20	6.20	6.10	6.20
Phytate phosphorus[8]	3.28	3.28	3.28	3.28	3.33	3.33	3.28	3.33
Available phosphorus	2.80	2.82	2.82	2.82	2.87	2.87	2.82	2.87

[1] Hamlet Protein A/S, Horsens, Denmark.

[2] Skretting Australia, Cambridge, Tasmania, Australia.

[3] South American fish meal (Skretting Australia, Cambridge, Tasmania, Australia).

[4] Supplied (mg kg^{-1} diet): 10.0, Vitamin A acetate; 12.0, Vitamin D3 powder; 200, Rovimix E50; 4.0, Menadione sodium bisulphate; 8.0, Riboflavin; 43.48, Calcium D-pantothenate; 20.0, Nicotinic acid; 0.02, Vitamin B12; 0.3, d-Biotin; 2.0, Folic acid; 2.25, Thiamin HCl; 7.32, Pyridoxine HCl; 600.0, Myo-inositol.

[5] Supplied (mg kg^{-1} diet): 23.3, $CuSO_4$ $5H_2O$; 363.1, $FeSO_4$ $7H_2O$; 61.5, $MnSO_4$ H_2O; 0.66, Na_2 SeO_3; 131.9, $ZnSO_4$ $7H_2O$; 1.44, KI; 9.54, Co SO_4 $7H_2O$.

[6] L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia Ltd, Sydney, Australia).
[7] BASF, Homebush, NSW, Australia.

[8] 54% of total phosphorus in SPC is phytate phosphorus

Table 4.2. Mineral and trace element composition of diets (n=2, Mean ± S.E.M) (P, phytase supplemented; Pre, phytase pre-treated)

Parameter	Unit	Diet							
		Control	P 250	P 500	P 1000	P 4000	Pre 250	Pre 500	Pre 1000
Minerals									
Ca	(%)	0.39	0.41	0.41	0.42	0.41	0.41	0.41	0.39
		0.02	0.01	0.05	0.05	0.00	0.01	0.00	0.02
Mg	(%)	0.21	0.21	0.21	0.21	0.21	0.21	0.21	0.21
		0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00
Elements									
Cu	(mg kg^{-1})	30.47	27.37	27.84	30.93	28.26	30.64	30.24	30.19
		0.48	0.13	0.13	0.23	0.41	0.48	0.14	0.28
Mn	(mg kg^{-1})	48.26	46.72	48.26	49.68	48.87	50.57	48.49	47.20
		1.30	0.87	0.53	0.36	0.71	0.89	0.27	0.65
Sr	(mg kg^{-1})	15.12	15.08	15.23	16.16	16.02	15.82	15.36	15.10
		0.38	0.19	1.25	1.35	0.29	1.96	0.15	0.19
Zn	(mg kg^{-1})	66.81	64.49	68.49	66.60	68.17	67.64	65.66	66.99
		0.89	1.06	0.77	0.38	2.47	1.55	1.50	2.04

(Ca, calcium; Cu, copper; Mg, magnesium, Mn, manganese; P, phosphorus; Sr, strontium; Zn, zinc)

4.4. Results

4.4.1. Feed intake and growth performance

Atlantic salmon fed diets supplemented with at least 4000 U kg^{-1} phytase had significantly greater feed intake and weight gain than fish fed the control diet (Table 4.3). There were no significant differences in feed intake and weight gain between the pre-treated diets or between these and the diets supplemented with 1000 and 4000 U kg^{-1} phytase. There were no significant differences in feed intake and weight gain between diets supplemented between 250 to 4000 U kg^{-1} phytase. Feed intake and weight gain were significantly lower in the control diet compared to the diets supplemented with 4000 U kg^{-1} phytase or pre-treated. Feed efficiency ratio (FER) did not differ between the treatments and ranged from 0.74 to 0.81. There were no differences in fish survival between treatments (96-100%) and differences in survival were due to fish escapes during the first week of trial.

4.4.2. Composition of fish

There were no significant differences in final whole-body dry matter and crude protein content (Table 4.4). Body ash was higher in fish fed diets supplemented with at least 1000 U kg^{-1} phytase in comparison with control diet (Table 4.4). There were no significant differences in body ash between the pre-treated diets or between these and the diets supplemented with 500 to 4000 U kg^{-1} phytase. Body ash was significantly lower in the control diet and there were no significant differences between control diet and diets supplemented with up to 500 U kg^{-1} phytase. Also, there were no significant differences between diets supplemented with 250, 500 and 1000 U kg^{-1} phytase or diet pre-treated with 250 U kg^{-1} phytase.

Body phosphorus was higher in fish fed diets supplemented with at least 500 U kg^{-1} phytase in comparison with control diet (Table 4.4). There were no

significant differences in body phosphorus between pre-treated diets or between these diets and the diets supplemented with 1000 and 4000 U kg^{-1} phytase. There were no significant differences in body phosphorus between control diet and the diet supplemented with 250 U kg^{-1} phytase. Also, there were no significant differences between diets supplemented with 250 and 500 U kg^{-1} phytase. There were no significant differences between the diets supplemented with 500 and 1000 U kg^{-1} phytase or pre-treated with 500 U kg^{-1} phytase.

There was a clear separation of diets in relation to bone ash. There were no significant differences in bone ash between the pre-treated diets or between these diets and the diets supplemented with 1000 and 4000 U kg^{-1} phytase (Table 4.4). Bone ash was significantly lower in fish fed the control diet or diets supplemented with up to 500 U kg^{-1} phytase.

There were no significant differences in bone phosphorus between the pre-treated diets or between these and diets supplemented with 1000 and 4000 U kg^{-1} phytase (Table 4.4). Bone phosphorus was significantly lower in the control diet and diet supplemented with 250 U kg^{-1} phytase in comparison with pre-treated diets or diets supplemented with 1000 and 4000 U kg^{-1} phytase. There were no significant differences in bone phosphorus between the supplemented diets with 250 and 500 U kg^{-1} phytase and control diet. There were no significant differences in bone phosphorus between the diets supplemented with 500 and 1000 U kg^{-1} phytase or pre-treated with 250 U kg^{-1} phytase.

Whole-body ash and phosphorus and bone ash and phosphorus increased in a non-linear pattern with graded levels of supplemental phytase (Figs. 4.2 and 4.3)

4.4.3. Minerals and trace elements digestibility

There were significant differences between different treatments in magnesium, phosphorus and zinc digestibility, but there were no significant differences

between different diets for calcium, copper, manganese and strontium digestibility (Table 4.5)

4.4.3.1. Phosphorus

Phosphorus digestibility markedly increased with increasing addition of phytase supplement (Fig. 4.1). Phosphorus digestibility for the control diet was significantly lower than other treatments. There were no significant differences in phosphorus digestibility between the pre-treated diets or between these and diet supplemented with 4000 U kg^{-1} phytase. There were no significant differences between the diets supplemented with 250 and 500 U kg^{-1} phytase. Furthermore, there were no significant differences in phosphorus digestibility between diets supplemented with 1000 U kg^{-1} phytase or diet pre-treated with 250 U kg^{-1} phytase.

4.4.3.2. Magnesium

Magnesium digestibility increased with increasing level of supplemented phytase (Table 4.5). There were no significant differences in Mg digestibility between the pre-treated diets or these diets and the diets supplemented with 1000 or 4000 U kg^{-1} phytase. Mg digestibility was significantly different between the control diet and the diets supplemented with at least 4000 U kg^{-1} phytase. There were no significant differences between the control diet and the diets supplemented with phytase up to 1000 U kg^{-1} phytase. Also, there were no significant differences in Mg digestibility between the diets supplemented with 250, 500, and 1000 U kg^{-1} phytase and the diets pre-treated with 250 and 500 U kg^{-1} phytase.
Using as low as 250 U kg^{-1} phytase for SPC pre-treatment was sufficient to improve feed intake, weight gain, body phosphorus, body ash, bone ash and phosphorus.

4.4.3.3. Zinc

There were no significant differences in Zn digestibility between the pre-treated diets or between these diets and the diets supplemented with 1000 and 4000 U kg^{-1} phytase (Table 4.5). Zn digestibility was significantly different between the control diet and the diets supplemented with at least 500 U kg^{-1} phytase. Zn digestibility was significantly lower for the control diet and there were no significant differences between this diet and the diet supplemented with 250 U kg^{-1} phytase. Also, there were no significant differences between diets supplemented with 250 and 500 U kg^{-1} phytase.

Correlations between mineral digestibility values were analysed (Table 4.6). The highest correlations were observed between phosphorus and zinc (r=0.95) and phosphorus and magnesium (r=0.90). The lowest correlation was observed between copper and other minerals.

Table 4.3. The performance of Atlantic salmon fed different diets (mean ± S.E.M, n=3) (P, phytase supplemented; Pre, phytase pre-treated)

Parameter	Unit	Diets								
		Control	P 250	P 500	P 1000	P 4000	Pre 250	Pre 500	Pre 1000	*P*
Initial weight	(g)	45.57	45.74	45.41	45.63	45.61	45.35	45.43	45.53	*ns*
		0.09	0.10	0.02	0.13	0.11	0.04	0.19	0.02	
Final weight	(g)	82.35^{a}	84.32^{ab}	84.5^{ab}	89.1^{abc}	93.49^{bc}	94.62^{c}	94.74^{c}	94.46^{c}	*<0.05*
		0.82	0.84	0.85	0.89	0.93	0.95	0.95	0.94	
Weight gain	(g)	36.77^{a}	38.59^{ab}	39.09^{ab}	43.48^{abc}	47.89^{bc}	49.26^{c}	49.21^{c}	48.93^{c}	*<0.05*
		1.59	2.52	3.36	0.12	0.86	1.51	1.21	1.87	
Total feed intake	(Kg DM)	1.00^{a}	1.04^{ab}	1.02^{ab}	1.14^{abc}	1.18^{bc}	1.23^{c}	1.21^{c}	1.24^{c}	*<0.05*
		0.009	0.003	0.002	0.003	0.003	0.05	0.03	0.08	
FER	($g\ g^{-1}$ DM)	0.74	0.74	0.77	0.76	0.81	0.80	0.81	0.79	*ns*
		0.026	0.052	0.064	0.002	0.016	0.011	0.011	0.043	
Survival	(%)	98.33	100	100	96.67	100	98.33	96.67	98.33	*ns*

Means with same letter are not significantly different (Tukey multiple comparison)

Table 4.4. Chemical composition (% wet weight), whole-body phosphorus content, bone ash and bone phosphorus of Atlantic salmon fed experimental diets (P, phytase supplemented; Pre, phytase pre-treated) (mean ± S.E.M, n=3, five fish pooled per replicate)

Parameter	Unit	Diet								
		Control	P 250	P 500	P 1000	P 4000	Pre 250	Pre 500	Pre 1000	*P*
Dry matter	(%)	33.81	33.83	33.71	33.66	33.68	33.67	33.72	33.52	*ns*
		0.25	0.10	0.15	0.11	0.07	0.23	0.07	0.13	
Crude protein	(%)	17.21	17.42	17.27	17.02	17.34	17.19	16.96	17.2	*ns*
		0.19	0.08	0.07	0.09	0.19	0.07	0.03	0.18	
Total lipid	(%)	10.60	10.66	10.51	10.32	10.44	10.56	10.38	10.69	*ns*
		0.12	0.15	0.23	0.21	0.21	0.19	0.07	0.12	
Ash	(%)	5.45^{a}	5.63^{ab}	5.99^{abc}	6.57^{bc}	6.82^{c}	6.60^{bc}	6.68^{c}	6.85^{c}	*<0.05*
		0.08	0.13	0.17	0.23	0.29	0.07	0.13	0.23	
Body phosphorus	(%)	1.23^{a}	1.29^{ab}	1.32^{bc}	1.37^{cd}	1.43^{d}	1.39^{d}	1.37^{cd}	1.43^{d}	*<0.05*
		0.011	0.006	0.033	0.006	0.014	0.011	0.014	0.008	
Bone ash	(%)	44.58^{a}	45.85^{a}	46.65^{a}	51.26^{b}	52.13^{b}	51.93^{b}	52.13^{b}	51.57^{b}	*<0.05*
		0.14	1.07	0.64	0.12	0.0.43	0.14	0.23	0.81	
Bone phosphorus	(%)	10.54^{a}	10.53^{a}	10.85^{ab}	11.21^{bc}	11.68^{c}	11.45^{bc}	11.82^{c}	11.52^{c}	*<0.05*
		0.18	0.05	0.06	0.04	0.08	0.05	0.31	0.04	

Initial group (mean ± S.E.M; $n = 10$): 30.86 ± 0.35% DM; 15.06 ± 0.43% crude protein; 12.67 ± 0.12% total lipid; 1.86 ± 0.03% ash, 1.43 ± 0.02% phosphorus DM. or (0.44 ± 0.01% phosphorus in wet weight)

Means with same letter are not significantly different (Tukey multiple comparison)

Table 4.5. Apparent digestibility (%) of minerals and trace elements in Atlantic salmon fed different diets (P, phytase supplemented; Pre, phytase pre-treated)

Parameter	Unit	Diet								
		Control	P 250	P 500	P 1000	P 4000	Pre 250	Pre 500	Pre 1000	*P*
Minerals										
AD_{Ca}	(%)	-15.51	0.58	-0.17	1.21	11.90	13.75	-4.27	2.63	*ns*
		5.84	4.77	2.88	5.55	0.76	5.03	12.12	3.82	
AD_{Mg}	(%)	53.56 ^{a}	56.54 ^{ab}	56.99 ^{ab}	59.99 ^{abc}	68.82 ^{c}	64.21 ^{bc}	64.44 ^{bc}	67.86 ^{c}	*<0.05*
		2.89	2.18	2.10	1.09	2.29	2.50	1.23	0.63	
Elements										
AD_{Cu}	(%)	55.07	48.57	49.09	37.44	47.71	51.00	53.77	51.07	*ns*
		0.78	3.32	1.70	21.04	5.04	2.21	6.23	1.67	
AD_{Mn}	(%)	18.34	17.57	16.84	21.71	31.88	20.46	16.44	32.22	*ns*
		4.97	4.97	1.92	2.04	5.47	3.14	6.43	6.66	
AD_{Sr}	(%)	7.93	10.44	7.96	10.07	18.43	11.76	3.47	11.65	*ns*
		4.76	4.60	2.16	2.79	4.37	2.17	6.62	0.72	
AD_{Zn}	(%)	58.18 ^{a}	61.35 ^{ab}	67.88 ^{b}	75.23 ^{c}	80.82 ^{c}	81.29 ^{c}	77.25 ^{c}	81.29 ^{c}	*<0.05*
		2.39	1.17	0.92	0.34	2.40	0.78	1.32	0.28	

Means with same letter are not significantly different (Tukey multiple comparison)
(Ca, calcium; Cu, copper; Mg, magnesium, Mn, manganese; P, phosphorus; Sr, strontium; Zn, zinc)

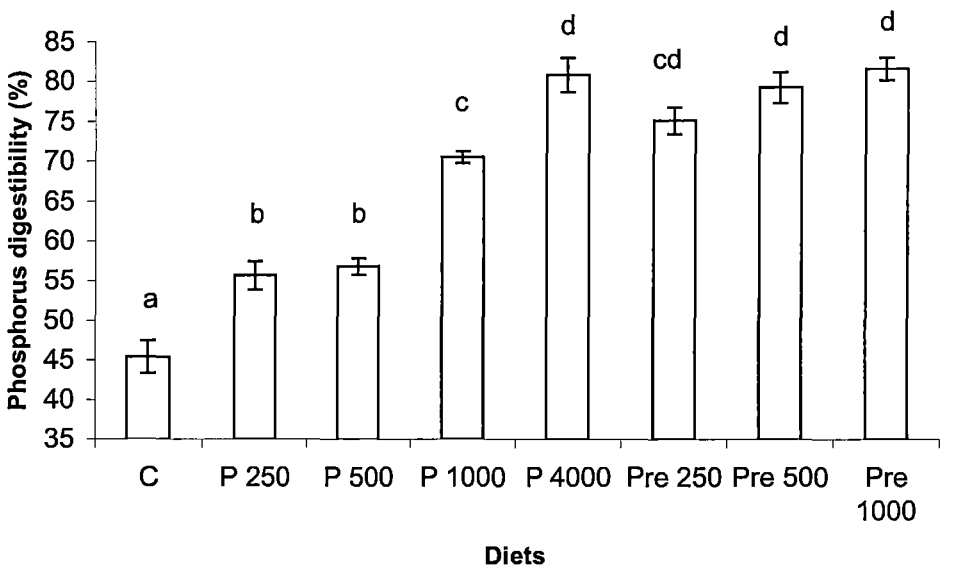

Figure 4.1. Phosphorus digestibility (%) in Atlantic salmon fed experimental diets for 12 weeks (P, phytase supplemented; Pre, phytase pre-treated) (mean ± S.E.M, n=3). Means with same letter are not significantly different (Tukey multiple comparison).

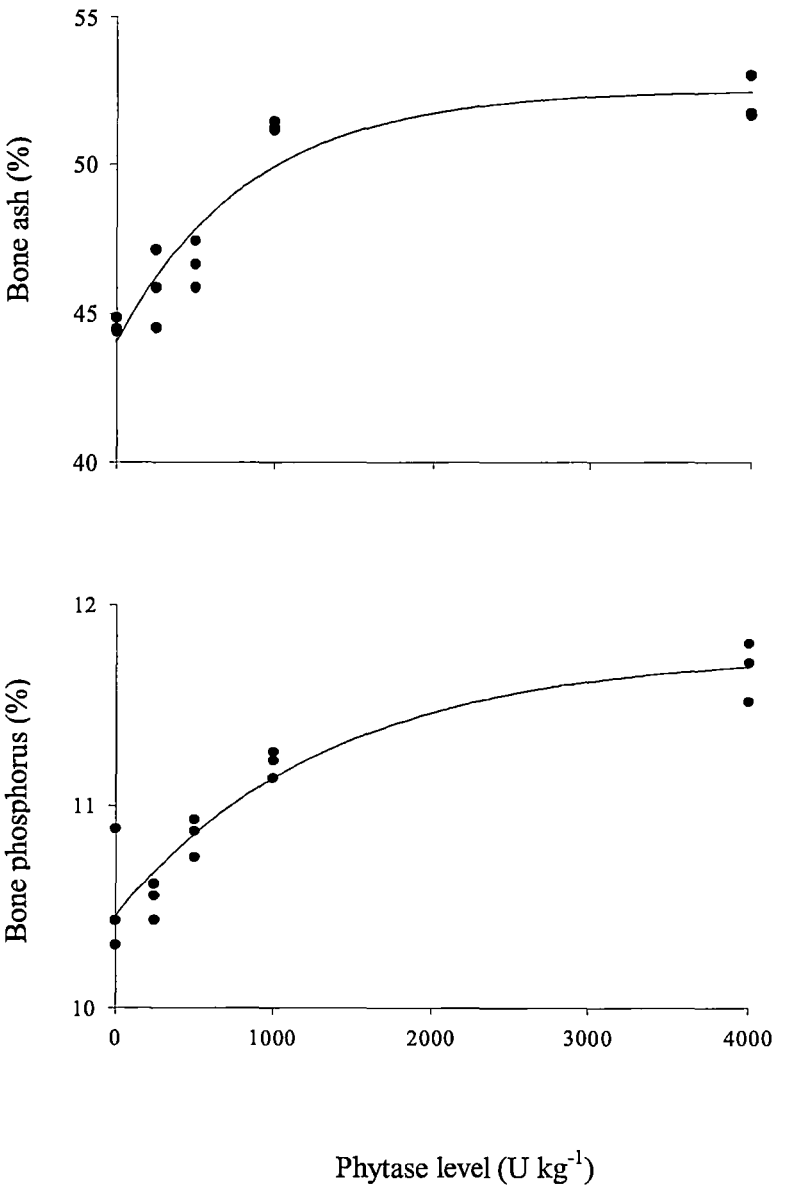

Figure 4.2. Non-linear relationship between bone ash [y=44.06+8.46(1-exp (-0.0012x)); r^2=0.88] and bone phosphorus [y=10.45+1.30(1-exp (-0.0007x)); r^2=0.88] and phytase level in Atlantic salmon fed SPC based diet supplemented with multiple levels of phytase

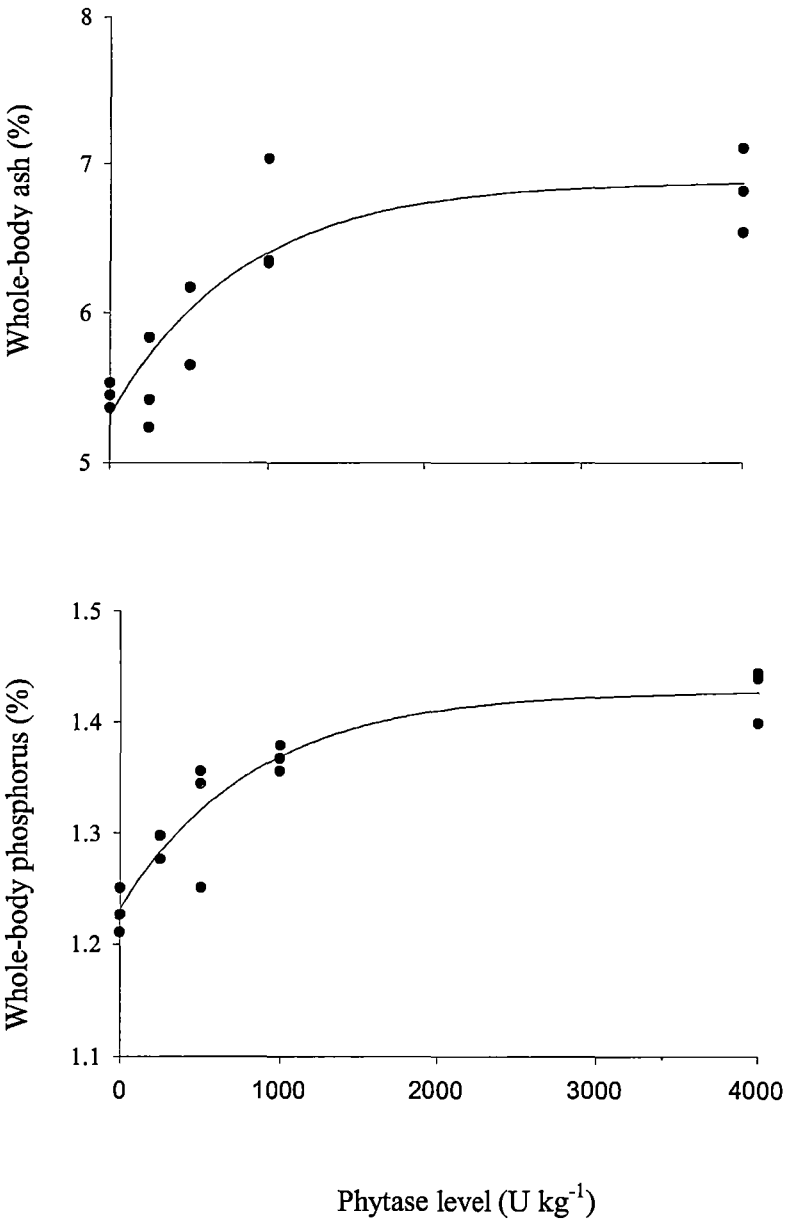

Figure 4.3. Non-linear relationship between whole-body ash [y=5.32+1.56(1-exp (-0.0012x)); r^2=0.79] and whole-body phosphorus [y=1.23+0.195(1-exp (-0.0012x)); r^2=0.88] and phytase level in Atlantic salmon fed SPC based diet supplemented with multiple levels of phytase

Table 4.6. Correlation matrix (r) of mineral and trace element digestibility in Atlantic salmon (n=24)

	AD						
AD	Ca	Mg	P	Cu	Mn	Sr	Zn
Mg	0.62**						
P	0.56**	0.90**					
Cu	0.27	0.18	0.01				
Mn	0.46*	0.66**	0.49*	0.14			
Sr	0.85**	0.61**	0.44*	0.26	0.71**		
Zn	0.55**	0.84**	0.95**	-0.07	0.44*	0.43*	

* Correlation significant at $P < 0.05$

** Correlation significant at $P < 0.01$

4.5. Discussion

4.5.1. Feed intake and growth performance

The results showed that pre-treatment of ingredients was more effective than supplementation, using phytase pre-treatment at 250 U kg^{-1} was as effective as adding 1000 U kg^{-1} phytase directly to the diets. Some studies have shown that weight gain increased in salmonids fed phytase (Cain and Garling, 1995; Hauler and Carter, 1997; Carter and Hauler 1998b). A few studies have used multiple supplementation of phytase (Schäfer et al., 1995; Jackson et al., 1996; Li and Robinson, 1997; Forster et al., 1999; Papatryphon et al., 1999; Sugiura et al., 2001) but no studies have been published on Atlantic salmon. Forster et al. (1999) supplemented different levels of phytase in a canola protein concentrate (CPC) based diet. They concluded that 4500 U kg^{-1} diet (the highest level in their study) was needed to improve phosphorus availability. Supplementation of a rainbow trout diet with 1000 U kg^{-1} phytase increased feed intake and weight gain without improving FCR (Rodehutscord and Pfeffer, 1995). In contrast, catfish fed diets supplemented with 250 U kg^{-1} (Jackson et al., 1996) to 500 U kg^{-1} phytase (Li and Robinson, 1997) had greater feed intake and weight gain in comparison with fish fed diet without phytase. Differences probably relate to several factors but temperature was considered the most important (Hardy, 1998), because efficacy of phytase depends on temperature.

In the present study, the fish fed less than 1000 U kg^{-1} phytase supplemented diets in comparison with other diets had between 14 and 24% lower feed intake. Phytase increased feed intake by 33, 10 and 21% in rainbow trout, channel catfish and Atlantic salmon, respectively (Rodehutscord and Pfeffer, 1995; Jackson et al., 1996; Carter and Hauler, 1998b). However, there has not yet been a satisfactory explanation for increased feed intake in animals fed phytase containing diets. This phenomenon has also been observed in terrestrial monogastric animals. There were no differences in FER between different treatments in the present study, this

suggests that increased feed intake was largely responsible for increased growth performance. The increased feed intake and weight gain following phytase inclusion may partly be explained by the fact that fish loose weight when fed phosphorus deficient diet (Lall, 2002). In this study phytase increased available dietary phosphorus to above the requirement (see below).

The lowest level used for SPC pre-treatment in the present study was 250 U kg^{-1}, so it was not possible to determine whether a lower level of phytase would have been effective. Cain and Garling (1995) observed higher weight gain in rainbow trout fed phytase-treated soybean meal. Higher feed intake and weight gain at lower levels of phytase for pre-treatment of ingredients in comparison to direct phytase inclusion may be attributed to more efficient hydrolysis of phytic acid in pre-treatment (Vielma et al., 2002). In the present study, SPC was treated under optimum conditions (pH and temperature), so the efficacy of phytase during pre-treatment was greater than when in the fish.

Growth increased with phytase supplementation and pre-treatment in the present study. Dietary available phosphorus was less than 50% of total dietary phosphorus and less than 50% of the salmonid phosphorus requirement (NRC, 1993) and the level of other minerals was more than salmonid requirement (NRC, 1993) in the present study. Dietary available phosphorus or non-phytate phosphorus, level of total phosphorus, level of phytate phosphorus in the diet and level of phytase all influence response to phytase (Selle et al., 2000; Kornegay, 2001). Basal diet in Chapter 3 was marginally deficient in available phosphorus and the level of plant protein was lower than the present experiment. Mineral level in the diet in the present study was lower than the basal diet in Chapter 3, so improvement in growth performance was not observed in experiment Chapter 3. Improvement in phosphorus, zinc and magnesium digestibility and utilisation in phytase supplemented or pre-treated diets might lead to better intake and growth in the present study.

In the present experiment, the level of plant protein source (SPC) was purposefully high in comparison with commercial diets. In studies with phytase, dietary phosphorus level should be low and phytic acid level should be high in order to force the fish to utilise phosphorus as much as possible (Rodehutscord and Pfeffer, 1995). So, the optimal dose of 1000 U supplemented phytase kg^{-1} diet that was determined in the present experiment may change in different nutritional conditions. Furthermore, the level of plant meal protein in current practical diets for carnivorous fish like salmon is lower than the level in the present experiment, so it would be expected 1000 U supplemented phytase kg^{-1} diet would be sufficient for inclusion in salmon practical diet. This value is equivalent to1667 U kg^{-1} SPC, 4 times more than the amount needed for ingredient pre-treatment.

4.5.2. Composition of fish

Whole-body phosphorus, bone ash and bone phosphorus were higher in fish fed pre-treated diets or diets supplemented with more than 1000 U kg^{-1} phytase. There was a non-linear (exponential) relationship between bone phosphorus, bone ash, whole-body phosphorus and whole-body ash and phytase supplementation levels; such a relationship was observed in terrestrial monogastric animals (Kornegay, 2001).

The fish fed diets with more than 500 U kg^{-1} supplemented phytase had significantly higher body ash than other lower supplements, and comparable to fish fed pre-treated soy protein concentrate. Van Weerd et al. (1999) found increasing level of body phosphorus and ash with increasing phytase supplementation (15 - 1000 U kg^{-1}) and there was a plateau at 750 U kg^{-1} in African catfish (*Clarias gariepinus*). Increased body ash and phosphorus (Vielma et al., 1998; Storebakken et al., 1998, Schäfer et al., 1995), body ash (Lanari et al., 1997) and body phosphorus (Masumoto et al., 2001) were observed in fish fed phytase containing diets. Whole-body and bone ash represent mineral status, increased whole-body and bone ash and phosphorus were possibly due to increased P, Zn, and Mg digestibility in fish fed phytase containing diets.

Comparison of phosphorus and ash content of body and bone of salmon that received enough phosphorus in the present experiment with results of Chapter 3 showed that these values are higher in the present experiment and this might be due to the size of fish (see Chapter 7).

4.5.3. Minerals and trace element digestibility

In the present study, lower digestibility of some cations was observed in the control diet and mineral digestibility increased with increasing level of phytase. Since phytate binds to divalent and trivalent mineral ions, leading to these ions becoming unavailable, these results indirectly confirmed presence of binding between mineral and phytate and showed positive effect of phytase on hydrolysing these bonds.

The highest positive correlation was observed between Zn and P apparent digestibilities. The lowest correlations were between Cu and other mineral and trace element apparent digestibilities. Mineral interaction may influence their bioavailability (Davis and Gatlin, 1996). There is no study on mineral apparent digestibility correlation in Atlantic salmon to compare with the results of the present study.

4.5.3.1. Calcium

There were no differences between treatments in Ca digestibility. There were no differences in Ca digestibility of Atlantic salmon fed phytase containing diet in comparison with control diets (Carter and Hauler, 1998b). There were some negative values for Ca digestibility and generally digestibility values for Ca were low. Negative values for Ca apparent digestibility indicated that fish faecal Ca excretion was more than dietary intake (Sugiura et al., 1999). Fish are able to absorb Ca from the water (NRC, 1993) and regulate Ca via the gills (Lall, 1989).

4.5.3.2. Magnesium

In the present study, apparent digestibility for Mg increased with increasing level of phytase in supplemented diet and was highest in the highest inclusion level and pre-treated SPC diets. Mg apparent digestibility was higher in fish fed phytase containing plant meal based diets in comparison with control diets (Storebakken et al., 1998; Sugiura, et al., 2001). Mg digestibility of different ingredients increased when phytase was used with ingredients in a reference diet (Cheng and Hardy, 2002). In contrast, no differences in Mg apparent digestibility observed in rainbow trout fed phytase containing diets (Vielma et al., 1998). No differences observed in Sr and Mn digestibility by using phytase in the diet (Storebakken et al., 1998).

4.5.3.3. Phosphorus

In the present study, phosphorus digestibility increased with increasing level of phytase whereas pre-treated diets had similar digestibility values. When rainbow trout were fed a CPC diet with different levels of supplemented phytase (0, 500, 1500 and 4500 U kg^{-1}), phosphorus digestibility was only improved by supplementation of the highest level of phytase (Forster et al., 1999). Sugiura et al. (2001) used phytase supplemented soybean meal diets with 0, 500, 1000, 2000 and 4000 U kg^{-1} as well as soybean meal pre-treated with 200 U phytase kg^{-1} ingredient in rainbow trout. Apparent digestibility of phosphorus increased with increasing level of supplemented phytase with maximum of 90% at 4000 U kg^{-1} that was comparable with phosphorus apparent digestibility in phytase pre-treated diet (93%). Hughes and Soares (1998) used different levels of phytase (0, 800, 1300 and 2400 U kg^{-1}) in striped bass diet and concluded that 800 U kg^{-1} diet improved phosphorus digestibility but optimal tissue phosphorus may need at least 1000 U kg^{-1}.

When comparing apparent digestibility values between different studies, care should be taken, because dietary level of nutrient and nutrient interaction with

other nutrients as well as differences in the species and experimental conditions may influence apparent digestibility (Papatryphon and Soares, 2001). Digestible phosphorus for diets supplemented with more than 1000 U kg^{-1} phytase and phytase pre-treated SPC diet was between 4.30 to 4.98 g kg^{-1} diet, and was sufficient for bone mineralisation and growth improvement in Atlantic salmon.

4.5.3.4. Zinc

Zn digestibility was lower in fish fed control diet and fish fed diets containing less than 1000 U kg^{-1}. Phytate binds with Zn and reduces its availability (Gatlin and Wilson, 1984; Richardson et al., 1985; Gatlin and Phillips, 1989; Satoh, et al., 1989). Inclusion of 1.5% sodium phytate significantly reduced Zn bioavailability in blue tilapia (*Oreochromis aureus*) (McClain and Gatlin, 1988). Zn digestibility increased when phytase is used in plant meal based diets (Carter and Hauler, 1998a; Storebakken et al., 1998). Also Zn digestibility of different ingredients increased when phytase was used with ingredients in a reference diet (Cheng and Hardy, 2002). Apparent digestibility of Zn was significantly higher in the diets containing low phytate corn in comparison with diet containing ordinary corn (Sugiura et al., 1999). Zn and Ca apparent digestibility increased by phytase pre-treatment of soybean meal or phytase inclusion of diet at levels 500-4000 U kg^{-1} (Sugiura et al., 2001). Zn utilisation was increased by phytase treatment of soy protein concentrate (Vielma et al., 2002). Zn digestibility also marginally improved in striped bass (*Morone saxatilis*) fed a high phytate diet (Papatryphon et al., 1999). However, in contrast, Bransden and Carter (1999) did not find differences in Zn digestibility between phytase containing diet and control diet and phytase did not increase Zn digestibility in Rainbow trout (Vielma et al., 1998).

In the present study, however Zn level in the diets was above salmonids requirement (NRC, 1993), with increasing level of supplemented phytase, Zn digestibility increased despite the fact that when excess amount of nutrients are used in the diets, the absorption coefficient is decreased (Papatryphon et al.,

1999). Gatlin and Wilson (1984) showed that when a diet with 1.1% natural phytate content used in channel catfish, Zn requirement was about 7 times more than catfish requirement for available Zn. So, even dietary Zn level was more than requirement in the present study, the presence of phytic acid and its binding with Zn probably reduced its availability and increased Zn requirement level.

4.6. Conclusion

Both phytase pre-treatment and supplementation influenced feed intake, growth performance, body composition and nutrient digestibility in the present study. Pre-treatment was more effective than supplementation; at least 1000 U phytase kg^{-1} diet was needed for supplementation whereas 250 U phytase kg^{-1} diet was enough using pre-treatment.

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CHAPTER 5

INTAKE AND APPARENT DIGESTIBILITY OF PHOSPHORUS IN ATLANTIC SALMON (*SALMO SALAR*, L.)

5.1. Abstract

The effect of phosphorus intake on apparent digestibility of phosphorus in Atlantic salmon was investigated in a 6-week trial. Six semipurified diets were formulated to contain phosphorus levels ranging form 3.7 to 21.0 g kg^{-1} (DM basis). Each diet was fed to triplicate groups of fish. The salmon become infected with *Flexibacter* and experiment was terminated after 6 weeks. However, prior to termination, phosphorus digestibility was measured by settlement on days 29, 30, 31. Phosphorus digestibility ranged between 22 and 86% and decreased with increasing dietary phosphorus concentration (r^2=*0.82*). Consequently, the digestible phosphorus content of the diets was 4.45 ± 0.35 g kg^{-1} despite the fact that dietary phosphorus ranged from well below to well above the requirement level.

Keywords: Atlantic salmon, Phosphorus digestibility, Phosphorus intake

Note: *originally, this experiment was designed to assess phosphorus requirement of Atlantic salmon smolt at high temperature feeding a low FCR diet. Unfortunately the fish become infected with Flexibacter and the experiment was terminated after 6 weeks. Therefore, it was decided to analyse existing data on phosphorus digestibility and write the chapter accordingly.*

5.2. Introduction

Phosphorus is an essential mineral that is involved in nutrient metabolism as well as a key component of skeletal tissues (NRC, 1993; Lall, 2002). Phosphorus flux divides into three pathways: phosphorus retention as growth; and losses via the faeces and urine (Lall, 1991; Sugiura et al., 2000). Environmental phosphorus load (excretion) can be controlled by restricting the level of phosphorus in the diet, limiting the use of ingredients supplying excess phosphorus and increasing feed efficiency. Dietary calcium concentrations influence phosphorus availability in some fish (Nakamura, 1982), however high dietary calcium intake did not affect phosphorus digestibility in Atlantic salmon (Vielma and Lall, 1998a).

Generally, phosphorus digestibility is relatively low (< 50%) and most of the excess phosphorus (60-90%) will be excreted via the faeces. Faecal phosphorus losses in rainbow trout and brown trout constituted 55-73% of total wastes (Dosdat et al., 1998) and generally, faecal phosphorus loss is higher than excretory losses (Cho et al., 1991; Dosdat et al., 1998). Changes in diet formulation may significantly change phosphorus availability (Riche and Brown, 1999). If the original dietary phosphorus was high then decreased dietary phosphorus may increase phosphorus availability (Storebakken et al., 2000). Generally, faecal phosphorus will increase with increasing phosphorus intake (Riche and Brown, 1996; Rodehutscord, 1996; Satoh et al., 1997; Vielma and Lall, 1998b; Coloso et al., 2001). Solid and dissolved phosphorus waste decreased with decreasing dietary phosphorus in rainbow trout (Green et al., 2002). When dietary phosphorus increased from suboptimal level to requirement level, plasma phosphate increased, but when dietary phosphorus increased above requirement level, plasma phosphate did not increase due to phosphorus homeostasis in Atlantic salmon (Vielma and Lall, 1998b). Avila et al. (2000) found that concentration of inorganic phosphorus in the luminal fluid was the same at different levels of dietary phosphorus when dietary inorganic phosphorus was less than the requirement, but when dietary phosphorus level increased beyond

requirement level, luminal fluid phosphorus increased with increasing level of dietary phosphorus.

In the present study the effect of phosphorus intake on phosphorus digestibility was investigated in Atlantic salmon. Six levels of dietary phosphorus from a deficient level up to three times more than established requirement (NRC, 1993) were used as treatments. This experiment was undertaken to assess the effect of dietary phosphorus on phosphorus digestibility and faecal excretion of phosphorus in Atlantic salmon.

5.3. Materials and methods

5.3.1. Experimental diets

Six semipurified diets were formulated to be isonitrogenous (490 g kg^{-1} crude protein) and isoenergetic (21.13 MJ kg^{-1}gross energy). Graded levels of inorganic phosphorus (diabasic sodium phosphate [Na_2HPO_4]) were added to the diets to make diets with phosphorus content ranging from 3.7 to 21.0 g kg^{-1} total phosphorus. Purified ingredients, Casein (ICN Biomedicals, Australasia Pty. Ltd. Seven Hills NSW, Australia), Gelatin (ICN Biomedicals, Australasia Pty. Ltd. Seven Hills NSW, Australia) and essential amino acids (arginine, histidine, lysine, methionine, phenylalanine and threonine, [Sigma Chemical Co., Castle Hill, NSW, Australia]) were used as the source of protein in the diets (Shearer et al., 1993). Pre-gelatinized starch was used as the carbohydrate source. Disodium phosphate (Na_2HPO_4) was substituted in place of alpha cellulose to obtain six levels of supplemented phosphorus in the diets. The composition of the experimental diets is shown in Table 5.1.

Dry ingredients were mixed in a Hobart mixer and then mixed with water for 10 min before being pelleted (pellet diameter: 4 mm) with a laboratory pelletizer (California Laboratory Pellet Mill) and dried at 30 °C for 24h, then stored in a cold room (4 °C) until fed.

5.3.2. Growth experiment

The experiment was performed at the School of Aquaculture, University of Tasmania. Atlantic salmon (out of season smolts) were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia) and transferred to the Aquaculture Centre, School of Aquaculture. Fish were kept in 300-l circular tanks in a recirculation system in seawater where water quality was maintained through physical and biological filters (Sajjadi and Carter, 2004). Fish were maintained on a commercial feed (4mm Salmon, Skretting, Cambridge, Tasmania, Australia) for four weeks in the seawater to acclimate the smolts to the salt water and complete smoltification. Water replacement rate in the system was about 10% d^{-1} and water flow through the tanks was 8-10 l min^{-1}. Salinity was 32.7 ± 1.1 ppt during the experiment period. Oxygen concentration was 90.0 ± 0.5% of saturation, average water temperature was 18.4 ± 0.2 °C and a photoperiod (16L: 8D) was in effect over the 42-day experiment.

At the beginning of experiment, fish were anaesthetised in benzocaine (50 mg l^{-1} ethyl-p-aminobenzoate, Sigma Chemical Co., Castle Hill, NSW, Australia) and the wet weight and fork length of individual fish measured. Twenty fish (118.0 ± 0.2 g, mean ± S.E.M, n=144) were allocated to each tank. Triplicate groups of fish were fed each experimental diet to satiation twice daily at 0900 and 1700 h, satiation was judged to have been reached when approximately 20 pellets were not eaten and lost via the outlet water. Uneaten pellets were collected by a mesh collector placed in the outlet water (Helland et al., 1996) and feed intake calculated by subtracting the number of uneaten from supplied pellets. Feed intake was monitored in this way every day throughout the experiment. Fish in each tank were anaesthetised, counted and bulk-weighed every three weeks. Fish were not fed on the day of weighing to avoid the inclusion of ingested feed in the weight measurement. At the end of the 6-week experiment, the fish were fasted for 24 h and the wet weight and fork length measured for all individual fish.
Feed efficiency ratio (FER) was calculated as:

FER (g g^{-1} DM) = total weight gain (g) / total feed intake (g DM) [5.1]

5.3.3. Apparent digestibility

Apparent digestibility (AD) was measured during the growth experiment. Ytterbium oxide (1 g kg^{-1}) was added to the diets as an inert marker (Sugiura et al., 1998). The fish were fed the diets containing ytterbium oxide for one week and then faecal samples were collected by Guelph-type settlement collectors (Carter and Hauler, 2000) for three days. One hour after feeding at 1600 h, the collectors were washed of uneaten food and faeces collected from that time until 1 h before next feeding time (0900h). Faeces were frozen, freeze-dried and used in the analysis of yttrium oxide and nutrients (see below). The faecal samples from the three collection days were pooled in equal weights for each tank and frozen at –20 °C until analysis. AD was calculated according to:

AD (%) = 100 – [100 (% I diet / % I faeces) × (% N faeces / %N diet)] [5.2]

(Maynard and Loosli, 1969), where I is the inert marker and N is the nutrient.

5.3.4. Chemical analysis

Chemical analysis of diets was performed according to standard methods: dry matter (freeze dry to constant weight); ash (AOAC, 1995); crude fat (Bligh and Dyer, 1959); crude protein (Kjeldahl using a selenium catalyst [N × 6.25]) and energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid). For analysis of the ytterbium marker in diets and faeces, 5 ml of HNO_3 was added to 100 mg samples in digestion tubes and boiled. After cooling, the samples were diluted to 10 ml with distilled water and ytterbium content of diets and faeces

measured by flame atomic absorption. Phosphorus in the diet and faeces was analysed by the molybdovanadate method (AOAC, 1995).

5.3.5. Statistical analysis

Mean values are reported ± Standard Error of the Mean (S.E.M). Comparison between means was by one-way ANOVA after confirming the normality and homogeneity of variance (SPSS, version 11.5). Multiple comparison was by Tukey. Differences were considered significant at $P < 0.05$. The relationship between dietary phosphorus and phosphorus digestibility was modelled using regression analysis, and best-fit curve was chosen according to its estimated reliability with checking R-square value (Microsoft Excel, 2000).

Table 5.1. Ingredient and chemical composition of experimental diets

Ingredient composition g kg^{-1}	P4	P6	P8	P11	P17	P21
Casein	400	400	400	400	400	400
Gelatin	100	100	100	100	100	100
Pre-gel starch	120	120	120	120	120	120
Fish oil	228	228	228	228	228	228
Amino acid mix[1]	41	41	41	41	41	41
Vitamin mix[2]	3	3	3	3	3	3
Mineral mix[3]	3	3	3	3	3	3
Stay C[4]	3	3	3	3	3	3
Choline chloride	8	8	8	8	8	8
Sipernat ® 50S	17	17	17	17	17	17
Carboxymethyl cellulose	20	20	20	20	20	20
Alpha cellulose	55	44.4	35	26	2	0
Na_2HPO_4	0	10.6	20	29	53	75
Ytterbium (III) oxide	1	1	1	1	1	1
Yttrium (III) oxide	1	1	1	1	1	1
Total	1000	1000	1000	1000	1000	1000
Chemical composition (g kg^{-1}DM)						
Dry matter (g kg^{-1})	924	940	924	937	916	930
Crude protein	490	489	490	489	488	486
Crude fat	233	234	232	235	234	234
Gross energy (MJ kg^{-1} DM)	21.13	21.14	21.13	21.15	21.13	21.14
Ash	30.3	39.5	48.5	57.3	79.8	98.0
Phosphorus (calculated)	3.37	5.68	7.74	9.70	14.87	19.74
Phosphorus (measured)	3.70	6.16	7.96	10.59	16.76	20.96

[1] Arginine, 10g; Histidine, 2g; Lysine, 10g; Methionine, 4g, Phenylalanine, 5g; Threonine, 10g.
[2] Supplied (mg kg^{-1} diet): 15.00, Vitamin A acetate; 18.00, Vitamin D3 powder; 300.00, Rovimix E50; 6.00, Menadione sodium bisulphate; 12.00, Riboflavin; 65.22, Calcium D-pantothenate; 30.00, Nicotinic acid; 0.03, Vitamin B12; 0.45, d-Biotin; 3.00, Folic acid; 3.37, Thiamin HCl; 10.98, Pyridoxine HCl; 900.00, Myo-inositol.
[3] Supplied (mg kg^{-1} diet): 70, $CuSO_4$ $5H_2O$; 1089.3, $FeSO_4$ $7H_2O$; 184.5, $MnSO_4$ H_2O; 1.98, Na_2SeO_3; 395.82, $ZnSO_4$ $7H_2O$; 4.32, KI; 28.62, Co SO_4 $7H_2O$.
[4] L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia Ltd, Sydney, Australia).

5.4. Results

5.4.1. Growth performance

The fish become infected with *Flexibacter* sp. and the feed intake and growth were low (Table 5.2). Mean weight gain was from 6.2 to 15.0 g $fish^{-1}$ during 42-day period of trial. There were no differences in feed intake, growth, FER and survival between different treatments (Table 5.2). The coefficient of variation (CV) of weight substantially increased during the six-week trial (Fig. 5.3). Mean of coefficient of variation of initial weight was 9.39 ± 0.29%, and increased to 37.86 ± 1.93%. Survival was between 83.3 and 95.8%. Generally, mortality was not high during 6-week trial and a total of 9.7% of fish died during this period irrespective of treatment. The incidence of disease symptoms in the fish was 49.4% (Fig. 5.1 and 5.2). In addition, some fish showed a deformed lower jaw and shortened operculum. There was an overall incidence of 4.7 ± 1.65% of jaw deformity, with no treatment effect.

5.4.2. Disease

After transferring the fish to seawater and during the smoltification period, fish appeared to loose a considerable amount of scales (personal observation, relative to previous experiments). Circular shallow erosions, especially on lateral and dorsal surfaces of the skin but also the fins and head, were observed. Fin rot, especially of pectoral and dorsal fins was also observed. Erosion and haemorrage was also observed in buccal cavity, jaw and abdominal part of body. Water samples were taken 3 times (days 1, 12, 30) from the system and plated on culture plates of Marine Sheiths Agar (MSA) and *Vibrio* Agar. Vibrio plates were positive on all 3 occasions. Also, on all MSA plates, there was positive growth for *Flavobacteria*. Disease symptoms indicated that there was bacterial infection most possibly a mixture of *Flexibacter* and *Vibrio* spp.. Unfortunately, identification

using microbiological techniques was not completed in order to identify the disease.

5.4.3. Phosphorus digestibility

Apparent digestibility of phosphorus was significantly ($P<0.05$) different between different diets (Table 5.3). There were no significant differences in phosphorus digestibility between diets P4 and P6. Also, there were no significant differences between diets P6 and P8 or P8 and P11. There were no significant differences between diets P11, P17 and P21 (Table 5.3).

Furthermore, phosphorus digestibility decreased with increasing dietary phosphorus concentration (r^2=0.82) (Fig. 5.4). There was no relationship between phosphorus digestibility (%) and phosphorus intake (mg g fish^{-1} day^{-1}) (r^2=0.05). Fig. 5.5 shows the level of digestible phosphorus in fish fed different diets. Except the lowest dietary phosphorus, digestible phosphorus was the same in fish fed different dietary phosphorus. Digestible phosphorus content of the diets was about 4.45 ± 0.35 g kg^{-1}. The relationship between digestible phosphorus (mg g fish^{-1} day^{-1}) and phosphorus intake (mg g fish^{-1} day^{-1}) has been shown in fig.5.6.

Table 5.2

The performance of Atlantic salmon fed diets containing different phosphorus levels for 42 days (mean ± S.E.M, n=3)

Parameter	Unit	Phosphorus level						
		P4	P6	P8	P11	P17	P21	*P*
Initial weight	(g)	117.7	118.1	118.2	117.0	118.7	118.2	*ns*
		0.6	0.4	0.5	0.4	0.6	0.6	
Final weight	(g)	125.6	131.9	123.7	132.0	125.7	124.4	*ns*
		7.5	8.6	2.5	8.9	8.9	2.4	
Weight gain	(g)	7.9	13.8	5.5	15.00	7.0	6.2	*ns*
		7.3	8.4	3.0	9.1	8.4	1.8	
Total feed intake	(kg DM)	0.2	0.20	0.16	0.21	0.17	0.16	*ns*
		0.03	0.06	0.02	0.04	0.02	0.01	
FER	($g\ g^{-1}$	0.26	0.46	0.20	0.47	0.17	0.27	*ns*
	DM)	0.0.23	0.18	0.07	0.29	0.23	0.07	
Survival	(%)	95.8	95.8	87.5	87.5	83.3	91.7	*ns*

Table 5.3
Apparent digestibility (%) of phosphorus (P) for Atlantic salmon fed diets containing different phosphorus levels for 42 days (mean ± S.E.M, n=3)

Parameter	Unit	Phosphorus level						
		P4	P6	P8	P11	P17	P21	*P*
AD_P	(%)	86.34^{a} 2.50	73.44^{ab} 2.34	53.18^{bc} 1.63	43.03^{cd} 7.77	26.17^{d} 3.19	21.67^{d} 8.64	*< 0.05*

Table 5.4
Occurrence of deformed jaw and short operculum (%) in Atlantic salmon fed diets containing different phosphorus levels for 42 days (mean ± S.E.M, n=3)

Parameter	Unit	Phosphorus level						
		P4	P6	P8	P11	P17	P21	*P*
Deformed jaw	(%)	4.17 4.17	4.17 4.17	6.66 6.66	4.17 4.17	4.76 4.76	0	*ns*
Short operculum	(%)	4.17 4.17	0	0	0	0	0	*ns*

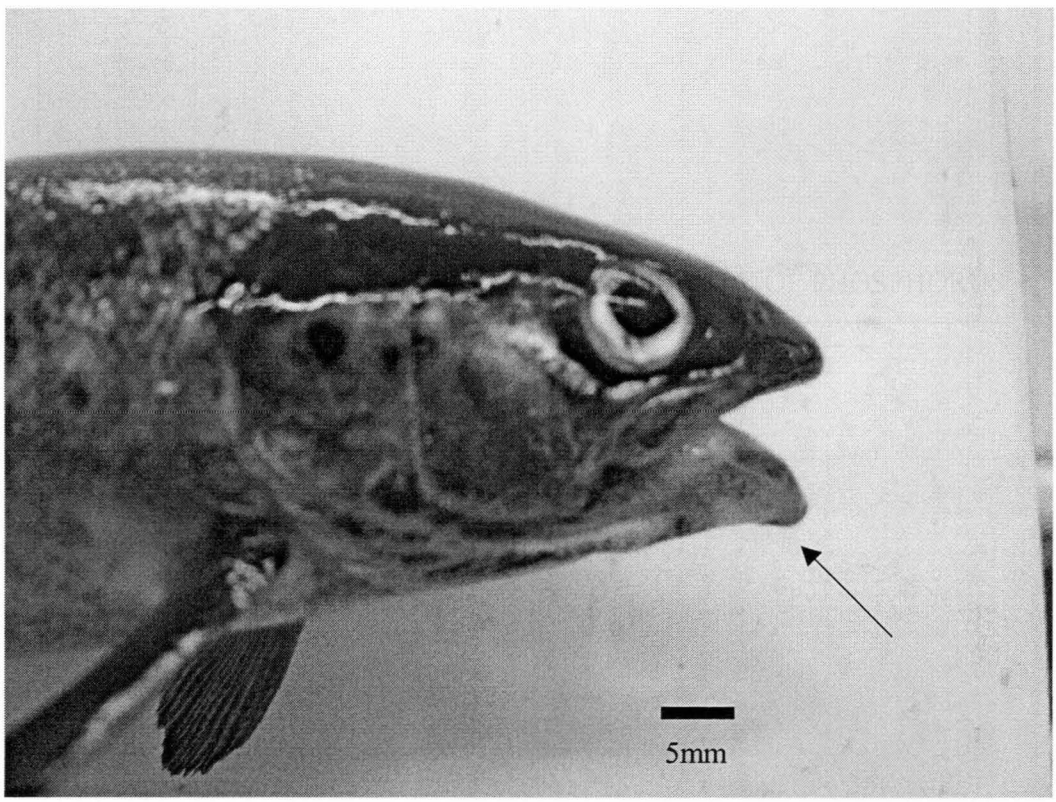

Figure 5.1. Deformed lower jaw in Atlantic salmon transferred to seawater at high temperature (day 42)

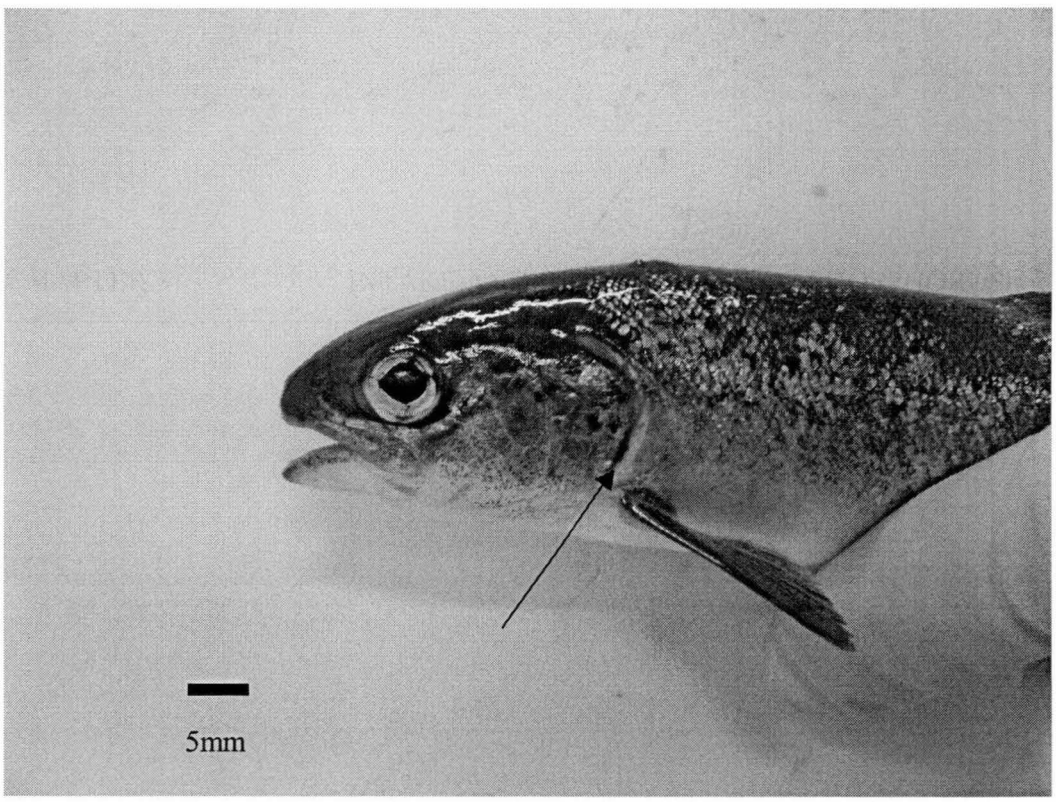

Figure 5.2. Shortened operculum in Atlantic salmon transferred to seawater at high temperature (day 42)

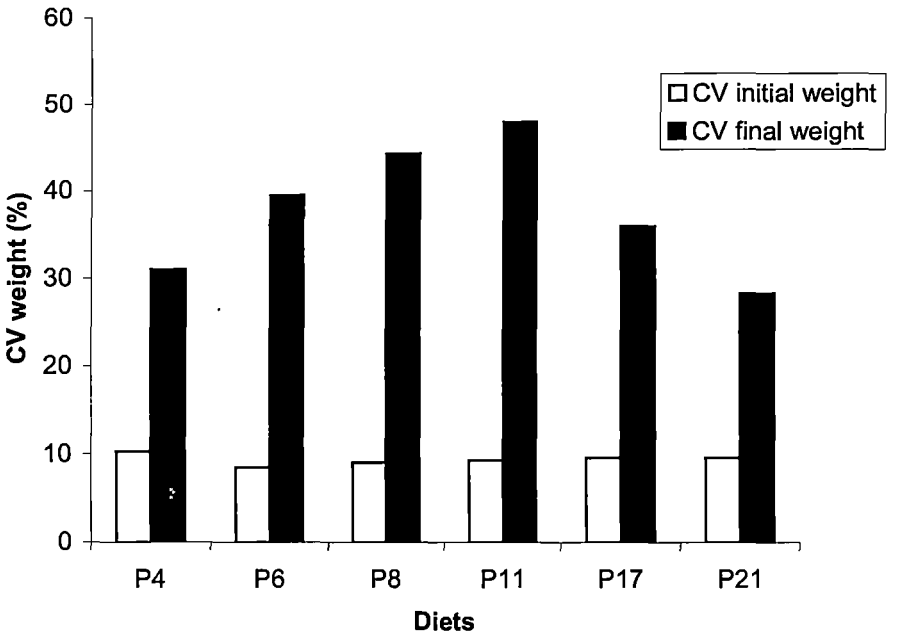

Figure 5.3. Inter-individual differences in initial weight (CV initial weight) and final weight (CV final weight) in fish fed different diets (mean; n=3)

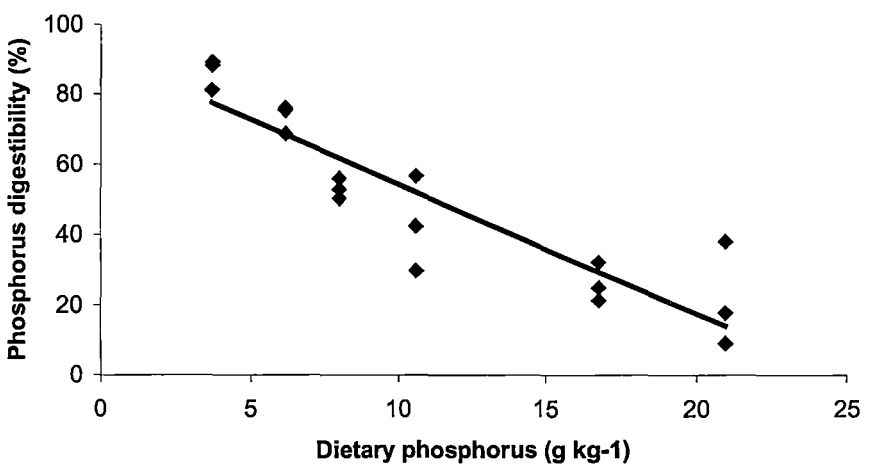

Figure 5.4. Correlation between dietary phosphorus (%) and apparent digestibility of phosphorus in fish fed different diets (y=-3.68x+91.24; n=18; r^2=0.82)

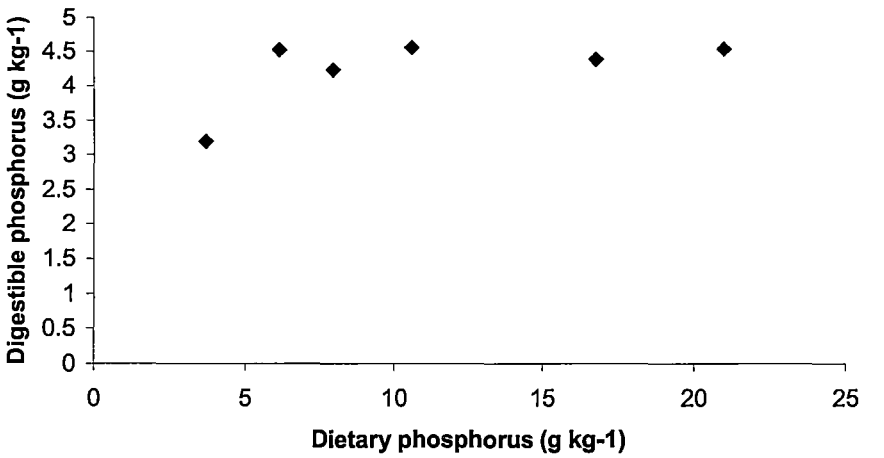

Figure 5.5. Digestible phosphorus values for Atlantic salmon as a function of dietary phosphorus concentration

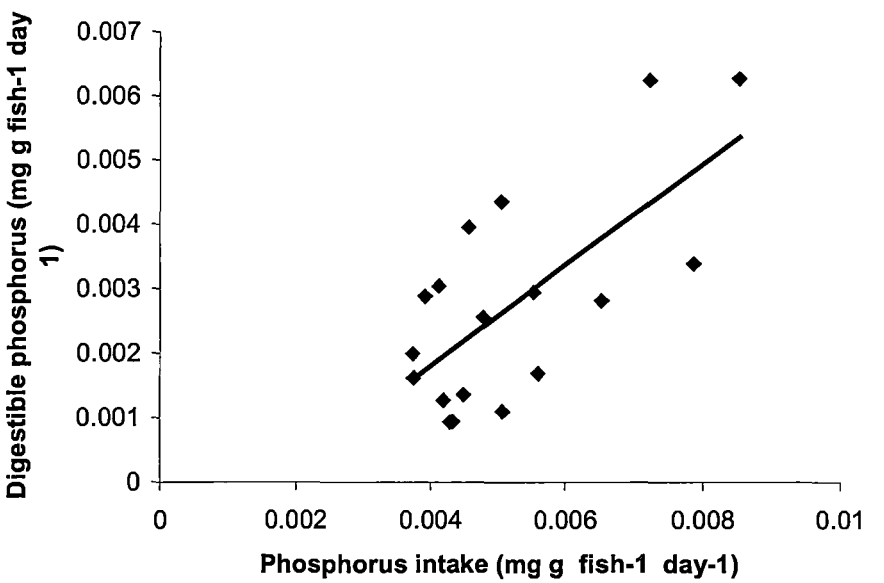

Figure 5.6. Correlation between phosphorus intake (mg g $fish^{-1}$ day^{-1}) and digestible phosphorus (mg g $fish^{-1}$ day^{-1}) (y=0.78x-0.0013; n=18; r^2=0.47)

5.5. Discussion

5.5.1. Growth performance and disease

The fish did not grow well and feed intake and FER were very low during the experiment. Variation within treatments was high and there were no significant differences in these criteria between different diets. This was most likely due to bacterial infection of fish. The first outbreak of *Flexibacter* in Tasmanian salmon farms occurred during 1988-1989 at high temperature (21° C) (Handlinger et al., 1997). Water temperature in the present study was 18° C and incidence of infection was not significantly different among the fish fed different dietary phosphorus. Thus, phosphorus deficiency was not thought to be the cause of the disease. Phosphorus deficiency did not have substantial negative effects on the immune system of whitefish (*Coregonus laveratus* L.) (Jokinen et al., 2003).

Approximately 5% of fish had a deformed lower jaw. This situation was observed previously in Atlantic salmon in Tasmania (Jungawalla, 1991; Hughes, 1992; Sadler et al., 2001) and Chile (Roberts et al., 2001). Several factors such as genetics, environmental conditions, presence of heavy metals, and nutritional deficiencies may cause lower jaw deformity or other skeletal deformity (Hickey, 1972; McKay and Gjerde, 1986). Sadler et al. (2001) observed higher incidence of skeletal deformities in triploid Atlantic salmon in Tasmania and suggested that one of the reasons might be the lower capacity of triploid fish to metabolise, absorb and utilise the nutrients such as dietary minerals and vitamins. Roberts et al. (2001) suggested that this pathological symptom might be a sign of phosphorus deficiency in Atlantic salmon. In the present study, there were no significant differences in occurrence of lower jaw deformity in fish fed different level of dietary phosphorus and the incidence was low (5%). However, the trial was very short, used relatively large fish which suffered the disease outbreak as noted.

5.5.2. Phosphorus digestibility

Apparent digestibility of nutrients and some amino acids decreased when fish were infected by *Aeromonas salmonicida* (Neji et al., 1993; Neji and de la Noüe, 1998). No information on the effect of disease on phosphorus digestibility was found. Apparent phosphorus digestibility was the highest in the fish fed the lowest dietary phosphorus and lowest in fish fed the highest dietary phosphorus. According to NRC (1993), phosphorus requirement of Atlantic salmon is 6.0 g kg^{-1} diet. The level of digestible phosphorus in the present experiment was between 3.2-4.6 g kg^{-1}. Excluding the lowest phosphorus diet, digestible phosphorus in other treatments was between 4.2 and 4.6 g kg^{-1} with a mean value of 4.45 g kg^{-1}. Despite the widely different levels of phosphorus in the diets, from well below requirement (3.7 g kg^{-1}) to well above it (21.0 g kg^{-1}), digestible phosphorus was relatively similar across the different diets. Regulation of phosphorus absorption occurs in the intestine and phosphorus absorption rate decreased with dietary phosphorus concentration (Avila et al., 2000).

An interesting observation from the present study was the negative relationship between dietary phosphorus concentration and apparent digestibility of phosphorus in the fish. Rodehutscord et al. (2000) found a non-linear correlation between phosphorus intake and faecal phosphorus excretion. They also found non-linear correlation between phosphorus intake and phosphorus apparent digestibility. Riche and Brown (1996) found that phosphorus apparent digestibility increased with increasing level of dietary phosphorus up to requirement level and then decreased beyond the requirement level in rainbow trout. In the present study there was not a non-linear relationship between dietary phosphorus concentration and phosphorus apparent digestibility and the relationship was linear.

5.6. Conclusion

Phosphorus digestibility in sick fish negatively correlated with phosphorus intake in Atlantic salmon.

5.7. References

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CHAPTER 6

EFFECT OF RATION ON NUTRIENT DIGESTIBILITY IN ATLANTIC SALMON (*SALMO SALAR*, L.)

6.1. Abstract

A digestibility trial was conducted to examine the effect of ration on dry matter, gross energy, crude protein and phosphorus digestibility in Atlantic salmon. Duplicate groups of fish were fed 0.25, 0.5, 0.75, 1.0, 1.25 and 1.9% BW day^{-1}. The faeces were collected by guelph-type collectors for five successive days. Dry matter, protein and phosphorus digestibilities were all significantly ($P < 0.05$) affected by ration. Dry matter digestibility was significantly lower in fish fed 0.25% BW day^{-1} in comparison with fish fed 0.5, 0.75 and 1% BW day^{-1}. Protein digestibility was significantly lower in fish fed 0.25% BW day^{-1} in comparison with 1.25% BW day^{-1}. Phosphorus digestibility was significantly lower in fish fed 0.25% BW day^{-1} in comparison with all other treatments except 1% BW day^{-1}. There were no significant differences for energy digestibility between fish fed different rations. The main effect was reduced digestibility at the lowest ration with no obvious relationship between ration and digestibility above this. This was explained by a relatively higher loss of endogenous faecal nitrogen and phosphorus at sub-maintenance ration.

Keywords: Atlantic salmon, Phosphorus digestibility, Ration

6.2. Introduction

Considerable information is available on nutritional effects on digestibility particularly in relation to nutrient and ingredient composition. In contrast, biological and environmental factors affect nutrient digestibility, but their effects are not well defined and results are contradictory (De Silva and Anderson, 1995; Fernández et al., 1998; Bureau et al., 2002). Endogenous (biological) factors such as animal size and age or exogenous (environmental) factors such as temperature and salinity may also affect the digestibility (Jobling, 1993; Jobling, 1994; De Silva and Anderson, 1995; Guillaume and Choubert, 2001; Bureau et al., 2002). Studies on the effect of ration on nutrient digestibility in fish are limited (Windell et al., 1978; Henken et al., 1985; Cho and Kaushik, 1990; Cui et al., 1994; Cui et al., 1996; Yamamoto et al., 2001) and sometimes controversial (Fernández et al., 1998).

Digestibility is generally assumed independent of ingestion rate (Guillaume et al., 2001). Feed intake did not affect dry matter, gross energy, crude protein, lipid or phosphorus apparent digestibility (Cho and Kaushik, 1990; Fernández et al., 1998). However, some studies have shown that ration size influenced nutrient digestibility (Windell et al., 1978). Rainbow trout fed the highest ration had significantly lower digestibility for dry matter, energy and carbohydrate but not for protein and lipid digestibility. Apparent digestibility of dry matter, energy, protein or lipid decreased with increasing ration (Henken et al., 1985; Yamamoto et al., 2001). In contrast, Cui et al. (1996) found higher energy digestibility at the highest ration and they did not find differences among other rations.

The purpose of this study was to investigate the effect of ration on nutrient, especially phosphorus, digestibility in Atlantic salmon. Six rations were selected from below maintenance to slightly greater than satiation level.

6.3. Materials and methods

6.3.1. Experimental diets

The diet was formulated to contain 48% crude protein, 22.5 MJ kg^{-1}gross energy and 0.7% phosphorus (Table 6.1). All nutrient requirements of Atlantic salmon were satisfied based on values for salmonids (NRC, 1993). Phosphorus concentration was set at the requirement level (0.7%). South American fish meal (Skretting, Cambridge, Tasmania, Australia), blood meal (Skretting, Cambridge, Tasmania, Australia), and casein (New Zealand Dairy Board, Wellington, New Zealand) were used as protein sources in the diet. Dietary ingredients were mixed using a Hobart mixer, pelleted with a laboratory pelletizer (California Laboratory Pellet Mill) and the pellets dried at 30 °C for 24 h. The diets were refrigerated at 4 °C until use.

6.3.2. Fish maintenance and feeding regime

The experiment was performed at the School of Aquaculture, University of Tasmania. Atlantic salmon (*Salmo salar* L.) were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia) and transferred to the Aquaculture Centre, School of Aquaculture. Fish were kept in 300-l circular tanks in a recirculation system where water quality was maintained through physical and biological filters (Bransden et al., 2001). Fish were maintained on a commercial feed (3mm Salmon, Skretting, Cambridge, Tasmania, Australia) for seven days. Water replacement rate in the system was about 20% d^{-1} and water flow through the tanks was 8-10 l min^{-1}. Oxygen concentration was 90.0 ± 0.7% of saturation, average water temperature was 14.9 ± 0.8 °C and a natural photoperiod (approximately, 9L: 15D) was in effect over the trial.
After the acclimation period, the fish were fed to satiation with the experimental diet for ten days and feed intake was determined for two successive days and the data used to set the ration for the experiment. At the beginning of experiment, fish

were anaesthetised in benzocaine (50 mg l^{-1}) and the wet weight of individual fish measured. Twenty fish (156.4± 0.20 g) were kept in each tank. Rations were set at 0.25, 0.5, 0.75, 1.0, 1.25 and 1.9% BW day^{-1}. Duplicate groups of fish were fed each ration twice daily at 0900 and 1600 h by automatic feeders.

Feed efficiency ratio (FER) was calculated as:

FER (g g^{-1} DM) = total weight gain (g) / total feed intake (g DM) [6.1]

At the end of 10-day experiment, the fish were fasted for 24 h and the wet weight measured for individual fish.

6.3.3. Apparent digestibility

Apparent digestibility (AD) was measured over five successive days. Ytterbium oxide (1 g kg^{-1} diet) was added to the diets as an inert marker (Sugiura et al., 1998). The fish were fed the diets containing ytterbium oxide for nine days and on days 5, 6, 7, 8 and 9 faecal samples were collected by Guelph-type settlement collectors (Carter and Hauler, 2000). One hour after feeding at 1600 h, the collectors were washed of uneaten food and faeces collected from that time until 1 hour before next feeding time (0900 h). Faeces were frozen and then freeze-dried and used for the analysis of ytterbium oxide and nutrients (see below). The faecal samples from the five collection days were pooled in equal weights for each tank and frozen at –20 °C until analysis. AD was calculated according to Maynard and Loosli (1969):

AD (%) = 100 – [100 (% I diet / % I faeces) × (% N faeces / %N diet)]

Where I is the inert marker and N is the nutrient.

6.3.4. Chemical analysis

Chemical analysis of diets and faeces were performed according to standard methods: dry matter (freeze dry to constant weight); ash (AOAC, 1995); crude fat (Bligh and Dyer, 1959); protein (Kjeldahl using a selenium catalyst [N × 6.25]) and energy (bomb calorimeter: Gallenkamp Autobomb, calibrated with benzoic acid). For analysis of the ytterbium marker in diets and faeces, 5 ml of HNO_3 was added to 100 mg samples in digestion tubes and boiled. After cooling, the samples were diluted to 10 ml with distilled water and ytterbium content of diets and faeces measured by flame atomic absorption (Chapter 4). Phosphorus in the diet and faeces were analysed by the molybdovanadate method (AOAC, 1995).

6.3.5. Statistical analysis

Mean values are reported ± Standard Error of the Mean (S.E.M). Comparison between means was by one-way ANOVA after confirming the normality and homogeneity of variance (SPSS, version 11.5). Multiple comparison was by Tukey. Differences were considered significant at $P < 0.05$. The relationship between ration-growth and ration- FER were modelled using regression analysis, and best-fit curve was chosen according to its estimated reliability with checking R-square value (Microsoft Excel 2000). First, second, third and forth order regression analyses were conducted using SigmaPlot 2000 to investigate the relationship between ration and nutrient digestibility. Correlation matrices of nutrient digestibility were compared by Pearson correlation coefficient (SPSS, version 11.5).

Table 6.1. Ingredient and chemical composition of experimental diet

Ingredient composition (g kg^{-1})	Diet
Casein	272
Fish meal	200
Blood meal	110
Pre-gelatinized starch	150
Fish oil	165
Alpha cellulose	50
Bentonite	32
Carboxymethyl cellulose	10
Mineral mix[1]	3
Vitamin mix[2]	3
Choline chloride	1
Stay C[3]	3
Ytterbium oxide	1
Chemical composition (g kg^{-1} DM)	
Dry matter (g kg^{-1})	955
Crude protein	480
Crude fat	216
Ash	73
Gross energy (MJ kg^{-1} DM)	22.42
Total Phosphorus (g kg^{-1} DM)	7.8

[1]Supplied (mg kg^{-1} diet): 70, $CuSO_4$ $5H_2O$; 1089.3, $FeSO_4$ $7H_2O$; 184.5, $MnSO_4$ H_2O; 1.98, Na_2SeO_3; 395.82, $ZnSO_4$ $7H_2O$; 4.32, KI; 28.62, Co SO_4 $7H_2O$.

[2]Supplied (mg kg^{-1} diet): 15.0, Vitamin A acetate; 18.0, Vitamin D_3 powder; 300, Rovimix E50; 6.0, Menadione sodium bisulphate; 12.0, Riboflavin; 65.22, Calcium D-pantothenate; 30.0, Nicotinic acid; 0.03, Vitamin B12; 0.45, d-Biotin; 3.00, Folic acid; 3.37, Thiamin HCl; 10.98, Pyridoxine HCl; 900, Myo-inositol.

[3]L-Ascorbyl-2-polyphosphate (Roche Vitamins Australia Ltd, Sydney, Australia).

6.4. Results

Initial feed intake was 1.7% BW day^{-1}, so rations were selected across a broad range from below maintenance (0.25% BW day^{-1}) to slightly greater than satiation (1.9% BW day^{-1}). Weight gain was negative in fish fed 0.25% BW day^{-1} and significantly ($P < 0.05$) lower than fish fed 1.0, 1.25 and 1.9% BW day^{-1} (Table 6.2). Growth was positive at 0.5% BW day^{-1} ration and the relationship between ration and growth was curvilinear (Fig. 6.1). FER was negative in fish fed 0.25% BW day^{-1} and significantly lower than fish fed 1.25 and 1.9% BW day^{-1} (Table 6.2). Ration-FER relationship was curvilinear and FER peaked at the range of 1.25-1.5% BW day^{-1} ration and then decreased (Fig. 6.2).

Ration had no significant effect on apparent digestibility for gross energy (Table 6.3). The apparent digestibility for phosphorus, dry matter and protein between fish fed different rations were significantly different (Table 6.3). A 0.5 % BW day^{-1} ration was approximately the maintenance ration for these fish. Fish fed 0.5% BW day^{-1} ration had significantly higher dry matter digestibility in comparison with 0.25, 1.25 and 1.9% BW day^{-1}. Apparent digestibility for dry matter was lower for fish fed 0.25% in comparison with fish fed 0.5,0.75 and 1% BW day^{-1} ration. There were no significant differences in dry matter digestibility between fish fed 0.25, 1.25 and 1.9% ration. There were no significant differences in dry matter digestibility for fish fed 0.75, 1, 1.25 and 1.9% BW day^{-1} ration and nor between 0.5, 0.75 and 1% BW day^{-1} ration (Table 6.3). Protein digestibility was lower for fish fed 0.25% BW day^{-1} in comparison with fish fed 1.25% BW day^{-1}. There were no significant differences between other treatments for phosphorus digestibility. Phosphorus digestibility was lower in fish fed 0.25% BW day^{-1} in comparison with 0.5, 0.75, 1.25 and 1.9% BW day^{-1}. There were no significant differences in phosphorus digestibility between fish fed 0.25 and 1% BW day^{-1} and no significant differences between 1% BW day^{-1} and other treatments. There was a tendency for digestibility to be lower in 0.25% BW day^{-1} (lowest ration).

Correlation matrix of nutrient digestibility has been shown in Table 6.4. Highest correlation was found between crude protein digestibility and phosphorus digestibility (r=0.82). Correlation between crude protein and dry matter digestibility (r=0.72) and phosphorus and dry matter digestibility (r=0.79) were significantly high (P<0.01).

Although there were significant differences between digestibility at different rations, there were no clear relationships over the range of rations (Fig. 6.3). Digestibility data for dry matter, protein and phosphorous were best described (highest *r2* value) by third order polynomial regression but these showed relatively flat relationships with ration. Regression analysis was not used with energy because there were no differences between rations.

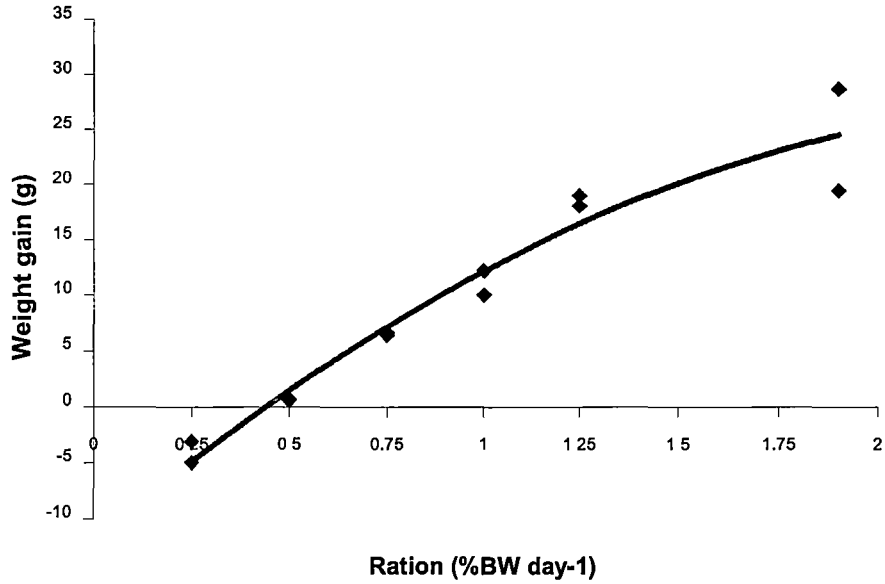

Figure 6.1. The relationship between ration and weight gain in Atlantic salmon ($y=-5.413x^2+29.497x-11.94$; $r^2=0.95$)

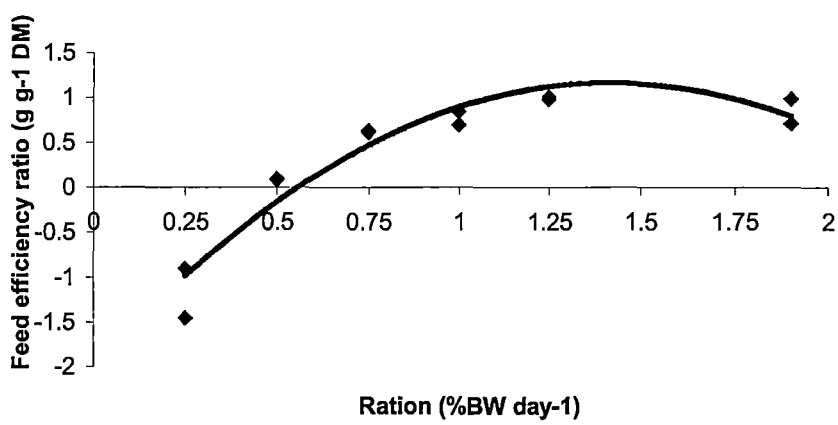

Figure 6.2. The relationship between ration and feed efficiency ratio in Atlantic salmon ($y=-1.5918x^2+4.4992x-2.009$; $r^2=0.92$)

Table 6.2. The growth performance of Atlantic salmon fed different rations (mean ± S.E.M, n=2)

Parameter	Unit	Ration (%BW day^{-1})						
		0.25	0.5	0.75	1.00	1.25	1.9	*P*
Initial weight	(g)	156.3 0.05	156.6 0.35	156.5 0.25	156.4 0.10	156.5 0.30	156.2 0.20	*ns*
Final weight	(g)	152.2^{a} 1.00	157.2^{ab} 0.35	163.0^{b} 0.15	167.5^{bc} 1.00	175.1^{cd} 0.15	180.3^{d} 4.40	*< 0.05*
Weight gain	(g)	-4.03^{a} 0.94	0.64^{ab} 0.05	6.51^{ab} 0.12	11.13^{bc} 1.09	18.52^{cd} 0.46	24.10^{d} 4.63	*< 0.05*
FER	(g g^{-1} DM)	-1.29^{a} 0.30	0.10^{b} 0.01	0.68^{bc} 0.01	0.84^{bc} 0.08	1.09^{c} 0.02	0.93^{c} 0.15	*< 0.05*
Survival	(%)	100	100	100	100	100	100	*ns*

Means with same letter are not significantly different (Tukey multiple comparison)

Table 6.3. Apparent digestibility (%) for dry matter (DM), energy (KJ), crude protein (CP) and phosphorus (P) in Atlantic salmon fed different rations (mean ± S.E.M, n=2)

Parameter	Unit	Ration (%BW day^{-1})						
		0.25	0.50	0.75	1.00	1.25	1.90	*P*
AD_{DM}	(%)	79.51^{a} 0.12	82.94^{c} 0.59	89.10^{bc} 0.04	81.43^{bc} 0.46	80.91^{ab} 0.01	80.92^{ab} 0.06	*< 0.05*
AD_{KJ}	(%)	88.56 0.79	89.58 1.30	89.65 0.32	88.63 0.63	89.02 0.75	88.69 0.87	*ns*
AD_{CP}	(%)	92.77^{a} 0.08	94.79^{ab} 0.75	94.87^{ab} 0.37	97.84^{ab} 0.36	95.10^{b} 0.04	94.74^{ab} 0.22	*< 0.05*
AD_{P}	(%)	39.81^{a} 1.49	55.88^{b} 3.75	52.28^{b} 1.13	49.76^{ab} 0.44	51.30^{b} 1.02	54.79^{b} 0.47	*< 0.05*

Means with same letter are not significantly different (Tukey multiple comparison)

Table 6.4. Correlation (r) matrix of nutrient digestibility in Atlantic salmon (n=12)

	AD			
AD	DM	kJ	CP	P
kJ	0.54			
CP	0.72*	0.47		
P	0.79*	0.41	0.83*	

* Correlation significant at $P < 0.01$ (selected to reduce probability of Type I error)

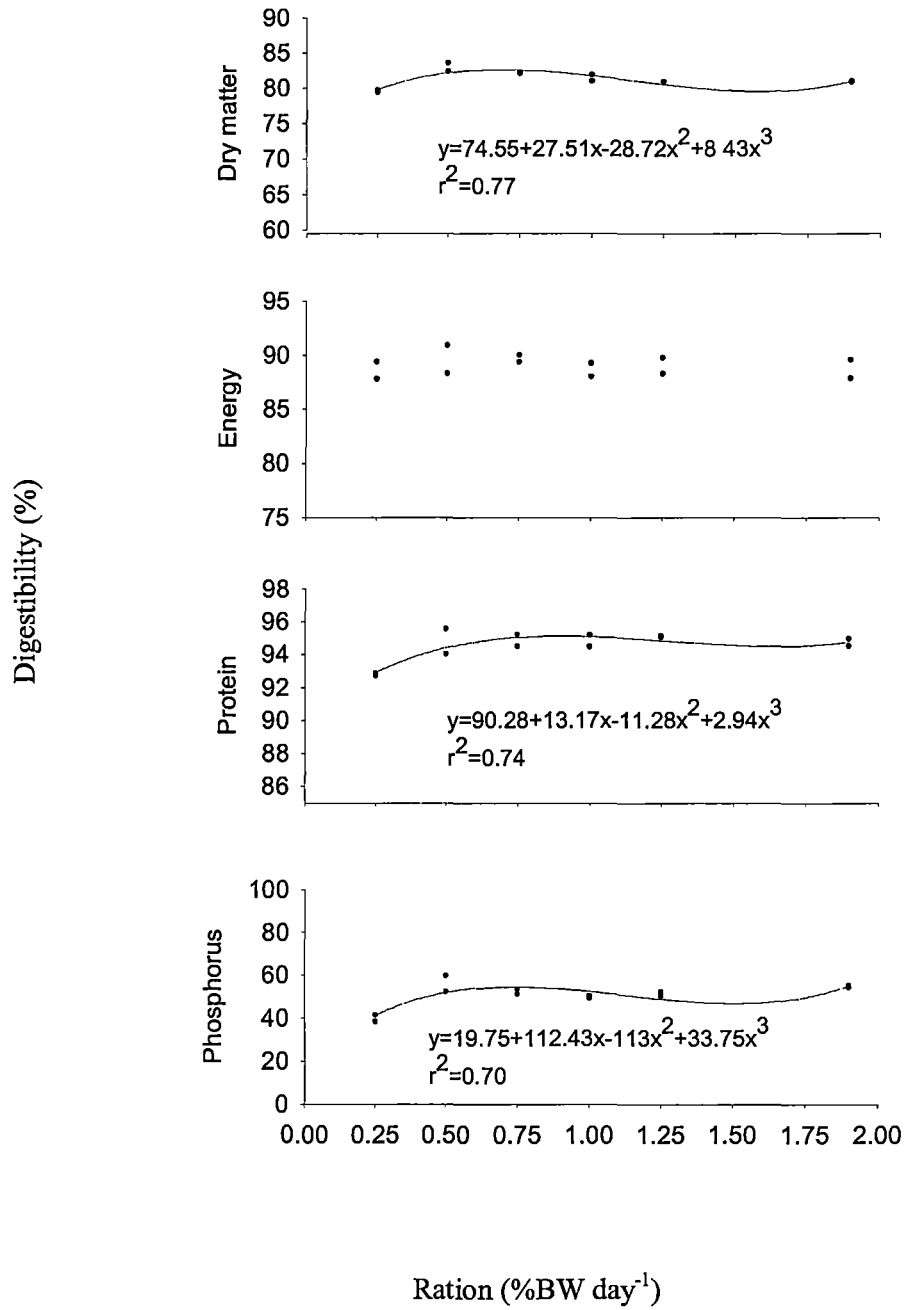

Figure 6. 3. The relationship between ration (%BW day^{-1}) and apparent digestibility (%) of nutrient in Atlantic salmon

6.5. Discussion

The diet used here had previously been shown to promote growth for Atlantic salmon, when salmon were fed to satiation, the SGR and FER were 1.30 and 1.22 respectively (Sajjadi and Carter, 2004). Generally, growth trials are conducted for 8-12 weeks, depending on fish size and growth rate. In the present study, despite the short length of the experiment, there were significant differences in growth and FER between different treatments. The fish fed 0.25% BW day^{-1} ration had a negative growth rate and feed efficiency ratio (FER). Regression relationships between ration-growth and ration-FER were typical (Jobling, 1994) as would be expected. In most fishes, the relationship between ration and wet weight growth is non-linear (Brett and Groves, 1979; Carter et al., 2001), but linear relationships were observed in some studies (Cui et al., 1994, 1996). In the present study, with increasing level of feed intake, growth increased and reached to plateau at highest ration (in excess). Maintenance ration was about 0.5% BW day^{-1}. FER increased with increasing level of feed intake and reached to a plateau and then decreased.

Ration influenced nutrient digestibility in the present study, but only in a minor way and there were no clear trends with increasing ration. The fish fed 0.25% BW day^{-1} ration had lowest digestibility values for protein, dry matter and phosphorus in the present study. Ration had no effect on energy digestibility. Ration level had no significant effect on apparent protein digestibility (Windell et al., 1978; Andrews, 1979; Storebakken and Austreng, 1987; Chakraborty et al., 1995) or lipid digestibility but significantly reduced energy, carbohydrate and dry matter digestibility in fish fed the highest ration (Windell et al., 1978).

Strong correlations were found between digestibility of protein and digestibility of dry matter. A large fraction of ingested dry matter is protein, so this would be expected. The high correlation between phosphorus digestibility and protein and dry matter digestibility could not be easily explained although it has also been observed in gilthead sea bream (*Sparus aurata*) (Fernández et al., 1998).

Henken et al. (1985) determined gross energy, dry matter and protein digestibility using three different methods in African catfish (*Clarias gariepinus*) and nutrient digestibility negatively correlated with feeding level. ADC for energy in rainbow trout (Andrews, 1979) dry matter and protein in Nile tilapia (*Oreochromis niloticus*) (Xie et al., 1997) and fat in agastric common carp (*Cyprinus carpio*) (Yamamoto et al., 2001) negatively correlated with increasing ration level. Yamamoto et al. (2001) found that protein and starch digestibility slightly decreased with increasing ration size. In contrast, Cui et al. (1994) found lower digestibility for protein at the lowest ration size and higher digestibility for dry matter at highest ration size in grass carp (*Ctenopharyngodon idella*) and there was a tendency for digestibility to increase at higher rations that was in agreement with the present study. Increasing ration level of highly digestible diets would not affect nutrient digestibility or have small effect on them. However, a substantial decrease in digestibility would be expected in low quality diets. This is one reason why the major effect of ration on digestibility results have been contradictory (Fernández et al., 1998).

Apparent protein digestibility may be lower at lower rations due to a relatively higher loss endogenous faecal nitrogen (Bureau et al., 2002). Generally, apparent protein digestibility is 2-3% lower than true digestibility due to endogenous faecal nitrogen, so when low protein diet are fed the endogenous faecal nitrogen will constitute a larger part of the total faecal nitrogen (Jobling, 1994). Lower protein digestibility (3%) in fish fed lowest ration in the present study is likely to be explained by this. Similarly, it is proposed that the lower phosphorus digestibility is attributed to relatively higher endogenous faecal phosphorus excretion.

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CHAPTER 7

GENERAL DISCUSSION

7.1. Overview of study

Feed ingredients of plant origin are being used at higher inclusion rates in aquafeeds for intensive aquaculture. The presence of phytate in plant protein ingredients is a major factor that limits their inclusion in aquafeeds. Successful application of phytase to increase phosphorus utilisation in poultry and pig diets has been reported (Nelson et al., 1968, 1971; Simons et al., 1990). While, inclusion of phytase in the terrestrial monogastric animals has been widely studied, fewer studies have been done on fish. Very limited information on the use of phytase in Atlantic salmon (*Salmo salar*, L.) feeds motivated this research. This study aimed to provide information on the effect of phytate and phytase within the context of understanding more about phosphorus utilisation in Atlantic salmon. Phosphorus is an essential nutrient for fish: It is a major component of bone, nucleic acid and cell membrane and directly involved in the cellular energy transformations as well as macronutrients metabolism (NRC, 1993). Phosphorus deficiency leads to poor growth, feed efficiency and bone mineralization (Sugiura et al., 2004), while insufficient phosphorus utilisation in fish leads to environmental pollution. Almost all phosphorus discharge from fish farms originates from feed, so dietary phosphorus manipulation or feeding management may influence phosphorus utilisation and excretion (Cho et al., 1994; Cho and Bureau, 1997; Cho and Bureau, 2001). Dietary phosphorus concentration and digestibility of phosphorus are the main factors affecting phosphorus load from aquaculture (Cho and Bureau, 2001).

Both purified phytic acid and natural sources of phytate (plant protein sources) were used in the present study to assess their effect on fish performance and utilisation of phosphorus and other nutrients. Phytase pre-treatment, supplementation and multiple levels of phytase were used in a series of experiments. Phytase neutralised negative effects of phytate and increased feed intake, growth performance and phosphorus and mineral utilisation from plant meal based diets for Atlantic salmon and substantially reduced environmental

phosphorus load. Furthermore, the effect of dietary phosphorus concentration and feed intake on phosphorus digestibility was investigated in the present study. Dietary phosphorus concentration and feed intake had significant effect on phosphorus digestibility and excretion. Phosphorus digestibility decreased with increasing dietary phosphorus content.

7.2. Purified phytic acid

Purified phytic acid was used in this study to investigate the direct effect of phytic acid and phytase on Atlantic salmon, this was to ensure observations were independent of other components in plant ingredients. Although purified phytic acid had been used in diets of different fish species (Spinelli et al., 1983; Richardson et al., 1985; Gatlin and Phillips, 1989; Satoh et al., 1989; Hossain and Jauncey, 1993; Usmani and Jafri; 2002), it had not been fed to Atlantic salmon. This study confirmed purified phytic acid had some of the negative effects on Atlantic salmon that found in other fish species. At the concentration added it did not affect feed intake and growth but did reduce protein and phosphorus digestibility (Chapter 2). The concentration of phytic acid (8g kg^{-1}) used was calculated to reflect inclusion from plant protein sources. The level of phytic acid in the diet was too low to affect growth performance (Satoh et al., 1989; Gatlin and Phillips, 1989; Richardson et al., 1985; Usmani and Jafri, 2002). Francis et al. (2001) suggested that salmonids are able to tolerate up to 5-6 g phytate kg^{-1} diet, while carp is sensitive to these levels. Feed intake was not affected by either phytic acid or phytase inclusion in the diet. High dietary phytate levels resulted in impaired feed intake and growth which may have been partly related to phytate complexing with cations (Chapter 1) and a reduction of Zn and protein bioavailability (Spinelli et al., 1983; Richardson, 1985; McClain and Gatlin, 1988).

It is recommended that different levels of phytic acid (more than 8 g kg^{-1} - the level that was used in this study) be preferably used in a purified diet to assess the effect of phytic acid on growth performance. However regarding current inclusion

rate of plant meals in aquafeeds, a high level of phytate would not be expected. Using extremely high levels of phytate would probably show its negative effect on performance on an experimental scale.

7.3. Phytase and digestibility of nutrients

Despite increased energy or protein digestibility in some studies with poultry, pig and fish fed diets containing phytase (Yi et al., 1996; Morz et al., 1994; Storebakken et al., 1998), it was not observed in the present study, except in Chapter 2 where there was a negative effect of phytate on protein digestibility. Despite statistical differences in protein digestibility, biologically it does not seem make a difference.

Phytate can bind with starch (Thompson and Yoon, 1984; Thompson, 1988). Phytase inclusion in terrestrial monogastric animals improved apparent metabolizable energy (AME) (Rajas and Scott, 1969; Farrell et al., 1993), but energy digestibility improvement has not been reported in fish (however it is difficult to make comparisons). Generally, in contrast to poultry and pig, carbohydrate and starch digestibility in Atlantic salmon is low (Stone, 2003). Starch is not the primary source of energy in Atlantic salmon, so the effect of phytase may not be reflected in energy digestibility in fish.

Almost all studies that used phytase in animals (including fish) diets, observed increased phosphorus digestibility (Cain and Garling 1995; Rodehutscord and Pfeffer, 1995; Schäfer et al., 1995; Jackson et al., 1996; Eya and Lovell, 1997; Li and Robinson, 1997; Hughes and Soares, 1998; Lanari et al., 1998; Storebakken et al., 1998; Vielma et al., 1998; Sajjadi and Carter, 2004). A clear positive effect of phytase on phosphorus digestibility was demonstrated in all experiments (Chapters 2,3 and 4). When available phosphorus level in the diet was more than the requirement level the effect on digestibility was not observed. With increasing level of phosphorus beyond the requirement, digestibility and utilisation decreased (Chapters 3 and 5). Using phytase in the diets with different levels of non-phytate

phosphorus has not been reported in fish, but in poultry it has been shown that with increasing level of non-phytate phosphorus, the efficacy of phytase decreases (Kornegay, 1999). Due to intestinal regulation of the phosphorus absorption in rainbow trout (Avila et al., 2000) and homeostatic regulation of phosphorus in salmon (Vielma and Lall, 1998) fish should be fed below their phosphorus requirement level when the phytase efficacy is assessed (Kemme et al., 1997). Dietary available phosphorus should be lower than requirement and total phosphorus should meet the requirement. Excess amount of minerals (more than NRC recommendation) in the diet should be avoided.

7.4. Phytase efficacy

The response to phytase depends on: phytase level in the diet; total phosphorus in the diet; dietary phytate level and its source; Ca and the Ca: P ratio; the intrinsic level of phytase in ingredients and their processing; pelleting methods (Kemme et al., 1997; Kornegay, 2001); water temperature and fish species (Hardy, 1998; Bransden and Carter, 1999; Sugiura et al., 2001).

Phytase supplementation and phytase pre-treatment had positive effects on feed intake, growth, phosphorus and mineral digestibility and utilisation in the present study. Phytase pre-treatment of ingredients was more effective than supplementation. Phytase pre-treatment of ingredients was conducted at optimum conditions (pH and temperature) for phytase activity in order to maximise the effectiveness of this method.

Multiple levels of phytase in fish feed has been explored in a very limited number of studies (Schäfer et al., 1995; Jackson et al., 1996; Li and Robinson, 1997; Forster et al., 1999; Papatryphon et al., 1999; Sugiura et al., 2001), so despite poultry and pigs studies, it is difficult to construct a response curve to different level of phytase in fish diet (Kornegay, 2001). Use of phytase at different levels in different species of fish with different plant ingredients are needed to make phosphorus dose-response model. In the present study, at least 1000 U kg^{-1}

phytase was needed to improve feed intake, growth and nutrient utilisation in Atlantic salmon fed soy protein concentrate (SPC) based diet, but 250 U kg^{-1} was enough when used for pre-treatment.

Partial degradation of phytate was observed during the diet preparation in Chapter 3, but phytate degradation was thought to mainly occur in the stomach of salmon. Conditions in gastrointestinal tract of agastric fish such as carp is not optimum for phytase activity (Schäfer et al., 1995; Bransden and Carter, 1999), so increasing the efficacy of phytase in feed during the diet preparation or pre-treatment of ingredient may be more efficient in the diet of these species in comparison to salmonids.

7.5. Phosphorus response criteria

Phosphorus requirement for maximum growth and bone mineralisation is different in rainbow trout (Rodehutscord, 1996). In the present study different criteria were used to assess phosphorus status of Atlantic salmon. Growth, whole-body phosphorus, bone phosphorus and bone ash were used as response criteria (Chapters 3 and 4). Results of Chapter 3 clearly showed that the more sensitive response criteria, showing differences before other parameters, were bone and whole-body ash and phosphorus. This is because reduced growth rates and mortality occur only at advanced stages of phosphorus deficiency, before growth or survival is impaired, the bone phosphorus and whole-body phosphorus are affected (Baeverfjord et al., 1998). These issues should be considered in phosphorus requirement studies, however it is not clear that maximising bone mineralisation is necessary for maintenance of health and performance of fish in the long period of time (Rodehutscord, 1996; Åsgård and Shearer, 1997).

Concentrations of most elements depend on fish size and decrease with increasing size (Storebakken et al., 1998). Whole-body phosphorus content in larger salmon (300 g) was less than small salmon (90 g) when the results of Chapters 3 and 4 were combined (Fig. 7.1). Baeverfjord et al. (1998) reported that initial whole-

body phosphorus of Atlantic salmon (112 g) was significantly lower than final whole-body phosphorus (three times heavier than initial weight). Furthermore, whole-body phosphorus concentration declined as rainbow trout grew from juvenile stage to adult (Shearer, 1984; Cain and Garling, 1995). Whole-body phosphorus in each chapter of this study was shown as percentage of dry matter, but in figure 7.1 whole-body phosphorus has been shown on a wet basis. In the present study, the water contents of fish fed different diets in each experiment were not significantly different. Data from different experiments of this study and other studies were compared (Fig. 7.1). The water contents of fish were different, so according to Shearer (1994), wet weight was used to eliminate the effect of dry matter on the results.

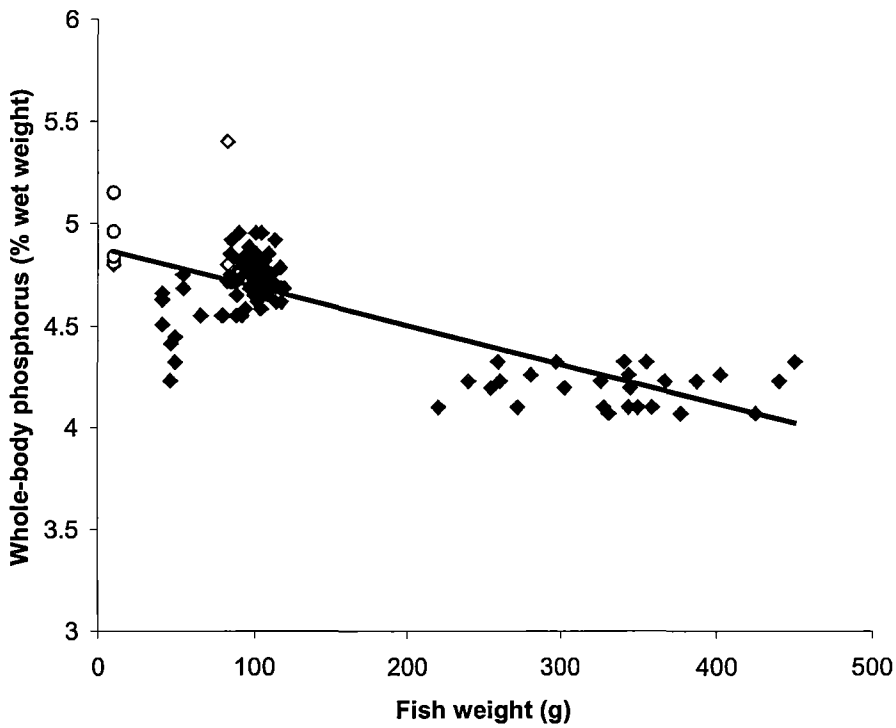

Figure 7.1. The relationship between fish size and body phosphorus content (% wet weight) in Atlantic salmon ($y = -0.0019x + 4.8839$; $r^2=0.62$) (♦ data from Chapters 3 and 4 initial and final fish that fed sufficient phosphorus; o data from El-mowafi et al. 1997; ◇ data from Nordrum et al. 1997)

7.6. Phosphorus utilisation

Results indicated that phosphorus was poorly available from both a canola meal and a soy protein concentrate and that pre-treatment or supplementation of phytase reduced the effects of phytate by increasing the availability of phosphorus and other minerals. Understanding phosphorus dynamics in relation to digestibility, optimising phosphorus utilisation and reducing phosphorus discharge is important. In the present study, phosphorus digestibility decreased with increasing dietary phosphorus. However, it should be noted that this result related to infected fish, interestingly the results were in agreement with other studies (Vielma and Lall, 1998; Sugiura et al., 1999; Avila et al., 2000; Rodehutscord et al., 2000). In contrast, some studies showed that when dietary phosphorus set to meet the requirement of phosphorus for growth, non-faecal phosphorus excretion is very low (Rodehutscord, 1996; Vielma and Lall, 1998), but when dietary phosphorus set to meet the requirement for phosphorus deposition, non-faecal phosphorus substantially increases (Rodehutscord, 1996; Sugiura et al., 2000). At this time, due to limited data available about phosphorus digestibility and relatively large experimental errors in micronutrient digestibility measurement in fish, it is very difficult to achieve a definitive conclusion about phosphorus digestibility pattern in relation to dietary phosphorus concentration (Cho and Bureau, 2001).

Feed intake influenced nutrient digestibility including phosphorus. Phosphorus and protein digestibility were lower at lowest ration (less than maintenance). It is likely that a greater share of endogenous faecal nitrogen and phosphorus excretion was the cause of lower digestibility values for protein and phosphorus at the lowest ration.

7.7. Practical outcomes

Commercial salmon feeds typically have 1% or more phosphorus to meet the requirement (Hardy, 2000). When plant meal based diets are fed to salmon, around two thirds of the phosphorus is unavailable. Clearly, one of the major issues when plant protein sources are used is the high level of phytate phosphorus, requiring dietary supplementation of phosphorus. The result of present study showed that the use of supplementary phosphorus could be reduced by using phytase with plant meals. Using phytase would not currently reduce the cost of the feed but it would reduce the phosphorus load in the water and enhance the value of plant meals to replace fish meal. The addition of phytase in plant protein based aquafeeds will be a very useful strategy to reduce phosphorus waste. Based on the results of this study and previous studies, it is clear that phytase is effective in increasing the utilisation of phytate. So, by using phytase, the increase of plant protein sources in the diet without decreasing fish performance and increasing faecal phosphorus will be possible.

In order to develop low- pollution aquafeeds, further studies are required to reduce the feed cost and optimise the fish growth. One of the most expensive minerals is supplemental phosphorus in fish feed (Cheng and Hardy, 2002), so supplementation of phytase reduces the need for phosphorus and also other minerals such as Zn supplementation. Several technologies are available to nutritionists to formulate low phosphorus, environmentally "friendly" diets for fish. Reducing the dietary phosphorus level and adding phytase to the diet is one of the most effective strategies that can be used to reduce phosphorus excretion. Phytase is arguably the most successfully used supplementary enzyme.

7.8. Future research directions

It is strongly recommended purified phytate is used at levels of more than 0.8% (the level was used in this study) to investigate effect of phytic acid on Atlantic

salmon growth performance. The effects of feed extrusion technology on phytate is another area of research to investigate whether extrusion reduces the phytate content of diet. Because phytase is heat liable and can not withstand high temperatures of extrusion, it is recommended that trials are done with extruded pellets top-sprayed with liquid phytase. Also, for commercialisation of application of phytase in the aquafeeds, diets containing phytase should be used in farm based trials to confirm laboratory based results such as the results of the present study.

However, phytase supplementation is more convenient for practical use and pre-treatment is less practical and may result in additional cost, but phytase pre-treatment is more effective. Plant protein concentrates are increasingly used in aquafeeds but phytate content of plant protein concentrates is higher than the original plant meal. Investigation on the inclusion of phytase in plant protein concentrates is necessary. Another area of interest is inclusion of low phytic acid grain and legume crops into fish diet. Use of low phytate crops has the potential to reduce the phytase and phosphorus supplementation in the fish diets (Sugiura et al., 1999; Overturf et al., 2003). Studies with poultry showed that using phytase with low phytate crops is a suitable method for phosphorus excretion management (Waldroup et al., 2000; Yan et al., 2000).

The phosphorus requirement of smolting salmon (the experiment that could not be completed due to fish disease) is a very interesting area that needs to be investigated especially at the elevated temperatures. The present study (Chapters 3 and 5) showed that phosphorus digestibility was negatively correlated with dietary phosphorus. There is limited information about the relationship between dietary phosphorus and phosphorus digestibility and more investigations are required in this field and account should be taken of the different methods for assessing digestibility. Also, the effect of endogenous phosphorus, organic phosphorus and inorganic phosphorus on digestibility should be measured. Finally, it would also be of interest to consider measuring true phosphorus digestibility rather than apparent digestibility.

7.9. Overall Summary

The most important findings and recommendations from this study were:

- Keeping the dietary available phosphorus at or around requirement level is necessary in phosphorus digestibility trials, excess phosphorus beyond requirement level reduces the digestibility values.

- Bone ash and phosphorus were more sensitive criteria than growth to assess phosphorus deficiency in Atlantic salmon.

- Ration (feed intake) influenced nutrient, including phosphorus, digestibility in Atlantic salmon.

- Dietary phosphorus was the most important dietary factor to affect phytase efficacy.

- A 1% purified phytate dietary inclusion had no effect on growth performance but reduced phosphorus and protein digestibility.

- Although, purified phytate reduced protein digestibility it had no effect on trypsin activity.

- Phytase was successfully used in both canola meal and soy protein concentrate (SPC) based diets and had positive effects on utilisation of phosphorus and other mineral.

- At least 1000 U phytase kg^{-1} diet is needed for inclusion with a SPC based diet to improve growth and nutrient utilisation of Atlantic salmon, whereas only 250 U kg^{-1} was enough for pre-treatment of SPC.

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