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This thesis is dedicated to my beloved grandfather Songxue Pan (1927-2011)

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Ås, October 2011

Yuexing ZHANG

List of Abbreviations

AA = Amino acid

ADC = Apparent digestibility coefficient

CPC = Canola protein concentrate

Ca = Calcium

C-MIX = Mixture of canola and potato protein concentrates and amino acids

DE = Digestible energy

DM = Dry matter

DP = Digestible protein

DSPC = Dephytinized soy protein concentrate

EAA = Essential amino acid

EAAP = Essential amino acid profile

FM = Fish meal

FCR = feed conversion ratio

FI = Feed intake

IP_6 = *myo*-inositol hexaphosphate

LPC = Lupin protein concentrate

L/P ratio = Ratio between LPC and PPC

Met = Methionine

Mg = Magnesium

MJ = Megajoule

NSP = Non-starch polysaccharide

N = Nitrogen

P = Phosphorous

PL = Phospholipid

P-MIX = Mixture of pea and potato protein concentrates, and amino acids

PPC = Pea protein concentrate

Potato PC = Potato protein concentrate

SGR = Specific growth rate

S-MIX = Mixture of soy protein concentrate, oil and amino acids

SPC = Soy protein concentrate

TAA = Total amino acid

Trp = Tryptophan

WG = Weight gain

Zn = Zinc

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List of Papers

The following papers are included in this thesis. They will be referred to by their roman numerals.

I. **Zhang, Y.,** Øverland, M., Sørensen, M., Penn, M., Mydland, L.T., Shearer, K.D., Storebakken, T. Optimal inclusion of lupin and pea protein concentrates in the extruded diets for rainbow trout (*Oncorhynchus mykiss*). Submitted to Aquaculture.

II. **Zhang, Y.,** Øverland, M., Xie, S., Dong, Z., Lv, Z., Xu, J., Storebakken, T. Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fish meal in extruded diet for juvenile black sea bream *Acanthopagrus schlegeli.* Submitted to Aquaculture.

III. **Zhang, Y.,** Penn, M., Øverland, M., Shearer, K.D., Sørensen, M., Mydland, L.T., Storebakken, T. Optimizing plant protein combinations in fish meal-free diets for rainbow trout (*Oncorhynchus mykiss*) by a mixture model. In manuscript.

IV. **Zhang, Y.,** Denstadli, V., Øverland, M., Storebakken, T. Incubation of soybean protein concentrate with phytase improves the nutritional value of a fish meal-free diet for rainbow trout (*Oncorhynchus mykiss*). In manuscript.

Abstract

Zhang, Y. 2011. Optimal use of plant protein concentrates in extruded feeds for carnivorous fish. Norwegian University of Life Sciences, Philosophiae Doctor Thesis, 2011: 53, ISSN: 1503-1667, ISBN: 978-82-575-1016-9

The main objective of the research presented in this thesis was to evaluate multiple amino acid-supplemented plant protein concentrates as the main protein source in diets for carnivorous fish. This objective was approached experimentally by: 1) Determining the effects of using plant protein concentrates to provide 30 %, 50 %, and 95 % of total protein in extruded diets for rainbow trout and black sea bream on growth performance, feed utilization, and nutrient digestibility and retention; 2) Examining if combinations of different plant protein concentrates diminishes the negative effects of anti-nutritional factors associated with single plant protein sources when fed to carnivorous fish, thereby facilitating higher dietary plant protein inclusions; 3) Evaluating the effects of dietary inclusion of plant protein concentrates on fish physiology and health; 4) Assessing the efficiency of pre-treatment of plant protein concentrates with phytase to improve utilization of minerals and macronutrients in rainbow trout; 5) Quantifying the changes in loss of nutrients to the environment by replacing high-quality fish meal with untreated or dephytinized plant protein concentrates. The dietary plant protein concentrates used in this thesis were all supplemented with multiple limiting amino acids and taurine.

Four experiments were conducted, and the results are reported in four papers. **Paper I** (**Optimal inclusion of lupin and pea protein concentrates in extruded diets for rainbow trout (*Oncorhynchus mykiss*)**) focuses on the response of rainbow trout to nine extruded diets. These diets included eight plant protein based diets formulated using four mixtures of lupin (LPC) and pea protein concentrates (PPC) (L/P ratio, 3:0, 2:1, 1:2 and 0:3) with two dietary inclusion levels (300 or 500 g plant protein kg^{-1} dietary protein) and one diet using LT-fish meal as the sole protein source (FM diet). The same nine diets, but with a lower level of oil coating, were also studied in black sea bream in **Paper II** (**Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fish meal in extruded diet for juvenile black sea bream (*Acanthopagrus schlegeli*)**) In **Paper III** (**Optimizing plant protein combinations in fish meal-free diets for rainbow trout (*Oncorhynchus mykiss*) by a mixture model**) three plant

protein premixtures (P-MIX, C-MIX, and S-MIX) were prepared by mixing four protein concentrates. These were pea protein concentrates (PPC), canola protein concentrate (CPC), potato protein concentrate, and soy protein concentrate (SPC). Seven plant protein based diets were formulated based on a mixture design using P-MIX, C-MIX, and S-MIX alone or in combinations to provide > 95% of the dietary protein. These diets were supplemented with 5% krill products as feeding attractant. One diet using LT-fish meal as the sole protein source (FM diet) was also produced. All diets were designed to contain equal amounts of digestible protein and digestible energy, and fed to rainbow trout. In **Paper IV** (**Incubation of soy protein concentrate with phytase improves the nutritional value of a fish meal-free diet for rainbow trout (Oncorhynchus mykiss)**), two of the diets from **Paper III** (the FM and SPC diets), and one additional experimental diet based on dephytinized SPC (DSPC diet) were fed to rainbow trout.

In **Papers I** and **II,** the trout grew from 58 to 180 g during the 62 days of feeding, and the black sea bream grew from 13 to 46 g during 60 days. No significant differences in growth rate among dietary treatments were seen for either species. The only body composition parameter significantly affected by the diets in both experiments was ash, which could be ascribed to the presence of phytic acid in the plant protein concentrates. The highest inclusion of 500 g plant protein concentrates kg^{-1}, and the diets with the highest L/P ratio resulted in higher feed intake (FI) in black sea bream. No significant effects of diet on feed intake were observed in rainbow trout. The average feed conversion ratio (FCR) was 0.72 g dry matter intake $(g\ gain)^{-1}$ in rainbow trout, and 1.13 g g^{-1} in black sea bream. The diets with the highest L/P ratio resulted in higher FCR in both species, due to the higher content of non-starch polysaccharides in LPC than in PPC. In rainbow trout, the apparent digestibility of starch was reduced as a consequence of increasing dietary LPC at both inclusion levels, while the digestibility of lipid was increased with increasing dietary LPC only at high inclusion level. The diet with the highest inclusion of PPC resulted in reduced maltase activity in the intestines of both trout and sea bream. Trout fed the diet with the highest inclusion of PPC tended to have a slight decrease in mucosal fold height and a slight increase in fold fusion. The energy retentions did not significantly differ between the FM and plant protein diets in either species. Several of the plant protein-concentrate based diets resulted in similar or significantly higher nitrogen retentions in both species. The conclusion of the two papers was that any combination of essential amino acid (EAA)-fortified LPC and PPC

can be efficiently used when total dietary plant protein inclusion is limited to 300 g kg^{-1}. At higher inclusion, PPC seemed preferable. The reduced mineralization and tendencies of change in the intestinal physiology caused by the LPC and PPC require further attention.

In **Papers III** and **IV** the trout grew from 61 to 214 g during 72 days of feeding, without significant differences in weight gain among dietary treatments. In both experiments, fish fed the plant protein based diets had significantly higher feed intake, FCR, and metabolic nitrogen (N) loss than those fed the FM diet. Digestibility of most nutrients other than mineral elements, and body composition did not significantly differ from the fish fed the FM diet. The digestibility of energy and retentions of both N and energy were significantly lower in trout fed the plant concentrate diets. Fish fed diets with P-MIX, containing protein from pea and potato, exhibited inflammatory changes of mild or moderate severity in the distal intestine.

The mixture model predicted different optimal diet formulations based on different response criteria. A combination of P-MIX and C-MIX gave most efficient feed conversion. The digestibility of N and amino acids were maximized when S-MIX was used alone. The digestibility of lipid and energy were maximized by a combination of P-MIX and S-MIX. Retention of ingested N was most efficient when combining P-MIX and S-MIX, while the highest retention of digested N was obtained by a combination of P-MIX and C-MIX. Using C-MIX alone supported the highest digestibility and retention of P, and whole-body concentrations of ash, P, Ca and Mg. The reason was that the CPC in C-MIX had been incubated with phytase by the producer. Dephytinization of the SPC also resulted in significant improvements in the utilization of dietary P, as well as Ca and Mg. In addition, dephytinization of the SPC resulted in lower FCR, and increased digestible energy concentration in the diet. Both metabolic losses of N and faecal loss of energy were higher for the plant protein diets than for the FM diet, while the faecal loss of P was higher for the FM diet. Dephytinization of SPC led to a reduction in faecal and metabolic loss of N and P, and faecal loss of energy. Considerable benefits both to secure the welfare of the fish and to minimize losses of P and N into water can thus be achieved by the use of dephytinized plant protein concentrates in fish feeds.

Sammendrag

Hovedmålet med forskningen presentert i denne avhandlingen var å undersøke anvendeligheten av ulike planteproteinkonsentrater anriket med flere begrensende aminosyrer, som hovedkilde i fôr til kjøttetende fisker. Følgende eksperimentelle tilnærminger ble benyttet for å nå dette målet: 1) Undersøke effekten på vekst, fôrutnyttelse, fordøyelighet og retensjon av næringsstoffer når planteproteinkonsentrater utgjør 30, 50 og 95 % av proteinet i ekstruderte fôr til både regnbueørret og black sea bream; 2) Finne ut hvorvidt kombinasjoner av ulike planteproteinkonsentrater kan redusere de negative effektene av antinæringsstoffer som forekommer i enkelte fôrmidler fra planter, og derved gjøre det mulig å øke mengden planteprotein i fiskefôr; 3) Vurdere effekten av planteproteinkonsentrater på fiskens fysiologi og helse; 4) Måle effekten av å forbehandle planteproteinkonsentrater med fytase for å øke utnyttelsen av mineraler og hovednæringsstoffer hos regnbueørret; 5) Kvantifisere endringene i tap av næringstoffer til miljøet som oppnås ved å bytte ut fiskemel av høy kvalitet som eneste kilde til protein i fôret med intakte eller fytasebehandlede planteproteinkonsentrater. Alle planteproteinkonsentratene som ble benyttet i dette arbeidet ble anriket med begrensende aminosyrer og taurin.

Fire forsøk ble utført, og resultatene er rapportert i fire manuskript. **Paper I** (**Optimal inclusion of lupin and pea protein concentrates in extruded diets for rainbow trout (*Oncorhynchus mykiss*)**) fokuserer på responsen av ni ekstruderte fôr. Disse fôrene ble formulert ved å lage fire blandinger med lupin (LPC) og erteproteinkonsentrat (PPC) (L/P ratio, 3:0, 2:1, 1:2 og 0:3) og med to ulike nivåer av innblanding (300 eller 500 g planteprotein kg^{-1} fôrprotein), og et fôr med LT fiskemel som eneste proteinkilde (FM diet). I **Paper II** (**Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fish meal in extruded diet for juvenile black sea bream (*Acanthopagrus schlegeli*)**), ble responser av de samme fôrene som ble benyttet i **Paper I** undersøkt i black sea bream, men med lavere fettinnhold. I Paper **III** (**Optimizing plant protein combinations in fish meal-free diets for rainbow trout (*Oncorhynchus mykiss*) by a mixture model**) ble tre blandinger av planteproteinkonsentrater (P-MIX, C-MIX og S-MIX) fremstilt ved å blande fire planteproteinkonsentrater. Disse var proteinkonsentrater fra erter (PPC), canola raps (CPC), potet og soyabønner (SPC). Sju fôr basert på planteproteiner ble formulert med utgangspunkt i et mixture design. P-MIX, C-MIX og S-

MIX ble benyttet alene, eller i kombinasjon slik at de utgjorde mer enn 95 % av fôrets protein. Fôrene inneholdt også 5 % krillprodukter for å stimulere fôropptak. Et fôr som inneholdt LT fiskemel som eneste proteinkilde (FM diet) ble også produsert. Alle fôrene var formulert til å inneholde samme mengde fordøyelig protein og energi, og ble fôret til regnbueørret. I **Paper IV** (**Incubation of soy protein concentrate with phytase improves the nutritional value of a fish meal-free diet for rainbow trout (*Oncorhynchus mykiss*)**), ble to av fôrene fra **Paper III** (FM og SPC) og et forsøksfôr basert på SPC med redusert innhold av fytinsyre (DSPC diet) gitt til regnbueørret.

I **Papers I** og **II** vokste ørreten fra 58 til 180 g i løpet av 62 fôringsdager, og black sea bream vokste fra 13 til 46 g i løpet av 60 dager. Ingen signifikante forskjeller i veksthastighet ble observert for noen av artene. Det eneste målet for kroppssammensetning som ble signifikant påvirket av fôrene, var askeinnholdet. Dette kan tilskrives innholdet av fytinsyre i plantekonsentratene. Det høyeste innblandingsnivået av planteprotein i fôret (500 g kg^{-1}), og fôrene med høyest L/P ratio førte til økt fôropptak hos black sea bream. Ingen av fôrene gav signifikant effekt på fôropptak hos regnbueørret. Gjennomsnittet for fôrutnyttelse (FCR) hos regnbueørret var 0,72 g fôrtørrstoff (g tilvekst)$^{-1}$, og 1.13 g g^{-1} hos black sea bream. Fôrene med høyest L/P ratio førte til høyere FCR hos begge arter, på grunn av at LPC inneholdt mer ufordøyelige polysakkarider enn de andre proteinkonsentratene. Fordøyelighet av stivelse hos regnbueørret ble redusert ved økende innblanding av LPC, mens fettfordøyeligheten økte med økende innhold av LPC ved høyeste innblandingsnivå av planteprotein i fôret. Fôret med mest PPC førte til redusert aktivitet av maltase i tarmen hos både ørret og bream. Ørret som fikk fôr med mest PPC tenderte til å ha noe lavere høyde på tarmtottene, og en svak økning i sammenslåtte tarmtotter. Retensjonen av energi var ikke signifikant forskjellig for FM og fôrene med planteprotein hos noen av artene. Flere av fôrene med planteproteinkonsentrat resulterte i tilsvarende eller høyere retensjon av nitrogen hos begge arter. Konklusjonen fra de to manuskriptene var at LPC og PPC, supplert med aminosyrer, utnyttes effektivt når total innblanding begrenses til 300 g (kg fôr)$^{-1}$. Ved høyere innblanding virket det som PPC hadde fordeler. Den reduserte mineraliseringen og tendensene til endringer i tarmfysiologien forårsaket av LPC og PPC krever videre oppfølging.

I **Papers III** og **IV** vokste ørreten fra 61 til 214 g i løpet av 72 fôringsdager. Det var ikke signifikante forskjeller mellom fôrene for veksthastighet. I begge forsøkene hadde fiskene som fikk fôr med planteproteiner signifikant høyere fôropptak, FCR, og metabolsk tap av nitrogen (N) enn de som fikk fôr med FM. Fordøyelighet av andre næringsstoffer enn mineraler, og kroppssammensetning var ikke signifikant forskjellig fra fisk som fikk fôr med FM. Fordøyelighet av energi, og retensjon av både N og energi var signifikant lavere hos ørret som fikk fôr med planteproteiner. Fisk som fikk fôr med P-MIX, som inneholdt protein fra erter og potet, hadde mild til moderat betennelse i baktarmen.

Modellen for å analysere resultater fra mixture design gav ulike prediksjoner for optimal fôrformulering når ulike responskriteria ble benyttet. Kombinasjon av P-MIX og C-MIX førte til mest effektiv fôrutnyttelse. Fordøyelighet av N og aminosyrer var mest effektiv når S-MIX ble benyttet alene. Fordøyelighet av fett og energi ble maksimert ved en kombinasjon av P-MIX og S-MIX, mens høyest retensjon av fordøyd N ble oppnådd med å kombinere P-MIX og C-MIX. Bruk av C-MIX alene førte til høyest fordøyelighet og retensjon av P, og innhold av P, Ca, Mg og aske i fiskekroppen. Grunnen var at CPC i C-MIX hadde blitt innkubert med fytase av produsenten. Nedbrytning av fytinsyre i SPC førte også til signifikant forbedring i utnyttelsen av P, Ca og Mg. I tillegg førte hydrolyse av fytinsyre i SPC til lavere FCR, og økt konsentrasjon av fordøyelig energi i fôret. Innkubering av SPC med fytase førte til en klar reduksjon i tap av både P og N i feces og som metabolske tap, og reduserte også tap av energi i feces. Betydelige fordeler, både for å sikre fiskens velferd og redusere utslipp and P og N til vannet, kan oppnås ved å benytte planteproteinskonsentrater med redusert innhold av fytinsyre i fôr til fisk.

1. General introduction

The proportion of fish meal use in fish feeds is predicted to decrease, because the amount of fish meal from wild fish is limited to 5-7 million tons a year (Chamberlain, 2011), while demand for fish feed resources is expected to continue growing rapidly (Tacon and Metian, 2008). Plants represent a highly abundant source of protein for use in food and feed. Typically, the world production of soybeans in 2010 was 258 million tons (Soystats, 2011), while that of rapeseeds was 61 million tons (Agricomodityprices, 2011). Plant protein sources with low degree of processing, such as defatted soybean meal, defatted rapeseed cake, and lupin kernel meal, have been widely used in fish feeds. Energy- and nutrient dense diets for carnivorous fish, however, have limited formulation space for this type plant protein sources. This is both due the presence of anti-nutritional factors found in these ingredients (Francis et al., 2001), and their high content of indigestible carbohydrates (Knudsen, 1997). Plant protein concentrates generally contain less of these factors. Protein concentrates from rapeseed (Thiessen et al., 2004), lupin (Glencross et al., 2011), potato (Refstie and Tiekstra, 2003), pea (Øverland et al., 2009), and soybeans (Kaushik et al., 1995; Storebakken et al., 1998a; 2000b) have shown promising results for use in salmonid diets. Thus, the use of plant protein concentrates has gained increasing interests by the feed industries.

Plant protein concentrates are produced by different methods. Air classification is commonly used to produce pea protein concentrate. This involves fine grinding, and separation of fractions high in starch and protein based on different settling points in an air stream due to different densities (Schutyser and van der Goot, 2011). Soy, lupin and rapeseed protein concentrates are often produced by extraction, either with hot water or in combination with ethanol. The defatted and de-hulled seed is ground prior to extraction off soluble, indigestible sugars and non-starch polysaccharides (Karnofsky, 1980). One fortunate effect of this process, is that the components in the soybean causing enteritis in the distal intestine of salmonids is extracted along with the carbohydrates (van den Ingh et al., 1991; van den Ingh et al., 1996), improving the usefulness of this ingredient in fish feed. The plant protein products with highest concentration are produced by precipitation of the proteins from an aqueous solution. One example is soy protein isolate, produced by iso-electric focusing or filtration of soy proteins (Alibhai et al., 2006).

Plant protein concentrates, however, have several limitations for direct use in fish feeds. All plants are deficient in essential amino acids, when compared to the requirements of fish. Typically, the first limiting amino acid in soy protein is methionine, while lysine is the second limiting. For most other plant proteins lysine is the first limiting amino acids (NRC, 2011). Salmonids and other carnivorous fish species can efficiently utilize crystalline amino acids (Espe et al., 2006). The frequent or even continuous feeding is preferred to minimize the difference in absorption between peptide-bound and crystalline EAA (Yamada et al., 1981; Cowey and Walton, 1988; Kaushik and Seiliez, 2010). Thus, amino acid deficiencies can be overcome by supplementing the diets with essential amino acids. Other essential nutrients may also become deficient when plant proteins account for the majority of protein in fish feeds. One example is taurine, a sulphur-containing derivative from methionine, can be also provided by fish meal, does not exist in plant-derived ingredients. Several fish species have lacking ability to synthesize taurine (Goto et al., 2003; Takagi et al., 2008; 2011) and recent findings show that rainbow trout benefits from dietary taurine supplement when given a diet with high proportion of plant proteins (Gaylord et al., 2006). Thus, taurine should be supplemented jointly with essential amino acids to diets with high content of plant proteins.

One important reason for using fish meal is that it is a feeding stimulant (Kousoulaki et al., 2009). Several plants contain bitter and detractive components such as alkaloids in lupin (Serrano et al., 2011) and soyasaponins in soy (Bureau et al., 1998). Some of these may be not always completely removed during the processing of the concentrate. Other marine products have strong attractant effects to fish. One of these is the krill, and several experiments have demonstrated increased feed intake and growth rates by using krill meal or krill hydrolysates, both in diets based on fish meal and in feeds with high concentration of plant ingredients (Oikawa and March, 1997).

All seeds contain phytic acid. This anti-nutrient cannot be removed by air classification, and it may even be concentrated by extraction to produce plant protein concentrates. Phytic acid has high concentration of phosphorous, which is not available to monogastric animals. It also chelates di- and trivalent cations in the intestine, making these unavailable for absorption (Storebakken et al., 2000a). Experiments have shown that phytic acid in soy protein concentrate can result in incomplete mineralization of hard tissues in salmonids (Storebakken et al., 1998a)

and fish meal based diets supplemented with phytic acid may introduce spinal deformities (Helland et al., 2006). Phytic acid can be hydrolyzed by including phytase in the feed if fed to warm water fish or coldwater fish at temperatures exceeding 10-15^{o}C (Vielma et al., 1998; Carter and Sajjadi, 2011). At lower temperatures, the effect of dietary phytase is minimal. Thus, incubation of plant concentrates with phytase (Storebakken et al., 1998a; Vielma et al., 2002; Denstadli et al., 2007) should be considered before using including them in the diets.

Nitrogen is the limiting nutrient for algal growth is seawater, while phosphorous limits growth in freshwater. The main nitrogen pollutants from fish farming are water soluble ammonia from deamination of amino acids and urea from catabolism of nucleic acids, and particulate loss of faeces. Faecal loss is the main source of pollution with phosphorous. Uneaten feed may also represent a significant source of pollution, but it can be largely eliminated by the use of feeds with high technical quality (Sørensen et al., 2010; Aas et al., 2011), and by correct feeding (Storebakken and Austreng, 1987). In order to minimize the impact of fish farming on the environment, it is of high importance to simultaneously minimize pollution from water soluble, metabolic loss, from faeces, and from uneaten feed.

All feeds are in practice mixtures, while feed ingredient research has largely focused on single ingredients. The use of mixture design can be helpful to determine if the synergetic effects which can increase the performance or desirability of feed may become significant when mixing dietary ingredients. Mixture models also facilitate the determination of optimal mixtures or feed formulations, based on given response criteria. Such designs have been widely used in the chemical (Akalin et al., 2010; Lin et al., 2010), pharmaceutical (Mahdhi et al., 2010; Malzert-Freon et al., 2010) and food industries (Karaman et al., 2011) to optimize processes or formulations. Only few studies using mixture models to optimize fish and shrimp feed have been reported (Ruohonen et al., 2003; 2007; Forster et al., 2010; Draganovic et al., 2011).

2. Objectives of the research

The main objective of the research behind this thesis was to determine the nutritional value of multiple amino acid-supplemented plant protein concentrates as the main protein sources in diets for the carnivorous rainbow trout and black sea bream. The main response criteria were growth performance, feed utilization, nutrient digestibility and retention, fish health and environmental impacts. The sub-objectives were:

- To determine the effect of using plant protein concentrates to provide 30 %, 50 %, and 95 % of total protein in extruded diets for rainbow trout and black sea bream on growth performance, feed utilization, and nutrient digestibility and retention (**Papers I, II, III** and **IV**).

- To determine if combining different plant protein concentrates diminishes negative effects associated with a single plant protein source in feed for carnivorous fish, and thereby facilitates higher use (**Papers I, II** and **III**).

- To evaluate the effect of dietary inclusion of plant protein concentrates on fish physiology and health (**Papers I, II, III** and **IV**).

- To determine the efficiency of pre-treatment of plant protein concentrates with phytase to improve utilization of mineral elements and macronutrients in rainbow trout (**Papers III** and **IV**).

- To determine the pollution load to the water when plant protein concentrates partly or largely replaced fish meal in diets for rainbow trout and black sea bream (**Papers I, II III** and **IV**), and to quantify the effects of phytase pre-treated dietary plant protein concentrates on the phosphorus and nitrogen pollution from fish farming (**Papers III** and **IV**).

3. Main results and discussion

3.1 Feed production and physical pellet quality

The experimental diets used in the experiments were based on two separate feed productions, in a semi-industrial twin screw extrusion line. The diets used for **Papers I** and **II** were from the same batches of extruded diets (uncoated). Nine extruded diets were formulated, including eight plant protein-based experimental diets made from four mixtures of lupin protein concentrate (LPC) and pea protein concentrate (PPC) (L/P ratio, 3:0, 2:1, 1:2 and 0:3) with two dietary inclusion levels (300 or 500 g plant protein kg^{-1} dietary protein) and one diet with LT-fish meal as the sole protein source (FM diet). The experimental diets were designed to be isonitrogenous and isolipidic. The diets for rainbow trout were coated with higher levels of oil than those for black bream.

Three essential amino acid (EAA) and taurine-fortified plant protein pre-mixtures (P-MIX, C-MIX, and S-MIX) were prepared by mixing four plant protein concentrates (PPC, canola protein concentrate (CPC), potato protein concentrate, and soy protein concentrate (SPC)) for the experiment reported in **Paper III**. Eight diets were formulated. These included seven plant protein based diets, formulated according to a mixture design, using P-MIX, C-MIX, and S-MIX alone or in combinations to provide > 95% of the dietary protein. One diet was produced with LT-fish meal as the sole dietary protein source (FM diet). All diets were designed to contain equal amounts of digestible protein and digestible energy. The FM diet and the diet with 95% of dietary protein from soy protein concentrate (SPC) were also used as controls for **Paper IV**. The experimental diet used in this experiment had the same formulation as the SPC diet, except the SPC had been incubated with phytase.

For each batch, feed extrusion, feeding rate, water addition to the preconditioner and extruder, and extruder screw speed (RPM) were the main adjustable parameters to optimise the bulk density to approximately 520 g l^{-1} in pellets prior to drying and vacuum coating. In **Paper I**, durability and hardness increased with increasing concentration of PPC and a corresponding decrease of LPC at the low plant protein inclusion level. This effect was not significant at the high inclusion level. In **Paper III,** the PPC and P+C diet, based on the combination of PPC, potato PC and CPC had the highest durability and hardness. These results were consistent with previous findings (Øverland et al., 2009). The presence of starch in PPC, the higher amylose to

amylopectin ratio in the pea starch than in the wheat starch, and the high binding property of un-denatured pea protein (Øverland et al., 2009) are the main factors contributing to this. The potato PC and CPC, however, also contributed to the physical quality of this diet. The FM diet had the lowest durability and hardness, and the highest expansion and water stability. The main reason for this was the high inclusion of starch in the FM diet (Sørensen et al., 2011).

3.2 Feed intake, growth and feed utilization

Replacement of LT-fish meal with 407 g kg^{-1} PPC or 439 g kg^{-1} LPC did not negatively affect feed intake (FI) of rainbow trout (**Paper I**). Neither inclusion level of plant protein combinations based on PPC and LPC, nor the ratio between LPC and PPC (L/P ratio) significantly affected FI. In the experiment with black sea bream, the dietary inclusion of 433 g kg^{-1} PPC did not impair the FI, and the dietary inclusion of 467 g kg^{-1} LPC even resulted in a significantly higher FI than the FM diet (**Paper II**). The diets with the highest level of LPC (L/P ratio = 3:0) resulted in significantly higher FI than the diets with less LPC.

Several of the diets with 95% of dietary protein from plant protein concentrates produced higher FI than the FM control diet, and none of the diets with combinations of plant protein concentrates produced reduced FI in rainbow trout (**Paper III**). Phytase pre-treatment of SPC did not significantly affect the FI of trout (**Paper IV**). These results illustrate that partial replacement of high quality amino acid-supplemented plant protein concentrates did not limit FI in either of the carnivorous fish species. The level of deterrent components like saponins in PPC or alkaloids in LPC did not negatively affected FI of the two carnivorous fish species. Krill products are known feeding attractants that improve the palatability of the feed (Oikawa and March, 1997; Olsen et al., 2006; Kousoulaki et al., 2009). Dietary energy density also appeared to affect the FI of both black sea bream and rainbow trout (Kaushik, 1998). Black sea bream fed the LPC-rich diet seemed to increase their FI to compensate for the lower digestible energy level in these diets compared to the PPC-rich diets. Trout fed the plant protein-based diets seemed to increase their FI to obtain similar digestible energy intakes to the trout fed the FM diet, resulting in comparable growth.

Rainbow trout obtained an average weight gain (WG) of 210 % after a 62-day feeding period (**Paper I**), and black sea bream obtained an average WG of 251% after a 60-day feeding period (**Paper II**). The WG of both species did not differ significantly among diets. Neither the inclusion level of plant protein nor the L/P ratio caused significant differences in WG of trout, while diets with 50 % plant protein resulted in significantly lower WG in sea bream than the diets with 30 %. No significant effect of L/P ratio on WG of sea bream was found. Rainbow trout fed diets with 95 % of protein from plant protein concentrates obtained a comparable WG to those fed the FM diet (**Paper III**). Phytase pre-treatment did not significantly increase the WG of trout fed the diet with 95 % of protein from SPC (**Paper IV**). The rapid growth achieved and absence of significant differences among diets in WG could be attributed to both high and comparable feed intake and efficient feed conversion of plant protein concentrates based diets supplemented with multiple EAA. This illustrates that rapid growth can be obtained in carnivorous fish, fed diets with inclusion levels of plant protein concentrates ranging from moderate to 95% of dietary protein.

Rainbow trout obtained an average feed conversion ratio (FCR) of 0.72 g DM ingested (g gain)$^{-1}$ in the first experiment (**Paper I**). Most plant protein-based diets had significantly lower feed efficiency than the FM diet, except for the diet with 30 % of protein from PPC. Black sea bream obtained an average FCR of 1.13 g DM ingested (g gain)$^{-1}$(**Paper II**). The diets with low plant protein inclusion and the diet with 50% of protein from PPC gave comparable FCR to the FM diet. In both species, the diets with high plant protein inclusion resulted in significantly lower feed efficiency than the diets with low inclusion. The diets with the most LPC (L/P ratio = 3:0) resulted in significantly lower feed efficiency than the diets with less LPC. Such high feed efficiency was mainly due to the dietary inclusion of LPC and PPC. These were highly digestible for salmonids (Øverland et al., 2009; Glencross et al., 2011), and were supplementation with multiple amino acids so that digested amino acids could be efficiently utilized for protein synthesis. The effect of L/P ratio on feed efficiency was mainly due to the different nutrient digestibilities between PPC and LPC.

The higher FCR in the black sea bream than the rainbow trout may reflect a species difference, or result from higher maintenance at higher water temperature. The diets with plant protein concentrates for black sea bream, however, only contained 143-164 g lipid (kg DM)$^{-1}$, while the

diets for rainbow trout with the same protein sources contained 191-220 g lipid kg^{-1}. Corresponding dietary protein concentrations were 522-534 g kg^{-1} in diets for sea bream, and 463-485 g kg^{-1} in diets for rainbow trout. With the trout diets, this resulted in DP/DE ratios ranged from 21.1-22.6 g DP (MJ DE)$^{-1}$. These values are higher than the optimum for 60 g rainbow trout (Green and Hardy, 2008). Digestibility assessment was not successful in black sea bream, thus dietary DP/DE ratios were not calculated. The optimal DP/DE ratio is reduced as fish grow larger (NRC, 2011), and the bream weighed 13 g, while the trout weighed 58 g at the beginning of the experiment. The diet fed to the black sea bream, however, contained more protein and less lipid than what is optimal for rainbow trout (Austreng, 1979). Thus, it is possible that sub-optimal balance between protein and energy in the diet for black sea bream also contributed to the higher FCR observed in this species. The clarification of these questions, however, requires additional comparative research.

Using plant protein combinations to provide of 95 % dietary protein instead of FM resulted in higher FCR values than the FM diet (**Paper III**). This was probably due to the decreased energy digestibility of plant protein-based diets. Significant interactions for feed efficiency were found among the dietary plant protein concentrates. These interactions showed synergetic effects on the feed efficiency. This may be explained by the more balanced nutritional profile when a greater variety of proteins with different origins and nutritional properties were included in diet. Dephytinization of the SPC improved the FCR by 0.11 g DM intake (g gain)$^{-1}$, and the FCR value of the DSPC diet was only 0.04 g intake (g gain)$^{-1}$ lower than that of the FM diet (**Paper IV**). This was mainly due to the decreased intake of both N and energy, and the decreased fecal N and energy loss together with the decreased metabolic N loss.

3.3 Nutrient digestibilities

Apparent digestibilities of nutrients were only evaluated in **Papers I**, **III** and **IV**. Collection of faeces by stripping (Austreng, 1978) was not feasible in juvenile black sea bream with a weight of approximately 45 g (**Paper II**).

In **Paper I**, all the diets with 30 % or 50 % of the dietary protein provided by LPC and PPC alone or in combinations resulted in comparable or higher digestibilities (ADC) of N, EAA and

lipid than the FM diet. The ADC values were also similar or higher than previously reported for FM based diets (Sørensen et al., 2002), PPC based diets (Øverland et al., 2009) and LPC based diets (Glencross et al., 2011) fed to salmonids. The EAA supplementations contributed to the high EAA digestibilities, since crystalline amino acids are more efficiently absorbed in fish than peptide-bound amino acids in several feed ingredients (Ambardekar et al., 2009). Extruding the diets caused gelatinization of the starch, and subsequent high digestibilities (Glencross et al., 2011). The extrusion of the feed is lenient with respect to amino acid digestibilities (Sørensen et al., 2002), but high temperatures employed during processing of the ingredient may significantly reduce the digestibility of these nutrients (Opstvedt et al., 1984). Consequently, the high ADC of N, Met, Trp at higher plant protein inclusions, may be ascribed to a combination of lenient heat treatment during production of the LPC, virtually no heat employed when producing PPC, and a high level of EAA supplementation.

The ADC of starch decreased with increasing inclusion and thereby with decreasing dietary concentration of starch. This is in contrast to expectations with decreasing ADC of starch with increasing intake (Bergot and Breque, 1983). Increasing L/P ratio also caused decreased ADC of starch, in spite of a corresponding increase in dietary starch level. The ADC of lipid also increased with increasing dietary starch, and increased faecal output of undigested starch. This is also contrary to previous findings (Storebakken et al., 1998b). Thus, the ADC of starch and lipid seemed to depend on the composition of the protein concentrates rather than dietary starch concentration. The type of starch may have been one factor. The diets with most PPC, resulting in highest ADC of starch, contained pea starch. The diets with high content of LPC had a high proportion of wheat starch. The LPC also contained significantly more non-starch polysaccharides (NSP) than the PPC. NSP is not only indigestible to fish, but can also negatively affect the digestion and absorption of other nutrients (Sinha et al., 2011).

In **Paper III**, all the diets with plant protein concentrates, except the CPC and SPC diets, provided comparable ADC of N, lipid, and starch to the FM diet. ADC of N was higher for the SPC than the FM diet, while ADC of lipid was lower. ADC of N in the CPC diets was lower than that of the FM diet. ADC of energy was significantly higher in the FM diet than in any of the plant protein based diets. Compared to the FM diet, the CPC diet resulted in a significantly lower ADC of N and the SPC diet resulted in a significantly higher ADC of N but a significantly lower

ADC of lipid. Reduced lipid digestibility has been observed previously in Atlantic salmon fed a diet with SPC (Refstie et al., 2001). The low lipid and energy digestibilities of the SPC diet may have been caused by low level of phospholipids (PL) in the SPC. PL is efficiently extracted with alcohol, used to remove the soluble carbohydrates during processing of the SPC. PL is important for emulsifying lipids during digestion (Tocher et al., 2008), and ADC of lipid had previous been increased by adding PL to a diet with defatted soybean meal when fed to rainbow trout (Hung et al., 1997).

The high digestibility values of the diets with plant proteins emphasize their potential for use in highly digestible fish feeds. The variability in ADC values obtained for different combinations of plant protein concentrates, however, does not identify a preferable one to the others. Typically, the S-MIX should be used alone if the target was to optimize ADC of N and amino acids, while a combination of P-MIX and S-MIX was preferred if the target was to optimize the ADC of lipid and energy.

C-MIX was only preferred if the target was to maximize ADC of P. This was due to fact that the CPC used had already been dephytinized. Phytic P in the canola meal is almost completely converted into highly digestible inorganic P during the manufacturing of CPC (Thiessen et al., 2004). While the low ADC of P in the SPC diet was mainly caused by the fact that the dietary P presented as phytate (IP6) which is almost indigestible to salmonid (Denstadli et al., 2006b). The results presented in **Paper IV**, showed that incubation of SPC with phytase which reduced the concentration of IP6 from 21.2 g kg^{-1} in the SPC to 11.2 g kg^{-1} in the DSPC, improved the ADC of P by 31%. The DSPC diet also resulted in a 61% higher ADC of P than the FM diet.

3.4 Nutrient retentions

Both the EAA balance and the digestible protein to digestible energy (DP/DE) ratio strongly influence protein utilization (Green and Hardy, 2008). In **Paper I**, most of the plant protein based diets resulted in significantly higher retention of both ingested and digested N compared to FM when fed to trout. In **Paper II**, the plant protein based diets were comparable to the FM diet in terms of retention of N in black sea bream. This may be due to the use of highly digestible plant protein concentrates and the multiple EAA supplementations in these diets. The higher

dietary crude protein level and higher DP/DE ratio in the FM diet may also have contributed to the lower N retention. Increasing the inclusion level of plant proteins reduced N retention, while increasing the L/P ratio significantly increased the N retention in black sea bream. Nitrogen retention, thus appears to be inversely related to feed intake in this species, and the fish generally had higher feed intake of diets containing lower starch and higher intake of NSP. Thus, the differences in N retention may indicate that the digestible energy was controlling feed intake in black sea bream, and that the protein intake in fish that ate the most was in excess of the requirement. This effect was not found in rainbow trout (**Paper I**).

In **Papers III** and **IV**, however, fish fed the FM diet had significantly higher ingested and digested N retentions than those fed plant protein based diets. The high retention of ingested N in fish fed the FM diets was supported by the low N intake. Both the retentions of ingested and digested N were fitted using quadratic models (**Paper III**). Three components (P-MIX, C-MIX and S-MIX) had similar effects on retentions of both ingested and digested N. The significant interaction between P-MIX and S-MIX indicates that using plant protein concentrates from different sources may contribute to a well balanced EAA profile.

Most of the plant protein based diets resulted in significantly lower retention of ingested energy in rainbow trout than the FM diet (**Paper III**). This was mainly due to the reduced digestibility of energy in the plant protein based diets. The phytase pre-treatment of the SPC significantly increased the retention of ingested energy (**Paper IV**).

Most of the plant protein based diets resulted in significantly higher percent-wise retention of ingested P than the FM diet (**Paper III**). This is well supported by the significantly higher P intake and faecal P loss in the FM diet fed fish than fish fed the plant protein based diets. The SPC diet resulted in the lowest ingested P retention among all the plant protein based diets (**Paper III**), and the phytase pre-treatment significantly increased the retention of both ingested and digested P (**Paper IV**). In **Paper III**, a linear model was used to fit the digested P retentions and C-MIX had the strongest effect. This was due to the fact that the CPC used had been dephytinized.

3.5 Fish whole body composition

The main differences between fish meal based diets and diets with plant protein concentrates, and among plant protein concentrate diets were related to mineralization. Typically, the highest dietary level combination of PPC and LPC resulted in reduced whole-body ash content, compared to the lower inclusion level, both in the black sea bream and the rainbow trout (**Papers I** and **II)**. The highest level of dietary plant protein also resulted in lower ash content in the black sea bream, compared to those fed the FM diet (**Paper II**).

In **Paper III,** only a trend (P=0.066) was seen for whole-body ash. However, trout fed the diets containing PPC or SPC at the highest inclusion levels (PPC and SPC diets), and the diet containing the combination of these two (P+S diet), had reduced P and Ca contents compared to the fish fed the FM diet. The CPC containing diets resulted in comparable concentration of P and Ca compared to fish fed the FM diet. Compared to the FM diet, the CPC and C+S diets resulted in significantly higher Mg and Zn contents, respectively. The PPC diet resulted in significantly lower concentration of both Mg and Zn. The reduced availabilities of P, Ca, Mg and Zn is attributed to the presence of phytic acid in PPC and SPC. Likewise, the improved mineral utilization from the CPC is explained by the dephytinized of this protein concentrate by the producer. Phytic P in the canola meal is almost completely hydrolyzed into highly digestible inorganic P during the manufacturing of CPC (Thiessen et al., 2004). Correspondingly, the phytase pre-treatment of SPC reported in **Paper IV,** reduced the concentration of IP6 from 21.2 g kg^{-1} in SPC to 11.2 g kg^{-1} in DSPC. The chelating effect of phytate on di- and trivalent cations (Duffus and Duffus, 1991) was consequently diminished, and the whole body concentrations of P, Ca and Mg, increased as the concentration of digestible P increased from 3.81 g kg^{-1} to 5.18 g kg^{-1}.

The only other parameter that was significantly influenced by the diets was whole-body protein content, which was higher in trout fed the SPC, P+C and P+S diets than those fed the FM diet (**Paper III)**. No differences in whole-body water, lipid and ash contents were seen among these diets. Furthermore, the difference was not ascribed to any particular plant protein concentrate or concentrate mix. Thus, the design of the experiment does not facilitate the explanation to the differences in whole-body protein.

3.6 Fish physiology and health

In both black sea bream and rainbow trout, the plasma triacylglycerol levels were not affected by the dietary inclusion of plant protein concentrates (**Papers I** and **II**). Increasing dietary LPC concentration, however, gave a hypocholesterolemic effect in rainbow trout. This may have been due to the increased ratio of dietary Arg/Lys that resulted from the increasing L/P ratio at both inclusion levels (Sugano et al., 1984; Carroll, 1991) and the existence of conglutin γ in white lupin (Sirtori et al., 2004). The hypocholesterolemic effects were observed in rainbow trout only, indicating that these effects may have been species specific.

In **Papers III** and **IV**, significantly lower plasma P concentrations were found in fish fed the plant protein-based diets compared to the FM diet. This is associated with a higher dietary P level in the FM diet than in the plant protein-based diets. The CPC diet resulted in a higher plasma P concentration than the other plant protein-based diets (**Paper III**). The plasma P concentration was significantly elevated in fish fed the DSPC diet (**Paper IV**). This higher plasma P concentration of fish fed the CPC and DSPC diets, can be explained by the hydrolysis of phosphate from phytic acid (Thiessen et al., 2004; Denstadli et al., 2006a; Denstadli et al., 2007).

The diet with 50 % dietary protein from PPC resulted in the lowest ($P < 0.05$) activity of maltase in the distal intestines (DI) of both species (**Papers I** and **II**). This may indicate mild changes associated with the mechanism that resulted in inflammation in DI at higher dietary PPC levels (Penn et al., 2011).

Low hepatosomatic indices (HSI) and low hepatocyte vacuolization were observed in trout fed the diet with 50 % dietary protein from LPC (**Paper I**). This is normally associated with accumulations of glycogen (Hilton and Atkinson, 1982) and was most likely caused by a lower digestible starch intake than in fish fed the other diets. No somatic indices changes were, however, found in black sea bream fed the different diets (**Paper II**).

No obvious histological differences were found in the MI or DI in trout fed the diet with 50 % of dietary protein from LPC (**Paper I**). No inflammation was observed in the DI of trout fed the diet containing 407 g kg^{-1} PPC, only a slight decrease in mucosal fold height and a slight increase in fold fusion were observed (**Paper I**). Fish fed fish meal-free diets containing 147-295

g kg^{-1} PPC, however, exhibited clear signs of inflammation in the DI (**Paper III**). These findings are consistent with previous reports of air classified PPC causing DI inflammation in Atlantic salmon when included in the feed at high levels (Penn et al., 2011). The PPC used in the two experiments was from the same batch, thus the DI inflammation cannot be caused by the inconsistent of PPC quality. The main difference between the diets used for **Papers I** and **III** was that the diets used for **Paper I** contained fish meal. The diets used for **Paper III** did not contain any fish meal, but contained small amounts of krill products and taurine. Krill meal with the water soluble fraction from krill did not cause changes in intestinal tissues, even when used as the sole source of dietary protein for Atlantic salmon (Hansen et al., 2011). Thus, the results may indicate that trout fed a fish meal-free diet are more sensitive to the agents causing enteritis in the distal intestine than fish fed a diet with fish meal.

3.7 Nitrogen and phosphorus excretions

For the purpose of comparison, both faecal and metabolic losses have been calculated per kg of gain. In **Paper I**, most plant protein-based diets resulted in significantly higher metabolic loss of nitrogen than the FM diet, except for the HLP2 and HLP3 diets. This can be largely explained by the DP/DE ratio (22.6 g MJ^{-1} of the FM diet and 21.2-21.5 g MJ^{-1} of the various plant protein diets). In rainbow trout, it has been reported that an increased DP/DE ratio was associated with decreasing N retention and increasing N excretion (Green and Hardy, 2008). Higher metabolic N loss was observed at high plant protein inclusion compared to low inclusion. This may be due to the more balanced digestible EAA profile at lower inclusion levels, which may facilitate a higher N retention (Green and Hardy, 2008).

The FM diet resulted in significantly lower faecal N loss than the diets containing CPC (except for the P+C+S diet, the diet with the lowest inclusion of CPC) (**Paper III**). This reflects low N digestibility of the CPC. The main factors causing this low digestibility may have been the relatively high fibre content in the CPC (Mwachireya et al., 1999), and high temperature employed during the processing of this protein concentrate (Opstvedt et al., 1984; Aslaksen et al., 2006).

The FM diet resulted in the lowest metabolic N-loss, while the loss from the SPC diet was the highest. The faecal N-loss did not significantly differ between the two diets (**Paper III**). Incubation of SPC with phytase significantly reduced both the faecal and metabolic N losses, when compared to both the SPC and FM diets (**Paper IV**). The reduced faecal loss may be explained by configurational changes in the soy protein during incubation and subsequent extrusion and drying. The marginally higher faecal N-loss from the FM diet may both be a product of drying temperature (Opstvedt et al., 1984), and the existence of indigestible non-protein N in FM (Opstvedt et al., 2000). The lower metabolic loss of the FM diet may have been an effect of reduced DP:DE ratio (Green and Hardy, 2008), thus saving protein from being deaminated for energy production.

In **Paper III**, the FM diet produced significantly higher fecal P excretion than the plant protein-based diets. This was due to the higher dietary P content, and the low digestibility of P in the fish meal. The SPC diet resulted in significantly higher fecal P excretion than other plant protein-based diets. This is reflected by the low P digestibility in this diet, due a high proportion of the dietary P being present as phytate, which is almost indigestible to salmonids (Denstadli et al., 2006b). The CPC diet resulted in the lowest fecal P excretion among all the diets, because the CPC had been dephytinized. The metabolic P loss of fish fed the PPC and P+C+S diets were less than zero. This could be associated with the low digestible P level in these diets (0.16 and 0.20 g $(MJ\ DE)^{-1}$, respectively), which were lower than the recommended level (0.25 g $(MJ\ DE)^{-1}$) for trout (Rodehutscord, 1996). Trout may absorb a small amount of P from the re-circulated water through the gills and intestinal tract (Winpenny et al., 1998).

Incubation of the SPC with phytase reduced faecal excretion and metabolic loss of P to levels significantly below those obtained with the SPC and FM diets (**Paper IV**)**.** The reduction in IP6-concentration of the SPC from 22.2 to 11.2 g kg^{-1} also significantly decreased the chelating effects of phytic acid, thereby improving the absorption of other cationic elements such as Ca and Mg. A 130% fecal excretion of Ca indicated absorption and intestinal excretion of Ca from the water. The ratio among Ca, P, Mg and Zn in bones is constant, and the whole-body concentration of Ca seemed limiting for bone mineralization in trout fed the SPC diet. This was probably a result of IP6 chelating the Ca in the intestine. The Ca deficiency in turn resulted in high metabolic loss of P. This effect was eliminated by dephytinization of the SPC, and the diet

with DSPC resulted in similar whole-body P concentration as the FM diet, despite the 35% lower concentration of P in the feed.

4. Main conclusions

- Both LPC and PPC are promising dietary protein sources for rainbow trout and black sea bream. Any combination of EAA-fortified LPC and PPC can be efficiently used in extruded diets for both species, when total dietary plant protein inclusion is limited to 300 g kg^{-1}. At higher inclusion levels, pure PPC appeared preferable for rainbow trout, and combinations with more PPC were preferred for black sea bream, due to less efficient feed conversion caused by the LPC.

- Rainbow trout had a good appetite and grew rapidly when fed diets with 95% of the protein from plant protein concentrates, with multiple EAA and taurine supplementations and using krill meal and the water soluble fraction of krill as feed attractant.

- The interactions among plant protein concentrates in diet had synergetic effects on feed efficiency and nitrogen retention of fish. Increasing the diversity of plant protein sources in the diet or using a combination instead of single protein ingredient may balance the dietary EAA, leading to higher protein utilization.

- Mixture modeling is a useful tool that can help optimize the use of plant protein concentrates in feed for rainbow trout. Different optimal plant protein combinations can be predicted based on different response criteria.

- Using dephytinized SPC as the predominant protein source in a rainbow trout diet, can improve feed utilization, increase bone mineralization and reduce N and P excretion into the water compared with using untreated SPC.

5. Future perspectives

The results illustrate that the majority of dietary protein in feed for carnivorous fish can be supplied by plants. There are still several questions, related to the use of plant protein concentrates in carnivorous fish feed, that need to be addressed.

1) The reduced mineral utilization will be the major limiting factor associated with the efficient use of plant protein concentrate to provide high percentage of dietary protein for both species. The strategies to solve this problem should be species specific. For rainbow trout and other fish reared in cold water, using phytase to pre-treat the ingredient with high phytate content is an efficient way. For black sea bream and other warm water species, other more cost-efficient process such as dietary phytase supplementation can also be tested.

2) The impaired lipid digestibility is a limiting factor for using SPC as the primary protein source of fish meal-free diets for rainbow trout. The mechanisms involved in this should be determined, and strategies to solve this problem should be developed.

3) The mild to moderate inflammatory changes found in the distal intestine of rainbow trout fed the diets with PPC, and eventually potato protein concentrate, may represent a limitation to the use of these ingredients. Firstly, there is a need to determine if both feed ingredients are responsible for these changes, or only the PPC that has been found to cause similar changes in Atlantic salmon. Secondly, the causes for these changes in the intestine should be identified, and subsequently eliminated.

4) Other processing strategies should be developed to reduce the high level of NSP in LPC. The factors causing reduced starch digestibility when using this ingredient should be identified, and subsequently eliminated during production of LPC.

5) The use of mixture models proved useful for optimizing the use of plant protein concentrates in diets for fish. Mixture methodology should be further pursued in feed ingredient evaluation, aimed at developing feed formulation programs that more accurately predict the consequences of dietary ingredient mixtures rather than the economical and nutritional values of individual ingredients only.

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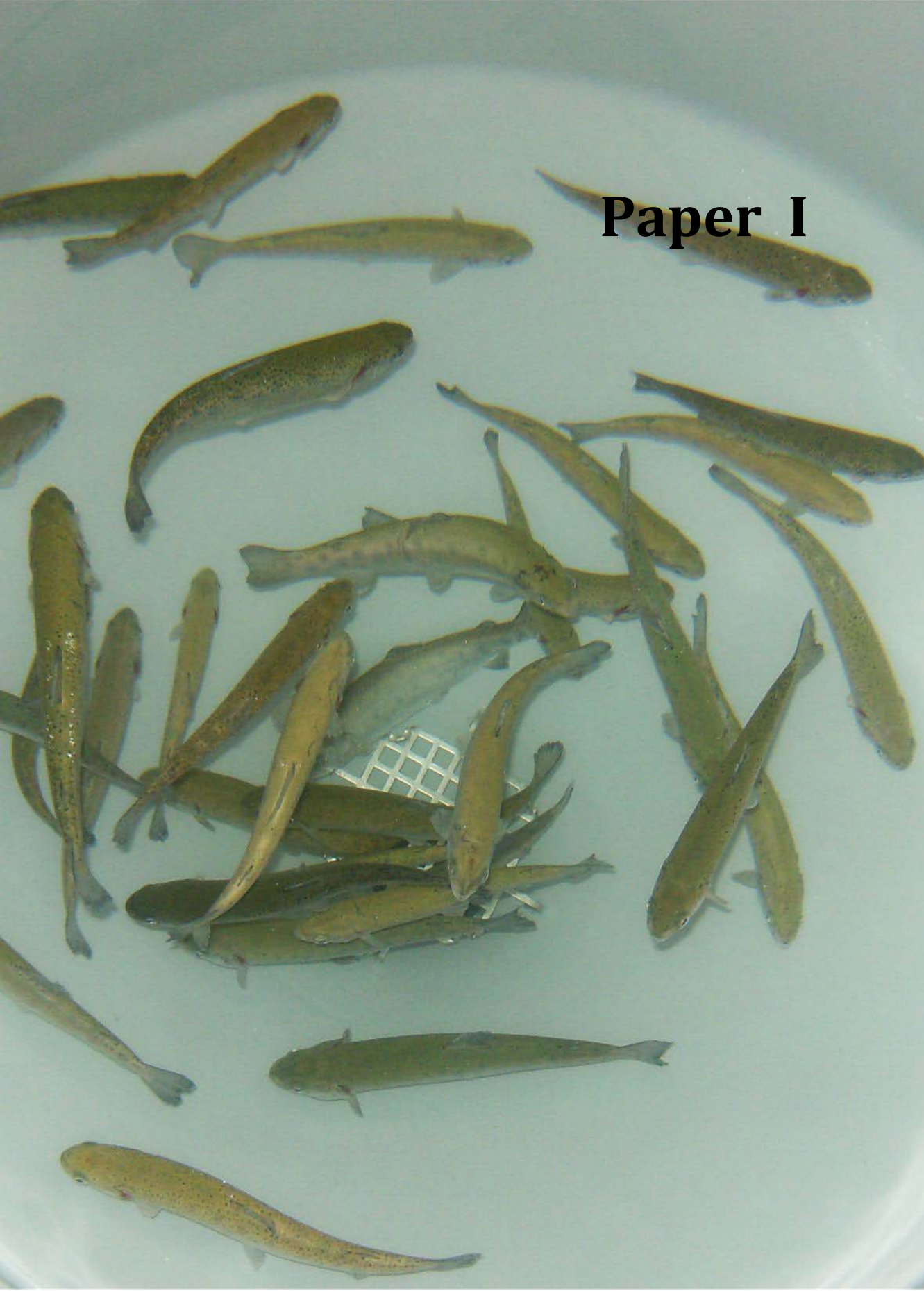
Paper I

Optimal inclusion of lupin and pea protein concentrates in extruded diets for rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The aim of this experiment was to determine the optimal inclusion level and ratio of lupin (LPC) and pea protein concentrates (PPC) in multiple essential amino acid-supplemented diets for rainbow trout (*Oncorhynchus mykiss*). Nine extruded diets, including eight plant protein based diets formulated using four mixtures of LPC and PPC (L/P ratio, 3:0, 2:1, 1:2 and 0:3) with two dietary inclusion levels (300 or 500 g plant protein kg^{-1} dietary protein) and one diet with LT fish meal as the sole protein source (FM diet) were used. Duplicate tanks of 58 g trout reared in 9^{o}C water were fed the diets for 62 days, followed by a 20-day digestibility experiment. No mortality occurred, an average weight gain (WG) of 210% and an average feed conversion ratio (FCR) of 0.72 g ingested dry matter (g gain)$^{-1}$ was obtained. Plant protein inclusion level or L/P ratio did not significantly affect feed intake, WG, body composition (except ash), or retention of nitrogen

(N) or energy. High inclusion of plant protein concentrates resulted in significantly higher FCR, apparent digestibility (ADC) of N, lipid, methionine (Met) and tryptophan (Trp) and plasma triacylglycerols level than low inclusion. However, ADC of dry matter, starch and histidine, whole body ash content and plasma cholesterol levels were reduced. The diets with the highest L/P ratio (3:0) resulted in significantly higher FCR than the other diets with less LPC. ADC of starch was reduced by increasing dietary LPC. The diets with the most PPC (L/P ratio = 0:3) inclusion resulted in significantly higher ADC of dry matter, N, starch and energy than other diets with less PPC, and higher plasma cholesterol level than diets with L/P ratio of 3: 0 and 1:2. Lipid digestibility was increased by increasing LPC at the 500 g protein kg^{-1} dietary protein inclusion level, but not for 300 g kg^{-1}. The diet with most LPC also resulted in lower trypsin activity in mid-intestine digesta than diets with less LPC and the FM diet. No intestinal inflammation was observed for any of the dietary treatments. However, trout fed the diet with highest inclusion of PPC tended to have a slight decrease in mucosal fold height and a slight increase in fold fusion. In conclusion, any combination of LPC and PPC with essential amino acid-supplementation can be efficiently used when total plant protein inclusion is limited to 300 g kg^{-1} crude protein in extruded diets for rainbow trout. At higher inclusion, PPC appears to be a preferable source of protein.

Keywords: Rainbow trout; White lupin protein concentrate; Pea protein concentrate; Growth; Nutrient utilization; Brush border enzyme; Histology; Soybean meal-induced enteritis

Introduction

Pea and lupin have already proved their potential as feed ingredients for salmonids, such as rainbow trout (Burel et al., 1998; Glencross et al., 2002; 2003b; 2004a; 2008) and Atlantic salmon (Carter and Hauler, 2000; Glencross et al., 2004b; Refstie et al., 2006; Aslaksen et al., 2007). There are, however, still challenges associated with the use of these two legumes at high concentration in diets for salmonids. These include imbalanced essential amino acid (EAA) composition (Glencross et al., 2003a), poor palatability (Tulli et al., 2007), and the presence of anti-nutritive factors (ANFs) (Francis et al., 2001). Furthermore, both seeds have low protein and energy density due to high contents of carbohydrates such as starch, indigestible oligosaccharides and non-starch polysaccharides (Evans et al., 1993; Drew et al., 2007). Balanced amino acid composition can be obtained by supplementing limiting EAA in diets for salmonids (Gomez-Requeni et al., 2004). Ingredient processing, such as de-hulling (Glencross et al., 2007), thermal treatment (Davies and Gouveia, 2008), soaking (Ogunji et al., 2008), fermentation (Chango et al., 1993) and enzyme treatment (Farhangi and Carter, 2007) can, to some extent, reduce the ANFs in the seed. Furthermore, genetic improvement of plants, such as lupin, has successfully produced varieties with lower contents of detractive alkaloids, which improves palatability of these feed ingredients (Petterson, 2000).

Improving the nutritional quality of lupins and peas through processing allows for an increased inclusion in fish diets. Removal of indigestible carbohydrates by aqueous extraction of lupin and air classification of peas results in products that are efficiently utilized by salmonids (Carter and Hauler, 2000; Thiessen et al., 2003; Øverland et al., 2009). In contrast to extraction with hot water and alcohol, which efficiently removes the factors causing soybean meal induced enteritis

in the distal intestine in salmonids (van den Ingh et al., 1991; Krogdahl et al., 2003), processing by air classification is not adequate to remove similar factors in pea protein concentrate, that cause enteritis at high inclusion levels (Penn et al., 2011). Mixing plant-protein ingredients with different nutritional characteristics can improve the dietary nutritional balance and overcome adverse effects on palatability (de Francesco et al., 2004; 2007). It remains unknown whether a mixture of different plant protein sources will overcome the adverse effects of ANFs in fish. Recent findings, however, show that soybean-meal induced enteritis can be prevented by adding bacterial protein to a soybean meal-based diet for Atlantic salmon (Romarheim et al., 2011).

The aim of the present experiment was to determine the effects of increasing levels and ratio of lupin protein concentrate (LPC) and pea protein concentrate (PPC) in multiple amino acid-supplemented diets on growth, digestibility, intestinal enzyme activity and histology of rainbow trout.

Materials and Methods

This study consisted of a 62-day feeding experiment and a subsequent 20-day digestibility experiment.

2.1 Ingredients and diets

The LPC was derived from white lupine (*Lupinus albus*), produced by dehulling, milling, aqueous extraction of lupine seeds to remove sugars and soluble non-starch polysaccharides,

heating and spray-drying. Pea protein concentrate was produced from yellow field pea (*Pisum sativum* L.) by dehulling, fine grinding and air-classification. The chemical composition of these two plant concentrates and the LT fish meal used in the diets is shown in Table 1. The LPC and PPC were each supplemented with the first-three limiting EAA (lysine, methionine, and tryptophan / threonine) to balance the EAA profile to that of LT fish meal.

A 2 x 4 factorial design was used in the present study, where the factors were inclusion level of plant protein concentrate (300 or 500 g plant protein kg^{-1} dietary protein) and mixing ratio between EAA-supplemented LPC and PPC in diets (L/P ratio at 3:0, 2:1, 1:2 and 0:3). The diets were isonitrogenous (463 – 485 g crude protein (CP) kg^{-1}) and isolipidic (191 - 208 g crude lipid (CL) kg^{-1}). In addition, a diet with LT fish meal as the sole source of protein (FM diet) was produced with 538 g CP and 228 g CL kg^{-1}, formulated to keep the same ratio between protein and lipid as the diets with plant protein sources. Yttrium oxide was used as a marker for digestibility measurement, in accordance with Austreng et al. (2000). Feed formulation and chemical composition are shown in Table 2.

All the diets were manufactured at the Centre for Feed Technology, at UMB, Ås, Norway. All the dry ingredients were ground through a 0.80-mm screen, mixed, preconditioned and extruded in a twin screw extruder with 2.0 mm die. The extrusion process was optimized to obtain a bulk density > 516 g l^{-1} in the pellets before drying, in order to facilitate slow sinking of the feed after drying and coating with lipid. The diameter of the dies was 2.8 mm. Pellets were dried to 930 g dry matter kg^{-1} and then coated with fish oil in a Forberg (Larvik, Norway) 6-l mini-coater. The equipment used for feed processing, monitoring of extrusion parameters, and methods for

assessment of physical pellet quality have been described in detail by Øverland et al. (2009). Briefly, breaking force and pellet width were determined with a texture analyzer, pellet length with a calliper, and pellet durability was estimated with a Holmen pellet tester (Borregaard Lignotech, Warringtom, UK).

2.2 Fish feeding and sampling

The 62-day feeding experiment was carried out in the Fish Nutrition Laboratory at UMB, with an indoor recirculation system. Before the feeding experiment started, the fish were deprived of feed for 48 h, and then a total of 720 juvenile rainbow trout with an average weight of 58 g were randomly assigned to 18 cylindrical 200-l fibreglass tanks, forty fish per tank. Each tank was supplied with freshwater at a flow rate of 6 - 7 l min^{-1}, and constant light was maintained. During the feeding experiment, water temperature ranged from 7.9 to 8.8 ºC, with a mean of 8.2 ºC. Dissolved oxygen levels were above 6.0 mg l^{-1} in the outlet water, based on daily measurements. Each diet was fed to fish in duplicate tanks, and the trout were fed three times per day (08:00, 14:00 and 20:00 h), 40 min per feeding using automatic band feeders.

Fish were fed ten percent in excess, based on average feed intake over the previous 3-day period. Uneaten feed was sieved from the outlet water and feed intake was monitored by the method of Helland et al. (1996), except that uneaten feed was collected immediately after each meal. Before the start of the experiment, 2 × 5 fish from the holding tank were euthanized with an overdose of MS-222, and stored at -20 ºC for whole body analysis. Fish were anaesthetized with MS-222 (90 mg l^{-1}) and batch-weighed in the beginning (Day 0) and the middle (Day 31) of the experiment.

At the end of feeding experiment, five fish were randomly sampled from each tank for blood and tissue samples for histology. The fish were weighed individually, and blood was collected from the caudal vein with heparinised vacutainers, kept on ice until centrifuged at 3000*g for 10 min. Then plasma was aliquoted into two separate Eppendorf tubes, frozen in liquid N_2 and kept at -80^{o}C until analysis.

The intact gastrointestinal (GI) tracts of the same fish were removed, and divided into 4 regions: stomach (ST), pyloric region (PR, from the distal side of the pyloric sphincter to distal-most caecum), mid intestine (MI, from distal side of the pyloric region to distal intestine) and distal intestine (DI, starting with the increase in intestine diameter and ending with anus). All the intestinal sections were opened longitudinally, and tissue samples of 1 cm^2 were taken from ST, MI, and DI wall, fixed in phosphate buffered formalin (4%; pH 7.4) for 24 h, and then transferred to 70% ethanol for storage until processing. The liver was also removed from the same fish, weighed, and 1 cm^2 × 0.5 cm histological samples were taken following the same procedure. Another 10 fish were taken from each tank, weighed individually, dissected, and eviscerated. Surface fat and connective tissue were carefully removed. The contents (digesta) from the PR, MI and DI sections were collected in Eppendorf tubes separately, frozen in liquid N_2 and kept at -80^{o}C for bile acid and trypsin activity analysis. The respective tissue walls of MI and DI were placed in pre-weighed containers, frozen in liquid N_2 and kept at -80 ºC for the determination of brush border leucine amino peptidase (LAP) and maltase activities. Three fish were taken from each tank, weighed individually, killed by a blow to the head, cut open to remove the intestinal contents, and stored at -20 ºC for whole body analysis. The remaining 22 fish in each tank were batch-weighed and kept for 3 rounds of faeces stripping. In each round,

fish were fed using the same procedure as in the feeding experiment for 6 d, then anaesthetized by MS-222, and stripped for faeces by the method of Austreng (1978). All the faecal samples from the same tank were pooled and stored at -20 ºC prior to analysis. Only the samples from the fish fed the FM diet and the diets with 500 g plant protein kg^{-1} crude protein inclusion were measured for trypsin activity and bile acid concentration in the intestinal contents from PI, MI and DI, and brush border enzyme activities in MI and DI. Only the samples from the fish fed the FM diet and the diets with the highest inclusion of LPC (HLP1) and PPC (HLP4) were used for histological evaluation.

2.3 Analyses

The initial and final whole body samples were homogenized with dry ice (CO_2) in a food processor and freeze-dried. Pooled faeces samples were freeze-dried and ground with a pestle and mortar. Fish scales were removed prior to analysis. Feed ingredients, feeds, and freeze-dried faeces samples were analyzed for dry matter (EU 71/393), Kjeldahl nitrogen (N) (EU 93/28), lipid (HCl hydrolysis and diethylether extraction (EU 98/64)), ash (EU 71/250) and minerals (ICP-AES/ICP-MS) (Nordic Committee on Food Analysis (NMKL) method 161) and starch (AOAC enzymatic method 996.11). Freeze-dried whole body samples were analyzed for proximate composition using the same methods, except that no HCl hydrolysis was employed. Gross energy was measured by bomb calorimetry (Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA). Amino acid (except tryptophan) analysis of all samples was according to EC (1998) on a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK). Tryptophan was analysed according to EC (2000) on a Dionex Summit HPLC system, with a Shimadzu RF-535

fluorescence detector. Yttrium oxide concentration in feed and faeces was determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete digestion of the homogenized and dried samples in HNO_3 after cooking in a microwave oven for 1 h. Plasma cholesterol and triacylglycerols were analysed by Cobas® Integra enzyme kits and automatic analyser equipment (Cobas Mira, Hoffman-La Roche & Co., Basel, Switzerland). Bile acids in the digesta samples were analysed by enzymatic assay (Bio-Stat Ltd, Stockport, UK). Activities of brush-border membrane bound leucine amino peptidase (LAP) and maltase were analyzed as described by Krogdahl et al. (2003). Trypsin activity of digesta samples was determined colorimetrically according to methods described by Kakade et al. (1973) and Krogdahl et al. (2003), using the substrate Na-benzoyl-dl-arginine-*p*-nitroanilide (BAPNA) (Sigma no. B-4875; Sigma Chemical Co., St. Louis, MO, USA), and a curve generated from a standardized bovine trypsin solution.

2.4 Histological evaluation

Processing of the histological tissues was done at the Section for Anatomy and Pathology of the Norwegian School of Veterinary Science (Oslo, Norway) using standard histological techniques. The MI was sectioned transversely, whereas the DI sample was sectioned longitudinally (i.e. perpendicular to the macroscopically complex folds; approximately 5 μm thick) and stained with haematoxylin and eosin (HE). Blind histological examination was performed using light microscopy. Two independent evaluations were performed. Tissue morphology was evaluated according to the descriptions of Amin et al. (1992) and Baeverfjord and Krogdahl (1996).

2.5 Calculations and statistical analysis

Expansion ratio (%) of pellets was calculated as: 100 × ((diameter of extrudate − die diameter) × die diameter^{-1}). Feed intake (FI) was estimated by subtracting uneaten feed from the amount fed on a dry matter basis. Recovery of uneaten feed was estimated as described by Helland et al. (1996). Weight gain (WG, %) was calculated as: $WG = 100 \times (FBW - IBW) \times IBW^{-1}$, where FBW and IBW represent final body weight and initial body weight, respectively. Feed conversion ratio (FCR) was calculated as: $FI \times (FBW - IBW)^{-1}$, where FI is feed intake. Apparent digestibility coefficients (ADC_N) of individual nutrients and energy were calculated as: $100 \times (1 - (Y_d \times Y_f^{-1} \times N_f \times N_d^{-1}))$, where Y_d and Y_f represent the concentration of yttrium in the diet and faeces, N_d and N_f represent the concentration of individual nutrients or energy in the diet and faeces, respectively. Faecal excretions (F_N) were calculated as: $100 - ADC_N$. Nutrient and energy retentions (R_N) were calculated as: $100 \times (N_1 \times FBW - N_0 \times IBW) \times (N_d \times FI)^{-1}$, where N_0 and N_1 represent the nutrient or energy concentration in the initial and final whole fish samples (pooled samples of 3 fish per tank), respectively. Metabolic loss of nutrients and energy were calculated as: $FI_N - (F_N + R_N)$, where FI_N represent the nutrient or energy intake. Hepatosomatic index (HSI) was calculated as: $HSI = 100 \times W_l \times W_f^{-1}$, where W_l and W_f represent liver weight and fish weight, respectively. Analytical residue in ingredient and diet was calculated as: dry matter – (crude protein + lipid + starch + ash).

The results were analysed using the GLM procedure of SAS statistical software (SAS, 1999). One-way analysis of variance (ANOVA) was used to compare effects of the FM diet with those of the plant protein diets. Factorial ANOVA used to analyse the effects of plant protein inclusion level and L/P ratio. Significant ($P < 0.05$) interactions between inclusion level and L/P ratio were rationalized by regression analysis of L/ (L + P) within inclusion level, if at least one of the main

effects were significant (Snedecor and Cochran, 1967). The results were expressed as the means and pooled standard errors of means (S.E.M). Duncan's multiple-range test was used to rank significant differences among diets in the one way ANOVA and main effects in the factorial ANOVA.

3. Results

3.1 Extrusion parameters and physical pellet quality

Mainly, feeding rate and water addition to the preconditioner were the two adjustable parameters used to optimise bulk density (Table 3). Consequently, torque and specific mechanical energy (SME) varied among the diets. Overall more water was needed for the diets with 500 g plant protein kg^{-1} dietary protein compared to 300 g kg^{-1}, and the water addition was slightly increased with the proportion of LPC in diet. Least water was added to the process when the FM diet was extruded and this diet also generated lower SME and torque compared to the diets with high content of PPC. For the diets with 300 g plant protein kg^{-1} dietary protein inclusion, a linear increase of durability with the increasing PPC was observed, while for the diets with 500 g protein kg^{-1} dietary protein inclusion, such effects on durability was not as pronounced. Durability of the pellets was mainly associated with torque. The effects of inclusion level and L/P ratio on the breaking point showed the same trend as the durability, but less distinct. Expansion ratio ranged between 39-52 % among the diets and was affected by ingredient composition and extruder parameters.

3.2 Growth and feed utilization

No mortality occurred, and all of the fish had high feed intake and grew well. Average weight gain was 210 % and the average FCR was 0.72 g DM ingested (g gain)$^{-1}$. Both during the first 30 days and over the whole period, feed intake (FI) and weight gain (WG) did not differ significantly among diets (Table 4). Neither inclusion level of plant protein sources nor the ratio between LPC and PPC (L/P ratio) caused significant differences in FI or WG. The FCR of trout fed the FM diet was not significantly different from that of the fish fed the LLP3, LLP4, HLP2, or HLP 4 diets during the first 30 days and the LLP4 diet for the whole experiment. All other diets resulted in significantly higher FCR than the FM diet.

Trout fed the diets with 500 g plant protein kg^{-1} dietary protein inclusion had significantly higher FCR than those fed the diets with 300 g kg^{-1} inclusion (Table 4). The main effects of L/P ratio were significant, indicating higher FCR for the diets with the most LPC (L/P ratio = 3:0). Significant interaction between plant protein inclusion level and L/P ratio for FCR was seen for the first 30 days of feeding (Table 4, Fig. 1). The interaction is rationalized by a quadratic response at the inclusion level of 300 g plant protein kg^{-1} dietary protein, where the FCR levelled off at higher L/P ratios. The response at the inclusion level of 500 g kg^{-1} was quadratic also, but the FCR value was significantly increased for the highest L/P ratio.

3.3 Digestibility

The apparent digestibility coefficient (ADC) of crude protein (Table 5) of fish fed the FM diet was lower ($P < 0.001$) than that of fish fed the LLP4, HLP3 and HLP4 diets, but not significantly different from that of fish fed the other diets. The ADC of lipid of fish fed the FM diet was significantly lower than that of fish fed the LLP4, HLP1 and HLP2 diets. The ADC of starch and

energy of fish fed the FM diet were not significantly different from that of fish fed the LLP4 and HLP4 diets, but significantly higher than that of fish fed the other diets. The ADC of crude protein and lipid were significantly higher for the 500 g plant protein kg^{-1} dietary protein inclusion level than for the 300 g kg^{-1} (Table 5). The ADC of energy was not affected by inclusion level. The diets with most PPC (L/P ratio = 0:3) resulted in significantly higher ADC of crude protein and energy than the diets with less PPC. The diets with most LPC (L/P ratio = 3:0) gave a significantly higher ADC of lipid than that of the diets with L/P ratio of 1:2, but did not differ from the other treatments. A significant interaction between plant protein inclusion level and L/P ratio for ADC of lipid was seen (Fig.2). At high plant protein inclusion level, the ADC of lipid increased with the increasing L/P ratio, while this was not significant at the low inclusion level.

The ADC of starch was significantly higher at 300 g plant protein kg^{-1} dietary protein inclusion than at 500 g kg^{-1} (Table 5; Fig. 3). A significant interaction between inclusion level and L/P ratio for ADC of starch was seen where the ADC of starch declined at a greater rate at 500 g plant protein kg^{-1} dietary protein inclusion than at 300 g kg^{-1}. The ADC of starch decreased in a linear manner from more than 97 % to 86 % with increasing levels of LPC in the diets (Fig. 4). There was a significant negative relationship between the analytical residue and ADC of starch for both the 300 and 500 g plant protein kg^{-1} dietary protein inclusion levels (Fig. 5).

Compared to the FM diet, the EAA Arg, Lys, Met and Phe were significantly more efficiently digested in the plant protein diets (Table 6). Also Trp was more efficiently digested in the plant protein diets, except for the LLP2 and LLP3 diets. The same was found for Thr, except for the

LLP3 and HLP3 diets and for Val, except for the LLP3, HLP2 and HLP3 diets. Leu was more efficiently digested in the LLP1, LLP4, HLP1 and HLP4 diets, Ile in the LLP1, LLP4 and HLP4 diets, and His in the LLP4 and HLP4 diets. All plant protein diets had similar or higher EAA (including Cys) digestibility than the FM diet.

Higher inclusion of plant protein in the diets resulted in significantly lower ADC of His, but higher ADC of Met and Trp. The ratio between LPC and PPC significantly affected the ADC of His, Ile, Lys, Met, Phe, Trp, Thr, and Cys. The diets with most PPC (L/P ratio = 0:3) resulted in significantly higher ADC of His than the diets with less PPC, and significantly higher ADC of Ile, Lys, Phe, Trp, and Thr than the diet with L/P ratio of 1:2. The diets with most LPC (L/P ratio = 3:0) resulted in significantly higher ADC of Met, Phe and Trp than treatments with L/P ratio of 1:2. The diets with L/P ratio of 1:2 resulted in significantly lower ADC of Cys than other diets. No interaction between inclusion level and L/P ratio was found for EAA and Cys digestibility. Regression analysis did not reveal any consistent relationships between ADC of Cys and any of the extrusion or physical quality parameters.

3.4 Body composition, nutrient retention and metabolic loss

No significant differences were found in whole body composition among diets (Table 7). Neither the inclusion level of plant protein nor the L/P ratio resulted in significant differences in whole body composition except for ash, which was significantly lower in the fish fed the diets with 500 g plant protein kg^{-1} crude protein than with 300 g kg^{-1}.

Retention of both ingested and digested nitrogen of the trout fed the FM diet were significantly

lower than for those fed the plant protein diets, except for the ingested nitrogen retention of those fed the LLP3 and HLP1 diets and the digested nitrogen retention of those fed the LLP3, HLP1, and HLP4 diets (Table 8). Retentions of ingested and digested energy did not significantly differ among diets. The inclusion level of plant protein sources and the L/P ratio did not significantly affect the retention of ingested or digested nitrogen or energy. The FM diet resulted in higher metabolic loss of nitrogen. The metabolic loss of nitrogen was higher in trout fed the FM diet than any of the dietary treatments with 300 g plant protein kg^{-1} dietary protein, and the fish fed the HLP2 and HLP3 diets. The metabolic loss of nitrogen was higher for the 500 g plant protein kg^{-1} dietary protein inclusion level than for the 300 g kg^{-1}. A significant interaction between inclusion level and L/P ratio for metabolic nitrogen loss was found (Table 8). This was rationalized by a parabolic effect of L/P ratio at the high inclusion, while no significant effect was seen at low inclusion (Fig. 6).

The EAA profile of whole body samples were evaluated only in fish fed the HLP1, HLP4, and FM diets (Table 9). The whole body EAA profile of the initial and final samples were similar, and also close to the profile of LT- fish meal, except for the level of His which was lower in LT-fish meal. In each treatment, the retentions of digestible Arg and Trp were lowest and His was highest among all EAAs. The retentions of digestible Arg and Phe in trout fed the FM diet were significantly higher than that of trout fed HLP1 and HLP4 diets.

Plasma triacylglycerol level did not differ significantly among dietary treatments (Table 7). Plasma cholesterol level was significantly higher in trout fed the FM diet than in trout fed the plant protein diets. Inclusion of 500 g plant protein kg^{-1} dietary protein resulted in significantly

higher triacylglycerols level, but lower cholesterol level than inclusion of 300 g kg^{-1}. The dietary treatments with L/P ratio = 3:0 resulted in significantly lower plasma cholesterol level than L/P = 2:1 and L/P = 0:3, while L/P = 1:2 was intermediate.

Generally, both trypsin activity and bile acid concentration in intestinal content decreased throughout the intestine (Table 10). Only the trypsin activity in the content of mid-intestine was significantly affected by L/P ratio, and fish fed the HLP1 diet had the lowest and those fed the HLP3 diet had the highest value. Digestibility of tryptophan decreased with increasing trypsin activity (TA) in the mid intestine up to a TA of 250 U $(mg\ DM)^{-1}$, and levelled out at higher TA levels (Fig. 7). The bile acid concentration in the contents of different intestinal sections was not affected by the L/P ratio. Leucine aminopeptidase (LAP) activity in the intestinal tissue was not significantly affected by the L/P ratio (Table 10). No significant differences were seen for maltase activity (MA) in MI by ANOVA. A significant quadratic decrease in MA with increasing L/P ratio (LPR, expressed by L/ (L+P)) ($MA = 4\ E\text{-}4\ LPR^2 + 7.94\ E\text{-}2\ LPR + 15.675$, $R^2 = 0.58$) was seen. Significant differences were seen for MA in DI, where the FM diet resulted in a lower value than the HLP2 diet. In contrast to what was found in MI, MA in DI increased with increasing L/P ratio ($MA = -1\ E\text{-}3\ LPR^2 + 1.08\ E\text{-}2\ LPR + 31.916$, $R^2 = 0.76$).

3.5 Histology of liver and intestine

No obvious histological differences were found in the MI and DI in trout fed the FM or the LPC diet. No inflammation was observed in trout fed the PPC diet, but a slight decrease in mucosal fold height and a slight increase in fold fusion were seen. Hepatosomatic Index (HSI) was significantly higher in trout the FM diet than in trout fed the HLP1 and HLP4 diets (Table 11).

Only the trout fed the HLP1 diet, however, had lower hepatocyte vacuolization compared to those fed the FM and HLP 4 diets.

4. Discussion

The present experiment has clearly demonstrated the potentials of LPC and PPC as major protein sources in extruded diets for rainbow trout. The rainbow trout reared in cold (8.2 ºC) freshwater obtained an average weight gain of more than 210 % after a feeding period of nine weeks. This growth rate is comparable to the growth in similar studies reported by Glencross et al. (2006) and Thiessen et al. (2003) who used smaller fish (≈ 35 g) and higher water temperatures (15 - 17 ºC). The rapid growth achieved could be attributed to both high feed intake and efficient feed conversion. The high feed intake and absence of significant differences among diets in the present experiment are consistent with previous work with LPC or PPC used individually in diets for rainbow trout (Thiessen et al., 2003; Glencross et al., 2006) and Atlantic salmon (Carter and Hauler, 2000; Øverland et al., 2009; Penn et al., 2011).

Bitter components in plant protein sources are known to be the main limiting factors for feed intake in salmonids. Saponins are the main bitter components present in pea. Saponins are not removed by air-classification, and may even be concentrated in PPC compared to pea meal (Drew et al., 2007). Two types of saponins, both with bitter taste, are present in pea, DDMP and saponin B. The content of the two saponins in pea varies among varieties (0.7 - 1.5 g kg^{-1} and 0 - 0.4 g kg^{-1}, respectively) (Heng, 2005). Lupin alkaloids have also been reported to possess a strong anti-palatability effect in rainbow trout (Serrano et al., 2011). White lupin seeds are,

however, not among the bitter varieties, and the total lupin alkaloid concentration may range from 120 (Petterson, 2000) to 500 mg kg^{-1} seed (Chango et al., 1993). Lupanine dominates in white lupin, accounting for 70% of total alkaloids. The remainders are albine (15%), multiflorine (3%), 13-OH lupanina (8%) and angustifoline (1%) (Wink et al., 1995). Rainbow trout can tolerate dietary concentrations of more than 100 mg kg^{-1} lupinine before feed intake is reduced (Serrano et al., 2011). The high feed intake in the present experiment demonstrated that neither the level of saponins in PPC nor alkaloids in LPC negatively affected feed intake of rainbow trout, even at inclusion levels as high as 407 g PPC or 439 g LPC kg^{-1} diet.

The apparent digestibility of protein and individual amino acids was similar to the values obtained with rainbow trout fed a LT fish meal based diet in freshwater using the same faecal collection method (Sørensen et al., 2002), except that digestibility of Trp was higher in the current experiment. Higher digestibility values for crude protein in lupin products than in high-quality fish meal have been reported in rainbow trout (extruded lupin (Burel et al., 2000); *L. angustifolius* and *L. luteus* protein isolates (Glencross et al., 2010); extruded *L. angustifolius* and *L. luteus* protein concentrates (Glencross et al., 2011)). Higher digestibility of crude protein and individual amino acids in the same type of pea protein concentrate has previously been reported in Atlantic salmon (Øverland et al., 2009). This high digestibility of protein and the lack of influence of the feed processing parameters on the ADC of Cys illustrates that the heat processing of both plant protein concentrates has been lenient, and that lupin and pea have low protease inhibitor activity (Valdebouze et al., 1980). Excessive heating results in reduced protein digestibility due to increased cross linking among amino acids, Maillard reaction, and loss of availability of amino acids with an ε-amino group, and oxidation of sulphur-containing amino

acids (Opstvedt et al., 1984). Protease inhibitors in legume seeds reduces protein digestion through inhibiting trypsin or chymotrypsin (Domash et al., 1993), but also increases endogenous faecal loss by binding to these proteases in a way that they are not hydrolyzed and reabsorbed (Krogdahl et al., 1994). Both trypsin and chymotrypsin are rich in Cys. The high ADC for all amino acids except Cys can also be related to the extrusion of the diets, because extrusion unfolds globular seed proteins, thus making these proteins more available to the proteases and thereby increasing their digestibility (Camire, 1991).

The higher digestibility of Met, Thr and Trp at 500 g plant protein kg^{-1} crude protein inclusion compared to 300 g kg^{-1} inclusion may partly be rationalized with higher supplementation of these amino acids in crystalline form. However, Lys did not show a similar response. The ADC of Thr was also similar at L/P of 3:0 and 0:3, despite no supplementation at L/P = 3:0 and relatively high supplementation at L/P = 0:3. Thus, at least the high digestibility of Thr is ascribed to the plant protein concentrates rather than the supplementation with crystalline amino acids.

The efficient utilization of the plant protein sources for growth in the present experiment supports previous findings with rainbow trout, and suggests that supplementation with limiting EAA is efficient (Sanchez-Lozano et al., 2009). The similar retention efficiency of Lys, Met and Thr in fish fed the diets with 500 g plant protein kg^{-1} crude protein derived from LPC, or PPC and the FM diet indicates that the feeding strategy and EAA supplementation was effective. Feeding three times per day at 5h intervals was sufficient to compensate for the reduced efficiency of protein synthesis (Zarate and Lovell, 1997) due to faster absorption of synthetic amino acids over protein-bound amino acids (Ambardekar et al., 2009). Previous studies with

rainbow trout have concluded that frequent or even continuous feeding is preferred to minimize the difference in absorption between peptide-bound and crystalline EAA (Batterham, 1974; Kaushik and Seiliez, 2010).

The higher retention of digested nitrogen, and lower metabolic loss in several of the groups of fish fed the plant protein-based diets, are largely explained by the DP/DE ratio (22.6 g MJ^{-1} for the FM diet and 21.2-21.5 g MJ^{-1} for the various plant protein diets). Recent results indicate that the optimal DP/DE ratio is similar to or lower than 18 g MJ^{-1} in trout of similar size (Green and Hardy, 2008). The utilization of digestible energy for growth was similar between the fish fed the FM and all the plant protein diets, which is explained by similar ratios between nitrogen and lipid, and nitrogen and starch in the diets. The retention data strongly suggest that all EAA were fed in excess of the requirement. Furthermore, the consistency and magnitude in metabolic loss of His for the FM, HLP1 and HLP4 diets shows that the EAA balance was not an important factor to explain differences in nitrogen retention.

The EAA profile of LT-fish meal was close to the EAA profile of rainbow trout whole body, except for His. The higher efficiency in using His for growth suggests that this EAA is the first limiting amino acid in LT-fish meal for rainbow trout. The retention of His was also highest in both diets with high levels of EAA-supplemented plant protein concentrates, and the value for the diet with most LPC closely resembled that of the FM diet. This indicates that His also became the limiting EAA under the current regime of EAA supplementation. The other EAA retention values fell within a narrow range from 43 to 48%, indicating that each of them was equally utilized for protein synthesis.

The overall lipid digestibility in the trout was high, and of the same magnitude as previously observed with rainbow trout in freshwater fed diets with fish oil as the main source of lipid (Morken et al., 2011). The analytical residue, indicating the level of insoluble non-starch polysaccharides (NSP) (Knudsen, 1997), was 4-6 times higher in the plant protein concentrate diets than in the FM diet, and increased with increasing amounts of LPC in the feed. NSP in digesta is known to negatively affect the digestion and absorption of other nutrients (Sinha et al., 2011). Soluble NSP can increase the intestinal viscosity and thereby decreasing the rate of passage and entrap soluble components in the intestine such as bile acids (Pasquier et al., 1996), impairing digestion and absorption of lipid (De Lange, 2000). However, lipid digestibility was higher in fish fed the two diets with the highest LPC inclusion (HLP1 and HLP2) than those fed the FM diet. This supports the assumption that the NSP remaining in LPC after extraction were insoluble. This assumption is further supported by the lack of effect of LPC on the bile acid concentration in digesta from the different intestinal sections of the trout. Generally, soluble NSP bind with bile salts, reducing their ability to emulsify lipids (Ebihara and Schneeman, 1989).

The hypocholesterolemic effect observed with increasing dietary LPC is consistent with previous findings (Sirtori et al., 2004; Refstie et al., 2006). There are several hypotheses for the hypocholesterolemic effect of lupin. The findings from Lindahl et al. (1957), Newman et al. (1958) and Griminger and Fisher (1958) have shown that the presence of saponins in legumes can form insoluble complexes with cholesterol or bile acids in the intestine, preventing their re-absorption. However, only trace amounts of saponins are found in white lupin (Ruiz et al., 1995) and the inclusion of purified soya saponin in the diet for Atlantic salmon did not result in a

significant reduction in plasma cholesterol (Sørensen et al., 2011). Carroll (1991) and Sugano et al. (1984) suggested that the cholesterol-reducing effect of lupin can be attributed to the amino acid profile of the plant protein, especially the high Arg/Lys ratio. In our experiment, the increased dietary ratio of Arg/Lys resulting from the increasing L/P ratio at both inclusion levels could also be an explanation. Sirtori et al. (2004) showed that even though conglutin γ is a minor protein component in white lupin, it has a strong hypocholesterolemic effect in rats, which is associated with stimulation of LDL receptors. Thus, it is possible that this protein has contributed to the reduced plasma cholesterol in trout.

In the present experiment, the dietary starch level ranged from 66 -117 g kg^{-1}, which was within the capacity of starch digestion by rainbow trout (Krogdahl et al., 2005). This and the fact that extrusion gelatinized the starch and made it available for enzymatic hydrolysis, explains the high starch digestibilities found for all diets. These levels are in keeping with the starch digestibilities observed in rainbow trout by Morken et al. (2011). A significant positive correlation between starch digestibility and starch level in the diet was found, which was in contrast to the inverse relationship often found in rainbow trout (Bergot and Breque, 1983). This positive correlation was not in agreement with the differences in digestibility of starch sources observed by Øverland et al. (2009), where wheat starch was more efficiently digested than pea starch by Atlantic salmon. The linear reduction of starch digestibility with increasing inclusion of LPC could not be explained by starch source, starch level, feed processing, or physical feed quality. Furthermore, previous results obtained with Atlantic salmon did not show any reduction in digestibility of starch when dietary lupin kernel meal was compared with other plant protein sources (Aslaksen et al., 2007). The reduction in starch digestibility was unlikely due to the presence of amylase

inhibitor in the LPC, because this is virtually absent in both sweet and bitter lupins (Embaby, 2010). Increasing dietary concentration of NSP may have reduced the starch digestibility, because NSP have been reported to impair the starch digestion by affecting the intestinal distribution of pancreatic α-amylase (Leenhouwers et al., 2006) and its activity (Slaughter et al., 2002) and maltase activity in the brush border. The activities of α-amylase in the digesta were not measured, but the maltase activity in the mid-intestine was reduced in trout fed the two highest levels of dietary LPC compared to those fed the HLP1 and HLP2 diets. However, the fish fed the FM diet also had intestinal maltase activity similar to those fed the two highest levels of dietary LPC, in spite of a much lower content of NSP.

Inclusion of LPC did not cause significant morphological changes in the intestine of rainbow trout, even at very high inclusion levels. This is consistent with previous findings (Refstie et al., 2006). The lack of inflammation found in the DI of the trout fed the diets with PPC is in contrast to the observations by Penn et al. (2011) whom reported that 350 g kg^{-1} inclusion of PPC induces enteritis in the DI of Atlantic salmon. This difference in response is in keeping with previous observations that rainbow trout are less sensitive than Atlantic salmon to the components causing soybean meal induced enteritis in the DI (Refstie et al., 2000). The slight decrease in mucosal fold height and slight increase in fold fusion, and the reduced maltase activities in DI of the fish fed the diet with the highest PPC inclusion may be indications of mild changes associated with the mechanism that results in inflammation at higher dietary PPC levels. This condition may worsen with prolonged feeding, as observed when soybean meal is fed to salmonids (Baeverfjord and Krogdahl, 1996). Thus, caution should be taken when using high levels of PPC in diets for trout.

The hepatocyte vacuolization and increased liver weight (higher HSI) is associated with storage of glycogen or lipid and is most likely associated with the high feed intake and high energy content in the feed. Thus, the observed lower degree of hepatocyte vacuolization in trout fed the LPC diet compared with those fed the FM and PPC diets coincide with the higher energy intake of the fish fed the FM and PPC diets.

Conclusion

Both lupin and pea protein concentrates were shown to be useful dietary protein sources for rainbow trout. PPC had a higher nutritional value than LPC, mainly due to the lower NSP content. The results suggested that any combination of LPC and PPC with EAA-supplementation can be efficiently used when total plant protein inclusion is limited to 300 g kg^{-1} crude protein in extruded diets for rainbow trout. At higher inclusion level, PPC seemed to be the preferable source of protein. This indicates that the benefit of mixing PPC and LPC may be limited at high inclusion level.

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Table 1 Composition of fish meal (FM), lupin protein concentrate (LPC), and pea protein concentrate (PPC) used in the diets

	FM[1]	LPC[2]	PPC[3]
Composition, kg^{-1}			
Dry matter, g	913	945	904
In dry matter			
Crude protein, g	742	500	549
Crude fat, g	88	89	42
Starch, g	-	6	87
Ash, g	140	38	58
Analytical residue[4], g	30	367	264
Essential amino acids (EAA)[5], g $16g^{-1}N$			
Arg	5.61	8.14	8.74
His	1.83	1.77	2.65
Ile	3.81	3.66	4.32
Leu	6.72	6.18	7.21
Lys	7.48	4.04	7.07
Met	2.64	0.67	0.90
Phe	3.56	3.26	4.73
Thr	3.90	3.04	3.73
Trp	0.99	0.64	0.99
Val	4.15	3.19	4.66
Total EAA	40.7	34.6	45.0
Non-essential amino acids (NEAA)[5], g $16g^{-1}N$			
Ala	5.33	2.66	4.27
Asp	8.89	8.81	11.24
Cys	0.88	1.09	1.40
Glu	13.15	17.52	16.83
Gly	4.85	2.91	4.22
Pro	3.55	3.33	4.14
Ser	4.18	4.54	4.93
Tyr	2.82	3.90	3.52
Total NEAA	43.7	44.8	50.6

[1] Norse LT-94®, low-temperature dried fish meal, Norsildmel, Bergen, Norway.
[2] NaProLup PO54®, Lupin protein concentrate, derived from white lupins (*Lupinus albus*), NaProFood, Bruckberg, Germany.
[3] PPC 55 PELLET, Pea protein concentrate, derived from yellow field pea (*Pisum sativum* L.), AgriMarin AS, Stavanger, Norway.
[4] Dry matter – (crude protein + crude fat + starch + ash).
[5] Presented in dehydrated form.

Table 2 Diet formulation and analyzed chemical composition (based on dry matter)

Diets	F M	LLP1	LLP2	LLP3	LLP4	HLP1	HLP2	HLP3	HLP4
Ingredients, g kg^{-1}									
Fish meal	664.0	410.0	410.0	410.0	410.0	290.0	290.0	290.0	290.0
LPC	-	263.0	175.0	88.0	-	439.0	292.0	146.0	-
PPC	-	-	81.0	163.0	244.0	-	136.0	272.0	407.0
Fish oil[1]	181.0	158.0	163.0	167.0	172.0	154.0	162.0	170.0	178.0
Wheat	150.9	155.3	158.2	160.0	162.9	96.9	101.4	104.8	109.3
Premix[2]	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Y_2O_3[3]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
L-Lys[4]	-	5.8	4.7	3.6	2.6	9.6	7.8	6.1	4.3
DL-Met[5]	-	3.2	3.1	3.0	2.9	5.4	5.2	5.1	4.9
L-Trp[6]	-	0.6	0.4	0.2	-	1.0	0.6	0.3	-
L-Thr[7]	-	-	0.5	1.0	1.5	-	0.8	1.6	2.5
Analyzed content, kg^{-1}									
Dry matter, g	954	941	951	943	948	948	954	957	942
In dry matter									
Crude protein, g	538	473	472	485	478	463	470	467	485
Crude fat, g	228	200	201	191	200	220	208	204	196
Starch, g	106	93	101	106	117	66	82	93	101
Ash, g	100	70	72	75	80	62	68	69	71
Analytical residue[8], g	28	164	154	143	125	189	172	167	147
Gross energy, MJ	24.0	23.6	23.5	23.7	23.5	23.9	23.6	23.7	23.6
EAA[9], g $(16gN)^{-1}$									
Arg	5.12	6.16	6.29	6.15	5.79	6.69	6.09	5.66	5.98
His	1.73	1.85	2.08	1.93	1.84	1.83	1.77	1.73	1.87
Ile	3.49	3.59	3.74	3.56	3.40	3.57	3.35	3.16	3.33
Leu	6.06	6.16	6.19	6.23	5.96	6.14	5.80	5.49	5.77
Lys	6.52	6.43	6.70	6.70	6.57	6.47	6.31	6.10	6.53
Met	2.36	2.30	2.38	2.38	2.35	2.33	2.36	2.26	2.32
Phe	3.30	3.32	3.45	3.52	3.44	3.28	3.17	3.11	3.44
Thr	3.63	3.53	3.71	3.78	3.74	3.41	3.44	3.42	3.70
Trp	1.22	1.13	1.13	1.15	1.27	1.10	1.04	1.07	1.25
Val	3.90	3.83	4.26	3.96	3.80	3.61	3.54	3.44	3.71
NEAA[9], g $(16gN)^{-1}$									
Ala	4.69	4.05	4.20	4.31	4.20	3.61	3.69	3.60	3.87
Asp	7.98	8.12	8.23	8.40	8.17	8.33	7.93	7.65	8.15
Cys	0.81	0.87	0.91	0.85	0.84	0.93	0.85	0.79	0.81
Glu	12.53	14.08	13.58	13.66	12.96	15.05	13.64	12.49	12.70
Gly	4.42	3.94	4.05	4.09	3.97	3.61	3.59	3.44	3.62
Pro	3.44	3.48	3.48	3.58	3.40	3.41	3.28	3.13	3.32
Ser	3.68	3.91	3.87	3.91	3.80	4.09	3.79	3.56	3.69
Tyr	2.36	2.76	2.68	2.59	2.38	3.02	2.65	2.36	2.41

[1] Silfas, Karmsund, Norway.
[2] Per kg diet: vitamin A: 2000 IU, vitamin D_3: 1200 IU; vitamin E: 160 mg; vitamin K_3: 8 mg; vitamin B_1: 12 mg; vitamin B_2: 20 mg; vitamin B_3: 60 mg; vitamin B_5:24 mg; vitamin B_6: 12 mg; vitamin B_9: 4 mg; vitamin B_{12}: 0.016 mg; vitamin C: 100 mg; Biotin: 0.2 mg; Ca: 876 mg; Cu: 4 mg; Co: 0.8 mg; I: 2.4 mg; Mn:12 mg; Zn: 96 mg.
[3] Metal Rare Earth Limited, Shenzhen, China.
[4] *L*-Lysine-HCl, 99% feed grade, CJ Indonesia, Jakarta, Indonesia.
[5] Rhodimet® NP 99, *DL*-methionine, 99% feed grade, Adisseo Brasil Nutricao Animal Ltda, Sao Paulo, Brazil.
[6] TrypAMINO®, *L*-tryptophan, 98 % feed grade, Evonik Fermas S.R.O., Slovenska L'upca, Slovakia.
[7] *L*-Threonine, 98.5 % Feed Grade, Ajinomoto Eurolysine S.A.S., Paris, France.
[8] Dry matter – (crude protein + crude fat + starch + ash).
[9] Presented in dehydrated form.

1 Table 3 Extrusion parameters and physical quality of the pellets

Diets	F M	LLP1	LLP2	LLP3	LLP4	HLP1	HLP2	HLP3	HLP4
Extruder parameters									
Feeding rate, kg h^{-1}	182	192	192	184	182	199	192	192	184
Water addition[1], kg h^{-1}	32.2	42.0	42.0	34.5	32.2	49.5	42.0	42.0	34.5
Steam addition[1], kg h^{-1}	7.5	7.5	6.2	7.5	7.5	7.5	7.5	8.8	7.5
SME[2], Wh kg^{-1}	73.0	71.1	78.1	74.2	81.9	62.4	73.4	87.1	83.2
Torque, Nm	281	259	284	289	315	236	267	317	324
Revolution screws, rpm	453	504	505	453	453	504	504	504	453
Die temperature, °C	124	122	126	127	129	112	127	116	128
Physical quality									
Length, mm	4.9	5.4	5.7	4.9	5.2	5.4	5.2	5.6	5.1
Diameter, mm	2.9	3.0	3.0	3.0	3.0	2.8	3.0	3.0	3.0
Expansion, %	43.3	48.9	51.6	50.0	49.5	39.4	49.5	51.0	47.4
Durability, %	65.1	63.7	68.4	71.4	74.5	67.9	64.0	69.9	81.5
Breaking point, N	13.9	14.0	16.4	16.2	20.3	13.8	13.6	16.6	21.3
Bulk density, (g l^{-1})	523	517	518	518	516	521	521	519	537

2 [1] In conditioner.
3 [2] Specific mechanical energy.

Table 4 Growth performance and feed utilization of rainbow trout fed the experimental diets

One way ANOVA model:						
	Feed intake, g DM fish^{-1}		Weight gain, % of initial body weight		Feed conversion ratio, g DM ingested (g gain)$^{-1}$	
Diet[1]	0-30 days	0-62 days	0-30 days	0-62 days	0-30 days	0-62 days
LLP1	38.7	90.4	90.7	213.5	0.74^{b}	0.73^{b}
LLP2	39.1	89.8	90.1	211.1	0.75^{b}	0.73^{b}
LLP3	38.5	88.3	90.5	210.9	0.73^{bc}	0.72^{bc}
LLP4	38.9	87.9	96.2	216.9	0.70^{c}	0.70^{cd}
HLP1	37.6	85.6	80.3	188.4	0.81^{a}	0.78^{a}
HLP2	39.5	91.3	95.0	217.0	0.72^{bc}	0.73^{b}
HLP3	39.5	91.3	92.5	213.6	0.74^{b}	0.74^{b}
HLP4	38.3	87.2	89.8	208.6	0.73^{bc}	0.72^{bc}
FM	38.0	86.7	93.8	219.9	0.70^{c}	0.68^{d}
Pooled SEM	1.0	2.5	3.2	7.0	0.01	0.01
ANOVA P < F	0.90	0.71	0.14	0.21	0.002	0.002
Factorial ANOVA model:						
	Feed intake		Weight gain		Feed conversion ratio	
	0-30 days	0-62 days	0-30 days	0-62 days	0-30 days	0-62 days
Factor						
Inclusion[2]						
300	38.8	89.1	91.8	213.1	0.73^{y}	0.72^{y}
500	38.7	88.8	89.4	206.9	0.75^{x}	0.74^{x}
L/P ratio[3]						
3:0	38.1	88.0	85.5	201.0	0.77^{r}	0.76^{r}
2:1	39.3	90.5	92.5	214.0	0.73^{s}	0.73^{s}
1:2	39.0	89.8	91.5	212.3	0.73^{s}	0.73^{s}
0:3	38.6	87.6	93.0	212.7	0.71^{s}	0.71^{s}
Pooled SEM	1.0	2.2	3.3	7.0	0.01	0.01
ANOVA P < F						
Inclusion[2]	0.90	0.87	0.32	0.25	0.039	0.011
L/P ratio[3]	0.72	0.52	0.17	0.29	0.006	0.008
Inclusion × L/P ratio	0.72	0.37	0.15	0.19	0.011	0.15

[1] For diet codes see Table 2. Different superscript letters [a, b, c, d] within a column indicate significant ($P < 0.05$) difference among diets.

[2] Inclusion level of plant protein sources (g plant protein kg^{-1} dietary protein). Different superscript letters [x, y] within a column indicate significant ($P < 0.05$) difference between inclusion levels.

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in the diets. Different superscript letters [r, s] within a column indicate significant ($P < 0.05$) differences among L/P ratios.

Table 5 Apparent digestibility (%) of macronutrients and energy of rainbow trout fed the experimental diets

One way ANOVA model:				
	Protein	Lipid	Starch	Energy
Diet[1]				
LLP1	87.7^{ef}	95.8^{ab}	90.5^{d}	82.8^{c}
LLP2	87.4^{f}	95.4^{ab}	92.2^{c}	83.1^{bc}
LLP3	88.0^{def}	94.3^{c}	94.5^{b}	83.1^{bc}
LLP4	89.6^{b}	96.0^{a}	98.3^{a}	85.4^{ab}
HLP1	88.8^{bcd}	96.4^{a}	86.0^{e}	81.3^{c}
HLP2	88.3^{cde}	96.1^{a}	91.1^{cd}	82.6^{c}
HLP3	89.2^{bc}	95.9^{ab}	93.9^{b}	83.3^{bc}
HLP4	90.7^{a}	95.4^{ab}	97.4^{a}	86.6^{a}
FM	88.0^{def}	94.9^{bc}	98.3^{a}	87.2^{a}
Pooled S.E.M.	0.3	0.3	0.5	0.8
ANOVA P < F	<0.001	0.018	<0.001	0.005
Factorial ANOVA model:				
	Protein	Lipid	Starch	Energy
Factor				
Inclusion[2]				
300	88.1^{y}	95.3^{y}	93.8^{x}	83.6
500	89.2^{x}	95.9^{x}	92.1^{y}	83.4
L/P ratio[3]				
3:0	88.2^{st}	96.1^{r}	88.2^{u}	82.1^{s}
2:1	87.8^{t}	95.7^{rs}	91.6^{t}	82.8^{s}
1:2	88.6^{s}	95.1^{s}	94.2^{s}	83.2^{s}
0:3	90.1^{r}	95.6^{rs}	97.8^{r}	86.0^{r}
Pooled S.E.M.	0.2	0.3	0.5	0.7
ANOVA P < F				
Inclusion[2]	< 0.001	0.013	< 0.001	0.73
L/P ratio[3]	< 0.001	0.035	< 0.001	0.003
Inclusion × L/P ratio	0.93	0.023	0.011	0.41

[1] For diet codes see Table 2. Different superscript letters $^{a, b, c, d, e, f}$ within a column indicate significant (P < 0.05) difference among diets.

[2] Inclusion level of plant protein sources (g plant protein kg^{-1} dietary protein). Different superscript letters $^{x, y}$ within a column indicate significant (P < 0.05) difference between inclusion levels.

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in the diets. Different superscript letters $^{r, s, t, u}$ within a column indicate significant (P < 0.05) differences among L/P ratios.

1 Table 6 Apparent digestibility (%) of EAA and cysteine of rainbow trout fed the experimental diets

One way ANOVA model:											
	Arg	His	Ile	Leu	Lys	Met	Phe	Trp	Thr	Val	Cys
Diet[1]											
LLP1	94.6^{ab}	88.1^{cde}	91.9^{ab}	93.2^{a}	94.3^{ab}	93.6^{b}	92.0^{abc}	91.7^{bc}	91.0^{ab}	92.0^{a}	77.9^{a}
LLP2	94.6^{ab}	89.7^{b}	91.6^{abc}	92.7^{ab}	94.2^{ab}	93.2^{bc}	91.4^{abc}	90.8^{def}	90.8^{ab}	92.1^{a}	78.0^{a}
LLP3	94.5^{ab}	89.5^{bc}	90.9^{abc}	92.6^{ab}	94.1^{b}	92.7^{c}	91.0^{c}	90.2^{ef}	90.2^{bc}	90.9^{abc}	74.0^{bc}
LLP4	95.0^{ab}	91.5^{a}	92.2^{a}	93.5^{a}	94.9^{a}	93.3^{bc}	91.9^{abc}	91.9^{b}	91.7^{a}	91.9^{ab}	77.5^{ab}
HLP1	94.8^{ab}	86.9^{e}	91.5^{abc}	93.1^{a}	94.6^{ab}	94.7^{a}	92.4^{a}	92.9^{a}	91.5^{ab}	91.8^{ab}	76.0^{abc}
HLP2	94.4^{ab}	87.5^{de}	90.8^{bc}	92.6^{ab}	94.1^{b}	93.9^{b}	91.6^{abc}	91.1^{cd}	90.8^{ab}	90.9^{abc}	74.9^{abc}
HLP3	94.3^{b}	89.0^{bcd}	90.7^{bc}	92.5^{ab}	94.2^{ab}	93.5^{bc}	91.3^{bc}	90.9^{de}	90.5^{abc}	90.6^{bc}	73.1^{c}
HLP4	95.1^{a}	91.6^{a}	92.0^{ab}	93.3^{a}	95.0^{a}	93.8^{b}	92.3^{ab}	92.0^{b}	91.7^{a}	91.7^{ab}	77.3^{ab}
FM	92.8^{c}	88.5^{bcde}	90.5^{c}	91.9^{b}	93.3^{c}	90.9^{d}	88.7^{d}	90.1^{f}	89.3^{c}	90.3^{c}	75.1^{abc}
Pooled S.E.M.	0.2	0.5	0.4	0.3	0.2	0.2	0.3	0.2	0.4	0.4	1.0
ANOVA P < F	0.001	<0.001	0.064	0.060	0.011	<0.001	0.001	<0.001	0.023	0.046	0.005
Factorial ANOVA model:											
	Arg	His	Ile	Leu	Lys	Met	Phe	Trp	Thr	Val	Cys
Factor											
Inclusion[2]											
300	94.7	89.7^{x}	91.7	93.0	94.4	93.2 ^{y}	91.6	91.1^{y}	90.9	91.7	76.9
500	94.7	88.8^{y}	91.3	92.9	94.5	94.0 ^{x}	91.9	91.7^{x}	91.1	91.3	75.3
L/P ratio[3]											
3:0	94.7	87.5^{t}	91.7^{rs}	93.2	94.5^{rs}	94.1^{r}	92.2^{r}	92.3^{r}	91.3^{rs}	92.0	77.0^{r}
2:1	94.5	88.6st	91.2^{rs}	92.7	94.1^{s}	93.5^{rs}	91.5^{rs}	90.9^{s}	90.8^{rs}	91.5	76.5^{s}
1:2	94.4	89.2^{s}	90.8^{s}	92.6	94.2^{s}	93.1^{s}	91.2^{s}	90.5^{s}	90.4^{s}	90.7	73.6^{s}
0:3	95.1	91.6^{r}	92.1^{r}	93.4	95.0^{r}	93.5^{rs}	92.1^{r}	92.0^{r}	91.7^{r}	91.8	77.4^{r}
Pooled S.E.M.	0.2	0.5	0.4	0.3	0.2	0.3	0.4	0.2	0.4	0.4	1.0
ANOVA P < F											
Inclusion[2]	0.92	0.026	0.18	0.49	0.64	0.002	0.23	0.006	0.51	0.14	0.075
L/P ratio[3]	0.086	< 0.001	0.050	0.073	0.030	0.021	0.046	< 0.001	0.043	0.067	0.026
Inclusion × L/P ratio	0.79	0.22	0.86	0.97	0.89	0.70	0.95	0.15	0.88	0.60	0.57

[1] For diet codes see Table 2. Different superscript letters [a, b, c, d, e, f] within a column indicate significant ($P < 0.05$) difference among diets.

[2] Inclusion level of plant protein sources (g plant protein kg^{-1} dietary protein). Different superscript letters [x, y] within a column indicate significant ($P < 0.05$) difference between inclusion levels.

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in the diets. Different superscript letters [r, s, t] within a column indicate significant ($P < 0.05$) differences among L/P ratios.

Table 7 Whole body composition (g kg^{-1}), total plasma cholesterol (TC) and triacylglycerols (TG) concentrations of rainbow trout fed the experimental diets

One way ANOVA model:							
	Whole body					Plasma	
	Dry matter	Crude protein	Lipid	Ash	Energy Value (MJ kg^{-1})	TC, (mM)	TG, (mM)
Diet[1]							
LLP1	300	164	112	23.2	8.1	8.95^{de}	6.20
LLP2	299	164	114	22.1	8.2	10.90^{bc}	6.90
LLP3	298	162	111	22.1	8.1	9.55^{cde}	5.00
LLP4	299	166	110	22.1	8.0	11.30^{b}	5.10
HLP1	308	164	125	20.0	8.5	8.05^{e}	5.50
HLP2	304	166	118	21.8	8.3	9.35^{de}	7.95
HLP3	301	167	112	21.1	8.2	8.90^{de}	7.35
HLP4	301	164	113	22.3	8.1	9.70^{cd}	7.50
FM	301	162	114	23.3	8.1	13.50^{a}	8.30
Pooled S.E.M.	4	2	4	0.7	0.2	0.44	0.79
ANOVA P < F	0.71	0.59	0.46	0.13	0.81	<0.001	0.10
Factorial ANOVA model:							
	Whole body					Plasma	
	Dry matter	Crude protein	Lipid	Ash	Energy Value	TC	TG
Factor Inclusion[2]							
300	299	164	112	22.3^{x}	8.1	10.18^{x}	5.80^{y}
500	304	165	117	21.3^{y}	8.3	9.00^{y}	7.08^{x}
L/P ratio[3]							
3:0	304	164	118	21.6	8.3	8.50^{t}	5.85
2:1	302	165	116	22.0	8.2	10.13^{rs}	7.43
1:2	299	164	112	21.6	8.1	9.23^{st}	6.18
0:3	300	165	111	22.2	8.0	10.50^{r}	6.30
Pooled S.E.M.	4	2	4	0.7	0.2	0.43	0.59
ANOVA P < F							
Inclusion[2]	0.14	0.53	0.13	0.047	0.29	0.005	0.016
L/P ratio[3]	0.69	0.95	0.40	0.74	0.67	0.007	0.12
Inclusion × L/P ratio	0.84	0.43	0.60	0.12	0.73	0.63	0.091

[1] For diet codes see Table 2. Different superscript letters $^{a, b, c, d, e}$ within a column indicate significant (P < 0.05) difference among diets.

[2] Inclusion level of plant protein sources (g plant protein kg^{-1} dietary protein). Different superscript letters $^{x, y}$ within a column indicate significant (P < 0.05) difference between inclusion levels.

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in the diets. Different superscript letters $^{r, s, t}$ within a column indicate significant (P < 0.05) differences among L/P ratios.

Table 8 Retentions (%) and metabolic loss of nitrogen and energy (g N or MJ kg^{-1} gain) in rainbow trout fed the experimental diets

One way ANOVA model:	Retention				Metabolic loss	
	Nitrogen		Energy			
	Ingested	Digested	Ingested	Digested	Nitrogen	Energy
Diet[1]						
LLP1	47.2^{abc}	53.8^{ab}	51.9	62.8	22.3^{cd}	5.32
LLP2	46.9^{bc}	53.7^{abc}	53.0	63.7	22.3^{cd}	5.18
LLP3	45.9^{bcd}	52.2^{bcd}	52.9	63.6	23.5^{bcd}	5.15
LLP4	49.8^{a}	55.6^{a}	54.3	63.7	21.2^{d}	5.08
HLP1	44.8^{cd}	50.5^{cd}	52.3	64.2	25.4^{ab}	5.43
HLP2	48.2^{ab}	54.5^{ab}	54.0	65.5	21.9^{d}	4.89
HLP3	48.2^{ab}	54.1^{ab}	51.9	62.3	22.6^{cd}	5.49
HLP4	46.6^{bc}	51.4^{bcd}	52.8	61.1	24.6^{abc}	5.71
FM	43.5^{d}	49.4^{d}	54.7	62.7	26.1^{a}	5.31
Pooled S.E.M.	0.8	0.9	2.1	2.3	0.7	0.36
ANOVA P < F	0.010	0.016	0.97	0.95	0.006	0.86
Factorial ANOVA model:						
	Nitrogen		Energy		Metabolic loss	
	Ingested	Digested	Ingested	Digested	Nitrogen	Energy
Factor						
Inclusion[2]						
300	47.4	53.8	53.0	63.4	22.3^{y}	5.18
500	46.9	52.6	52.7	63.2	23.6^{x}	5.38
L/P ratio[3]						
3:0	46.0	52.2	52.1	63.5	23.9	5.37
2:1	47.5	54.1	53.5	64.6	22.1	5.04
1:2	47.0	53.1	52.4	62.9	23.0	5.32
0:3	48.2	53.5	53.6	62.4	22.9	5.39
Pooled S.E.M.	0.9	1.0	1.9	2.2	0.7	0.35
ANOVA P < F						
Inclusion[2]	0.44	0.12	0.84	0.92	0.030	0.45
L/P ratio[3]	0.15	0.32	0.81	0.78	0.18	0.71
Inclusion × L/P ratio	0.031	0.037	0.90	0.71	0.030	0.61

[1] For diet codes see Table 2. Different superscript letters $^{a, b, c, d}$ within a column indicate significant ($P < 0.05$) difference among diets.

[2] Inclusion level of plant protein sources (g plant protein kg^{-1} dietary protein). Different superscript letters $^{x, y}$ within a column indicate significant ($P < 0.05$) difference between inclusion levels.

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in the diets.

Table 9 Essential amino acid (EAA) profile of LT-fish meal protein and rainbow trout whole body protein and, retention of digestible EAA and metabolic loss of EAA in rainbow trout fed the HLP1, HLP4, and FM diets

	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Trp	Val
EAA profile, (A/E ratio)[1]										
LT-fish meal	12.6	4.1	8.6	15.1	16.9	5.9	8.0	8.8	2.2	9.3
Initial	12.5	5.5	8.5	14.4	16.1	5.9	8.1	9.1	2.3	9.7
Final										
HLP1	12.2	5.6	8.6	14.4	16.4	6.0	8.1	9.2	1.6	9.8
HLP4	12.4	5.7	8.6	14.3	16.1	6.0	8.2	9.2	1.8	9.8
FM	12.4	5.4	8.7	14.4	16.3	5.9	8.2	9.2	1.5	9.8
EAA retention, %										
HLP1	29.7[a]	56.0	41.6	39.3	42.5	42.6	41.6[a]	46.1	18.8	46.7
HLP4	33.4[a]	52.1	43.5	41.0	40.4	42.7	40.0[a]	42.2	21.0	45.0
FM	43.0[b]	58.5	45.8	43.1	44.8	45.4	46.4[b]	47.5	18.2	46.7
Pooled S.E.M.	1.0	1.5	1.2	1.3	1.0	0.8	0.9	1.3	4.3	1.2
ANOVA[2] P < F	0.006	0.18	0.24	0.31	0.15	0.18	0.044	0.17	0.90	0.62
Metabolic loss, g kg^{-1} gain										
HLP1	16.38[a]	2.57	7.01	12.74	12.93	4.65	6.51[a]	6.18	3.05	6.49
HLP4	13.05[b]	2.82	5.96	10.93	12.72	4.29	6.56[a]	6.75	3.12	6.44
FM	9.59[c]	2.25	6.07	11.23	11.91	4.15	5.56[b]	6.03	3.16	6.65
Pooled S.E.M.	0.26	0.13	0.20	0.36	0.33	0.09	0.15	0.21	0.19	0.22
ANOVA P < F	< 0.001	0.12	0.061	0.070	0.22	0.055	0.030	0.18	0.92	0.79

[1] (Each EAA content/ total EAA content including Cys and Tyr) × 1000.

[2] Different superscript letters [a. b] indicate significant (P < 0.05) difference among treatments.

Table 10 Trypsin activity and bile acid concentration in contents of different intestinal sections, and leucine aminopeptidase (LAP) and maltase activities in intestinal sections of rainbow trout fed the experimental diets with 500 g plant protein kg^{-1} crude protein inclusion and the FM diet (n=2)

	Trypsin activity (U $(mg\ DM)^{-1}$)			Bile acid ($mg\ (g\ DM)^{-1}$)			LAP activity ($mmol\ h^{-1}(kg\ BW)^{-1}$)		Maltase activity ($\mu mol\ min^{-1}(kg\ BW)^{-1}$)	
	PI	MI	DI	PI	MI	DI	MI	DI	MI	DI
Diet										
HLP1	405	187 ^{c}	64.9	181	110	30.1	49.1	219	12.0	31.3^{ab}
HLP2	419	247 ^{b}	92.4	206	120	34.1	51.4	231	12.5	33.1^{a}
HLP3	326	309 ^{a}	95.3	199	127	32.5	55.5	213	13.3	26.1^{c}
HLP4	304	233 ^{b}	109.9	181	124	38.5	61.3	199	15.7	23.3^{c}
FM	265	248 ^{b}	123.6	194	139	54.2	51.8	210	11.4	26.7^{bc}
Pooled S.E.M.	37	10	15.7	11	9	5.6	6.7	23	1.1	1.3
ANOVA P < F	0.12	0.003	0.24	0.48	0.37	0.13	0.73	0.89	0.17	0.017

Different superscript letters $^{a.\ b.\ c}$ indicate significant ($P < 0.05$) difference among treatments.

Table 11 Hepatosomatic Index and the degree of hepatocyte vacuolization in fish fed the HLP1, HLP4 and FM diets

	Hepatosomatic Index	Degree of hepatocyte vacuolization		
		Low	Moderate	High
Diet				
HLP1	1.29^{b}	6	4	0
HLP4	1.31^{b}	0	9	1
FM	1.46^{a}	1	7	2
Pooled S.E.M.	0.04			
ANOVA P < F	0.038			

Different superscript letters $^{a.\ b}$ indicate significant ($P < 0.05$) difference among treatments.

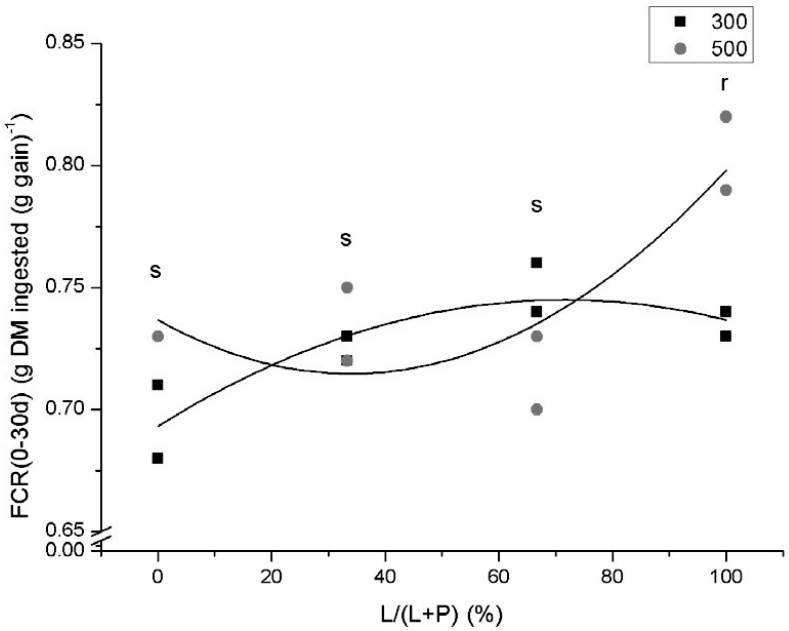

Figure 1.

Feed conversion ratio (FCR) in rainbow trout during the first month of feeding diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/ (L + P)). FCR_{300} = (-1.01 E-5) LPR^2 + 0.00145 LPR + 0.693, P_{model} = 0.022, R^2 = 0.69; FCR_{500} = (1.91 E-5) LPR^2 – 0.0013 LPR + 0.737, P_{model} = 0.045, R^2 = 0.60. Different superscript letters $^{r, s}$ indicate significant ($P < 0.05$) differences in main effects among the 4 different L/P ratios.

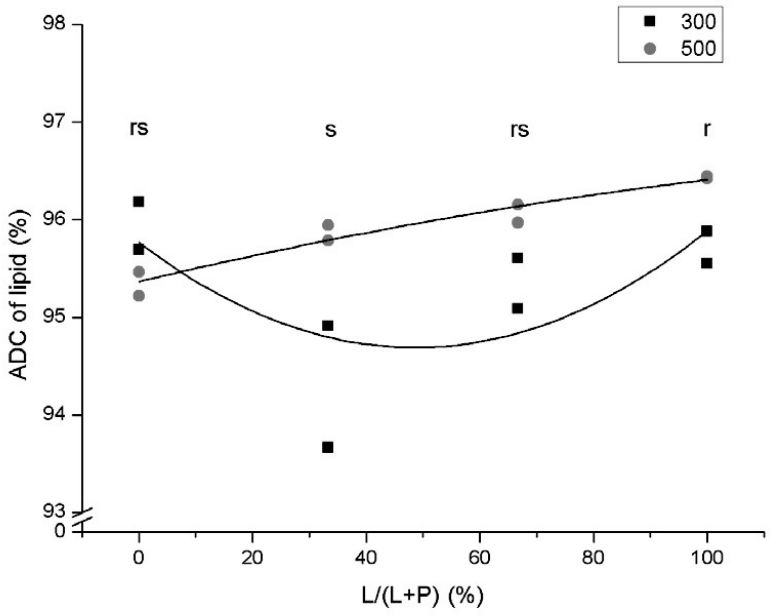

Figure 2.

Lipid digestibility (ADCli) of rainbow trout fed diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/ (L + P)). $ADCli_{300} = (4.54\ E\text{-}4)\ LPR^2 - 0.044\ LPR + 95.8$, $P_{model} = 0.20$, $R^2 = 0.27$; $ADCli_{500} = (-3.40\ E\text{-}5)\ LPR^2 + 0.0138\ LPR + 95.4$, $P_{model} = 0.001$, $R^2 = 0.91$. Superscript letters $^{r, s}$, see Fig. 1.

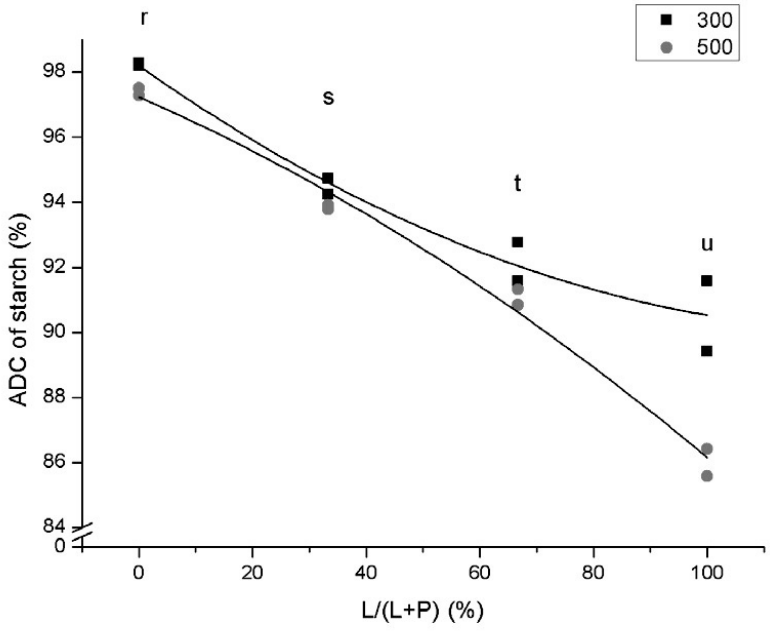

Figure 3.

Starch digestibility (ADCst) of rainbow trout fed diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/ (L + P)). $ADCst_{300} = (4.67\ E\text{-}4)\ LPR^2 - 0.123\ LPR + 98.2$, $P_{model} < 0.001$, $R^2 = 0.94$; $ADCst_{500} = (-3.48\ E\text{-}4)\ LPR^2 - 0.076\ LPR + 97.2$, $P_{model} < 0.001$, $R^2 = 0.99$. Different superscript letters $^{r, s, t, u}$, see Fig. 1.

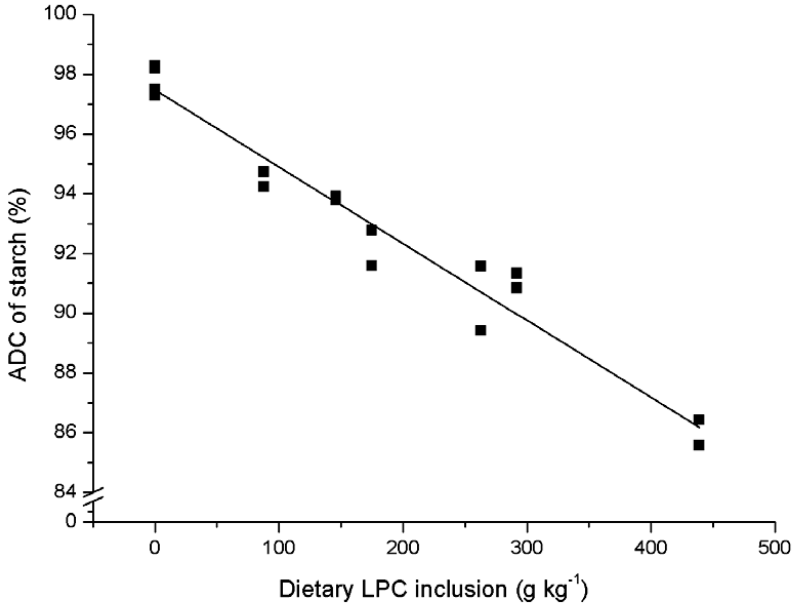

Figure 4.

Starch digestibility (ADCst) of rainbow trout fed diets with different inclusion of LPC. ADCst = -0.0257 LPC + 97.5, $P_{model} < 0.001$, $R^2 = 0.95$.

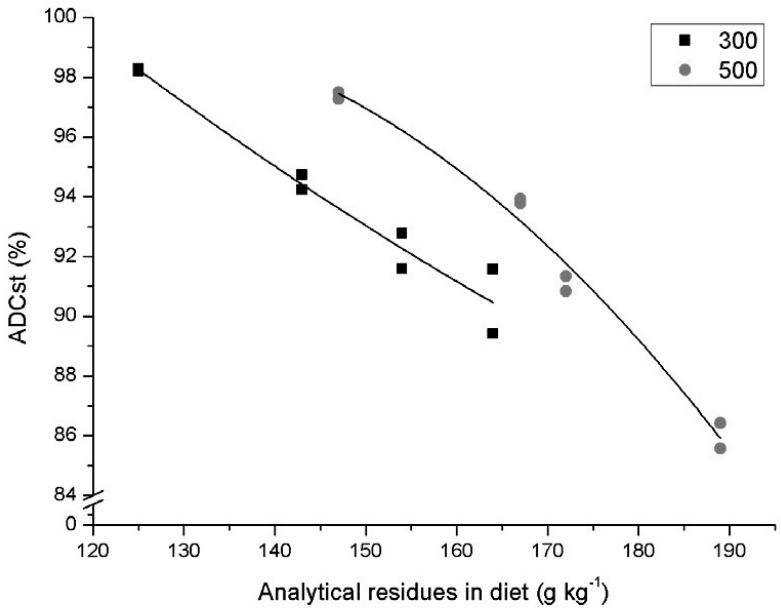

Figure 5.

Starch digestibility (ADCst) of rainbow trout fed diets included 300 and 500 g kg^{-1} plant protein which implied different analytical residues (RSD) in the diet. $ADCst_{300}$= (6.46 E-4) RSD^2 – 0.387 RSD + 137, $P_{model} < 0.001$, R^2= 0.94; $ADCst_{500}$ = -0.0028 RSD^2+ 0.664 RSD + 60.2, $P_{model} < 0.001$, R^2=0.98.

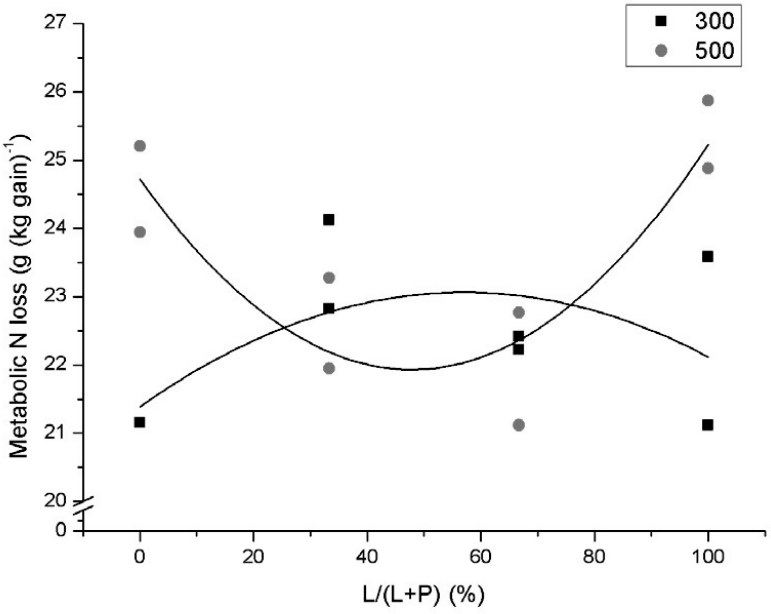

Figure 6.

Metabolic nitrogen loss (MNL) of rainbow trout fed diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/ (L + P)). MNL_{300} = (-5.14 E-4) LPR^2 +0.0587 LPR + 21.4, P_{model} = 0.35, R^2 = 0.08; MNL_{500} = 0.00121 LPR^2- 0.116 LPR + 24.7, P_{model} = 0.024, R^2 = 0.68.

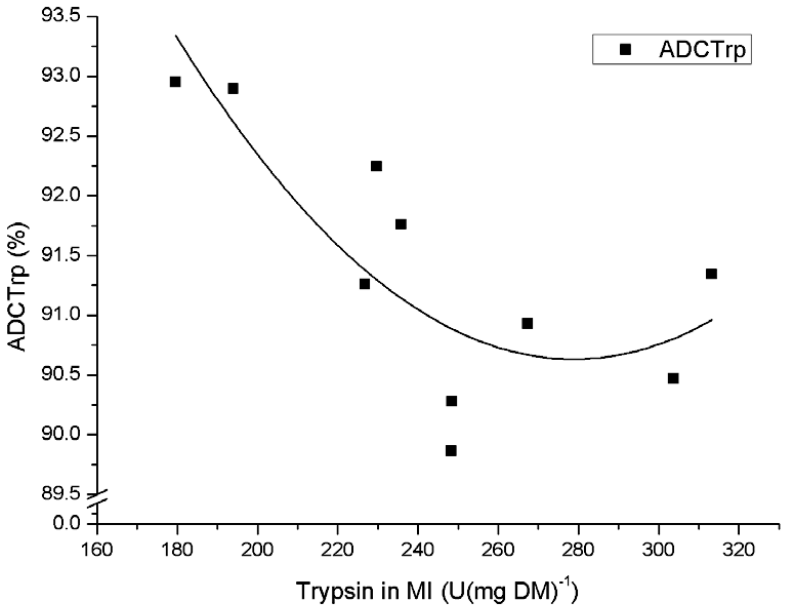

Figure 7.

Regression of tryptophan digestibility (ADC_{Trp}) on trypsin activity (TA) in digesta from mid-intestine of the rainbow trout. $ADC_{Trp} = (2.75\ E\text{-}4)\ TA^2 - 0.154\ TA + 112$, $P_{model} = 0.019$, $R^2 = 0.59$.

Paper II

Mixtures of lupin and pea protein concentrates can efficiently replace high-quality fish meal in extruded diet for juvenile black sea bream (*Acanthopagrus schlegeli)*

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Abstract

A 60-day feeding experiment was carried out to investigate the effects of including lupin protein concentrate (LPC) and pea protein concentrate (PPC) in multiple essential amino acid-supplemented extruded diets for black sea bream (*Acanthopagrus schlegeli*). Nine diets, including eight diets formulated to contain four mixtures of LPC and PPC (L/P ratio, 3:0, 2:1, 1:2 and 0:3) with two dietary inclusion levels (300 or 500 g plant protein kg^{-1} dietary protein) and one diet with high-quality fish meal as the sole protein source (FM diet) were fed to 18 tanks of 13-g black sea bream. Growth performance, nutrient

utilization, and brush-border membrane bound maltase activities were evaluated. An average weight gain (WG) of 251% and an average feed conversion ratio (FCR) of 1.13 g ingested dry matter (g gain)$^{-1}$ were obtained. Neither plant protein inclusion level nor L/P ratio significantly affected body composition (except ash), fish somatic indices or plasma parameters. The high inclusion of 500 g kg^{-1} resulted in significantly higher feed intake during the first month and FCR during the whole period. The WG, whole body ash content, and nitrogen (N) and energy retentions of these fish were, however, significantly lower than that of the fish fed diets with low plant protein inclusion (300 g kg^{-1}). The highest LPC inclusion (L/P ratio = 3:0) resulted in significantly higher feed intake and FCR, and lower N retention than the treatments with less LPC, but did not affect the growth rates or energy retentions. The diet with the highest PPC inclusion resulted in significantly reduced maltase activity in distal intestine. Any combination of LPC and PPC in essential amino acid-supplemented extruded diets, accounting for up to half of dietary protein, can be used without impairing fish growth. At higher inclusion, combinations with more PPC are preferred, due to less efficient feed conversion caused by the LPC.

Keywords: White lupin concentrate; Pea protein concentrate; Mixture; Growth; Nutrient utilization; Brush border maltase; Black sea bream

Introduction

Aquaculture of black sea bream (*Acanthopagrus schlegeli*) is increasing in East Asia. The main traditional source of feed for this species is 'trash fish', leading to a series of problems such as unbalanced and incomplete nutrient composition, contamination by unsafe transport and handling, water pollution, and growth of pathogenic bacteria. Other seabream species, like gilthead sea bream (*Sparus aurata*) can successfully utilize extruded feeds with a lipid level around 20% (Grigorakis et al., 2009). Because of physiological similarities between the black and gilthead seabream, it can be expected that a nutritionally balanced extruded diet also is applicable for black sea bream. Such balanced diets will contribute to more economical and ecological farming of this species. Published nutritional studies on black sea bream has up to now mainly focused on the basic nutrients requirements, including protein, essential amino acids, vitamins and phosphorous (Shao et al., 2008; Peng et al., 2009). There is, however, limited published information concerning the nutritional value of different feed protein ingredients in the diet for black sea bream.

Lupin and pea are legumes with high potentials as sources of protein in diets for salmonid fish (Burel et al., 1998; Carter and Hauler, 2000; Glencross et al., 2002; 2003; 2004a; 2004b; Refstie et al., 2006; 2008). These plant protein sources have also demonstrated their potentials in diets for temperate and warm water fish such as European sea bass (Gouveia and Davies, 1998;2000; Adamidou et al., 2009), milkfish (Borlongan et al., 2003), and silver perch (Booth et al., 2001). In gilthead sea bream, dietary inclusion of unprocessed lupin and pea meals are limited to 20% and 30% of the dietary protein,

respectively (Pereira and Oliva-Teles, 2002; 2004). The low dietary inclusion level can be related to the low protein content, imbalanced amino acid composition and presence of anti-nutritional factors (ANF) in the lupin and pea.

Removing the indigestible carbohydrates by extraction from lupin and the starch by air classification from peas, result in lupin (LPC) and pea protein concentrates (PPC) with high nutrient digestibilities, that can be efficiently used in diets for salmonids (Carter and Hauler, 2000; Thiessen et al., 2003; Glencross et al., 2006; Øverland et al., 2009). PPC can provide 40% protein in the diet for gilthead sea bream diet without any negative effects on growth and feed utilization when replacing fish meal as a source of protein (Sanchez-Lozano et al., 2011). Combining PPC and rice protein concentrate rather than using PPC alone, allowed an increase of PPC up to 60% of the protein in diets for gilthead sea bream (Santigosa et al., 2008). Zhang et al. (submitted) showed that any combination of LPC and PPC with multiple essential amino acid-supplementation can be efficiently used when total plant protein inclusion is limited to 300 g kg^{-1} crude protein in extruded diets for rainbow trout (*Oncorhynchus mykiss*). A higher inclusion of 500 g kg^{-1} PPC appeared to be a preferable source of protein.

The aims of the present experiment were to 1) determine the effect of including LPC and PPC in extruded high energy diets on the growth, nutrient utilization and intestinal enzyme activities of juvenile black sea bream, 2) investigate if the combination of these ingredients allowed a higher dietary inclusion rate than when applied separately, and 3)

determine the optimal combination of LPC and PPC in extruded diets for juvenile black sea bream.

2. Materials and Methods

2.1 Ingredients and diets

The LPC was derived from white lupine (*Lupinus albus*), produced by dehulling, milling, aqueous extraction of lupine seeds to remove sugars and soluble non-starch polysaccharides (NSP), heating and spray-drying. The PPC was produced from yellow field pea (*Pisum sativum* L.) by dehulling, fine grinding and air-classification. The chemical composition of these two plant concentrates and LT fish meal has been previously reported by Zhang et al. (submitted). The LPC and PPC were each supplemented with the first-three limiting essential amino acids to balance the essential amino acid profile to that of LT fish meal. A 2 × 4 factorial design was used in the present experiment, where the factors were inclusion level of plant protein concentrate (300 or 500 g protein kg^{-1} dietary protein), and ratio between essential amino acid-supplemented LPC and PPC in the diets (L/P ratio at 3:0, 2:1, 1:2 and 0:3). The diets were isonitrogenous (530 g crude protein (CP) kg^{-1}) and isolipidic (160 g crude lipid (CL) kg^{-1}). In addition, a diet with LT fish meal as the sole source of protein (FM diet) was produced with 570 g CP and 180 g CL kg^{-1}, and formulated to keep the same ratio between protein and lipid ratio as the 8 diets with plant protein sources. Yttrium oxide was used as a marker for digestibility measurement (Austreng et al., 2000). Feed formulation and chemical composition are shown in Table 1. Feed processing and equipment are described in detail by Zhang et al. (submitted). All dry ingredients were

ground, mixed, preconditioned and extruded in a twin screw extruder with 2.0 mm dies and the pellets were dried and coated with fish oil in a vacuum coater.

2.2 Fish and feeding

The experiment was conducted at the Joint Laboratory of Nutrition and Feed for Marine Fish, Marine Fisheries Research Institute of Zhejiang Province (Putuo, Zhoushan, China). The black sea bream juveniles were obtained from a hatchery in Fodu (Putuo), acclimated in an indoor concrete pond for three weeks, and fed a commercial diet (52% CP, 8% fat). Before the start of the experiment, 900 bream with an initial weigh of 13 g were depleted of feed for 24 hours, anaesthetized with MS-222 (90 mg l^{-1}), batch-weighed, then randomly assigned to 18 circular 500-l tanks, fifty fish per tank. Each tank was supplied with sand-filtered seawater at a flow rate of 1.5 l min^{-1} and additional aeration via air stone. A natural photoperiod (13 h light, 11 h dark) were applied throughout the feeding period. The average water temperature and salinity were 25.0 ºC and 29 g l^{-1}, respectively. Each diet was fed to fish in duplicate tanks and all the fish were fed three times per day, at 06:30, 11:30, and 17:30. Before each feeding, the water flow was stopped, while continuous aeration was maintained. The fish were fed by hand for 1 hour. After each feeding, all uneaten feed particles remained intact, and the number of uneaten pellets in the bottom of each tank were counted and siphoned out immediately. The amount of uneaten feed was set by multiplying the number of uneaten pellets with the average pellet weight for each feed (counting 4 × 100 pellets). The daily feeding rate was tentatively set 10% in excess based on the average feed intake over the last 3-day feeding,

but the fish received more feed if they showed signs of feed intake at the end of the one hour meals. The feeding experiment lasted for 60 days.

2.3 Sampling

Before the start of the experiment, 2 × 8 fish (depleted of feed for 24 h) from the acclimation pond were killed by overdose of MS-222, and kept at -20 ºC for whole body analysis. Fish were anaesthetized with MS-222 (90 mg l^{-1}) and batch-weighed in the beginning (Day 0) and the middle (Day 31) of the experiment. At the end of the feeding experiment, five fish were randomly sampled from each tank for blood samples. The fish were weighed individually, blood was collected from the caudal vein by a 1-ml disposable syringe with a 27-gauge needle, and kept on ice until centrifuged at 3000*g for 10 min. The plasma was aliquoted into two EP tubes, frozen in liquid N_2, and kept at -80 ºC until analysis. Another three fish were taken from each tank, individually measured for weight and length, and then killed by a blow to the head. The breams were cut open to remove the intestinal contents, and then the whole viscera, liver and carcass were weighed separately. Ten fish were taken from each tank, weighed individually, then the intact gastrointestinal tracts were gently removed and divided into 3 regions as follows: stomach, mid intestine (MI, from distal side of the stomach region to distal intestine) and distal intestine (DI, from the start of the last fold of intestine until the anus). Surface fat and connective tissue were carefully removed. The intestinal tissue walls of MI and DI were placed in pre-weighed EP tubes, frozen in liquid N_2 and kept at -80 ºC for the determination of brush border maltase activity.

2.4 Chemical analysis

The initial and final fish samples were autoclaved at 120 ºC for 20 min, homogenized and oven-dried at 70°C. The dried whole fish samples and feed samples were analyzed for dry matter, crude protein, lipid, ash, and energy. Dry matter was determined by drying at 105 ºC to constant weight (AOAC 1984). Crude protein content was measured using 2300 Kjeltec Analyzer Unit (Foss, Tecator, Sweden). Lipid was determined by petroleum ether extraction using a Soxtec system (Soxtec 2055, Foss Analytical, Denmark), ash by combustion at 550°C, and gross energy by bomb calorimetry (Phillipson Microbomb Calorimeter; Gentry Instruments Inc., Aiken, SC, USA). Minerals in feed samples were determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete digestion of the homogenized and dried samples in HNO_3 after cooking in a microwave oven for 1 h. For each measurement, duplicate samples were analyzed. Plasma cholesterol and triacylglycerols were analyzed by RSBIO® kits (Shanghai Rongshen Biotech Co., Ltd. Shanghai, China) and spectrophotometer micro-plate reader (PowerWave XS, BioTek Instruments Inc., Winooski, VT, USA). Activities of brush-border membrane bound maltase in MI and DI was analyzed as described by Krogdahl et al. (2003). Only the samples from the fish fed the FM diet and the diets with 500 g plant protein kg^{-1} crude protein inclusion were measured for maltase activity.

2.5 Calculations and statistical analyses

Feed intake (FI) was calculated by subtracting uneaten feed from fed feed on a dry matter basis. Weight gain (WG, %) was calculated as: $WG = 100 \times (FBW - IBW) \times IBW^{-1}$, where IBW and FBW are the initial and final body weight of individual fish (tank mean),

respectively. Feed conversion ratio (FCR) was calculated as: $FCR = FI \times (FBW - IBW)^{-1}$. Nitrogen or energy retention was calculated as: Retention (%) = $100 \times (N_1 \times FBW - N_0 \times IBW) \times (N_d \times FI)^{-1}$, where N_0 and N_1 represent the nitrogen or energy content in the initial and final whole fish samples (pooled samples of 3 fish per tank), respectively. Hepatosomatic index (HSI, %) was calculated as: HSI = 100 × (weight of liver) × (total fish weight)$^{-1}$. Viscerosomatic index (VSI, %) was calculated as: VSI = 100 × (visceral weight) × (fish weight)$^{-1}$. Condition factor (CF) was calculated as: CF = 100 × (fish weight) × (body length)$^{-3}$, where weight is expressed in g and length is in cm.

Each tank was considered an experimental unit. The results were analysed using the GLM procedure of SAS statistical software (SAS, 1999). One-way analysis of variance (ANOVA) was used to compare effects of the FM diet with those of the diets with plant protein. Factorial ANOVA used to analyze the effects of plant protein inclusion level and L/P ratio. Significant ($P < 0.05$) interactions between inclusion level and L/P ratio were rationalized by regression analysis of L/ (L + P) within inclusion level, provided that at least one of the main effects were significant (Snedecor and Cochran, 1967). The results were expressed as the means and pooled standard errors of means (S.E.M). Duncan's multiple-range test was used to rank significant differences among diets in the one way ANOVA and main effects in the factorial ANOVA.

3. Results

3.1 Growth and feed utilization

All fish had good appetite and grew well on all diets. The biomass was more than tripled after a 60-day feeding period, achieving an average weight gain (WG) of 251%. Only one fish died during the experimental period; one fed the HLP3 diet on day 14. An average feed conversion ratio (FCR) of 1.13 g DM ingested (g gain)$^{-1}$ was achieved (Table 2). Both during the first month and the whole period, the feed intake (FI) of the fish fed the FM diet was significantly lower than that of the fish fed the HLP1 diet, and did not differ significantly from those fed the other diets. No significant difference was found among diets for growth (WG). During the first month, the FCR for the LLP3, LLP4 and HLP4 diets did not significantly differ from that of the FM diets, while the FCR for the other diets were significantly higher. Over the whole feeding period, the FCR for the LLP1, LLP3, LLP4 and HLP4 diets did not differ from that of FM diet, while the others were significantly higher.

The FI during the first month was significantly higher for the bream fed the diets with 500 g plant protein kg^{-1} protein than those fed the diets with 300 g plant protein kg^{-1}. During both feeding periods, the diets with most LPC (L/P ratio = 3:0) resulted in significantly higher FI than the diets with less LPC. Diets with 500 g plant protein kg^{-1} resulted in significantly lower WG than the diet with 300 g kg^{-1}. No significant effect of L/P ratio on WG was found. Higher dietary inclusion of plant protein resulted in significantly higher FCR during both feeding periods, and diets with the highest LPC ratio (L/P ratio = 3:0) resulted in significantly higher FCR compared to diets with less LPC during both feeding periods. The FCR for the diets with L/P ratio of 1:2 and 0:3 during the first month and those with L/P ratio of 2:1, 1:2 and 0:3 during the whole

feeding period did, however, not differ significantly from each other. Significant interactions were seen between inclusion level and L/P ratio for FI during the first month and FCR both during the first month and over the whole period. This was due to a steep and significant increase in FI with increasing dietary LPC at 500 g plant protein kg^{-1} inclusion, while, this effect was not significant for 300 g kg^{-1} (Table 2, Figs. 1, and 2). A similar interaction was seen for FCR during the first month of feeding (Fig. 3).

3.2 Body composition and nutrient retention

No significant difference was found in whole body composition among diets except for ash (Table 3). The ash content in the fish fed the FM diet was significantly higher than those fed the dietary plant protein based diets, except for the LLP3 and LLP4 diets. Neither inclusion level nor the L/P ratio resulted in significant differences in whole body composition, except for ash, which was significantly lower in fish fed the diets with 500 g kg^{-1} plant protein inclusion than with 300 g kg^{-1} inclusion.

The nitrogen (N) retention of fish fed the FM diet was significantly higher than those fed the HLP1 and HLP2 diets, but did not differ from those fed the other diets (Table 4). Both N and energy retentions of fish fed diets with inclusion of 500 g plant protein kg^{-1} crude protein were significantly lower than those fed diets with 300 g kg^{-1} inclusion. The diets with lower LPC levels (L/P ratio = 1:2 or 0:3) resulted in significantly higher N retention than diets with the highest LPC level (L/P ratio = 3:0).

3.3 Fish somatic indices, plasma parameters and intestinal maltase activity

No significant difference was found for the somatic indices, HIS, VSI, and CF, or the plasma parameters, total plasma cholesterol and triacylglycerol levels among the different diets (Table 5). Also, none of these parameters were significantly affected by plant protein inclusion level or L/P ratio. No significant differences were seen for maltase activity in MI, but the activity in DI was significantly lower in fish fed the HLP4 diet (Table 6).

4. Discussion

The high growth rate obtained in the present experiment was consistent with previous findings in this species with similar fish size and rearing conditions (Shao et al., 2008; Peng et al., 2009). The similar growth rates among fish fed the FM diet and those fed the other plant protein based diets, showed that using multiple amino acid supplemented diets with LPC or PPC alone or in combinations could provide 50% of dietary protein for black sea bream without impairing growth.

Adequate feed intake is a precondition to guarantee a precise nutritional evaluation of plant proteins in fish feed. A feed intake reduction has been observed when including high levels of plant protein concentrates in the diet for gilthead sea bream (Kissil et al., 2000). This may be related to the removal of palatable constituents derived from FM and the presence of detractive compounds in plant-derived ingredients. The high feed intake in the present experiment was consistent with previous findings with LPC and PPC in rainbow trout (Zhang et al., submitted). Further, the comparable high feed intake of the

FM and the PPC-rich diets was consistent with findings with PPC in gilthead sea bream (Sanchez-Lozano et al., 2011). The current results show that the concentration of detractive components, such as alkaloids from lupin and saponins from pea were not sufficient to impair feed intake, even at a dietary inclusion level of 467 and 433 g kg^{-1} LPC and PPC respectively.

The dietary energy density also affects the feed intake of fish (Kaushik, 1998). In the present experiment, fish fed the LPC-rich diet seemed to increase their feed intake in response to compensate for the lower digestible energy level in these diets compared with the PPC-rich diets. Similarly, the interaction between inclusion level and L/P ratio for FI during the first month is clearly a result of the reduced energy density with increasing LPC in the diets.

The feed conversion values in the present experiment suggest more efficient utilization of the feed by black sea bream than recorded by Shao et al. (2008) and Peng et al. (2009).These values show more efficient feed conversion than found in gilthead sea bream fed lupin and pea meal based diet (Pereira and Oliva-Teles, 2002; 2004) and PPC based diet (Sanchez-Lozano et al., 2011). The aqueous extraction and air-classification mainly removes the soluble NSP from the lupin seed meal and carbohydrates from the pea meal, while the other ANF may still exist. However, extrusion is known both to improve protein utilization by inactivating heat labile ANF, and by unfolding globular storage proteins to facilitate access of the digestive enzymes. In addition, extrusion results in gelatinization of the starch, which is necessary for efficient digestion. Thus, the

high performance of the bass obtained in the present experiment can both be related to the nutritional qualities of the LPC and PPC (Carter and Hauler, 2000; Refstie et al., 2006; Øverland et al., 2009) and the use of extrusion to produce the diets, which improves the utilization of protein and starch (Sørensen, 2003).

The main advantage of PPC over LPC as a dietary protein source seems to be the lower content of NSP. This is in keeping with previous observations (Carter and Hauler, 2000; Zhang et al., submitted). NSP are almost indigestible for fish due to the absence of α-galactosidase and β-xylanase in the digestive tract (Kuz'mina, 1996; Bansleben et al., 2008). In addition, the undigested NSP in digesta can negatively affect the digestion and absorption of other nutrients (Sinha et al., 2011). Soluble NSP have a viscous nature and can bind to the intestinal brush border and form a thick unstirred water layer adjacent to the mucosa to block the access of substrates to brush border enzymes, and reduce nutrient digestibilities by increasing the intestinal viscosity. A major reason for processing lupin into LPC was to remove the soluble carbohydrate fraction, thus soluble NSP was not the major reason for the preference of PPC over LPC at high inclusion rate.

The whole body composition of the black sea bream in the present experiment was similar to that reported by Peng et al. (2009) for fish fed a fish oil control diet. Whole-body ash content (WBA) was significantly related to dietary P (DPh) concentration ($WBA = -0.10\ DPh^2 + 4.27\ DPh + 11.2$; $R^2 = 0.66$, $P = 0.0001$), but not to dietary phytate concentration. This indicate that dietary P was mainly limiting for bone mineralization, not for the soft tissues growth, as fish growth was not impaired by reduced dietary P-

concentrations. Dietary P-supply also explains the reduction in whole-body ash when inclusion of plant protein concentrates was increased from 300 to 500 g kg^{-1} crude protein.

There is no published information available on N and energy retentions for black sea bream. However, the average value of N retention in the present experiment was at least 10% units higher than those reported on gilthead sea bream (Sanchez-Lozano et al., 2009;2011). This may by associated with the use of multiple amino acid supplementation in our experiment, which could improve the balance of essential amino acids in diet, and consequently increase the N utilization of fish fed the plant protein based diets (Sanchez-Lozano et al., 2009). The significantly lower N retention in fish fed HLP1 and HLP2 compared with the other diets may have been related to the high inclusion of LPC. The design of the experiment does not, however, provide explanations to weather this was caused by the high NSP content, differences in nutrient digestibilities, or other differences between the two plant protein concentrates. The protein retention efficiencies indicate that essential amino acids were provided in excess by all diets. Thus, inadequate amino acid supply was not a plausible explanation to the differences in N retention.

Hughes (1991) and Lairon (1996) reported that NSP of legume seeds was an effective cholesterol-reducing agent. A clear hypocholesterolemic effect was also observed in our previous experiment with rainbow trout (Zhang et al., submitted). The absence of hypocholesterolemic effect in the present experiment indicates that such effect may be species specific.

The brush border enzymes are responsible for the final stages of hydrolysis of protein and starch. Their activities do not only indicate the capacity of digestion but also the integrity of intestinal structure, especially in the distal part. The reduction of maltase activity in DI is in keeping with our previous finding with rainbow trout (Zhang et al., submitted). The trout also had a slight decrease in mucosal fold height and a slight increase in fold fusion in DI of fish fed diet with the highest level of PPC. This may indicate mild changes associated with the mechanism that resulted in inflammation in DI at higher dietary PPC levels (Penn et al., 2011). Histological studies are, however, needed to find out if the reduced maltase activity is related to changes in the integrity of the distal intestine.

To conclude, both LPC and PPC are promising dietary protein sources for black sea bream. Any combination of LPC and PPC in essential amino acid-supplemented extruded diets, accounting for up to half of dietary protein, can be used without impairing fish growth. At higher inclusion, combinations with more PPC are preferred, while high inclusion of LPC resulted in less efficient feed conversion.

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Table 1 Diet formulation and analyzed chemical composition (based on dry matter)

Diets	F M	LLP1	LLP2	LLP3	LLP4	HLP1	HLP2	HLP3	HLP4
Ingredients, g kg^{-1}									
Fish meal[1]	706.0	436.0	436.0	436.0	436.0	309.0	309.0	309.0	309.0
LPC[2]	-	280.0	186.0	94.0	-	467.0	311.0	155.0	-
PPC [3]	-	-	86.0	173.0	260.0	-	145.0	289.0	433.0
Fish oil[4]	121.0	98.0	103.0	107.0	112.0	94.0	102.0	110.0	118.0
Wheat	160.9	165	168	170.0	173	103	108	112	116
Premix[5]	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3	4.3
Y_2O_3[6]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
L-Lys[7]	-	6.1	5.0	3.9	2.7	10.2	8.3	6.4	4.6
DL-Met[8]	-	3.4	3.3	3.2	3.1	5.7	5.6	5.4	5.2
L-Trp[9]	-	0.6	0.4	0.2	-	1.0	0.7	0.3	-
L-Thr[10]	-	-	0.5	1.0	1.6	-	0.9	1.7	2.6
Analyzed content, kg^{-1}									
Dry matter, g	939	943	934	936	945	936	950	933	940
In dry matter									
Crude protein, g	575	529	534	534	533	522	522	531	528
Crude fat, g	174	163	164	152	143	161	157	152	149
Starch, g	110	100	107	110	121	69	84	98	107
Ash, g	107	78	81	83	89	70	76	78	77
Gross energy, MJ	23.5	23.1	23.1	23.0	22.2	23.0	22.5	22.8	22.1
Phosphorous, g	16.3	11.0	11.3	11.6	12.7	8.9	9.8	10.6	11.2
Phytic acid, IP6, g	1.47	3.02	3.49	4.83	5.61	4.19	4.47	6.07	8.40

[1] Norse LT-94®, low-temperature dried fish meal, Norsildmel, Bergen, Norway.
[2] NaProLup PO54®, Lupin protein concentrate, derived from white lupins (*Lupinus albus*), NaProFood**,** Bruckberg, Germany
[3] PPC 55 PELLET, Pea protein concentrate, derived from yellow field pea (*Pisum sativum* L.), AgriMarin AS, Stavanger, Norway.
[4] Silfas, Karmsund, Norway.
[5] Per kg diet: vitamin A: 2000 IU, vitamin D_3: 1200 IU; vitamin E: 160 mg; vitamin K_3: 8 mg; vitamin B_1: 12 mg; vitamin B_2: 20 mg; vitamin B_3: 60 mg; vitamin B_5:24 mg; vitamin B_6: 12 mg; vitamin B_9: 4 mg; vitamin B_{12}: 0.016 mg; vitamin C: 100 mg; Biotin: 0.2 mg; Ca: 876 mg; Cu: 4 mg; Co: 0.8 mg; I: 2.4 mg; Mn:12 mg; Zn: 96 mg.
[6] Metal Rare Earth Limited, Shenzhen, China.
[7] *L*-Lysine-HCl, 99% feed grade, CJ Indonesia, Jakarta, Indonesia.
[8] Rhodimet® NP 99, *DL*-methionine, 99% feed-grade, Adisseo Brasil Nutricao Animal Ltda, Sao Paulo, Brazil.
[9] TrypAMINO®, *L*-tryptophan, 98 % feed-grade, Evonik Fermas S.R.O., Slovenska L'upca, Slovakia.
[10] *L*-Threonine, 98.5 % Feed Grade, Ajinomoto Eurolysine S.A.S., Paris, France.

Table 2 Growth performance and feed utilization of black sea bream fed the experimental diets

One way ANOVA model						
	Feed intake, g DM fish^{-1}		Weight gain, % of initial body weight		Feed conversion ratio, g DM ingested (g gain)$^{-1}$	
	0-30 days	0-60 days	0-30 days	0-60 days	0-30 days	0-60 days
Diet[1]						
LLP1	15.0^{bc}	37.2^{bc}	104	259	1.12^{bc}	1.10^{bcde}
LLP2	15.5^{b}	37.5^{bc}	110	258	1.08^{cd}	1.11^{bcd}
LLP3	14.2^{cd}	36.0^{bc}	109	267	1.00^{de}	1.03^{de}
LLP4	14.1^{cd}	35.9^{bc}	105	255	1.04^{cde}	1.10^{cde}
HLP1	18.0^{a}	40.5^{a}	96	226	1.44^{a}	1.38^{a}
HLP2	15.5^{b}	38.0^{ab}	99	241	1.20^{b}	1.21^{b}
HLP3	14.1^{cd}	35.5^{bc}	96	236	1.13^{bc}	1.16^{bc}
HLP4	13.9^{d}	34.5^{c}	100	241	1.07^{cde}	1.10^{bcde}
FM	14.6^{bcd}	36.2^{bc}	116	280	0.97^{e}	1.00^{e}
Pooled SEM	0.3	0.9	5	10	0.03	0.03
ANOVA P < F	< 0.001	0.025	0.16	0.080	0.045	0.046
Factorial ANOVA model						
	Feed intake		Weight gain		Feed conversion ratio	
	0-30 days	0-60 days	0-30 days	0-60 days	0-30 days	0-60 days
Factor						
Inclusion[2]						
300	14.7^{y}	36.6	107^{x}	260^{x}	1.06^{y}	1.08^{y}
500	15.3^{x}	37.1	97^{y}	236^{y}	1.21^{x}	1.21^{x}
L/P ratio[3]						
3:0	16.5^{r}	38.8^{r}	100	243	1.27^{r}	1.24^{r}
2:1	15.5^{s}	37.7^{r}	104	249	1.14^{s}	1.16^{s}
1:2	14.1^{t}	35.7^{s}	102	252	1.07^{t}	1.09^{s}
0:3	14.0^{t}	35.2^{s}	102	248	1.05^{t}	1.09^{s}
Pooled SEM	0.3	0.8	5	9	0.03	0.03
ANOVA P < F						
Inclusion[2]	0.016	0.40	0.025	0.005	< 0.001	< 0.001
L/P ratio[3]	< 0.001	0.005	0.83	0.77	< 0.001	0.007
Inclusion × L/P ratio	0.002	0.072	0.86	0.641	0.012	0.020

[1] For diet codes see Table 1. Different superscript letters $^{a, b, c, d, e}$ within a column indicate significant ($P < 0.05$) difference among diets.

[2] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein). Different superscript letters $^{x, y}$ within a column indicate significant ($P < 0.05$) difference between inclusion levels.

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets. Different superscript letters $^{r, s, t}$ within a column indicate significant ($P < 0.05$) differences among L/P ratios.

Table 3 Whole body composition of black sea bream fed the experimental diets (g kg^{-1})

One way ANOVA model	Moisture	Crude protein	Lipid	Ash	Energy (MJ kg^{-1})
Diet[1]					
LLP1	667	176	113	46.6^{bc}	8.74
LLP2	664	176	115	46.3^{bc}	8.02
LLP3	669	181	101	49.6^{ab}	8.11
LLP4	663	176	113	49.6^{ab}	8.22
HLP1	670	174	112	40.9^{c}	8.06
HLP2	668	177	113	45.4^{bc}	8.05
HLP3	676	177	102	45.0^{bc}	7.91
HLP4	682	173	104	46.0^{bc}	7.99
FM	676	181	92	55.3^{a}	7.36
Pooled S.E.M.	7	2	5	2.2	0.35
ANOVA P < F	0.57	0.17	0.13	0.043	0.49

Factorial ANOVA model	Moisture	Crude protein	Lipid	Ash	Energy
Factor					
Inclusion[2]					
300	666	177	110	48.0^{x}	8.27
500	674	175	108	44.3^{y}	8.00
L/P ratio[3]					
3:0	669	175	113	43.8	8.40
2:1	666	176	114	45.9	8.04
1:2	672	179	101	47.3	8.01
0:3	673	175	108	47.8	8.11
Pooled S.E.M.	7	2	6	1.7	0.37
ANOVA P < F					
Inclusion[2]	0.13	0.27	0.55	0.015	0.33
L/P ratio[3]	0.71	0.16	0.18	0.16	0.70
Inclusion × L/P ratio	0.61	0.71	0.79	0.56	0.81

[1] For diet codes see Table 1. Different superscript letters $^{a, b, c}$ within a column indicate significant ($P < 0.05$) difference among diets.

[2] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein). Different superscript letters $^{x, y}$ within a column indicate significant ($P < 0.05$) difference between inclusion levels.

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets.

Table 4 Nitrogen and energy retentions (%) of black sea bream fed the experimental diets

One way ANOVA model		
	Nitrogen	Energy
Diet[1]		
LLP1	30.3^{abc}	39.9
LLP2	29.8^{bc}	35.7
LLP3	33.3^{a}	39.1
LLP4	30.3^{abc}	38.9
HLP1	24.2^{d}	29.6
HLP2	28.2^{c}	34.1
HLP3	29.2^{bc}	34.6
HLP4	29.7^{bc}	37.8
FM	32.1^{ab}	35.0
Pooled S.E.M.	0.3	0.9
ANOVA P < F	0.004	0.11
Factorial ANOVA model		
	Nitrogen	Energy
Factor		
Inclusion[2]		
300	30.9^{x}	38.4^{x}
500	27.8^{y}	34.0^{y}
L/P ratio[3]		
3:0	27.2^{s}	34.8
2:1	29.0^{rs}	34.9
1:2	31.3^{r}	36.8
0:3	30.0^{r}	38.3
Pooled S.E.M.	1.0	2.2
ANOVA P < F		
Inclusion[2]	0.002	0.025
L/P ratio[3]	0.023	0.38
Inclusion × L/P ratio	0.089	0.23

[1] For diet codes see Table 1. Different superscript letters $^{a, b, c, d}$ within a column indicate significant ($P < 0.05$) difference among diets.

[2] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein).

[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets. Different superscript letters $^{r, s}$ within a column indicate significant ($P < 0.05$) differences among L/P ratios.

Table 5 Somatic indices, and total plasma cholesterol (TC) and triacylglycerols (TG) concentrations of black sea bream fed the experimental diets

One way ANOVA model	Somatic indices			In plasma	
	HSI[4]	VSI[5]	CF[6]	TC (mM)	TG (mM)
Diet[1]					
LLP1	1.41	9.35	3.15	6.84	15.0
LLP2	1.48	8.31	3.83	6.89	20.7
LLP3	1.35	7.44	3.82	7.03	16.8
LLP4	1.49	8.37	3.84	6.33	10.5
HLP1	1.59	8.23	3.72	7.04	19.8
HLP2	1.40	8.10	3.88	7.58	19.3
HLP3	1.42	7.64	3.92	7.70	15.7
HLP4	1.35	8.22	3.71	7.60	13.3
FM	1.23	7.46	3.75	7.70	17.7
Pooled S.E.M.	0.08	0.53	0.32	0.66	0.6
ANOVA P < F	0.22	0.38	0.82	0.80	0.96
Factorial ANOVA model					
	HSI	VSI	CF	TC	TG
Factor					
Inclusion[2]					
300	1.43	8.38	3.66	6.77	15.7
500	1.44	8.05	3.81	7.48	17.0
L/P ratio[3]					
3:0	1.50	8.79	3.43	6.94	17.4
2:1	1.44	8.20	3.86	7.24	20.0
1:2	1.39	7.54	3.87	7.37	16.3
0:3	1.42	8.30	3.78	6.97	11.9
Pooled S.E.M.	0.08	0.56	0.34	0.68	4.2
ANOVA P < F					
Inclusion[2]	0.84	0.44	0.55	0.18	0.67
L/P ratio[3]	0.52	0.25	0.56	0.91	0.34
Inclusion × L/P ratio	0.21	0.69	0.77	0.88	0.85

[1] For diet codes see Table 2.
[2] Inclusion level of plant protein sources (g protein kg^{-1} dietary protein).
[3] Mixing ratio between essential amino acid-supplemented LPC and PPC in diets.
[4] Hepatosomatic index.
[5] Viscerosomatic index.
[6] Condition factor.

Table 6 Maltase activities in intestinal sections of black sea bream fed the experimental diets with 500 g plant protein kg^{-1} crude protein inclusion and the FM diet (n=2)

	Diet						
	HLP1	HLP2	HLP3	HLP4	FM	Pooled S.E.M.	*ANOVA* P < F
Mid intestine							
µmol h^{-1}(g tissue)$^{-1}$	0.326	0.352	0.309	0.472	0.408	0.043	0.17
µmol h^{-1}(kg BW)$^{-1}$	19.9	18.2	17.1	13.0	16.8	1.9	0.26
µmol h^{-1}(mg protein)$^{-1}$	129	133	105	112	129	14	0.61
Distal intestine							
µmol h^{1}(g tissue)$^{-1}$	0.305^{a}	0.331^{a}	0.322^{a}	0.163^{b}	0.312^{a}	0.027	0.033
µmol h^{-1}(kg BW)$^{-1}$	8.22^{a}	9.02^{a}	7.31^{a}	4.47^{b}	6.38^{ab}	0.74	0.044
µmol h^{-1}(mg protein)$^{-1}$	63.9^{bc}	74.3^{ab}	69.6^{ab}	51.6^{c}	86.3^{a}	4.5	0.020

Different superscript letters $^{a.\ b.\ c}$ indicate significant (P < 0.05) difference among treatments.

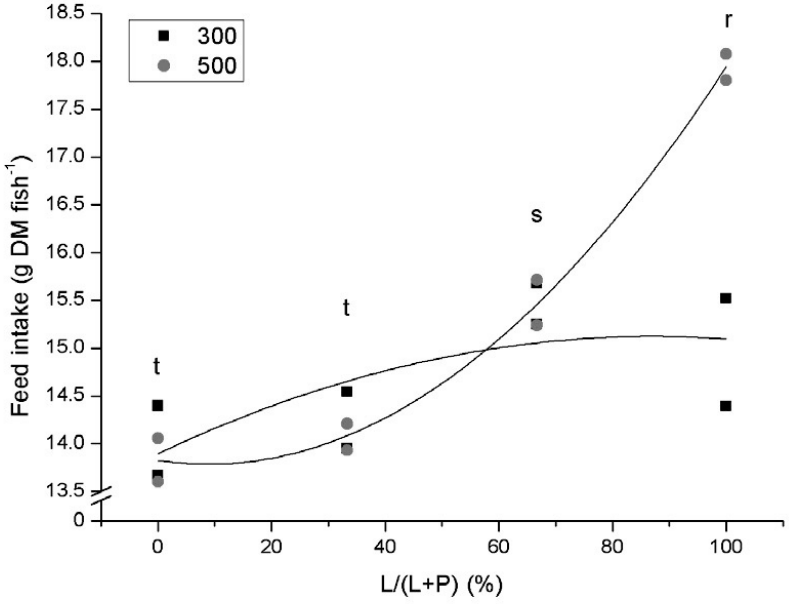

Figure 1 Feed intake (FI) of black sea bream during the first 30-day of feeding diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/ (L + P)). FCR_{300} = (-1.62 E-4) LPR^2 + 0.0282 LPR + 13.9, P_{model} = 0.18, R^2 = 0.29; FI_{500} = (5.01 E-4) LPR^2 - 0.00889 LPR + 13.8, P_{model} < 0.001, R^2 = 0.98. Different superscript letters $^{r, s, t}$ indicate significant (P < 0.05) differences in main effects among the 4 different L/P ratios.

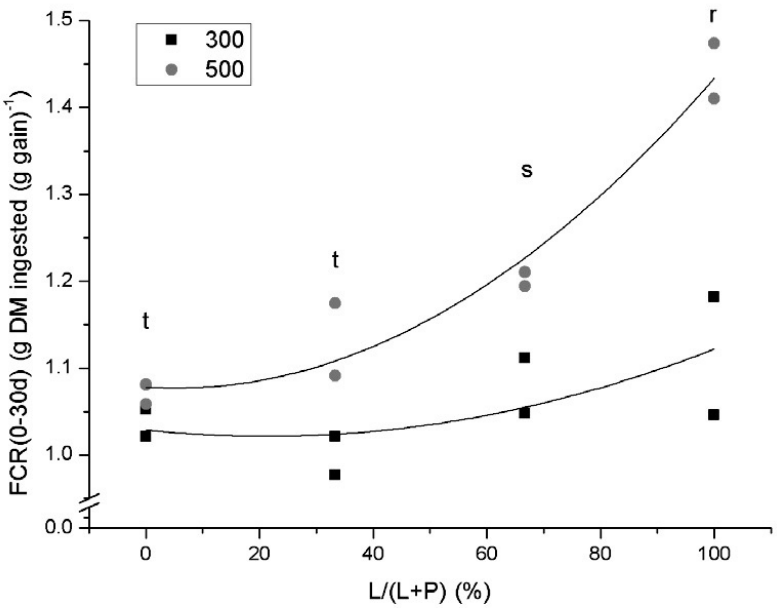

Figure 2. Feed conversion ratio (FCR) in black sea bream during the first 30-day of feeding diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/ (L + P)). FCR_{300} = (1.62 E-5) LPR^2 - (6.82 E-4) LPR + 1.03, P_{model} = 0.231, R^2 = 0.22; FCR_{500} = (3.97 E-5) LPR^2 - (4.17 E-4) LPR + 1.08, P_{model} < 0.001, R^2 = 0.93. Different superscript letters $^{r, s, t}$ indicate significant (P < 0.05) differences in main effects among the 4 different L/P ratios.

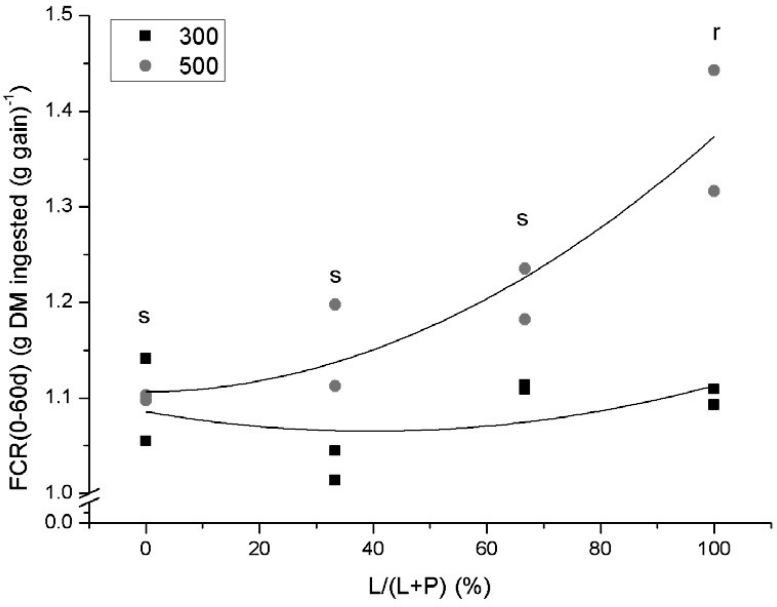

Figure 3.

Feed conversion ratio (FCR) in black sea bream during the whole 60-day of feeding diets with 300 and 500 g kg^{-1} of total crude protein from LPC and PPC, and with different ratios of protein from LPC and PPC (LPR, expressed by L/ (L + P)). FCR_{300} = (1.31 E-5) LPR^2 – 0.00104 LPR + 1.09, P_{model} = 0.57, R^2 = 0; FCR_{500} = (2.61 E-5) LPR^2 + (6.06 E-5) LPR + 1.11, P_{model} = 0.008, R^2 = 0.80. Different superscript letters $^{r, s}$ indicate significant ($P < 0.05$) differences in main effects among the 4 different L/P ratios.


Paper III

Optimizing plant protein combinations in fish meal-free diets for rainbow trout (*Oncorhynchus mykiss*) by a mixture model

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Abstract

The aim of this experiment was to define the optimal mixtures of plant protein concentrates in an extruded, fish meal free diet for rainbow trout. The response criteria were growth, feed utilization, nutrient digestibilities (apparent digestibility coefficients, ADCs) and retentions, body composition and intestinal histology. Three essential amino acid (EAA) and taurine-fortified plant protein mixtures (P-MIX, C-MIX, and S-MIX) were prepared by mixing four plant protein concentrates (pea protein concentrate (PPC), canola protein concentrate (CPC), potato protein concentrate, and soy protein concentrate (SPC)). Seven plant protein based diets were formulated based on a mixture design using P-MIX, C-MIX, and S-MIX alone or in combinations to provide > 95% of the dietary protein. The diets were supplemented with 5% krill products as

attractant. One fish meal based diet using LT-fish meal as the sole dietary protein source (FM diet) was also produced. All diets were balanced to contain equal amounts of digestible protein (400 g kg^{-1}) and digestible energy (21 MJ kg^{-1}). Each diet was fed to duplicate tanks of 61 g rainbow trout reared in 9 ^{o}C water for 72 days. Fish fed the plant protein based diets had significantly ($P<0.05$) higher feed intake, feed conversion ratios (FCR), ADC of phosphorus (P), retention of ingested P, and metabolic nitrogen (N) loss than those fed the FM diet. Growth rates, ADC of N, most EAA, cysteine and total amino acids (except for the CPC and SPC diets), lipid (except for the SPC diet) and starch, body composition of dry matter, lipid, ash and gross energy, and metabolic energy loss did not significantly differ from the fish fed the FM diet. The ADC of energy, ingested N and energy retentions, digested N retention, and plasma phosphorus concentrations were significantly lowered in trout fed the plant concentrate diets. Fish fed the diet with half of the dietary plant protein or more from the P-MIX exhibited inflammatory changes of mild or moderate severity in the distal intestine. Using the combination of P-MIX and C-MIX as the main dietary protein source instead of single mixture resulted in a higher digested N retention. Due to lack of differences in growth rate, there was no available model to define an optimal mixture for growth. Based on the predicted models, a combination of P-MIX and C-MIX led to the most efficient feed conversion. ADC of N and individual amino acids were highest when S-MIX was used alone. The most efficient ADC of lipid and energy were obtained by a combination of P-MIX and S-MIX. Using C-MIX alone supported the highest ADC and retention of P, and whole-body concentrations of ash, P, Ca and Mg, due to the dephytinized CPC. Retention of ingested N was most efficient when a combination of P-MIX and S-MIX was used, while retention of ingested N was most efficient for a combination of P-MIX and C-MIX.

In conclusion, the mixture model proved useful to optimize combinations of plant protein concentrates in a fish meal-free diet for rainbow trout.

Keywords: pea protein concentrate, canola protein concentrate, potato protein concentrate, soy protein concentrate, protein combinations, mixture design, rainbow trout, distal-intestinal inflammation

Introduction

Both the amount and variety of plant protein concentrates used in fish feeds are increasing. Mainly due to the removal of most of the indigestible carbohydrate, plant protein concentrates contain more protein than the unprocessed plant ingredient and can thus provide a higher level of protein in the diets. There are, however, still challenges associated with high or total fish meal replacement by plant proteins, such as reduced feed intake, growth, and feed utilization and poor nutrient digestibility. Most of these can be attributed to low diet palatability, imbalanced essential amino acids (EAA), and the presence of anti-nutritional factors (ANF) in plant protein concentrates.

The low palatability can be ascribed to reduced dietary attractants mainly provided by the fish meal (Kousoulaki et al., 2009), but also due to deterrent components contained in plant-derived ingredients (Bureau et al., 1998; Serrano et al., 2011). Krill meal, krill hydrolysate, and the water soluble fraction of krill contain various water soluble fractions, such as free amino acids, peptides, small proteins and minerals, which can be used as dietary attractants for rainbow trout (Oikawa and March, 1997).

Imbalanced EAA also limits total fish meal replacement in salmonid diets. Typically, grain proteins have lysine (Lys) as the first limiting EAA, while the first limiting EAA in legumes is methionine or Lys. The ability of salmonids to utilize crystalline amino acids is well documented (Kaushik and Seiliez, 2010), and EAA supplementation to plant-based salmonid diets is successful provided that the fish is fed frequently to obtain an overlap in absorption of peptide-bound and supplemented crystalline EAA (Zhang et al., submitted). In addition, other nutrients such as taurine (Tau), which is provided by fish meal, does not exist in plant-derived ingredients, and may also limit total fish meal replacement in salmonid diets (Gaylord et al., 2006).

All commonly used plant protein ingredients contain ANF. These include enzyme inhibitors and lectins, hormone analogues and other metabolically active components, as well as toxins or detractants that are specific to each plant (Francis et al., 2001). Several of these ANF may be inactivated or removed through production of protein concentrates. For examples, enzyme inhibitors and lectins are inactivated by the heating step of the process. The components responsible for soybean-induced enteritis in the distal intestine of salmon and trout are removed by extraction of defatted soy to produce soy protein concentrate (van den Ingh et al., 1996). The effect is not necessarily the same when protein concentrates are produced by other methods. For example, pea protein concentrate produced by air classification causes enteritis in the distal intestine of salmon, similar to that caused by soybean meal (Penn et al., 2011). Some ANF may even be concentrated during production of plant protein concentrates. One example is phytic acid, the storage medium for phosphate in most seeds. Phytic acid is indigestible and is, thus, not an available source of phosphate to salmonids. Another main challenge with phytate is that it binds di- and trivalent cations, and examples show that dietary soy protein concentrate, high in phytate, both induces poor mineralization (Storebakken et al., 1998) and spinal deformities (Helland et al.,

2006) in Atlantic salmon. The necessity to remove phytate in a fish meal free diet had been addressed in a separate paper (Zhang et al., manuscript 4).

Using the combinations of protein-ingredients with various nutritive characters in the diet can improve the dietary nutrient balance. It can also dilute or even mitigate the adverse effect of ANF from single ingredients (Romarheim et al., 2011) or detractive components and consequently allow a higher inclusion in the diet for carnivorous fish species.

Mixture design is a useful statistical experimental design and analysis tool. In a mixture design, the variables are the proportions of the components in the mixture rather than the absolute amount that they can be summed to 1 (or 100%). The measured response is assumed to depend only on these proportions. Mixture models allow interpolation to determine the mixture that will produce a desired maximum or minimum response. Such designs have been widely used in the chemical (Akalin et al., 2010; Lin et al., 2010), pharmaceutical (Mahdhi et al., 2010; Malzert-Freon et al., 2010) and food industries (Karaman et al., 2011) to optimize processes or formulations. Only few studies using mixture models to optimize fish and shrimp feed have been reported (Ruohonen et al., 2003; 2007; Forster et al., 2010; Draganovic et al., 2011).

The aims of the present experiment were to 1) investigate if rainbow trout could utilize diets with 95% of protein from plant protein concentrates supplemented with EAA to eliminate the amino acid imbalance and krill meal and the water soluble fraction from krill as feeding attractant to avoid the possible feed intake depression, 2) define the optimal combinations of plant protein concentrates, based on different criteria such as growth rates, feed conversions, and digestibility with the help of mixture models, and 3) evaluate the effect of extreme dietary levels of plant protein concentrate on health effects in fish and determine if using combinations instead of single

ingredient could facilitate better fish health.

Materials and Methods

This study consisted of a 72-day feeding experiment and a subsequent 10-day digestibility experiment.

2.1 Ingredients and experimental design

Four plant protein-concentrates; pea protein concentrate (PPC), potato protein concentrate with low solanidine glycoalkaloids (SGA) content (potato PC), dephytinized canola protein concentrate (CPC) and soy protein concentrate (SPC) were used. The PPC was produced from yellow field pea (*Pisum sativum* L.) by dehulling, fine grinding and air-classification. The Potato PC was obtained from potato by wet grinding, extraction, thermal coagulation, drying, and SGA removal. The CPC was produced from canola meal by grinding, glucosinolates degradation, extraction, dephytinization, and drying. The SPC was produced from soy white flakes by aqueous-ethanol extraction, heating, and drying. The chemical compositions of these plant protein concentrates are shown in Table 1.

Three plant protein mixtures (P-MIX, C-MIX, and S-MIX) were formulated to contain 600 g digestible protein kg^{-1} and 16 MJ digestible energy kg^{-1} (Table 2) by blending PPC and Potato PC, CPC and Potato PC, SPC and canola oil. The first-two limiting EAA were supplemented to each plant protein mixture to meet the requirements of rainbow trout (Arg, Thr and Met: Rodehutscord et al. (1995b; a); other EAA: (NRC, 2011)). A three-component, Simplex-

Centroid design was applied and seven plant protein based fish meal-free diets were formulated using different combinations of P-MIX, C-MIX, and S-MIX to provide 95% of total dietary protein (Table 3). According to mixture design, the proportion of each plant protein premixture (P-MIX, C-MIX, or S-MIX) in these combinations was varied from 0 to 100 %, but the summed proportions of three premixtures were kept constant as 1 (or 100%) A FM control diet, using LT-fish meal, as the sole source of protein, was also used. All diets were designed to be formulated to contain equal amounts of digestible protein (400 g kg^{-1}) and digestible energy (21 MJ kg^{-1}) (Table 4). Digestible crude protein, essential amino acids (EAA), and energy values of all ingredients were based on the chemical composition analysis and nutrient digestibilities from published and unpublished values. Values for potato PC were obtained from Refstie and Tiekstra (2003), CPC from Thiessen et al. (2004) and Drew et al. (unpublished), PPC and SPC from Drew et al. (unpublished), LT-fish meal from Anderson et al. (1995), krill meal from Hansen et al. (2011), and wheat from Gaylord et al. (2008; 2010). Values obtained by collecting faeces using Guelph sedimentation method (Cho and Slinger, 1979) were transformed to values obtained by manual stripping (Austreng, 1978), assuming 5% higher estimates obtained by using the settling method (Drew et al. (unpublished)).

2.2 Feed manufacturing

The diets were produced at the Centre for Feed Technology, at UMB, Ås, Norway. All the dry ingredients were ground in a hammer mill through a 0.80-mm screen, mixed, preconditioned and extruded in a twin screw extruder with 2.0 mm die. The krill water solubles were added in the conditioner by using a pump with a fixed pumping rate between liquid and feeding rate of mixed

mash. The extrusion process was optimized (Table 5) to obtain a bulk density > 520 g l^{-1} in the pellets before drying, in order to facilitate slow sinking of the feed after drying and coating with lipid. Pellets were dried to 930 g dry matter kg^{-1} and then coated with fish oil in a Forberg (Larvik, Norway) 6-l mini-coater. The equipment used for feed processing, monitoring of extrusion parameters, and methods for assessment of physical pellet quality have been described in detail by Øverland et al. (2009) and Zhang et al. (submitted). Briefly, breaking force and diameter was determined with a texture analyzer, pellet length with a calliper, and pellet durability was estimated on uncoated pellets with a Holmen pellet tester (Borregaard Lignotech, Warringtom, UK). Water stability was determined according to the method described by Baeverfjord et al. (2006).

2.3 Fish feeding and sampling

The 72-day feeding experiment was carried out in the Fish nutrition laboratory at UMB, with an indoor recirculation system. The rainbow trout (*Oncorhynchus mykiss*) were deprived of feed for 48 h, and then a total of 720 juveniles with an average weight of 61 g were randomly assigned to 18 cylindrical 200-l fibreglass tanks, with forty fish per tank. Each tank was supplied with freshwater at a flow rate of 6-7 l min^{-1} and additional aeration via air stone. Constant light was maintained. The water temperature ranged from 6.5 to 11.8 ºC, with a mean of 9.1 ºC. Dissolved oxygen remained above 6.0 mg l^{-1} in the outlet water, based on daily measurements. Each diet was fed to fish in duplicate tanks, and the trout were fed three meals per day (08:00, 14:00 and 20:00 h), 40 min per feeding using automatic band feeders. The fish were fed 10 % in excess, based on average feed intake over the previous 3-day period. Uneaten feed was sieved from the

outlet water and feed intake was monitored by the method of Helland et al. (1996), except that the uneaten feed was collected immediately after each meal. Before the start of the experiment, 2 × 5 fish from the holding tank were euthanized with an overdose of MS-222, and stored at -20 ºC for whole body analysis. Fish were anaesthetized with MS-222 (90 mg l^{-1}) and batch-weighed in the beginning (Day 0), and the middle (Day 37) of the experiment.

At the end of feeding experiment, blood and tissue samples for histology were obtained from five fish per tank. The fish were weighed individually. Blood was collected from the caudal vein with heparinised vacutainers, kept on ice until centrifuged at 3000*g for 10 min. Then plasma was aliquoted into two separate Eppendorf tubes, frozen in N_2 and kept at -80^{o}C until analysis. The intact gastrointestinal (GI) tracts of the same fish were removed, and divided into 4 regions as follows: stomach (ST), pyloric region (PR, from the distal side of the pyloric sphincter to distal-most caecum), mid intestine (MI, from distal side of the pyloric region to distal intestine) and distal intestine (DI, starting with the increase in intestine diameter and ending with anus). All the intestinal sections were opened longitudinally. Tissue samples of 1 cm^2 were taken from MI and DI walls, fixed in phosphate buffered formalin (4%; pH 7.4) for 24 h, and then transferred to 70% ethanol for storage until processing. Another three fish were taken from each tank, weighed individually, and killed by a blow to the head. The gut was opened to remove the intestinal content, and then guts and carcasses were stored at -20^{o}C for whole body analysis. The remaining 22 fish in each tank were batch-weighed and kept for collection of faeces. In each of the two collections, fish were fed using the same procedure used in the feeding experiment for 5 d, then anaesthetized by MS-222, and stripped for faeces by the method of Austreng (1978). All the faecal samples from the same tank were pooled and stored at -20^{o}C prior to analysis.

2.4 Analyses

The initial and final whole body samples were homogenized with CO_2 ice in a food processor and freeze-dried. Pooled faeces samples were freeze-dried and ground with a pestle and mortar. Fish scales were removed prior to analysis. Feed ingredients, feeds, and freeze-dried faeces samples were analyzed for dry matter (EC 71/393), Kjeldahl nitrogen (N) (EC 93/28), lipid (HCl hydrolysis and diethylether extraction (EC 98/64)), ash (EC 71/250), minerals (ICP-AES/ICP-MS) (Nordic Committee on Food Analysis (NMKL) method 161) and starch (AOAC enzymatic method 996.11). Freeze-dried whole body samples were analyzed for proximate composition using the same methods, except that no HCl hydrolysis was employed for lipid extraction. Gross energy was measured by bomb calorimetry (Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA). Amino acid (except tryptophan) and taurine analyses were according to EC (98/64) on a Biochrom 30 amino acid analyser (Biochrom Ltd., Cambridge, UK). Tryptophan was analyzed according to EC (2000/45) on a Dionex Summit HPLC system, with a Shimadzu RF-535 fluorescence detector. Yttrium oxide concentration in feed and faeces was determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete digestion of the homogenized and dried samples in HNO_3 after cooking in a microwave oven for 1 h. The plasma samples were weighted, dried at 80 ^{o}C and then ashed at 550 ^{o}C before mineral analysis (ICP-AES/ICP-MS).

2.5 Histological evaluation

Processing of the histological tissues was done at the Section for Anatomy and Pathology of the Norwegian School of Veterinary Science (Oslo, Norway) using standard histological techniques.

The MI samples were sectioned transversely, whereas the DI samples were sectioned longitudinally (i.e. perpendicular to the macroscopically visible circular folds; approximately 5 μm thick) and stained with haematoxylin and eosin (HE). Blind histological examination was performed using light microscopy. Tissue morphology was evaluated according to the descriptions of Amin et al. (1992) and Baeverfjord and Krogdahl (1996).

2.6 Calculations and statistical analysis

Expansion ratio (%) of pellets was calculated as: 100 × ((diameter of extrudate − die diameter) × die diameter^{-1}). Feed intake (FI) was estimated by subtracting uneaten feed from the amount fed on a dry matter basis. Recovery of uneaten feed was estimated as described by Helland et al. (1996). Weight gain (WG, %) was calculated as: $WG = 100 \times (FBW - IBW) \times IBW^{-1}$, where FBW and IBW represent final body weight and initial body weight, respectively. Feed conversion ratio (FCR) was calculated as: $FI \times (FBW - IBW)^{-1}$, where FI is feed intake. Apparent digestibility coefficients (ADC_N) of individual nutrients and energy were calculated as: $100 \times (1 - (Y_d \times Y_f^{-1} \times N_f \times N_d^{-1}))$, where Y_d and Y_f represent the concentration of yttrium in the diet and faeces, N_d and N_f represent the concentration of individual nutrients or energy in the diet and faeces, respectively. Nutrient and energy retentions (R_N) were calculated as: $100 \times (N_1 \times FBW - N_0 \times IBW) \times (N_d \times FI)^{-1}$, where N_0 and N_1 represent the nutrient or energy concentration in the initial and final whole fish samples (pooled samples of 3 fish per tank), respectively. Metabolic loss of nutrients and energy were calculated as: $FI_N - (F_N + R_N)$, where FI_N represent the nutrient or energy intake. Hepatosomatic index (HSI) was calculated as: $HSI = 100 \times W_l \times W_f^{-1}$, where W_l and W_f represent liver weight and fish weight, respectively.

One-way analysis of variance (ANOVA) using the GLM (SAS, 1999) was used to compare effects of the FM diet with those of the diets with plant proteins. The results were expressed as the means and pooled standard errors of means (S.E.M). Duncan's multiple range test was used to rank significant differences ($P<0.05$) among diets. Mixture methodology was used to address the relationships between the responses and proportion of each plant protein mixture in the combination by the software Design Expert (ver. 8.0.5b; Stat-Ease, Inc., Minneapolis, MN, USA). In the mixture methodology, each mixture (P-MIX, C-MIX, and S-MIX) was transformed to the corresponding pseudo component (the percentage of each plant protein premixture in the total plant protein combination). One of three regression models with different polynomial orders was applied to model the responses with varied pseudo components. The equations of these models were:

Linear: $Y = a_1 P + a_2 C + a_3 S$

Quadratic: $Y = a_1 P + a_2 C + a_3 S + a_{12} P*C + a_{13} P*S + a_{23} C*S$

Special-cubic: $Y = a_1 P + a_2 C + a_3 S + a_{12} P*C + a_{13} P*S + a_{23} C*S + a_{123} P*C*S$

The intercepts were set to zero, Y represents the different response variables, P, C and S represent the pseudo components of P-MIX, C-MIX and S-MIX, respectively, and $a_1 \ldots a_{123}$ represent the estimated regression coefficients. The best fitting model was chosen automatically by the program based on the following criteria: 1) The highest order of polynomial of model was chosen based on the results of sequential model sum of squares; 2) Only the model with insignificant lack-of-fit was available for estimation; 3) The available model with maximized adjusted and predicted R^2-values were chosen as the best fitting model. The contour plots generated from the predicted equations for the selected parameters were superimposed to obtain the optimum combinations.

3 Results

3.1 Extrusion parameters and physical quality of feed pellet

The extrusion parameters and feed physical quality are shown in Table 5. Bulk density of the pellets were optimised by adjusting feeding rate of mash from holding bin to the preconditioner, amount of water added to the preconditioner and extruder, steam injection into the preconditioner as well as extruder speed (RPM). Consequently, the dependent parameters specific mechanical energy as well as temperature and pressure in front of the die varied among diets. Generally, the P+C diet had the highest durability and breaking force, the PPC diet had similar values to the P+C diet, and the FM diet had the lowest values. The FM diet had the highest expansion and water stability, the water stability of the PPC diet was similar to the FM diet, the SPC diet had the lowest stability.

3.2 Growth and feed utilization

One fish died during the experiment (during mid-weighing). All diets were well accepted, and the fish grew from 61 g to an average weight of 214 g over the whole feeding period. Fish fed the FM diet had a significantly lower FI than those fed all the plant protein based diets except the P+S diet (Table 6). Fish fed the P+S diet had the lowest FI among all the plant protein diets, with significantly lower FI than those fed the CPC and SPC diets. No significant differences in WG were found among fish fed the different diets. Fish fed the FM diet had significantly lower FCR

than those fed plant protein based diets. Fish fed the P+C and P+C+S diets had significantly lower FCR than those fed the CPC and SPC diets.

According to the program criteria, no model was available to fit the FI or WG data. The FCR data was fitted using a quadratic polynomial model (Table 7). The prediction indicated that both C-MIX and S-MIX had an equal effect on FCR, and that they were more effective than the P-MIX. Significant interactions were found both between C-MIX and P-MIX, and between C-MIX and S-MIX. Both interactions had antagonistic effects on the FCR. Based on this model, the predicted optimal combination of the three plant protein mixtures that minimized the FCR (0.78 g DM ingested (g gain)$^{-1}$) was 64% P-MIX, 36% C-MIX and 0% S-MIX (Fig. 1).

3.3 Nutrient digestibilities

The ADC of N was significantly higher in the FM diet than in the CPC diet, but significantly lower in the SPC diet (Table 6). The ADC of lipid was significantly higher in the FM diet than in the SPC diet, but was not significantly different from the other diets. Fish fed the FM diet had significantly higher ADC of energy but lower ADC of phosphorus than those fed the plant protein based diets.

Fish fed the SPC diet had significantly higher ADC of N than those fed the other plant protein based diets, except for the P+S diet. They also had significantly lower ADC of lipid and P than those fed the other plant protein based diets. Furthermore, ADC of energy was significantly lower in the SPC diet than the other plant protein based diets, except for the C+S diet. Fish fed the CPC diet had significantly lower ADC of N than those fed the other plant protein based diets, except for the P+S diet. The ADC of phosphorus was significantly higher in the CPC diet

compared to the other plant protein based diets. No significant differences were found in ADC of starch.

Fish fed the SPC diet had the highest ADC of EAA except for Thr, and the highest ADC of Cys and total amino acids (TAA). Fish fed the CPC diet had the lowest ADC of EAA, Cys and total TAA. The ADC of most EAA, Cys and TAA of fish fed the FM diet did not differ significantly from that of fish fed the SPC diet, except for the significantly lower ADC of Arg and Met. The ADC of nutrients in the P+C, P+S, C+S diets were the average of the diets based on each single mixture.

The ADC of N, EAA, Cys, and TAA of fish fed the different diets were fitted with linear models (Table 7). The equations indicate that the S-MIX had the strongest effect on these digestibilities, followed by P-MIX and C-MIX. The ADC of lipid and energy were fitted using quadratic models, where P-MIX and C-MIX had similar effects, and S-MIX had a lesser effect. Significant interactions were found between P-MIX and C-MIX, and between C-MIX and S-MIX. Both interactions showed synergetic effects on the ADC of lipid. A significant interaction between P-MIX and S-MIX was also found, which showed a synergetic effect on the ADC of energy. A special-cubic model fitted the ADC of P, and the equation indicated that C-MIX had the strongest effect, followed by P-MIX and S-MIX. Significant interactions were found between P-MIX and C-MIX and between P-MIX and S-MIX. Both interactions showed synergetic effects on the ADC of P. Significant interactions showing antagonistic effects on ADC of P, however, were found between C-MIX and S-MIX and among the three components.

3.4 Whole body composition and plasma mineral concentration

No significant differences were found in the body compositions among the treatments except for crude protein (Table 8). The crude protein content in fish fed the FM diet was significantly lower than that of those fed the SPC, P+C, and P+S diets, but not significantly different from that of fish fed the other diets. The P and calcium (Ca) content of fish fed the FM diet, did not differ significantly from that of fish fed any of the CPC containing diets. Fish fed the CPC diet had the highest P and Ca content, which were significantly higher than that of fish fed the PPC, SPC, and P+S diets. Fish fed the FM diet had intermediate Mg content, which did not significantly differ from that of fish fed other plant protein based diets except for the CPC and PPC diets. Fish fed the CPC diet had the highest Mg content, while fish fed the PPC diet had the lowest. Fish fed the FM diet also had intermediate Zn content, which was not significantly different from that of fish fed plant protein based diets except for the C+S and PPC diets. Fish fed the C+S diet had the highest Zn content, while fish fed the PPC diet had the lowest..

The whole body crude protein content was fitted by a special-cubic model (Table 9). The content of ash, P, Ca, Mg, and Zn were fitted by linear models. The equations indicated that C-MIX had the strongest effect on ash, P, Ca and Mg content, P-MIX and S-MIX had similar effects but less than C-MIX. The C-MIX and S-MIX had similar effects on Zn content, whereas P-MIX had a lesser effect.

In fish plasma, no significant differences in the concentration of Ca and Mg were found among fish fed the different diets (Table 8). Fish fed the FM diet had significantly higher P concentration than those fed the plant protein based diets. Fish fed the CPC diet had significantly higher P concentration than those fed the other plant protein based diets, except for the P+C diet. The Zn concentration of fish fed the FM diet was significantly lower than that of fish fed the CPC and C+S diets, but did not differ from that of fish fed the other diets. Fish fed the CPC and

PPC diets had the highest and lowest Zn concentration, respectively. The plasma P and Zn concentrations were best fitted using linear models, and C-MIX had the strongest effect on both (Table 9).

3.5 Nutrient retentions

Fish fed the FM diet had significantly higher ingested and digested N retentions than those fed plant protein based diets (Table 8). The P+S and P+C diets gave the highest ingested N and digested N retentions, respectively, among all the plant protein based diets. The ingested N retention of fish fed the P+S diet was significantly higher than that of fish fed the PPC and SPC diets. The digested N retention of fish fed the P+C diet was significantly higher than that of fish fed the PPC and CPC diets. Fish fed the CPC and SPC diets had the lowest ingested and digested N retentions, respectively.

The ingested energy retention of fish fed the FM diet was significantly higher than that of fish fed the plant protein based diets, except for the P+C+S diet. Fish fed the P+C+S diet had significantly higher ingested energy retention than fish fed the SPC and CPC diets. No significant difference in digested energy retention was found among fish fed the different diets.

Fish fed the FM diet had significantly lower ingested P retention than those fed the plant protein based diets, except for those fed the SPC diet. Fish fed the SPC diet had the lowest ingested P retention among all the fish fed plant protein-based diets. Digested P retentions exceeded 100% in fish fed the PPC and P+C+S diets, but no significant differences in were found among diets.

Both the ingested and digested N retentions of fish were fitted using quadratic models (Table 9). Three components had similar effects on both N retentions, and a significant interaction was found between P-MIX and C-MIX, which had a synergetic effect on both ingested and digested N retentions. A significant interaction between P-MIX and S-MIX was found, which showed a synergetic effect on digested N retention only. The digested P retentions were fitted using a linear model, and C-MIX had the strongest effect. Based on the digested N retention model, the predicted optimal combination of the three plant protein mixtures with maximized digested N retention (53.9 %) was 55% P-MIX, 45% C-MIX and 0% S-MIX (Fig. 2).

3.6 Nitrogen, energy and phosphorus budget

The N, energy and P budgets are summarized in Table 10. For each kg production, trout fed the FM diet had lower N intake and metabolic N loss than the plant protein-based diets. The amount of N partitioned into growth from the FM diet was significantly lower than that from the SPC, P+C, and P+S diets. Fish fed the CPC diet had significantly higher N intake and faecal N loss than those fed the other diets. Fish fed the P+S diet had significantly higher N used for growth than those fed the other diets. Fish fed the FM diet had significantly lower faecal N loss than those fed the CPC, P+C, and C+S diets, but did not differ significantly from those fed the other diets. Fish fed the SPC diet had the lowest faecal N loss but the highest metabolic N loss among those fed plant protein based diets. The faecal N loss was significantly lower than that of the fish fed the PPC, CPC, P+C, and C+S diets, while the metabolic N loss was significantly higher than that of fish fed the P+C and P+C+S diets. Fish fed the P+C diet had the lowest metabolic N loss among those fed plant protein based diets.

For the energy budget, for each kg production, trout fed the FM diet had significantly lower energy intake and faecal loss than those fed plant protein based diets. Fish fed the P+C diet had significantly lower energy intake than those fed the diets based on a single plant protein mixture. Fish fed the SPC diet had significantly higher faecal energy loss than those fed other diets. No significant differences in the energy partition for growth and metabolic loss were found among fish fed different diets.

For the P budget, for each kg production, trout fed the FM diet had significantly higher P intake and fecal P excretion than those fed plant protein-based diets. The P partition for growth of fish fed the FM diet was significantly higher than that of fish fed the PPC, SPC, and P+S diets. Fish fed the SPC diet had significantly higher P intake and fecal P excretion than those fed other plant protein-based diets. Fish fed the CPC diet had the lowest fecal P excretion and the highest P partition for growth. These values were significantly lower and higher, respectively, than that of fish fed other diets, except for the fecal P excretion of fish fed the P+C diet. Fish fed the PPC diet had the lowest P intake and P partition for growth, and both were significantly lower than that of fish fed the other diets, except for the P partition for growth of fish fed the SPC, P+S, and P+C+S diets. The metabolic P loss of fish fed the PPC and P+C+S diets were less than zero.

3.7 Histology of distal intestine

Clear differences were found among fish fed the different diets both in distal intestine tissues. Fish fed the FM diet had distal intestine tissue that appeared normal (Fig. 3), with tall mucosal folds and thin lamina propria and submucosa. Low to moderate numbers of intraepithelial leukocytes (IELs) were noted. Enterocytes were highly vacuolated (supranuclear absorptive

vacuoles) with basally located nuclei. Samples from the fish fed the SPC diet were similar to those fed the FM diet. Fish fed the PPC diet exhibited inflammatory changes of moderate severity (Fig. 3 and 4). Mucosal folds were shorter than normal; lamina propria and submucosa were widened with leukocyte infiltration. Enterocyte supranuclear absorptive vacuolization was reduced to absent, and large abnormal (i.e. pathological) vacuoles were occasionally seen in the epithelium. Increased numbers of mitotic figures were observed in the basal areas of mucosal folds. Other groups also exhibited changes qualitatively similar to those described above, but of lesser severity. Table 11 summarizes the numbers of individuals in each dietary treatment displaying changes based on severity of the changes observed. On average, the severity of the changes can be classified as follows: Fish fed the PPC diet had moderate changes. Fish fed the P+C diet had mild to moderate changes. Fish fed the CPC, P+S, and P+C+S diets had mild changes. Fish fed the C+S, SPC, and FM diets were normal.

4. Discussion

The present experiment has demonstrated that, it is possible to fully replace LT-fish meal with PPC, potato PC, CPC, and SPC individually or in combinations in extruded diets for rainbow trout without adverse effects on growth and body composition. Such replacement, however, requires multiple supplementations of EAA and Tau, and using small amounts of highly palatable ingredients such as krill meal and water soluble fractions of krill as feeding attractants.

The physical quality of feed was affected by ingredient properties and extrusion parameters. The PPC alone or combined with CPC (P+C diet) showed the best physical quality in terms of durability and breaking force compared to the other diets. This was consistent with previous

findings (Øverland et al., 2009; Zhang et al., submitted), and is rationalized by the presence of starch in PPC and a high amylose to amylopectin ratio in the pea starch. Furthermore, the pea protein is not denatured during the process of air classification, which may also contribute to better binding (Øverland et al., 2009). The FM diet had the lowest durability and breaking force but the highest expansion and water stability. This could be associated with the high dietary wheat inclusion (Sørensen et al., 2011).

The rainbow trout reared in cold (9.1 ºC) freshwater obtained an average weight gain of more than 251 % after a 72-d feeding period. Such rapid growth is comparable to the growth observed in our previous experiment (Zhang et al., submitted) with smaller fish size (58 g) and lower water temperatures (8.2 ºC). The rapid growth rate and absence of significant differences among the diets can be attributed to both high feed intake and efficient feed conversion.

The FI of fish was not impaired even when the dietary inclusion of plant protein mixtures reached as high as 600 g kg^{-1}. The FI of fish fed most plant-based diets was even higher than that of fish fed the FM diet. Such a promising finding was consistent with the previous studies in which inclusion of plant protein at 407 g kg^{-1} of PPC (Zhang et al., submitted), 490 g kg^{-1} of CPC (Thiessen et al., 2004), or 620 g kg^{-1} of SPC (Kaushik et al., 1995) in rainbow trout diets, or 210 g kg^{-1} of potato PC with low SGA in Atlantic salmon diets (Refstie and Tiekstra, 2003) did not negatively affect the feed intake and growth of fish. In our experiment, the maximum dietary inclusion levels were 295 g kg^{-1} PPC, 295 g kg^{-1} potato PC, 294 g kg^{-1} CPC, and 560 g kg^{-1} SPC, respectively. Thus, inclusions of individual plant protein concentrates were lower than the maximum levels reported in the literature, with the exception of potato PC. The use of krill meal and krill hydrolysate has been reported to significantly increase the feed intake of salmonids (Oikawa and March, 1997; Olsen et al., 2006). The water soluble fraction of krill is generated

during krill meal production. It contains various water soluble molecules and particles, and these compounds have been shown to have a positive feed intake effect on fish (Kousoulaki et al., 2009). The supplementation of krill meal and the water soluble fraction of krill in the plant protein based diets in the present experiment have improved feed palatability, and consequently increased feed intake. In addition, the dietary digestible energy density has a considerable impact on feed intake (Kaushik, 1998). All the plant protein based diets had lower digestible energy content than the FM diet in the present experiment. The plant protein based diet fed fish may have increased their feed intake to obtain equal digestible energy intake as the FM diet fed fish to maintain comparable growth. This was further confirmed by the energy budget. For each kg weight gain of fish, the energy partition for growth and metabolic loss was comparable among fish fed the different diets. The energy intake and fecal loss of the fish fed plant protein based diets was higher than that of the fish fed the FM diet. The lower FI of fish fed the P+S diet than that of the fish fed the CPC and SPC diets could also be explained by the lower faecal energy loss in these fish.

The N digestibility in the SPC diet fed fish was higher than that previously reported in studies with Atlantic salmon (Storebakken et al., 1998; 2000; Denstadli et al., 2007), but comparable to that reported in rainbow trout (Mambrini et al., 1999). Heat liable protease inhibitors and heat stable indigestible carbohydrates, saponins and antigens were removed during the manufacturing of SPC (Lusas and Riaz, 1995). Thus, SPC contains a higher level of good quality protein compared to raw soy products, which is highly digestible to salmonids (Aksnes and Opstvedt, 1998; Refstie et al., 2001). The highest digestibilities of EAA, Cys, and TAA in the SPC diet fed fish and the highest coefficiency of S-MIX in the linear models of these digestibilities further supports this. Both the PPC and dephytinized CPC are promising dietary protein sources for

salmonids with high digestibility. The N digestibility in fish fed the PPC diet was comparable to that previously reported in salmonids (Øverland et al., 2009; Penn et al., 2011; Zhang et al., submitted). The N digestibility of fish fed the CPC diet was, however, lower than Thiessen et al (2004) reported in rainbow trout. This may be due to the lower N and higher fiber content of the CPC used in our experiment, where the high fiber content may have decreased the nutrient and energy digestibility (Mwachireya et al., 1999). Both the PPC and the CPC diets contained the same amount of low-SGA potato PC, which also has been reported to be highly digestible to salmonids (Refstie and Tiekstra, 2003). However, the coefficient for ADC of N, EAA, Cys, and TAA of C-MIX was lower than P-MIX in the models. This indicates that the proteins in the P-MIX were more digestible than in the C-MIX. The lower N and EAA digestibilities in the CPC diet fed fish than the PPC diet fed fish also confirmed this. This may be mainly due to the different processing methods applied in the manufacturing of PPC and CPC. No thermal treatment was involved in PPC production, but high temperature was applied in the processing of the canola meal to obtain the CPC. The heat treatment may decrease the protein and amino acid digestibilities. This may be partly due to the formation of disulphide bonds between protein-chains during the heat treatment, making them resistant to proteolytic hydrolysis (Opstvedt et al., 1984; Aslaksen et al., 2006). The very low Cys digestibility in the CPC diet fed fish support this. The lower N digestibility of the FM diet than the SPC diet indicate the presence of non-protein nitrogen compounds such as biogenic amines in the fish meal (Opstvedt et al., 2000). This is suggested by the lower TAA content in the FM diet than in the plant protein based diets. The lower ADC of Arg in the FM diet fed fish than the SPC diet fed fish may be due to the heat treatment during FM manufacturing (Aksnes and Mundheim, 1997). The lower ADC of Met in

the FM diet fed fish than in the SPC diet fed fish could be partially due to the DL-Met supplementation in the SPC diet.

The higher N retention of fish fed the FM diet than of fish fed the other diets was likely due to the well balanced dietary available EAA in the LT- fish meal (Zhang et al., submitted) and the lowest ratio of digestible protein to digestible energy (DP/DE). Both the EAA balance and the DP/DE ratio strongly influence protein utilization (Green and Hardy, 2008). The higher ingested N retention of fish fed the P+S diet than those fed the PPC and SPC diets is explained by its higher N digestibility than the PPC diet and lower DP/DE than the SPC diet. Because of the identical DP/DE among the PPC, CPC, and P+C diets, the higher digested N retention of fish fed the P+C diet could be mainly attributed to a more balanced digestible EAA profile, maximizing protein synthesis. This was confirmed by the higher N partitioning for growth and lower metabolic N loss of fish fed the P+C diet than the other two diets. The synergetic effect of P-MIX and S-MIX on the digested N retention could also be explained by the more balanced digestible EAA profile when a greater variety of proteins with different origins and nutritional properties were included in the diet. It can be concluded that increasing the diversity of plant protein sources in the diet or using a combination instead of single protein ingredient may balance the dietary EAA, leading to higher protein utilization. The low ingested N retention in fish fed the CPC diet and the low digested N retention in fish fed the SPC diet were mainly due to the low N digestibility and high dietary DP/DE, respectively.

The decreased lipid digestibility in fish fed the SPC diet was consistent with previous reports on salmonids (Mambrini et al., 1999; Penn et al., 2011). This decrease in lipid digestibility was not likely caused by canola oil inclusion because the maximum proportion of canola oil in the total dietary lipid was only 9%. Canola oil is a promising alternative lipid source for salmonids, and

can comprise 65% and 47% of the total dietary lipid for rainbow trout and Atlantic salmon, respectively, without compromising growth and feed utilization (Dosanjh et al., 1998; Drew et al., 2007). As a conjugator of bile acids, Tau plays an important role in lipid digestion. In the present experiment, the Tau level in the SPC diet was 7.15 g kg^{-1}, which was higher than that in the FM diet (4.81 g kg^{-1}) because of the extra Tau supplementation. Therefore, the decreased lipid digestibility could not be ascribed to the dietary Tau level. The decreased lipid digestibility, however, may have been caused by low content of phospholipids (PL) in the SPC diet. PL are important to emulsify lipids during digestion (Tocher et al., 2008) and lipid digestibility is increased by adding PL to a diet with defatted soybean meal when fed to rainbow trout (Hung et al., 1997). The variation found in energy digestibility was mainly attributed to the lipid digestion. In the present experiment the models of digestibilities of lipid and energy indicated that P-MIX and C-MIX were comparably digestible on an energy basis.

The high energy digestibility together with the low energy intake of fish fed the FM diet, which was caused by the low dietary energy level and low feed intake, resulted in higher ingested energy retention. The lowest ingested energy retention, in fish fed the SPC diet, was due to the lowest energy digestibility which was confirmed by the high faecal energy loss. No significant differences were found in digested energy retention and metabolic energy loss, indicating that the metabolism of digested energy was not affected by the dietary treatment.

The higher P digestibility in fish fed the CPC and P+C diets compared to those fed the other diets, together with the higher coefficients of C-MIX than P-MIX and S-MIX in the phosphorus digestibility model, indicate that CPC had the highest P availability. The higher whole body P, Ca, and Mg content and higher plasma P and Zn concentrations of fish fed the CPC containing diets may indicate a higher bone mineralization than those fed the PPC, SPC, and P+S diets. This

was mainly due to the high digestible P and low phytate content in the dephytinized CPC. Phytic P in the canola meal is almost completely converted into highly digestible inorganic P during the manufacturing of CPC (Thiessen et al., 2004). The chelating effect of phytate on di- and trivalent mineral ions (Duffus and Duffus, 1991) which may further induce an impaired mineral utilization in fish was also diminished with the reduced phytate content in the diet. The higher coefficiency of C-MIX than P-MIX and S-MIX in the models for whole body ash, P, Ca and Mg content also support this. The lowest ADC of P in fish fed the FM diet was mainly due to the high P level in this diet compared to the other plant protein based diets which resulted in the lowest ingested P retention. The lower P digestibility and low ingested P retention in fish fed the SPC diet compared to those fed the other plant protein based diets together with the lowest coefficiency of S-MIX were mainly due to the high phytic P content in the SPC diet. Although SPC has a lower phytate (IP6) content than PPC (21.2 vs. 23.1 g kg^{-1}, on a dry matter basis), the ADC of P of the SPC diet fed fish was still lower than the PPC fed fish. This may be attributed to the lower P content in the PPC diet (Table 3) or the lower IP6 content in potato PC. The phytate content in potato PC was unknown in our experiment, therefore, the estimated the IP6 content in potato PC may be lower than that of SPC based on the report of Phillippy et al. (2004). This may result in a lower IP6 content in P-MIX than in S-MIX. The higher coefficiency of P-MIX than S-MIX in the model also supports this. The nutritional effects of dephytinization of SPC is addressed in a separate paper (Zhang et al. manuscript 4)

The over 100% digested P retentions and negative metabolic P loss of fish fed the PPC and P+C+S diets could be associated with an inadequate available dietary P level (0.16 and 0.20 g MJ^{-1} DE, respectively), which was lower than the recommended level (0.25 g MJ^{-1} DE) for trout (Rodehutscord, 1996). Trout may absorb a small amount of P from the re-circulated water

through the gills and intestinal tract (Winpenny et al., 1998). Thus, in the P-MIX, or combination of P-MIX, C-MIX, and S-MIX based fish meal-free diets with high dietary energy density for rainbow trout grown from 60 g to 220 g, a supplementation of MCP of more than 10 g kg^{-1} should be used.

Fish fed diets containing high levels of pea protein meal exhibited clear signs of inflammation in the distal intestine. These findings are consistent with previous reports of air classified pea protein concentrate causing distal intestine inflammation in Atlantic salmon when included in the feed at high levels (Penn et al., 2011), or when included in diets with soyasaponins supplementation (Chikwati et al., 2011). Interestingly, mild inflammation was also observed in fish fed diets containing canola protein and combinations of canola and pea protein. Canola protein has not previously been reported to cause inflammation when fed to salmonids. However, these diets also contained potato protein. Only a few reports exist regarding the use of potato protein in diets for fishes. Refstie and Tiekstra (2003) reports favorable use of potato protein with low glycoalkaloid content in diets for Atlantic salmon regarding feeding and growth, but tissue histology was not reported. Therefore we cannot exclude the possibility that potato protein may have affected intestinal tissue histology alone, or in combination with other ingredients in the current study.

Conclusions

Rainbow trout could utilize diets with 95% of protein from plant protein concentrates with multiple EAA supplementations and using krill meal and the water soluble fraction of krill as feed attractant, without depressing feed intake or growth. Using combinations of plant protein

concentrates can dilute the adverse effect of ANF from a specific ingredient. The mixture model is a useful method to optimize the plant protein concentrates in feed for rainbow trout.

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Table 1. Composition of fish meal and the four plant protein concentrates used in the experimental diets

Ingredient	Fish meal (LT-94)[1]	Pea protein concentrate (PPC)[2]	Potato protein concentrate (potato PC)[3]	Canola protein concentrate (CPC)[4]	Soy protein concentrate (SPC)[5]
Composition, g kg^{-1}					
Dry matter (DM), g	911	890	912	950	944
In DM					
Crude protein, g	749	550	844	595	702
Crude fat, g	99	46	9	72	1
Starch, g	-	52	4	-	8
Ash, g	157	66	6	87	74
Essential amino acids (EAA)[6], g $16g^{-1}N$					
Arg	5.27	7.74	4.45	5.11	6.41
His	1.87	2.43	2.13	2.42	2.50
Ile	3.69	3.82	4.92	3.62	4.14
Leu	6.26	6.29	8.75	6.18	6.64
Lys	6.92	6.63	6.97	4.38	5.53
Met	2.42	0.85	1.89	1.70	1.18
Phe	3.37	4.39	5.66	3.57	4.45
Thr	3.65	3.38	5.22	3.67	3.52
Trp	0.73	0.84	1.13	1.16	1.14
Val	4.03	4.05	5.61	4.29	4.06
Total EAA	38.22	40.42	46.73	36.08	39.56
Non-essential amino acids (NEAA)[6], g $16g^{-1}N$					
Ala	4.89	3.43	3.86	3.46	3.42
Asp	8.09	9.77	10.59	6.27	9.93
Cys	0.81	1.11	1.28	1.19	1.17
Glu	12.65	15.25	9.97	14.31	16.80
Gly	4.57	3.26	3.80	3.67	3.22
Pro	3.14	3.41	4.06	4.33	4.14
Ser	3.60	4.27	4.73	3.53	4.46
Tyr	2.47	3.12	4.64	2.52	3.04
Total NEAA	40.22	43.63	42.94	39.27	46.18
Total AA	78.39	83.33	88.59	74.40	84.66

[1] Norse LT-94®, low-temperature dried fish meal, Norsildmel, Bergen, Norway.

[2] PPC 55 PELLET, Pea protein concentrate, AgriMarin AS, Stavanger, Norway. Analyzed IP6 content: 23.1 g kg^{-1}.

[3] PROTASTAR®, Potato protein concentrate, AVEBE FEED, Veendam, Holland.

[4] CanPro-60, Canola protein concentrate, Can Pro Ingredients Limited, SK, Canada.

[5] Soycomil® R, ADM Specialty Ingredients Europe, Koog aan de Zaan, Holland. Analyzed IP6 content: 21.2 g kg^{-1}.

[6] Presented in dehydrated form.

Table 2. Composition of the plant protein premixtures used in the experiment

Mixture	P-MIX	C-MIX	S-MIX
Ingredients, g kg^{-1}			
Pea protein concentrate	491	-	-
Potato protein concentrate	491	490	-
Canola protein concentrate	-	490	-
Soy protein concentrate	-	-	932
Canola oil[1]	-	-	38
DL-Met[2]	13	10	14
L-Lys[3]	6	-	16
L-Arg[4]	-	11	-
Estimated Digestible Protein (DP), g kg^{-1}	599	606	605
Estimated Digestible Energy (DE), MJ kg^{-1}	15.8	17.2	15.9

[1] Using 39.5 MJ kg^{-1} as the energy value.

[2] Rhodimet® NP 99, *DL*-methionine, 99% feed grade, Adisseo Brasil Nutricao Animal Ltda, Sao Paulo, Brazil.

[3] *L*-Lysine-HCl, 99% feed grade, CJ Indonesia, Jakarta, Indonesia.

[4] *L*-Arginine, 98.5%, Sigma-Aldrich Logistik GmbH, Steinheim, Germany.

Table 3. Mixture composition in the plant protein based diets formulated with P-MIX, C-MIX, and S-MIX in a three-component, Simplex-Centroid design

Diet		Original components, g kg^{-1}			Pseudo-components[1]		
		P-MIX	C-MIX	S-MIX	P-MIX	C-MIX	S-MIX
1	PPC	600	0	0	1	0	0
2	CPC	0	600	0	0	1	0
3	SPC	0	0	600	0	0	1
4	P+C	300	300	0	0.5	0.5	0
5	P+S	300	0	300	0.5	0	0.5
6	C+S	0	300	300	0	0.5	0.5
7	P+C+S	200	200	200	0.33	0.33	0.33

[1] The proportion of each plant protein premixture in total plant protein combination.

Table 4. Feed formulation and analyzed chemical composition (based on dry matter)

Diet	PPC	CPC	SPC	P+C	P+S	C+S	P+C+S	FM
Ingredients, g kg^{-1}								
Fish meal	-	-	-	-	-	-	-	520
Deshelled Krill meal[1]	35	35	35	35	35	35	35	-
Krill water solubles[2]	15	15	15	15	15	15	15	-
P-MIX	600	-	-	300	300	-	200	-
C-MIX	-	600	-	300	-	300	200	-
S-MIX	-	-	600	-	300	300	200	-
Fish oil[3]	235	215	220	225	227	218	223	180
Wheat	96	116	111	106	104	113	108	280
Premix[4]	4	4	4	4	4	4	4	4
MCP[5]	10	10	10	10	10	10	10	-
Y_2O_3[6]	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Taurine[7]	5	5	5	5	5	5	5	-
Estimated DP, g kg^{-1}	398	404	402	401	400	403	402	406
Estimated DE, MJ kg^{-1}	20.7	21.0	20.6	20.9	20.7	20.8	20.8	21.2
Analyzed content, kg^{-1}								
Dry matter (DM), g	938	966	943	950	945	947	950	971
In DM								
Crude protein, g	466	484	450	483	460	479	467	430
Lipid, g	217	226	252	234	231	239	234	243
Starch, g	108	91	90	94	90	90	90	191
Ash, g	34	42	57	40	47	50	45	86
Gross energy, MJ	25.2	25.4	24.1	25.0	24.4	24.9	25.0	23.9
EAA[8], g $(16gN)^{-1}$								
Arg	5.68	6.16	6.07	6.03	5.97	6.40	5.95	5.31
His	2.25	2.33	2.41	2.29	2.33	2.42	2.32	2.08
Ile	3.90	4.02	3.57	3.96	3.68	3.82	3.77	3.37
Leu	7.57	7.81	6.29	7.69	6.90	7.15	7.12	6.32
Lys	7.22	6.02	6.95	6.62	7.09	6.60	6.60	6.55
Met	2.84	2.90	2.64	2.91	2.75	2.87	2.78	2.40
Phe	4.96	4.80	4.15	4.83	4.49	4.55	4.54	3.52
Thr	4.53	4.83	3.50	4.64	3.99	4.24	4.20	3.80
Val	4.76	5.04	3.80	4.88	4.19	4.48	4.45	4.11
Cys, g $(16gN)^{-1}$	1.18	1.28	1.11	1.25	1.14	1.21	1.21	0.93
TAA[8], g $(16gN)^{-1}$	87.6	88.0	83.6	88.0	85.2	87.2	85.4	80.3
Tau, g kg^{-1}	6.51	7.19	7.15	7.41	7.06	7.38	7.16	4.81
Minerals, g kg^{-1}								
Phosphorous, P	6.12	7.30	8.14	7.15	7.37	7.90	7.32	13.02
Calcium, Ca	3.56	5.58	5.33	4.94	4.67	5.69	4.73	17.47
Magnesium, Mg	1.31	2.55	2.84	1.99	2.13	2.69	2.24	1.82
Zinc, Zn	0.12	0.13	0.12	0.13	0.12	0.13	0.12	0.13

[1] Krill meat pellet, Aker Biomarine, Oslo, Norway.

[2] Krill flavor concentrate, Aker Biomarine, Oslo, Norway.

[3] Silfas, Karmsund, Norway.

[4] Per kg diet: vitamin A: 2000 IU, vitamin D_3: 1200 IU; vitamin E: 160 mg; vitamin K_3: 8 mg; vitamin B_1: 12 mg; vitamin B_2: 20 mg; vitamin B_3: 60 mg; vitamin B_5:24 mg; vitamin B_6: 12 mg; vitamin B_9: 4 mg; vitamin B_{12}: 0.016 mg; vitamin C: 100 mg; Biotin: 0.2 mg; Ca: 876 mg; Cu: 4 mg; Co: 0.8 mg; I: 2.4 mg; Mn:12 mg; Zn: 96 mg.

[5] MCP22, mono-calcium phosphate, feed grade, Suntran Industrial Group Ltd., Anhui, China.

[6] Metal Rare Earth Limited, Shenzhen, China.

[7] Taurine-JP8, Qianjiang Yongan Pharmaceutical Co., Ltd., Hubei, China.

[8] Presented in dehydrated form.

1 Table 5. Extrusion processing parameters and feed pellet physical quality

Diet	PPC	CPC	SPC	P+C	P+S	C+S	P+C+S	FM
Extruder parameters								
Feeding rate, kg h^{-1}	150	130	100	150	130	130	130	125
Water addition in extruder, kg h^{-1}	10.5	6.4	17.0	6.4	6.4	11.9	6.4	0
Water addition in conditioner, kg h^{-1}	21.2	32.5	28.0	30.0	35.1	34.8	35.1	17.5
Steam addition in conditioner, kg h^{-1}	8.5	7.7	5.0	9.0	6.5	6.5	13.3	6.3
Krill stick water addition in extruder, kg h^{-1}	7.1	6.1	4.9	7.1	6.2	6.2	6.2	0
Pressure in front of die, bar	50.8	49.4	38.7	47.7	50.8	51.5	52.3	40.0
SME[1], Wh kg^{-1}	82.5	110.9	83.6	77.8	76.1	78.8	83.4	69.6
Revolution screws, rpm	350	454	318	349	307	326	343	274
Torque, Nm	94.0	90.6	83.7	91.3	93.3	93.9	92.1	79.5
Die temperature, °C	117	124	110	115	117	121	113	121
Cutter speed, rpm	2606	2963	2510	2956	2866	2865	2856	2208
Physical quality								
Length, mm	5.9	4.9	5.3	6.1	5.8	5.9	5.8	4.9
Diameter, mm	3.2	3.1	3.4	3.0	3.2	3.2	3.1	3.6
Expansion, %	60	55	70	50	60	60	55	80
Durability, %	90.5	78.5	79.1	91.8	87.0	88.6	78.5	58.5
Breaking point, N	33.8	20.3	30.9	35.3	28.5	27.7	25.9	18.1
Water stability, %	85.6	84.2	62.6	77.9	83.2	74.1	76.0	86.5

2 [1] Specific mechanical energy.

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Table 6. Growth performance, feed utilization, and nutrient digestibilities of rainbow trout fed the experimental diets

Diet	PPC	CPC	SPC	P+C	P+S	C+S	P+C+S	FM	Pooled S.E.M.[1]	*ANOVA* P < F
Feed intake, g DM fish^{-1}										
	123^{ab}	130^{a}	131^{a}	127^{ab}	118^{bc}	124^{ab}	128^{ab}	112^{c}	2.7	0.016
Initial weight, g	61.1	60.8	61.0	61.5	60.9	60.8	61.4	60.5	0.47	0.79
Weight gain, % of initial weight										
	244	248	249	264	236	249	260	257	6.6	0.20
Feed conversion ratio (FCR), g DM ingested (g gain)$^{-1}$										
	0.82^{abc}	0.86^{ab}	0.87^{a}	0.78^{c}	0.82^{abc}	0.82^{bc}	0.80^{c}	0.72^{d}	0.014	0.001
Apparent digestibility coefficients (ADC), %										
Nitrogen	86.5^{c}	82.1^{d}	91.4^{a}	84.6^{cd}	91.1^{ab}	85.7^{c}	87.6^{c}	87.9^{bc}	1.01	0.002
Lipid	93.6^{a}	90.9^{a}	80.6^{b}	93.3^{a}	95.2^{a}	91.6^{a}	94.7^{a}	96.3^{a}	2.19	0.018
Starch	94.3	94.5	92.2	95.3	94.9	93.9	95.1	94.1	0.89	0.38
Energy	82.7^{bc}	80.5^{bc}	76.0^{d}	82.6^{bc}	83.9^{b}	79.1^{cd}	83.4^{b}	88.3^{a}	1.12	0.002
Phosphorus	55.2^{d}	73.4^{a}	46.8^{f}	68.3^{b}	51.4^{e}	58.8^{c}	57.0^{cd}	40.1^{g}	1.06	< 0.001
EAA										
Arg	92.5^{cde}	91.0^{e}	97.0^{a}	92.2^{de}	95.6^{ab}	93.7^{cd}	94.1^{bc}	93.9^{bcd}	0.52	0.001
His	86.0^{de}	82.2^{f}	92.8^{a}	84.3^{ef}	90.7^{ab}	86.9^{cde}	88.0^{bcd}	89.8^{abc}	0.99	0.001
Ile	84.8^{cd}	79.1^{e}	93.9^{a}	82.8^{de}	90.5^{ab}	85.3^{cd}	87.3^{bc}	91.6^{a}	1.20	< 0.001
Leu	89.3^{c}	84.9^{d}	94.8^{a}	88.1^{c}	92.8^{ab}	88.9^{c}	90.6^{bc}	93.6^{ab}	0.89	0.001
Lys	89.8^{cd}	84.7^{e}	95.2^{a}	88.3^{d}	93.8^{ab}	89.9^{cd}	91.4^{bc}	93.9^{ab}	0.87	< 0.001
Met	92.5^{cd}	90.6^{e}	96.7^{a}	91.7^{de}	95.1^{b}	93.4^{c}	93.9^{bc}	93.1^{cd}	0.45	< 0.001
Phe	89.6^{cd}	85.1^{e}	95.0^{a}	88.3^{d}	92.9^{ab}	89.3^{cd}	90.9^{bcd}	91.9^{abc}	0.94	0.002
Thr	85.3^{cd}	82.2^{d}	90.6^{ab}	84.2^{cd}	89.7^{ab}	85.8^{cd}	87.3^{bc}	91.6^{a}	1.05	0.002
Val	84.9^{c}	79.9^{d}	92.9^{a}	82.8^{cd}	89.7^{ab}	84.7^{c}	86.8^{bc}	91.5^{a}	1.16	0.001
Cys	74.0^{cd}	64.4^{e}	84.1^{a}	69.2^{de}	80.9^{ab}	73.4^{cd}	75.5^{bcd}	78.7^{abc}	1.83	0.001
TAA	88.2^{c}	84.0^{d}	93.3^{a}	86.7^{cd}	91.9^{ab}	88.2^{c}	89.6^{bc}	91.8^{ab}	0.95	0.002

[1] Pooled standard error of means. Different superscript letters $^{a, b, c, d, e, f}$ indicate significant ($P < 0.05$) differences among treatments.

Table 7. Models for feed conversion and nutrient digestibilities in rainbow trout fed the experimental diets

Response	Type of model	Equation of model[1]	P_{model}	R^2	Goal	Max (Min) value	Optimal composition (%)			Desirability
							P-MIX	C-MIX	S-MIX	
FCR	Quadratic	FCR = 0.8 P + 0.86 C + 0.86 S - 0.21 P*C - 0.18 C*S	0.002	0.82	Min	0.78	64	36	0	0.94
ADC (%)										
Nitrogen	Linear	ADC_N = 87.5 P + 81.8 C + 91.8 S	< 0.001	0.83	Max	91.8	0	0	100	1.0
Lipid	Quadratic	ADC_L = 93.9 P + 91.2 C + 80.6 S + 31.7 P*C + 22.8 C*S	0.004	0.80	Max	96.5	76.4	0	23.6	1.0
Energy	Quadratic	ADC_E = 83 P + 81.3 C + 76.3 S + 18.8 P*S	0.001	0.79	Max	85.0	72.1	0	27.9	1.0
Phosphorus	Special-cubic	ADC_P = 55.2 P + 73.4 C + 46.8 S + 15.8 P*C + 1.5 P*S - 5.1 C*S - 77.3 P*C*S	< 0.001	0.99	Max	73.4	0	100	0	0.92
EAA										
Arg	Linear	ADC_{Arg} = 93 P + 91 C + 97.2 S	< 0.001	0.89	Max	97.2	0	0	100	0.98
His	Linear	ADC_{His} = 86.7 P + 82 C + 93.1 S	< 0.001	0.89	Max	93.1	0	0	100	0.98
Ile	Linear	ADC_{Ile} = 85.7 P + 79.1 C + 94 S	< 0.001	0.90	Max	94.0	0	0	100	0.97
Leu	Linear	ADC_{Leu} = 90 P + 85 C + 94.8 S	< 0.001	0.87	Max	94.8	0	0	100	0.95
Lys	Linear	ADC_{Lys} = 90.7 P + 85.1 C + 95.6 S	< 0.001	0.89	Max	95.6	0	0	100	1.0
Met	Linear	ADC_{Met} = 92.8 P + 90.6 C + 96.8 S	< 0.001	0.92	Max	96.8	0	0	100	0.95
Phe	Linear	ADC_{Phe} = 90.3 P + 85.3 C + 95 S	< 0.001	0.87	Max	95.0	0	0	100	0.97
Thr	Linear	ADC_{Thr} = 86.2 P + 82.1 C + 91 S	< 0.001	0.80	Max	91.0	0	0	100	0.97
Val	Linear	ADC_{Val} = 85.6 P + 79.6 C + 92.7 S	< 0.001	0.89	Max	92.7	0	0	100	0.95
Cys	Linear	ADC_{Cys} = 74.8 P + 64.1 C + 84.5 S	< 0.001	0.89	Max	84.5	0	0	100	0.97
TAA	Linear	ADC_{TAA} = 88.9 P + 84 C + 93.6 S	< 0.001	0.86	Max	93.6	0	0	100	0.97

[1] P, C and S represent the pseudo components of P-MIX, C-MIX and S-MIX (the percentage of each plant protein premixture in total plant protein combination), respectively.

Table 8. Whole body composition, plasma mineral concentrations, and nutrient retentions of rainbow trout fed the experimental diets

Diet	PPC	CPC	SPC	P+C	P+S	C+S	P+C+S	FM	Pooled S.E.M.[1]	P < F
In whole body, kg^{-1}										
Dry matter, g	320	315	309	311	315	311	324	311	4.6	0.35
Crude protein, g	164^{d}	166^{cd}	169^{ab}	168^{bc}	172^{a}	164^{d}	165^{d}	164^{d}	0.8	0.001
Lipid, g	136	123	120	123	124	124	137	121	4.4	0.14
Ash, g	19	22	19	21	19	21	20	22	0.8	0.066
Energy, MJ	8.82	8.64	8.33	8.40	8.60	8.43	8.85	8.33	0.139	0.14
P, g	3.44^{c}	4.17^{a}	3.46^{c}	3.85^{abc}	3.45^{c}	3.90^{ab}	3.71^{bc}	4.01^{ab}	0.118	0.014
Ca, g	2.83^{c}	4.25^{a}	2.86^{c}	3.51^{abc}	2.82^{c}	3.69^{abc}	3.33^{bc}	3.78^{ab}	0.259	0.033
Mg, mg	252^{e}	282^{a}	262^{cde}	271^{abc}	253^{de}	275^{ab}	265^{bcd}	265^{bcd}	3.5	0.003
Zn, mg	15.5^{c}	21.2^{b}	21.7^{b}	18.9^{bc}	19.2^{bc}	27.2^{a}	22.3^{b}	19.5^{b}	1.16	0.004
In plasma, kg^{-1}										
P, mg	28.7^{c}	37.9^{b}	23.6^{c}	31.3^{bc}	23.3^{c}	23.3^{c}	27.7^{c}	51.5^{a}	2.27	< 0.001
Zn, mg	10.8^{e}	18.5^{a}	15.8^{abc}	15.1^{bcd}	12.3^{de}	16.3^{ab}	14.6^{bcd}	13.1^{cde}	0.84	0.004
N retention, %										
Ingested	43.5^{cd}	40.6^{e}	44.5^{cd}	45.7^{bc}	47.0^{b}	42.7^{d}	44.9^{bc}	53.7^{a}	0.62	< 0.001
Digested	50.3^{c}	49.4^{c}	48.7^{c}	54.0^{b}	51.6^{bc}	49.9^{c}	51.3^{bc}	61.1^{a}	0.99	< 0.001
Energy retention, %										
Ingested	46.0^{bc}	42.4^{c}	42.3^{c}	45.6^{bc}	46.0^{bc}	44.1^{bc}	47.7^{ab}	51.0^{a}	1.23	0.015
Digested	55.6	52.6	55.6	55.2	54.8	55.8	57.2	57.8	1.57	0.49
P retention. %										
Ingested	59.2^{a}	63.6^{a}	42.8^{cd}	64.3^{a}	49.2^{bc}	56.3^{ab}	57.7^{ab}	38.3^{d}	2.66	0.001
Digested	107.2	86.9	91.5	94.1	95.6	95.7	101.3	95.5	4.92	0.28

[1] Pooled standard error of means. Different superscript letters $^{a, b, c, d, e}$ indicate significant ($P < 0.05$) differences among treatments.

Table 9. Models for whole body composition, plasma mineral concentrations, and nutrient retentions of rainbow trout fed the experimental diets

Response	Type of model	Equation of model[1]	P_{model}	R^2	Goal	Max value	Optimal composition (%) P-MIX	C-MIX	S-MIX	Desirability
In whole body, kg^{-1}										
Crude protein, g	Special-cubic	WB_{CP} = 164 P + 166 C + 169 S + 12 P*C + 20 P*S – 13 C*S – 100 P*C*S	0.001	0.93	Max	172	38.1	0.0	61.9	0.91
Ash, g	Linear	WB_{Ash} = 18.8 P + 22.5 C + 18.9 S	< 0.001	0.76	Max	22.5	0.0	100.0	0.0	0.99
P, g	Linear	WB_{P} = 3.45 P + 4.21 C + 3.48 S	< 0.001	0.83	Max	4.21	0.0	100.0	0.0	0.99
Ca, g	Linear	WB_{Ca} = 2.8 P + 4.28 C + 2.9 S	< 0.001	0.79	Max	4.28	0.0	100.0	0.0	0.92
Mg, mg	Linear	WB_{Mg} = 252 P + 285 C + 261 S	< 0.001	0.85	Max	285	0.0	100.0	0.0	0.98
Zn, mg	Linear	WB_{Zn} = 15.5 P + 21.2 C + 21.8 S	0.001	0.88	Max	27.1	0.0	48.8	51.2	0.92
In plasma, kg^{-1}										
P, mg	Linear	PLA_{P} = 27.9 P + 35.3 C + 20.6 S	0.004	0.63	Max	25.0	0.0	79.8	20.2	0.59
Zn, mg	Linear	PLA_{Zn} = 10.7 P + 18.5 C + 15.2 S	< 0.001	0.86	Max	26.8	0.0	61.3	38.7	0.85
Retentions (%)										
Ingested N	Quadratic	Ret_{Ni} = 43.6 P + 40.6 C + 44.5 S + 13.2 P*C + 10.5 P*S	< 0.001	0,88	Max	46.7	45.6	0	54.4	0.88
Digested N	Quadratic	Ret_{Nd} = 51 P + 49.6 C + 49.5 S + 14.3 P*C	0.027	0.59	Max	53.9	55.0	45.0	0	0.85
Ingested P	Linear	Ret_{Pi} = 59.5 P + 65.8 C + 43.2 S	< 0.001	0.82	Max	65.8	0	100	0	0.98

[1] P, C and S represent the pseudo components of P-MIX, C-MIX and S-MIX, respectively.

Table 10. Nitrogen, energy and phosphorus budget of rainbow trout fed the experimental diets

Diet	PPC	CPC	SPC	P+C	P+S	C+S	P+C+S	FM	Pooled S.E.M.[1]	P < F
Nitrogen, g kg^{-1} gain										
Intake	61.5^{b}	66.8^{a}	62.4^{b}	60.4^{b}	60.5^{b}	62.6^{b}	59.7^{b}	49.8^{c}	1.03	< 0.001
Faecal loss	8.3^{bc}	12.0^{a}	5.4^{d}	9.3^{ab}	5.4^{d}	8.9^{ab}	7.4^{bcd}	6.1^{cd}	0.73	0.002
Growth	26.8^{d}	27.1^{cd}	27.8^{b}	27.5^{bc}	28.4^{a}	26.7^{d}	26.8^{d}	26.7^{d}	0.18	0.001
Metabolic loss	26.4^{abc}	27.7^{ab}	29.3^{a}	23.5^{c}	26.7^{abc}	27.0^{abc}	25.5^{bc}	17.0^{d}	1.02	< 0.001
Energy, MJ kg^{-1} gain										
Intake	20.8^{ab}	21.9^{a}	20.9^{ab}	19.5^{c}	20.1^{bc}	20.3^{bc}	20.0^{bc}	17.3^{d}	0.35	< 0.001
Faecal loss	3.6^{bcd}	4.3^{ab}	5.0^{a}	3.4^{bcd}	3.2^{d}	4.2^{abc}	3.3^{cd}	2.0^{e}	0.27	0.001
Growth	9.5	9.3	8.8	8.9	9.2	9.0	9.5	8.8	0.18	0.077
Metabolic loss	7.6	8.4	7.1	7.2	7.6	7.1	7.1	6.4	0.37	0.13
Phosphorus, g kg^{-1} gain										
Intake	5.05^{g}	6.31^{cd}	7.06^{b}	5.58^{f}	6.07^{de}	6.46^{c}	5.85^{ef}	9.43^{a}	0.100	< 0.001
Faecal loss	2.26^{e}	1.67^{f}	3.75^{b}	1.77^{f}	2.95^{c}	2.66^{d}	2.52^{d}	5.65^{a}	0.080	< 0.001
Growth	2.98^{c}	4.01^{a}	3.02^{c}	3.59^{ab}	2.98^{c}	3.64^{ab}	3.38^{bc}	3.61^{ab}	0.149	0.009
Metabolic loss	-0.20	0.63	0.28	0.22	0.14	0.16	-0.04	0.17		

[1] Pooled standard error of means. Different superscript letters $^{a, b, c, d, e, f, g}$ indicate significant (P < 0.05) differences among treatments.

Table 11. Ten individuals per diet group are classified according to the severity of alterations in distal intestine (DI) tissue

Diet	PPC	CPC	SPC	P+C	P+S	C+S	P+C+S	FM
Severity of histological changes in DI								
Normal	0	2	9	0	3	7	1	10
Mild	3	7	1	5	5	3	8	0
Moderate	7	1	0	5	2	0	1	0
Severe	0	0	0	0	0	0	0	0

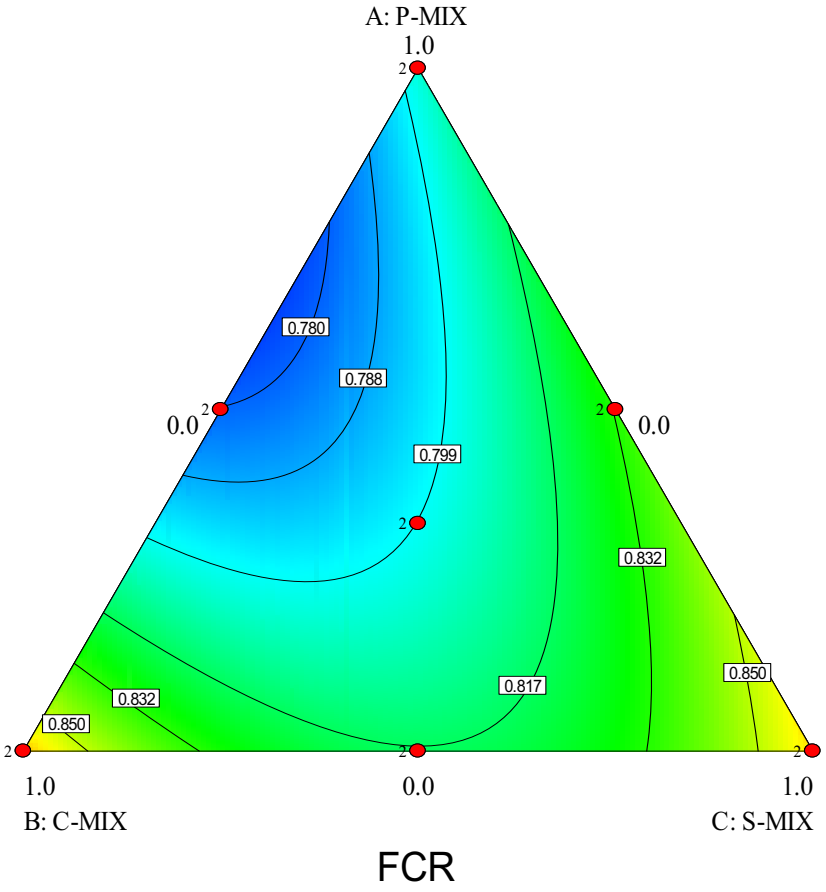


Fig. 1

Contour plot of feed conversion ratio (FCR, g DM ingested (g gain)$^{-1}$) of rainbow trout fed fish meal-free diets containing different combinations of P-MIX, C-MIX, and S-MIX. Each dot represents the duplicated design points (plant protein combinations). The predicted best combination is 64% P-MIX, 36% C-MIX and 0% S-MIX and the predicted best FCR value is 0.776 g DM ingested (g gain)$^{-1}$.

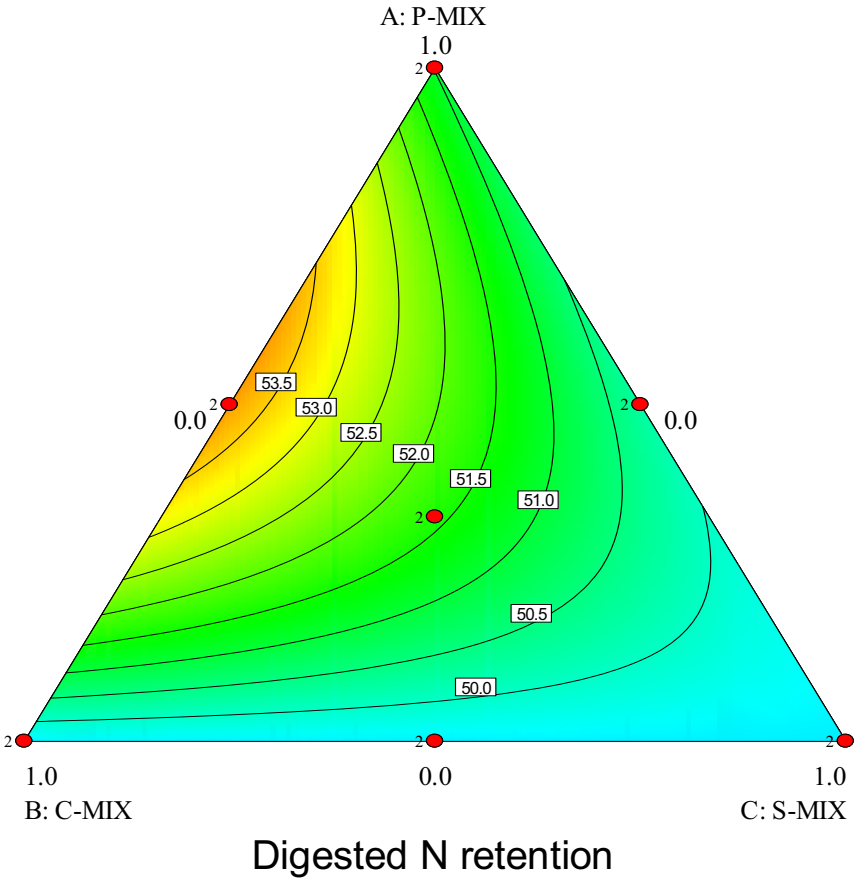


Fig. 2

Contour plot of digested nitrogen (N) retention (%) of rainbow trout fed fish meal-free diets containing different combinations of P-MIX, C-MIX, and S-MIX. Each dot represents the duplicated design points (plant protein combinations). The predicted best combination is 55% P-MIX, 45% C-MIX and 0% S-MAX and the predicted best digested N retention is 53.9 %.

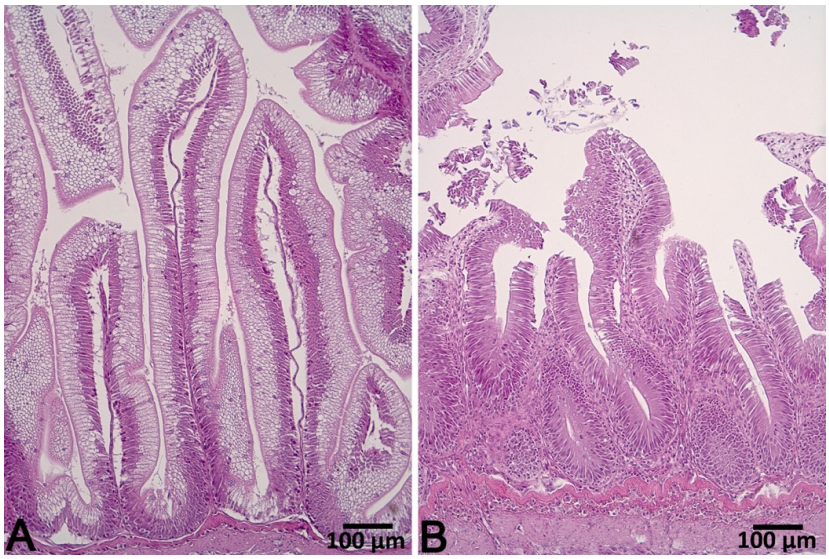


Fig. 3

Distal intestine histology showing (A) normal appearance of a FM diet fed fish and (B) abnormal appearance of a PPC diet fed fish. The abnormal tissue exhibits shorter mucosal folds, fusion between adjacent folds (bridging) and reduced enterocyte vacuolization.

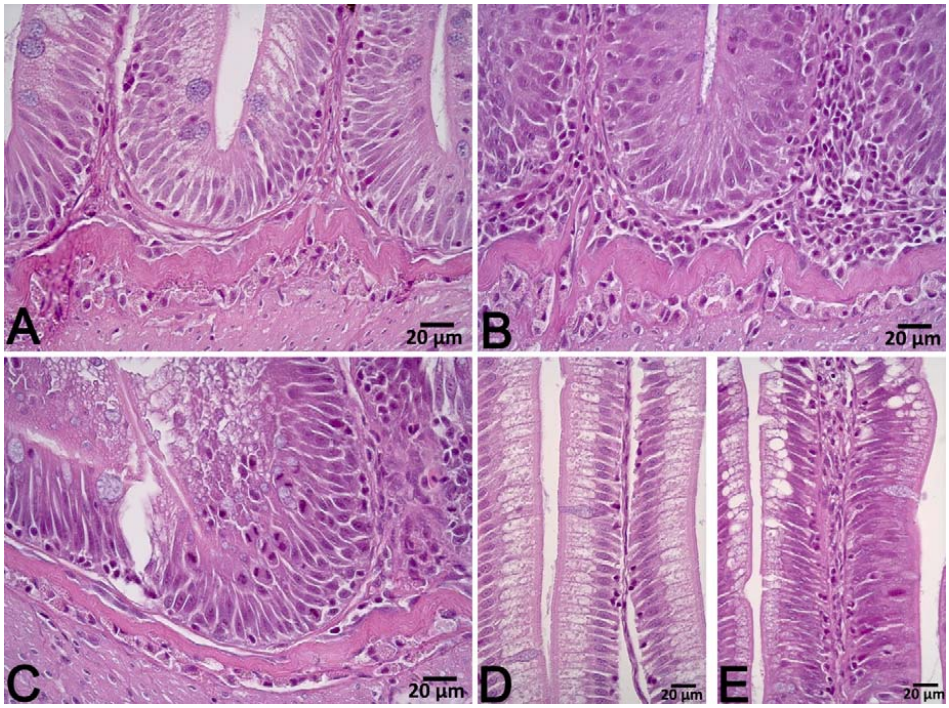
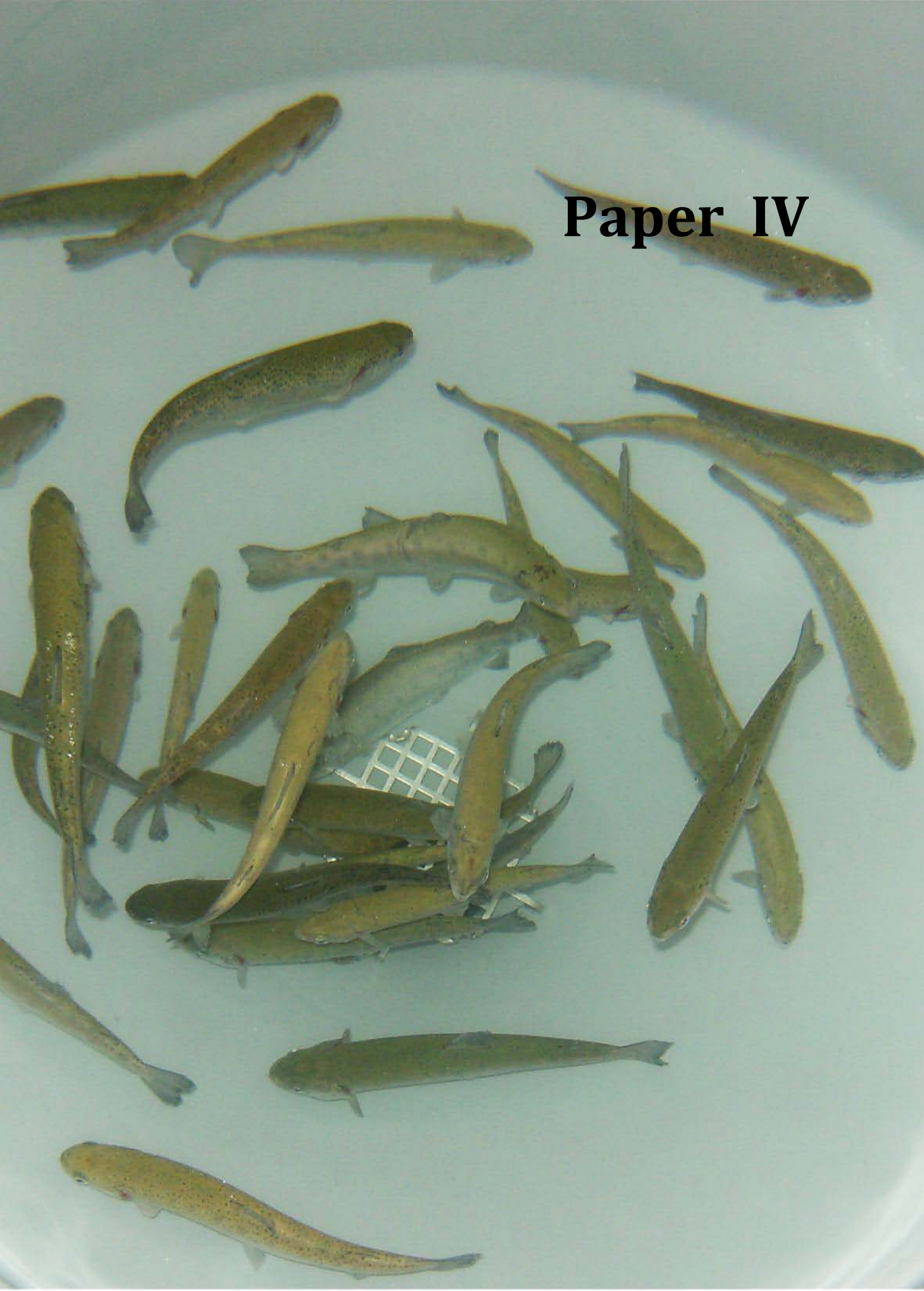


Fig. 4

Photomicrographs of distal intestinal tissue showing (A) normal submucosa from a SPC diet fed fish; (B) abnormal submucosa from a PPM diet fed fish showing leukocyte infiltration into the submucosa and basal lamina propria; (C) increased mitotic figures in the basal area of mucosal folds in a P+C diet fed fish; (D) normal epithelium from a FM fed fish; (E) and abnormal epithelium from a PPM diet fed fish showing increased width and hypercellularity of the lamina propria, increased intraepithelial leukocytes, areas of enterocytes with reduced or absent absorptive vacuoles, and areas of enterocytes with large abnormal (i.e. pathological) vacuoles.


Paper IV

Incubation of soy protein concentrate with phytase improves the nutritional value of a fish meal-free diet for rainbow trout (*Oncorhynchus mykiss*)

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Abstract

The aim of this experiment was to determine the efficiency of dephytinization to improve the nutritional value of soy protein concentrate (SPC) when this feed ingredient accounted for 95% of crude protein in a diet for rainbow trout. Two control diets, one based on LT fish meal (FM diet) and one based on SPC (SPC diet) and one experimental diet based on dephytinized SPC (DSPC diet) were used. The DSPC was produced from the same SPC as used in the SPC diet by incubation with phytase. Incubation with phytase reduced the concentration of phytate in the SPC from 22.2 to 11.2 g kg^{-1}. The SPC and DSPC diets were enriched with crystalline methionine and lysine, taurine, phosphate, and 50 g kg^{-1} of a mixture of krill meal and water soluble material from krill. Each of the three extruded diets was fed to two groups of rainbow trout with an initial weight of 61 g, reared in freshwater with an average temperature of 9.1^{o}C,

for 72 days. The average weight gain (WG) was 255%, and no significant differences were seen among diets for weight gain or whole-body composition of dry matter, crude protein, lipid, ash, or energy, retention of digested energy, or faecal excretion of Mg and Zn. Compared to the SPC diet, dephytinization significantly ($P < 0.05$) improved feed conversion, apparent digestibility of phosphorus (P), whole-body P, Ca, Mg content, plasma P concentration, whole-body retentions of ingested and digested nitrogen (N) and P, as well as retention of ingested energy. Compared to the FM diet, the DSPC diet resulted in significantly higher FCR, digestibilities of N and P, whole-body Mg, retention of ingested P, and faecal excretion of Ca. Feed intake, digestibilities of total amino acids, lipid and starch, whole body concentrations of P, Ca, Mg and Zn, and retentions of ingested energy and digested P did not differ significantly between the DSPC and FM diets. The digestibility of energy, plasma P-concentration, and retentions of ingested and digested N were significantly lower in trout fed the DSPC diet compared to those fed the FM diet. In conclusion, dephytinization of SPC increased utilization of P and other essential mineral elements, facilitating safe replacement of high-quality fish meal. Pre-treatment of SPC with phytase also increased the nutritional value of SPC as a source of dietary protein and energy. The marginally lower FCR obtained by feeding FM compared to DSPC was mainly rationalized by a more favourable ratio between digestible protein and energy in the FM diet.

Keywords: Phytase pre-treatment; Soy protein concentrate; Fish meal-free diet; Nitrogen budget; Energy budget; Phosphorus budget; Rainbow trout

1. Introduction

Soy protein concentrate (SPC) is increasingly used as a source of protein in salmonid feeds, because of its high protein content, relatively balanced amino acid content, high digestibility, and lack of negative effect on the distal intestine of salmonids (Storebakken et al., 2000). Higher feed intake and similar growth rate can be achieved by replacing LT-fish meal with 95% of protein from SPC in diets for rainbow trout (Zhang et al, manuscript 3).

A limitation for using high levels of SPC in diets for salmonids, is the presence of high amounts of phytic acid (*myo*-Inositol hexaphosphate, IP6), which is in the range of 15-20 g kg^{-1} (Storebakken et al., 2000). Phytate-phosphorus (P) is unavailable to salmonids. In addition, IP6 chelates di- and trivalent cationic minerals such as zinc, iron and magnesium at alkaline conditions making them unavailable for absorption (Duffus and Duffus, 1991; Storebakken et al., 2000; Denstadli et al., 2006a). The poor availability of P results in high fecal excretion which in turn may contribute to eutrophication in receiving freshwater bodies. Imbalanced uptake of essential mineral elements caused by IP6 result in incomplete mineralization of hard tissues in salmonids (Storebakken et al., 1998) and may introduce spinal deformities (Helland et al., 2006). This is challenging both from an ethical and from a product quality point of view.

Dietary supplementation of phytase to facilitate hydrolysis of IP6 in the digestive tract of salmonids is feasible when they are reared at temperatures well above 10^{o}C (Vielma et al., 1998; Carter and Sajjadi, 2011). A high dosage of phytase is, however, required even at a water temperature at 15^{o}C (Carter and Sajjadi, 2011). Most commercial phytases have the maximum activity near 40 to 50 ^{o}C, and are not efficient at low temperatures. Effects of phytase in feed for salmonids reared at temperatures below 10^{o}C have been virtually absent (Denstadli et al., 2007).

If high amounts of plant protein concentrates such as SPC are to be used in diets for salmonids in cold water, IP6 should be hydrolyzed prior to feeding to the fish. This can be done by incubation and subsequent drying of the dephytinized SPC (Storebakken et al., 1998; Carter and Sajjadi, 2011), or integrated in the feed production line directly after the incubation (Denstadli et al., 2007).

The aim of this experiment was to quantify the effects of incubation with phytase when SPC accounted for 95% of the dietary protein in a fish meal-free diet for rainbow trout. The main criteria were the utilization of P and other essential mineral elements, and secondary the utilization of macro-nutrients and energy.

2. Materials and methods

This study consisted of a 72-day feeding experiment and a subsequent 10-day digestibility experiment.

2.1 Phytase incubation of soy protein concentrate and feed production

The soy protein concentrate (SPC) used in the present study was Soycomil® R, provided by ADM (Koog aan de Zaan, Holland). SPC was dephytinized by incubating SPC with phytase, as described by Denstadli et al. (2006b) with some modification. Briefly, 150 kg of SPC was pre-heated to 40°C by steam injection in a 400-l twin shaft mixer with an external heating socket (Tatham, Rochdale, UK). Subsequently, 30 g of phytase powder (Natuphos 5000, *Aspergillus* 3-phytase, with a minimum specific activity of 5,000 FTU g^{-1}, BASF, Ludwigshafen, Germany)

was diluted by 270 g ground wheat, and mixed with the preheated SPC for another 5 min. Then, 120 l of 60°C tap water was sprayed onto the mixture through a nozzle during mixing to obtain a final temperature around 50°C. After 120 min of incubation the incubated SPC was pelleted by use of the final section of a twin screw extruder without any thermal processing. Further, the dephytinized SPC (DSPC) pellets were air-dried in a fluid bed hot air drier, developed by The Centre of Feed Technology at UMB, and ground by a hammer mill through a 0.8 mm screen. The feed formulations are shown in Table 1. Three diets were made: A fish meal (FM) diet, a SPC control diet, and an experimental diet where DSPC replaced SPC. The SPC and DSPC diets were supplemented with methionine, lysine, and taurine, and included 50 g kg^{-1} of a mixture of krill meal and partly dehydrated water soluble material from Antarctic krill (*Euphausia superba*). Feed processing and equipment are described in detail by Zhang et al. (manuscript 3), and results concerning the FM and SPC control diets were obtained from the same study. The dry ingredients were ground, mixed, preconditioned and extruded in a twin screw extruder with 2.0 mm dies. Finally, the pellets were dried and added fish oil in a vacuum coater.

2.2 Fish feeding and sampling

Fish keeping routines and facilities are described in detail by Zhang et al. (manuscript 3). Rainbow trout, with a mean initial weight of 61 g were kept in an indoor circulated water system, with water temperatures ranging from 6.5 to 11.8 ℃ (mean of 9.1 ℃). Each diet was fed to trout in two cylindrical 200-l fibreglass tanks, with 40 fish per tank. Each diet was fed three times per day (08:00, 14:00 and 20:00 h) by automatic belt feeders, with approximately ten percent excess feeding. Uneaten feed was sieved in a collection system from the outlet water and feed intake

was monitored and determined following the principles of Helland et al. (1996), with the exception that uneaten feed was collected 10 min after every meal in order to secure high recovery of the pellets.

Before the start of the experiment, 2 × 5 fish from the holding tank were obtained for whole body analysis. Fish were batch-weighed at the start of the trial (day 0), and half way through the experiment (day 37). At the end of the experiment, all the fish were weighed individually. Blood was collected from the caudal vein with heparinized vacutainers, kept on ice, centrifuged at 3000*g for 10 min, and plasma was frozen until analysis. Another three fish were taken from each tank, weighed individually, killed by a blow to the head and gut opened to remove digesta. Empty guts and carcasses then were pooled and stored frozen until whole body analysis. The remaining 22 fish in each tank were weighed and kept for a time repeated faecal stripping by the method of Austreng (1978). Faecal samples from the same tank were pooled, frozen and freeze dried prior to analysis.

2.3 Analyses

The initial and final whole body samples were homogenized with CO_2 ice in a food processor and freeze-dried. Freeze dried faeces were ground with a pestle and mortar, and fish scales were removed prior to analysis. Feed ingredients, feeds, and freeze-dried faeces were analyzed for dry matter (Commission dir. 71/393/EEC), Kjeldahl nitrogen (N) (Commission dir. 93/28/EEC), lipid (HCl hydrolysis and diethyl ether extraction (Commission dir. 98/64/EC)), starch (AOAC enzymatic method 996.11), ash (Commission dir. 71/250/EEC) and minerals (ICP-AES/ICP-MS) (Nordic Committee on Food Analysis (NMKL) method 161). Freeze-dried whole body samples

were analyzed for proximate composition using the same methods, except that no HCl hydrolysis was carried out prior to lipid extraction. Gross energy was measured by bomb calorimetry (Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA). Yttrium oxide concentration in feed and faeces was determined by inductively coupled plasma mass spectroscopy (ICP-MS) after complete digestion of the homogenized and dried samples in a H_2O_2/HNO_3 mixture (2:1, v/v) after cooking in a microwave oven for 1 h. Phytic acid was determined according to the method described by Carlsson et al. (2001). The plasma samples were weighed and dried at 80 ^{o}C, and then combusted at 550 ^{o}C before mineral analysis (ICP-AES/ICP-MS).

2.4 Calculations and statistical analysis

Feed conversion ratio (FCR) was calculated as: $FI \times (FBW - IBW)^{-1}$, where FI is feed intake, and FBW and IBW represent final and initial body weights, respectively. Apparent digestibilities of individual nutrients and energy were calculated as: $100 \times (1 - (Y_d \times Y_f^{-1} \times N_f \times N_d^{-1}))$, where Y_d and Y_f represent the concentration of yttrium in the diet and faeces, N_d and N_f represent the concentration of an individual nutrient or energy in the diet and faeces, respectively. Faecal excretion of minerals was calculated as follows: $100 \times (1 - (Y_d \times Y_f^{-1} \times N_f \times N_d^{-1}))$. Nutrient retentions (%) were calculated as $100 \times (N_1 \times FBW - N_0 \times IBW) \times (N_d \times FI)^{-1}$, where N_0 and N_1 represent the nutrient concentration in the initial and final whole fish samples, respectively. The metabolic loss of N, energy and P was calculated as the difference between the values of intake and faecal loss plus deposition.

One-way analysis of variance (ANOVA) was used to statistically analyse the data, by the GLM procedure in SAS statistical software version 9.1 (SAS Institute, Cary, NC). Results are

presented as means and the pooled standard errors of means (S.E.M). Duncan's multiple range test was used to rank significant ($P<0.05$) differences among dietary treatments.

3. Results

3.1 Diets

The phytase pre-treatment reduced the concentration of phytic acid (IP6) from 21.2 g kg^{-1} in SPC to 11.2 g kg^{-1} in DSPC. The estimated concentration of starch increased by 19 g $(kg\ DM)^{-1}$ as a result of the incubation with phytase.

3.2 Growth and feed utilization

The feed intake of the trout fed the DSPC diet was intermediate between those fed the FM and SPC diets (Table 2). The fish grew from 61 g to 216 g on average during the whole feeding period. Only one fish fed the SPC diet died during the experiment. The growth rate of trout fed the DSPC diet was not significantly different from those fed the FM and SPC diets. FCR was significantly different among all three diets. Dephytinization of the SPC improved the FCR by 0.11 g DM intake $(g\ gain)^{-1}$, while the FCR value for the FM diet was 0.04 g intake $(g\ gain)^{-1}$ more efficient than that for DSPC.

3.3 Nutrient digestibilities

Dephytinization of the SPC did not significantly affect digestibilities of nitrogen, total amino acids, lipid, starch or energy (Table 3). The apparent digestibilities of the FM and DSPC diets did not significantly differ for total amino acids, lipid or starch. Trout fed the SPC and DSPC diets had significantly higher digestibility of N and lower digestibility of energy than fish fed the FM diet. Phytase treatment significantly improved the digestibility of P by 31%. The digestibility of P in fish fed the DSPC diet was 61% higher than that of fish fed the FM diet.

3.4 Whole-body composition and mineral concentration in plasma

No significant differences in whole-body proximate composition were seen among the dietary treatments (Table 4). Phytase treatment of SPC significantly increased whole-body concentrations of P, Ca and Mg, but not that of Zn. Compared to trout fed the FM diet, those fed the DSPC diet had significantly higher whole-body Mg, while the values for P and Ca did not significantly differ. In the plasma, the concentrations of Ca, Mg and Zn were not significantly different among diets, while the P concentration was significantly elevated in fish fed the DSPC diet, whereas the plasma P concentration in the FM diet fed fish was 89% higher.

3.5 Nutrient retention and mineral excretion

Fish fed the DSPC diets had significantly higher retention of N as a percentage of ingested and digested N and higher retention of ingested energy compared to fish fed the SPC diet. The retention of N was highest ($P < 0.05$) in fish fed the FM diet (Table 5). The retention of digested energy did not differ significantly among fish fed the three different diets.

Trout fed the DSPC diet had the highest retention of P as a percentage of ingested, which was significantly higher than that of the fish fed both the SPC and FM diets. Fish fed the DSPC diet also had significantly higher retention of digested P than fish fed the SPC diet, but did not differ significantly from fish fed the FM diet. Fish fed the DSPC diet had a significantly lower fecal Ca excretion than fish fed the SPC diet, but higher than that of trout fed the FM diet. No significant differences were found in fecal Mg and Zn excretion among fish fed three different diets.

3.6 Nitrogen, energy and phosphorus budget

The N, energy and P budgets of rainbow trout fed different diets are summarized in Table 6. The DSPC diet fed fish had a significantly lower intake of N than fish fed the SPC diet (8.1 g less), but higher than fish fed the FM diet. The DSPC diet fed fish had the lowest ($P < 0.05$) fecal N excretion (0.7 and 1.4 g less than fish fed the SPC and FM diets, respectively) and lower non-fecal N excretion than the SPC diet fed fish (6.7 g less); the FM diet fed fish had the lowest non-fecal N excretion and the SPC diet fed fish had a significantly higher N partition for growth than both the DSPC and FM diets fed fish.

The DSPC diet fed fish had significantly lower energy intake and faecal loss than the SPC diet fed fish (2.6 and 1.5 g less, respectively), but still higher ($P < 0.05$) than the FM diet fed fish. Energy for growth partition and metabolic energy loss did not differ among fish fed three different diets.

The DSPC diet fed fish had the lowest ($P < 0.05$) P intake (3.06 and 0.69 g less than the FM and SPC diets fed fish, respectively), the lowest fecal P excretion (3.2 and 1.3 g less than the FM and

SPC diets fed fish, respectively), and the lowest metabolic P loss (0.91 and 0.20 g more than the FM and SPC diets fed fish, respectively). The SPC diet fed fish had the lowest P partition for growth, which was 0.59 and 0.82 g kg^{-1} gain lower than the FM and DSPC diets fed fish, respectively.

4. Discussion

This experiment demonstrated that phytase pre-treatment of SPC was useful to improve the nutritional value when used in a fish meal-free diet by increasing the mineral uptake of rapidly growing rainbow trout to a level similar to that of fish fed high quality fish meal as the sole source of protein. The rate of IP6 degradation and corresponding improvement in digestibility of P are consistent with previous reports (Denstadli et al., 2007). It is probable that an even higher rate of IP6 degradation could have been achieved by increasing the phytase concentration to more than 1,000 FTU kg^{-1} during incubation (Carter and Sajjadi, 2011), or allowing the incubation to last for more than two hours. In a previous incubation experiment with a mixture of extracted soy and wheat (3:1 ratio), a dosage of 2500 FTU kg^{-1} reduced the concentration of IP6 by 86% at semi-moist conditions (Denstadli et al., 2006b). The observed increments in whole body P, Ca and Mg, and plasma P concentrations were also in accordance with previous findings in rainbow trout (Vielma et al., 2002). The digestible P level increased from 3.81 g kg^{-1} to 5.18 g kg^{-1} by releasing highly digestible phosphate from indigestible IP6. The efficacy of the liberated phosphate is further illustrated by the DSPC diet containing the same concentration of digestible P (5.2 g P kg DM^{-1}) as the FM diet, even though the total concentration of P in this diet only was 65% of that in the FM diet. The difference in plasma P concentration between trout fed the FM

and DSPC diets was higher than what could be expected from the digestibility of P. This may be due to different uptake kinetics between P from the fish meal and from the MCP and hydrolyzed IP6. The higher P level in plasma of fish fed the FM diet compared to the DSPC diet may indicate different uptake kinetics between the two ingredients.

The reduction of IP6 in turn decreased the chelating effect on other minerals such as Ca and Mg, and increased their dietary availability and absorption in fish intestine. The difference in Ca utilization between fish fed the DSPC diet and SPC diet demonstrated that the chelating effect of phytate on Ca utilization had been reduced.

The concentration of whole-body P in trout fed the SPC diet was lower than in fish fed the FM and DSPC diets. The Ca: P ratios in fish whole body were 0.94 for the FM diet fed fish, 0.83 for SPC and 0.98 for DSPC diets fed fish. In addition to dietary Ca, the fish absorbed Ca from the water and excreted this via the intestine, as illustrated by the fecal excretion of Ca being 130% of dietary Ca intake. The non-dietary Ca uptake was, however, not sufficient to compensate for reduced intestinal absorption caused by IP6 in the SPC diet. The ratio between Ca, P, Mg and Zn in bones is constant. Thus, Ca became limiting for bone mineralization, and the low retention of digested P in fish fed the SPC diet was a consequence of Ca deficiency. This is consistent with the findings of (Vielma and Lall, 1998) who demonstrated a positive effect of increasing dietary Ca on bone mineralization, only when the dietary P content was below the requirement level. In the present experiment, the available P in the FM and DSPC diets were 0.25 and 0.27 g MJ^{-1} digestible energy (DE), respectively. This is similar to the optimal P requirement for rainbow trout with the similar size (0.25 g MJ^{-1} DE) (Rodehutscord, 1996). The available P level in the SPC diet was 0.21 g MJ^{-1} DE, and thus below the optimal level. The supplementation of 10 g MCP kg^{-1} in the SPC diet was deficient in Ca and P for rainbow trout grown from 60 g to 220 g,

while it was sufficient for the DSPC diet. In rainbow trout, N retention decreases and metabolic loss of N increased with dietary DP/DE ratios increasing from 18 to 24 g DP MJ^{-1} (Green and Hardy, 2008). In our experiment, the FM diet had the lowest DP/DE ratio (17.9 g MJ^{-1}), followed by the DSPC diet (21.0 g MJ^{-1}) and the SPC diet (22.5 g MJ^{-1}). The regression of DP/DE ratio (DPER) on metabolic N loss (MNL) was: MNL= 0.243 $(DPER)^2$ – 7.37 DPER + 71.3 (R^2 = 0.88, P = 0.042). This emphasizes that the DP/DE ratio was a main factor in describing variations in metabolic nitrogen loss.

To conclude, pre-treating SPC with phytase reduced the phytic acid concentration from 22.2 to 11.2 g kg^{-1}. This dephytinization resulted in improved feed utilization, increased bone mineralization and reduced N and P excretion into the water compared with using untreated SPC as the predominant source of dietary protein.

5. Acknowledgements

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Table 1. Feed formulation and analyzed chemical composition

	FM diet	SPC diet	DSPC diet
Formulation[1], g kg^{-1}			
LT fish meal	520	-	-
Partly deshelled krill meal	-	35	35
Partly dehydrated water soluble from krill	-	15	15
Soy protein concentrate (SPC)	-	556	-
Dephytinized SPC[2]	-	-	556
Rape seed oil	-	24	24
Fish oil	180	220	220
Wheat	280	105	105
Vitamin and micro mineral premix	4	4	4
Mono calcium phosphate	-	10	10
Y_2O_3	0.1	0.1	0.1
L-lysine	-	8.2	8.2
DL-methionine	-	9.5	9.5
Taurine	-	5	5
Analyzed content, kg^{-1}			
Dry matter (DM), g	971	943	944
In DM			
Crude protein, g	430	450	449
Crude fat, g	243	252	255
Starch, g	191	90	109
Ash, g	86	57	59
Analytical residue[3], g	50	151	128
Gross energy, MJ	23.9	24.1	24.3
Minerals, g			
Phosphorous, P	13.0	8.1	8.4
Calcium, Ca	17.5	5.3	6.1
Magnesium, Mg	1.8	2.8	2.7
Zinc, Zn	0.13	0.12	0.12

[1] For detailed information about the feed ingredients, see Zhang et al. (manuscript 3).

[2] Pre-treated with 1000 FTU kg^{-1} SPC.

[3] DM - (crude protein + crude fat + starch + ash)

Table 2. Growth performance and feed utilization of rainbow trout fed the experimental diets

	FM diet	SPC diet	DSPC diet	Pooled S.E.M.[1]	P > F
Feed intake, g DM fish^{-1}					
0-72d	112^{b}	127^{a}	123^{ab}	2.9	0.042
Weight gain, % of initial weight					
0-72d	257	249	260	6.1	0.49
FCR, g DM ingested (g gain)$^{-1}$					
0-72d	0.72^{c}	0.87^{a}	0.76^{b}	0.004	< 0.001

[1] Pooled standard error of means. Different superscript letters $^{a, b, c}$ indicate significant (P < 0.05) difference among treatments.

Table 3. Apparent digestibilities (%) of macronutrients, total amino acids, energy, and phosphorous of rainbow trout fed the experimental diets

	FM diet	SPC diet	DSPC diet	Pooled S.E.M.[1]	P > F
Nitrogen	87.9[b]	91.4[a]	91.4[a]	0.30	0.006
Total AA	91.8	93.3	93.5	0.44	0.12
Lipid	96.3	80.6	87.3	4.80	0.21
Starch	94.1	92.2	93.1	1.25	0.60
Energy	88.3[a]	76.0[b]	80.3[b]	1.68	0.031
Phosphorous	40.1[c]	46.8[b]	61.5[a]	0.80	0.031
DP/DE ratio[2], g MJ^{-1}	17.9[a]	22.4[b]	21.1[b]	0.41	0.010

[1] Pooled standard error of means. Different superscript letters [a, b, c] indicate significant ($P < 0.05$) difference among treatments.

[2] Digestible protein (digestible energy)$^{-1}$.

Table 4. Whole body composition and plasma mineral concentrations of rainbow trout fed the experimental diets

	Initial[1]	FM diet	SPC diet	DSPC diet	Pooled S.E.M.[2]	P > F
In whole body, kg^{-1}						
Dry matter, g	282	311	309	317	4.4	0.46
Crude protein, g	157	164	169	165	0.9	0.062
Lipid, g	99	121	120	128	3.9	0.42
Ash, g	24	22	19	22	1.2	0.24
Energy, MJ kg^{-1}	7.09	8.33	8.33	8.64	0.179	0.47
P, g	4.56	4.01^{a}	3.46^{b}	4.04^{a}	0.095	0.040
Ca, g	4.97	3.78^{a}	2.86^{b}	3.94^{a}	0.177	0.042
Mg, mg	299	265^{b}	262^{b}	282^{a}	1.4	0.004
Zn, mg	22.4	19.5	21.7	21.3	0.82	0.27
In plasma, kg^{-1}						
P, mg		51.5^{a}	23.6^{c}	27.3^{b}	0.85	< 0.001

[1] Initial samples were not included in the statistical analysis.

[2] Pooled standard error of means. Different superscript letters $^{a, b, c}$ indicate significant ($P < 0.05$) difference among treatments.

Table 5. Nitrogen, energy and phosphorus retentions and mineral fecal excretions of rainbow trout fed the experimental diets

	F M diet	SPC diet	DSPC diet	Pooled S.E.M.[1]	P > F
Nitrogen retention, % of dietary intake					
ingested	53.7[a]	44.5[c]	49.7[b]	0.41	0.001
digested	61.1[a]	48.7[c]	54.3[b]	0.37	< 0.001
Energy retention, % of dietary intake					
ingested	51.0[a]	42.3[b]	50.4[a]	1.14	0.021
digested	57.8	55.6	62.4	1.79	0.16
Phosphorous retention, % of dietary intake					
ingested	38.3[c]	42.8[b]	60.3[a]	0.54	< 0.001
digested	95.5[a]	91.5[b]	98.0[a]	0.68	0.015
Fecal excretions, % of dietary intake					
Calcium	99[c]	130[a]	109[b]	1.8	0.003
Magnesium	36.7	48.3	47.9	3.12	0.13
Zinc	79.1	74.5	72.9	3.31	0.48

[1] Pooled standard error of means. Different superscript letters [a, b, c] indicate significant ($P < 0.05$) difference among treatments.

Table 6. Nitrogen, energy and phosphorus budget of rainbow trout fed the experimental diets.

	F M diet	SPC diet	DSPC diet	Pooled S.E.M.[1]	P > F
Nitrogen, g kg^{-1} gain					
Intake	49.8^{c}	62.4^{a}	54.3^{b}	0.24	< 0.001
Faecal loss	6.1^{a}	5.4^{a}	4.7^{b}	0.17	0.021
Growth	26.7^{b}	27.8^{a}	27.0^{b}	0.18	0.046
Metabolic loss	17.0^{c}	29.3^{a}	22.6^{b}	0.21	< 0.001
Energy, MJ kg^{-1} gain					
Intake	17.3^{c}	20.9^{a}	18.3^{b}	0.08	< 0.001
Faecal loss	2.0^{c}	5.0^{a}	3.5^{b}	0.28	0.012
Growth	8.8	8.8	9.2	0.24	0.46
Metabolic loss	7.06	6.44	5.57	0.375	0.14
Phosphorus, g kg^{-1} gain					
Intake	9.43^{a}	7.06^{b}	6.37^{c}	0.042	< 0.001
Faecal loss	5.65^{a}	3.75^{b}	2.45^{c}	0.096	< 0.001
Growth	3.61^{b}	3.02^{c}	3.84^{a}	0.033	< 0.001
Metabolic loss	0.17^{ab}	0.28^{a}	0.08^{b}	0.026	0.026

[1] Pooled standard error of means. Different superscript letters $^{a, b, c}$ indicate significant ($P < 0.05$) difference among treatments.