Effects of increasing replacement of dietary fishmeal with plant protein sources on growth performance and body lipid composition of Atlantic salmon (*Salmo salar* L.)

J. Pratoomyot[1,*], E.Å. Bendiksen[2], J.G. Bell[1] and D.R.Tocher[1]

[1]*Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland, UK.*
[2]*BioMar AS, Nordregt. 11, N-7484 Trondheim, Norway.*

Keywords: Fish meal; plant proteins; growth performance; composition; Atlantic salmon

*, Correspondence to: Jarunan Pratoomyot, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, United Kingdom. Tel. No: +44 1786 467993; Fax No: +44 1786 472133; Email: jarunan.pratoomyot@stir.ac.uk

Abstract

The effects of high levels of replacement of dietary fish meal (FM) by mixtures of plant protein (PP) sources on growth performance, lipid composition, protein and lipid digestibility and fatty acid profile were investigated in Atlantic salmon, *Salmo salar*. Experimental diets containing 35% protein and 28% lipid were formulated with a low level of FM that was replaced by increasing levels of PP resulting in four diets of 25/45 ((% FM/% PP, F25), 18/50 (F18) 11/55 (F11) and 5/60 (F5). Dietary oil was supplied by a fish oil (FO) and rapeseed oil blend at a ratio of ~40/60 so this formulation was effectively a dual replacement of FO and FM. Diets were supplemented with crystalline amino acids, to compensate for the reduction in indispensible amino acids due to reduced FM content, and all diets were supplemented with lecithin. Salmon, initial weight 1.30 ± 0.1 kg, were fed one of the four experimental diets for 19 weeks. Feed consumption decreased as PP inclusion in diets increased, probably as a result of reduced palatability. Fish fed the F18, F11 and F5 diets had significantly lower final body weights than fish fed the F25 diet, with SGR decreased by 5 %, 11 % and 23 %, respectively. The lower growth as FM inclusion in diets decreased was associated with decreased feed intake throughout the trial. In contrast, nutrient utilization was significantly affected in the first phase with increased FCR and decreased PER as FM inclusion decreased. However, there were no significant differences in these parameters in the second phase suggesting that there was metabolic adaptation to the diets. Changes in feed physical texture and/or chemical olfactory attractants possibly reduced the palatability of the diets. Essential fatty acid composition, in particular EPA, DHA and ARA in salmon flesh and liver were not negatively affected by dietary treatment and there was some evidence of increased retention and/or synthesis of LC-PUFA.

1. Introduction

Atlantic salmon *Salmo salar* are an important high value, carnivorous fish species generally farmed in intensive systems and fed high-energy extruded feeds containing high quality protein. The protein content of feed for farmed salmon has traditionally been marine fish meals (FM) derived from industrial, reduction fisheries (Hardy, 1996; Sargent and Tacon, 1999; Pike, 2005). It is clear that FM (and fish oil, FO) supplies from these finite fisheries are strictly limited and, if aquaculture continues to expand worldwide, the requirements for FM and FO will soon exceed global supplies (FAO, 2006). The constraints that utilization of these marine products impose has resulted in increasing investigation of alternative protein and oil sources in aquafeeds to sustain aquaculture development.

Many studies have investigated replacement of FM in feeds with a variety of plant protein (PPs) at different levels of inclusion for a range of fish including Atlantic salmon (Storebakken et al., 1998a,b; Refstie et al., 2000, 2001; Carter and Hauler, 2000; Opstvedt et al., 2003; Mundheim et al., 2004; Dias et al., 2005). Wheat gluten can substitute up to 40 % of FM in feeds for salmon and trout (Hardy, 1996), and partial substitution of FM with soybean meal at levels up to 30 - 40 % showed no reduction in growth of various species (Smith et al., 1995; Nengas et al., 1996; Robaina et al., 1997; Opstvedt et al., 2003; Kaushik et al., 2004; Dias et al., 2005). Substitution of FM with soybean protein concentrate up to 80 % or 100 % in feeds for halibut (Berge et al., 1999) and rainbow trout *Oncorhynchus mykiss* (Kaushik et al., 1995) showed no adverse effects on growth performance or nutrient utilization. Addition of pea protein concentrate, corn gluten, sunflower meal, or dehulled peas at up to 30 % of total protein showed no adverse effects on growth performance or carcass composition in salmonids and sea bream (Mente et al., 2003; Thiessen et al., 2003; Gill et al., 2006; Lozano et al., 2007). A blend of soybean meal and corn gluten meal could be used at up to 69 % of total protein replacement without any negative effect on growth and feed intake in cod (Albrektsen et al., 2006). However, total replacement of FM with PP affected growth

77 performance of rainbow trout (Gomes et al., 1995) and Atlantic salmon (Espe et al., 2006),
78 although substitution of FM in feeds close to 100 % was possible in salmon with no negative
79 effect on growth if the amino acid profile was well balanced and if feed intake was
80 comparable to a high FM feed (Espe et al., 2007).

81 In most of the above studies FO still constituted the major lipid source in the feeds.
82 However, FO supply is more pressured and, thus, imminently more limiting than FM and,
83 currently, VOs are considered the most sustainable alternatives for FO replacement in
84 aquafeeds due to the steadily increasing production, high availability and stable prices
85 (Fountoulaki et al., 2009). Several studies have shown that the use of VO to replace FO in
86 aquafeeds at levels of > 50% replacement for all species, or indeed complete replacement in
87 the case of salmon, is now feasible in practical feeds without affecting growth of fish, but
88 does significantly impact on tissue fatty acid composition and metabolism (Bransden et al.,
89 2003; Torstensen et al. 2004; Izquierdo et al., 2005; Pratoomyot et al., 2008; Petropoulos et
90 al., 2009). Therefore, replacing FM and FO with alternative non-marine ingredients can affect
91 not only production parameters such as growth, but also nutritional quality including fillet
92 fatty acid composition.

93 In the present study, the effects of dual substitution of FM and FO were investigated in
94 adult Atlantic salmon of initial weight of 1.3 kg that were grown to market size (> 3 kg) over
95 a period of 19 weeks on diets with 60 % of dietary FO replaced by rapeseed oil, and
96 increasing proportions of FM substituted by PPs (a mixture of sunflower meal, corn gluten
97 meal, soybean meal, and wheat gluten). The level of FO substitution represented the upper
98 level of FO replacement currently used in commercial ongrowing diets. The control diet
99 contained 25 % FM and 45 % PP, which also represented the current minimum commercial
100 level of FM inclusion. Three further diets had FM inclusion reduced to 18, 11 and 5 %, with
101 PP inclusion increased to 50, 55 and 60 %, of the diet. Effects on growth performance, feed
102 utilization efficiency, protein and fat digestibility, sustainability index, and lipid and fatty acid
103 compositions of flesh and liver were investigated.

2. Materials and methods

2.1. Diets and animals

Four diets were formulated to satisfy the nutritional requirements of salmonid fish (National Research Council, 1993), and manufactured at Biomar TecCentre, Brande, Denmark. All diets contained 35 % crude protein and 28 % crude lipid and were formulated to fixed digestible protein and digestible energy contents of 308 g kg^{-1} and 20.5 MJ kg^{-1}, respectively. The control diet was formulated to represent the maximum level of PP inclusion currently in commercial use and contained 45 % PP (a blend of sunflower and corn gluten meals, and soybean protein concentrate) and 25 % FM (Diet F25) (Table 1). The remaining three diets followed a regression with PP inclusion increased to 50 %, 55 % and 60 % and FM inclusion reduced to 18 %, 11 % and 5 % of total diet, diets F18, F11 and F5, respectively. All diets were coated with a 60:40 blend of rapeseed oil and FO. All diets were supplemented with crystalline amino acids, lecithin and carophyll pink as sources of amino acids, phospholipid and pigments (Table 1). The proximate composition, lipid class composition and fatty acid composition of the diets are shown in Tables 1-3, respectively.

One thousand eight hundred Atlantic salmon (*Salmo salar* L.) of initial mean weight 1.3 ± 0.1 kg were randomly distributed among 12 cages of 125 m^3 (5 × 5 × 5 m) with 150 fish/cage at the Marine Harvest Fish Trials Unit, Ardnish, Scotland, and fed one of the four diets in triplicate cages. The experiment was conducted over 19 weeks from October 2007 to February 2008 under natural photoperiod. Fish were fed to apparent satiation by a combination of manual feeding and automatic feed hoppers (Arvo-tec, Sterner Arvo-tec UK, Inverness, Scotland). Daily feed intake was determined in each cage from the difference between the feed ration (1 or 2 meals depending on temperature and day-length) per day and the mass of uneaten pellets registered 15-45 min after each meal in a waste feed lift-up system. Mortalities, feed consumption and waste feed were recorded daily.Mortalities, feed

consumption and waste feed were recorded daily.

2.2 Sampling protocols

Fish were bulk weighed at the initiation, at the end of week 8 and at the termination of the trial, week 19. At the end of the trial, 2 fish per pen (6 fish per dietary treatment) were anaesthetized with metacaine sulphonate (MS222; 50 mg/L) and killed by a blow to the head. Flesh samples were taken from the Norwegian Quality Cut and were homogenized in a food processor after removal of skin and bones and stored at -20 ^{o}C prior to lipid analysis. Livers were also collected from the six fish and a 1-2g sample placed into glass vials containing chloroform/methanol (2:1, by vol.) for analysis of lipid class and fatty acid composition, and the remaining portion immediately frozen on dry ice (for lipid content). Both liver samples were then stored at -20 ^{o}C prior to analysis.

2.3. Proximate composition and pigment analyses

Diets were ground prior to determination of proximate composition according to standard procedures (AOAC, 2000). Moisture contents were obtained after drying in an oven at 110 ^{o}C for 24 h and ash content determined after incineration at 600 ^{o}C for 16 h. Crude protein content was measured by determining nitrogen content (N × 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, U.K), and crude lipid content determined after acid hydrolysis followed by Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus, Foss, Warrington, U.K). Dietary crude fiber content was analysed as outlined in EU DIR 92/89m. Feed and flesh carotenoid pigments were extracted and analyzed by HPLC essentially according to the method of Barua et al. (1993), as described in detail previously (Pratoomyot et al., 2008). Feed samples were digested with Maxatase enzyme (International Biosynthetics, Rijswijk, Netherlands) prior to extraction and analysis.

155

156 *2.4. Apparent digestibility analyses*

157

158 Yttrium oxide (Y_2O_3) was determined by inductively coupled plasma-optical emission
159 spectrometry (ICP-OES). The diet (0.2-0.5g) or faeces (0.1g) were weighed into pre-cleaned
160 beakers and 4 ml of concentrated nitric acid added. The beakers were covered with clean
161 watch glasses and placed in a fume cupboard for 24h. The partially digested samples were
162 placed on a hotplate and boiled for 1h before being transferred quantitatively to pre-cleaned
163 25 ml volumetric flasks and made to volume with 2% v/v nitric acid. The digested samples
164 were then analysed by ICO-OES using a Varian 725-ES instrument. Standards of between 0.5
165 and 120 mg/L Y were prepared as calibrants and the Y signal was monitored at two different
166 wavelengths. Apparent digestibility coefficients (ADC) were estimated according to the
167 formula:

168 $$ADC = 100\text{-}100*((Y_{feed}/Y_{faeces})*(N_{faeces}/N_{feed}))$$

169 where Y_{feed} = Yttrium oxide in feed, Y_{faeces} = Yttrium in faeces, N_{faeces} = nutrient in faeces,
170 N_{feed} = nutrient in feed. All data were based on calculated dry weight of the samples.

171

172 *2.5. Lipid and fatty acid analysis*

173 Total lipid of flesh and liver was extracted according to the method of Folch et al.
174 (1957). Approximately 1 g of flesh homogenate or liver was placed in 20 ml of ice-cold
175 chloroform/methanol (2:1, by vol) and homogenized with an Ultra-Turrax tissue disrupter
176 (Fisher Scientific, Loughborough, U.K.). The non-lipid and lipid layers were separated by
177 addition of 5 ml of 0.88 % (w/v) KCl and allowed to separate on ice for 1 h. The upper non-
178 lipid layer was aspirated and the lower lipid layer dried under oxygen-free nitrogen. The lipid
179 content was determined gravimetrically after drying overnight in a vacuum desiccator.

Lipid class composition of diet and tissues was determined by high-performance thin-layer chromatography (HPTLC) using 10 x 10 cm HPTLC plates (VWR, Lutterworth, England. Approximately 10 μg of total lipid was applied as 2 mm streaks, 1 cm from the bottom, and the plates developed in methyl acetate/isopropanol/ chloroform/methanol/0.25 % aqueous KCl (25:25:25:10:9, by vol.) to two-thirds up the plate. After desiccation for 20 min, the plate was fully developed with isohexane/diethyl ether/acetic acid (85:15:1, by vol.) and placed in a vacuum desiccator for 20 min. The lipid classes were visualized by charring at 160 ^{o}C for 15 min after spraying with 3 % (w/v) aqueous cupric acetate containing 8 % (v/v) phosphoric acid and quantified by densitometry using a CAMAG-3 TLC scanner (version Firmware 1.14.16) (Henderson and Tocher, 1992). Scanned images were recorded automatically and analyzed by computer using winCATS Planar Chromatography Manager, version 1.2.0).

Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transesterification at 50 ^{o}C for 16 h according to the method of Christie (1993). Extraction and purification of FAME was carried out as described by Tocher and Harvie (1988). The FAME were separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50^{o}C. Hydrogen was used as carrier gas and temperature programming was from 50 ^{o}C to 150 ^{o}C at 40 ^{o}C min^{-1} and then to 230 ^{o}C at 2.0 ^{o}C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19)

2.6. Formulae, calculations and statistical analysis

Feed consumption (g/day) = feed intake (g) x [number of fish x days]$^{-1}$

205 Feed Conversion (FCR) = feed intake (g) x [final biomass – initial biomass + dead fish]$^{-1}$

206 Hepatosomatic Index (HSI, %) = 100 × [weight of liver (g)] x [weight of fish (g)]$^{-1}$

207 Protein efficiency ratio (PER) = [final mean weight (g) - initial mean weight (g)] x [crude

208 protein fed (g)]$^{-1}$

209 Specific growth rate (SGR, % day) = 100 × [ln (final mean weight) – ln (initial mean

210 weight)] x days^{-1}

211 Thermal growth coefficient (TGC) = 1000 x [(final wt)$^{1/3}$ – (initial wt)$^{1/3}$ x (degree days)$^{-1}$

212 Visceromatic Index (VSI, %) = 100 × [weight of viscera (g)] x [weight of fish (g)]$^{-1}$

213

214 All data are presented as means ± SD (n value as stated). The effects of dietary treatment on
215 growth performance were analyzed by one-way analysis of variance (ANOVA) followed,
216 where appropriate, by Tukey's post hoc test. The relationship between dietary treatment and
217 chemical composition was analyzed by regression analysis. Percentage data and data
218 identified as non-homogeneous (Levene's test) or non normality (Shapiro-Wilks's test) were
219 subjected to arcsine transformation before analysis. ANOVA and regression analysis were
220 performed using a SPSS Statistical Software System version 14 (SPSS inc, 2005). Differences
221 were regarded as significant when $P < 0.05$ (Zar, 1999).

222

223 **3. Results**

224 *3.1. Diet compositions*

225 Formulating on fixed digestible protein and digestible energy will result in some small
226 variance in dietary fat and protein content depending upon recipe compositions, and level and
227 availability of nutrient and energy from different raw materials. The main differences in
228 proximate compositions of the diets were that lipid and the nitrogen-free extract (NFE) were
229 slightly lower and higher, respectively, in the diets with highest FM replacement, with levels
230 in diets F11 and F5 being significantly different to those in diets F25 and F18 (Table 1). The

majority of lipid supplied by the diets was neutral lipid, predominantly triacylglycerol (TAG), and there were no significant differences in total polar and neutral lipid levels between the treatments (Table 2). There were no significant differences between the diets in polar lipid composition with all diets containing around 8 – 9 % of polar lipid, mainly phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI) / phosphatidylserine (PS). All diets contained approximately 54 % total monoenes, predominantly 18:1n-9 (oleic acid), with around 16 % saturated fatty acids, mainly 16:0, and 30 % polyunsaturated fatty acids (PUFA), with half of that being 18:2n-6 and the remainder being n-3 PUFA, 18:3n-3, and the long-chain PUFA (LC-PUFA), eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) (Table 3). There were no significant differences in total saturated fatty acids, total mononenes, total n-6, total n-3 and total PUFA among dietary treatments. However, there were some small but significant differences in proportions of specific fatty acids among the dietary treatments. Thus, the proportions of 14:0, 16:1n-7, 20:1n-9, 22:1n-9/11 and DHA decreased as FM inclusion decreased, and percentages of 18:1n-9 and 18:3n-3 increased as PP inclusion increased in the diets.

3.2. Growth performance

There were no significant differences in initial weight of fish (Table 4). After 19 weeks, the overall growth performance of fish revealed that final body weight and weight gain were significantly reduced by FM replacement (Fig. 1), resulting in reduced SGR and TGC (Fig. 2). The decreased growth was associated with decreased feed consumption, as the level of FM inclusion decreased (Fig. 1). Protein efficiency (PER) showed a tendency to decrease with increased inclusion of PP but FCR was unaffected (Fig. 2). There were virtually no mortalities in the trial and no significant differences in hepato-somatic index (HSI) among treatments

(data not shown), but viscero-somatic index (VSI) was significantly lower in fish fed the F25 diet compared to those fish fed F11 and F5 diets (Fig. 1).

Similar trends in feed consumption, body weight, weight gain, SGR and TGC, as described above for the overall trial, were observed in both growth phases of the trial, at average temperatures of 11 ^{o}C and 7 ^{o}C during weeks 0-8 and 8 – 19, respectively (Table 4). In contrast, FCR was significantly affected by diet during the first phase in weeks 0-8, being significantly increased as dietary FM inclusion decreased (Table 4). Similarly, PER significantly decreased during the first phase of the trial as FM inclusion decreased. In both cases, these effects were not observed in the second phase when diet had no significant effects on FCR and PER (Table 4).

3.3 Fish in:Fish out ratios of the feeds

The weights (kg) of FM and FO utilized to produce one kg of farmed salmon were calculated from the data for FCR and diet FM and FO contents. Thus, the weight of FM used in the present study were 258, 187, 114 and 57 g per kg of salmon produced when fish were fed the F25, F18, F11 and F5 diets, respectively. Similarly, the amounts of FO used were 119, 121, 123 and 135 g per kg of salmon when fed the F25, F18, F11 and F5 diets, respectively. Dividing these data by 225 and 50 representing the weights (g) of FM and FO obtained from one kg of pelagic feed fish, based on the average reduction yields of 22.5 % and 5 % for FM and FO, respectively (Tacon and Meitan, 2008), provides an estimate of the ratio of kg feed fish:kg salmon produced, or Fish in:Fish out (Fi:Fo) ratio (Fig. 3). The data show that reducing FM inclusion clearly reduced the feed fish required for the FM input, but that even with 60 % replacement, FO input is the major contributor to feed fish utilization.

3.4. Protein and lipid digestibilities

281 The apparent digestibility coefficients (ADC) of protein and fat were significantly affected by
282 the levels of dietary FM and PPs (Fig. 4). The ADC of protein significantly increased from
283 82.6 % to 85.3 %, whereas the ADC of fat significantly decreased from 94.6 % to 90.5 %
284 with decreasing dietary FM and increasing dietary PP inclusion.
285
286 *3.5. Lipid and fatty acid compositions of salmon flesh and liver*
287 Lipid content of the flesh varied between 11.6 and 13.2 % of wet weight and was unaffected
288 by diet (Table 5). Although the lipid content of liver also showed no statistically significant
289 differences between dietary treatments, there was a clear trend for liver lipid to decrease with
290 decreasing FM inclusion, reducing from 7.1 % in fish fed the highest level of FM down to 5.2
291 % in fish fed the lowest FM inclusion (Table 5). However, there were no significant effects of
292 diet on the proportions of total polar and neutral lipids, or the relative percentages of any
293 individual lipid classes in liver. There were some minor differences in polar lipid class
294 composition in flesh, but these were of doubtful biological or physiological significance. The
295 pigment content of the flesh was also unaffected by dietary treatment.
296 The gross fatty acid composition of flesh reflected the diet compositions, with over 50 %
297 total monoenes, predominantly 18:1n-9, around 17 % saturated fatty acids, mainly 16:0, and
298 over 30 % PUFA with 18:2n-6 being the most abundant followed by DHA, EPA and 18:3n-3
299 (Table 6). DHA was retained at a higher concentration in the flesh than provided in the diet.
300 The levels of 20:1n-9 and 22:1n-9/11 in the flesh decreased with decreasing FM inclusion
301 similar to the dietary trend but other differences were not related directly to dietary levels.
302 Thus, 16:0 and 16:1n-7 increased as FM inclusion decreased, whereas proportions of 18:2n-6
303 and 18:3n-3 decreased and the levels of desaturated and elongated products including
304 arachidonic acid (20:4n-6, ARA), EPA and 22:5n-3, increased with decreasing FM inclusion
305 (Table 6). The fatty acid composition of liver showed more variability between treatments but

306 was generally similar to the diet compositions, with monoenes, particularly 18:1n-9,
307 predominating with around 15 % saturated fatty acids, mainly 16:0, and 33 - 38 % PUFA
308 (Table 7). As with flesh, the proportion of DHA was much higher in liver lipids than dietary
309 lipids, and was the predominant PUFA followed by 18:2n-6, EPA and 18:3n-3. Decreasing
310 FM inclusion resulted in slightly reduced 14:0 and, particularly, reduced proportions of 20:1n-
311 9 and 22:1n-9/11 in liver total lipid. In contrast to flesh, diet had no major effect on liver
312 18:2n-6 or 18:3n-3 levels, but ARA and 22:5n-3 were significantly increased and there were
313 trends of increasing EPA and DHA in response to decreasing dietary FM inclusion (Table 7).
314
315 **4. Discussion**
316 The regressive reduction of FM from 25 % to 5 % in the diets by progressively increasing
317 replacement with mixed PP sources (sunflower meal, soybean protein concentrate, corn
318 gluten, and wheat gluten) did not affect the survival of Atlantic salmon (mortality less than
319 1%) indicating that the experimental diets did not have any major negative effects on fish
320 health. However, the dietary treatments significantly affected growth performance of salmon
321 in the present study. As FM inclusion decreased from 25 % to 5 % there was a progressive
322 reduction in growth resulting in final weights being reduced by 5 %, 13 % and 22 % in fish
323 fed 18, 11 and 5 % FM, respectively, compared to fish fed 25% FM. Moreover, SGR for the
324 fish fed the F18, F11 and F5 diets was reduced by 5 %, 11 % and 23 %, and TGC by 5 %, 16
325 % and 27 %, respectively, compared to fish fed the control F25 diet. Despite the lower growth
326 performance compared to the control diet, the fish fed the F18 and F11 diets showed weight
327 gains, TGCs and SGRs in a similar range to salmon of similar size fed high FM diets (Lie et
328 al., 1986; Karalazos et al., 2006; Pratoomyot et al., 2008; Torstensen et al., 2008).
329 Therefore, the results obtained in the present study were supported by previous studies
330 showing that replacing high levels of FM with PPs reduced growth in salmon (Opstvedt et al.,
331 2003; Mundheim et al., 2004). Level of replacement is crucial, as partial replacement of up to

80 % of FM in diets showed no adverse effects on growth of Atlantic salmon (Berge et al., 1998; Sveier et al., 2001; Opstvedt et al., 2003; Espe et al., 2006), whereas total replacement of FM by a mixture of PPs lowered the growth performance (Espe et al., 2006). In the present study growth retardation was observed in salmon fed diets containing FM inclusion levels of 18 % and lower in diets where there was simultaneous replacement of 60 % of FO with VO (rapeseed oil). In the previous studies, the levels of dietary FO used were between 22 and 30 % of the total diet, which was much higher than the level of FO used in the present study, which was around 12 % (with VO 18%) of the total diet. The effect of FM replacement on growth is, therefore, likely dependent not only upon the level of FM replacement, but also on the level of FO in the diet. Supporting this, in another study investigating dual replacement of FM and FO, Atlantic salmon fed a diet with 80 % of the FM replaced by alternative protein sources along with 70 % FO replaced by VO (a linseed/rapeseed/palm oil blend) showed significant growth reduction, whereas diets with substitution of 40 % FM and 70 % VO, or 80 % FM and 35 % FO showed no negative effects (Torstensen et al., 2008). However, studies in gilthead sea bream reported that there was no difference in growth when fish were fed diets containing 15% FM and high levels of PPs, and either 0 %, 33 % or 66% of the dietary FO replaced by a VO blend (Benedito-Palos et al., 2008, 2009).

Reduced growth in salmonids at high dietary inclusion levels of PP has been associated with various factors including increased digestible and indigestible carbohydrate levels (starch/fibre levels) (Hemre et al. 2003; Opstvedt et al. 2003), reduced feed palatability and presence of anti-nutrients (Krogdahl et al. 1994; Francis et al. 2001), and imbalanced dietary amino acid concentration (Espe et al. 2006; 2007). In the present study, the NFE (N-free extract plus fiber) level increased as the level of PP inclusion increased, but also the feed intake was reduced by feeding the diets with increased PPs. Plant meals containing significant amounts of carbohydrate may have detrimental effects on Atlantic salmon performance

(Waagbo et al., 1994; Hemre et al., 1995), and so the increased NFE in the high PP diets may have contributed to the lower growth. The NFE value encompasses both digestible and indigestible carbohydrate, and high energy (fat and digestible carbohydrate) levels can lead to improved utilization of ingested protein through increased contribution of the non-protein sources for energy provision (Cho and Kaushik, 1985,1990). However, salmon have a low capacity to utilize carbohydrate and there was no positive effect on PER of increased levels of PP (and increased NFE). Furthermore, indigestible carbohydrate/fibre may partially limit metabolic capacity of the distal intestine epithelium resulting in the lower ADC of fat.

It is particularly noteworthy that feed intake was affected by feeding the diets with reduced levels of FM inclusion in the present study with Atlantic salmon. The reduced consumption of diets containing high PPs was clearly correlated with the lower growth observed in fish fed these diets. Previous studies showed that even moderate reductions in feed intake in fish may severely affect cumulative nutrient absorption and growth in a given period (Refstie et al., 1998, 2001; Storebakken et al., 1998a,b; Carter and Hauler, 2000; Espe et al., 2006). Indeed, the results were consistent with previous studies that reported that increasing replacement of FM by PP in diets for salmonids resulted in reduced growth performance that was caused by reduced feed intake (Kaushik et al., 2004; Epse et al., 2006). Previous studies have reported that Atlantic salmon require time to adapt before accepting high PP diets (Storebakken et al., 1998a,b; Torstensen et al., 2008), but are able to compensate after being fed a restricted feed intake (Johansen et al., 2001; Mundheim et al., 2004). Thus, salmon could perhaps increase feed consumption after a period of reduced feed consumption as a compensatory adaptation although this was not the case in the present study, over 19 weeks, where the effect on feed intake and the consequent effects on growth were observed throughout the trial. It is probable that the reduced intake of the diets with decreased FM and increased PP was due, at least in part, to changes in taste and palatability. It was

382 shown that increasing both the quality and level of FM inclusion enhanced palatability and
383 feed efficacy (Webster et al., 1999), and so reducing FM and changing the composition of
384 ingredients likely influences the flavor, reducing palatability of the diets and diminishing the
385 appetite of the fish. A similar incremental reduction in SGR and TGC was seen in cod when
386 FM was reduced by up to 28 %, and replaced by full fat soya, with reduced diet palatability
387 the likely cause (Karalazos et al., 2007). The palatability of diets and feed acceptance can be
388 improved and enhanced by inclusion of relatively minor amounts of specific feed attractants
389 including krill meal, or hydrolysates of fish protein and squid in diets (Espe et al., 1999, 2006;
390 Dias et al., 2005; Olsen et al., 2006).

391 Although reduced feed intake was the major consequence of the reduction in dietary
392 FM inclusion, there were also apparent effects on nutrient utilization between salmon fed high
393 and low FM inclusion. FCR was increased and PER decreased by decreasing dietary FM
394 inclusion in the first phase of the trial while there were no significant differences between any
395 of the dietary treatments in these parameters in the second phase of the trial. This indicates
396 that there was metabolic adaptation to the diets that improved nutrient utilization of high PP
397 diets in the later stages of the study. Generally, in addition to high carbohydrate contents, a
398 major potential consequence of diets containing high PPs is unbalanced amino acid profiles.
399 Protein utilization can be reduced when dietary lysine is limited, but it was not observed when
400 dietary methionine was limited (Rodehutscord et al., 1997; Epse et al., 2007, 2008). Lewis
401 and Kohler (2008) suggested that dietary amino acid imbalance resulted in elevated FCR and
402 liposomatic index of sunshine bass fed diets with dietary FM inclusion reduced from 24 to 8
403 %. Therefore in the present study, high dietary PP and reduced feed consumption could have
404 resulted in an inadequate amino acid balance to support maximum growth. This may have
405 limited protein deposition and lipid utilization and resulted in increased perivisceral lipid
406 deposition as evidenced by increased VSI in fish fed the lower levels of FM. Several studies

have demonstrated that high PP inclusion in diets did not effect protein utilization if dietary amino acids were balanced and feed intake was not significantly reduced (Espe et al., 2006, 2007, 2008). Amino acid balance can be improved by the addition of crystalline amino acids to diets (Rodehutcord et al., 1995; Espe et al., 1999). In the present study, feeds were formulated to mimic FM composition, and to meet amino acid requirements of Atlantic salmon (NRC 1993), and so crystalline amino acids (lysine, threonine, methionine) were supplemented. This may suggest the effects on FCR and PER are more related to reduced feed intake than to imbalanced amino acid composition.

The apparent digestibilities of protein (90 – 95 %) and fat (80 – 83 %) measured in the present study were comparable to those reported in previous studies in Atlantic salmon (Opstvedt et al., 2003; Mundheim et al., 2004; Aslaksen et al., 2007), but lower than digestibilities reported in rainbow trout (Cho & Kaushik, 1990). This is likely due to differences in the sources and levels of ingredients, the method for faeces collection and, of course, fish species. In previous studies on smaller Atlantic salmon, increasing dietary PPs as replacement for up to 50 % of FM reduced the ADCs of protein and fat, and protein retention, and the authors concluded that protease inhibitors and interaction between fat and carbohydrate fractions reduced protein and lipid digestibility (Opstvedt et al. 2003; Mundheim et al. 2004). Although the ADC of fat was decreased as the level of dietary PPs increased in the present study, the positive correlation of ADC of protein was contrary to the previous data. The present data suggest that protein availability of the refined PP sources (e.g. wheat gluten) may be as high or higher than FM. This effect may actually be greater than observed as the reduced feed intake in fish fed the high PP would tend to underestimate protein digestibility since endogenous gut loss could be expected to be higher (protein content of faeces increases) when feed intake is lower.

Nutritional quality of fish products is important with respect to human consumption, particularly in terms of flesh fatty acid composition and the content of the health beneficial n-3 LC-PUFA, EPA and DHA. The strong relationships between tissue fatty acid composition and dietary lipid are well documented (Torstensen and Froyland, 2000; Rosenlund et al., 2001; Bell et al., 2002, 2004). In the present study, it was noteworthy that substituting FM with very high levels of PP did not reduce levels of EPA and DHA in the flesh below those observed in salmon fed the control F25 diet. Indeed, there were clear trends, some significant, showing that all the major bioactive LC-PUFA, ARA, EPA and DHA tended to increase in flesh and liver of fish fed increased PP. There were also indications of 18:2n-6 and 18:3n-3 decreasing in flesh as PP inclusion increased. These changes in salmon tissue PUFA composition cannot be adequately explained merely by dietary fatty acid compositions as ARA and EPA were constant in the diets and DHA decreased with increasing PP inclusion. Therefore, the effects observed are likely due to changes in metabolism. For instance, some of the effect in liver may be partly related to reduced lipid levels in liver where TAG decreased and phospholipid increased. However, other metabolic effects may include differential oxidation of fatty acids and increased retention of LC-PUFA as PP inclusion increased. When fatty acids are provided at low concentrations in diets, they tend to be preferentially retained or deposited in tissue (Bell et al., 2003, 2004). Levels of ARA, EPA and, especially, DHA were higher in flesh, and especially liver, than in the diets suggesting selective retention, whereas levels of 18:2n-6 and 18:3n-3 in tissues were less than in the diet suggesting that these fatty acids were selectively utilized for energy (Henderson and Sargent, 1985; Henderson, 1996; Caballero et al., 2002) and/or for synthesis of longer chain, more unsaturated products. Therefore, increased desaturation of 18:2n-6 and 18:3n-3 to LC-PUFA may also be a factor. The fatty acid composition of flesh may also reflect another metabolic effect as the increasing proportions of 16:0 and 16:1 with increasing PP inclusion may reflect

456 increased lipogenesis in the fish, possibly as a result of decreased feed intake. In contrast, the
457 tissue levels of some fatty acids did reflect dietary levels, with 20:1 and 22:1 both decreasing
458 in liver and flesh as FM inclusion decreased.

459 The calculation of approximate Fi:Fo ratios for the feeds used in the present trial clearly
460 demonstrated how dietary FO impacts more on the sustainability issue than the utilization of
461 FM. In these low FM diets, even replacing 60 % of FO still results in quite high Fi:Fo figures.
462 The data also clearly show the great impact that a relatively small difference in FCR makes
463 (Naylor et al., 2009), as the higher FCR in fish fed diet F5 is clearly reflected in an increase in
464 the Fi:Fo ratio for FO use. These data also show that feed formulated with 25 % FM can
465 produce salmon with an Fi:Fo approaching 1 for FM use, at least in the present study with an
466 FCR close to 1 and estimating the yield of FM at 22.5 % of wet weight of feed fish. However,
467 the data also show that assuming an FCR of 1.0, FO substitution would have to be at least 80
468 % in salmon diets formulated with 30 % dietary lipid, and nearer 90 % in diets formulated
469 with 40 % total lipid, for the Fi:Fo ratio to approach 1 for FO, as the feeds used here with
470 60% substitution (FO at 12 % of total diet) still have an Fi:Fo ratio of around 2.4.
471

472 **5. Conclusion**

473 Atlantic salmon showed lower growth performance when dietary FM inclusion was
474 reduced from 25 % to 5 % by increased substitution with PPs. The fish consumed less feed as
475 FM inclusion in diets decreased and this effect was observed throughout the trial. Nutrient
476 utilization was significantly affected in the first phase with increased FCR and decreased PER
477 as FM inclusion decreased. However, there were no significant differences in PER and FCR
478 at the end of the trial suggesting that there was metabolic adaptation and no amino acid
479 limitation in the diets. Changing feed ingredients may have affected dietary physical texture,
480 chemical olfactory attractants or introduced negative taste factors that reduced the palatability

of the diets. Enhancing the palatability of the diets by adding additional feed attractants or avoiding negative taste components may help to minimize effects on feed intake. Essential fatty acid composition, in particular EPA, DHA and ARA in flesh and liver were not negatively affected by dietary treatment and there was some evidence of increased retention and/or synthesis of LC-PUFA. The overall conclusion is that successful replacement of FM is dependent on finding the right replacers and strategy to maintain palatability of the feed and appetite.

6. Acknowledgement

Jarunan Pratoomyot was funded by a Royal Thai Government Scholarship.

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724 Figure legends
725

726 Fig. 1. Feed intake, specific growth rate (SGR) and feed conversion efficiency (FCR) in
727 Atlantic salmon fed the experimental diets. Values are mean ± SD (n = 3). Values for each
728 parameter with different superscript letters are significantly different as determined by
729 ANOVA ($p < 0.05$). Diets F25, F18, F11 and F5 were formulated with 25, 18, 11 and 5 %
730 fishmeal, respectively, and increasing proportions of alternative protein sources as described
731 in the Methods.
732

733 Fig. 2. Final weight (kg), thermal growth coefficient (TGC) and protein efficiency (PER) in
734 Atlantic salmon fed the experimental diets. Values are mean ± SD (n = 3). Values for each
735 parameter with different superscript letters are significantly different as determined by
736 ANOVA ($p < 0.05$). Diets F25, F18, F11 and F5 were formulated with 25, 18, 11 and 5 %
737 fishmeal, respectively, and increasing proportions of alternative protein sources as described
738 in the Methods.
739

740 Fig. 3. Amount (kg) of feed fish used per kg of salmon produced. Data were calculated from
741 the known dietary fishmeal and fish oil contents of the experimental feeds (F25, F18, F11 and
742 F5), feed conversion ratios of salmon fed each feed, and assuming yield from feed fish of 22.5
743 % for fishmeal and 5 % for fish oil (Tacon and Metian, 2008).
744

745 Fig. 4. Apparent digestibility coefficients (ADC %) for total protein and fat in salmon fed the
746 diets containing 25 % (F25), 18 % (F18), 11 % (F11) and 5 % (F5) fishmeal. Values are mean
747 ± SD (n = 3). Values (columns) for each nutrient with different superscript letters are
748 significantly different as determined by ANOVA ($p < 0.05$).
749

750 Table 1. Feed formulation (g kg^{-1}) and analyzed compositions (%) of the experimental diets.
751

Feed ingredients		F25	F18	F11	F5
Fishmeals[1]	(67/10)[2]	250	180	110	50
Sunflower expeller	(37/10)[2]	115	77	40	-
Corn gluten	(62/2)[2]	85	135	175	215
Soy concentrate	(60/2)[2]	85	135	175	225
Wheat gluten	(77/3)[2]	-	2	18	20
Rapeseed oil[3]		173	175	178	180
Fish oil [4]		116	117	118	120
Binders		160	160	160	160
Micronutrients [5]		11.95	17.59	23.59	28.99
L-lysine[6]		0.62	1.72	3.44	4.26
L-threonine[6]		-	-	0.43	0.67
DL-methionine[6]		0.57	1.03	1.56	2.01
Lecithin		5.0	5.0	5.0	5.0
Astaxanthin		0.40	0.40	0.40	0.40
Antioxidant[7]		4.25	4.25	4.25	4.25
Analysed composition					
Crude protein (N x 6.25)		34.3 ± 0.4 [b]	35.1 ± 0.3 [a]	35.0 ± 0.1 [a]	34.7 ± 0.3 [ab]
Crude lipid		29.8 ± 0.1 [a]	29.5 ± 0.1 [a]	27.9 ± 0.1 [b]	27.3 ± 0.2 [c]
Moisture		6.7 ± 0.1 [b]	6.0 ± 0.0 [d]	6.2 ± 0.0 [c]	6.9 ± 0.0 [a]
Ash		6.0 ± 0.1 [a]	5.6 ± 0.0 [b]	5.2 ± 0.1 [c]	4.8 ± 0.0 [d]
Crude fiber		3.5	3.3	2.6	3.0
NFE[8]		19.7 ± 0.3 [b]	20.5 ± 0.4 [b]	23.1 ± 0.4 [b]	23.3 ± 0.5 [a]

752 [1] Peruvian fishmeals produced from *Anchoveta*

753 [2] Figures in parentheses are crude protein/crude lipid values, respectively.

754 [3] Non-GM double-low rapeseed oil

755 [4] North-Atlantic standard fish oil

756 [5] Vitamin and mineral premixes with limestone carrier added according to the commercial
757 standards of BioMar AS

758 [6] Purified (99%) crystalline amino acids

759 [7] Blend of antioxidants and starch carrier added according to the commercial standards of
760 BioMar AS

761 [8] NFE (nitrogen free extract) calculated by subtraction, 100 - (crude protein + crude fat +
762 moisture + ash + crude fiber)

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763 Table 2. Lipid class composition (percentage of total lipid) and pigment content
764 (g kg^{-1}) of the experimental diets
765

Parameters	F25	F18	F11	F5
Lipid classes				
PC	2.8 ± 0.2	2.7 ± 0.2	2.1 ± 0.3	1.9 ± 0.6
PE	3.5 ± 0.6	3.6 ± 0.5	3.9 ± 0.6	3.2 ± 1.1
PI/PS	1.5 ± 0.3	2.0 ± 0.4	3.1 ± 0.6	2.8 ± 0.2
Sphingomyelin	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.1	nd
Lyso-PC	0.1 ± 0.0	0.1 ± 0.0	tr	nd
Polar lipid	8.2 ± 0.8	8.6 ± 0.7	9.3 ± 1.4	7.9 ± 1.0
Neutral lipid	91.8 ± 0.8	91.4 ± 0.7	90.7 ± 1.4	92.1 ± 1.0
Triacylglycerol	74.2 ± 1.8	72.7 ± 0.7	73.9 ± 1.4	75.6 ± 1.0
Sterol	8.5 ± 0.6	8.6 ± 0.5	6.9 ± 0.4	6.9 ± 0.3
Free fatty acid	9.1 ± 1.5	10.1 ± 0.7	9.9 ± 0.8	9.6 ± 0.8
Steryl ester	tr	tr	tr	tr

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767 Results are means ± SD (n = 4). There were no significant differences between
768 feeds for any parameter as determined by ANOVA. nd, not detected; PC,
769 phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol;
770 PS, phosphatidylserine; TNL, total neutral lipids; TPL, total polar lipids, tr, trace.
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794 Table 3. Fatty acid compositions (percentage of total fatty acids) of the diets
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Parameters	F25	F18	F11	F5
14:0	2.6 ± 0.0 [a]	2.6 ± 0.0 [a]	2.5 ± 0.0 [b]	2.3 ± 0.0 [c]
16:0	8.7 ± 0.1[b]	9.1 ± 0.1 [a]	9.1 ± 0.2 [a]	8.6 ± 0.1[b]
18:0	2.7 ± 0.1	2.8 ± 0.1	2.6 ± 0.1	3.1 ± 0.4
20:0	0.5 ± 0.0 [b]	0.5 ± 0.0 [b]	0.5 ± 0.0 [b]	0.6 ± 0.1[a]
22:0	0.9 ± 0.0 [b]	1.0 ± 0.1 [a]	0.8 ± 0.1[b]	1.3 ± 0.4 [a]
Total saturated[1]	15.5 ± 0.3	16.1 ± 0.2	15.7 ± 0.1	16.1 ± 0.8
16:1n-7	3.0 ± 0.0[a]	3.0 ± 0.0 [a]	2.8 ± 0.1[b]	2.6 ± 0.1[c]
18:1n-9	38.4 ± 0.3[b]	38.8 ± 0.1[b]	41.1 ± 0.7[a]	41.4 ± 0.6[a]
18:1n-7	2.7 ± 0.0	2.7 ± 0.1	2.7 ± 0.2	2.7 ± 0.2
20:1n-9	4.5 ± 0.0[a]	3.8 ± 0.1[b]	3.6 ± 0.0[c]	3.3 ± 0.1[d]
22:1n-11	4.6 ± 0.0 [a]	3.7 ± 0.1[b]	3.4 ± 0.0 [c]	3.0 ± 0.1[d]
22:1n-9	0.7 ± 0.0 [a]	0.7 ± 0.0[a]	0.6 ± 0.0[b]	0.6 ± 0.0[b]
Total monoenes[2]	54.6 ± 0.1	53.4 ± 0.2	54.8 ± 1.0	54.3 ± 0.5
18:2n-6	15.0 ± 0.1	15.4 ± 0.1	15.1 ± 0.3	15.1 ± 0.2
20:3n-6	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:4n-6	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
Total n-6 PUFA[3]	15.7 ± 0.1	16.1 ± 0.1	15.8 ± 0.3	15.8 ± 0.3
18:3n-3	5.6 ± 0.1[b]	5.7 ± 0.1[b]	5.8 ± 0.2[b]	6.2 ± 0.1[a]
18:4n-3	1.0 ± 0.1[a]	0.9 ± 0.0 [ab]	0.8 ± 0.1[b]	0.8 ± 0.0[b]
20:5n-3	4.1 ± 0.1	4.3 ± 0.1	3.9 ± 0.3	3.9 ± 0.1
22:5n-3	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
22:6n-3	3.0 ± 0.1[a]	2.9 ± 0.0[a]	2.5 ± 0.2[b]	2.3 ± 0.1[b]
Total n-3 PUFA[4]	14.2 ± 0.3	14.4 ± 0.2	13.7 ± 0.8	13.8 ± 0.4
Total PUFA	29.9 ± 0.4	30.5 ± 0.3	29.4 ± 1.1	29.6 ± 0.5

796 Results are means ± SD (n = 6). Values within a row with different superscript letters
797 are significantly different as determined by ANOVA. [1]Totals include 15:0 present at
798 up to 0.2 %; [2]Totals include 16:1n-9, 20:1n-7 and 24:1n-9 present at up to 0.3%;
799 [3]Totals include 18:3n-6, 20:2n-6, 20:3n-6 and 22:5n-6 present at up to 0.3 %;
800 [4]Totals include 20:4n-3 present at up to 0.2 %.
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