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Screening study to predict the optimum inclusion of air classified faba bean protein concentrate in feeds for Atlantic salmon (*Salmo salar*)

C. De Santis[a*], K. Ruohonen[b], D.R. Tocher[a], S.A.M. Martin[c], E. Król[c], C.J. Secombes[c], J.G. Bell[a], A. El-Mowafi[b], V. Crampton[b]

[a] Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, Scotland UK

[b] EWOS Innovation, 4335 Dirdal, Norway

[c] Institute of Environmental and Biological Sciences, University of Aberdeen, Tillydrone Avenue Aberdeen AB24 2TZ, Scotland UK

[*] Corresponding author: christian.desantis@stir.ac.uk

Abstract

The limited availability of marine ingredients means that new and improved raw materials with high potential to replace fishmeal (FM) are required. Faba bean (*Vicia faba*) is a legume with good potential that has previously been tested in fish species with some promising results. The present study aimed to determine whether an air-classified faba bean protein concentrate (BPC, 615 g kg^{-1} crude protein content) could offer improved or favourable growth performance and physiological responses compared to the main commercially used protein sources, FM and soy protein concentrate (SPC), in Atlantic salmon (*Salmo salar*). The trial investigated the performance of 16 feeds formulated with varying FM/SPC/BPC proportions using a mixture design approach. Salmon parr of average weight 1.47 g were used as a model. The trial lasted eight weeks and also included high FM (560 g kg^{-1}) and high defatted soybean meal (360 g kg^{-1}) feeds as negative and positive controls respectively, for the assessment of gut inflammation. The results demonstrated conclusively that total inclusion levels of BPC ranging from 50 to 200 g kg^{-1}, partially replacing SPC and/or FM, displayed the greatest potential to be beneficial in terms of fish performance and nutrient composition with increased growth, protein content, fat content and ash. In addition to favourable whole-body composition parameters, it was found that inclusions of BPC below 340 g kg^{-1} of feed did not cause detrimental effects such as the gut inflammation observed in fish fed the high soybean meal control. High Inclusion level (450 g kg^{-1}) of BPC caused a mild gut inflammation that was not as severe as that caused by the feed with high soybean meal. The results of this screening study indicate that BPC derived from faba beans can be a valuable alternative protein source in Atlantic salmon feeds. The data provided a platform to model the optimum range of BPC inclusion levels in combination with FM and SPC for further investigation in commercially relevant fish and conditions.

Keywords: faba bean protein concentrate, air classification, fishmeal replacement, mixture models, enteritis

Highlights of the manuscript (3-4 lines max 85 characters)

1. Performance of Atlantic salmon fed air-classified faba bean protein concentrates was tested
2. Optimum range of inclusion with potential commercial application was identified
3. High inclusion of air-classified faba beans cause mild gut inflammation
4. Gut inflammation caused by faba bean was not as severe as that caused by soybean

1. Introduction

The steady decline or stagnation of wild-caught fisheries and the consequent limited availability of marine ingredients such as fishmeal (FM) allied with the growth of farmed fish production have stimulated the increasing use of plant products as protein sources for formulated fish feed (Gatlin *et al.*, 2007, pp. 551-579). Due to the nutritional requirement of fish and manufacturing constraints, plant products with high protein content and amino acid compositions similar to that of FM are extremely sought after. In particular, soy protein concentrate (SPC) processed from defatted, alcohol washed soybeans has proved to be an excellent source of protein efficiently utilized by carnivorous fish such as salmonids (Glencross *et al.*, 2005, pp. 211-220). Along with good nutritional profiles, SPC has the advantage, unlike many other plant protein concentrates, of being available in sufficient volumes to fulfil the rising demand and allow a high level of inclusion in commercial European salmonid and marine fish feeds. As a result, modern commercially available feeds for species such as Atlantic salmon (*Salmo salar*) often contain 200-250 g kg^{-1} SPC as a dominating substitute of FM, meaning that feed manufacturers heavily rely upon this plant product as a source of replacement (Ytrestøyl *et al.*, 2014,).

Heavy reliance upon a single ingredient can have important nutritional and commercial implications. Nutritionally, the abundant use of a single vegetable/plant alternative as a replacement to FM can translate into higher concentration of specific types of anti-nutritional factors (ANFs) that potentially exceeds the level physiologically tolerable by the fish. Several adverse effects have been reported in fish fed plant-derived ANFs, including amongst other reduced nutrient digestibility, pancreatic hyperactivity and inflammation of the distal intestine (RW.ERROR - Unable to find reference:174; Baeverfjord and Krogdahl, 1996, pp. 375-387; Gu *et al.*, 2014, pp. 432-444; Knudsen *et al.*, 2007, pp. 2261-2267; Kortner *et al.*, 2012, pp. 101-6148-8-101). It is well known that different plant products may vary in content and composition of ANFs, with unrefined or defatted soybean meal containing arguably the highest levels (Francis *et al.*, 2001, pp. 197-227). In this respect, using a combination of plant protein sources might increase the number of ANF species in the feed while reducing the concentration of each individual ANF. This may enable fish to cope better with lower levels of a mixture of ANFs rather than higher levels of any individual factor. In addition to biological/nutritional benefits, being able to rely upon multiple raw

materials might translate into greater flexibility when formulating the feeds, into increased purchasing power and also into mitigating the negative effects of any undesirable between-batch differences upon final feed quality. All these characteristics are highly desirable from a commercial perspective. For the above-mentioned reasons it is critically important to investigate new and improved raw materials with high potential to be used as a protein source in fish feeds.

Faba bean (*Vicia faba* L.) also known as field bean, horse bean or broad bean is a legume with good potential as a protein substitute for inclusion in salmon feeds. Faba bean crops are grown in a variety of climates including both in Northern Europe and North America (FAO STAT, 2009,) and they can be successfully used in crop rotation to help reduce the use of nitrogen fertilisers made from fossil fuel sources. A significant proportion of the ANF present in faba beans are concentrated in the seed coat, which is removed in the process of de-hulling (Vidal-Valverde *et al.*, 1998, pp. 140-145). Meals from whole or de-hulled faba beans (FBM) have been investigated as replacement for FM or soybean meal (SBM) in feeds for omnivorous and carnivorous fish species such as Nile tilapia (*Oreochromis niloticus*) and rainbow trout (*Oncorhynchus mykiss*) fingerlings with attractive results (Azaza *et al.*, 2009, pp. 174-179; Gaber, 2006, pp. 986-993; Ouraji *et al.*, 2013, pp. 161-165). One important limitation of FBM, however, is the relatively low protein concentration (~30%) that does not allow a formulation that easily fulfils the total dietary protein required in modern commercial feeds for carnivorous species. To overcome this limitation, additional processing is required in order to obtain a product with higher protein concentration.

The process of fine-grinding and air-classification has been utilized previously to produce a protein concentrate from faba beans (herein termed bean protein concentrate, BPC) with a highly desirable profile (i.e. 55-60% protein content) for inclusion in commercial salmon feeds (Gunawardena *et al.*, 2010, pp. 660-670). The advantage of the air classification technology is that there is no requirement for solvents or acids, thus making it a relatively simple and low-cost process. Such simplicity also means that ANFs are not likely to be degraded, but may co-purify with proteins and possibly be present in higher concentration in the final BPC compared with the original de-hulled material. Despite the potential advantages associated with the use of faba beans in combination with air-classification technology, there have been no reported studies assessing the applicability of this product,

individually or in combination with other plant protein products, as an ingredient for Atlantic salmon feeds. In contrast, air classification was applied to another legume, the field pea (*Pisum sativum*), and tested in salmon feeds. Promising results were reported when inclusions of pea protein concentrates with up to 50% crude proteins were moderate (i.e. 200 g kg^{-1}) but higher levels (350 g kg^{-1}) in combination with other plant products induced gut inflamation (Øverland *et al.*, 2009, pp. 305-311; Penn *et al.*, 2011, pp. 267-273).

The present study was the first aiming to provide evidence to support the commercial application of air classified BPC in salmon feeds. In particular, this research sought to determine whether mixtures of different protein sources including air classified BPC, SPC and FM could offer improved growth and favourable physiological responses compared to feeds formulated using only FM and SPC. In particular, and as a first step before working with seawater fish, a screening trial, using salmon parr, investigating the performance of 16 experimental feeds and two controls was performed. The primary aim was to identify the ideal range of BPC inclusion levels that could be later tested in conditions more relevant to a commercial environment. Small fish were used as a model justified by the dramatic reduction of costs that allowed a large number of feeds to be tested in parallel and not for a specific evaluation of the life-stage requirements. The feeding trial utilized a mixture design approach which allowed a structured investigation of varying proportions of blends of FM, BPC and SPC. The effects on growth performance, body nutrient composition, survival and fish health, specifically gut histology, were determined. Total inclusion levels of BPC ranging from approximately 50 to 200 g kg^{-1}, partially replacing SPC and/or FM, displayed the greatest potential to be beneficial in terms of fish performance and nutrient composition with increased growth, protein content, fat content and ash. Histological evidence of detrimental effects on gut health was only observed at high Inclusion level (450 g kg^{-1}) of BPC however the inflammation was not as severe as that caused by the feed with high soybean meal.

2. Materials and Methods

2.1. Ingredients, design and feed composition

In this experiment, feeds were designed using a mixture design approach with three factors. This experimental design was indicated as the most appropriate strategy to maximize the information on interactions between feed components (Ruohonen and Kettunen, 2004, pp. 145-151). The main advantage of the mixture design approach is the possibility to empirically predict the response to any combination of the blend, exclude those with the least promising potential and focus only on those displaying sub-optimal performances. To apply this particular experimental design, feeds required to be formulated using a variable and a fixed component. The fixed component (constituting 350 g kg^{-1} of the feed) was the same for all feeds and not expected to affect the observed response, whilst the variable component (constituting 650 g kg^{-1} of the feed) included the raw materials under examination (Table 1). The fixed component comprised FM Norse LT-94 (120 g kg^{-1}), wheat gluten (80 g kg^{-1}), tapioca (63 g kg^{-1}), fish oil (62 g kg^{-1}), mineral, vitamin and pigment premixes (25 g kg^{-1}) (the source of each ingredient is reported in Tables 1 & 2). In each blend of the mixture design, the proportion of each raw material summed to 100 % (or 1000 g kg^{-1}). Due to the different composition of the raw materials used (Table 1), three analogues or pre-mixes were formulated containing either FM, SPC or BPC along with other materials to allow them to be substituted for each other without altering the nutrient composition of the final feeds. Analogues were given the abbreviations FM_a, SPC_a and BPC_a, respectively (Table 2). From this point forward the suffix “a” is used to distinguish the analogue from the raw material on which it was based. The analogues were formulated to have identical digestible proteins, digestible energy, and the amino acids lysine, methionine + cystine and threonine (Table 3). Thus, since both SPC and BPC had a low concentration of some indispensable amino acids (especially methionine) compared to that of FM, synthetic amino acids were also added. Tapioca meal and mono calcium phosphate were used as diluents. The independent variable used for statistical modelling of the sixteen experimental feeds was the mix of analogues used in each of the feeds and expressed as a proportion of the total variable component.

The design of the experiment is illustrated in Fig. 1.

, where each dot represents a feed and the numbers within the mixture space show the relative proportion of each analogue to the variable component of the feed. Upper and lower limits for the inclusion of each analogue were set prior the experimental design and the optimal design solved using the D-optimality criterion (Hardin and Sloane, 1993, pp. 339-369). Experimental feeds were named "XX : YY : ZZ" where XX, YY and ZZ represented respectively the percent of FM_a, SPC_a and BPC_a used in the variable component of the feed (XX + YY + ZZ always equals 100). The composition of the 16 experimental feeds is given in Table 4. Two reference feeds, termed "FMref" and "HiSBM" were also produced to assist in the assessment of soybean meal induced enteritis. The FMref was intended to act as a negative control, with a high inclusion of fishmeal but no extracted soybean included in this feed. Alternatively, HiSBM (360 g kg^{-1} extracted SBM) was used as a positive control for gut inflammation. The specific formulation of these reference feeds is shown in Table 5.

2.2. Fish rearing and sampling

The feeding trial was conducted at the facilities of EWOS Innovation (Dirdal, Norway). Tank size was 0.6 m x 0.6 m containing approximately 60 L and supplied with well aerated freshwater, with the mean water temperature of 13°C (range 12.7–13.2°C). Fish were transferred to the allocated tanks (approximately 150 fish per tank) and allowed to acclimate to their new environment for two weeks before they were bulk weighed at the initiation of the trial (average weight 1.47 g), and fed experimental or reference feeds. Mortalities were counted daily, and bulk weights were recorded fortnightly. At the end of the eight weeks trial, the number of fish per tank was recorded and the initial numbers calculated in retrospect from the final number plus mortalities. Each of the 16 experimental feeds was fed to four replicate tanks while the two reference feeds were fed to three replicate tanks. Feed was given in excess of appetite. For whole body proximate analysis, three batches of fifty fish were randomly selected from the source tank (at the start of the study) and from each experimental tank (end of the study). In addition, for the FMref feed, 90 fish were sampled from each tank and pooled by weight class into three groups (small, medium, large) of 30 fish per tank. This was done in order to estimate the dependency of body composition parameters from fish weight. Prior to sampling, fish were starved for 24

h. The average whole body weight of each group was recorded and fish frozen at -20°C prior to whole body proximate analyses.

For histological analyses and determination of gut inflammation, intestinal samples were dissected from a subset of treatments chosen to have a fixed amount of FM_a (20%) and increasing level of BPC_a substituting SPC_a (20:80:00, 20:60:20, 20:40:40, 20:20:60, 20:00:80). In addition, the two reference treatments FMref and HiSBM were also sampled as negative and positive enteritis controls, respectively. The gastrointestinal tract from 10 randomly selected fish from each tank on the above-mentioned treatments was removed. The distal intestine was cleaned from adipose tissue, fixed in phosphate-buffered formalin and then stored in 70 % ethanol at room temperature for subsequent histological examination.

2.3. Biochemical compositions of raw materials, feeds and fish

Crude protein in raw materials and in the whole fish body were analysed using a Leco TruMac N machine (Leco Corporation, Michigan, USA) to measure nitrogen content and then converted to protein using a factor of 6.25. Fat and starch in raw materials and fat and moisture in whole fish body were determined by Near Infra-red equipment (NIR XDS rapid content analyser, FOSS, Hilleroed, Denmark) after calibration with traditional methods to produce an in-house (EWOS Innovation) model. Amino acid composition was taken as the average of several published sources, corrected where necessary to the amino acid content as a proportion of analysed crude protein. Samples of feeds were ground before determination of proximate composition according to standard procedures (AOAC, 2000). Moisture content was obtained after drying in an oven at 110 ˚C for 24 h and ash content determined after incineration at 600˚C for 16 h. Crude protein content was measured by determining nitrogen content (N x 6.25) using automated Kjeldahl analysis (Tecator Kjeltec Auto 1030 analyzer, Foss, Warrington, UK), and crude lipid content determined gravimetrically after Soxhlet lipid extraction (Tecator Soxtec system 2050 Auto Extraction apparatus).

2.4. Histological examination

Tissues were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to standard histological techniques. Transverse sections (5 µm) of the

distal gut were cut with a microtome and mounted onto microscope slides. These sections were then subjected to haematoxylin and eosin (H&E) staining, combined with Alcian Blue staining at pH 2.5 to enhance the contrast between goblet cells and supranuclear vacuoles. All sections were digitized at a total magnification of 50, 100 and 200x, using a Zeiss Axioskop 40 light microscope equipped with a ProgRes microscope camera and the associated image capture software (ProgRes Capture Pro 2.8.8 for Windows PC). The resultant images were randomised to ensure blinded examination and scored using a semi-quantitative system previously developed to assess the degree of SBM-induced enteritis in Atlantic salmon (Urán *et al.*, 2009, pp. 733-744). This system consists of six histological parameters (sub-epithelial mucosa, mucosal folds, lamina propria, eosinophilic granulocytes, goblet cells and supranuclear vacuoles), with each parameter scored on a scale from 1 (no enteritis) to 5 (severe enteritis).

2.5 Statistical methods

Scheffe's mixture polynomials with increasing complexity (null, linear, quadratic and special cubic models) were fitted to the weight gain and body composition data, and the most plausible model was selected on the basis of likelihood ratio tests. Ordinary model diagnostics were carried out. All the models were fitted with the *lm* function of the R language (R Core Team, 2013,). Histology scores were modelled with multilevel ordinal regressions, which were used because multiple fish were sampled from each tank. Ordinal regression was used because the scores are ordered multinomial variates. The estimation was conducted with the ordinal package (Christensen, 2012,) of the R language. Four nested mixture polynomial models were used to describe growth and body composition parameters data for the fish fed the 16 experimental feeds. These models were: *model 1*) only the mean response; *model 2)* linear mixture model; *model 3*) quadratic mixture model and *model 4*) special cubic mixture model. Detailed results of the statistical tests for models used in this investigation are provided as supplementary material (Supplementary Tables 1-4). The most plausible model was chosen based on the principle of parsimony. Clustering of the histology scores was carried out by the EM algorithm that does not require a pre-set number of clusters but finds the number of clusters from the data. This analysis was carried out with the WEKA data mining tools (Hall *et al.*, 2009,).

3. Results

3.1. Survival

Mortality was very low for fish fed the FMref feed where only one fish died. Survival was generally good also for fish fed the experimental feeds with only four tanks showing more than ten mortalities. However, mortalities also included fish that escaped the tank by jumping through the gap between the automatic feeder and the tank covering and so are not associated with feed quality.

3.2. Growth

By the end of the trial, fish fed the FMref feed had grown to an average of 14.4 g corresponding to nearly a 10-fold weight gain in the 8 weeks period. This growth indicated good quality conditions and stock and thus observed differences in performance were likely to reflect the nutritional quality of the feeds. For weight data the special cubic model was chosen as the most plausible. The contour plot of the cubic model showed that there was a relatively large plateau without a steep drop that was indicative of maximum growth performance, 11.02 g/fish (Fig. 2). The model indicated that the optimum inclusion level of BPC would be 31% FM_a, 48% SPC_a and 21% BPC_a (200.8 g kg^{-1} FM, 268.9 g kg^{-1} SPC, 117.4 g kg^{-1} BPC). On the plot, starting from any point of the left edge of the design space and moving right horizontally is equivalent to substituting BPC_a in place of SPC_a on a 1:1 basis, that is without any change in FM_a. It was evident that such a substitution in the use of plant protein sources gave an increase in weight gain until an inclusion level of about 20% BPC_a (111.8 g kg^{-1} BPC). The observed benefits on growth were not evident at 40% BPC_a (223.6 g kg^{-1} BPC) and weight gain at this level of inclusion was similar to that at 0% BPC_a (with FM_a inclusion unchanged). At levels of inclusion higher than 40% BPC_a (223.6 g kg^{-1} BPC) a reduction in weight gain was evident and the most detrimental effects were observed above 60% BPC_a (335.4 g kg^{-1} BPC).

3.3. Body composition

Whole body composition analyses indicated that there was a wide variation in the raw data between treatments as well as considerable within-treatment variation (data not shown). In order to reduce this variation and distinguish the effect associated with weight of fish rather than the feed on proximate composition, the three size groups of fish fed FMref (small, medium, large) were used to predict the dependency of whole body proximate parameters from weight. These data were used to generate a predictive model that indicated that for every 1 g increase in weight, fat increased by approximately 12.2%, moisture decreased by 16.1% and ash decreased by 0.56% (supplementary Table 2). In contrast, protein and phosphorus composition did not show any dependency on fish weight. In other words, fish preserved body protein content but increase fat content at the expense of moisture and, to a very small extent, ash content. These relationships were calculated for fish fed the FMref diet and were assumed to also apply to the remaining experimental feeds.

Nested models of increasing complexity (as for the weight gain analyses above) were fitted and compared to examine the effects of feed composition on whole body proximate composition parameters. The likelihood ratio tests suggested that the quadratic mixture model (model 3) was the most plausible for both protein and fat. Analysis of body protein composition revealed the presence of a large plateau where proteins represented approximately 16.5% of whole body weight (Fig. 3). This value decreased gradually as composition moved towards the edges and more dramatically towards the right edge of the figure where concentrations of BPC_a were higher. Whole body fat concentration was also affected by feed composition. As fat content was significantly dependent on body weight, body weight itself was provided as a co-variate into the model (Fig. 4). Using weight as a co-variate may, however, over-compensate the adjustment and actually explain some of the effects of weight. Whatever the merits of using weight as a co-variate, it was clear that there was a region depicting higher fat content located approximately in the centre of plot. A decreased level of fat content was obtained by adding either more BPC_a or SPC_a to the feed composition (moving to the right or left of the plot, respectively). This effect was less evident when weight was used as a co-variate. In addition, a slight increase in body fat composition was also noted in response to modifying the amount of FM_a in the feed.

3.4. Histological evaluation of gut health

Six different aspects of enteritis development were scored via histological analyses including sub-epithelial mucosa (SM), lamina propria (LP), eosinophilic granulocytes (EG), mucosal folds (MF), goblet cells (GC) and superanuclear vacuoles (SV). In most fish on all dietary treatments at least 70 % of the samples received a score 1 for SM, score 2 for LP, score 3 for EG and scored 2 or 3 for MF (supplementary Fig. 2). These features of soybean-induced enteritis clearly did not develop in this study and were not statistically different between treatments, including positive and negative controls. In contrast, for GC and SV a significantly higher score was detected using ordinal regression. Specifically, fish fed HiSBM and 20:00:80 had significantly higher scores for GC and SV compared to those fed FMref (Fig. 5 & Fig. 6). Fish fed all other experimental treatments (that is where BPC_a was less or equal than 60 %) received scores generally lower than those of fish fed the FMref.

4. Discussion

The present study investigated whole-body performance and intestinal health of Atlantic salmon fed diets formulated with air-classified faba bean protein concentrate (BPC) and represented the first milestone towards the commercial application of this ingredient in salmon feed manufacture. A mixture design using salmon parr as a model enabled the screening of a 16 different feed formulations differing in raw ingredient proportions and provided an empirical model to predict fish performance in response to various combinations of dietary FM, SPC and BPC. It was demonstrated that BPC inclusion levels ranging from approximately 50 to 200 g kg^{-1} of feed partially replacing SPC and/or FM displayed the greatest potential to be beneficial in terms of fish growth performance and nutrient composition, and were unlikely to cause detrimental effects such as gut inflammation. These results provided an empirically determined range of "optimal" FM, SPC and BPC inclusion levels (proportions) in salmon feed that should be investigated further in commercially relevant conditions.

The primary finding of the present study was the beneficial effects observed on several performance parameters (i.e. weight gain, protein and fat content) in response to moderate BPC inclusion (50-200 g kg^{-1}) substituting FM and/or SPC. A recent study reported on the response to dietary de-hulled faba bean meal (FBM) in a salmonid species, rainbow trout (Ouraji *et al.*, 2013, pp. 161-165). Interestingly, it was found that moderate inclusion of FBM replacing mainly SBM and, to a minor extent FM, was beneficial for growth in a similar way to that observed in the present study in Atlantic salmon. The authors modelled, utilizing broken-line analysis, that an inclusion of 141 g kg^{-1} FBM represented the optimum for maximizing performance in rainbow trout. Whole body proximate compositions were also positively affected in trout where an increase in protein content from 16.1 % to 16.6 % was reported as well as an increase of fat and ash content. In contrast to the results in salmonids, dietary whole FBM did not improve growth in freshwater species such as Nile tilapia (*Oreochromis niloticus*) (Azaza *et al.*, 2009, pp. 174-179). However, moderate inclusions of FBM did not negatively affect tilapia performance and dramatically reduced production costs and detrimental effects were only evident at high inclusion levels. These results strongly support our hypothesis that fish might cope better with lower levels of a mixture of ANFs rather than higher levels of any individual ANF. This is evident from the fact

that, while moderate dietary inclusions of faba bean products such as BPC or FBM can lead to beneficial effects on fish performance, significant growth impairment and detrimental effects are generally observed when higher levels are used.

Herein, it was also shown that inclusion of high proportions of BPC (i.e. > 500 g kg^{-1}) were detrimental to fish performance, resulting in a weight gain about 3.5 g less than that obtained with moderate inclusions (5-200 g kg^{-1}), which was approximately 11 g/fish during the 8 weeks period. It is unlikely that this reduced performance can be associated with the different nutrient profiles of the raw materials used as these were carefully balanced in the feeds. While the complete amino acid profile was not analyzed in this study, it was verified that some of the most limiting and important amino acids such as methionine, threonine and lysine, were similar and that the feeds had comparable levels of digestible protein and energy. Alternatively, the most likely explanation is that the observed detrimental effects may have been caused by levels of one or more species of ANFs. The production process for the BPC used in this trial was to de-hull the dry beans, finely grind them and separate the larger, starch-rich particles from the smaller, protein-rich particles in a stream of air. No solvents and very little heating were involved, suggesting that the ANFs generally present in the native kernels may have co-purified with the protein fraction.

Faba beans are known to contain ANFs, although the levels might be substantially lower than that of soybean (El-Shemy *et al.*, 2000, pp. 515-524), and some ANFs may be largely eliminated due to the fact that they are contained in the hull (Vidal-Valverde *et al.*, 1998, pp. 140-145). Important levels of tannins (tannic acid and condensed tannins) for example were found in the FBM tested in Nile tilapia which was not de-hulled (Azaza et al., 2009). Specifically, condensed tannins are one of the ANF mainly present in the hull (Alonso et al., 2000). We may hypothesize that the BPC used in the present study is less likely to contain this particular class of tannins as hulls were removed during the manufacturing process. However, condensed tannins appear to be not as detrimental for fish growth and performance as tannic acid, which is instead more abundant in the seed and can dramatically affect the feed intake in fish as shown for carp (*Cyprinus carpio*) (Becker and Makkar, 1999;Ferruzzi et al., 2010). In the present study, feed intake was not measured but future seawater trials should test the possibility that reduced growth may be simply associated with reduced feed intake.

Other possible candidate ANFs that might have affected growth include the glucosides, vicine and convicine (Duc G., 1991;LATTANZIO et al., 1983), or haemagglutinins (Bhatty and Christison, 1984), as these are thermostable and known to be associated with the protein fraction. Phytic acid, saponins or other phenolic compounds were also found to be present in faba bean (Azaza et al., 2009). Some of these ANFs such as saponins are well-studied and associated with intestinal inflammation or enteritis. In the present study, a reference treatment (feed) including SBM (HiSBM) that was known to induce this pathology was included to compare its effects to those caused by the raw materials tested (i.e. SPC, FM and BPC). Using an established assessment method to score fish "blind", those fish fed the HiSBM feed clearly developed gut inflammation or enteritis, but this condition did not occur with the fish fed the FMref feed or with the fish fed high SPC and no BPC. However, when BPC was included in the feeds at the highest concentration (447.2 g kg^{-1}) fish developed signs of gut inflammation, although these were not as severe or comparable to the condition caused by the HiSBM feed. It was however interesting that fish fed intermediate treatments containing a mix SPC-BPC content were generally not different from the FMref. On the contrary, treatments with mix SPC-BPC inclusion showed a marginal improvement at least based on SV scores (significant at inclusion of 111.8 g kg^{-1} and 335.4 g kg^{-1}).

It is noteworthy that air-classified pea protein concentrate also induced inflammation of the distal intestine in Atlantic salmon when included above a level of 350 g kg^{-1} of feed, but not at lower (130 g kg^{-1}) levels of inclusion (Penn et al., 2011). Similarly to what observed in the present study using BPC, the pea protein concentrate did not cause symptoms as severe as those induced by high levels of SBM. These results may indicate that the air classification technology is perhaps not particularly effective in removing ANFs present at high levels, but can produce protein concentrates at low cost that are of acceptable quality if used at moderate inclusion levels in feeds. When fed SBM, Atlantic salmon parr do not develop gut inflammation as severe as that observed in post-smolts and we therefore cannot exclude the possibility that different results might be observed in seawater even using similar levels of inclusion (Bakke, 2012; Crampton *et al*., 2014).

The primary aim of the present study was to determine if an opportunity existed to reduce dietary inclusion levels of SPC and/or FM by using air-classified BPC through screening a large number of feeds using Atlantic salmon parr as a model. It can be concluded that an

inclusion level of BPC ranging from 50 to 200 g kg^{-1} of diet substituting SPC and FM increased weight gain and reduced the incidence of intestinal inflammation in Atlantic salmon. The data generated from this investigation will be used to identify an appropriate range of replacement levels to be applied in an in-depth seawater study. Investigation of feeds formulated with a similar range of BPC inclusion levels as those mentioned above in post-smolt in saltwater will clarify if similar results can be obtained in more commercially relevant conditions, supporting the interest generated in BPC as a new and appropriate raw material. Furthermore, in addition to the performance parameters presented in this study, further analyses of hepatic and intestinal transcriptomes will further characterise the biochemical and molecular responses of salmon to BPC. These data will provide further insight into other physiological effects of BPC including those not evident or measurable at macroscopic level. These data will further define the parameters that should be more closely monitored in long-term commercial trials.

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Legends to Figures

Figure 1. A schematic diagram showing the design of the study, with red dots representing 16 experimental feeds. FM_a, fish meal analogue; SPC_a, soy protein concentrate analogue; BPC_a, faba bean protein concentrate analogue. Numbers within the mixture space show the proportion that each analogue contributes to the variable component of the feed. The variable component contributed to 65% of the total feed ingredients.

Figure 2. Contour plot showing weight gain (g/fish) calculated using the special cubic model. The feeds in the study are shown by the symbol ”X”. The contour lines join points of equal growth. Lighter colours denote regions with higher weight gain. For graphical representation of the raw data see supplementary figure 1.

Figure 3. Contour plot showing the whole body protein content of fish calculated using the quadratic mixture model. The feeds in the study are shown by the symbol ”X”. Lighter colours denote regions with higher protein content.

Figure 4. Contour plots describing the percentage of fat in the whole body of fish depending on the inclusion of analogues in the final feed. Whole body weight was used in the model as a co-variate. For results that do not take body weight into account as a co-variate see Supplementary Figure 2. Lighter colours denote regions with higher fat content.

Figure 5. Modelled effect of feeds on the goblet cells (GC) and supranuclear vacuoles (SNV). Mean and 95% confidence interval are shown. Positive number means a more severe effect. Red dotted line denotes the mean score assigned to fish fed HiSBM while blue dotted line that of fish fed FMref. For visualization of raw scores of all parameters measured in this study see supplementary figure 2.

Figure 6. Representative histological images of distal intestine from Atlantic salmon fed with FMref (A), BPC_a80 (B) and HiSBM (C) diets for 8 weeks. The images show the progression of goblet cells (GC) and supranuclear vacuoles (SV) from basal levels (A, scattered GC and large SV) through the increased number of GC and reduced size of SV (B) to highly abundant GC and extinction of SV (C), indicating a healthy gut (no enteritis), mild enteritis and severe enteritis, respectively. The differences in GC and SV histological scores between the three dietary treatments were significant at $p < 0.05$ (for details, see Results). Scale bar = 200 µm.

Table 1. Composition in g kg^{-1} of the fish meal, soya and bean protein concentrates used in the experimental feeds.

	FM[a]	SPC[b]	BPC[c]
Crude Protein	705.9	627.8	615.8
Crude Fat	95	29	27
Starch	-	67	3.3
Selected Amino Acid composition			
Lysine	53.3	38.5	40.4
Methionine	19.1	8.3	4.2
Cystine	6.5	8.8	7.8
Threonine	58.7	24.6	21.8
Tryptophan	6.7	7.2	4.3

[a] Norse LT-94, Norsildmel AS, Bergen, Norway

[b] Imcopa International, Av. das Araucárias 5899, CEP 83.707-000, Araucária, Paraná, Brazil.

[c] Fabaqua, Sotexpro, La Croix Forzy, Bermericourt, 51220, France.

Table 2. Composition in g kg^{-1} of the three analogues termed FM_a, SPC_a and BPC_a.

	FM_a	SPC_a	BPC_a
Fishmeal[1]	788	-	-
Soy Protein Concentrate[1]	-	862	-
Bean Protein Concentrate[1]	-	-	860
Tapioca[2]	100	-	2
Mono calcium phosphate[3]	112	52	52
Methionine[4]	-	6	9
Lysine 78%[5]	-	10	10
L-Threonine[6]	-	-	3
Fish oil[7]	-	70	64
Sum	1000	1000	1000

[1] See previous table for source.

[2] Hoff Norsk Potatindustri, Gjøvik, Norway

[3] Tessenderlo Group, Brussels, Belgium

[4] Evonik Industries AG, Essen, Germany

[5] Ajinomoto Eurolysine S.A.S. Paris, France.

[6] Evonik Industries AG, Essen, Germany

[7] Norsildmel A/S, Bergen

Table 3. Calculated nutrient content (g kg^{-1} unless otherwise stated) of the three analogues termed FM_a, SPC_a and BPC_a.

	FM_a	SPC_a	BPC_a
Crude Protein	556	555	551
Digestible Protein	500	499	496
Crude fat	76	95	88
Starch	87	17	28
DE (kJ/g)	155	155	153
Lysine	42	41	43
Methionine	15	13	13
Cystine	5	8	6
Threonine	23	21	22
%DE from Protein	76.1	76.1	76.8

DE = Digestible Energy

Table 4. Composition of the variable component (65%) of the feed in g kg^{-1} of the 16 experimental feeds. See Table 2 for the specific composition of the analogues; see Fig. 1 for a schematic layout of these feeds. For detailed explanation of feed nomenclature see paragraph 2.1.

	10:90:00	20:80:00	31:69:00	40:60:00	00:90:10	20:60:20	00:75:25	40:31
FM_a	65	130	201	260	-	130	-	260
SPC_a	585	520	449	390	585	39.00	484	200
BPC_a	-	-	-	-	65	130	166	190
Sum	650	650	650	650	650	650	650	650
	07:55:38	20:40:40	20:20:60	40:00:60	00:34:66	20:00:80	00:10:90	10:00
FM_a	47	130	130	260	-	130	-	65
SPC_a	355	260	130	-	222	-	65	-
BPC_a	248	260	390	390	428	520	585	585
Sum	650	650	650	650	650	650	650	650

The fixed ingredients totalled 350 g kg^{-1} and were the same for all the above feeds and comprised the following, units in g kg^{-1}: Fishmeal (Norse LT-94) 120, Wheat Gluten 80, Tapioca 63, Fish Oil 62, Mineral, vitamin and pigment premixes 25.

To convert the amount of analogue into the actual amount of raw material used it is sufficient to multiply the amount (g kg^{-1}) showed in table 4 by the corresponding amount (g kg^{-1}) of raw material in the specific analogue of interest reported in Table 2. It follows, for example, that the content of BPC in the feed 20:00:80 was 520 g kg^{-1} x 860 g kg^{-1} = 447.2 g kg^{-1}. Note: when calculating FM inclusion a further 42 g kg^{-1} is to be added due to the amount of FM included in the fixed component of the feed.

Table 5. Composition in g kg^{-1} of the two reference feeds used in the assessment of soybean meal induced enteritis.

	FMref	HiSBM
Fishmeal	560	440
wheat gluten	80	-
SPC	160	-
Extracted soybean meal	-	360
Tapioca	80	80
Fish oil	90	90
Premixes	30	30
Sum	1000	1000

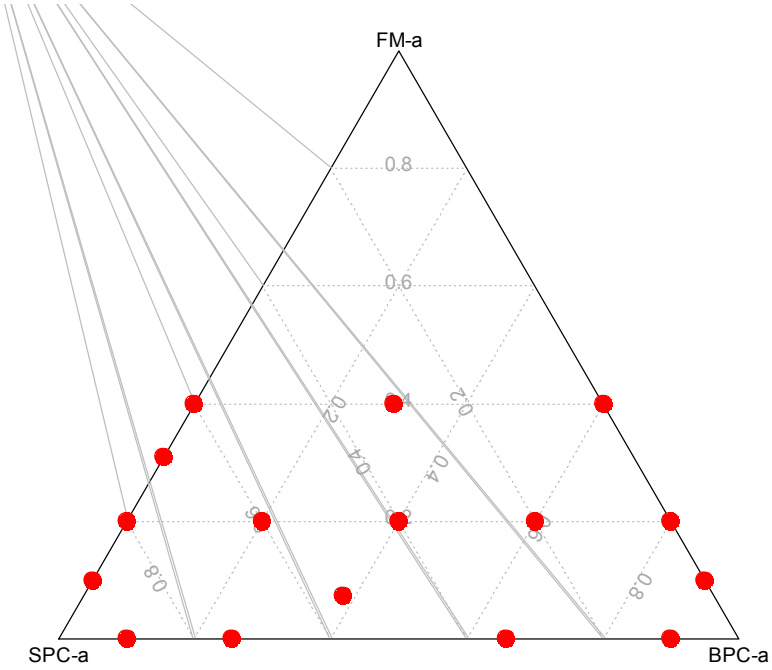
Table 6. Analysed proximate and calculated amino acid composition of feeds in g kg^{-1}. Note protein, fat, ash and starch are on a dry matter basis.

Moisture	65	74	73	70	74	96	78	115	86
Protein	560	560	560	561	549	554	554	561	554
Oil	125	131	120	120	123	121	125	122	119
Ash	101	101	108	116	93	104	92	115	98
Starch	84	84	94	98	85	89	76	92	78
Lysine	34	34	34	34	34	34	34	35	35
Methionine	12	12	12	12	12	12	12	12	12
Cystine	7	7	7	7	7	7	7	6	7
M+C	19	19	19	19	19	19	19	19	19
Threonine	19	19	19	19	19	19	19	19	19
	20:40:40	20:20:60	40:00:60	00:34:66	20:00:80	00:10:90	10:00:90	FM ref	HiSBM
Moisture	80	84	53	50	75	87	93	87	107
Protein	559	557	561	555	559	558	564	597	547
Oil	120	121	114	121	123	125	125	167	143
Ash	104	105	117	93	104	94	98	94	100
Starch	87	81	81	66	68	63	75	75	80
Lysine	35	35	35	35	35	35	35	37	34
Methionine	12	12	12	12	12	12	12	13	11
Cystine	7	6	6	7	6	6	6	6	5
M+C	19	18	18	18	18	18	18	20	16
Threonine	19	19	20	19	19	19	19	22	19

See text for meaning of Feed abbreviation.

"M+C" means methionine plus cysteine.

Fig. 1.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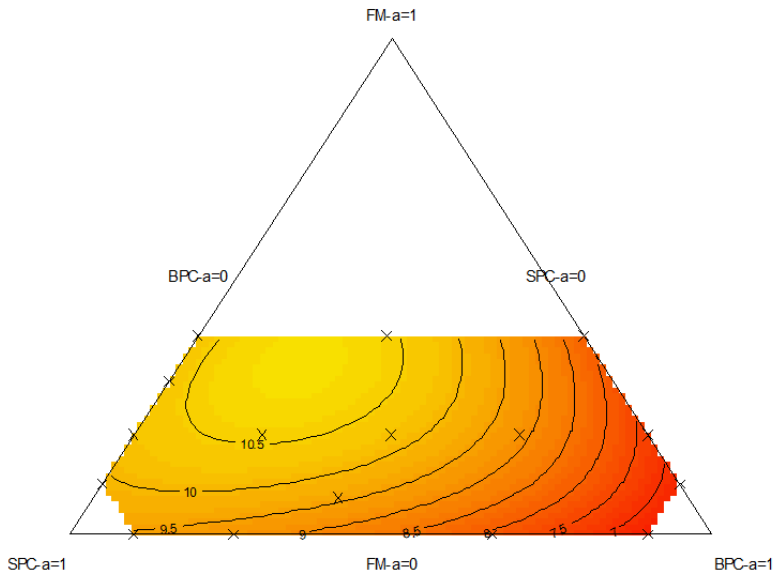
0.4
0.2
SPC-a
BPC-a

Fig. 2.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BPC-a=0
SPC-a=0
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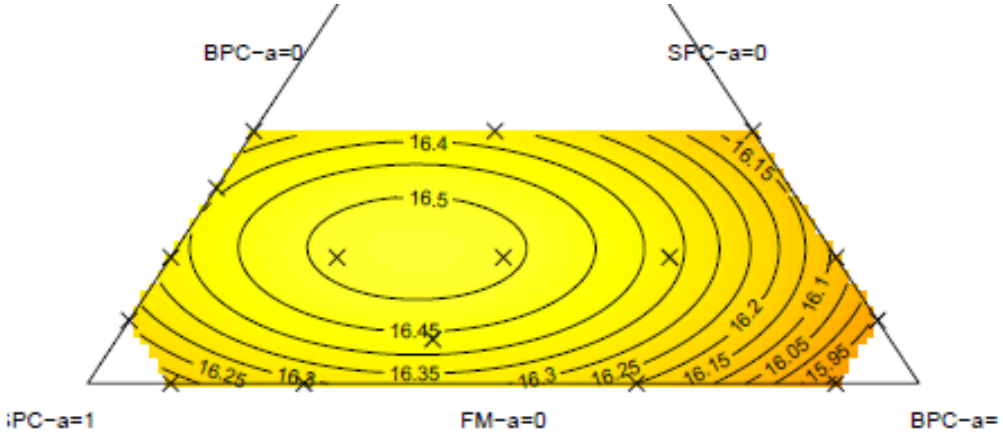
10
9.5
9
8.5
8
7.5
7
SPC-a=1
FM-a=0
BPC-a=1

Fig. 3.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16.45
16.35
16.3
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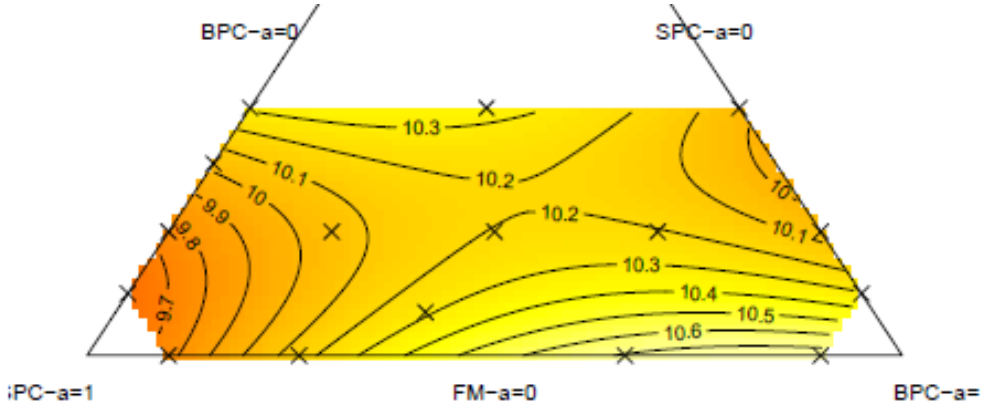
16.15
16.1
16.05
15.95
iPC−a=1
FM−a=0
BPC−a=

Fig. 4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SPC−a=0
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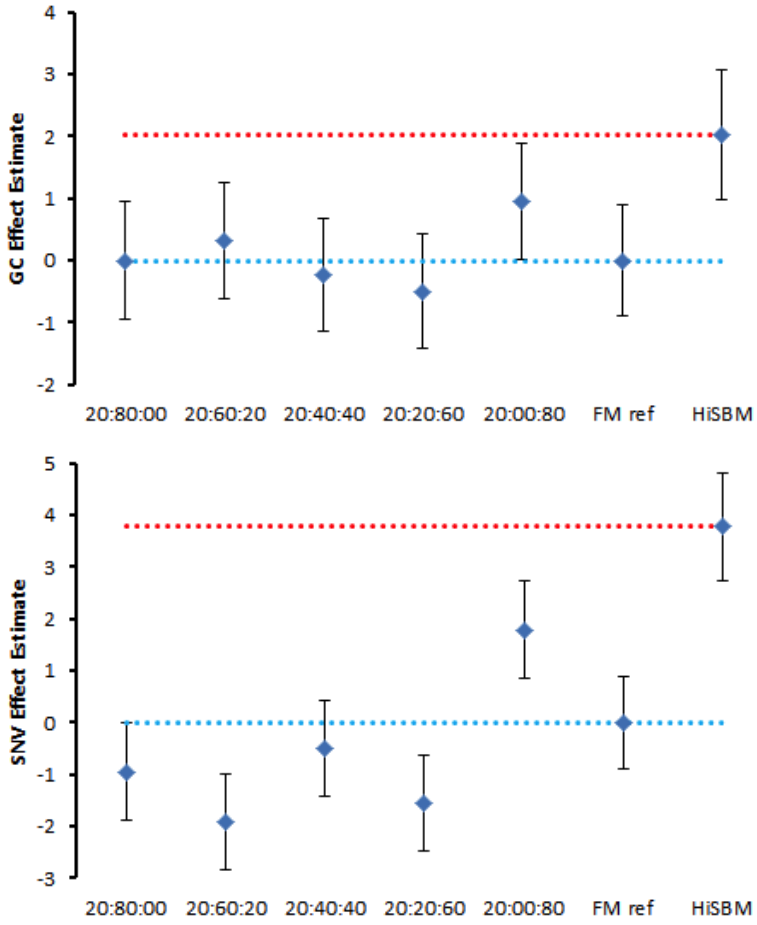
10.2
10.1
10
9.9
9.8
9.7
10.4
10.5
10.6
iPC−a=1
FM−a=0
BPC−a=

Fig. 5.


GC Effect Estimate
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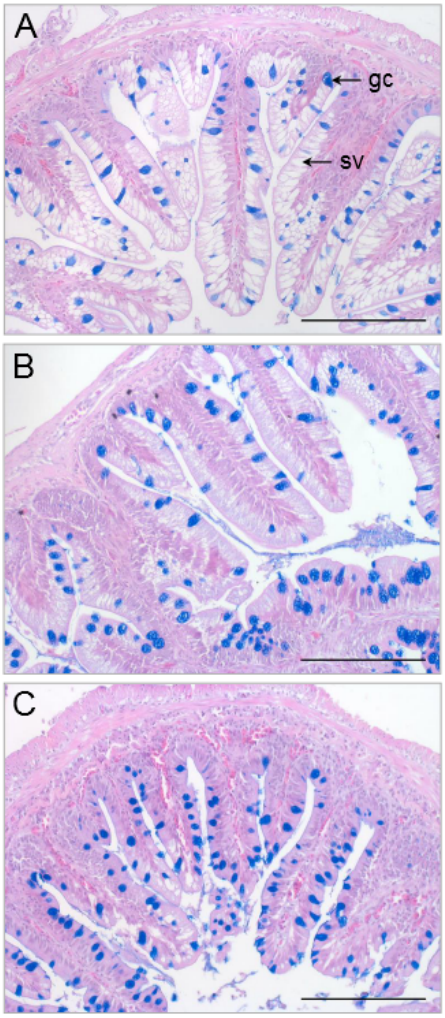
3
2
1
0
-1
-2
20:80:00
20:60:20
20:40:40
20:20:60
20:00:80
FM ref
HiSBM
SNV Effect Estimate
5
4
3
2
1
0
-1
-2
-3
20:80:00
20:60:20
20:40:40
20:20:60
20:00:80
FM ref
HiSBM

Fig. 6.



Supplementary Material

Supplementary Table 1. *Statistical analysis of the nested polynomial models tested to describe weight gain of the fish fed the 16 experimental feeds.*

Model	Res Df	RSS	Df	SumofSq	F	Pr (>F)	
1	61	136.949					
2	59	46.205	2	90.744	91.8982	< 2.2e-16	***
3	56	30.121	3	16.084	10.8592	1.047e-05	***
4	55	27.155	1	2.966	6.0083	0.01744	*

"Res Df" is residual degrees of freedom, "RSS" is residual sum of squares, "Df" is additional model degrees of freedom, SumofSq" is the sum of squares, "F" is the F-statistic score and "Pr (>F)" is the probability that the change in F score could have occurred by chance. the order of rows is sequential following the nested structure of the models. Asterisks represent standardised methods to describe Pr (>F).

Supplementary Table 2. *Results of statistical analysis for the effect of weight upon proximate composition of the three size groups of fish from three individual tanks fed the FMref feed. Where a significant (P<0.05) relationship was detected, the slope and its standard error are shown.*

Nutrient	intercept	Std err, intercept	slope	Std err, slope
Protein	16.48	0.09	-	-
Fat	9.56	0.38	0.122	0.024
Moisture	73.25	0.22	-0.161	0.014
Ash	2.30	0.009	-0.0056	0.0005
Phosphorus	0.343	0.002	-	-

Supplementary Table 3. *Statistical analysis of the nested polynomial models tested to describe whole body protein content of fish.*

Model	Res Df	RSS	Df	SumofSq	F	Pr(>F)	
1	63	8.50					
2	61	7.71	2	0.79	3.53	0.036	*
3	58	6.53	3	1.18	3.50	0.021	*
4	57	6.38	1	0.15	1.33	0.25	

See Supplementary Table 1 for meaning of column headings.

Supplementary Table 4. *Statistical analysis of the nested polynomial models tested to describe whole body fat content of fish. The upper part of the table shows the analysis when whole body weight is not offered as a co-variate whilst the lower part shows the results when it is offered.*

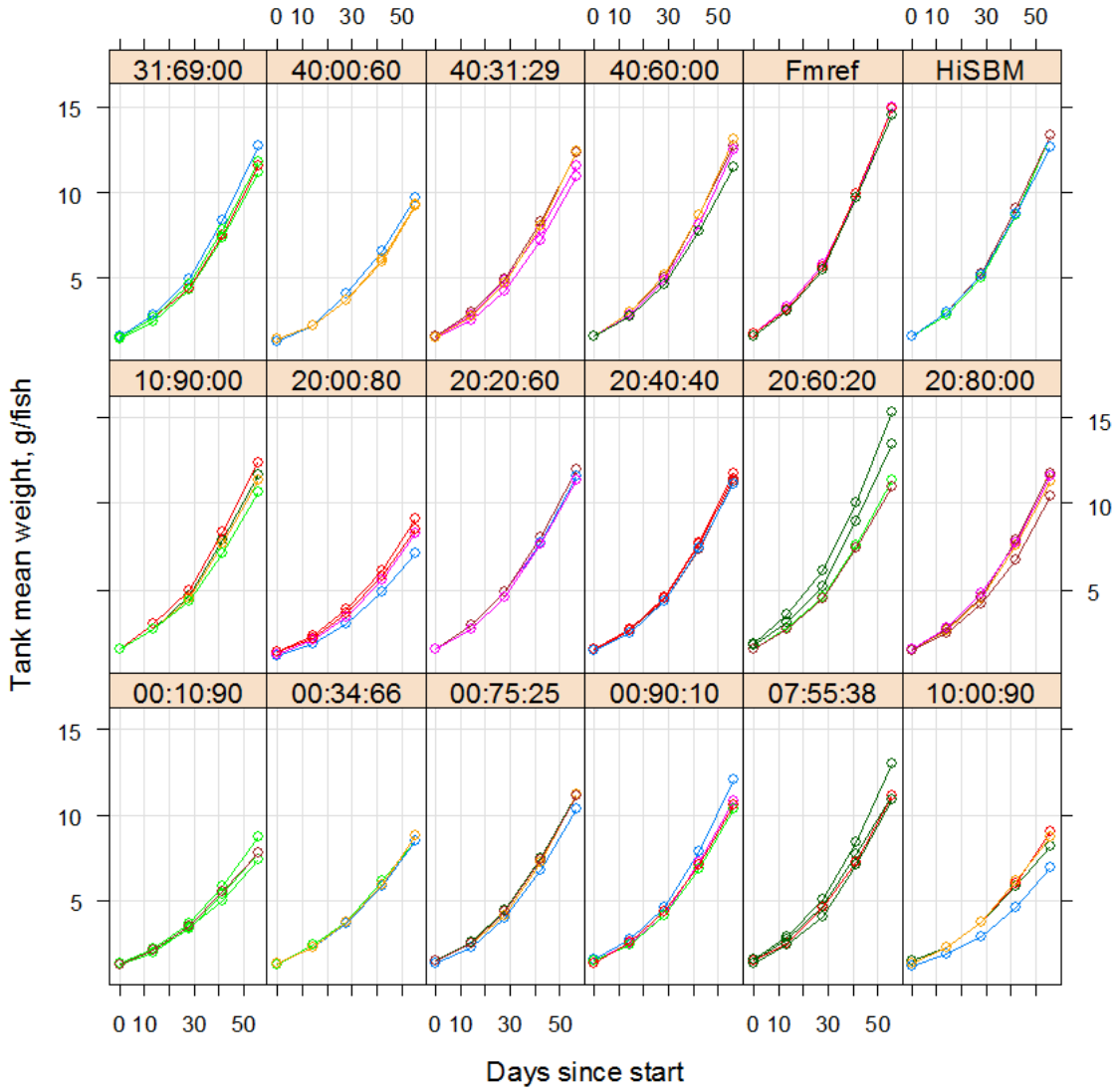
*Whole body weight of fish **not** offered as a co-variate*

Model	Res Df	RSS	Df	SumofSq	F	Pr(>F)	
1	63	9.4					
2	61	8.9	2	0.55	2.3	0.105	.*
3	58	7.1	3	1.75	5.0	0.004	**
4	57	6.9	1	0.42	3.6	0.06	.

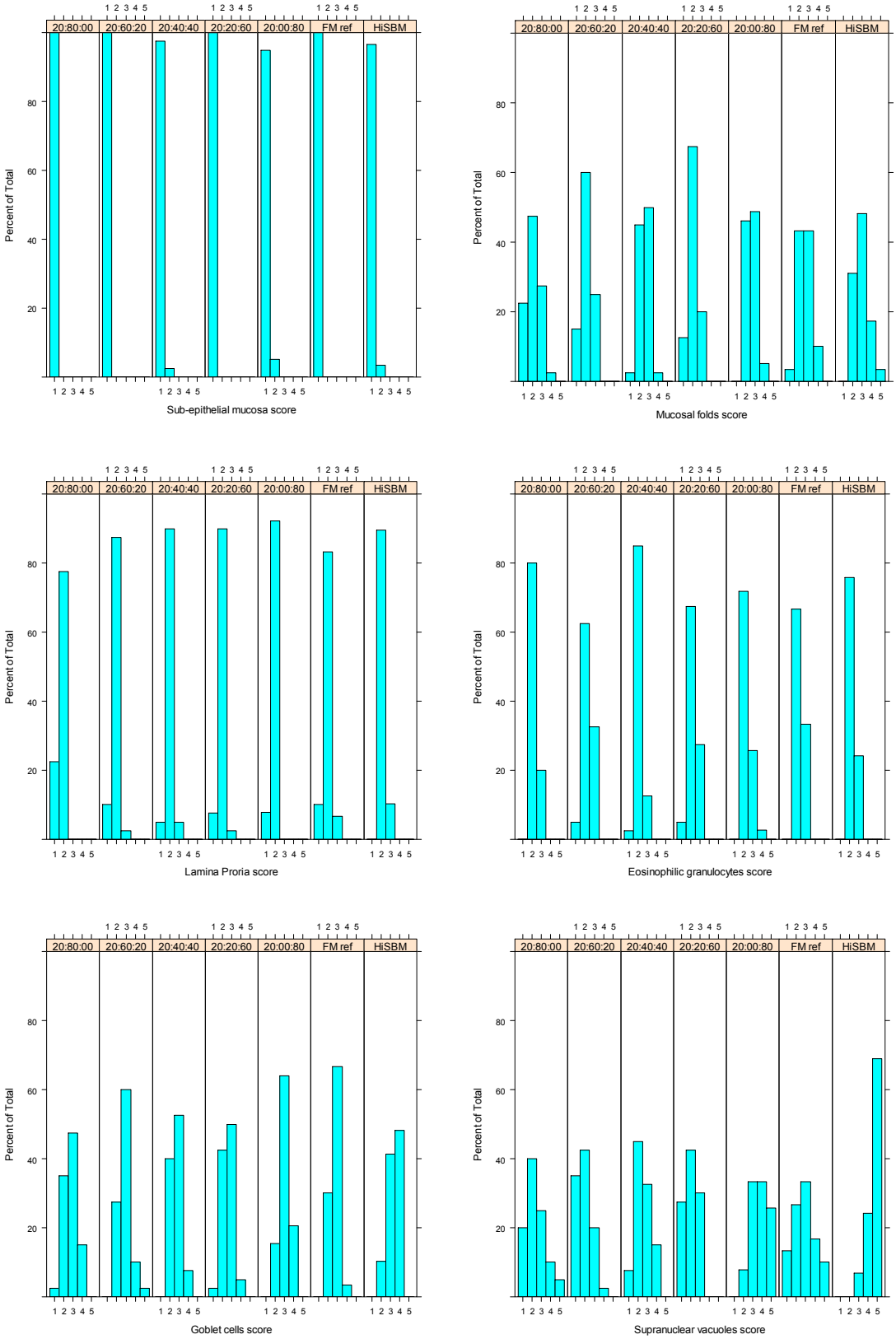
Whole body weight of fish offered as a co-variate

Model	Res Df	RSS	Df	SumofSq	F	Pr(>F)	
1	62	8.3					
2	57	7.2	5	1.1	2.2	0.066	.
3	51	5.0	6	2.3	4.0	0.002	**
4	49	4.7	2	0.3	1.5	0.224	.

See Supplementary Table 1 for meaning of column headings.



Supplementary Figure 1. Panel plot of raw data showing growth of fish. Different colour lines denote data from different tanks.



Supplementary Figure 2. Histograms summarising the scores of SM, MF, LP, EG, GC and SNV for each of the seven treatments on which fish were assessed. To avoid cluttering the charts, scores are not detailed by replicate tank. Fish from four replicate tanks were assessed for the experimental feeds and three replicate tanks for the two reference feeds.