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The effects of increasing dietary levels of amino acid-supplemented soy protein concentrate and constant dietary supplementation of phosphorus on growth, composition and immune responses of juvenile Atlantic salmon (*Salmo salar* L.)

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Abstract Diets with 50 (SPC50), 65 (SPC65) and 80 % (SPC80) substitution of prime fish meal (FM) with soy protein concentrate (SPC) were evaluated against a commercial type control feed with 35 % FM replacement with SPC. Increases in dietary SPC were combined with appropriate increases in methionine, lysine and threonine supplementation, whereas added phosphorus was constant among treatments. Diets were administered to quadruplicate groups of 29 g juvenile Atlantic salmon were exposed to constant light, for 97 days. On Day 63 salmon were subjected to vaccination. Significant weight reductions in SPC65 and SPC80 compared with SPC35 salmon were observed by Day 97. Linear reductions in body cross-sectional ash, Ca/P ratios, and Ca, P, Mn and Zn were observed at Days 63 (prior vaccination) and 97 (34 days post-vaccination), while Mg presented a decrease at Day 63, in salmon fed increasing dietary SPC. Significant reductions in Zn, Ca, P and Ca/P ratios persisted in SPC65 and SPC80 compared with SPC35 salmon at Day 97. Significant haematocrit reductions in SPC50, SPC65 and SPC80 salmon were observed at Days 63, 70 and 97. Enhanced plasma haemolytic activity, increased total IgM, and a rise in thrombocytes were demonstrated in SPC50 and SPC65 salmon on Day 97, while increased lysozyme activity was demonstrated for these groups on Days 63, 70 and 97. Leucocyte and lymphocyte counts revealed enhanced immunostimulation in salmon fed with increasing dietary SPC at Day 97. High SPC inclusion diets did not compromise the immune responses of salmon, while SPC50 diet also supported good growth without compromising elemental concentrations.

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Introduction

As yet, plant proteins have been the most economically viable alternatives to fish meal (FM) (Ytrestøyl et al. 2015), representing 37 % of salmon diets, which

translates to approximately 67 % of protein from plants (Ytrestøyl et al. 2015). From this percentage, about 38 % is covered by soy protein concentrate (SPC) (Ytrestøyl et al. 2015). However, the ever-increasing FM prices compared with the stable or decreasing soybean prices could potentially improve even more the cost efficiency of SPC over FM, making SPC the dominant protein source in salmon feeds (FAO 2015). SPC is an exceptionally digestible protein source with high protein content and favourable amino acid profile compared with FM (Dersjant-Li 2002; Ytrestøyl et al. 2015). It is produced by aqueous alcohol extraction of the soluble carbohydrates in defatted soy flakes, thus increasing their protein content while reducing the concentrations of anti-nutritional factors (ANFs) such as trypsin inhibitors, lectins, saponins and soy antigens linked to enteritis (Drew et al. 2007). Studies have demonstrated no changes in the growth of Atlantic salmon fed diets with up to 75 % substitution of FM by SPC (Refstie et al. 1998; Storebakken et al. 1998, 2000; Refstie et al. 2001). Moreover, it has been reported that diets with even 100 % substitution of FM with SPC did not promote any incidences of soybean-induced enteritis in salmonids (Krogdahl et al. 2000; Escaffre et al. 2007).

Additional information is required regarding the effects of increasing dietary plant protein levels on the growth performance, proximate and elemental compositions of Atlantic salmon (NRC 2011; Prabhu et al. 2013, 2014). Overall, juvenile salmon appear to be more sensitive to dietary soy protein inclusion due to the increasing presence of phytic acid in soy products and the immature state of their digestive tract (Storebakken et al. 1998, 2000; Burr et al. 2012). Phytic acid bound on the protein fraction of soybeans is the main ANF found in SPC (Storebakken et al. 1998). Almost three-fourths of the P in soybeans is found in the form of phytic acid, which not digestible by peptic enzymes, thus decreasing the availability of P (Francis et al. 2001). Phytic acid inhibits protein hydrolysis and forms complexes with positively charged cations, proteins and amino acids, reducing their availability for the fish (Francis et al. 2001; Riche and Garling 2004). Muscle is the main storage tissue for most nutrients, while skeleton consists in the principal elemental store in fish. Therefore, any changes in nutrient retention could be reflected in the composition of these tissues. Herein, body cross-sections from the

end of the dorsal fin to the start of the anal fin including muscle, bone, skin and scales [in a similar manner as Norwegian quality cutlet (NQC) samples are utilised for flesh quality control in market size salmon] were used for the compositional assessment of both tissues as a whole. Elemental data were compared with whole-body concentrations which are generally used as the golden standard in order to assess this novel methodological approach (Shearer et al. 1994; Helland et al. 2005, 2006).

In general, there is a lack of information regarding the effects of high dietary levels of plant proteins on the overall health status of Atlantic salmon. Previously, Krogdahl et al. (2000) reported increased lysozyme and total IgM levels in the intestinal mucosa of seawater-adapted Atlantic salmon maintained on feeds with 30 % of dietary protein from soy products (SBM and SPC) compared with FM-fed salmon. Moreover, SPC-fed salmon demonstrated improved resistance against *Aeromonas salmonicida* compared with their FM- and soybean meal (SBM)-fed counterparts. While studies on the effects of dietary plant proteins on the immune status of Atlantic salmon are still scarce, the majority of these were focused on the effects of these feedstuffs on the innate immune responses of fish that have not been stimulated immunologically (Thompson et al. 1996). However, determining immune responses shortly or later post-immunisation (via vaccination in the present study) may highlight dietary modifications that were not evident before (Thompson et al. 1996).

Vaccination in the present study was primarily used in order to provide immunological stimulation and secondarily to reproduce commercial conditions, as most farmed salmon are vaccinated for disease prevention during the freshwater stage (Poppe and Koppang 2014). In fish subjected to vaccination, normal skeletal elemental deposition could be decreased if these nutrients are required for metabolic and immunomodulatory processes (Lall 2003; Kiron 2012). Overall, the negative effects of vaccination on bone development and mineralisation depend on fish size at vaccination (Berg et al., 2006; Grini et al. 2011), the type of the vaccine (Aunsmo et al. 2008), and the culture conditions (Grini et al. 2011). With regard to the latter variable, studies have shown that both vaccinated and non-vaccinated salmon exposed to high water temperatures (15–16 °C) (used in order to boost salmon growth) are more prone to changes in

their vertebral structure and composition which translates to a higher prevalence of skeletal pathologies (Ytteborg et al. 2010; Grini et al. 2011). Exposure to continuous light is another common commercial practice, targeting the prevention of maturation and/or smoltification in juvenile salmon but has also been associated with an enhancement in salmon growth (Kråkenes et al. 1991; Bromage et al. 2001; Berrill et al. 2003; Stefansson et al. 2007; 2008). Fjelldal et al. (2005, 2006) reported that Atlantic salmon post-smolts reared in seawater cages under a continuous light regime, promoting faster growth, demonstrated lower vertebral mineralisation compared with fish exposed to natural photoperiod. Therefore, salmon exposure to intensive rearing regimes, aiming faster growth, could negatively affect skeletal mineralisation and development.

The main aim of the study was to assess the growth and health of juvenile Atlantic salmon fed increasing dietary SPC levels (50, 65 and 80 % of dietary protein from SPC) against a commercial type control diet with 35 % of protein from SPC, under constant light, a temperature of 13 °C and an intensive feeding regime. While the commercial implementation of salmon diets with higher than 65 % of protein from SPC is improbable, due to the high price of this ingredient compared to other FM alternatives, they were used in this context in order to amplify responses, which could highlight nutritional deficiencies. Health was evaluated by assessing immune responses and body cross-sectional composition prior to (Day 63) and post-vaccination with a commercial vaccine [Day 97/34 days post-vaccination (dpv)].

Materials and methods

Diets and growth trial

Four experimental diets were prepared. These were formulated so that aqueous alcohol-extracted SPC (Imcopa, Paraná, Brazil) supplied 35, 50, 65 and 80 % of total dietary protein (termed SPC35, SPC50, SPC65 and SPC80, respectively) with high-quality FM providing the rest of the dietary protein in all cases. Diet formulations are provided in Table 1. SPC35 served as the control diet. The rationale behind the use of SPC35 as a control treatment was to mimic commercial salmon dietary compositions with inclusion of plant proteins. In

this case, SPC as a sole plant-derived FM substitute was chosen due to its premium nutritional value. In previous trials, SPC35 treatment had been tested against EWOS commercial feed formulations and it was found to perform equally well as its commercial counterparts (personal communication Viv Crampton). The diets were manufactured by EWOS Innovation, Dirdal, Norway, and were formulated so that protein/fat ratios were constant, whereas lysine, methionine, and threonine were supplemented to give the same AA/protein ratios across dietary treatments. The amount of supplemented dicalcium phosphate was constant among the diets, representing 3 % of all experimental dietary formulations, exceeding Atlantic salmon phosphorus (P) requirement (Lall 2003).

The feeding trial was conducted at EWOS Innovation facilities in Dirdal, Norway. For the trial, fresh water was supplied to 16 square tanks with rounded corners each containing approximately 60 litres of water. Water temperature was maintained at 13 ± 1 °C, whilst photoperiod was constant (24 h of light). The diffused oxygen level of inlet water to the tanks was constantly 90–100 % saturation. A total of one thousand nine hundred and twenty (1920 fish) unvaccinated S0 Atlantic salmon parr (from a commercial SalmoBreed strain selected for improved growth performance) of an average weight of 29 g (i.e. 120 fish $\times$ tank^{-1}) were randomly allocated to the 16 tanks. Fish were acclimatised to experimental tanks for 28 days prior to commencing the trial, during which time they were fed a commercial EWOS freshwater diet for salmonids (EWOS micro). During the study period, quadruplicate tanks of fish were fed one of the four experimental diets. Fish were fed with automatic belt feeders, continuously every 435 for 20 s. Feeding time and period within feed intervals were the same for the acclimation and trial period. The daily ration depended on the trial period and fish appetite. A daily ration of 3.5 % of tank biomass was recorded during the acclimation period, while this proportion was decreased to 2.75, 2.5 and 1.5 % of tank biomass during the first, second and third month of the trial period, respectively. A high daily feed ration was used to avoid differences in feed intake often associated with satiation feeding of plant substituted diets (Refstie et al. 1998).

The growth trial was conducted for a total of 97 days, at which time all fish in the tanks were removed and bulk-weighed post-anaesthetisation (metacaine, 30 mg $\times$ l^{-1}) at Days 36, 63 and 97. At

Table 1 Formulation and calculated chemical composition of experimental diets with varying soy protein concentrate levels

	2 mm				3 mm			
	SPC35	SPC50	SPC65	SPC80	SPC35	SPC50	SPC65	SPC80
Feed composition ($\times kg^{-1}$)								
Fish meal[a] (g)	462.86	344.15	230.64	121.98	462.86	344.15	230.64	121.98
SPC[b] (g)	274.64	384.25	489.16	590.97	274.64	384.25	489.16	590.97
Tapioca[c] (g)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Di-calcium phosphate[d] (g)	30.00	30.00	30.00	30.00	30.00	30.00	30.00	30.00
Vitamin and mineral premixes[e] (g)	3.55	3.55	3.55	3.55	3.55	3.55	3.55	3.55
Vitamin C 35 %[e] (g)	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
$MgSO_4$[d] (g)	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Methionine[f] (g)	2.10	3.50	4.50	5.50	2.10	3.50	4.50	5.50
Lysine 78 %[f] (g)	1.80	2.40	2.80	3.30	1.80	2.40	2.80	3.30
L-Threonine[f] (g)	0.60	0.70	0.90	0.98	0.60	0.70	0.90	0.98
Ultralec[g] (g)	10.00	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Fish oil[h] (g)	113.00	120.00	127.00	132.26	113.00	120.00	127.00	132.26
Chemical composition ($\times kg^{-1}$)								
Dry matter (g)	937.4	938.7	932.5	911.9	920.6	907.7	921.3	931.2
In dry matter basis								
Crude protein (g)	504.2	502.1	491.7	471	501.2	488.9	491.2	485.4
Crude fat (g)	175.6	166.2	157.6	144.4	174.3	158.5	151.4	146.1
Crude protein: crude fat ratio	2.9	3	3.1	3.3	2.9	3.1	3.2	3.4
Phytic acid (g)	11.9	13.5	14.6	15.3	11.6	13.1	14.2	15.2
Phytic acid-bound P (g)	3.4	3.8	4.1	4.3	3.3	3.7	4.0	4.3
Ash (g)	108.4	106.3	103	97.1	106.2	102.8	100.9	100.1
Phosphorus (P) (g)	16.6	16.0	15.3	14.6	16.4	16.0	15.5	14.7
Calcium (Ca) (g)	33.4	26.8	22.5	19.0	31.8	27.8	23.1	18.5
Ca/P ratio	2	1.7	1.5	1.3	1.9	1.7	1.5	1.3
Zn (mg)	295.4	285.3	273.4	265.9	287.00	285.5	275.4	268.4
Mg (g)	2.18	2.32	2.39	2.44	2.21	2.34	2.39	2.43
Mn (mg)	83.54	87.32	86.99	86.78	83.87	87.45	86.32	85.89

SPC35 diet with 35 % of dietary protein soy protein concentrate (SPC), SPC50 diet with 50 % of dietary protein from SPC; SPC65 diet with 65 % of dietary protein from SPC, SPC80 diet with 80 % of dietary protein from SPC

* The concentrations of phytic acid and phytic acid-bound P were estimated using a Megazyme Phytate/Total Phosphorus Assay kit (Megazyme, Ireland) following the protocols provided by the company and were then corrected according to the total dietary P values estimated via ICP/MS

[a] Fish meal (Egersund Sildoljefabrikk, Norway) with an apparent protein digestibility coefficient (ADC protein) of 90.2 %

[b] SPC (~62 % crude protein) (Imcopa, Paraná, Brazil) with an apparent protein digestibility coefficient (ADC protein) of 90.8 % (Anti-trypsins <3.0 mg × g^{-1}, Fibre <5.0 mg × g^{-1}, Lectins <0.1 μg × g^{-1}, Saponins = 0 %, Glycinin <3.0 μg × g^{-1}, β-conglycinin <1.0 μg × g^{-1}) (compositional analyses performed by an authorised external laboratory hired by Imcopa)

[c] Tapioca (Hoff Norske Potetindustrier, Gjøvik, Norway)

[d] Dicalcium Phosphate (Normin AS, Hønefoss, Norway)

[e] Vitamin premix and Mineral premix (EWOS AS, Bergen, Norway)

[f] Amino acids (Evonik Degussa International AG, Hanau, Germany)

[g] Ultralec: De-oiled lecithin powder (ADM, Decatur, USA)

[h] Fish Oil (Egersund Sildoljefabrikk, Norway)

Day 63 and after bulk weighing fish from each tank, blood from six individuals per tank was withdrawn for the evaluation of both haematological and immunological responses. After blood sampling, body cross-sections between the end of the dorsal fin and the start of the anal fin, from the same fish, were collected for the commencement of composition analyses in pools of six body cross-sections per tank (4 pools per treatment). Salmon were then intraperitoneally (i.p.) vaccinated with 100 μl of a commercial vaccine (AquaVac^{TM} Furovac, Intervet UK Ltd., Milton Keynes) against *A. salmonicida* in order to simulate commercially applied conditions and most importantly to stimulate immune responses. Thereafter, fish were sampled for haematological and immunological evaluation at Day 70 (7 days post-vaccination) and Day 97 (34 days post-vaccination), while body cross-sections were sampled at Day 97 of the trial.

Sample collection

Blood was withdrawn from the caudal vein of six fish per tank per dietary group on days 63, 70 and 94 after the start of the study (i.e. pre-vaccination and 7 and 34 dpv) using 1-ml syringes rinsed with heparin (10 IU $\times$ ml^{-1}; Sigma-Aldrich, Dorset, UK), and used to assess a variety of haematological and immunological parameters in dietary fish. Haematocrit values were determined for all sampled fish at each sampling point. Total and differential leucocyte counts were determined from only three fish per tank. Three blood smears were prepared for each fish for the determination of differential leucocyte counts. The smears were air-dried and subsequently stained with Rapid Romanowsky stain (Raymond A lamb, Eastbourne, UK) and examined at $1000\times$ magnification to determine the percentage of different leucocyte types and the numbers of these cells per ml of blood according to total leucocyte numbers. Two pooled blood samples per tank were then obtained (2 pools of 1.2 ml of blood from three individuals per tank, giving 8 pools of blood per treatment). The blood was centrifuged at $3000\times g$ for 20 min at 4 °C and pooled serum then aliquoted into seven Eppendorf tubes (around 40–50 μl per tube) and stored at −80 °C until used.

Head kidney samples (approximately 5 mm) from three individuals (from the same tank) were aseptically removed and pooled into plastic 5-ml bijoux containers containing 5 ml of ice-cold Leibovitz medium (L-15; Sigma-Aldrich) and 40 μl heparin (10 IU $\times$ ml^{-1}). Two pools per tank were used for the determination of the superoxide anion (O^{-2}) production by head kidney macrophages (HKMs).

Head kidney macrophage isolation and macrophage respiratory burst activity

For the isolation of HKMs, the method described by Korkea-aho et al. (2011) was used. The superoxide anion (O^{-2}) production by HKM suspensions was measured by the conversion of NBT (Sigma-Aldrich) to formazan, according to the method published by Secombes (1990) with some modifications described by Korkea-aho et al. (2011).

Determination of plasma protein and lysozyme activity

Protein content of plasma was determined by the Pierce BCA (bicinchoninic acid) protein assay kit (Thermo Scientific, IL, USA) based on the conversion of Cu^{2+} to Cu^{1+} under alkaline conditions (Biuret reaction) using bovine serum albumin (BSA) as a standard. Serum lysozyme activity was based on the lysis of lysozyme sensitive *Micrococcus lysodeikticus* as described by Korkea-aho et al. (2011).

Measurement of natural haemolytic activity (complement)

Plasma haemolytic activity used was based on a method described by Sirimanapong et al. (2014) with modifications including a 22 °C incubation temperature of the reaction mixture and the use of four double serial plasma dilutions for the determination of haemolytic activity starting from an 1:4 dilution.

Total plasma immunoglobulin M (IgM) assay

The level of IgM in sera of experimental fish was determined using an indirect enzyme-linked immunosorbent assay (ELISA) described by Sirimanapong et al. (2014), with modifications including the use of 1:500 and 1:1000 plasma dilutions, the application of a monoclonal mouse anti-trout/salmon IgM (F11-Aquatic Diagnostics Ltd, Stirling, Scotland) diluted 1:66 in antibody buffer (1 % BSA in LSWB)

for 1 h at 22 °C, and lastly the use of goat anti-mouse immunoglobulin-G labelled with horseradish peroxidase (HRP) (Sigma-Aldrich) diluted 1: 4000 in conjugate buffer (1 % BSA in LSWB) for 60 min at 21 °C.

Determination of antibody titres against A. salmonicida

An ELISA was used to measure the specific antibody response of Atlantic salmon to the A. salmonicida vaccine using a modification of the method outlined by Adams et al. (1995).

Anti-protease activity

The method used was designed to detect anti-protease activity in trout plasma and was based on the method described by Ellis (1990), modified for use in microtitre plates.

Calculations

Estimated feed conversion ratios (FCRs) in the current trial were based on the feed amount given to the fish and do not represent the actual FCR; therefore, they were not included in the current report or statistical analyses. For simplicity, growth performance was evaluated by monitoring the mean salmon weight from each tank and estimating the weight gain of the experimental salmon groups:

Weight gain (WG):

$$WG = \frac{\text{Biomass final}(g) - \text{Biomass initial}(g)}{\text{Number of fish}}$$

specific growth rate (SGR):

$$SGR = \left(\frac{\ln W1 - \ln W0}{t}\right) \times 100$$

thermal growth rate (TGC):

$$TGC = \left(\frac{\sqrt[3]{W1} - \sqrt[3]{W0}}{(t \times T)}\right) \times 100$$

where $W1$ is the group's average weight at each time point of the feeding trial (g) and $W0$ is the group's average initial weight (g). T is the water temperature (°C), and t is the duration of the experiment (number of days).

Proximate composition analysis

Cross-section samples obtained as described at the end of the "Diets and growth trial" section were stored frozen and then thawed prior to analysis. Feeds were homogenised prior to the analysis. Dry matter and moisture were determined according to standard methods (AOAC 1990) by oven-drying of both homogenised feeds and pooled body cross-sections, for 16 h at constant weight. After drying, the pooled cross-sections were homogenised and used for ash, crude protein, crude lipid and elemental determination. Pulverised body cross-section and feed samples were ashed in a muffle furnace at 550 °C according to (AOAC 1990). Crude protein was estimated by the Kjeldahl method using the Tecator Kjeltec System (AOAC 1990), and crude fat was determined using Soxhlet extraction with chloroform: methanol (2:1 v/v) (Christie 2003). Minerals and phosphorus from homogenised feed and dried body cross-section pools were determined using inductively coupled plasma mass spectroscopy, ICP–MS, with collision cell technology (CCT) (Thermo X Series 2). One hundred milligrams of pooled body cross-section homogenates was added to Teflon digestion tubes with 5 ml of 69 % nitric acid. The tubes were then put into a microwave digester (Mars Fish digester) for the initiation of the digestion process (Step 1: 21–190 °C for 10 min at 800 W; Step 2: 190 °C for 20 min at 800 W; Step 3: 190–21 °C for 30 min cooling period). Samples from digestion tubes were then poured into 10-ml volumetric flasks and made up to 10 ml with the addition of distilled water. Four hundred microlitres of the diluted digested material was poured into another plastic 10-ml sample tube and made up to a final volume of 10 ml with distilled water prior to measuring the elemental concentration within pooled dried carcass and bone homogenates using ICP–MS. Mineral concentration was calculated as $\mu g \times g^{-1}$ using the following equation:

$$\text{Elemental concentration} \left(\frac{\mu g}{g}\right)$$
$$= \frac{\text{Sample volume (ml)}}{1000} \times \frac{\text{Result from ICP}}{\text{Sample weight (g)}}$$

Dietary phytic acid-bound P levels were estimated using a Megazyme Phytate/Total Phosphorus Assay kit (Megazyme, Ireland). After estimating the % of P

bound to phytic acid from the total amount of P in the diet using the kit, the concentration of P bound to phytic acid and the amount of phytic acid were extrapolated from the concentration of P estimated via ICP/MS.

Statistics

Growth trajectories based on the mean weight estimates at Day 36/prior vaccination, Day 63/prior vaccination and Day 97/34 days post-vaccination were modelled as repeated measures (Crampton et al. 2010; Espe et al. 2012). Growth performance indices (mean daily weight gain, SGR and TGC) were also assessed in a similar manner. Moreover, a hierarchical (multilevel) statistical model was used for body cross-sections composition, haematological and composition data from each tank, since multiple observations from a single tank were available (several pools) (Espe et al. 2012; Nanton et al. 2012; Hartviksen et al. 2014). The statistical analysis was carried out with the help of the R language (R Core Development Team 2014) and its lme4 package (Bates et al. 2013). The statistical approach applied was model based. This means that to find whether any specific effect was statistically significant, data were fitted in three different models with increasing complexity where tank was included as a random effect, sampling time as a categorical variable and the percentage of protein from SPC as a continuous factor possibly having nonlinear effects:

1. a model with only sampling time (Tank considered as a random effect).
2. a model with sampling time and the % of dietary protein from SPC with their interaction (Tank considered as a random effect).
3. a model as above but with an additional quadratic effect of the % of dietary protein from SPC with interactions to reveal any nonlinearity in the response (Tank considered as a random effect).

The three models were nested and compared with a likelihood ratio test (LRT) that evaluated whether the improvement in the likelihood required a more complex model or whether the simpler model could be applied. Models demonstrating possibilities (P values <0.05) were selected for the description of data. The simplest possible model was adopted according to Occam's razor principle. The adopted model was demonstrated by plotting the expected mean response with 95 % confidence intervals. For a categorical effect, these were represented as points with error bars and for a continuous effect as a curve with shaded confidence region. The expected mean and 95 % confidence intervals were solved by a posterior simulation from the adopted statistical model ($n = 1500$ random draws were used throughout this study) (Gelman and Hill 2007).

Results

Growth performance

The mortalities among the four experimental groups of Atlantic salmon were negligible (<1 %) for all groups (Table 2). Figure 1 demonstrates the weight development of the four dietary groups of Atlantic salmon. The models with the linear effect of the dietary percentage of protein from SPC were favoured using the LRTs (likelihood ratio tests) for both expected mean weight of salmon and expected mean daily weight gain (WG) (Statistical models are presented in Fig. 2a, b). The expected mean values of the aforementioned growth parameters with their estimated 95 % confidence intervals (CI) are presented with different colour for each time point or period, respectively. More specifically, the black line (expected mean values) and its corresponding shaded region (estimated 95 % CI) represent the expected mean weight values for Day 36 and the expected mean WG for the pre-vaccination period from Day 0 to Day 36. The values in red represent the mean weight estimates for Day 63 and the expected mean daily WGs for the pre-vaccination period from Day 36 to Day 63, while values in blue demonstrate the corresponding values for Day 97 and the post-vaccination period, from Day 63 (day of vaccination) to Day 97 (34 dpv). Overall, increasing SPC levels resulted in lower mean weight and daily WG in Atlantic salmon juveniles at all time points of the study. Approximate reductions of 12 and 10 % in expected mean weight were observed in SPC80 compared with SPC35 salmon at Day 36 and 63 of the feeding trial, respectively, while an 8 % reduction in weight was demonstrated at Day 97 (34 dpv) between the two dietary groups (Fig. 2a). Moreover, SPC65 and SPC80 salmon exhibited significantly lower mean weights compared with the

Table 2 Performance data of juvenile Atlantic salmon dietary groups

Growth and survival	SPC35	SPC50	SPC65	SPC80	Model selected with $P < 0.05$**
Initial weight (g) (Day 0)	29.2 ± 0.49	28.8 ± 0.60	28.6 ± 0.89	28.8 ± 0.80	–***
Intermediate weight (g) (Day 36)	72.7 ± 3.92	69.1 ± 2.91	67.7 ± 6.80	65.7 ± 6.38	Linear
Intermediate weight (g) (Day 63)	122.8 ± 1.47	118.6 ± 4.23	110.7 ± 11.12	112.8 ± 5.66	
Final weight (g) (Day 97)	174.1 ± 3.56	169.4 ± 5.70	159.8 ± 9.76	162.4 ± 12.03	
Weight gain* (g × fish^{-1} × day^{-1}) (Days 0–36)	1.21 ± 0.10	1.12 ± 0.08	1.08 ± 0.17	1.03 ± 0.16	Linear
Weight gain* (g × fish^{-1} × day^{-1}) (Days 36–63)	2.05 ± 0.16	2.03 ± 0.07	1.76 ± 0.40	1.93 ± 0.09	
Weight gain* (g × fish^{-1} × day^{-1}) (Days 63–97)	1.48 ± 0.09	1.46 ± 0.08	1.43 ± 0.12	1.42 ± 0.21	
Feed given (g × fish^{-1}) (Days 0–36)	20.55 ± 0.42	20.24 ± 0.42	20.08 ± 0.62	20.22 ± 0.56	–
Feed given (g × fish^{-1}) (Days 36–63)	25.83 ± 0.65	25.06 ± 1.06	23.53 ± 2.42	23.83 ± 1.68	–
Feed given (g × fish^{-1}) (Days 63–97)	76.09 ± 1.26	73.14 ± 2.37	67.90 ± 7.76	70.11 ± 3.46	–
Mortalities (%)	0.8	0	0.8	0	–

Data for proximate composition represents mean ± SD for 4 pooled samples (dried, pulverised and blended pools of body cross-sectional homogenates—1 pool of 6 fish per tank). Data for performance represent mean ± SD for four replicate tanks

SPC 35 diet with 35 % of dietary protein from soy protein concentrate (SPC), *SPC50* diet with 50 % of dietary protein from SPC, *SPC65* diet with 65 % of dietary protein from SPC, *SPC80* diet with 80 % of dietary protein from SPC

* Weight gain (daily weight gain) (g fish^{-1} day^{-1}) = total weight of fish within treatment (g) × (Number of fish within treatment)$^{-1}$ × (number of trial days)$^{-1}$

** Raw data were fitted in models with increasing complexity where tank was included as a random effect, sampling time as a categorical variable and the percentage of protein from SPC as a continuous factor possibly having nonlinear (quadratic) effects. The linear model included sampling time and the % of dietary protein from SPC with their interaction, whereas the quadratic model was more complex including an additional quadratic effect of the % of dietary protein from SPC with interactions to reveal any nonlinearity in the studied response. The models were compared with a likelihood ratio test (LRT) to assess if the improvement in the likelihood required a more complex model or whether the simpler model was sufficient for the description of the data. Models demonstrating possibilities (*P* values) <0.05 were selected as the best for the description of these data

*** Not included in the model-based analysis

SPC35 group at all time points. In a similar manner, the reduction in daily WG observed in the high SPC inclusion dietary groups was improved at post-vaccination compared with the SPC35 group. This could be observed by the slope of the WG curve for the post-vaccination period when 8 % reduction in WG for SPC80 compared with SPC35 salmon was recorded. This slope was much shallower compared with the slopes obtained for the pre-vaccination periods, when 17 and 10 % reduction in WG in SPC80 compared with SPC35 salmon was observed (Fig. 2b). Overall, SPC65 and SPC80 salmon demonstrated significantly lower mean daily WG compared with the SPC35 group, for the first (Day 0–Day 63) and second (Day 63–Day 97) period of the feeding trial, while no differences were noted for the last bit of the study, further supporting the improved performance of fish receiving higher SPC levels compared with the SPC35

group. SGR and TGC values for the experimental groups did not present significant differences among the four dietary groups and were only affected by the study period and the developmental stage of the fish. Overall, Atlantic salmon experienced the highest SGR and TGC values during the first part of the study, while the lowest values were demonstrated at the last part of the study and after the vaccination of the fish, while intermediate SGRs and TGCs were exhibited for the second period of the study.

Proximate and elemental composition of pooled body cross-sections

No differences due to the inclusion of SPC in the feeds were demonstrated among dietary groups of salmon in terms of lipid, protein and moisture concentrations, whereas a linear reduction in body cross-section ash

Fig. 1 Observed weight development of the four dietary groups of Atlantic salmon juveniles. Mean salmon weight (g) per dietary treatment with standard deviations at each time point of the study
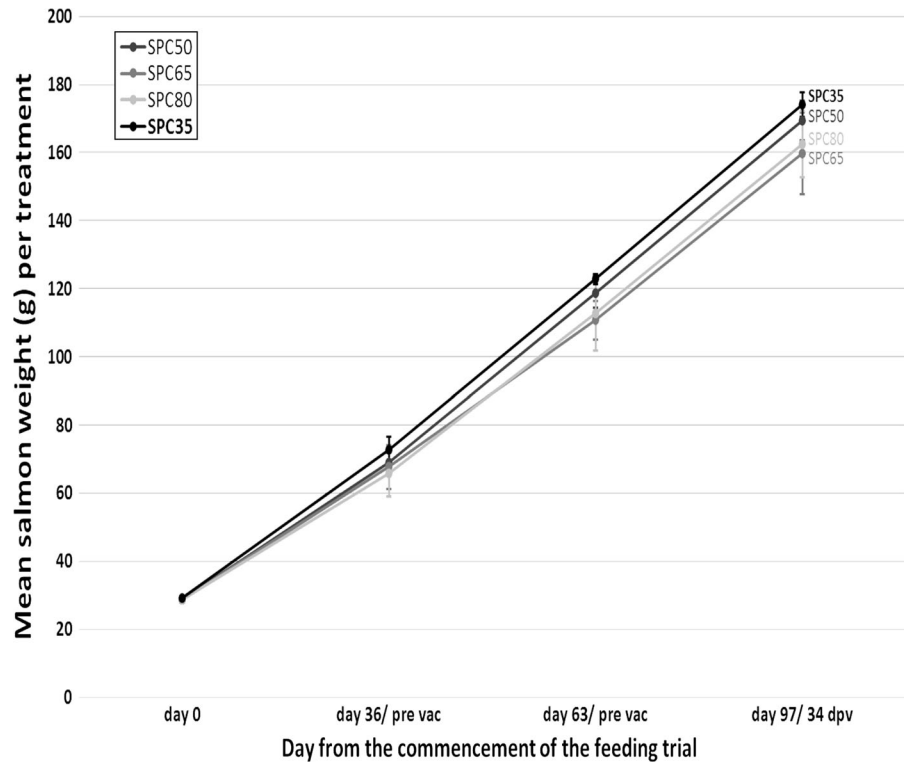
levels both prior to vaccination (Day 63) and at 34 dpv (Day 97) was demonstrated in salmon fed on high dietary levels of SPC (Model is plotted in Fig. 3a). Overall, moisture, lipid and protein concentrations were only affected by time. Expected ash values in salmon juveniles were affected linearly by the increased inclusion of SPC in the diets with the SPC80 group demonstrating a 13 % decrease in credible ash content compared to SPC35 salmon at Day 63. Lower ash levels were demonstrated at Day 97 (34 dpv) for all dietary groups. However, the groups receiving lower levels of dietary protein from SPC were the ones demonstrating the highest reduction in body cross-section ash levels. A 5 % reduction in expected ash concentration was demonstrated in SPC80 compared with the SPC35 salmon at Day 97. Raw data for ash and elemental composition in Atlantic salmon cross-sections are presented in Supplementary Table 1.

Expected calcium-to-phosphorus (Ca/P) ratio and calcium (Ca), phosphorus (P), manganese (Mn) and zinc (Zn) levels in pooled body cross-section samples were affected in a linear fashion by increasing levels of dietary SPC, since the model with linear description of the data was found to describe the data in a better

manner compared with the other two models (Models selected are presented in Figs. 3b–d, 4b, c). Therefore, the dietary groups receiving feeds with higher levels of SPC inclusion exhibited lower amounts of the above-mentioned elements. On the other hand, the model showing a quadratic effect of the percentage of dietary SPC improved the fit over the linear model for body cross-section expected Mg levels (Model selected is plotted in Fig. 4a). Overall, at Day 63, SPC35 salmon demonstrated significantly higher Ca/P ratio and Ca, P and Mg concentrations than the SPC50, SPC65 and SPC80 groups and higher amounts of Zn and Mn in comparison with the SPC65 and SPC80 groups.

In general, lower body cross-sectional elemental amounts were demonstrated for all dietary groups at Day 97 (34 dpv) compared with Day 63 (prior to vaccination). At this time point significantly lower Ca/P ratio and Ca, P and Zn concentrations were recorded for SPC65 and SPC80 salmon compared with the SPC35 group. Despite the observed reduction in body cross-section elemental concentrations at Day 97 compared to Day 63, an overall improvement in expected Ca/P ratio and Ca, P, Mg and Mn levels was demonstrated from the modelled based statistical analysis in vaccinated salmon receiving increased
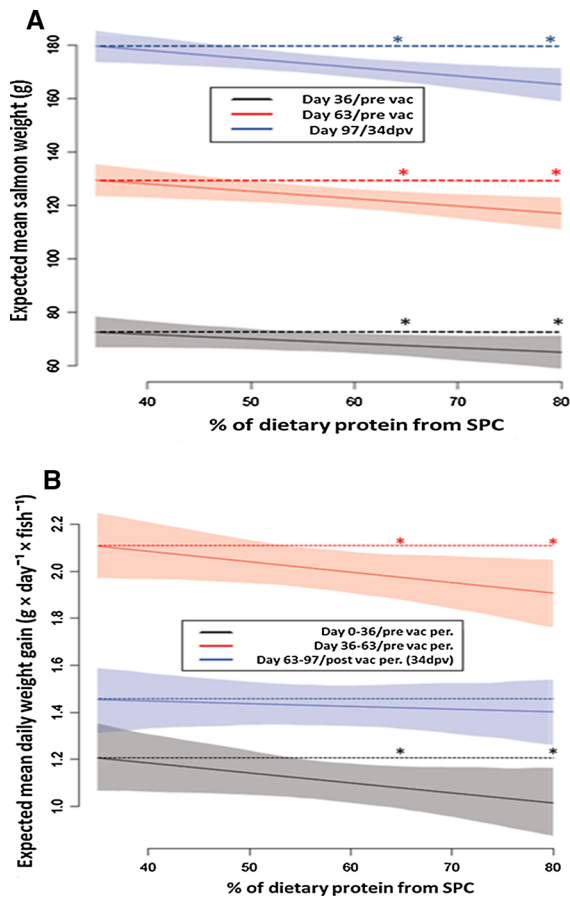
Fig. 2 Modelled representation of growth performance for the four dietary groups at each time point. **a** Expected mean salmon weight (g) and **b** expected mean daily weight gain per fish ($g \times day^{-1}$); (with 95 % CI) in relation to % SPC inclusion over the course of the study. *Black line* connects the expected mean weights of the four dietary groups at Day 36 in the first case and the expected mean daily weight gains over the period between Day 0 and Day 36 of the feeding trial (prior to vaccination); *Red line* connects the expected mean weights at Day 63 and the expected mean daily weight gains over the period between Day 36 and Day 63 of the feeding trial (prior to vaccination); *blue line* connects the expected mean weights at Day 97 of the feeding trial (34 days post-vaccination) and the expected mean daily weight gains over the period between Day 63 and Day 97 of the feeding trial (post-vaccination period). *Shaded regions of the same colours* indicate the 95 % confidence interval regions for these values (derived by posterior simulation of the model with n = 1500 random draws). *Dashed horizontal lines* denote the mean values for the salmon fed diets with 35 % of dietary protein from SPC. Mean values which lie outside the 95 % CI of the group receiving 35 % of dietary protein from SPC are considered significant. *Asterisks* denote significant differences between salmon fed 35 % of protein from SPC and salmon fed diets with higher percentage of dietary protein from SPC at different time points. (Color figure online)

dietary SPC. This was apparent from the slope connecting the expected mean values for the dietary groups at this time point (blue lines in Figs. 3b–d, 4a, c) which was much shallower compared to Day 63 and the fact that SPC35 salmon was the group demonstrating the greatest reduction for all elements except Zn compared with the other groups. Expected Zn was the only mineral with no marked improvement in the body cross-sections of Atlantic salmon fed increasing dietary SPC inclusions, at Day 97 (34 dpv).

Haematological and immunological responses

Raw data for the haematological and immunological responses of Atlantic salmon juveniles are presented in Supplementary Table 2. Haematocrit declined with increasing dietary proportions of SPC at all time points (Modelled response is presented in Fig. 5a). Significant differences were demonstrated between SPC80 compared with SPC35 salmon prior vaccination/PBS injection (since the expected confidence intervals given with the shaded regions for each treatment are overlapping with the expected mean values of the four groups). Overall lower haematocrits were obtained at 7 dpv. The decrease in haematocrit values with increasing SPC inclusion was much more noticeable at 7 dpv compared to the other sampling dates. At 7 dpv, haematocrit decreased significantly in the SPC50, SPC65 and SPC80 groups compared to SPC35 salmon. At 34 dpv despite the slight increment in haematocrit values exhibited in the former groups, salmon receiving diets with higher than 35 % of protein from SPC demonstrated significantly lower haematocrits compared with the SPC35 group. Higher haematocrit levels were recorded for all groups at 34 dpv in comparison with the previous time points.

Similar trends were obtained for leucocyte numbers during the two first time points (pre vac and 7 dpv) with decreasing numbers observed upon increased dietary SPC inclusion. Significant differences were revealed for the SPC65 and SPC80 groups compared with SPC35 salmon at both time points, while increased leucocyte counts were demonstrated for all dietary groups at 7 dpv compared to pre-vaccination levels. However, at 34 dpv leucocyte concentrations followed the opposite pattern, demonstrating increasing numbers in salmon fed diets with increasing dietary SPC inclusion (Modelled response is presented
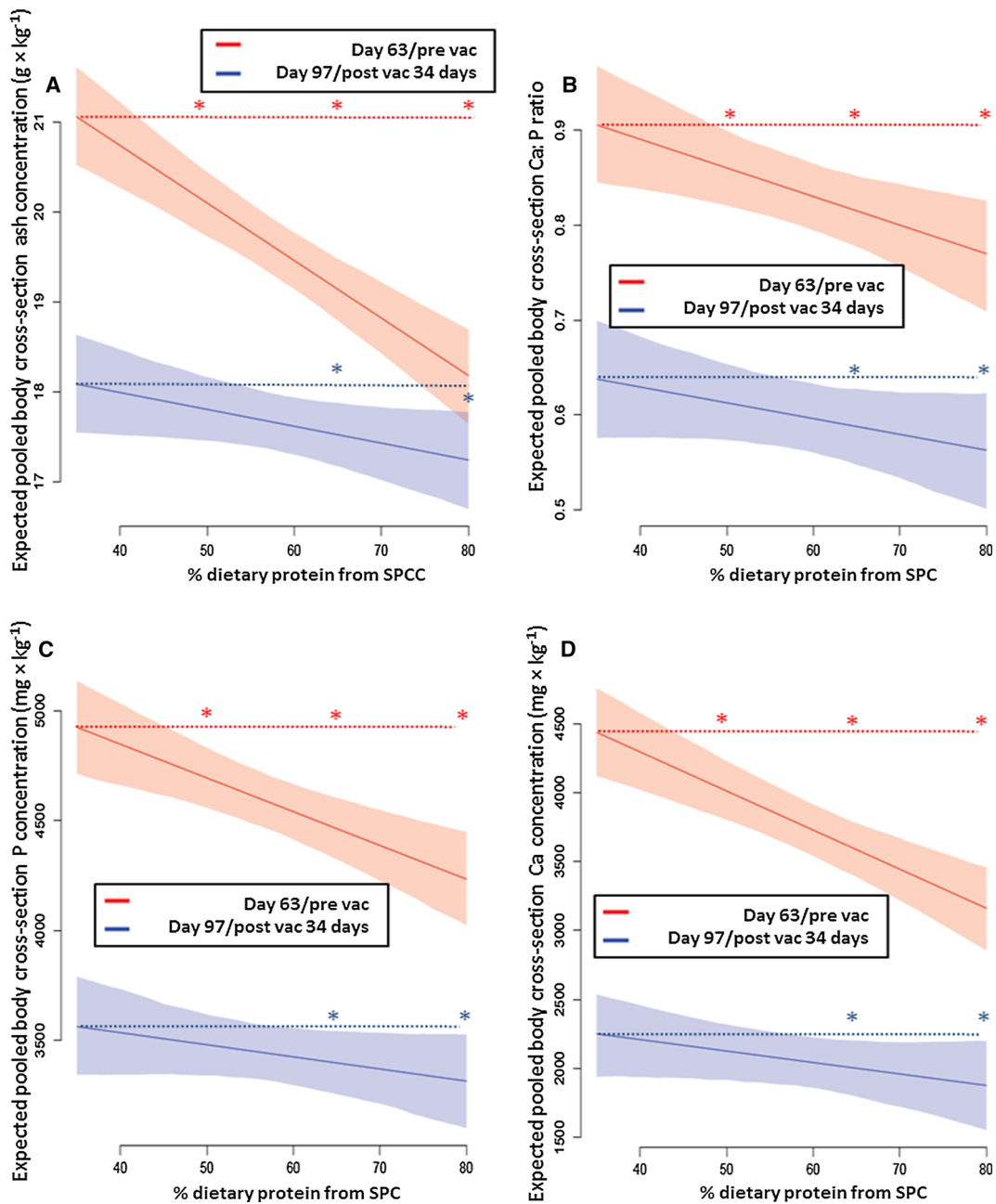
Fig. 3 Modelled ash, Ca/P ratio, P and Ca pooled body cross-section levels. Statistical models showing a significant linear effect of the percentage of protein from SPC on pooled body cross-section ash and elemental levels with P values <0.05; Expected (**a**) ash (g × kg^{-1}); (**b**) Ca/P ratio; (**c**) phosphorus (P) (mg × kg^{-1}); (**d**) calcium (Ca) (mg × kg^{-1}) (with 95 % CI) (y axis) in relation to % SPC inclusion (x axis) over the course of the study. *Red* and *blue lines* connect the expected mean values for each measurement at Day 63 post-feeding prior to vaccination and Day 97 post-feeding (34 days post-vaccination), respectively, while *shaded regions* of the *same colours* indicate the 95 % confidence regions for these values (derived by posterior simulation of the model with $n = 1500$ random draws). *Dashed horizontal lines of the aforementioned colours* denote the mean values for salmon fed diets with 35 % of dietary protein from SPC. Mean values which lie outside the 95 % CI of the group receiving 35 % of dietary protein from SPC are considered significant. *Asterisks of the same colours* denote significant differences between salmon fed 35 % of protein from SPC and salmon fed diets with higher percentage of dietary protein from SPC at different time points. (Color figure online)
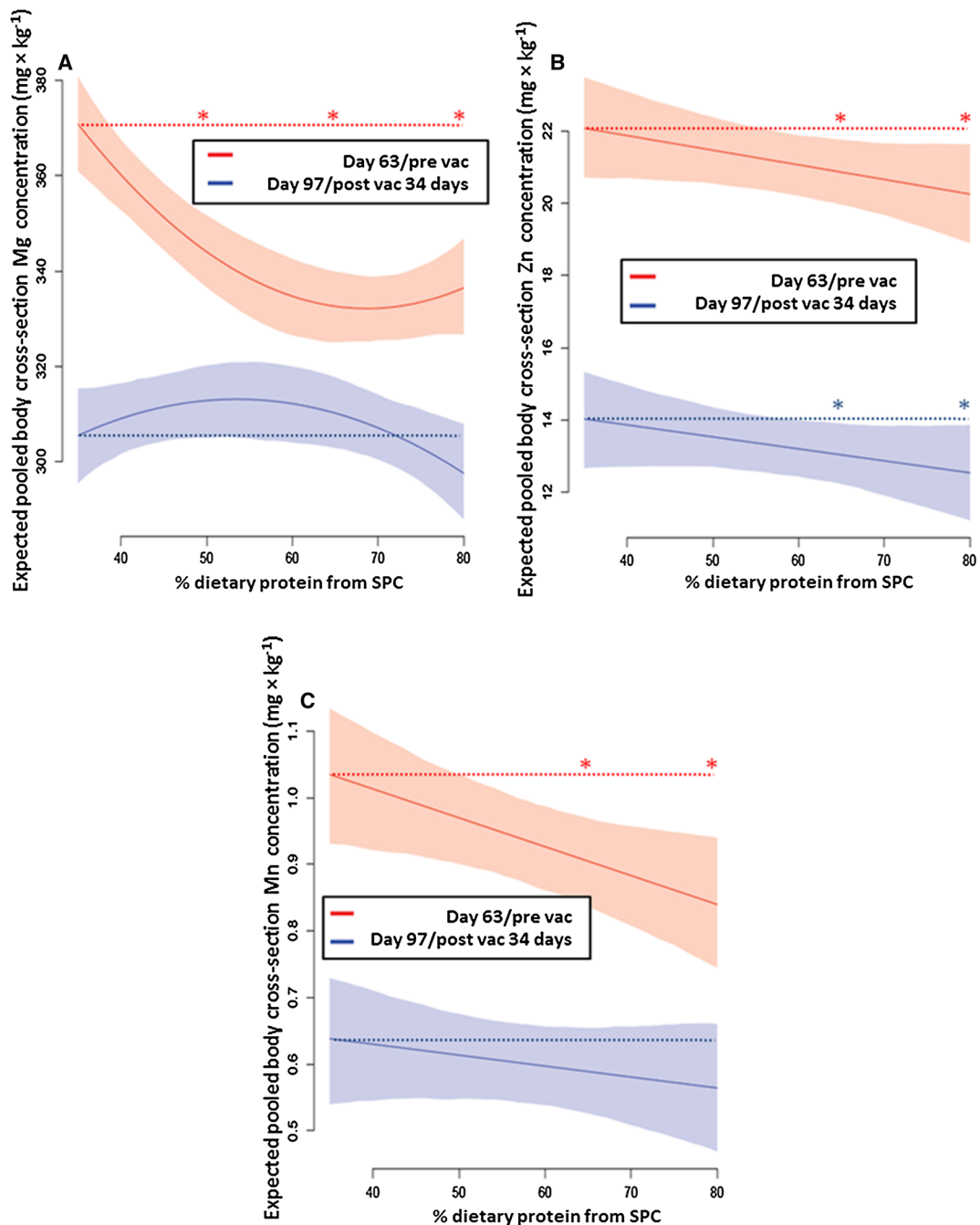
Fig. 4 Modelled Mg, Zn and Mn pooled body cross-section levels. Statistical models showing a significant quadratic or linear effect of the percentage of protein from SPC on pooled body cross-section elemental levels with P values <0.05; Expected (**a**) magnesium (Mg) (mg $\times$ kg^{-1}); (**b**) zinc (Zn) (mg $\times$ kg^{-1}); (**c**) manganese (Mn) (mg $\times$ kg^{-1}) (with 95 % CI) (y axis) in relation to % SPC inclusion (x axis) over the course of the study. *Red* and *blue lines* connect the expected mean values for each measurement at Day 63 post-feeding prior to vaccination and Day 97 post-feeding (34 days post-vaccination), respectively, while *shaded regions of the same colours* indicate the 95 % confidence regions for these values (derived by posterior simulation of the model with $n = 1500$ random draws). *Dashed horizontal lines of the aforementioned colours* denote the mean values for salmon fed diets with 35 % of dietary protein from SPC. Mean values which lie outside the 95 % CI of the group receiving 35 % of dietary protein from SPC are considered significant. *Asterisks of the same colours* denote significant differences between salmon fed 35 % of protein from SPC and salmon fed diets with higher percentage of dietary protein from SPC at different time points. (Color figure online)
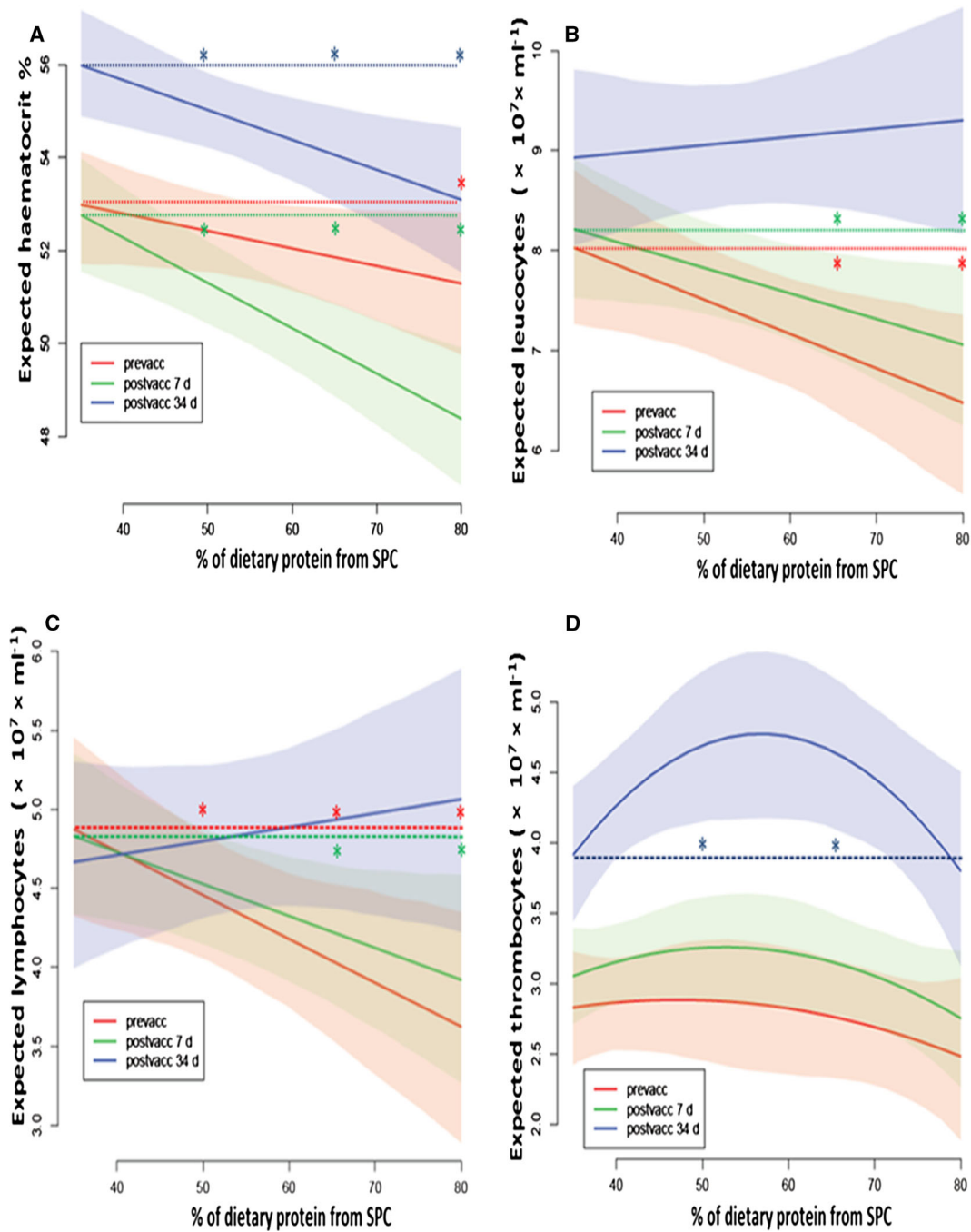
Fig. 5 Modelled haematological responses. Statistical models showing the linear (models selected with P values <0.05) effect of the percentage of dietary protein from SPC on: **a** expected haematocrit (%); **b** expected leucocytes ($\times 10^7 \times ml^{-1}$); **c** expected lymphocytes ($\times 10^7 \times ml^{-1}$); **d** expected thrombocytes ($\times 10^7 \times ml^{-1}$). *Red*, *green* and *blue lines* connect the expected mean values for each measurement prior to vaccination—pre vacc (Day 63 post-feeding), 7 days post-vaccination (7 dpv-Day 70 post-feeding) and 34 dpv (Day 97 post-feeding), respectively, while *shaded regions of the same colours* indicate the 95 % confidence regions for these values (derived by posterior simulation of the model with $n = 1500$ random draws). *Dashed horizontal lines of the aforementioned colours* denote the mean values for salmon fed diets with 35 % of dietary protein from SPC. Mean values which lie outside the 95 % CI of the group receiving 35 % of dietary protein from SPC are considered significant. *Asterisks of the same colours* denote significant differences between salmon fed 35 % of protein from SPC and salmon fed diets with higher percentage of dietary protein from SPC at different time points. (Color figure online)

in Fig. 5b). No significant differences in total leuco-cyte levels were recorded among the three dietary groups of salmon at this time point. Regarding the expected differential leucocyte numbers, lymphocytes and thrombocytes were the only types of leucocytes affected by increasing SPC inclusion. Lymphocytes followed similar trends to that of total leucocytes. Prior to vaccination and at 7 dpv, lymphocyte num-bers exhibited a linear reduction in salmon fed diets with increasing levels of SP. Significantly lower lymphocyte numbers were detected in SPC50, SPC65 and SPC80 salmon compared to SPC35 salmon before the vaccination of the fish. However, at 7 dpv lymphocyte numbers exhibited a slight increase in salmon fed on diets with higher than 35 % of total protein from SPC, whereas SPC35 salmon lympho-cytes presented a slight reduction. At this point significantly lower lymphocyte counts were demon-strated in SPC65 and SPC80 salmon in contrast to the SPC35 group of fish. At 34 dpv a linear increase in lymphocytes was observed in salmon fed with higher dietary amounts of SPC (Modelled response is presented in Fig. 5c). Nonetheless, no differences on lymphocyte levels were recorded among the three dietary groups at 34 dpv. On the other hand, throm-bocytes were affected curvilinearly in salmon fed increasing amounts of SPC with SPC50 and SPC65 salmon demonstrating the highest levels of thrombo-cytes and the SPC35 group having slightly higher thrombocyte numbers than SPC80 salmon, at all time points (modelled response is presented in Fig. 5d). However, no significant differences were revealed prior to and 7 dpv. Overall, thrombocytes increased upon vaccination with levels peaking at 34 dpv. At this point, significantly higher thrombocyte levels were demonstrated for the SPC50 and SPC65 groups compared with SPC35 salmon. Increasing dietary SPC inclusion had no significant effect on circulating granulocyte and monocyte levels of juvenile salmon. Neutrophilic granulocytes increased after vaccination peaking up at 7 dpv, while at 34 dpv the levels of these leucocytes decreased. Contrary to the results above, monocytes demonstrated a gradual increase post-vaccination, showing the highest levels 34 dpv.

Statistical models revealed no effect of increasing dietary SPC inclusion on the respiratory burst activity of both PMA-stimulated and non-stimulated HKMs. HKMs in general demonstrated increased respiratory burst activity a week after vaccination and a subse-quent reduction to pre-vaccination levels, at 34 dpv.

Increasing dietary SPC inclusion in the diets of Atlantic salmon parr had no significant effect on their plasma protein levels and plasma anti-protease activ-ity. Plasma protein concentrations demonstrated a sharp reduction at 7 dpv compared with pre-vaccina-tion levels. At 34 dpv plasma protein presented an increase compared with protein levels at 7 dpv. Nonetheless, protein levels at this time point were found to be lower than pre-vaccination values. Plasma anti-protease activity, on the other hand, presented a gradual decrease at the post-vaccination period com-pared to pre-vaccination levels, with the lowest activity obtained at 34 dpv. Plasma-specific antibody titres against *A. salmonicida* were only measured at 34 dpv, so there was no time effect to model. Thus, the plausible statistical models consisted of only the SPC inclusion effects. The likelihood comparison demon-strated no effect of dietary SPC inclusion, however.

Increasing SPC levels in the diets had a curvilinear effect on plasma lysozyme activity, total IgM levels and haemolytic activity (modelled responses are presented in Fig. 6a–c). Prior to vaccination, expected lysozyme activity appeared to be higher in salmon receiving the SPC50 and SPC65 dietary treatments compared with the salmon receiving SPC35 and SPC80. Significant differences were also demon-strated between the former two groups and SPC35 salmon. At 7 dpv a similar trend was observed; however, the levels of lysozyme activity were markedly higher than prior to vaccination. At this point significantly higher lysozyme activity was demonstrated for the SPC65 group of fish in contrast to SPC35 salmon. The pattern of lysozyme activity with respect to the level of dietary SPC inclusion was the same at 34 dpv, as seen with the previous time points; however, the activity was lower than prior to vaccination. Moreover, significantly higher activity was demonstrated in SPC50 and SPC65 salmon compared with the SPC35 group (modelled response is shown in Fig. 6a). Expected plasma IgM levels prior to vaccination demonstrated no differences among the four dietary groups of salmon. At both post-vaccina-tion points (7 dpv, 34 dpv), total plasma IgM concen-trations were found to be significantly higher for SPC50 and SPC65 salmon compared with SPC35 salmon, while similar levels were demonstrated for
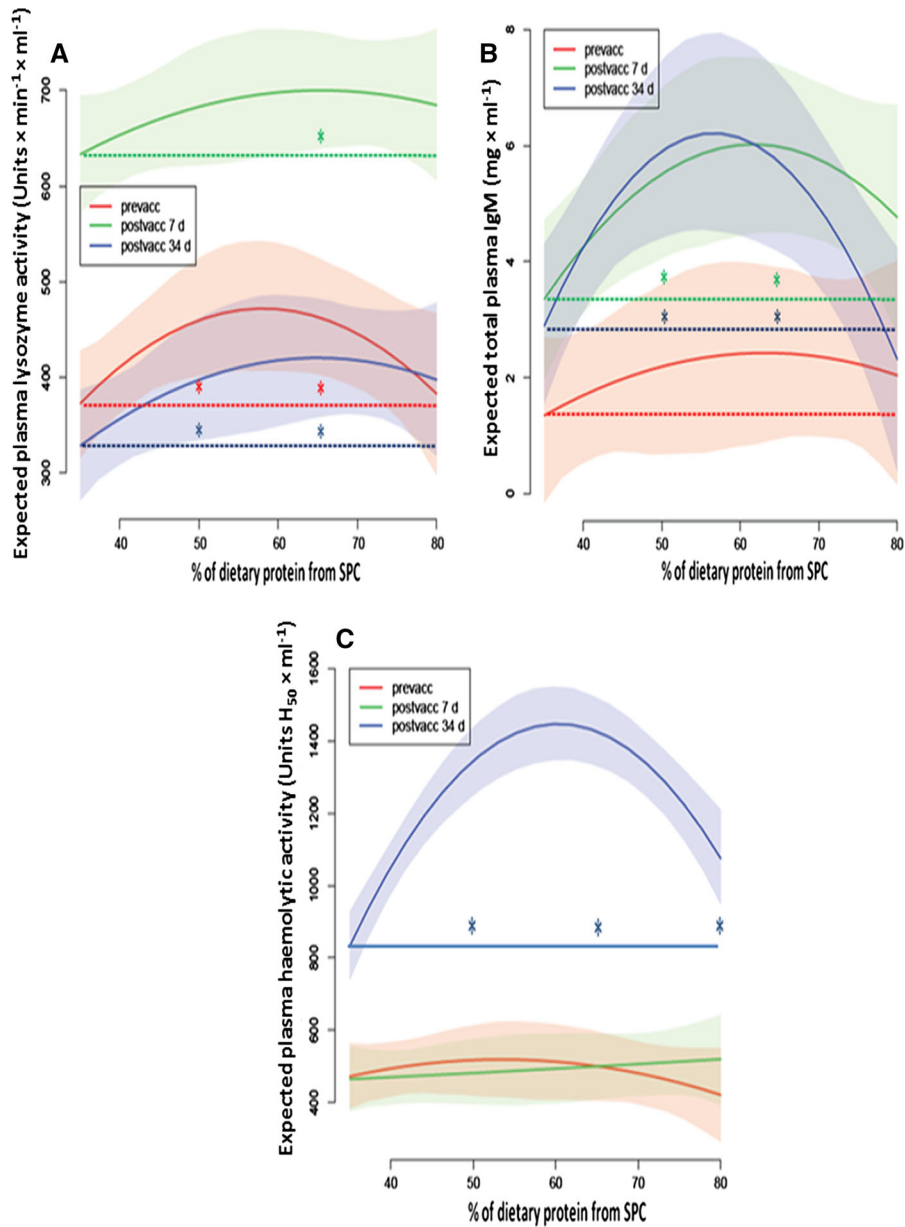
Fig. 6 Modelled immunological responses. Statistical models showing significant quadratic effect (models selected with P values <0.05) of the percentage of protein from SPC on: **a** expected lysozyme activity (Units $\times$ min^{-1} $\times$ ml^{-1}); **b** expected total plasma IgM (mg $\times$ ml^{-1}); **c** expected haemolytic activity (Units H_{50} $\times$ ml^{-1}). *Red*, *green* and *blue lines* connect the expected mean values for each measurement prior to vaccination—pre-vaccination (Day 63 post-feeding), 7 days post-vaccination (7 dpv-Day 70 post-feeding) and 34 dpv (Day 97 post-feeding), respectively, while *shaded regions of the same colours* indicate the 95 % confidence regions for these values (derived by posterior simulation of the model with $n = 1500$ random draws). *Dashed horizontal lines* of the aforementioned *colours* denote the mean values for salmon fed diets with 35 % of dietary protein from SPC. Mean values which lie outside the 95 % CI of the group receiving 35 % of dietary protein from SPC are considered significant. *Asterisks of the same colours* denote significant differences between salmon fed 35 % of protein from SPC and salmon fed diets with higher percentage of dietary protein from SPC at different time points. (Color figure online)

SPC35 and SPC80 salmon (modelled response is illustrated in Fig. 6b). Expected haemolytic activity demonstrated minor differences among the four experimental dietary groups of salmon prior to and at 7 dpv. However, at 34 dpv plasma haemolytic activity presented a salient increase in fish from all the dietary treatments with fish fed on medium levels of SPC inclusions possessing the highest values. Significant differences were denoted for the SPC50, SPC65 and SPC80 groups in comparison with SPC35 salmon at 34 dpv (modelled response is shown in Fig. 6c).

Discussion

Growth

Phytic acid in soybean products has been proven to reduce nutrient digestibility, inhibit the activity of gastrointestinal enzymes and compromise dietary intake, feed efficiency and fish growth (Denstadli et al. 2006; Kumar et al. 2012). Compositional analyses of the tested treatments demonstrated an increase in the phytic acid concentrations upon increased dietary inclusion of SPC which could explain the reductions in juvenile salmon growth performance (Denstadli et al. 2006). The reductions in salmon mean weight and daily WG were much more evident during the first (Day 0–Day 36) and second period (Day 36–Day 63), prior to salmon vaccination, while improved growth was observed at post-vaccination, during the last part of the study, indicating a slow but steady adaptation of juvenile salmon to diets with increasing levels of SPC. Burr et al. (2012) noted that the growth performance of Atlantic salmon parr fed diets in which protein was supplied from alternative protein blends improved with the age of the fish, with late-stage parr presenting improved growth. In the present study, despite the overall reduction in mean weight, an improvement in WG was witnessed for high SPC inclusion dietary salmon during the post-vaccination period. This was an indication that larger size Atlantic salmon can utilise the nutrients of these diets more efficiently either due to having a more developed digestive tract or due to the fact that they require a longer adaptation time to accept these diets, which is in accordance with the findings of Burr et al. (2012). Despite the observed significant reduction in the growth performance of the SPC80 compared with

the SPC35 group, numerically this decrease was not substantial. Overall, weight reduction in SPC80 compared with SPC35 salmon was only 8 % upon completion of the study and was much lower than weight differences observed in salmon fed diets entirely based on different FM varieties (Anderson et al. 1997).

The lower WG values observed at post-vaccination (Day 63–Day 97) compared with the pre-vaccination period from Day 36 to Day 63 (Fig. 2b), despite the longer duration and the larger size of the fish at the former phase, could be attributed to the stressful nature of the vaccination process reducing fish appetite and growth modulating hormones (Pickering 1993; Wendelaar Bonga 1997). Moreover, decreased energy expenditure for growth due to immune induction is expected at post-vaccination (Van Muiswinkel and Wiegertjes 1997; Melingen and Wergeland 2000).

Body cross-sectional composition

To overcome any differences attributed to dietary P limitations in SPC, 30 g $\times$ kg^{-1} of dicalcium phosphate was added in all experimental diets, exceeding the minimum reported dietary P requirements for Atlantic salmon which was previously estimated at around 10–11 g $\times$ kg^{-1} (Åsgard and Shearer 1997; NRC 2011; Prabhu et al. 2013). Analysis of the diets also confirmed that available P levels (total P–P bound to phytic acid) were found to be close to the reported requirement range, from 13.4–10.6 g $\times$ kg^{-1}, decreasing progressively upon increased SPC inclusion (Storebakken et al. 1998).

Body cross-sectional proximate and elemental composition analysis revealed a linear reduction in expected ash, Ca/P ratio and Ca, P, Mn and Zn content and an overall reduction of Mg in juvenile salmon fed on increasing dietary SPC levels prior to (Day 63) and 34 dpv (Day 97). Estimated body cross-sectional P, Ca, Mg and Mn concentrations at Day 63 were similar to the whole-body contents reported by Helland et al. (2005, 2006) for healthy juvenile Atlantic salmon of comparable size. Zinc levels at Day 63 were also found within the range reported by Helland et al. (2006); however, these values were closer to the lower end of this distribution, suggesting either limitations in the use of body cross-sections for the estimation of this mineral or the onset of Zn deficiency in salmon. Similarly, Ca/P ratio which usually lies close to 1:1 in

whole-body samples was found to range from 0.8 to 0.9 suggesting reduced skeletal calcification at Day 63 (Shearer et al. 1994; Helland et al. 2006). At Day 97 (34 dpv), elemental concentrations and Ca-P ratio were lower compared with the first time point and the whole-body values previously described by Helland et al. (2005, 2006), which could be an indication of salmon with subclinical deficiencies for all the above elements. Nonetheless, no apparent signs of elemental deficiencies were recorded for any of the dietary groups of salmon such as growth reductions, increased mortalities, lens cataracts, fin and skin erosions or morphological anomalies (Lall 2003).

Similar to our findings, Storebakken et al. (1998) reported decreased levels of whole-body ash, P, Ca Mg and Zn content and Ca/P ratio in salmon fed diets with 75 % of dietary protein from SPC and $30 \text{ g} \times \text{kg}^{-1}$ of supplementary dicalcium phosphate for 84 days compared with FM-fed salmon. Increased presence of phytic acid in diets with higher SPC content, negatively affects P availability (Storebakken et al. 1998; Lall 2003), as shown by the chemical compositions of the four diets used in the present study. Inadequate dietary uptake of phosphorus might lead to the inability of the fish to retain several minerals including Ca and Mg (Åsgard and Shearer 1997; Storebakken et al. 1998). In addition to that, the chelating effects of this substance on positively charged ions such as Ca, Mg, Mn and Zn and the formation of insoluble salts within the gut are the main reasons for the observed reduction of these minerals in whole-body samples (Francis et al. 2001; Lall 2003). Therefore, it is suggested that increased dietary SPC inclusion in salmon diets should be combined with an appropriate increment in dietary P supplementation. An alternative solution, of great potential, would be the use of graded levels of phytases which are enzymes that can break down phytic acid and their salts, increasing the availability of P and several other essential macro- and micronutrients from plant feedstuffs such as SPC (Storebakken et al. 1998; Cheng and Hardy 2003; Carter and Sajjadi 2011). Phytase can be applied prior pelleting or onto pellets (Denstadli et al. 2007; Wang et al. 2008). However, the high processing costs, the existent inconsistencies regarding the optimum doses of phytase in salmon plant-based feeds for the replacement of inorganic P, the inactivation of the enzyme at high temperatures required for pelleting (>80 °C) or certain storage

temperatures and the narrow optimum pH range for its activation are the main limiting factors for the current use of phytase in commercial salmon feeds (Carter and Sajjadi 2011; Kumar et al. 2012).

The present study shows that diets with higher than 50 % of dietary protein from SPC require higher than $30 \text{ g} \times \text{kg}^{-1}$ of dicalcium phosphate supplementation in order to promote both good growth and sufficient bone mineralisation. Studies on salmonids have shown that diets supplemented with phosphates of a low Ca/P ratio (≤ 1) are more digestible, improving the availability of P to fish (Aliphos, 2012). Therefore, monocalcium or monoammonium phosphates might be more appropriate inorganic P sources compared with dicalcium phosphate in high plant protein inclusion diets (Aliphos, 2012). Additional fortification of such diets with other nutrients (vitamins or minerals) should not be excluded (Prabhu et al. 2013, 2014; Torstensen 2014), with Zn being a predominant candidate mineral to consider for increased supplementation upon increased addition of plant proteins in salmon feeds as shown by the present data (Helland et al. 2006; Prabhu et al. 2014). In an era of dynamic changes in the protein sources used in aqua feeds, Atlantic salmon elemental requirements (NRC 2011) should be re-assessed in order to meet the requirements of salmon grown on diets with higher plant protein inclusions (Prabhu et al. 2013, 2014). Therefore, the assessment of elemental availability from commercially applied alternative dietary protein sources and identification of Atlantic salmon elemental requirements according to their growth rate and life stage, are of uttermost importance in order to proportionately balance the inclusion of these nutrients in their dietary premixes (Torstensen et al. 2008; Prabhu et al. 2013, 2014).

The reduction in body cross-sectional ash, Ca/P ratio and Ca, P, Mg, Mn and Zn levels detected at Day 97 (34 dpv) of the study compared to Day 63 (prior vaccination) seems to have a multifactorial cause. Storebakken et al. (1998) stated that reductions in body elemental concentrations at different production stages could be attributed to differences in fish size. Vaccination with injectable oil-based vaccines can also exert a negative effect on the mineral status of salmon (Berg et al. 2006, 2012; Grini et al. 2011). However, the negative impacts of injection vaccination on salmon skeletal mineral levels are often combined with intensive culture conditions inducing

faster growth (Grini et al. 2011; Fjelldal et al. 2012). Herein, the primary reason for the observed elemental reductions seems to be the enhanced growth performance of salmon, promoted by the application of continuous light (combined with favourable temperature of ~13 °C for salmon growth) and the intensive feeding regime which represent the commercial reality (Kråkenes et al. 1991; Fjelldal et al. 2005, 2006; Stefansson et al. 2008). These conditions have led to the achievement of almost double daily WG and ~1.6 greater weight in juvenile salmon compared to the study performed by Helland et al. (2006), despite the fact that fish have been subjected to vaccination during the last part of the trial. Fjelldal et al. (2006) reported that fast-growing Atlantic salmon post-smolts after 6 months under continuous light demonstrated lower mineral content and mechanical strength in vertebral bones than slower-growing salmon under natural light. Rungruangsak-Torrissen et al. (2009) added that increased growth in salmon by exposure to continuous light could be associated with reduced vertebral mineralisation and strength. Moreover, Hernandez et al. (2000) noted that during periods of fast growth, the actual time required for the bone matrix to be produced and mineralised could be decreased to a critical level, resulting to under-mineralised bony tissues. This was one of the proposed explanations for the higher incidence of vertebral deformities in fast-growing under-yearling Atlantic salmon smolts compared to slower-growing yearling smolts (Fjelldal et al. 2006). In addition, Stefansson et al. (2007, 2008) noted that juvenile Atlantic salmon reared under constant light (as a means of blocking salmon maturation and smoltification) could develop into "pseudo-smolts" exhibiting all the external changes, characterising smoltifying salmon but not the physiological changes required for seawater adaptation. Therefore, disturbances in ion and osmo-regulation of salmon due to "pseudo-smoltification" could have led to reduced elemental levels at Day 97.

Despite the observed decline in elemental levels at Day 97 compared to Day 63 in all dietary groups, salmon fed on higher dietary SPC levels demonstrated subsidiary decrements in body cross-sectional ash and elemental concentrations (Zn excluded) compared to Day 63 (Figs. 3, 4a, c). On the contrary, the decrements in body cross-sectional elemental levels were much more evident in the groups fed lower dietary SPC levels, with SPC35 salmon demonstrating the greatest reductions regarding ash and mineral concentrations by comparison to Day 63. The improvement in elemental retention observed in salmon fed diets with increasing dietary amounts of SPC could primarily be attributed to the overall delayed growth performance of these groups compared to SPC35 salmon. Fjelldal et al. (2010) reported that in the long-term, high plant protein inclusion feeds could have a positive impact on the skeletal health of Atlantic salmon post-smolts by delaying salmon growth. Possible extension of the study could have eliminated the elemental differences among the four dietary groups. In addition to the overall delayed growth, better assimilation of high SPC inclusion feeds (either through improved feed intake and/or digestibility) is suggested by the improvement in the growth performance (daily WG) of these groups, during the last period of the study (post-vaccination period from Day 63 to Day 97) (Fig. 2b). Earlier studies suggest that Atlantic salmon requires long adaptation periods in order to accept and start utilising efficiently high plant protein diets, after which they might be able to compensate growth and nutrient uptake (Torstensen et al. 2008; Burr et al. 2012). Moreover, according to Prabhu et al. (2013, 2014), the elemental requirement for WG in farmed fish differs from the requirement for proper skeletal mineralisation and these depend on both the growth rate and the life stage of the fish. Herein, it was evident that during the last period of the study, high SPC inclusion diets performed better in comparison with the first two periods and matched more closely the elemental requirements of the fish they were allocated to, for both growth and skeletal mineralisation compared to their SPC35 counterpart.

Haematological and immune responses

For Atlantic salmon, haematocrit values, which represent the oxygen carrying capacity of blood, normally range from 35 to 60 % (Hardie et al. 1990; Thompson et al. 1996). Haematocrit levels were found to be within this range in the present study. Decreasing haematocrit, however, was observed in salmon fed diets with increasing dietary SPC protein. Hemre et al. (2005) reported decreased erythrocyte size in Atlantic salmon fed diets with increased protein levels from soybeans, which could explain the lower haematocrit in these groups.

Increased circulatory leucocytes mainly attributed to simultaneous increments in both lymphocyte and

thrombocyte numbers reveal immune and haemostatic stimulation at 34 dpv for all dietary groups (Nikoskelainen et al. 2007; Silva et al. 2009). Overall, the state of leucocyto- and lymphocyto-penia in salmon fed increasing amounts of SPC prior to vaccination and 7 dpv could be accredited to poorer nutrition of these fish caused by the increasing presence of phytic acid (Fletcher 1997). Furthermore, it is possible that salmon fed on diets with moderate and high SPC inclusion, due reduced nutrient uptake, could have exhibited suppression of various pathways including the ones involved in systemic immunity and possibly haematology, utilising most of the dietary energy for growth (Tacchi et al. 2012). A slow but steady reversal of this trend, however, seems to occur upon vaccination (at 34 dpv), with salmon fed increasing dietary SPC inclusion investing more energy on immunostimulation which is revealed by the linear increase in lymphocyte numbers (Nikoskelainen et al. 2007). Moreover, increased haemostatic stimulation was exhibited in SPC50 and SPC65 compared to the SPC35 and SPC80 salmon, as suggested by the higher thrombocyte levels (haemostasis regulating cells) recorded for these groups at 34 dpv (Silva et al. 2009). The increase in granulocyte and monocyte levels at post-vaccination were in accordance with earlier reports by Chin and Woo (2005) and Nikoskelainen et al. (2007), demonstrating significant increases in the circulating numbers of these cells in salmonids vaccinated against different pathogens. However, no differences were exhibited among the dietary groups, suggesting that increasing dietary SPC does not compromise the production of these cells.

Respiratory burst of both PMA-stimulated and naive HKMs did not appear to be affected by dietary SPC inclusion. In accordance Bransden et al. (2001), reported unaffected neutrophil oxygen radical production in Atlantic salmon fed dehulled lupin meal at 40 % inclusion. Contrarily Burrells et al. (1999) demonstrated reduction in HKM respiratory burst activity in rainbow trout (Oncorhynchus mykiss) fed diets with 80 % substitution of FM with dehulled solvent extracted SBM. The decreased post-vaccination plasma protein levels observed in the dietary groups could be attributed to stress-induced reduction of fish appetite (Melingen and Wergeland 2000). Nevertheless, no differences in plasma protein concentrations were exhibited among the dietary groups of salmon. Reduced anti-protease activity has been

reported in salmon after vaccination or infection via intraperitoneal injection (Secombes and Olivier 1997), which is in agreement with the present findings. Similar to plasma protein levels, no differences in anti-protease activity were exhibited among the groups. Unaffected anti-protease activity in Atlantic salmon fed on diets with 40 % substitution of FM with dehulled lupin meal has been reported by Bransden et al. (2001). Unaltered lysozyme activity has been previously demonstrated in rainbow trout fed with up to 100 % of dietary protein from plant derived feedstuffs (Jalili et al. 2013). On the contrary, higher lysozyme activity was recorded in SPC50 and SPC65 salmon at all time points, while higher levels of total IgM were revealed at both time points post-vaccination compared to SPC35 salmon. In accordance with the present results, Rumsey et al. (1994) reported increased lysozyme activity in rainbow trout fed SBM diets. Increased circulatory total IgM levels are expected after salmon vaccination explaining the higher amounts at 7 and 34 dpv (Melingen and Wergeland 2000). Superior lysozyme activity in SPC50 and SPC65 salmon suggests stimulation of the cellular part of immunity and could be a favourable trait against disease outbreaks (Waagbø et al. 1993; Krogdahl et al. 2000). Moreover, increased circulating total IgM levels at post-vaccination for the aforementioned groups could improve the effectiveness of vaccination conferring higher protection against diseases (Krogdahl et al. 2000).

Plasma haemolytic activity as a consequence of the activation of complement factors (from both alternative and classical pathways) and/or other substances such as lectins and haemolysins (Alexander and Ingram, 1992) was not affected linearly by increasing dietary SPC levels. Prior to and 7 dpv, salmon from different dietary groups exhibited no differences in plasma haemolytic activity. Sitjà-Bobadilla et al. (2005) and Jalili et al. (2013) reported decreased alternative complement activity in gilthead sea bream (Sparus aurata) and rainbow trout, respectively, fed diets with 70 % protein from plant-derived products. The former study, however, exhibited an enhancement of alternative complement activity in gilthead sea bream fed 50 % of protein from vegetal ingredients. At 34 dpv, there was a marked increase in haemolytic activity in fish from all the dietary treatments with SPC50, SPC65 and SPC80 salmon exhibiting higher activity than SPC35 salmon suggesting higher

immunostimulation of these groups at 34 dpv while highlighting dietary modifications that were not evident prior to vaccination (Thompson et al. 1996; Nikoskelainen et al. 2002).

Conclusions

In conclusion, increasing substitution of high-quality FM with SPC at a percentage beyond 35 % of dietary protein reduced the growth performance and body cross-sectional elemental concentrations and enhanced several humoral immune responses of juvenile Atlantic salmon. Diets with 50 % of protein from SPC, appropriate supply of lysine, methionine and threonine and 30 g $\times$ kg^{-1} of P promoted similar growth and elemental composition to a control diet with 35 % of protein from SPC, mimicking commercial salmon formulations. Body cross-sectional elemental reductions due to the increasing dietary SPC levels, observed during the initial 63-day period, were improved at Day 97 (34 dpv). This seems to be both an effect of the long-term provision of juvenile salmon with increasing dietary SPC levels or an interaction of these diets with vaccination. Moreover, it was shown that exposure of Atlantic salmon to continuous light, intensive feeding regimes and vaccination may result in elemental deficiencies rendering developing salmon susceptible to skeletal deformities or diseases. Under these conditions slow-growing salmon fed on high SPC inclusion feeds can match their elemental requirements for both growth and bone mineralisation more efficiently than the faster growing SPC35 fish. Lastly the use of body cross-sections appears to be promising for the assessment of salmon elemental status; however, validation studies are required to demonstrate the correlation between whole-body and body cross-sectional compositions, from fish reared under the same conditions. While P involvement on growth and mineral retention in fish is well documented, the implication of P in fish immunity is not clear (Lall 2003). However, P could affect immunity either directly by supplying the energy required for immunological stimulation, or indirectly by influencing the digestion of other dietary elements including Zn, Mg or Ca, modulating important immune responses (Lall 2003; Kiron 2012). It is proposed that higher supplementation of P ($>$30 g $\times$ kg^{-1}) and several other nutrients (e.g. minerals and vitamins) in

diets with 65 % or over of protein from SPC could improve salmon growth, elemental retention and prevent potential compromises in immune responses. Further studies are required to assess the effect of high SPC inclusion diets on these aspects and whether the observed increments in immunological responses are translated into increased disease resistance.

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