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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Keywords: Atlantic salmon, body cross-sections, growth, mineral composition, soy protein concentrate

Abstract

Diets with 50 (SPC50), 65 (SPC65) and 80 % (SPC80) substitution of prime fishmeal (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 29g juvenile Atlantic salmon exposed to constant light, for 97 days. On Day 63 salmon were subjected to vaccination. Significant weight reductions in SPC65 and SPC80 compared to SPC35 salmon were observed by Day 97. Linear reductions in body cross-sectional ash, Ca:P ratios, 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 to 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.

1. 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 to 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 to 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 antinutritional 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. 2013). Phytic acid bound on the protein fraction of soybeans is the main antinutritional factor (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 the principal elemental store

76 in fish. Therefore, any changes in nutrient retention could be reflected in the composition of
77 these tissues. Herein, body cross-sections from the end of the dorsal fin to the start of the
78 anal fin including muscle, bone, skin and scales (in a similar manner as Norwegian quality
79 cutlet (NQC) samples are utilised for flesh quality control in market size salmon) were used
80 for the compositional assessment of both tissues as a whole. Elemental data were compared
81 with whole body concentrations which are generally used as the golden standard in order to
82 assess this novel methodological approach (Shearer et al. 1994; Helland et al. 2005; 2006).
83 In general, there is a lack of information regarding the effects of high dietary levels of
84 plant proteins on the overall health status of Atlantic salmon. Previously, Krogdahl et al.
85 (2000) reported increased lysozyme and total IgM levels in the intestinal mucosa of seawater
86 adapted Atlantic salmon maintained on feeds with 30 % of dietary protein from soy products
87 (SBM and SPC) compared to FM-fed salmon. Moreover, SPC-fed salmon demonstrated
88 improved resistance against *Aeromonas salmonicida* compared to their FM- and soybean
89 meal (SBM) fed counterparts. While studies on the effects of dietary plant proteins on the
90 immune status of Atlantic salmon are still scarce, the majority of these were focused on the
91 effects of these feedstuffs on the innate immune responses of fish that have not been
92 stimulated immunologically (Thompson et al. 1996). However, determining immune
93 responses shortly or later post immunisation (via vaccination in the present study) may
94 highlight dietary modifications that were not evident before (Thompson et al. 1996).
95 Vaccination in the present study was primarily used in order to provide immunological
96 stimulation and secondarily to reproduce commercial conditions, as most farmed salmon are
97 vaccinated for disease prevention during the freshwater stage (Poppe and Koppang 2014).
98 In fish subjected to vaccination, normal skeletal elemental deposition could be decreased if
99 these nutrients are required for metabolic and immunomodulatory processes (Lall, 2003;
100 Kiron 2013). 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 regards 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 (Ytterborg 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 1990; 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 to 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)).

2. Materials and methods

126 ***2.1. Diets and growth trial***

127 Four experimental diets were prepared. These were formulated so that aqueous-alcohol
128 extracted SPC (Imcopa, Paraná, Brazil) supplied 35, 50, 65 and 80 % of total dietary protein
129 (termed SPC35, SPC50, SPC65 and SPC80, respectively) with high quality FM providing
130 the rest of the dietary protein in all cases. Diet formulations are provided in Table 1. SPC35
131 served as the control diet. The rationale behind the use of SPC35 as a control treatment was
132 to mimic commercial salmon dietary compositions with inclusion of plant proteins. In this
133 case, SPC as a sole plant derived FM substitute was chosen due to its premium nutritional
134 value. In previous trials, SPC35 treatment had been tested against EWOS commercial feed
135 formulations and it was found to perform equally well as its commercial counterparts
136 (personal communication Viv Crampton). The diets were manufactured by EWOS
137 Innovation, Dirdal, Norway and were formulated so that protein: fat ratios were constant
138 whereas lysine, methionine and threonine were supplemented to give the same AA: protein
139 ratios across dietary treatments. The amount of supplemented dicalcium phosphate was
140 constant among the diets, representing 3% of all experimental dietary formulations,
141 exceeding Atlantic salmon phosphorus (P) requirement (Lall 2003).

142 The feeding trial was conducted at EWOS Innovation facilities in Dirdal, Norway. For
143 the trial, fresh water was supplied to 16 square tanks with rounded corners each containing
144 approximately 60 litres of water. Water temperature was maintained at 13 ± 1ºC, whilst
145 photoperiod was constant (24 h of light). The diffused oxygen level of inlet water to the
146 tanks was constantly 90-100 % saturation. . A total of one thousand nine hundred and twenty
147 (1920 fish) unvaccinated S0 Atlantic salmon parr (from a commercial SalmoBreed strain
148 selected for improved growth performance) of an average weight of 29 g (i.e. 120 fish ×
149 $tank^{-1}$) were randomly allocated to the 16 tanks. Fish were acclimatised to experimental tanks
150 for 28 days prior to commencing the trial, during which time they were fed a commercial

151 EWOS freshwater diet for salmonids (EWOS micro). During the study period quadruplicate
152 tanks of fish were fed one of the 4 experimental diets. Fish were fed with automatic belt
153 feeders, continuously every 435 seconds for 20 seconds. Feeding time and period within feed
154 intervals were the same for the acclimation and trial period. The daily ration depended on
155 the trial period and fish appetite. A daily ration of 3.5 % of tank biomass was recorded during
156 the acclimation period, while this proportion was decreased to 2.75, 2.5 and 1.5% of tank
157 biomass during the first, second and third month of the trial period, respectively. A high
158 daily feed ration was used to avoid differences in feed intake often associated with satiation
159 feeding of plant substituted diets (Refstie et al. 1998).

160 The growth trial was conducted for a total of 97 days, at which time all fish in the tanks
161 were removed and bulk weighed post anaesthetisation (metacaine, 30 mg × l^{-1}) at Days 36,
162 63 and 97. At Day 63 and after bulk weighing fish from each tank, blood from 6 individuals
163 per tank was withdrawn for the evaluation of both haematological and immunological
164 responses. After blood sampling, body cross-sections between the end of the dorsal fin and
165 the start of the anal fin, from the same fish were collected for the commencement of
166 composition analyses in pools of six body cross-sections per tank (4 pools per treatment).
167 Salmon were then intraperitoneally (i.p.) vaccinated with 100µl of a commercial vaccine
168 (AquaVac™ Furovac, Intervet UK Ltd., Milton Keynes) against *A. salmonicida* in order to
169 simulate commercially applied conditions and most importantly to stimulate immune
170 responses. Thereafter, fish were sampled for haematological and immunological evaluation
171 at Days 70 (7 days post vaccination) and 97 (34 days post vaccination) while body cross-
172 sections were sampled at Day 97 of the trial.

173 ***2.2. Sample collection***

174 Blood was withdrawn from the caudal vein of 6 fish per tank per dietary group on Days 63,
175 70 and 94 after the start of the study (i.e. pre vaccination, 7 and 34 dpv) using 1 ml syringes

rinsed with heparin (10 IU × 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 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 × g for 20 min at 4ºC and pooled serum then aliquoted into seven eppendorf tubes (around 40-50 µl per tube) and stored at -80ºC until used.

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

2.3. Head kidney macrophage isolation and macrophage respiratory burst activity

For the isolation of head kidney macrophages (HKMs) the method described by Korkea-aho et al. (2011) was used. The superoxide anion (O^{-2}) production by head kidney macrophage suspensions were 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).

2.4. 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).

2.5. 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.

2.6. 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.

2.7. 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).

2.8. Antiprotease 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.

2.9. 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):

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

Specific Growth Rate (SGR):

$$SGR = \left(\frac{lnW1 - lnW0}{t}\right) \times 100$$

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 timepoint 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).

2.10. Proximate composition analysis

Cross-section samples obtained as described at the end of the Section 2.1 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 were 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 µl of the diluted digested material were 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 µg × g^{-1} using the following equation:

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

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.

2.11. 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

271 weight gain, SGR and TGC) were also assessed in a similar manner. Moreover, a hierarchical
272 (multilevel) statistical model was used for body cross-sections composition, haematological
273 and composition data from each tank, since multiple observations from a single tank were
274 available (several pools) (Espe et al. 2012; Nanton et al. 2012; Hartviksen et al. 2014). The
275 statistical analysis was carried out with the help of the R language (R Core Development
276 Team 2014) and its lme4 package (Bates et al. 2014). The statistical approach applied was
277 model-based. This means that to find if any specific effect was statistically significant data
278 were fitted in three different models with increasing complexity where tank was included as
279 a random effect, sampling time as a categorical variable and the percentage of protein from
280 SPC as a continuous factor possibly having non-linear effects:

281 1. a model with only sampling time (Tank considered as a random effect).
282 2. a model with sampling time and % protein from SPC with their interaction (Tank
283 considered as a random effect).
284 3. a model as above but with an additional quadratic effect of % protein from SPC with
285 interactions to reveal any non-linearity in the response (Tank considered as a random
286 effect).

287 The three models were nested and compared with a likelihood ratio test (LRT) that evaluated
288 if the improvement in the likelihood required a more complex model or whether the simpler
289 model could be applied. Models demonstrating possibilities (P values) < 0.05 were selected
290 for the description of data. The simplest possible model was adopted according to Occam's
291 razor principle. The adopted model was demonstrated by plotting the expected mean
292 response with 95% confidence intervals. For a categorical effect these were represented as
293 points with error bars and for a continuous effect as a curve with shaded confidence region.
294 The expected mean and 95% confidence intervals were solved by a posterior simulation from

295 the adopted statistical model (n=1500 random draws were used throughout this study)
296 (Gelman and Hill 2007).

297 **3. Results**

298 ***3.1. Growth performance***

299 The mortalities among the four experimental groups of Atlantic salmon were negligible
300 (<1%) for all groups (Table 2). Fig. 1 demonstrates the weight development of the four
301 dietary groups of Atlantic salmon. The models with the linear effect of the dietary percentage
302 of protein from SPC were favoured using the LRTs (likelihood ratio tests) for both expected
303 mean weight of salmon and expected mean daily weight gain (WG) (Statistical models are
304 presented in Fig. 2A and 2B). The expected mean values of the aforementioned growth
305 parameters with their estimated 95% confidence intervals (C.I.) are presented with different
306 colour for each timepoint or period respectively. More specifically the black line (expected
307 mean values) and its corresponding shaded region (estimated 95 % C.I.) represents the
308 expected mean weight values for Day 36 and the expected mean WG for the pre vaccination
309 period from Day 0 to Day 36. The values in red represent the mean weight estimates for Day
310 63 and the expected mean daily WGs for the pre-vaccination period from Day 36 to Day 63,
311 while values in blue demonstrate the corresponding values for Day 97 and the post
312 vaccination period, from Day 63 (day of vaccination) to Day 97 (34dpv). Overall, increasing
313 SPC levels resulted in lower mean weight and daily WG in Atlantic salmon juveniles at all
314 timepoints of the study. Approximate reductions of 12 and 10% in expected mean weight
315 were observed in SPC80 compared to SPC35 salmon at Day 36 and 63 of the feeding trial
316 respectively, while an 8% reduction in weight was demonstrated at Day 97 (34dpv) between
317 the two dietary groups (Fig. 2A). Moreover, SPC65 and SPC80 salmon exhibited
318 significantly lower mean weights compared to the SPC35 group at all timepoints. In a similar
319 manner, the reduction in daily WG observed in the high SPC inclusion dietary groups was

improved at post-vaccination compared to 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 to SPC35 salmon was recorded. This slope was much shallower compared to the slopes obtained for the pre-vaccination periods, when 17 and 10% reduction in WG in SPC80 compared to SPC35 salmon was observed (Fig. 2B). Overall, SPC65 and SPC80 salmon demonstrated significantly lower mean daily WG compared to 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 to 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.

3.2. 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 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

345 from SPC were the ones demonstrating the highest reduction in body cross-section ash
346 levels. A 5% reduction in expected ash concentration was demonstrated in SPC80 compared
347 to the SPC35 salmon at Day 97.

348 Expected calcium to phosphorus (Ca:P) ratio, calcium (Ca), phosphorus (P),
349 manganese (Mn) and zinc (Zn) levels in pooled body cross-section samples were affected
350 in a linear fashion by increasing levels of dietary SPC, since the model with linear description
351 of the data was found to describe the data in a better manner compared to the other two
352 models (Models selected are presented in Fig. 3B, 3C, 3D, 4B and 4C). Therefore the dietary
353 groups receiving feeds with higher levels of SPC inclusion exhibited lower amounts of the
354 abovementioned elements. On the other hand, the model showing a quadratic effect of the
355 percentage of dietary SPC, improved the fit over the linear model for body cross-section
356 expected Mg levels (Model selected is plotted in Fig. 4A). Overall, at Day 63, SPC35 salmon
357 demonstrated significantly higher Ca: P ratio, Ca, P and Mg concentrations than the SPC50,
358 SPC65 and SPC80 groups and higher amounts of Zn and Mn in comparison to the SPC65
359 and SPC80 groups.

360 In general, lower body cross-sectional elemental amounts were demonstrated for all
361 dietary groups at Day 97 (34 dpv) compared to Day 63 (prior to vaccination). At this
362 timepoint significantly lower Ca: P ratio, Ca, P and Zn concentrations were recorded for
363 SPC65 and SPC80 salmon compared to the SPC35 group. Despite the observed reduction in
364 body cross-section elemental concentrations at Day 97 compared to Day 63, an overall
365 improvement in expected Ca:P ratio, Ca, P, Mg and Mn was demonstrated from the modelled
366 based statistical analysis in vaccinated salmon receiving increased dietary SPC. This was
367 apparent from the slope connecting the expected mean values for the dietary groups at this
368 timepoint (blue lines in Fig. 3B, 3C, 3D, 4A and 4C) which was much shallower compared
369 to Day 63 and the fact that SPC35 salmon was the group demonstrating the greatest reduction

for all elements except Zn compared to 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 (34dpv).

3.3. Haematological and Immunological responses

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 to 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 7dpv, haematocrit decreased significantly in the SPC50, SPC65 and SPC80 groups compared to SPC35 salmon. At 34dpv 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 to the SPC35 group. Higher haematocrit levels were recorded for all groups at 34 dpv in comparison to the previous timepoints.

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 to SPC35 salmon at both timepoints, while increased leucocyte counts were demonstrated for all dietary groups at 7dpv 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 in Fig. 5B). No significant differences in total leucocyte levels were recorded among the three dietary

395 groups of salmon at this timepoint. Regarding the expected differential leucocyte numbers,
396 lymphocytes and thrombocytes were the only types of leucocytes affected by increasing SPC
397 inclusion. Lymphocytes followed similar trends to that of total leucocytes. Prior to
398 vaccination and at 7 dpv, lymphocyte numbers exhibited a linear reduction in salmon fed
399 diets with increasing levels of SP. Significantly lower lymphocyte numbers were detected in
400 SPC50, SPC65 and SPC80 salmon compared to SPC35 salmon before the vaccination of the
401 fish. However, at 7 dpv lymphocyte numbers exhibited a slight increase in salmon fed on
402 diets with higher than 35% of total protein from SPC, whereas SPC35 salmon lymphocytes
403 presented a slight reduction. At this point significantly lower lymphocyte counts were
404 demonstrated in SPC65 and SPC80 salmon in contrast to the SPC35 group of fish. At 34 dpv
405 a linear increase in lymphocytes was observed in salmon fed with higher dietary amounts of
406 SPC (Modelled response is presented in Fig. 5C). Nonetheless, no differences on lymphocyte
407 levels were recorded among the three dietary groups at 34dpv. On the other hand,
408 thrombocytes were affected curvilinearly in salmon fed increasing amounts of SPC with
409 SPC50 and SPC65 salmon demonstrating the highest levels of thrombocytes and the SPC35
410 group having slightly higher thrombocyte numbers than SPC80 salmon, at all time- points
411 (Modelled response is presented in Fig. 5D). However, no significant differences were
412 revealed prior to and 7 dpv. Overall, thrombocytes increased upon vaccination with levels
413 peaking at 34 dpv. At this point significantly higher thrombocyte levels were demonstrated
414 for the SPC50 and SPC65 groups compared to SPC35 salmon. Increasing dietary SPC
415 inclusion had no significant effect on circulating granulocyte and monocyte levels of
416 juvenile salmon. Neutrophilic granulocytes increased after vaccination peaking up at 7 dpv,
417 while at 34 dpv the levels of these leucocytes decreased. Contrary to the results above,
418 monocytes demonstrated a gradual increase post-vaccination, showing the highest levels 34
419 dpv.

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

Increasing dietary SPC inclusion in the diets of Atlantic salmon parr had no significant effect on their plasma protein levels and plasma anti-protease activity. Plasma protein concentrations demonstrated a sharp reduction at 7 dpv compared to pre-vaccination levels. At 34 dpv plasma protein presented an increase compared to protein levels at 7 dpv. Nonetheless, protein levels at this timepoint 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 compared to pre-vaccination levels, with the lowest activity obtained at 34dpv. 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 demonstrated 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, 6B and 6C). At prior to vaccination, expected lysozyme activity, appeared to be higher in salmon receiving the SPC50 and SPC65 dietary treatments compared to the SPC35 and SPC80 salmon. Significant differences were also demonstrated between the former two groups and SPC35 salmon. At 7dpv 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

445 lower than prior to vaccination. Moreover, significantly higher activity was demonstrated in
446 SPC50 and SPC65 salmon compared to the SPC35 group (Modelled response is shown in
447 Fig. 6A). Expected plasma IgM levels prior to vaccination demonstrated no differences
448 among the four dietary groups of salmon. At both post vaccination points (7dpv, 34dpv),
449 total plasma IgM concentrations were found to be significantly higher for SPC50 and SPC65
450 salmon compared to SPC35 salmon while similar levels were demonstrated for SPC35 and
451 SPC80 salmon (Modelled response is illustrated in Fig.6B). Expected haemolytic activity
452 demonstrated minor differences among the four experimental dietary groups of salmon prior
453 to and at 7 dpv. However, at 34 dpv plasma haemolytic activity presented a salient increase
454 for fish from all the dietary treatments with fish fed on medium levels of SPC inclusions
455 possessing the highest values. Significant differences were denoted for the SPC50, SPC65
456 and SPC80 groups in comparison to SPC35 salmon at 34dpv (Modelled response is shown
457 in Fig.6C).

458 **4. Discussion**

459 ***4.1. Growth***

460 Phytic acid in soybean products has been proven to reduce nutrient digestibility, inhibit the
461 activity of gastrointestinal enzymes and compromise dietary intake, feed efficiency and fish
462 growth (Destandli et al., 2006; Kumar et al. 2012). Compositional analyses of the tested
463 treatments demonstrated an increase in the phytic acid concentrations upon increased dietary
464 inclusion of SPC which could explain the reductions on juvenile salmon growth performance
465 (Destandli et al. 2006). The reductions in salmon mean weight and daily WG were much
466 more evident during the first (Day 0 - Day 36) and second period (Day 36 – Day 63), prior
467 to salmon vaccination, while improved growth was observed at post vaccination, during the
468 last part of the study, indicating a slow but steady adaptation of juvenile salmon to diets with
469 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 to the SPC35 group, numerically this decrease was not substantial. Overall, weight reduction in SPC80 compared to 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 to 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).

4.2. Body cross-sectional composition

To overcome any differences attributed to dietary P limitations in SPC, 30 g × 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 × 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 × 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, 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 0.8-0.9 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 to the first timepoint, 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).

Similarly to our findings, Storebakken et al. (1998) reported decreased levels of whole body ash, P, Ca Mg, Zn and Ca:P ratio in salmon fed diets with 75% of dietary protein from SPC and 30 g × kg^{-1} of supplementary dicalcium phosphate for 84 days compared to 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

520 phosphorus might lead to the inability of the fish to retain several minerals including Ca and
521 Mg (Åsgard and Shearer 1997; Storebakken et al. 1998). In addition to that, the chelating
522 effects of this substance on positively charged ions such as Ca, Mg, Mn and Zn and the
523 formation of insoluble salts within the gut are the main reasons for the observed reduction
524 of these minerals in whole body samples (Francis et al. 2000; Lall 2003). Therefore, it is
525 suggested that increased dietary SPC inclusion in salmon diets should be combined with an
526 appropriate increment in dietary P supplementation. An alternative solution, of great
527 potential, would be the use of graded levels of phytases which are enzymes that can break
528 down phytic acid and their salts, increasing the availability of P and several other essential
529 macro- and micro-nutrients from plant feedstuffs such as SPC (Storebakken et al. 1998;
530 Cheng and Hardy 2003; Carter and Sajjadi 2011). Phytase can be applied prior pelleting or
531 onto pellets (Denstadli et al. 2007; Wang et al. 2008). However, the high processing costs,
532 the existent inconsistencies regarding the optimum doses of phytase in salmon plant based
533 feeds for the replacement of inorganic P, the inactivation of the enzyme at high temperatures
534 required for pelleting (>80°C) or certain storage temperatures and the narrow optimum pH
535 range for its activation are the main limiting factors for the current use of phytase in
536 commercial salmon feeds (Carter and Sajjadi 2011; Kumar et al. 2012).

537 The present study shows that diets with higher than 50% of dietary protein from SPC
538 require higher than 30 g × kg^{-1} of dicalcium phosphate supplementation in order to promote
539 both good growth and sufficient bone mineralisation. Studies on salmonids have shown that
540 diets supplemented with phosphates of a low Ca: P ratio (≤ 1) are more digestible, improving
541 the availability of P to fish (Aliphos 2012). Therefore, monocalcium or monoammonium
542 phosphates might be more appropriate inorganic P sources compared to dicalcium phosphate
543 in high plant protein inclusion diets (Aliphos 2012). Additional fortification of such diets
544 with other nutrients (vitamins or minerals) should not be excluded (Prabhu et al. 2013; 2014;

545 Torstensen 2014), with Zn being a predominant candidate mineral to consider for increased
546 supplementation upon increased addition of plant proteins in salmon feeds as shown by the
547 present data (Helland et al. 2006; Prabhu et al. 2014). In an era of dynamic changes in the
548 protein sources used in aqua-feeds, Atlantic salmon elemental requirements (NRC 2011),
549 should be re-assessed so that the requirements of salmon grown on diets with higher plant
550 protein inclusions are met (Prabhu et al. 2013; 2014). Hence, the assessment of elemental
551 availability from commercially applied alternative dietary protein sources and identification
552 of Atlantic salmon elemental requirements according to their growth rate and life stage, are
553 of uttermost importance in order to proportionately balance the inclusion of these nutrients
554 in their dietary premixes (Torstensen et al. 2008; Prabhu et al. 2013; 2014).

555 The reduction in body cross-sectional ash, Ca: P ratio, Ca, P, Mg, Mn, and Zn levels
556 detected at Day 97 (34 dpv) of the study compared to Day 63 (prior vaccination) seems to
557 have a multifactorial cause. Storebakken et al. (1998) stated that reductions in body
558 elemental concentrations at different production stages could be attributed to differences in
559 fish size. Vaccination with injectable oil-based vaccines can also exert a negative effect on
560 the mineral status of salmon (Berg et al. 2006; Grini et al. 2011; Berg et al. 2012). However,
561 the negative impacts of injection-vaccination on salmon skeletal mineral levels are often
562 combined with intensive culture conditions inducing faster growth (Grini et al. 2011; Fjelldal
563 et al. 2012). Herein, the primary reason for the observed elemental reductions seems to be
564 the enhanced growth performance of salmon, promoted by the application of continuous
565 light (combined with favourable temperature of ~13°C for salmon growth) and the intensive
566 feeding regime which represent the commercial reality (Kråkenes et al. 1991; Fjelldal et al.
567 2005; 2006; Stefansson et al. 2008). These conditions have led to the achievement of almost
568 double daily WG and ~1.6 greater weight in juvenile salmon compared to the study
569 performed by Helland et al. (2006), despite the fact that fish have been subjected to

570 vaccination during the last part of the trial. Fjelldal et al. (2006) reported that fast growing
571 Atlantic salmon post-smolts after 6 months under continuous light demonstrated lower
572 mineral content and mechanical strength in vertebral bones than slower growing salmon
573 under natural light. Rungruangsak-Torrissen et al. (2009) added that increased growth in
574 salmon by exposure to continuous light could be associated with reduced vertebral
575 mineralisation and strength. Moreover, Hernandez et al. (2000) noted that during periods of
576 fast growth, the actual time required for the bone matrix to be produced and mineralised
577 could be decreased to a critical level, resulting to under-mineralised bony tissues. This was
578 one of the proposed explanations for the higher incidence of vertebral deformities in fast
579 growing under-yearling Atlantic salmon smolts compared to slower growing yearling smolts
580 (Fjelldal et al. 2006). In addition, Stefansson et al. (2007; 2008) noted that juvenile Atlantic
581 salmon reared under constant light (as a means of blocking salmon maturation and
582 smoltification) could develop into "pseudo-smolts" exhibiting all the external changes,
583 characterising smoltifying salmon but not the physiological changes required for seawater
584 adaptation. Therefore, disturbances in ion and osmo-regulation of salmon due to "pseudo-
585 smoltification" could have led to reduced elemental levels at Day 97.

586 Despite the observed decline in elemental levels at Day 97 compared to Day 63 in all
587 dietary groups, salmon fed on higher dietary SPC levels demonstrated subsidiary decrements
588 in body cross-sectional ash and elemental concentrations (Zn excluded) compared to Day 63
589 (Fig. 3, 4A & 4C). On the contrary, the decrements in body cross-sectional elemental levels
590 were much more evident in the groups fed lower dietary SPC levels, with SPC35 salmon
591 demonstrating the greatest reductions regarding ash and mineral concentrations by
592 comparison to Day 63. The improvement in elemental retention observed in salmon fed diets
593 with increasing dietary amounts of SPC could primarily be attributed to the overall delayed
594 growth performance of these groups compared to SPC35 salmon. Fjelldal et al. (2010)

595 reported that in the long term, high plant protein inclusion feeds could have a positive impact
596 on the skeletal health of Atlantic salmon post-smolts by delaying salmon growth. Possible
597 extension of the study could have eliminated the elemental differences among the four
598 dietary groups. In addition to the overall delayed growth, better assimilation of high SPC
599 inclusion feeds (either through improved feed intake and/or digestibility) is suggested by the
600 improvement in the growth performance (daily WG) of these groups, during the last period
601 of the study (post-vaccination period from Day 63 to Day 97) (Fig. 2B). Earlier studies
602 suggest that Atlantic salmon requires long adaptation periods in order to accept and start
603 utilising efficiently high plant protein diets, after which they might be able to compensate
604 growth and nutrient uptake (Torstensen et al. 2008; Burr et al. 2011). Moreover, according
605 to Prabhu et al. (2013; 2014), the elemental requirement for WG in farmed fish differs from
606 the requirement for proper skeletal mineralisation and these depend on both the growth rate
607 and the life stage of the fish. Herein, it was evident that during the last period of the study,
608 high SPC inclusion diets performed better by comparison to the first two periods and
609 matched more closely the elemental requirements of the fish they were allocated to, for both
610 growth and skeletal mineralisation compared to their SPC35 counterpart.

611 ***4.3. Haematological and Immune responses***

612 For Atlantic salmon, haematocrit values, which represent the oxygen carrying capacity of
613 blood, normally range from 35 to 60 % (Hardie et al. 1990; Thompson et al. 1996).
614 Haematocrit levels were found to be within this range in the present study. Decreasing
615 haematocrit however, was observed in salmon fed diets with increasing dietary SPC protein.
616 Hemre et al. (2005) reported decreased erythrocyte size in Atlantic salmon fed diets with
617 increased protein levels from soybeans, which could explain the lower haematocrit in these
618 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 2011). 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, utilizing 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 to 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 on dehulled lupin meal at 40% inclusion. Contrarily Burrells et al. (1999) demonstrated reduction of 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 have been reported in salmon after vaccination or infection via intra-peritoneal injection (Secombes and Olivier 1997), which is in agreement with the present findings. Similarly 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 Brandsen 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 timepoints while higher levels of total IgM were revealed at both timepoints post vaccination compared to SPC35 salmon. In accordance to 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

669 haemolysins (Alexander and Ingram, 1992) was not affected linearly by increasing dietary
670 SPC levels. Prior to and 7 dpv, salmon from different dietary groups exhibited no differences
671 in plasma haemolytic activity. Sitja-Bobadilla et al. (2005) and Jalili et al. (2013) reported
672 decreased alternative complement activity in gilthead sea bream (*Sparus aurata*) and
673 rainbow trout respectively, fed diets with 70 % protein from plant derived products. The
674 former study however, exhibited an enhancement of alternative complement activity in
675 gilthead sea bream fed 50 % of protein from vegetal ingredients. At 34 dpv, there was a
676 marked increase of haemolytic activity in fish from all the dietary treatments with SPC50,
677 SPC65 and SPC80 salmon exhibiting higher activity than SPC35 salmon suggesting higher
678 immunostimulation of these groups at 34 dpv while highlighting dietary modifications that
679 were not evident prior to vaccination (Thompson et al. 1996; Nikoskelainen et al. 2002).

680 **4. Conclusions**

681 In conclusion, increasing substitution of high quality FM with SPC at a percentage beyond
682 35% of dietary protein reduced the growth performance and body cross-sectional elemental
683 concentrations and enhanced several humoral immune responses of juvenile Atlantic
684 salmon. Diets with 50% of protein from SPC, appropriate supply of lysine, methionine and
685 threonine and 30 g × kg^{-1} of P promoted similar growth and elemental composition to a
686 control diet with 35 % of protein from SPC, mimicking commercial salmon formulations.
687 Body cross-sectional elemental reductions due to the increasing dietary SPC levels, observed
688 during the initial 63-day period, were improved at Day 97 (34 dpv). This seems to be both
689 an effect of the long term provision of juvenile salmon with increasing dietary SPC levels or
690 an interaction of these diets with vaccination. Moreover, it was shown that exposure of
691 Atlantic salmon to continuous light, intensive feeding regimes and vaccination may result in
692 elemental deficiencies rendering developing salmon susceptible to skeletal deformities or
693 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.

Acknowledgments

This study was financially supported by the University of Stirling and EWOS Innovation. The authors would like to thank Mr. Graeme McWhinnie and Mr. William Struthers from the Institute of Aquaculture, University of Stirling for their technical assistance in proximate and elemental composition analyses. We would also like to thank the technical staff of EWOS Innovation for the feed manufacture, the performance of the feeding trial and their assistance during the samplings.

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Figure captions

Figure 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 timepoint of the study.

Figure 2. **Modelled representation of growth performance for the four dietary groups at each timepoint**. **(A)** Expected mean salmon weight (g) and **(B)** Expected mean daily weight gain per fish ($g \times day^{-1}$); (with 95% C.I.) 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 - 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 - 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 - 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% C.I. of the 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 timepoints.

Figure 4. 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 ($g \times kg^{-1}$); **(B)** Ca: P ratio; **(C)** Phosphorus (P) ($mg \times kg^{-1}$); **(D)** Calcium (Ca) ($mg \times kg^{-1}$) (with 95% C.I.) (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% C.I. 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 timepoints.

Figure 5. 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% C.I.) (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% C.I. 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 timepoints.

Figure 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% C.I. 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 timepoints.

Figure 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 –prevacc-(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% C.I. 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 timepoints.

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

Feed composition (× kg^{-1})	SPC35	SPC50	SPC65	SPC80	SPC35	SPC50	SPC65	SPC80
	2mm				3mm			
Fishmeal [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 (× 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

Abbreviations: SPC 35 - diet with 35 % of dietary protein soy protein concentrate (SPC); SPC 50 - diet with 50 % of dietary protein from SPC; SPC 65 - diet with 65 % of dietary protein from SPC; SPC 80 - diet with 80% of dietary protein from SPC.

[a] Fishmeal (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 % (Antitrypsins < 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 authorized 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).

* 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.

Table 2. Performance data of juvenile Atlantic salmon dietary groups.

Growth and survival	SPC35	SPC50	SPC65	SPC80	Linear	Quadratic
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	*P<0.05*	*NS*
Intermediate weight (g) (Day 63)	122.8±1.47	118.6±4.23	110.7±11.12	112.8±5.66	*P<0.05*	*NS*
Final weight (g) (Day 97)	174.1±3.56	169.4±5.70	159.8±9.76	162.4±12.03	*P<0.05*	*NS*
Weight gain*($g \times fish^{-1} \times day^{-1}$) (Days 0-36)	1.21±0.10	1.12±0.08	1.08±0.17	1.03±0.16	*P<0.05*	*NS*
Weight gain*($g \times fish^{-1} \times day^{-1}$) (Days 36-63)	2.05±0.16	2.03±0.07	1.76±0.40	1.93±0.09	*P<0.05*	*NS*
Weight gain*($g \times fish^{-1} \times day^{-1}$) (Days 63-97)	1.48±0.09	1.46±0.08	1.43±0.12	1.42±0.21	*P<0.05*	*NS*
Feed Given ($g \times fish^{-1}$) (Days 0-36)	20.55±0.42	20.24±0.42	20.08±0.62	20.22±0.56	-	-
Feed Given ($g \times fish^{-1}$) (Days 36-63)	25.83±0.65	25.06±1.06	23.53±2.42	23.83±1.68	-	-
Feed Given ($g \times fish^{-1}$) (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	*NS*	*NS*

Data for performance represent means ±SD for 4 replicate tanks.
Abbreviations: SPC 35 - diet with 35 % of dietary protein from soy protein concentrate (SPC); SPC 50 - diet with 50 % of dietary protein from SPC; SPC 65 - diet with 65 % of dietary protein from SPC; SPC 80 - 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) × $(\text{Number of fish within treatment})^{-1} * (\text{Number of trial days})^{-1}$.

Figure 1.

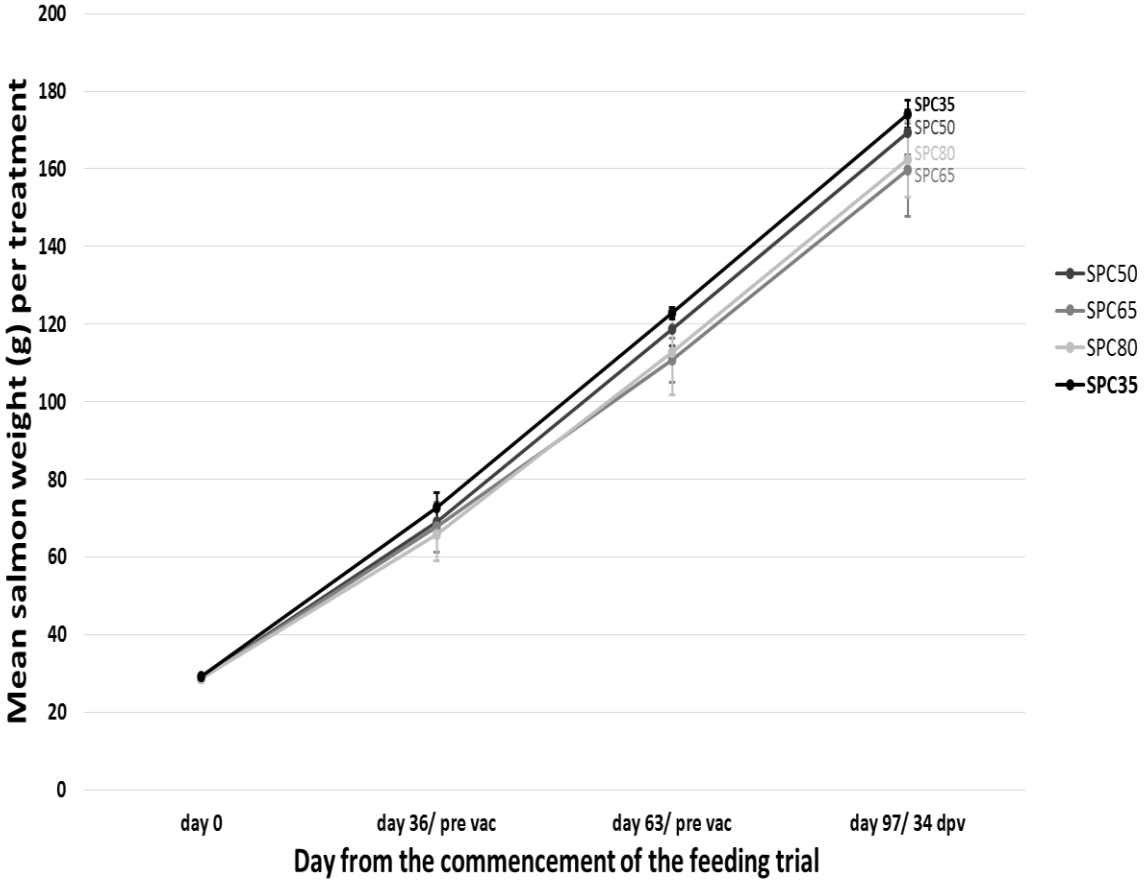

Mean salmon weight (g) per treatment
200
180
160
140
120
100
80
60
40
20
0
day 0
day 36/ pre vac
day 63/ pre vac
day 97/ 34 dpv
Day from the commencement of the feeding trial
SPC35
SPC50
SPC80
SPC65
SPC50
SPC65
SPC80
SPC35

Figure 2.

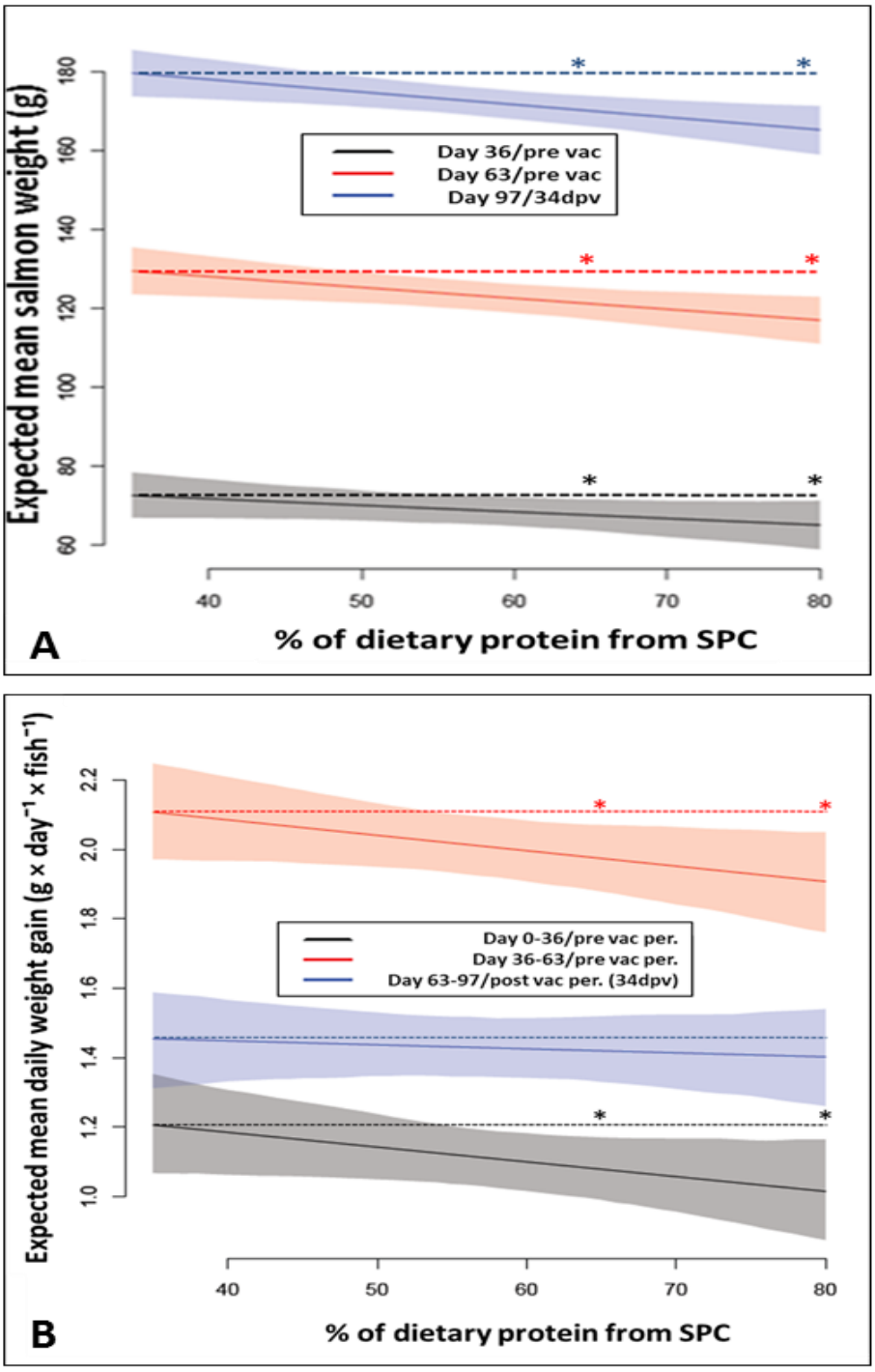

Expected mean salmon weight (g)
60
80
100
120
140
160
180
Day 36/pre vac
Day 63/pre vac
Day 97/34dpv
40
50
60
70
80
% of dietary protein from SPC
A
Expected mean daily weight gain (g × day⁻¹ × fish⁻¹)
1.0
1.2
1.4
1.6
1.8
2.0
2.2
Day 0-36/pre vac per.
Day 36-63/pre vac per.
Day 63-97/post vac per. (34dpv)
40
50
60
70
80
% of dietary protein from SPC
B

Figure 3.

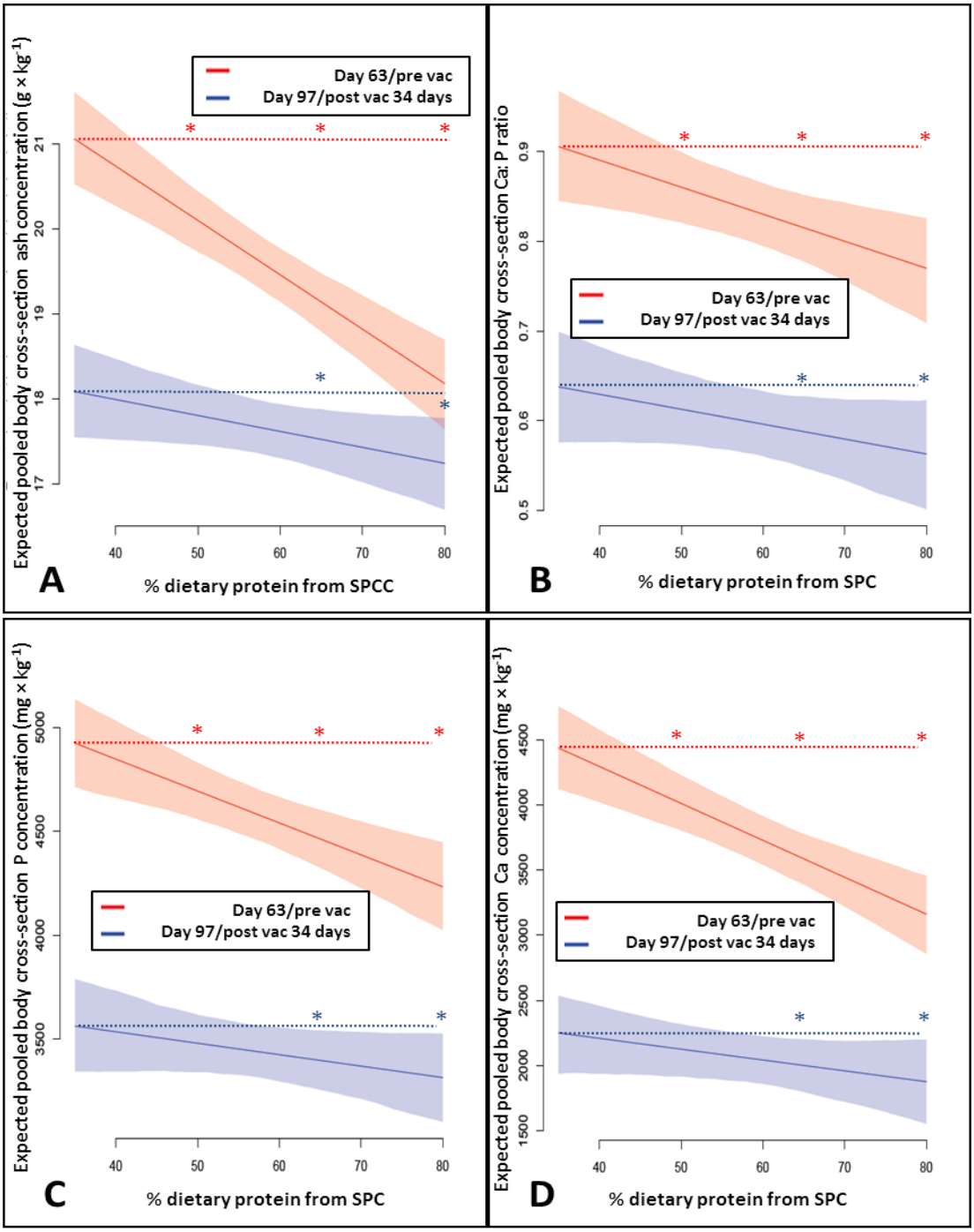

Expected pooled body cross-section ash concentration (g × kg-1)
Day 63/pre vac
Day 97/post vac 34 days
A
% dietary protein from SPCC
Expected pooled body cross-section Ca: P ratio
Day 63/pre vac
Day 97/post vac 34 days
B
% dietary protein from SPC
Expected pooled body cross-section P concentration (mg × kg-1)
Day 63/pre vac
Day 97/post vac 34 days
C
% dietary protein from SPC
Expected pooled body cross-section Ca concentration (mg × kg-1)
Day 63/pre vac
Day 97/post vac 34 days
D
% dietary protein from SPC

Figure 4.

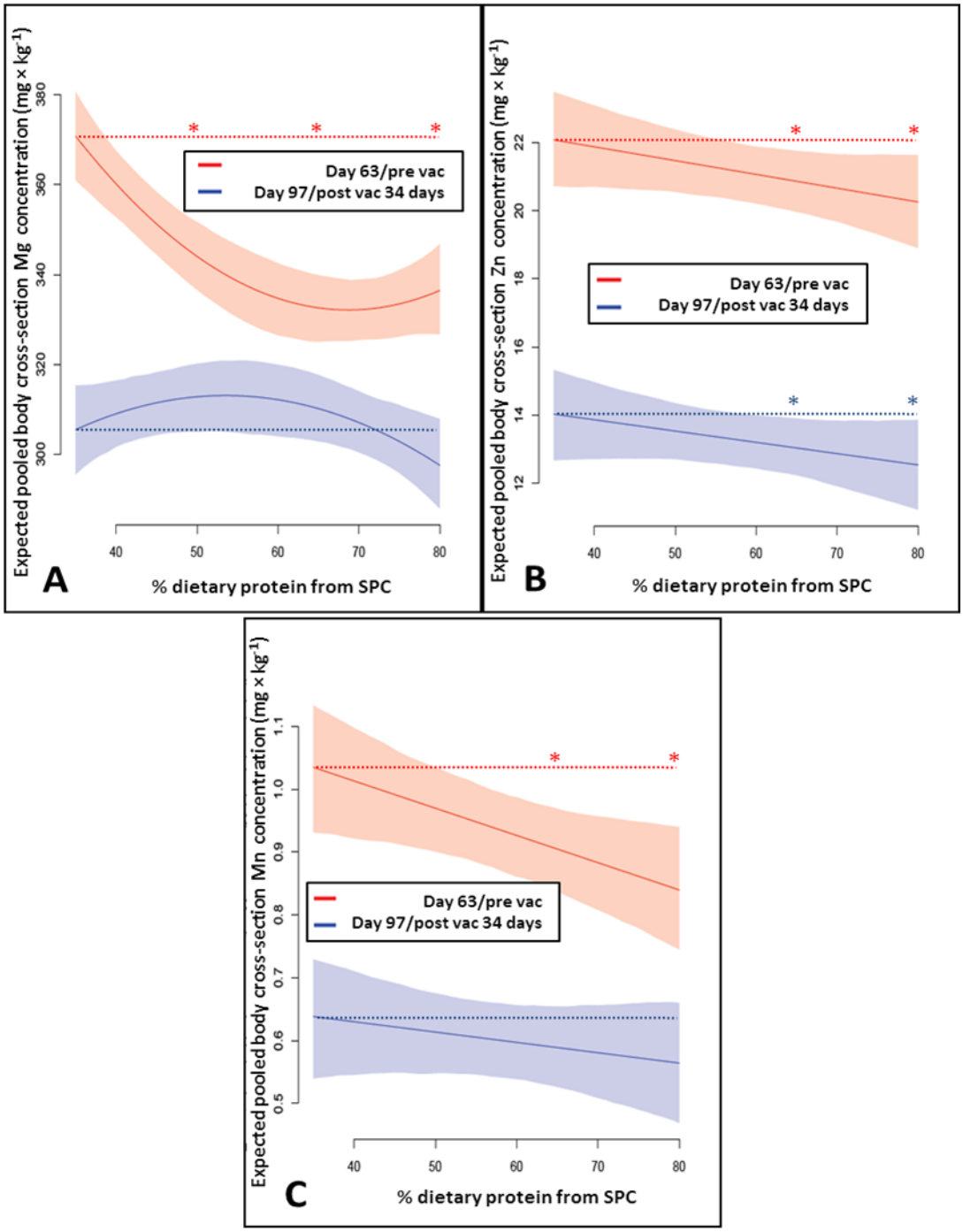

Expected pooled body cross-section Mg concentration (mg × kg⁻¹)
300
320
340
360
380
40
50
60
70
80
% dietary protein from SPC
Day 63/pre vac
Day 97/post vac 34 days
A
Expected pooled body cross-section Zn concentration (mg × kg⁻¹)
12
14
16
18
20
22
40
50
60
70
80
% dietary protein from SPC
Day 63/pre vac
Day 97/post vac 34 days
B
Expected pooled body cross-section Mn concentration (mg × kg⁻¹)
0.5
0.6
0.7
0.8
0.9
1.0
1.1
40
50
60
70
80
% dietary protein from SPC
Day 63/pre vac
Day 97/post vac 34 days
C

Figure 5.

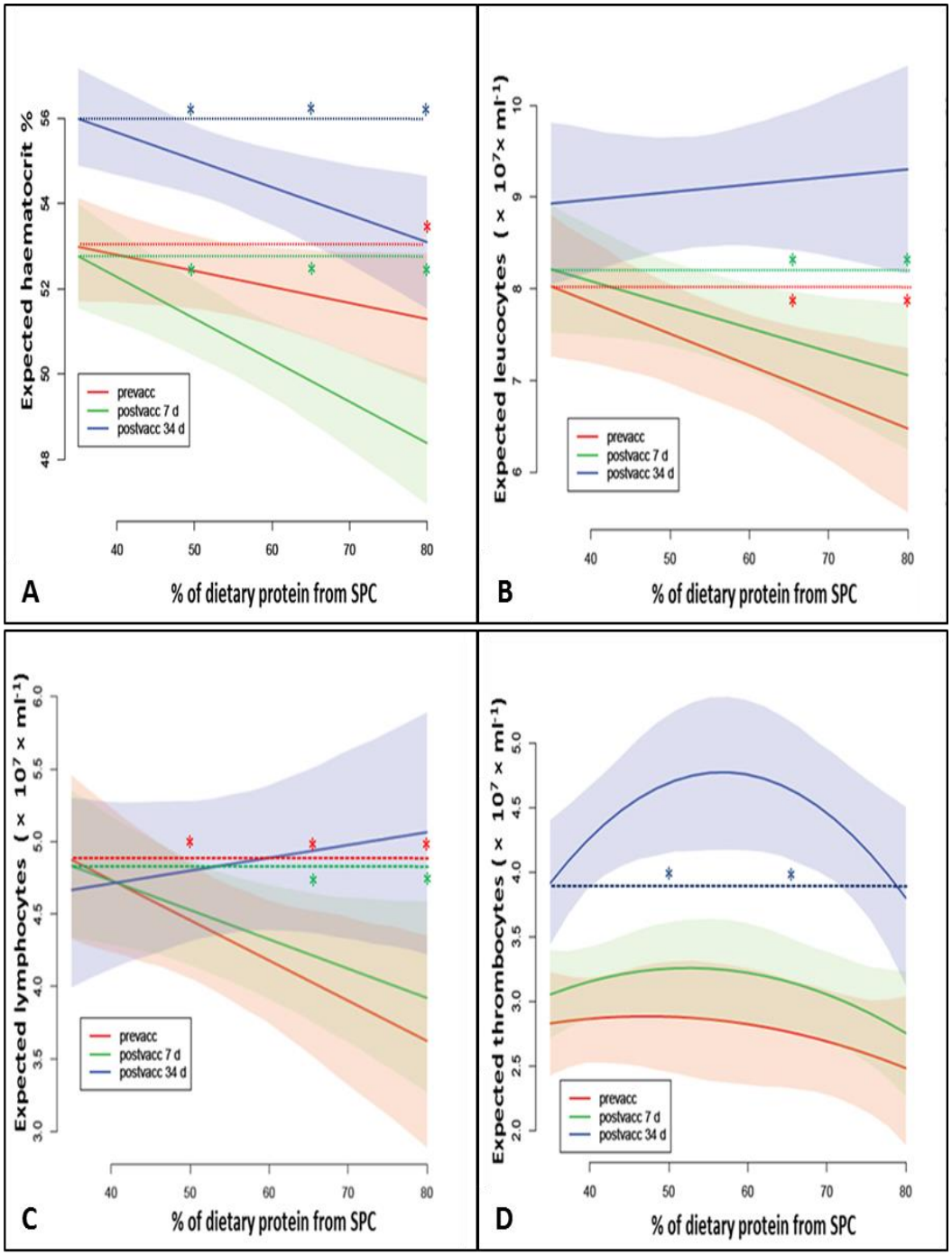
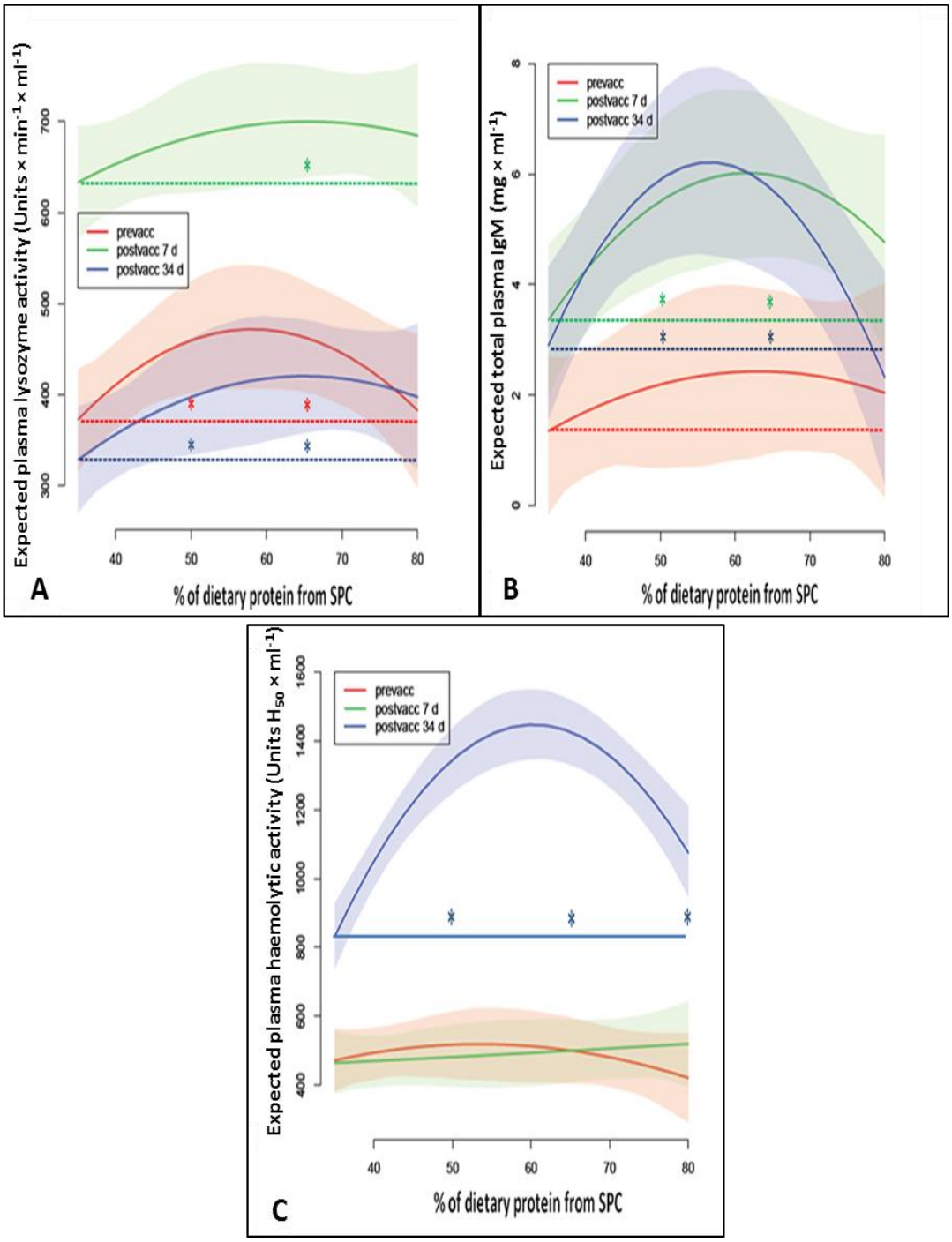

Expected haematocrit %
prevacc
postvacc 7 d
postvacc 34 d
% of dietary protein from SPC
A
Expected leucocytes (× 10⁷× ml⁻¹)
prevacc
postvacc 7 d
postvacc 34 d
% of dietary protein from SPC
B
Expected lymphocytes (× 10⁷ × ml⁻¹)
prevacc
postvacc 7 d
postvacc 34 d
% of dietary protein from SPC
C
Expected thrombocytes (× 10⁷ × ml⁻¹)
prevacc
postvacc 7 d
postvacc 34 d
% of dietary protein from SPC
D

Figure 6.


Expected plasma lysozyme activity (Units × min-1 × ml-1)
prevacc
postvacc 7 d
postvacc 34 d
A
% of dietary protein from SPC
Expected total plasma IgM (mg × ml-1)
B
% of dietary protein from SPC
Expected plasma haemolytic activity (Units H50 × ml-1)
C
% of dietary protein from SPC

Supplementary Data

Table 2. Ash and elemental composition of Atlantic salmon parr pooled body cross-sections

Mineral Composition (× kg^{-1})	SPC35	SPC50	SPC65	SPC80	Linear	Quadratic
Day 63(prior to vaccination)						
Ash (g)	2.12±0.06	2.00±0.03	1.90±0.06	1.83±0.08	*P<0.05*	*NS*
Phosphorus (P) (mg)	4887±208	4875±472	4220±102	4336±314	*P<0.05*	*NS*
Calcium (Ca) (mg)	4265±286	4269±511	3602±95	3069±704	*P<0.05*	*NS*
Ca: P ratio	0.87±0.03	0.87±0.03	0.85±0.01	0.70±0.01	*P<0.05*	*NS*
Magnesium (Mg) (mg)	369±11	351±12	320±4	345±22	*NS*	*P<0.05*
Manganese (Mn) (mg)	0.96±0.05	1.09±0.21	0.85±0.11	0.82±0.05	*P<0.05*	*NS*
Zinc (Zn) (mg)	22.02±0.67	22.29±2.78	19.41±0.88	20.88±1.64	*P<0.05*	*NS*
Day 97(34 days post vaccination)						
Ash (g)	1.77±0.02	1.85±0.05	1.74±0.05	1.71±0.09	*P<0.05*	*NS*
Phosphorus (P) (mg)	3480±25	3602±53	3439±114	3261±62	*P<0.05*	*NS*
Calcium (Ca) (mg)	2127±171	2328±190	2002±101	1816±89	*P<0.05*	*NS*
Ca: P ratio	0.61±0.04	0.65±0.05	0.58±0.02	0.56±0.04	*P<0.05*	*NS*
Magnesium (Mg) (mg)	305±5	315±5	308±12	298±4	*NS*	*P<0.05*
Manganese (Mn) (mg)	0.56±0.05	0.70±0.08	0.58±0.06	0.54±0.02	*P<0.05*	*NS*
Zinc (Zn) (mg)	13.29±1.28	14.60±1.11	13.19±0.67	12.06±0.58	*P<0.05*	*NS*

Data for ash and elemental composition are referred as means ± SD of 4 pooled samples (1 pool of 6 body cross-section homogenates per tank).
Abbreviations: SPC 35 - diet with 35 % of dietary protein from soy protein concentrate (SPC); SPC 50 - diet with 50 % of dietary protein from SPC; SPC 65 - diet with 65 % of dietary protein from SPC; SPC 80 - diet with 80% of dietary protein from SPC.

Supplementary Data

Table 3. Haematological and immunological responses of Atlantic salmon juveniles

PRE VACCINATION	SPC35	SPC50	SPC65	SPC80	Linear	Quadratic
Haematocrit (%)	53.0±4.5	51.9±4.7	51.8±4.7	51.3±4.4	*P<0.05*	*NS*
Leucocytes (× 10^7× ml^{-1})	6.7±2.3	7.5± 1.7	7.1± 0.9	6.4 ±2.0	*P<0.05*	*NS*
Lymphocytes (×10^7× ml^{-1})	4.2±1.6	4.3±1.1	4.1±0.6	3.6±1.4	*P<0.05*	*NS*
Thrombocytes (×10^7× ml^{-1})	2.2±0.9	3.0±1.0	2.6±0.7	2.7±1.2	*NS*	*P<0.05*
Granulocytes (×10^7× ml^{-1})	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.04	*NS*	*NS*
Monocytes (×10^7× ml^{-1})	0.02±0.02	0.02±0.02	0.02±0.02	0.02±0.03	*NS*	*NS*
Lysozyme act. (units × min^{-1}× ml^{-1} of plasma)	369.8±80.4	480.2±90.9	442.1±78.2	389.7±118.5	*NS*	*P<0.05*
Haemolytic act. (units H_{50} × ml^{-1} of plasma)	444.3±87.9	497.4±130.4	528.4±105.2	416.4±64.8	*NS*	*P<0.05*
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	0.2±0.2	0.2±0.1	0.2±0.1	0.3±0.1	*NS*	*NS*
Stimulated HKMs resp. burst (OD for 10^5 nuclei)	0.4±0.3	0.4±0.2	0.4±0.3	0.5±0.2	*NS*	*NS*
Total plasma protein (mg × ml^{-1} of plasma)	48.3±5.2	49.5±3.6	47.7±2.8	47.4±4.9	*NS*	*NS*
Antiprotease activity (Units TI_{75} × ml^{-1})**	845.6±96.6	908±39.8	855.7±60.2	891.6±67.4	*NS*	*NS*
Total plasma IgM (mg × ml^{-1} of plasma)	1.9±3.0	1.3±2.3	2.3±2.0	0.9±1.1	*NS*	*P<0.05*
7 DAYS POST VACCINATION						
Haematocrit (%)	52.2±5.1	49.4±4.3	51.6±6.7	47.8±4.3	*P<0.05*	*NS*
Leucocytes (× 10^7 × ml^{-1})	8.4±1.7	8.5±1.9	8.2±3.0	6.4±2.5	*P<0.05*	*NS*
Lymphocytes (×10^7× ml^{-1})	4.5±1.3	4.7±1.5	4.8±1.9	3.5±1.5	*P<0.05*	*NS*
Thrombocytes (×10^7× ml^{-1})	3.4±1.0	3.3±0.9	3.1±1.3	2.6±1.2	*NS*	*P<0.05*
Granulocytes (×10^7× ml^{-1})	0.4±0.3	0.4±0.3	0.4±0.3	0.3±0.2	*NS*	*NS*
Monocytes (×10^7× ml^{-1})	0.02±0.03	0.04±0.04	0.03±0.04	0.02±0.02	*NS*	*NS*
Lysozyme act. (units × min^{-1}× ml^{-1} of plasma)	639.4±216.0	719.4±75.1	662.0±184.1	698.63±97.0	*NS*	*P<0.05*

Haemolytic act. (units $H_{50} \times ml^{-1}$ of plasma)	446.0±96.1	447.2±108.1	529.0±133.7	507.6±209.1	*NS*	*P<0.05*
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	0.6±0.5	0.8±0.4	0.9±0.3	0.8±0.2	*NS*	*NS*
Stimulated HKMs resp. burst (OD for 10^5 nuclei)	0.9±0.8	1.0±0.6	1.1±0.4	1.1±0.3	*NS*	*NS*
Total plasma protein (mg × ml^{-1} of plasma)	42.1±5.7	39.5±2.6	40.3±3.1	37.3±3.5	*NS*	*NS*
Antiprotease activity (Units $TI_{75} \times ml^{-1}$)**	653.3±98.6	677.9±138.2	647.1±98.0	667.8±97.6	*NS*	*NS*
Total plasma IgM (mg × ml^{-1} of plasma)	3.6±2.3	4.9±3.6	6.5±4.1	3.8±2.9	*NS*	*P<0.05*
34 DAYS POST VACCINATION						
Haematocrit (%)	55.3±4.1	54.5±2.8	54.4±3.3	53.0±4.9	*P<0.05*	*NS*
Leucocytes (× 10^7× ml^{-1})	8.7±1.5	8.6±1.5	9.4±2.9	9.3±2.9	*P<0.05*	*NS*
Lymphocytes (×10^7× ml^{-1})	4.1±0.9	3.9±1.2	4.8±1.7	5.5±2.7	*P<0.05*	*NS*
Thrombocytes (×10^7× ml^{-1})	4.2±1.1	4.5±1.2	4.8±2.2	3.8±1.2	*NS*	*P<0.05*
Granulocytes (×10^7× ml^{-1})	0.4±0.2	0.2±0.1	0.3±0.2	0.2±0.1	*NS*	*NS*
Monocytes (×10^7× ml^{-1})	0.05±0.05	0.03±0.03	0.05±0.05	0.04±0.05	*NS*	*NS*
Lysozyme act. (units × min^{-1}× ml^{-1} of plasma)	438.4±68.4	373.3±96.3	450.4±62.7	390.0±78.9	*NS*	*P<0.05*
Haemolytic act. (units $H_{50} \times ml^{-1}$ of plasma)	1183.7±126.7	1344.6±242.2	1424.9±208.2	1072.4±126.9	*NS*	*P<0.05*
HKMs resp. burst (NBT) (OD for 10^5 nuclei)	0.2±0.1	0.3±0.2	0.2±0.1	0.3±0.2	*NS*	*NS*
Stimulated HKMs resp. burst (OD for 10^5 nuclei)	0.3±0.1	0.3±0.2	0.2±0.1	0.3±0.2	*NS*	*NS*
Total plasma protein (mg × ml^{-1} of plasma)	46.4±7.7	8.6±1.5	9.4±2.9	9.3±2.9	*NS*	*NS*
Antiprotease activity (Units $TI_{75} \times ml^{-1}$)**	596.6±15.9	604.0±16.8	600.6±30.2	598.9±11.6	*NS*	*NS*
Total plasma IgM (mg × ml^{-1} of plasma)	3.6±3.8	6.4±2.6	5.6±2.8	2.9±2.2	*NS*	*P<0.05*
Specific IgM (plasma titers)	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01	*NS*	*NS*

Values for immune responses are means ±SD of 8 pools of 2 fish per diet ±SD; haematocrit values are means ±SD of 24 fish; leukocyte and differential leucocyte counts are means ± SD of 12 fish per diet. Abbreviations: SPC 35 - diet with 35 % soy protein concentrate (SPC); SPC 50 - diet with 50 % SPC; SPC 65 - diet with 65 % SPC; SPC 80 - diet with 80% SPC.

**Units $TI_{75} \times min^{-1} \times ml^{-1}$ of plasma.