THE EFFECT OF LOW MARINE INGREDIENT FEEDS ON THE GROWTH PERFORMANCE BODY COMPOSITION AND HEALTH STATUS OF ATLANTIC SALMON (*Salmo salar*)

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by

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Declaration

I hereby declare that this thesis has been composed entirely by myself and has not been submitted for any other degree. Except where specifically acknowledged the work described in this thesis is the result of my own investigations.

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Abstract

Fish meals (FM) and fish oils (FO) are used extensively as the main protein and lipid sources respectively in industrially compounded salmon feeds, mainly due to their excellent nutritional properties. Nevertheless, several reasons dictate the utilisation of sustainable alternative protein and lipid sources and the subsequent reduction in the reliance on fishery-products in aquafeeds. Soy protein concentrate (SPC) is a very promising alternative to FM Hence, the main objective of the present thesis was to investigate the effects of the increased substitution of FM withSPC, lysine and methionine on the growth performance and immune responses of early and late stage Atlantic salmon parr prior to and after vaccination with commercial vaccines (Experiments II and V presented in Chapters 4 and 6). Furthermore the effects of increasing dietary levels of SPC with consistent and/or increasing dietary supplementation of phosphorus on the Atlantic salmon parr' body proximate and mineral composition or the skeletal mineral composition respectively, prior and post-vaccination with commercial vaccines; under continuous light or under 12 hours light: 12 hours dark photoperiod (Experiments I and V presented in Chapters 3 and 5 respectively). Lastly the impact of FM-, FO- and fishery-free diets on the growth, carcass proximate composition and immune status of Atlantic salmon post-smolts was investigated (Experiment III presented in Chapter 7). In the latter experiment six diets were tested including: two commercially applied marine based diets, one with partial inclusion of vegetable proteins (VPs) and oils (VOs) according to the EU standards (2011-12) (MBE) and one with partial inclusion of VPs, VOs and land animal-by product (ABP) proteins according to the non-EU standards (MBABP); a fully vegetable protein (VP) diet; a fully algal and vegetable oil (VO) diet; a marine-free VP and VO and algal oil diet (VP/VO) diet; and a marine-free diet with a mix of VPs and land ABP proteins and lipid from VOs and algal oils (MFABP).

The results of the Experiments I and II (Chapters 3 and 4) show that late Atlantic salmon parr can grow efficiently on SPC80 diets, however, they require longer periods to adapt to these diets compared to fish fed diets containing lower levels of SPC as a protein source. Decreased mineralisation of body cross-section was observed for salmon fed increasing dietary SPC. Vaccination improved mineralisation for the high dietary SPC salmon groups. However, continuous light exposure promoting fast growth appeared to be detrimental for Atlantic salmon body cross ash, Ca, Mg, Mn, P and Zn. Moreover, it was demonstrated that substitution of up to 50% of high quality FM protein with SPC and constantly added P has the minimum possible impact on late salmon parr growth, whereas it stimulates several immune parameters prior to immunisation. Immunostimulatory effects were also shown for the diets with higher dietary SPC levels. It is not clear if these results were an effect of increased FM replacement with SPC or not properly balanced levels of dietary P. The Experiments III and IV (Chapters 5 and 6) illustrated that early stage Atlantic salmon parr can accept diets with up to 58% protein from SPC without serious effects on body growth. However, higher levels can severely affect salmon growth performance. Moreover, it was shown that long-term feeding of salmon with increasing dietary SPC combined with increasing phosphate supplementation, alone or in combination with vaccination can actually be beneficial for Atlantic salmon parr bone mineralisation. However, mineralisation in vaccinated fish was higher than in PBS-injected fish. This could be linked to the slower growth of vaccinated salmon allowing their developing bones to mineralise properly. Changes in the modulation of the different components of the complement activity was revealed in Atlantic salmon fed increasing dietary levels of SPC. The modulation of complement activity was demonstrated at both studies utilising increasing dietary SPC concentrations, indicating that complement componenets are among the most prominent immunological markers upon dietary FM replacement with SPC. However,

overall no differences in total complement activity and therefore the immune capacity and resistance against *Aeromonas salmonicida* were observed among the salmon groups receiving increasing levels of SPC.

Lastly in Experiment V (Chapter 7) higher growth performance indices (weight gain, SGR and TGC) were evident in the MBE salmon compared to the MBABP group, salmon fed diets with complete eleimintion of FM or FO (VP and VO respectively) and fish fed two diets with total substitution of both marine derived feed ingredients, three months after the start of the feeding trial. Higher feed intake was demonstrated for both MBE and VP salmon compared to the other groups for the duration of the first period. The above results could have been influenced by discrepancies in the size of the fish at the start ofthe trial, revealing flaws in the experimental design. Both MBE and VP salmon groups also presented the highest feed conversion ratios, revealing the lowest efficiency in dietary nutrient utilisation in comparison to the rest of salmon which exhibited no differences in feed efficiency, revealing an overall better performance of the MBABP and diets with low levels of marine feedstuffs. Improved FI compared to the values of the first period and higher SGR and TGC values were demonstrated for salmon from the latter treatments compared to MBE and VP salmon, during the second part of the study, revealing compensatory growth for these groups. VP salmon demonstrated the highest and VO salmon the lowest condition factor values. The former finding might possibly be related with higher fat accumulation in the viscero-hepatic tissues. No differences were observed in carcass moisture, protein, fat and ash concentrations among the dietary groups of fish. Furthermore, no differences were demonstrated in terms of total and differential leucocyte counts, plasma haemolytic activity, plasma protein and total IgM levels, stimulated and non-stimulated HKM burst activity among the different dietary groups. However, lower haematocrit values were observed in the MB and VO-fed groups compared to the MFABP and VP/VO groups. Furthermore, decreased lysozyme

activity was observed for all diets in contrast to the control groups, whereas FM-free diets promoted plasma anti-protease activity. The former result could have been an effect of either immune or stress induction, whereas the second is regarded as an immunostimulatory effect. The results suggest that marine-oil, marine-protein and marine-free diets could be satisfactorily used for Atlantic salmon post-smolts without severe reductions in their innate immune responses, although longer adaptation periods might be required for the fish to fully accept these diets.

Abbreviations and acronyms

AA amino acid(s)
ABP animal by-products
AC (P) alternative complement (pathway)
ANF antinutritional factors
BAPNA N-a-benzoyl-DL-arginine-p-nitroanilide
BSA bovine serum albumin
Ca calcium
CC (P) classical complement (pathway)
cfu colony forming units
CO canola oil
CP crude protein
DAA dispensible amino acids
DHA docosahexaenoic acid (22:6n-3)
DLM dehulled lupin meal
DM dry matter
DMSO dimethyl sulfoxide
DSSM (dehulled solvent extracted soybean meal)
dpPBSinj days post PBS injection
dpi days post injection or days post PBS injection
dpv days post vaccination
EDTA ethylene diamino tetra-acetic acid
EGTA ethylene glycol tetra-acetic acid
ELISA enzyme linked immunosorbent assay
EPA eicosapentaenoic acid (20:5n-3)
FA fatty acid(s)
FCR feed conversion ratio
Fe Iron
FF fishery free
FI feed intake
FM fish meal
FO fish oil
FTU phytase units
G-CFB gelatine-complement fixation buffer
hct haematocrit
HKM head kidney macrophages
HPFM hydrolysed poultry feather meal
HSWB high salt wash buffer
HUFA Highly Unsaturated Fatty Acid(s)
IAA indispensable amino acids
ICP/MS Inductively Coupled Plasma/Mass-Spectrometry
i.m. intramuscular
i.p. intraperitoneal
IPNV infectious pancreatic necrosis virus
IU International Units
K condition factor

LC-PUFA long chain polyunsaturated fatty acids
LRT likelihood ratio test
LSWB low salt wash buffer
MAb monoclonal antibodies
MBE marine based diet according to European standards 2011-12 (inclusion of vegetable proteins and vegetable oils)
MBABP marined based diet with inclusion of animal by-products according to non-EU standards 2011-12 (combined inclusion of vegetable and animal by-product proteins and vegetable oils)
Mg magnesium
Mn manganese
n-3 omega-3 polyunsaturated fatty acids
n-6 omega-6 polyunsaturated fatty acids
NBT nitroblue tetrazolium
NSP non-starch polysaccharides
NQC Norwegian quality cuts
O.D. optical density
P phosphorus
PBS phosphate buffered saline
PMA phorbol myristate acetate
PUFA poly-unsaturated fatty acid(s)
RO rapeseed oil
rpm revolution per minute
SBM soy bean meal
SCO single cell oils
SGR specific growth rate
SPB sodium phosphate buffer
SPC soy protein concentrate
SPC35 diet with 35% of total dietary protein derived from SPC
SPC50 diet with 50% of total dietary protein derived from SPC
SPC58 diet with 58% of total dietary protein derived from SPC
SPC65 diet with 65% of total dietary protein derived from SPC
SPC80 diet with 80% of total dietary protein derived from SPC
SRBC sheep red blood cells
TC total complement
TGC thermal growth coefficient
TSA tryptic soy agar
TSB tryptic soy broth
TSE transmissible spongiform encephalopathies
V vanadium
VO vegetable oil(s)
VP vegetable protein(s)
VP/VO diet fully vegetal diet
WG weight gain
Zn zinc

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Chapter 1.

General Introduction

1.1 OVERVIEW OF WORLDWIDE AQUACULTURE WITH A FOCUS ON FARMED ATLANTIC SALMON PRODUCTION

1.1.1 Atlantic salmon culture

Salmonids, along with cyprinids, are the two most important groups of fish farmed in aquaculture (FAO, 2012), with Atlantic salmon, (*Salmo salar*, Linnaeus, 1758) representing the most important farmed salmonid (FAO, 2012). Farmed Atlantic salmon are hatched from eggs and raised in inland freshwater facilities to the smolt stage before being transferred for on-growing to floating sea cages or net pens, anchored in sheltered bays or fjords (FAO, 2004). The on-growing phase can be up to 16-18 months until salmon reach the desired marketable size, which usually varies between 2 to 6 kg (FAO, 2004). The production of farmed Atlantic salmon has presented an approximate 53-fold increase (from 39.000 tonnes to 2.067.000 tonnes) during the past 3 decades (Fig. 1 from FAO, 2014). The main points contributing to the large success of the Atlantic salmon aquaculture industry are: the carnivorous characteristics of the specie, the fact that it grows well under culture conditions, it has a relatively high commercial value, it adapts well to farming conditions outside its native environment and most importantly the early onset of selective breeding programs which led to the production of genetically improved salmon growing faster than wild salmon strains while requiring less feed (Thodesen et al., 1999; FAO, 2004).

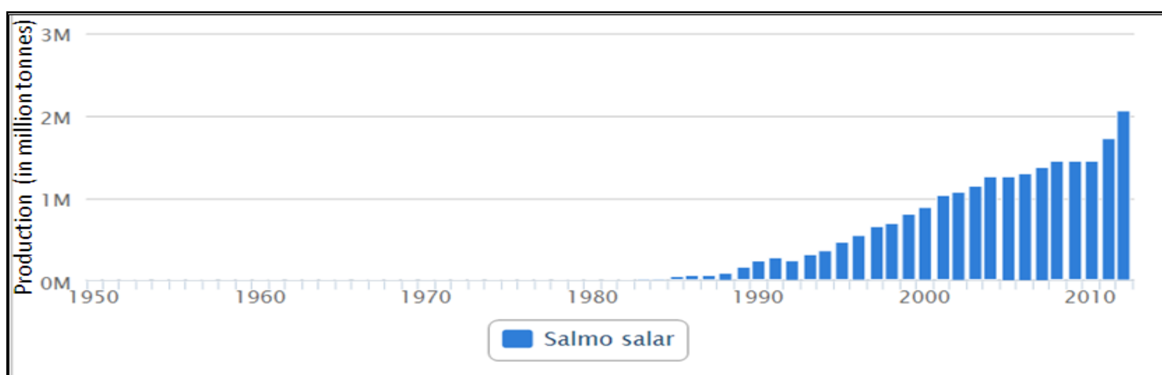

Figure 1 Total aquaculture production for Atlantic salmon from 1984 to 2012 (FAO, 2014).

1.1.2 Current and future status of salmonid aquaculture

Global farmed finfish production levels are still modest compared to worldwide poultry and swine production (Brugère and Ridler, 2004; FAO, 2012). However the aquaculture industry is the most rapidly growing sector of animal production globally, expanding at an average annual rate of more than 8 percent, from 5.2 million tonnes in 1981 to 62.7 million tonnes in 2011 over the past three decades (FAO, 2012). In contrast capture fisheries production has remained fairly stable for the past decade (FAO, 2012) (Fig.2). Moreover, as the global demand for aquatic products increases, it is believed that the production of farmed aquatic species will continue to rise over future decades (FAO, 2012). In 2012 world food fish (finfish, crustaceans, molluscs and other aquatic animals) aquaculture production was estimated at 66.6 million tonnes and almost 42.2 % of total food fish production (aquaculture and fisheries), while in 2013 FAO estimated that worldwide farmed food fish production rose by 5.8 % to 70.5 million tonnes (FAO, 2012). World farmed food fish production presented an average annual rate of expansion of 6.2% between 2000-2012, which was slower than in the periods 1980-1990 (10.8%) and 1990-2000 (9.5%) (FAO, 2012). Nonetheless, between 1980 and 2012, worldwide cultured food fish production volume increased at an average rate of 8.6% per annum. World aquaculture production more than doubled from 32.4 million tonnes in 2000 to 66.6 million tonnes in 2012 (FAO, 2012). By 2030 global aquaculture is expected to grow to approximately 93.6 million tonnes (World Bank, 2013) (representing a 24% increase compared to 2013 and an annual growth of 1.4%), supplying a bit more than half of global demand for aquatic products. While aquaculture expansion for the majority of countries within the European Union is predicted to be similar to that of worldwide aquaculture growth between 2000 and 2030, Norwegian aquaculture production (based mainly on marine cage aquaculture of Atlantic salmon) is estimated to increase by as much as 4% per annum until 2030 (World Bank, 2013). An overview of the

evolution of global fish production from 1984 until 2009 and followed the projections until 2030, underlining the increasingly significant role of farmed fish production in total fish production are presented in Fig. 2. Farmed Atlantic salmon's production, like other farmed salmonids, is expected to rise significantly over forthcoming decades (Brugère and Ridler, 2004; FAO, 2012).

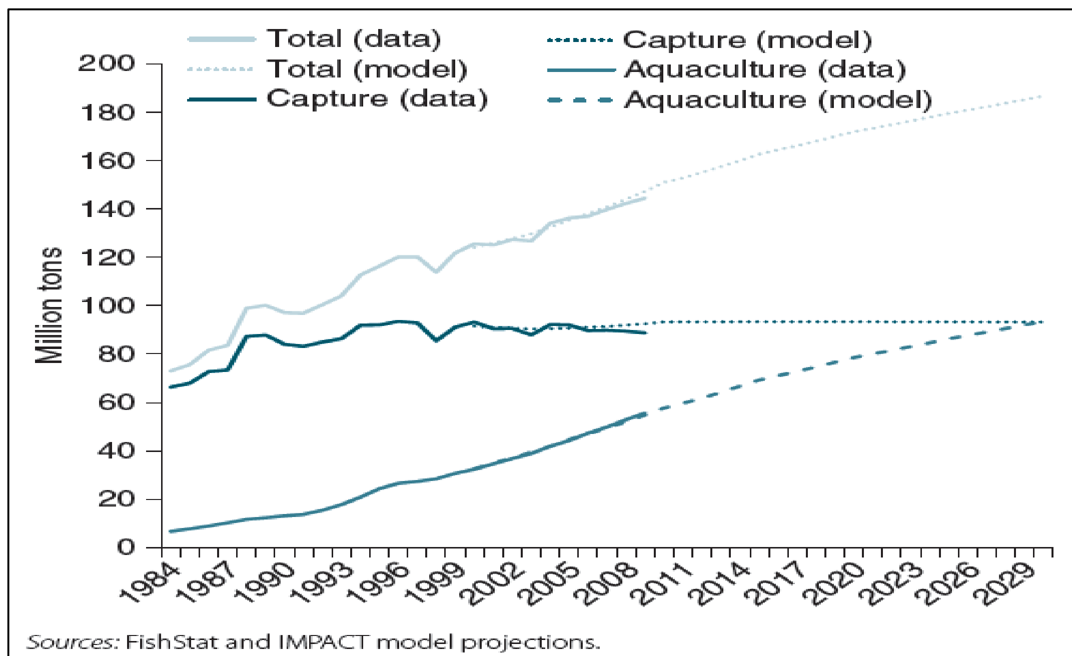

Figure 2 Evolution of global fish production from 1984-2009 (FAO, 2012).

1.2 NUTRITIONAL REQUIREMENTS OF ATLANTIC SALMON AND THE USE OF FISH PRODUCTS IN SALMON DIETS

1.2.1 Atlantic salmon nutritional requirements

Atlantic salmon, like most living organisms, have a requirement for a well-balanced mixture of indispensable (IAA) and dispensable amino acids (DAAs) (Wilson, 2002), lipids, essential and non-essential fatty acids (FAs), vitamins and essential elements for normal development and survival. Indispensable AAs (IAAs) required by Atlantic salmon include arginine (arg), histidine (his), isoleucine (ile), leucine (leu), lysine (lys), methionine (met), phenylalanine (phe), threonine (thr), tryptophan (trp) and valine (val) (reviewed by Brown

et al., 1997). AAs are essential for body growth as well as precursors for the synthesis of many bioactive proteins including enzymes, hormones, neurotransmitters and immune factors (D'Mello, 2003). Dietary lipids help to fulfil energy requirements in the form of essential (EFA) (i.e. linoleic acid and α-linolenic acid) and non-essential fatty acids (NEFA), while assisting the absorption of fat-soluble vitamins in the digestive tract (NRC, 1993). Moreover, FAs are involved in maintaining membrane integrity and serve as precursors for hormone-like compounds, the eicosanoids, which help to regulate inflammatory and immune processes (Bell et al., 1986; de Pablo et al., 2000). Lastly, chemical elements are required for the formation of the skeletal structure, the maintenance of homeostasis and regulation of the acid - base equilibrium, while they can act as salient components for the normal function of various hormones, enzymes and enzyme activators (Lall, 2003).

1.2.2 FM and FO production and nutritional value

Fishmeal (FM) is a nutrient-rich powder obtained after cooking, pressing, drying and grinding whole fish and fish by-products, while fish oil (FO) is normally the liquid extracted from squeezed cooked fish. FM and FO are primarily used as dietary ingredients for domestic animals (poultry, pigs, cattle, etc) and farmed aquatic species (Hertrampf and Piedad-Pascual, 2000; IFFO, 2010). They are generally manufactured from wild, short-lived, fast-growing, pelagic marine fish (forage fish) for which there is little or no demand for human consumption. The main fish species used for the production of FM and FO include anchovies, blue whiting, herrings, mackerels, capelin, pilchard, menhaden, sardines and sand eel (Hertrampf and Piedad-Pascual, 2000; Tacon et al., 2004; IFFO, 2010; FIN, 2010; 2012). The International Fishmeal and Fish Oil Organisation has estimated that in 2008, almost 75 % of all FM production was derived from forage fish, while the remaining 25% (1.23 million tonnes) came from by-products like fish trimmings from the seafood processing industry, with an estimated 6 million tonnes of food fish waste used for FM and FO production during

2009 (FAO, 2012). This amount is expected to grow as the processing of waste from farmed piscine species steadily increases (FAO, 2012).

FM is grouped into four product categories: “High quality” which is largely used by small scale aquaculture units for carnivorous marine or freshwater (FW) fish, “low temperature” (LT), which is characterised by its high digestibility, and is usually utilised as a feedstuff in industrially compounded salmon feeds and piglet production and “Prime” and “Fair Average Quality (FAQ)” which have much lower protein content, used as food additives pig and poultry feeds (IFFO, 2010; FIN, 2010; 2012). FM is recognized by nutritionists as a high-quality, very digestible feed ingredient used as an excellent source of protein and essential amino acids (EAA), EFA, minerals and vitamins (FAO, 1986; NRC, 1993). However, FM derived from different fish species can vary significantly in terms of chemical composition (Hertrampf and Piedad-Pascual, 2000). For instance, the protein content ranges from less than 60% to more than 70% in different types of FM, while the fat varies from 5% to 10% (NRC, 1993). On the other hand, FOs are an almost unique source of EFA and especially docosahexaenoic (DHA) and eicosapentaenoic acid (EPA), which constitute about 10% to 25% of total FAs. Moreover, FOs are very good energy sources due to their high content of hexadecanoic acid and monoenes such as octadecenoic acid, and in the case of Northern hemisphere FO, eicosenoic and docosenoic acid (NRC, 1993). FM and FO have always been a major protein and energy sources in aqua-feeds used for the feeding of carnivorous species (Sargent and Tacon, 1999).

1.2.3 Current and future production, consumption and demand

The quantities of landed pelagic fish and shellfish from capture fisheries destined for use as FM and FO for animal-feeds and other non-food purposes increased from 20.6 million tonnes in 1976 to 34.2 million tonnes in 1994 (a proportionate increase from 31.5 to 37.1 percent of total catch (FAO, 2012). Since 1995, these quantities have been decreasing both

in absolute terms and as a proportion of total catch (FAO, 2012). In 1995, 31.3 million tonnes of global capture fisheries landings were destined for non-food uses (33.9 percent of the total catch), however only 27.2 million tonnes (29.5 percent of total catch) were reduced into FM and FO. In 2009 total capture fisheries landings destined for FM and FO production were 22.8 million tonnes, which was equivalent to 25.7 percent of the total caught. Out of this 17.9 million tonnes (20.2 percent of total catch) were reduced into FM and FO (FAO, 2012).

Tacon, (2004), summarizing the global exploitation status of about 80% of total wild capture fisheries landings, stated that most of wild caught fish are either totally exploited, over-exploited or in a recovery state, after being over-exploited. Moreover, fully exploited fishes cannot be expanded further as they are already at, or very close to, their maximum sustainable harvest limits (Tacon, 2004), and hence, there is no space for further expansion of capture fisheries production. For several decades the proportion of the industrial fisheries catch (whole fish) fluctuated between 20 and 30 million tonnes. This means that the resources of wild feed grade fisheries have remained static or demonstrated a slight decline for the past two decades affecting FM and FO production, which reached approximately 5.5 and 1.2 million tonnes respectively in 2008 (Tacon and Metian, 2008). In 2009, aquaculture's share for FM and FO was estimated at 68 and 88% respectively with salmonid aqua-feeds consuming 13.7 and 16.9% of globally produced FM and FO respectively (FAO, 2012) (Fig. 3 and 4 respectively).

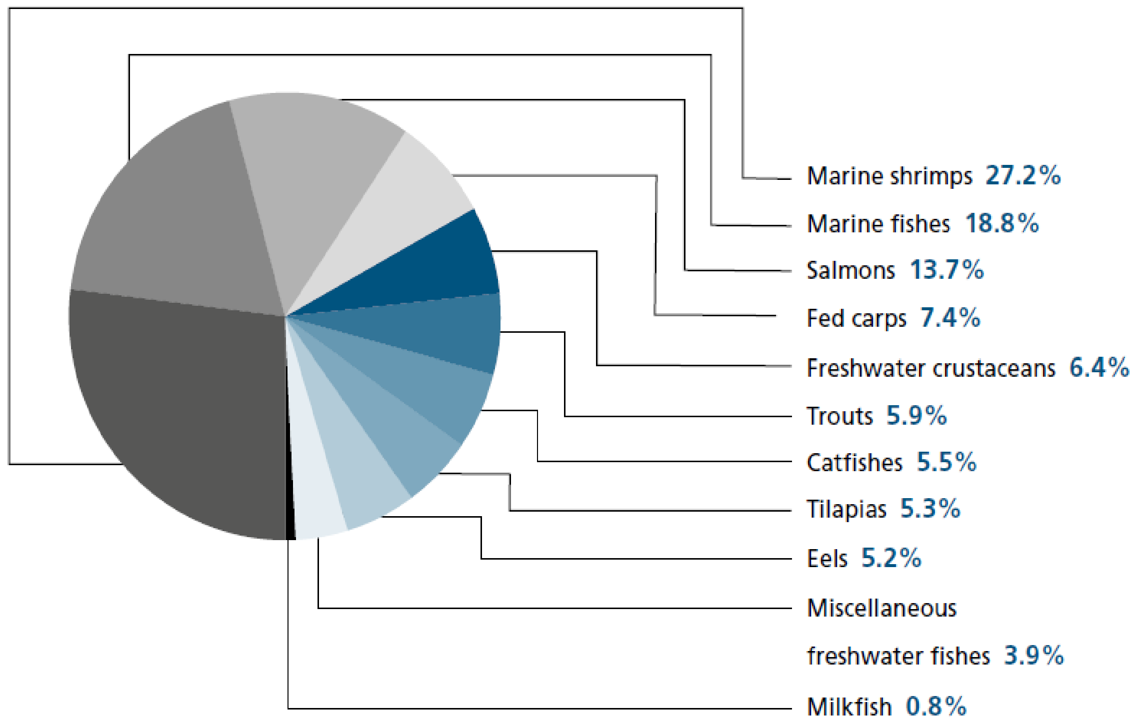

Figure 3 Global consumption of FM (percentage of total usage in compounded aqua feeds) by major aquaculture species groups in 2008 (FAO, 2012).

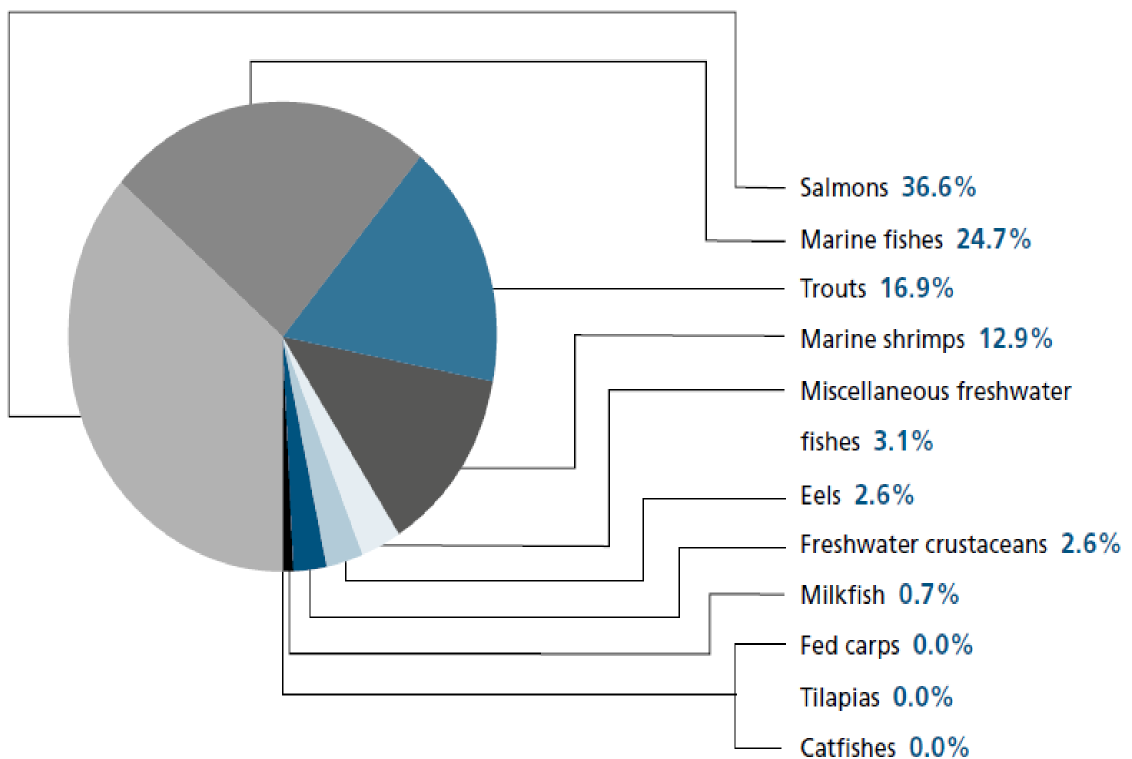

Figure 4 Global consumption of FO (percentage of total usage in compounded aqua feeds) by major aquaculture species groups in 2008 (FAO, 2012).

1.3 ATLANTIC SALMON FEEDS

1.3.1 Increasing substitution of FM and FO in salmon diets

On-growing Atlantic salmon are fed with pelleted, extruded diets of varying sizes depending on fish size (Helland et al., 1991; NRC, 1993; Lovell, 2002). Atlantic salmon feeds contain high amounts of protein, lipid and energy (Helland et al., 1991; Lovell, 2002). However, the trend over the last 20 years has been to increase the dietary lipid level at the expense mainly of protein but also of carbohydrates (Einen and Roem, 1997; Hillestad et al., 1998; Lovell, 2002; FAO, 2004; Solberg, 2004). Given the estimated significant growth of aquaculture during the following years and the steady production of forage fisheries, reliance of aquaculture on marine resources could severely impair the viability, growth and profitability of the sector (FAO, 2012). Therefore, in the last decade, cheaper, alternative ingredients to FM and FO available from other sources have been included in salmon feeds (Tacon and Metian, 2008; Crampton et al., 2010; Hatlen et al., 2013; Hatlen et al., 2014; Hartviken et al., 2014; Liland et al., 2014). The most commonly applied feedstuffs are mainly derived from grains and oilseeds and material recovered from livestock and poultry processing (rendered or slaughter by-products). Moreover, single cell (microbial) proteins and oils, and marine products from lower trophic levels (plankton and algae) have been successfully applied in salmon feeds, however their high production costs still limit their wider use in the aquafeed sector (Olsen et al., 2004). Today, FM inclusion levels in Atlantic salmon aqua-feeds span from 15% to 55% while FO levels range from 3% to 40%. These variations largely depend on the country they are manufactured and they partly reflect differences in the production systems employed as well as differences among farmed salmon breeds (DeSilva et al., 2012).

1.3.2 Alternatives to FM and FO

The utilisation of plant based feedstuffs as sustainable alternatives to marine meals in aqua-feeds has a great potential. The main advantages of plant proteins and oils are that they have high global availability at competitive prices compared to FM and FO, and their nutritional properties can satisfy the nutritional requirements of the fish (NRC, 1993). However, the use of plant based proteins in aqua-feeds requires that these feedstuffs possess certain nutritional properties, including low levels of non-starch polysaccharides (NSPs) and anti-nutritional factors (ANFs), relatively high protein content, a favourable AA profile, high nutrient digestibility and reasonable acceptability by fish (Naylor et al., 2009; Hardy, 2010). Quite often methionine and lysine, two indispensable amino acids (IAAs) found at low levels in several plant proteins, have to be supplemented in the form of crystalline AAs (Mambrini et al., 1999; Espe et al., 2007). Some plant-derived feedstuffs including oilseed meals and oils, plant protein concentrates and cereal by-product meals and oils have most of these desirable traits (FAO, 2012). Nowadays, the sharp increases in the prices of FM and FO, driven by the competition between livestock farming, aquaculture and various other food and medical sectors, have made the use of these refined ingredients more economical.

Animal by-products (ABPs) already play an important role as an ingredient in aquaculture feed pellets. Most of them have a reasonably balanced AA profiles. However, IAAs including methionine and lysine, which are usually limited in several animal by-products, should be supplemented as described previously (Kureshy et al., 2000). Regarding by-products from fish processing plants, there are some issues related to potential pathogens and contaminants that could be harmful to both fish and consumers, which need to be addressed through improved treatment of these ingredients prior to their incorporation into aquafeeds (Kureshy et al., 2000). An example of the importance of animal by-products as a feedstuff in fish feeds is given by fish producer Marine Harvest, who used pellets containing

12% poultry by-products and only 19% FM at their Chilean salmon farms in 2008 (Marine Harvest, 2010). The use of ABPs, in the European Union (EU), was until recently limited by legal restrictions with some exceptions as a means to prevent the inclusion of transmissible spongiform encephalopathies (TSE) (EU, 2003, 2001), however these restrictions have changed for aqua-feeds in Europe (EU, 2012). To date, many studies have evaluated the potential use of some of these terrestrial animal meals in the diets of several salmonids (Higgs et al., 1979; Steffens, 1994; Twibell et al., 2012; Hatlen et al., 2013; Hatlen et al., 2014; Hartviksen et al., 2014).

The potential use of zooplanktonic crustaceans as substitutes for FM and/or FO has been the focus of several studies (Storebakken, 1988; Olsen et al., 2006; Suontama et al., 2006), with the aims of mapping the sustainable harvesting potential (Aksnes and Blindheim, 1996; Melle et al., 2004; Skjoldal et al., 2004), the development of efficient harvesting techniques (Nicol and Foster, 2003) and industrial processes, and evaluating the nutritional properties of raw materials with respect to fish feed (Storebakken, 1988; Moran et al., 2006; Olsen et al., 2006; Suontama et al., 2006). The crustaceans of interest include copepods, euphausiids, krill and amphipods, grazing on phytoplankton (Suontama et al., 2006). However, this research is still at an early stage, but if it should prove commercially viable it could be a major catalyst for further aquaculture growth.

Another research area that recently has received a great deal of attention is the prospects of substituting FM with unicellular organisms such as yeast, moulds, bacteria, microalgae and fungi as additives to aquaculture feeds. Research in this field has demonstrated that partial replacement of marine-based feedstuffs with yeast and bacterial products does not have any negative impacts on fish survival, growth, or disease resistance (Murray and Marchant, 1986; Storebakken et al., 1998a; Romarheim et al., 2011). Similarly, FO could be replaced by single cell oils (SCO), extracted from microbial organisms grown

under heterotrophic conditions or even whole cell organisms (Carter et al., 2003; Miller et al., 2007). Nowadays the major advantage in the use of unicellular organisms is that there are several processes designed to produce industrial quantities under controlled and environmentally safe conditions. Moreover, the chemical composition of many microorganisms can be manipulated for the achievement of higher protein and lipid levels, through their enrichment with specific IAAs or FAs (Kangas et al., 1982; Sanchez et al., 1995; Day and Tsavalos, 1996). Although various studies have reported the incorporation of microalgae oils and meals in aquafeeds (Day et al., 1990; Zhou et al., 1991; Laing and Millican, 1992; Day and Tsavalos, 1996; Langden and Onal, 1999; Carter et al., 2003; Miller et al., 2007), this approach cannot be applied commercially for the replacement of marine-based feedstuffs, mostly due to their high production costs and culture inefficiency (Borowitzka, 1991; Chaumont, 1993; Wilkinson, 1998). As the technology continues to develop, higher oil yields and greater densities of cells in the fermentation broth will continue to drive the costs down to the point where perhaps these oils can compete with the less expensive marine oils. And if the price of FO continues to rise, it would stimulate further research to develop these alternative sources at a competitive price (Bimbo, 2007).

1.3.3 Challenges and limitations in the use of vegetal proteins and oils in aqua-feeds

Relative to FM, plant protein ingredients generally contain more indigestible organic matter, in the form of insoluble carbohydrates and fibre, leading to higher levels of fish excretion and waste. Moreover, certain elements in plant products, such as phosphorus (Lall, 2003), have limited uptake in fish. However, recent advances in fish nutrition, feeding, and dietary manipulation have significantly reduced the production of fish waste and increased substantially nutrient uptake and growth efficiency of cultured aquatic species (Hardy and Gatlin, 2002). Further advances in this area continue to be made through classic breeding, transgenic manipulation, exogenous enzyme treatment (e.g. phytase in salmonid feed)

(Forster et al., 1999), and post-harvest processing technologies that enhance the quality of plant protein concentrates (Barrows et al., 2008). Even without improved genetic lines and post-harvest processing of plant protein ingredients, numerous dietary manipulations have proven beneficial in increasing the utilisation of these feedstuffs in aquaculture diets. Some of these techniques include the blending of complementary ingredients for the achievement of amino acid profiles that better meet the metabolic requirements of the targeted species, or in some cases, supplementing commercially available forms of the most limiting AAs and elements such as synthetic methionine analogues (Forster and Dominy, 2006), various sulphur AA compounds (for methionine), or lysine (Gatlin et al., 2007) inorganic phosphates and other minerals (Lall, 2003).

FO substitution with vegetal oils (VOs) should be able to provide sufficient energy in the form of the preferred FAs. Saturated and monounsaturated FAs are preferred over PUFAs for energy generation in fish as suggested by previous studies investigating mitochondrial β oxidation (Henderson, 1996). Moreover, another negative impact of FO substitution with VOs is the lack of long-chain n-3 fatty acids, including EPA, docosapentaenoic acid and DHA, which represent up to 30% of total lipids and they are considered to be essential for optimal growth, development and for an efficient immune response in Atlantic salmon (Sargent et al., 2002). On the other hand VOs contain high levels of C18 PUFAs, which are found in moderate levels within FO (NRC, 1993; Hertrampf and Piedad-Pascual, 2000; Gatlin et al., 2007).

1.3.4 Vegetal oils and vegetal proteins in Atlantic salmon diets

Previous studies reported that total (Mambrini et al., 1999; de Francesco et al., 2004; Espe et al., 2006) or partial substitution of FM (Gomes, 1995; Kaushik et al., 2004) with vegetal proteins in salmonid diets can adversely affect feed intake and weight gain. Further, Adelizi et al. (1998) reported that rainbow trout (*Oncorhynchus mykiss*) fed on FM-free diets, presented lower weight gain, higher feed intake and increased FCR. Growth retardation for the diets with complete or partial substitution of FM could be attributed to changes in physical texture of the diets, reduced feed intake, the increment in ANFs including phytate and non-starch polysaccharides (NSPs) present in vegetal proteins (Francis et al., 2001), not properly balanced AA levels and the lack of some specific bioactive compounds in which FM-based diets are rich in (Espe et al., 2006). Moreover, significantly lower feed intake was observed in Atlantic salmon fed diets in which FO was totally replaced by rapeseed oil (RO) (Moya-Falcón et al., 2005). Furthermore, self-selecting feeding trials illustrated that rainbow trout prefer FO diets over those with VO (Geurden et al., 2005, 2007), which suggests that some fish do actively select diets according to the dietary source of lipids. However, despite any reductions in feed intake many studies on salmonids have demonstrated that VOs are good substitutes for FO in terms of growth (Bell et al., 2001; Rosenlund et al., 2001; Bell et al., 2002; Caballero et al., 2002; Tocher et al., 2003; Ng et al., 2004; Torstensen et al., 2004; Fonseca-Madrigal et al., 2005; Torstensen et al., 2005; Bell et al., 2006; Richard et al., 2006; Crampton et al., 2010; Hartviksen et al., 2014). Torstensen et al. (2008) reported reduced feed intake and growth performance in Atlantic salmon post-smolts fed on a diet with 80% VPs and 70% VOs for an initial period followed by a period of increased growth, which was concomitant with no reductions in feed intake. This is an indication that Atlantic salmon requires a longer adaptation time before fully accepting any feed treatment containing very low levels of FM and FO or even total substitution of FM and/or FO with alternative protein

or lipid sources of animal or plant origin (Torstensen et al., 2008). Moreover, it was proposed that Atlantic salmon have the ability to compensate growth after periods of restricted feed intake since these fish have a greater potential for growth (Johansen et al., 2001).

Espe et al. (2006) reported that condition factor was not significantly affected in Atlantic salmon post smolts fed on diets devoid of FM. On the contrary Torstensen et al.(2008) reported lower condition factor in Atlantic salmon maintained on diets with 80% of dietary protein from VP and moderate or high substitution of FO with VO (35 and 70% respectively) compared to fish fed the fishery based diet and the diet with moderate VP inclusion (40% of dietary protein from VP and 70% of dietary lipid from VO).

It was also reported that Atlantic salmon is able to digest and utilise VOs efficiently for energy production at low water temperatures (Torstensen et al., 2008), suggesting that VO can support good growth despite restricting feed intake, providing a protein sparing effect (Karalazos et al., 2007; Torstensen et al., 2008). Espe et al.(2006) demonstrated that condition factor was not significantly affected in Atlantic salmon fed on diets with complete replacement of FM with plant proteins.

Studies have demonstrated no effects of dietary VO on the carcass lipid composition of salmonids (Karalazos et al., 2007; Turchini and Francis, 2009), whereas others reported an increase in whole body lipid levels of VO fed salmonids (Turchini et al., 2003; Karalazos et al., 2011). In addition, Espe et al. (2006) reported lower levels of fat deposition in salmon fed on a VP diet compared to a marine based (MB) control group. Furthermore, mineral availability varies considerably among different sources and ingredients (Hilton, 1989). Mineral deficiencies in Atlantic salmon could be revealed as reduction in salmon tissue ash levels (Shearer et al., 1994). To avoid potential deficiencies, feed formulations including high levels of FM substitution are supplemented with inorganic minerals known to be available to fish. However, various compounds within applied feedstuffs lower the

availability of supplemented inorganic and non-supplemented organic dietary minerals through direct or indirect interactions (Sugiura et al., 1998). Mineral availability in commonly used feedstuffs; and potential interactions of different fractions of added feedstuffs on dietary organic mineral availability or their interactions with supplemented inorganic mineral sources have not been well researched (Sugiura et al., 1998).

1.3.5 Soy Protein Concentrate (SPC) in diets for salmonids

The most common method of manufacturing SPC for aqua-feed production is through aqueous ethanol or methanol extraction, which reduces the carbohydrate fraction (Peisker, 2001). A schematic representation of SBM and SPC production from soybeans by Peisker (2001) is illustrated in Fig.5. Alcohol extracted SPC (referred to as SPC from this point forward) contains approximately 65% crude protein, 1% fat and 6% ash (Peisker, 2001). Compared to SBM (~48% protein in solvent extracted, dehulled SBM), the protein content of SPC is more similar to that of FM, which typically ranges between 60-70% depending on the source species and efficiency of lipid extraction (NRC, 1993). With the exception of methionine, and potentially lysine, the EAA content of SPC compares favourably with FM (Masumoto et al., 1996). SPC also contains low levels of ANF; specifically trypsin inhibitors, lectins, saponins, soy antigens and oligosaccharides (Peisker, 2001; Russett, 2002). Lectin, saponins and trypsin inhibitors, which are the main anti-nutrients related with soybean induced intestinal enteritis (Krogdahl et al., 1995; van den Ingh et al., 1996; Krogdahl et al., 2000, 2003), are typically found in much lower concentrations than the concentrations reported to induce inflammation in salmonid fish (Hart et al., 2007; Russett, 2002). Moreover, many studies have shown no signs of enteritis in salmonids fed on diets with up to 100% substitution of FM with SPC (Bureau et al., 1998; Krogdahl et al., 2000; Escaffre et al., 2007). Because of its favourable traits, SPC has gained interest as a FM substitute in aqua-feeds. Several studies have shown that SPC produced mostly through

aqueous alcohol extraction of defatted soy flakes, are more acceptable or supported better growth than soybean meal for salmonids and other fish species (Murai, 1989; Shimeno et al., 1992; Olli et al., 1994; Kaushik et al., 1995). Regarding Atlantic salmon, literature studies showed that up to 75% of dietary protein (with methionine supplementation) could be replaced by SPC without negatively influencing growth performance (Olli et al., 1994; Refstie et al., 1998; Storebakken et al., 1998b; 2000). Most of the above nutritional studies regarding the effects of dietary SPC on Atlantic salmon to date have been performed in salmon post-smolts. Nonetheless, Brown et al.(1997) reported that early stage Atlantic salmon parr can efficiently utilise diets with up to 30% of protein from SPC without negative effects on their growth performance. Moreover, commercial trials by EWOS (personal communication) have shown that diets with up to 35% of protein from SPC can be utilised by Atlantic salmon fingerlings without any problems. However, it seems that the tolerance of salmon for plant feedstuffs depends on salmon size and stage. Burr et al. (2012) demonstrated that early stage Atlantic salmon parr are much more sensitive to dietary vegetal protein inclusion than late stage parr. To date, studies regarding the general performance of freshwater Atlantic salmon parr fed on increasing levels of dietary SPC are scarce.

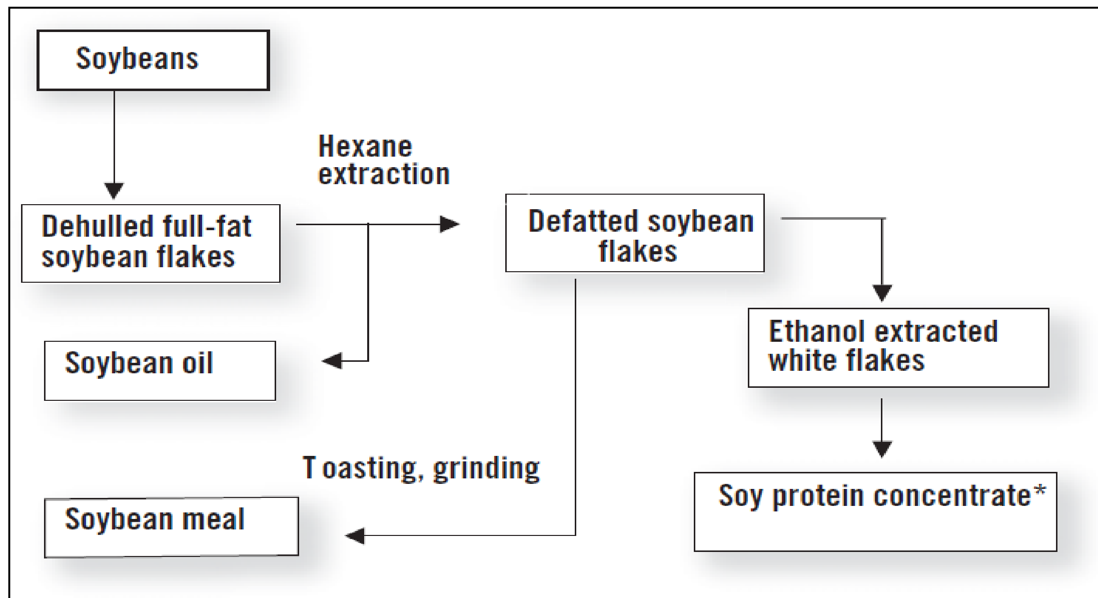

Figure 5 Production of soybean meal and soy protein concentrate (Peisker, 2001). The asterisks points at the product used for the formulations of the diets in the studies presented in Chapters 3, 4, 5, 6 and 7.

1.4 SKELETAL TISSUE MINERALISATION

1.4.1 Description of fish skeletal system and skeletal metabolism

Fish skeleton, like most vertebrates, represents a reservoir of Ca, P, and many other ions, which are in a state of continuous exchange with electrolytes present in body fluids (Lall, 2003). These functions require continuous remodelling of the skeletal tissue throughout the whole lifecycle of the fish. Skeletal remodelling involves three types of cells: osteoblasts (bone forming cells), osteocytes (cells entrapped inside the bone matrix) and osteoclasts (multinucleated bone resorbing cells). Most importantly, fish skeleton supports the structure of the body for normal posture, development and locomotion (Lall and Lewis-McCrea, 2007). Furthermore, other important functions of the skeletal system include the provision of sites for muscular attachment and the protection of fish' vital organs.

Fish skeletal tissues consist of calcium hydroxyapatite salts embedded in a matrix of type I collagen fibres. Therefore, bones are comprised of an organic bone matrix and inorganic minerals. Scales do not contain osteocytes, but instead they are covered by scleroblasts, which are involved in matrix production and mineralisation (Lall, 2003). Bones

containing osteocytes (cellular bones) and bones without the presence of them (acellular bones) have been identified in fish. Acellular bones are formed from osteoblasts moving away from the site of mineralisation, while bone deposition occurs (Moss, 1963). Salmonids and more specifically Atlantic salmon have cellular bones.

Fish skeletal elemental composition varies with the age of the bone. The inorganic phase of fish skeleton is poorly crystallized apatite (Neuman and Mulryan, 1968). Calcium, phosphate, and carbonate and small amounts of magnesium, manganese, zinc, strontium, citrate, fluoride, hydroxide, and sulphate are the main bone constituents (Lall, 2003). Although fish have the ability to derive most of these constituents from their aquatic environment, diet represents the main source for certain elements, including P and Mg, for the coverage of their nutritional requirements and ensure normal growth, bone development and health.

1.4.2 Essential elements for normal development with an emphasis on skeletal development

Macro and micro-elements in vertebrates are involved in a plethora of structural, catalytic and signal transductions, in which the functions of these two elemental classes overlap considerably. Similar to other vertebrates, Ca and P are the most abundant macro-elements present, displaying an important role in maintaining the fish skeletal structure and development. Moreover, many essential micro-elements, including magnesium, zinc and manganese, are required for normal bone formation and mineralisation (Lall, 2003).

1.4.2.1 Phosphorus and calcium

Despite the importance of Ca in bone formation and maintenance of skeletal structure, it is widely distributed in all soft tissues, having an integral role in muscle contraction, blood clot formation, transmission of nerve impulses across synapses, maintenance of cell membrane integrity and activation of several important enzymes. Furthermore, Ca in the cell membrane

is closely bound to phospholipids, regulating membrane permeability and nutrient uptake by the cells (Lall, 2003). Moreover, Ca and P are both involved in the maintenance of acid-base equilibrium (Lall, 2003).

Phosphate serves as a structural cell component, a factor in intermediate metabolism and a component of genetic material (Lall, 2003). Phospholipids constitute the major component of the cell membranes and intracellular organelles, while phosphate is also an integral constituent of nucleic acids (DNA and RNA) (Lall, 2003). Moreover, phosphorus is part of high energy phosphate esters such as adenosine phosphate (ATP), the hydrolysis of which can release high energy for metabolic processes and muscle contraction. In addition, phosphate is essential for carbohydrate, lipid and amino acid metabolism and in muscle and nervous tissue metabolism.

As mentioned previously, Ca and P are closely connected to the normal bone formation and mineralisation, while the stability of the vertebral bone is maintained by a solid form of calcium phosphate (Lall, 2003; Lall and Lewis-McCrea, 2007). Fish can derive Ca and P from the surrounding aquatic environment, while Ca requirements can be met by their ability to sequester this mineral from the water. On the contrary, the concentration of P in the water is very low making the dietary supply of this element the main source of P. In fish, gills are the major site of Ca regulation (Lall, 2003). Other sites of significant Ca regulation include the fins and oral epithelia and tissues (Lall, 2003). The absorbed Ca is then deposited in the bones, scales and skin. While scale chemical composition is similar to that of other bony tissues, Ca metabolism in scales is physiologically different compared to the other skeletal structures. Generally, the regulation of P is considered to be more crucial than that of Ca, since fish have to absorb, store, mobilise and conserve P in both fresh and saline aquatic environments (Lall, 2003; Lall and Lewis-McCrea, 2007). Absorbed P is mainly

accumulated in soft tissues (heart, liver, muscle, blood etc) and to a lesser degree in skeletal tissues.

In general, chemical analysis of the diets cannot be used to define the biological effectiveness of a nutrient. It has been shown that the actual bioavailability of an element can vary considerably when it is provided from different feed ingredients or even within the same feed component in different diets (Lall, 2003). Moreover, it has been reported that elemental bioavailability can be influenced by a wide range of factors. These include levels of mineral, the particle size, interactions with other dietary components (chelators or inhibitors) or nutrients, the physiological and pathological status of the animal and the type of feed processing (Lall, 2003). Dietary mineral bioavailability can differ depending on the molecular form of the element, its valence state and the number of ligands in the ingested dietary element (Lall, 2003). Furthermore, several mechanisms that are associated with the formation of insoluble and non-absorbable substances in the intestinal tract may inhibit or promote the mucosal absorption, transport and metabolism of an element in the body (Lall, 2003). Feedstuffs of animal origin, such as FM and meat meals, demonstrate the highest levels of Ca and P with P ranging between 1.5-3.2 % and 3.5-5.5 % in these ingredients respectively. In these feedstuffs, the bony tissues contribute significantly to the P content, where the major proportion is present as inorganic phosphates, whereas the remainder is found as organic phosphate complexes, which increase the availability of P to fish compared to the P forms found in plant feedstuffs.

Phosphorus in cereal grains and plant protein concentrates varies from 0.3-0.4 % and 0.5-1.4 % respectively. However, oilseeds including soybean store P as phytates, i.e., salts of phytic acid (inositol hexaphosphoric acid). The average amount of phytic acid in cereal and oilseed feedstuffs is 1-2% by weight, however certain varieties may contain higher levels of this compound (3-7%) (Lall, 2003). In soy products phytic acid, phosphatides and

inorganic P constitute approximately 75, 12 and 4% of the total P, respectively. Phytate is not digestible by fish, due to the lack of an endogenous enzyme (phytase) in the gut, which is able to break down the phytic acid to its moieties. Due to the high volume of negatively charged phosphate groups, phytate can reduce the availability of several positively charged ions, including Ca, Mg and Zn at high pH, by binding them and forming insoluble salts within the intestine (Francis et al., 2001). As mentioned before many dietary minerals are found as organic salt complexes, which have to be broken down by intestinal enzymes to be released. Phytate has also been shown to inhibit the function of intestinal enzymes, especially the ones involved in proteolysis (Francis et al., 2001; Denstadli et al., 2006), while inhibition of lipid digestibility has also been reported in rainbow trout (Mambrini et al., 1999). Furthermore, phytate has been proven to form insoluble complexes with proteins reducing their digestibility and availability (Francis et al., 2001). Lastly, non-starch polysaccharides (NSPs) and oligosaccharides in plant derived protein ingredients have been linked with reduced nutrient availability (including minerals) due to the formation of gelatinized solutions within the gut, entrapping nutrients and decreasing their availability, reducing the distribution of digestive enzymes and the flow on the mucosal layer (Storebakken et al., 2000, 1998b).

Dietary available P requirements for Atlantic salmon range between 6 to 10 g $\times$ kg^{-1} (Åsgard and Shearer, 1997). Many studies with mono-gastric animals have reported that an optimum dietary Ca:P ratio is important and that by increasing it, the absorption of P could be adversely affected, whereas a high P: Ca ratio may cause reductions in Ca absorption. Commercial feed trials have shown that diets with an adverse Ca: P ratio ($\leq$ 1) are preferred for salmonid nutrition due to their higher digestibility and increased availability of P (Aliphos, 2012).

Calcium deficiency is not very common in fish since this mineral can be obtained straight from the aquatic environment. However, P deficiencies can lead to reduced growth, decreased feed efficiency, reduced bone mineralisation, skeletal deformities and increased mortalities (Lall, 2003). Atlantic salmon fed on low dietary P possessed abnormally soft bones; wrinkly ribs and scoliotic spines (Baeverfjord et al., 1998). In general, P and other elemental deficiencies have been proposed as the main cause of vertebral deformities in farmed Atlantic salmon (Witten et al., 2005; Fjelldal et al., 2007; 2009a, 2009b). The reason for this, is the production of fish with under-mineralised bones which are soft, brittle and more prone to the effects of muscular contructions (Lall and Lewis-McCrea, 2007). Vertebral fusions and compressions are the most commonly occurring types of skeletal deformities (Witten et al., 2005, 2006, 2009), however, curvatures have been observed in farmed Atlantic salmon (Waagbø et al., 2000; Silverstone and Hammell, 2002; Fjelldal et al., 2004; Waagbø, 2006, 2008; Gil Martens et al., 2012). Moreover, a condition termed as hyper-dense vertebrae, caused by the replacement of the adipose tissue with ectopic cartilage within the trabecular network, giving vertebrae a denser appearance in radiographic images, has been reported in Atlantic salmon parr (Helland et al., 2006). The number of fish with this condition was found to increase when salmon parr were fed with increasing amounts of dietary phytate (Helland et al., 2006).

In P deficient fish exhibiting bone deformities, a decrease in bone mineralisation has been described, due to an increment in the amount of osteoid tissue and osteoclast numbers combined with a subsequent reduction in the number of osteoblasts (Takagi and Yamada, 1991; Roy, 2002). Hence, normal development and growth of bones are dependent on the amounts of dietary P, and even most importantly the levels of dietary available P. A deficiency or excessive intake of P can result in the formation of skeletal anomalies (Lall and Lewis-McCrea, 2007).

1.4.2.2 Magnesium, zinc and manganese

Magnesium (Mg) is an essential cofactor in a wide range of catabolic and anabolic enzymatic processes of the intermediary metabolism including the transfer of phosphate groups, the hydrolysis of phosphate and pyrophosphate groups, the oxidation of fatty acids and the synthesis of amino acids (Lall, 2003). However, Mg is also required in skeletal metabolism, osmoregulation and neuromuscular transmission (Lall, 2003). Most of the Mg in fish is located in the bone with the remainder being found within the cells of soft tissues. Fish in general can obtain Mg ions either from the aquatic environment or from dietary sources (Lall, 2003). Magnesium deficiency in Atlantic salmon include anorexia, reduced growth, sluggishness, high mortality and decreased body and bone Mg concentrations (El-Mowafi and Maage, 1998). Moreover, in rainbow trout Mg deficiency has been linked with nephrocalcinosis, vertebral deformities and degeneration of muscle fibres and epithelial cells of the pyloric cecum and gill filaments (Cowey et al., 1977; Ogino and Yang, 1978). Mg requirements for Atlantic salmon have been estimated to be around 0.04% (NRC, 1993). In general, Mg levels in freshwater are quite low (200 μM) to satisfy the metabolic requirements of fish, and Mg must be supplied in the diet (Lall, 2003). In rainbow trout it was shown that the Mg requirement is not influenced by an increase in the dietary Ca and P levels (Knox et al., 1981). Among food sources cereal grains contain less Mg (0.15-2%) than other commonly applied aqua-feed ingredients. In cereals, most of the Mg is found in the bran fraction. Vegetal proteins may contain 0.4-0.6% of Mg. Moreover skeletal tissues found in meat and fish meals contribute significantly to the Mg levels detected in these Mg rich feedstuffs.

Zinc is a cofactor in several metalloenzymes or has been shown to act as a catalyst for the regulation of specific Zn-dependent enzymes (Lall, 2003). Zn is in general involved in the regulation of a wide range of metabolic processes including carbohydrate, lipid and

protein metabolism, while it may also have a structural role in nucleoproteins and it is involved in the metabolism of prostaglandins (Lall, 2003). Zn is abundant in the bone tissue of the fish and like in other vertebrates it seems to have a role in the maintenance of bone mineral density and bone metabolism (Helland et al., 2006). In humans Zn is utilised in every step of bone metabolism and its deficiency result in osteoporosis (Molokwu and Li, 2006). Adequate amounts of Zn are required for the proper function of proteins within mammalian bones' organic matrix, while also Zn acts as a cofactor for the maintenance of osteoblast's proper function and is required for peak bone density (Molokwu and Li, 2006). Fish can derive Zn from both aquatic environment and dietary sources, however dietary Zn is more efficiently utilised (Lall, 2003). Zn deficiency signs in rainbow trout include growth depression, high mortality rates, lens cataracts, fin and skin erosions and short body dwarfism (Satoh et al., 1983). The dietary requirement of Atlantic salmon for Zn ranges between 37-67 mg $\times$ kg^{-1} (Maage and Julshamn, 1993). Moreover, among the dietary factors affecting dietary Zn uptake in the gut, Ca and P levels and Ca: P ratios, the presence of phytic acid, protein source, the form of Zn and Ca content are the most important (Lall, 2003). Most uncontaminated animal and fish feedstuffs contain 30-100 mg Zn $\times$ kg^{-1} of dry matter, while among plant feedstuffs cereal, grains contain 15 to 30 mg Zn $\times$ kg^{-1} and plant protein concentrates contain 40-80 mg Zn $\times$ kg^{-1}. Zinc sulphate ($ZnSO_4$) and nitric zinc ($ZnNO_3$) are effectively utilised by rainbow trout, as dietary Zn supplements in low Zn diets, containing white FM (referring to FM varieties containing no more than 6 % oil and not more than 4 % salt, mainly obtained from white fish or white fish waste such as filleting offal) eliminating the adverse effects of dietary Zn deficiency including dwarfism and cataracts (Satoh et al., 1983). Furthermore the presence of phytate in dietary oilseed protein ingredients has been shown to reduce Zn availability in Atlantic salmon diets (Storebakken et al., 1998b, 2000; Denstadli et al., 2006). It has been suggested that the higher supplementation of practical

feeds with Zn can increase Zn bioavailability, especially in diets with increased dietary levels of phytate, Ca and P (Lall, 2003).

Manganese (Mn) is an essential mineral widely distributed in all animal and fish tissues including the liver, muscle, kidney, gonads and skin, however the highest amounts of this mineral are found in the bones (Lall, 2003). Mn is mainly found in mitochondria rather than cytoplasm or other cell organelles. It is an integral cofactor for the activation of a large number of metalloenzymes or an essential constituent of certain metalloenzymes involved in carbohydrate, lipid and protein metabolism (Lall, 2003). Due to similar chemistry of Mn with Mg, many enzymes can be activated by both ions. In mammalian bones Mn seems to be involved in the proper function of bone cells and the synthesis of the organic matrix (Strause and Saltman, 1987). Fish can derive Mn either from the water or from their diet. It has been demonstrated that dietary Mn uptake can be adversely affected by the increased dietary levels of Ca or P. Mn deficiencies in rainbow trout have been linked with reduced growth, cataracts, and reduced skeletal mineralisation and vertebral malformations (Knox et al., 1981; Satoh et al., 1983; Yamamoto et al., 1983). Dietary Mn requirements for Atlantic salmon range between 7.5 and 10.5 mg $\times$ kg^{-1} (Maage et al., 2000). The Mn requirement for juvenile Atlantic salmon ranges between 2 to 15 mg$\times$kg^{-1}(Lall, 2003). High amounts of dietary P may lower the absorption of Mn, and thus increase the necessity for dietary Mn supplementation (Lall, 2003). Animal protein feedstuffs are generally low in Mn. For example, Mn concentrations in the most commonly used applied FMs vary from 3-70 mg Mn $\times$ kg^{-1} with the majority of them (capelin, herring and anchovy meal) demonstrating low levels of Mn (3-10 mg Mn $\times$ kg^{-1}) and menhaden meal being the richest source of Mn (~70 mg Mn $\times$ kg^{-1}) (Sugiura et al., 1998). Among plant feedstuffs, cereal proteins contain 8-50 mg Mn $\times$ kg^{-1} (Lall, 2003), while oilseed proteins contain 40-70

mg Mn × kg^{-1}. The availability of Mn differs in various dietary inorganic salt supplements (Lall, 2003).

1.4.3 Other biotic and abiotic factors affecting salmon elemental composition and normal vertebral development

Whole body and skeletal elemental amounts could be used as an index for the bone mineralisation and general health status of the fish (Shearer et al., 1994; Helland et al., 2005, 2006). Low bone mineralisation in on-growing and healthy salmon could be an index of subclinical deficiency in a range of elements, which in the long term could increase the prevalence of bone deformities (Fjelldal et al., 2007, 2009a, 2009b, 2012) or even worst, cause increased mortalities due to a negative elemental balance (Lall, 2003). Baeverfjord et al., (1998) demonstrated that Atlantic salmon have the homeostatic mechanism to manage the reduced dietary supply of P and possibly other elements, the dietary uptake of which is influenced by dietary P. This is done by sacrificing skeletal deposition of these elements to aid other more essential metabolic roles of these elements. Moreover, Fjelldal et al., (2009b) demonstrated that proper bone mineralisation during the early seawater phase, induced by supplementation with extra dietary minerals, prevents the development of vertebral deformities in on-growing under-yearling smolt transferred to seawater at high temperature and long day-length in August.

The intensive rearing conditions applied nowadays for the achievement of optimal growth rates, are associated with increased occurrence of production related conditions including reduced bone mineralisation, which could promote problems such as the ones described above. In addition the growing need of replacing FM in commercial aqua-feeds could initiate several nutritional imbalances, which could reduce bone mineralisation. A previous study by Fjelldal et al., (2010) revealed no negative effects of increasing dietary vegetal ingredients on bone deformities, which could be an indication of unaffected skeletal

mineralisation by vegetal feedstuffs. However, since bone development and mineralisation seems to be influenced by the developmental stage of salmon (Fjelldal et al., 2010) the use of such products in diets for Atlantic salmon parr could be detrimental for body and skeletal mineral levels. Nevertheless, several environmental and physiological factors related with intensive rearing conditions, have been associated with the problem. For example, the use of elevated temperatures and continuous light in order to supress early maturation and prevent smoltification, during the freshwater phase of Atlantic salmon are two widely applied techniques in salmon industry aiming to optimise salmon growth performance at this stage (Bromage et al., 2001; Berrill et al., 2003; Stefansson et al., 2007; Stefansson et al., 2008; Ytteborg et al., 2010; Grini et al., 2011). However, enhancement of salmon growth rate followed by the application of any of the two techniques has been associated with deprived bone mineralisation and increased prevalence of bone deformities (Fjelldal et al., 2004, 2005; Ytteborg et al., 2010; Grini et al., 2011). Moreover, vaccination used as a prophylactic method against certain diseases has also been linked with changes in bone mineralisation status and in some cases with increased prevalence of skeletal deformities (Berg et al., 2005; Grini et al., 2011; Berg et al., 2012).

The removal of the light sensitive pineal gland Fjelldal et al., (2004) and photoperiod manipulation (continuous light or increased exposure to light) (Fjelldal et al., 2005, 2006; Wargelius et al., 2009), inducing increased growth performance, have been shown to adversely affect vertebral bone mineralisation in Atlantic salmon, thus compromising the normal development of the vertebral body by increasing the prevalence of bone deformities.

One of the most commonly used prophylactic methods against several diseases in Atlantic salmon culture is the intra-peritoneal administration of oil adjuvanted vaccines to fish prior to seawater transfer. Some studies have shown that vaccination induces vertebral deformities in Atlantic salmon, visible at the time of harvest (Berg et al., 2006, 2012).

Moreover, Berg et al., (2012) demonstrated a correlation in the increased occurrence of bone deformities with reduced bone mineralisation. On the contrary, other studies have demonstrated increased bone mineralisation after vaccination in salmon under certain post-vaccination temperature regimes (Grini et al., 2011), while Berg et al., (2005) also reported increased bone mineralisation in vaccinated salmon. Further studies have also reported no signs of vertebral deformities in Atlantic salmon at the post vaccination period (Gil Martens et al., 2010; Grini et al., 2011). It is apparent that the effect of vaccination on bone health depends on the vaccination strategy used (Berg et al., 2006), especially the size of the fish during vaccination (Berg et al., 2006; Grini et al., 2011), the type of the vaccine applied (Aunsmo et al., 2008) and on-growing conditions (Fjelldal et al., 2005; 2006; 2009a, 2009b; Grini et al., 2011).

1.4.4 Dietary SPC effects on salmon elemental composition

Early studies by Storebakken et al., (1998b, 2000) have demonstrated that substitution of FM (61% of the diet) with SPC (50% SPC and 16% FM in the diet) in Atlantic salmon post-smolt diets can reduce whole body Ca, Mg and Zn levels, apparent digestibility coefficients of the same elements and retentions of P and Zn. This was an indication that increased amounts of phytate present within SPC based diets for Atlantic salmon could influence salmon's mineral composition and adversely affect bone mineralisation.

As mentioned before, P is the most critical chemical element when formulating fish feeds that contain a high level of oilseed proteins, such as SPC (Lall, 2003). The main concern regarding the use of SPC in salmon diets is the low P availability, the high prevalence of phytate, which is a heat-stable and non-soluble in alcohol chemical component and the presence of NSPs, which can cause problems in nutrient digestibility (Francis et al., 2001; Kraugerud et al., 2007). However, despite the reduced nutrient digestibility, Atlantic salmon fed on high SPC diets (75% of total protein) can maintain rapid somatic growth

developing subclinical Ca, P, Mg, Mn and Zn deficiencies (Baeverfjord et al., 1998; Storebakken et al., 2000), which can impair growth and increase mortalities at the clinical stage (Baeverfjord et al., 1998).

While research and commercial application of exogenous enzymes for the degradation of NSPs, have shown promise, a few studies have explored the potential of these enzymes in aqua-feeds. On the other hand, phytate levels can be decreased to some extent by taking advantage of the endogenous enzyme phytase during processing, such as soaking or fermentation at a suitable temperature and pH (Liener, 1994). The effects of supplemental phytase on nutrient utilisation or the growth of salmonids have been reported since the late 1990s (Vielma et al., 1998; Storebakken et al., 1998b; Vielma et al., 2000; Carter and Sajjadi, 2004; Cheng et al., 2004; Carter and Sajjadi , 2011). Several phytases have been applied both prior to pelletizing (pre-treatment of feedstuffs; dephytinization) ((Storebakken et al., 1998b; Vielma et al., 2002; Denstadli et al., 2007) and onto pellets (direct phytase supplementation) (Denstadli et al., 2007; Nwanna et al., 2008; Wang et al., 2008). Nevertheless, despite the great potential of the use of exogenous phytase in plant based aquafeeds, there is still a lack of information on the site of phytase activity on phytate within the gastrointestinal tract, while it has been reported that phytase has an optimum activity in two pH ranges i.e., alkaline and acidic pH (Debnath et al., 2005). Moreover, optimum phytase inclusion varied markedly between studies and depended on several important variables such as the plant ingredient and its dietary inclusion level, total available dietary phosphorus and Ca: P ratio and environmental conditions such as water temperature (Carter and Sajjadi, 2011). The aforementioned reasons combined with the high costs, inactivation of the enzyme at high temperatures required for pelleting (>80°C), inactivation under certain storage temperatures and the narrow optimum pH range (Debnath et al., 2005) are the main factors limiting the use of phytase in aqua-feeds.

To date the most common practice in commercial aquafeed industries regarding the enhancement of P and mineral availability in fish, is the supplementation of fish diets with phosphates and other minerals (Baeverfjord et al., 1998).

1.5 FISH IMMUNE SYSTEM

1.5.1 An overview of fish immune system

Fish use layered defences of increasing specificity in order to avoid infections. A general overview of the fish's immune system is presented in Fig.6. Fish infection pressure in the wild is much lower than in farmed populations, mainly due to the commonly followed intensive aquaculture practices. These practices often compromise the primary defence barriers (skin, gills and gut) composing local immune defences, through the formation of abrasions which increase the accessibility of pathogens to the tissues and the circulation (Kiron, 2012). Systemic defences take over at this stage, providing an immediate, but non-specific innate immune response. Nonetheless, pathogens are able to overcome the complex innate immune responses of fish that are generally weak, and finally the third layer of protection, the adaptive immunity, is activated. In this phase, the immune system adapts its response during an infection to improve its recognition of the pathogen. This improved response is then retained after the pathogen has been eliminated, in the form of immunological memory, which gives the adaptive immune system an opportunity to mount a faster and stronger attack against the pathogen each time the pathogen is encountered.

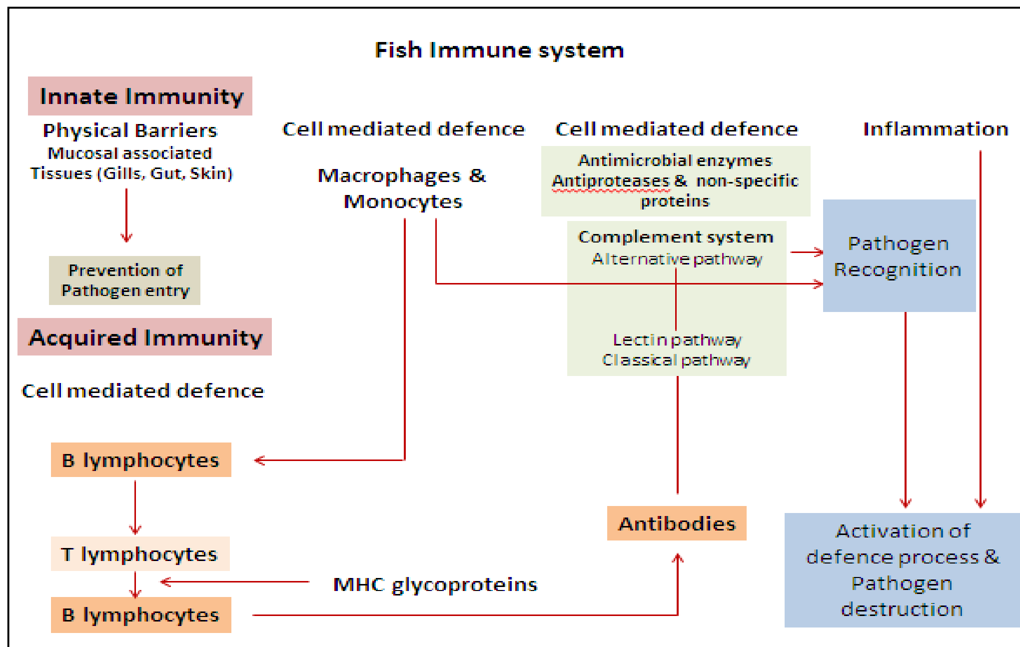

Figure 6 An overview of the fish immune system.

1.5.2 Mucosal associated lymphoid tissues

The mucosal associated tissues of the fish include the gut, the skin and the gills. These tissues are directly exposed to environmental pathogens and comprise the primary defence barriers. Mucus secreted by specialized cells of these tissues contains a wide range of antimicrobial factors inhibiting the infection by pathogenic organisms (Alexander and Ingram, 1992; Ruangsri et al., 2010). Gills are primarily a respiratory organ, but they are also involved in the immune defence of fish through the presence of tissues characterised by a wide range of leucocytes including macrophages, lymphocytes, neutrophils, mast cells and eosinophilic granulocytes (Reite and Evensen, 2006). Similarly to mammalian mucosa, lymphoid cell aggregations (T cells) are found at the base of fish gill filaments, which suggests gill involvement in the immune defence of fish (Haugarvøll et al., 2008). The gastrointestinal tract (GIT), primarily involved in nutrition, has also a role in local immunity. The gut lymphoid tissue in fish is a diffusely organized system, which is morphologically and functionally different to that of mammalian; containing a wide variety of leucocyte types necessary for local immune response (Georgopoulou and Vernier, 1986). Furthermore, the

GIT mucosal interface provides habitat for a diverse ecosystem of microorganisms, which is involved primarily in the nutrition, but also in the health of the host fish (Montalto et al., 2009). Lastly, the skin mucosal barrier serves basic roles of which protection from pathogens is one of the most important. Teleost fish are able to exhibit skin associated adaptive immunity, through their residual antibody secreting cells (ASCs), such as lipopolysaccharide (LPS) inducible B cells and non-replicating plasma cells producing immunoglobulins (Ig). Lastly, skin lymphoid tissues have been reported to contain several leucocyte types involved in innate and specific immune processes (Xu et al., 2013).

1.5.3 The immune organs in fish

The main immunological organs in fish are the anterior kidney (pronephros), the spleen, the thymus (Uribe et al., 2011). The anterior kidney is the largest site of haematopoiesis (Zapata et al., 2006) displaying a critical role in phagocytosis; antigen degradation/processing; and the production of immunoglobulin M (IgM) (Dannevig et al., 1994; Brattgjerd and Evensen, 1996). Furthermore, the concentration of melanomacrophage centres, which are aggregates of reticular cells, macrophages, lymphocytes, plasma cells and a variety of pigments in this organ, suggests its participation in antigen trapping and immunologic memory (Herraez and Zapata, 1986). The spleen has a secondary role compared to the anterior kidney in both the specific and non-specific defence mechanisms (Kiron, 2012). It is believed to be a major haemopoietic organ, whilst it also participates in the clearance of macromolecules, antigen degradation/processing and antibody production (Kiron, 2012). It is believed that the melanomacrophage centres, in which most of the spleen macrophages are found, are associated with antigenic stimulation, suggesting a possible engagement in immunologic memory (van Muiswinkel et al., 1991). Lastly the thymus, situated at the dorsolateral region of the gill chamber, has an important role in the generation of T lymphocytes and antibody producing B cells (Zapata and Amemiya, 2000).

1.5.4 Innate immune responses

1.5.4.1 Non-specific Immune Cells

Immune cells associated with innate immunity in fish consist of monocytes/macrophages, neutrophils and non-specific cytotoxic cells or natural killer cells. Monocytes/macrophages are probably the most important cell types in the fish immune response, not only by displaying a critical role of producing cytokines (Clem et al., 1985), but also as the primary cells involved in phagocytosis and pathogen killing (Shoemaker et al., 1997). Neutrophils are the primary cells involved in the initial stages of inflammation in fish, displaying similar roles as monocytes or macrophages (Manning, 1994). Non-specific cytotoxic cells are also present in teleosts and they respond to virus-infected host cells and protozoan parasites (Secombes, 1996).

1.5.4.2 Phagocytosis

Phagocytosis is one of the most primitive defence mechanisms that occur in fish. The first step in phagocytosis is the movement of the immune cell (monocyte or macrophage) in response to a foreign agent. This movement occurs through chemokinesis (non-directional movement of the phagocyte) or chemotaxis (directional movement of the phagocyte) (Weeks-Perkins and Ellis, 1995; Klesius and Sealey, 1996) in response to a bacterial or a viral antigen. After movement in response to a non-self agent, attachment occurs via lectins and is enhanced by opsonisation (Ainsworth, 1993). The next step of this process is the engulfment of the foreign agent by the cell membrane of the phagocyte to form an internal phagosome. The final steps of phagocytosis in fish include oxygen dependent or oxygen-independent killing mechanisms and lastly the digestion of the agent. Oxygen independent mechanisms involve low pH, lysozyme, and lactoferrin plus proteo- and hydro-lytic enzymes. The oxygen dependent mechanism, the so-called respiratory burst is characterised by an increased oxygen uptake, which is used for the generation of free oxygen and nitrogen

radicals known to be toxic for bacteria and protozoan parasites (Secombes, 1990). Analysis of this phagocytic activity (percentage of phagocytosis, and production of microbicidal oxygen free radicals or reactive oxygen species) in a range of leucocyte types (e.g. macrophages, monocytes and neutrophils), isolated from lymphoid organs, is considered a very reliable technique for the determination of the fish' ability to combat bacterial infections (Secombes, 1990).

1.5.4.3 Lysozyme

Lysozyme is considered a mucolytic enzyme of leucocytic origin (Saurabh and Sahoo, 2008), however, it was shown to be synthesized in the liver and several other extrahepatic tissues (Bayne and Gerwick, 2001). It is found on all body surfaces in contact with the environment including the skin, the gills and the GIT, whilst is also present in blood circulation (Ellis, 1999). This enzyme damages bacterial cell walls by catalyzing hydrolysis of 1,4-beta-linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in a peptidoglycan and between N-acetyl-D-glucosamine residues in chitodextrins and it is very effective in killing Gram-positive bacteria (Imoto et al., 1972; Saurabh and Sahoo, 2008).

1.5.4.4 Protease inhibitors

Protease inhibitors are present in fish serum and other body fluids. They have an essential role in the maintenance of body fluid homeostasis (Bowden et al., 1997), in acute phase reactions and defence against various pathogens producing proteolytic enzymes (Magnadottir, 2010). Protease inhibitors are divided in active-site inhibitors, which inactivate the active site of proteases by binding to it and the most widely studied is a2-macroglobulins (a2-Ms),which have high specificity, inhibition involving the encapsulation of bacterial and parasitic proteolytic enzymes (Armstrong, 2006).

1.5.5 Humoral Components involved in Innate and Adaptive Immunity

1.5.5.1 Complement

Complement is composed from several glycoproteins, synthesized mainly as inactive pro-enzymes, which function either as enzymes or as binding proteins after their activation by the presence of invading microorganisms and their secreted substances in the bloodstream (Boshra et al., 2006). Complement signals to the host fish the presence of potential pathogens, and engages in the annihilation and elimination of these pathogens through phagocytosis, cytolysis of the pathogens and inflammatory responses, mediates and enhances humoral immunity and lastly coordinates the development of adaptive immunity (Boshra et al., 2006). Complement is triggered by one or a combination of three pathways, the alternative, lectin and classical (Boshra et al., 2006). The alternative complement pathway, which is a major humoral component of the innate immune response facilitating chemotaxis, opsonisation and pathogen destruction (Boshra et al., 2006). Unlike the mammalian alternative complement system, quite high titres and increased capacity for the recognition of a wider range of non-self components is demonstrated in fish (Boshra et al., 2006). The classical complement pathway is an integral part of the specific humoral immunity of the fish, initiated by a complex between an antigen and an antibody, which activates the enzymatic cascade associated with this pathway (Gasque, 2004). Lastly, the lectin pathway is triggered by the formation of complexes between lectins including mannose binding lectin and ficolins, with sugar moieties located on the surface of invading microorganisms (Fujita, 2002; Turner, 2003). The interaction of lectins with microbial sugar moieties activates the enzymes related with these compounds. The initiation of this pathway is also independent of antibody and seems to operate in the crossroads of innate and adaptive immune response (Nikoskelainen et al., 2002).

1.5.5.2 Natural antibodies (Immunoglobulins)

Albeit antibodies are generally considered as components of acquired immunity, natural antibodies can also be classified as elements of the innate immune system and they are present in the serum of healthy vertebrates (Uribe et al., 2011). The former are produced in the presence of a specific antigen, which activates B cells for their formation, while the latter are constantly produced in the absence of general arrangement and without any particular antigenic stimulation (Magnadóttir, 2006). Natural antibodies are integral components of innate immunity providing immediate and broad protection against a wide range of bacterial and viral pathogens, while they are also linked to adaptive immunity (Uribe et al., 2011). In the case of salmonid fish, immunoglobulins are represented by the tetrameric IgM like immunoglobulin generally referred to in the literature as IgM and as of 2005, also IgT, another isotype which has been characterised in rainbow trout (Hansen et al., 2005) as explained in more detail below.

1.5.6 Adaptive Immunity

1.5.6.1 Cell-mediated adaptive immunity

The cells of the adaptive immune system in fish, like in mammals, are special types of leucocytes, called lymphocytes. B and T cells are the major types of lymphocytes. B cells are mainly derived from hematopoietic stem cells of the anterior kidney, while T cells are produced in the thymus (Clem et al., 1991). B cells and plasma cells are involved in the humoral immune response comparably to the mammalian analogous cells, being able to produce antibodies which they can bear on their cell membrane or secrete into body fluids (Zelikoff, 1998). However there are some noticeable discrepancies, which suggest a broader functional activity than that of mammals (Kiron, 2012). Teleost fish species contain three different immunoglobulin isotypes, IgM, IgD and the recently described IgT (Danilova et al., 2005; Hansen et al., 2005; Solem and Stenvik, 2006). In all species analysed so far IgM

is the most abundant Ig present in serum and until recently it was thought to be the only teleost fish Ig responding to antigenic stimulation (Warr, 1995; Solem and Stenvik, 2006). Zhang et al., (2010), however, have shown that IgT can also respond to antigenic stimulation. With regards to IgD, this Ig has only been characterised in catfish as having a monomeric form (Edholm et al., 2011), but has been cloned in several other fishes (wilson et al., 1997; Solem and Stenvik, 2006); whilst it was shown to lack the variable immune related region (Edholm et al., 2011), thus having an unclear role in immunity. Lastly IgT was first described in 2005 (Hansen et al., 2005) in rainbow trout and seems to be related with mucosal immunity (Zhang et al., 2010). Like B cells, teleostean T lymphocyte populations are analogous to the mammalian T cells. Nakanishi, (2002), first reported the presence of specific cytotoxic T lymphocytes in teleosts through the use of graft rejection and cell-mediated cytotoxicity studies. However, the recent advances in molecular biology and the application of genomic tools have led to the identification of several key T cell markers (CD4, CD8, CD3, CD28, CTLA-4) and cytokines, indicating the existence of discrete regulatory T subtypes including helper, memory, regulatory and natural killer T cells (Manning and Nakanishi, 1996; Castro et al., 2011). In general cell mediated adaptive immunity appears to be similar to that of higher vertebrates considering the wide range of cell types present as well as the activity of effector cells (Kiron, 2012).

1.5.6.2 Humoral components of adaptive Immunity

When B cells and T cells are activated and begin to replicate, some of their offspring will become long-lived memory cells. These memory cells remember each specific pathogen encountered and can mount a strong response if the pathogen is detected again. Memory IgM responses have been demonstrated in teleost fish, although they are substantially less enduring than those exhibited in mammals (Arkoosh and Kaattari, 1991; van Muiswinkel and Wiegertjes, 1997; Bromage et al., 2004; Zwollo et al., 2005), while they also require

much longer for the generation of significant antigen-specific response (Bromage et al., 2004; Zwollo et al., 2005). These are secreted by plasmablasts or plasma like cells (Bromage et al., 2004; Zwollo et al., 2005), which are mainly localized in the anterior kidney and they present poor affinity maturation of their IgM responses (Warr, 1997; Kaattari et al., 2002; Solem and Stenvik, 2006).

1.6 ASSESSMENT OF IMMUNE RESPONSES

1.6.1 Assessing the effects of nutrition on the immune response and disease resistance of fish

The fish immune system involves a wide variety of cell types with distinct characteristics and functions, and several humoral components, which govern several complex defence mechanisms both locally and systemically. Immunity is highly reliant on dietary provision of nutrients for the synthesis of all components orchestrating fish immunity (Kiron, 2012; Sanchez, 2005). To evaluate the status and functionality of the immune system, the measurement of an extended range of immunological parameters that are indicative of the fish innate or specific immunity is required.

The recent development of monoclonal antibodies (MAbs) against fish immunoglobulins have helped to implement the use of serological immunoassays for the measurements of total and specific immunoglobulins (Coll and Dominguez-Juncal, 1995). The measurement of total antibodies and specific antibody production *in vitro* is used to evaluate both innate and specific immune response respectively, in fish after immune stimulation (e.g. via vaccination), after subjecting the fish to different dietary treatments for example (Kiron, 2012). Despite the fact that vaccination and measurement of total and specific antibody generation could be a very informative technique for the evaluation of immune function and vaccine efficacy in nutritional studies, it is not frequently applied.

Another point to note is that there can be significant variation in antibody production between fish with both high and low responders being found in vaccinated populations (Wiegertjes et al., 1996; Schrøder et al., 2009). In general, antibody responses in fish are determined using techniques such as agglutination, precipitation or enzyme linked immune-sorbent assays (ELISA).

The use of *ex vivo* studies through the application of *in vitro* assays using cells isolated from specific tissues of fish subjected to a prescribed treatment, is another approach for the evaluation of immune defences (Kiron, 2012). However, the main disadvantage of *in vitro* assays is the removal of key components during the isolation procedure, which could potentially misrepresent the results *in vivo* (Kiron, 2012). However, the *ex vivo* studies have revealed differences, reflecting feed effects. Furthermore, the results of such studies help to confirm observations obtained from *in vivo* experiments (Kiron, 2012). The use of cells isolated from blood, lymphoid organs (head kidney, spleen or intestine) or the peritoneal cavity of fish (Rowley, 1990), which have been subjected to different dietary treatments, is the main way in which *ex vivo* studies are performed (Kiron, 2012). The analysis of the phagocytic activity of isolated leucocyte populations is considered a very reliable technique for the determination of the fish ability to combat bacterial infections (Secombes, 1990).

The examination of fish blood is also a very useful diagnostic tool in establishing the health status of fish stocks, and the diagnosis of early disease or stress (Hemre et al., 1995; Waagbø et al., 1988). A variety of techniques are used to investigate the various properties of fish blood such as the measurement of haematocrit (hct), total leucocytes and differential immune-related cell counts. Serum-based assays constitute another useful array of measurements for the evaluation of immune defences, primarily ones involved in innate immunity. The most commonly examined humoral (circulating) constituents are complement activity, lysozyme activity, total Ig, acute phase proteins etc (Kiron, 2012).

These immune parameters are the most indicative of dynamic *in vivo* immune defences in fish. Lysozyme is a salient constituent of fish innate immunity with antiviral, antibacterial and anti-inflammatory properties (Saurabh and Sahoo, 2008) influenced by a wide variety of factors including nutrition (Kiron, 2012). Anti-proteases are also important humoral components of the innate immunity influenced by several factors, including nutrition, and have been used to evaluate the fish's health status (Kaleeswaran et al., 2011). Complement is another important humoral component modulated by a wide range of extrinsic factors including nutrition in fish (Thompson et al., 1996; Panigrahi et al., 2005; Kiron, 2012). More specifically, the alternative complement activity can function at varying temperatures and can mediate the lysis of target erythrocytes from several animals (Yano et al., 1988, 1987), suggesting increased capacity for the recognition of a wider range of non-self compounds compared to the mammalian (Boshra et al., 2006).

Lastly, the most informative way for the evaluation of the effects that diets have on the general health status of fish is the application of pathogen challenges where either mortality or morbidity of fish is recorded, or a stress challenge with post-exposure stress mapped (Kiron, 2012). Challenge studies can be very useful since they are indicative of the integrated fish-defences (Kiron, 2012). To date many studies have used this technique to assess the effects of experimental aqua-feeds containing different dietary components on fish resistance to various diseases (Thompson et al., 1996; Bransden et al., 2001; Krogdahl et al., 2000; Kiron, 2012). The reliability of challenge experiments is very much dependent on their repeatability. However, contrary to the stressful and unfavourable environmental conditions fish encounter in sea cages or other exposed locations, laboratory challenge experiments are often described, based on controlled experimental conditions. This major difference could certainly influence an increased requirement for nutrients in order for the

fish to develop efficient defence mechanisms and cope under the unfavourable conditions of cage culture (Blazer, 1992; Lall and Olivier, 1993).

1.6.2 Effect of plant ingredients on fish immunity

1.6.2.1 The effect of plant proteins

Kumar et al. (2010) found unaffected leucocyte counts and haematocrit levels in rainbow trout (*Oncorhynchus mykiss*) when fish were fed on diets in which detoxified jatropha kernell meal replaced 50 and 62.5% of FM protein. Similarly, Jalili et al. (2013) reported no changes in blood parameters (haematocrit, leucocytes and differential leucocyte levels) in rainbow trout fed on 0, 40, 70 and 100% of protein from vegetable proteins (VPs). Rumsey (1993) demonstrated that rainbow trout fed on toasted SBM, containing high or low levels of antigenic proteins glycinin and β-conglycinin or SPC, presented an increased number of circulating leucocytes. This could be translated as an immunostimulatory effect of soy inclusion in trout diets or could be attributed to inflammatory and hypersensitivity processes, since the ANFs in the aforementioned soy products (including SPC) have been linked with intestinal enteritis (Krogdahl et al., 2000). However, Hemre et al. (2005) observed a significant reduction of cell size, measured as MCV, as the content of plant proteins increased in some of the dietary groups of Atlantic salmon fed soybean products Enlarged spleens were also noted in the soybean-fed salmon. Hemre et al. (2005) proposed that increased dietary inclusion of soybeans could be associated with the early release of immature erythrocytes.

Bransden et al.(2001) demonstrated that Atlantic salmon could be supplied diets with 40 % of total protein provided by dehulled lupin meal or a combination of dehulled lupin and hydrolysed poultry feather meal without any adverse effects to their innate immune responses (neutrophil oxygen radical production, lysozyme levels, total IgM), blood chemistry or resistance to *Vibrio anguillarum*. Similarly Jalili et al. (2013) found total serum

immunoglobulin levels, alternative complement activity, serum lysozyme concentration and resistance to *Yersinia ruckerii* to be unaffected in rainbow trout fed on diets with up 50 % protein from vegetable proteins (VPs). However, adverse effects were noted on serum total immunoglobulins and alternative complement activity when the trout were fed diets with higher than 70% of dietary protein from plant derived products. This was in agreement with the findings of Sitjà-Bobadilla et al. (2005) who found that the alternative complement activity was decreased in juvenile gilthead sea bream (*Sparus aurata*) fed on diets with over 75% of FM substitution with plant proteins. However, contrary to the results reported by Jalili et al. (2013), diets with up to 50% FM substitution with VP appeared to stimulate alternative complement activity in sea bream (Sitjà-Bobadilla et al., 2005). Discrepancies between the studies could be due to the use of different species, differences regarding the macro- and micro- nutrient balance of the diets or even the feedstuffs that were used in the diets.

Krogdahl et al.(2000) demonstrated increased resistance to *Aeromonas salmonicida* in Atlantic salmon fed on diets with 30% of total protein, provided by SPC, compared to the FM-fed control salmon and salmon fed on feeds where 30% of protein was provided by SBM. The increased resistance to furunculosis and the lower mortalities observed in SPC fed salmon challenged with *A. salmonicida* was believed to be influenced by the increased levels of polyspecific antibodies (IgMs) and lysozyme in the intestine of SPC fed salmon. These could serve as opsonins; promoting phagocytosis of the pathogen by activated macrophages (Krogdahl et al. 2000). It was thus proposed that SPC could have an immunostimulatory effect in Atlantic salmon.

Increased respiratory burst of head kidney leucocytes was noted in rainbow trout and juvenile sea bream fed on diets with 75 and 100% substitution of FM by a mixture of PP sources balanced with IAAs. Moreover, rainbow trout fed on low or high antigen-toasted

SBM or SPC demonstrated increased neutrophil, monocyte and macrophage activity, when they were assessed by oxidative radical production assays (Rumsey *et al.,* 1994). As mentioned earlier, this could be attributed to either an immunostimulatory effect of soy or an anti-inflammatory effect due to the promotion of intestinal enteritis in rainbow trout fed on such products or could even be a combination of both. Contrary to this, Burrells et al. (1999) found that dehulled solvent extracted soybean meal (DSSM), at inclusion rates of 10-50 % of total protein, had no significant effects on macrophage oxidative radical formation, whereas inclusion levels of up to 80% reduced macrophage respiratory burst activity. Restoration of macrophage respiratory burst activity was observed when fish were fed on diets with 89 % of protein from DSSM.

1.6.2.2 The effect of plant oils

Examination of the literature shows that substitution of the n-3 PUFA rich FOs with the n-6 PUFA rich VO can have both beneficial and in some instances, detrimental effects on Atlantic salmon immune response and disease resistance. For example, when Atlantic salmon was challenged with *A. salmonicida* or *Vibrio anguillarum,*VO fed Atlantic salmon was less resistant to infection when compared to the FO fed salmon (Thompson et al., 1996). In the same study no effects were seen on the non-specific immune parameters (i.e. haematocrit, leucocytes and differential leucocyte counts, head kidney macrophage phagocytic activity, head kidney macrophage intracellular and extracellular oxidative radical production, serum haemolysis, anti-protease activity, lysozyme concentration) of naive Atlantic salmon. It was then proposed by Thompson et al. (1996) that measuring defence mechanisms prior to immune stimulation (challenge or vaccination) only represents resting levels. In another study by Bransden et al. (2003) resistance to *V. anguillarum* was severely impaired in Atlantic salmon fed on diets where FO was substituted by sunflower oil at different levels. Similarly Balfry et al. (2006) have demonstrated unaffected innate immune

responses and decreased antibody titres against *V. anguillarum* and peripheral blood respiratory burst activity in Atlantic salmon fed on diets supplemented with up to 60% of the lipid as a mixture of canola oil and poultry fat compared to the FO control group. In line with the previous results, Thompson et al. (1996) showed that fish fed high levels of FOs had better survival when infected with *A. salmonicida* despite the fact that head kidney macrophage phagocytosis and bacterial killing activity was reduced in these salmon. On the other hand, Gjøen et al. (2004) demonstrated that Atlantic salmon fed on diets in which FO was completely replaced by soybean oil, presented no differences in phagocytic activity and resistance to *A. salmonicida* when compared to fully FO fed salmon. In a similar study using algal products, Carter et al. (2003) showed that the replacement of FO with algal oil, in canola-oil-based diets for Atlantic salmon, decreased their resistance to disease, whereas innate immune responses in salmon from different dietary groups remained unaffected. The reason why some diets presented improved disease resistance in salmon while others did not may be an indication of an optimal n-3/n-6 ratio promoting disease resistance, which have not yet been identified.

1.7 FURUNCULOSIS

Furunculosis is a highly infectious disease caused by the Gram negative bacterium *A. salmonicida* (Austin, 1997). Characteristic symptoms of the disease is the reddening or the presence of haemorrhages at the base of the fins, the presence of boils and haemorrhages on the body, the distention of the abdomen due to intestinal inflammation, exopthalmia and the increased mortality of the infected fish. Some of the most commonly occurring symptoms of the disease are illustrated in Fig. 7. The seriousness of furunculosis in Atlantic salmon farming was illustrated by the epidemic in 1991-1992, which led to the loss of ~10000 tonnes (about 25% of the total cultured salmon production) of Atlantic salmon in Scotland. *Aeromonas salmonicida* can affect Atlantic salmon in both the freshwater and saltwater

phases of their lifecycle (Austin, 1997). The major route of infection is poor water quality; however, it can also be linked with stress factors such as overcrowding, high temperatures and trauma (Austin, 1997). The site(s) of uptake of the pathogen into fish, although remaining the subject of conjecture, seems likely to include gills, mouth, anus and/or surface injury (Klontz, 1968; McCarthy, 1980; Hodgkinson et al., 1987).

Figure 7 Atlantic salmon parr with external signs of furunculosis. a) exopthalmia, b) furunculs and hemorrages at the base of the pelvic fins; c) furuncles and haemorrhage at the base of the pelvic fin (showing erosion) and distended abdomen; d) parr parr with an extended abdominal furuncle.

In a previous study Krogdahl et al. (2000) demonstrated increased resistance of SPC fed Atlantic salmon compared to SBM-fed and FM-fed control Atlantic salmon, challenged through cohabitation with *A. salmonicida*. During the challenge trial in which intraperitoneally infected cohabitants were used for the disease transmission, the bacteria were thought to enter the fishes' gastrointestinal tract via pellets dropped on the water surface of the tanks where they became coated with bacteria (Enger et al., 1992). Previously Michel

et al. (1990) reported enhanced resistance of rainbow trout against furunculosis despite the absence of *A. salmonicida*-specific antibodies, mainly due to the increased levels of poly-specific antibodies induced by the feed, serving as opsonins; enhancing macrophages to increase phagocytosis of the pathogen. Furthermore many studies have demonstrated that non-specific immune responses are crucial in resistance against furunculosis (Oliviet et al., 1985; Grinde, 1989; Møyner et al., 1993; Lamas and Ellis, 1994; Secombes and Olivier, 1997). The aforementioned studies were used as a reference to explain the increased survival in SPC-fed salmon in the study by Krogdahl et al. (2000), since relatively high total IgM levels were found in the mid-intestine of salmon fed the SPC diet. Since the exact route of infection has not yet been identified, an intraperitoneal injection of *A. salmonicida* was used for the infection of experimental Atlantic salmon parr in this study to describe the effects of increasing dietary SPC levels on the resistance of Atlantic salmon parr against furunculosis.

1.8 SALMON VACCINATION

1.8.1 An overview of salmon vaccination

Vaccination is the administration of antigenic material from a specific pathogen in order to stimulate an individual's immune system for the development of adaptiveimmunityagainst the pathogens. Fish vaccination is considered a very effective method of control against certain pathogens, aimed at the prevention or reduction of morbidity from infection and is now a common prophylactic practice in large scale salmon culture (Poppe and Koppang, 2014). These vaccines have been effective in reducing losses against furunculosis and other bacterial diseases (Sommerset et al., 2005). Vaccines are administered to fish in one of three ways, orally or by immersion or injection (Sommerset et al., 2005).

More specifically injection vaccination allows direct delivery of a small volume of antigen into the muscle (intramuscular or i.m. injection) or into the body cavity

(intraperitoneal or i.p. injection), allowing for more direct stimulation of a systemic immune response (Vinitnantharat et al., 1999). Injection vaccines normally include an oil-based or a water-based compound, known as adjuvant that serves to further stimulate the immune system (Tafalla et al., 2013). Over the past few decades a wide range of adjuvants have been tested in fish vaccines, including aluminium salts (Lillehaug et al., 1992), potassium aluminium salts (Horne et al., 1984; Mulvey et al., 1995) and hydrocarbon oils (Haugarvøll et al., 2010). The protection offered through this injection vaccination is much longer than by any other method (Poppe and Koppang, 2014). Another advantage is that multiple antigens from different pathogens (multivalent vaccines) can be delivered at the same time. Multivalent oil-adjuvanted injected vaccines are the most commonly used vaccines in salmon aquaculture nowadays (Poppe and Koppang, 2014).

1.8.2 Side-effects of fish vaccination

Vaccination in general causes pain (Poppe and Koppang, 2014) and induces stress, causing the reduction of circulating hormones related with the promotion of fish growth, while it also increases energy expenditure for immune induction (Pickering, 1993; van Muiswinkel and Wiegertjes, 1997; Wendelaar Bonga, 1997). Most administration forms may cause acute or prolonged side effects, but those caused by injection of oil-adjuvanted vaccines in salmon are by far the most severe. The acute side-effects can be divided into those resulting from poorly calibrated equipment, poor handling, anaesthesia, contamination of the vaccine or unhygienic vaccination processes and genuine side-effects caused by the vaccine itself (Lillehaug, 1989). For example, high levels of post-vaccination mortalities have been reported in Atlantic salmon due to the use of poorly calibrated equipment and/or techniques delivering the vaccine within internal organs (liver, spleen, intestine, swim bladder) or vessels and causing embolic lesions or necrosis in parts of these organs (Skrudland et al., 2002). The acute reduction in fish appetite and growth due to injection-vaccination is most

of the times correlated with the severity of the lesions at the site of injection (Poppe and Koppang, 2014). Furthermore, the post-vaccination effect on the fish feeding activity seems to depend on the size of the fish, water temperature, water quality and several other factors, while acute mortalities are related to poor equipment and handling techniques (Poppe and Koppang, 2014).

Chronic side effects of fish vaccination include long term loss of appetite, growth retardation, which may be recovered later in production (Berg et al., 2006), injection-site localized fibrinous peritonitis (Midtlyng, 1996; Mutoloki et al., 2006), extensive lesions on the abdomen, abdominal organs and muscle (Poppe and Breck, 1997) autoimmunity (Koppang et al., 2008; Haugarvøll et al., 2010) and lastly skeletal lesions and deformities (Berg et al., 2006; Aunsmo et al., 2008; Haugarvøll et al., 2010; Fjelldal et al., 2012)

1.9 AIMS OF THE STUDY

Several studies have investigated the effects of different levels of FM substitution with SPC on the growth performance of Atlantic salmon post-smolts (Olli et al., 1994; Refstie et al., 1998; Storebakken et al., 1998b, 2000). Furthermore, one published study has investigated the effects of diets with 30% of dietary protein from SPC on the disease resistance and intestinal immunity of seawater adapted Atlantic salmon (Krogdahl et al. 2000). A gap in information regarding the effects of increasing dietary levels of SPC on the growth performance, composition and general immune status of Atlantic salmon parr still exists. To date most of the reported nutritional studies focusing on immune responses relate to measuring innate immune responses of fish, which have not been stimulated immunologically though infection or vaccination. It has been proposed that measuring defence mechanisms prior to immune stimulation only represents resting levels (Thompson et al., 1996). However, measuring immune parameters shortly after immune stimulation may highlight the effects of dietary modifications that were not evident before. Vaccination is an

integral prophylactic practice in large scale salmon farming, which usually takes place during the sensitive parr stage, increasing salmon's resilience against commonly occurring lethal diseases such as furunculosis (Poppe and Koppang, 2014). Nonetheless, vaccination as mentioned previously has been linked with reduced feed intake, growth reduction, changes in bone composition and morphology and many other side effects (Poppe and Koppang, 2014). While most of the reported studies have focused solely on the effects of SPC diets on the performance, composition and immunity of naïve salmon, the present study aims to investigate the effect of the provision of diets with increasing dietary SPC prior and post -immunization with commercial vaccines, representing a more possible scenario.

The overall aim of the present study was to investigate the effects of FM replacement with SPC as well as FM-, FO- and fish-free aqua feeds on the growth, feed efficiency, composition and immune responses of cultured Atlantic salmon. The specific objectives were:

1) To investigate the effects of increasing dietary SPC levels combined with increased dietary supplementation of lysine and methionine and consistent dietary supply of P on the growth performance (weight gain) and proximate composition of juvenile Atlantic salmon exposed to continuous light and subjected to vaccination.

2) To assess the effects diets with increasing levels of SPC, methionine and lysine and constant dietary supplementation of phosphorus on the immune responses of juvenile Atlantic salmon prior and post-vaccination with a commercial *A. salmonicida* vaccine.

3) To explore the effects of increasing levels of SPC, lysine and methionine, combined with increasing dietary supplementation of phosphorus on growth and bone mineral composition of Atlantic salmon (*Salmo salar* L.) parr held under a 12h light: 12h dark photoperiod prior to and at post-vaccination or injection with Phosphate Buffered Saline (PBS) (control fish).

4) To evaluate the resistance and general immune response of vaccinated against *A, salmonicida* and PBS-injected Atlantic salmon parr fed on increasing levels of SPC and challenged against furunculosis in a controlled disease challenge laboratory.

5) To investigate the effects of FM-, FO- and fish-free diets on growth, feed efficiency, whole body proximate composition as well as the innate immune responses of farmed Atlantic salmon post smolts (seawater phase) compared to salmon fed commercially applied feed FM- and FO- based formulations.

Chapter 2.

General Materials and Methods

2.1 EXPERIMENTAL DIETS

The experimental diets for Experiments I, II, III, IV, V (described in Chapters 3, 4, 5, 6 and 7 respectively) were produced at the EWOS Innovation Technological Centre (Dirdal, Norway) as practical-type extruded pellets. The diets were formulated to meet all the known nutritional requirements of Atlantic salmon (NRC, 1993). The formulations, proximate and fatty acid compositions of the experimental diets used in each experiment, along with details regarding the feeding practices used, are described in the methodology of the relevant chapter. Prior to each feeding trial or during an initial acclimatisation period the fish were fed commercial extruded feeds produced by EWOS.

2.2 EXPERIMENTAL ANIMALS AND HUSBANDRY

The experiments were conducted in accordance with the guidelines relating to research on experimental animals from either the Norwegian Government (Chapters 3, 4 and 5) or the British Home Office (Chapters 4, 5). The experimental animals used in this study were Atlantic salmon parr for Chapters 3, 4, 6, 7, and Atlantic salmon post-smolts for Chapter 5. The fish for Chapters 3, 4 and 5 were held in enclosed tanks at the aquarium facilities of EWOS Innovation experimental facilities at Dirdal, Norway, whilst salmon parr used in Chapters 6and 7 at the aquarium facilities of the Institute of Aquaculture of the University of Stirling (Stirling, Scotland). The specific experimental conditions for each individual dietary trial are described in the specific methodology section of the relevant chapter.

2.3 SAMPLING PROCEDURES

2.3.1 Feed sampling

At the beginning of each feeding trial samples of diets were collected for analyses as described below. The diets were kept at 4ºC until analysed and used in the trials.

2.3.2 Fish sampling

Prior to any experimental procedure (e.g. weighing, measuring, vaccinating and challenging) all fish were anaesthetized using MS222 (Tricaine Mesylate, Sigma-Aldrich) (50 mg$\times$ l^{-1} for Atlantic salmon parr or smolts). After the experimental procedure the fish were placed in clean aerated water and allowed to recover (usually within 5 min) before being returned to their tank. Measurements of fish weight and length were made throughout the experiments. The fish were weighed to the nearest 0.1g. Where fish required to be sacrificed for blood and tissue sampling, they were anaesthetized with MS222 (100 mg $\times$ ml^{-1} for salmon parr in the first trial and 7 g $\times$ l^{-1} for Atlantic salmon growers in the second trial) or benzocaine (E1501, Sigma-Aldrich) (100 mg $\times$ ml^{-1} for salmon parr in the third trial).

2.3.3 Body cross-sections, carcass and vertebral samples for chemical analyses

Pools of body cross-sections between the end of the dorsal and the start of the anal fin were used for chemical analysis in the study presented in Chapter 3. Each pool was made using 6 individuals per tank. In the study shown in Chapter 5 Norwegian quality cut (NQC) fillet samples were used for proximate analyses. Four NQC samples from each tank were skinned, deboned and pooled together and then frozen until analysed. In the study presented in Chapter 6 the sampled fish were divided into 3 groups of 96 fish each, consisting of 96 naïve fish prior to vaccination or injection with 0.02 M phosphate buffered saline pH 7.3 (PBS), 96 salmon from 62 days post vaccination/PBS injection (dpv/dPBSinj) and 96 fish from 62 days post-vaccination (dpv/dPBSinj). After undergoing an initial external gross examination, fish were placed within trays and stored at -70°C. Salmon were defrosted prior to the analysis. The thawed samples were placed on trays, weighed individually, and transferred into an oven at 160°C for 30 min in order to soften the flesh. The flesh was then removed carefully with the help of a scalpel blade; the spine was excised and visually

examined for any obvious bone anomalies. The spine of each individual was then defatted in isohexane for 20 h, placed on small trays and weighed prior to any chemical analyses.

2.3.4 Tissue sampling for the evaluation of the immune responses

Blood was collected from the caudal vein into heparinised syringes. Two capillary tubes were filled with blood and haematocrit values measured after centrifugation of the capillary tubes at 5400×g for 20 min. Blood cell counts were determined as described below by placing 10 µl of blood into an 1.5 ml plastic eppendorf tube containing 990 µl of L-15 medium (Sigma-Aldrich) and immediately 100 µl of this cell suspension was pipetted into a second tube containing 900 µl of L-15 medium to achieve a blood dilution in L-15 of 1/1000. Finally after preparing two blood smears per sampled fish as described in Section 2.4.2, the remaining blood was centrifuged at 600 × *g* for 5 min at 4°C. A hundred and fifty microliters of plasma from 3 fish derived from the same tank were pooled together producing a 450 µl pooled plasma aliquot, which was further aliquoted into 7 aliquots of about 65 µl and stored at -80°C (2 plasma pools from 3 fish/tank). Anterior kidney samples were then sampled aseptically from each fish after partial decapitation to expose the head kidney area, which were placed in plastic bjoux vials containing ice-cold L-15 medium (Sigma-Aldrich) (2 pools of 3 head kidney samples per tank).

2.4 IMMUNOASSAYS

2.4.1 Head kidney macrophages respiratory burst and phagocytic activity determination

For the isolation of head kidney macrophages, the head kidney samples were teased through a 100 µm nylon mesh (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) into 2.5 ml L-15 containing 40 µl of heparin (10 IU×ml^{-1}). The mesh was rinsed with 2.5 ml of the medium and the cell suspension placed on ice. Levels of O^{-2} production by head kidney

macrophage suspensions were measured by the conversion of nitroblue tetrazolium (NBT; Sigma-Aldrich) to formazan, following the method of Secombes (1990) with some modifications described by Korkea-aho et al. (2011). One hundred µl of macrophage suspension was added to the 96-well plate (Iwaki, Tokyo, Japan), incubating at 15°C for 2 h to allow cell attachment. The supernatant was removed and wells washed three times with L-15. After washing, 100 µl of L-15 containing 1 mg $\times$ ml^{-1} NBT was added to three replicate wells, and this together with phorbol myristic acetate (1 µl $\times$ ml^{-1} PMA) was added to another three replicate wells, while 100 µl of lysis buffer (citric acid, 0.1 mol$\times$ l^{-1}; Tween 20, 1.0 % (v⁄v); crystal violet, 0.05 % (w⁄v); Sigma-Aldrich) was added to two additional replicate wells. The plate was incubated for 60 min at 15°C, the medium removed and cells fixed with 100 % (v⁄v) methanol for 2–3 min before washing three times with 70 % (v/v) methanol. The plates were air-dried before adding 120 µl of 2 M potassium hydroxide (Sigma-Aldrich) and 140 µl of 2 M dimethyl sulfoxide (Sigma-Aldrich) to each well to dissolve the resulting formazan. The absorbance was determined at 610 nm using an automated multi-mode microplate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA). The number of macrophages attached to the plate was determined by counting the average number of nuclei released by the addition of lysis buffer for two replicate wells. The number of released macrophage nuclei was achieved using a Neubauer chamber, by counting the number of nuclei in the 4 sets of the 16 corner squares from one grid. The total number of nuclei within the 4 sets of squares was then divided by 4 and then multiplied by the dilution factor giving the number of nuclei $\times$ 10^4 $\times$ ml^{-1}. The level of respiratory burst was expressed as an absorbance at 610 nm for 10^5 cells $\times$ $sample^{-1}$.The head kidney cell suspension was also used for the determination of phagocytic activity by head kidney macrophages. Duplicate 100-µl cell samples were placed on glass slides and incubated for 1 h at 15°C to allow macrophages to attach. After this time, non-adherent cells were removed by washing the slides three times

with L-15 medium. Baker's yeast resuspended in L-15 medium at 5 mg ml^{-1} (100 μl) was added to one of the samples on the microscope slide. An equal volume of L-15 medium was added to the other sample on the same slide as a negative control. Samples were incubated for 1 h at 15°C to allow phagocytosis to proceed. The slides were then washed three times with L-15 medium before 100-μl volumes of 100% methanol were added for 5 min. Slides were washed three times with 70% methanol and stained with rapid Romanowsky stain (Raymond A Lamb, Eastbourne, UK). The slides were viewed at × 1000 magnification, and 100 macrophages were counted per sample. The phagocytic activity was determined as the percentage of macrophages performing phagocytosis (% phagocytosis) and as the number of yeast cells engulfed by each macrophage (phagocytic index). Examples of HKMs fixed on glass slides under normal conditions and performing phagocytosis of yeast cells is depicted in Fig. 8.

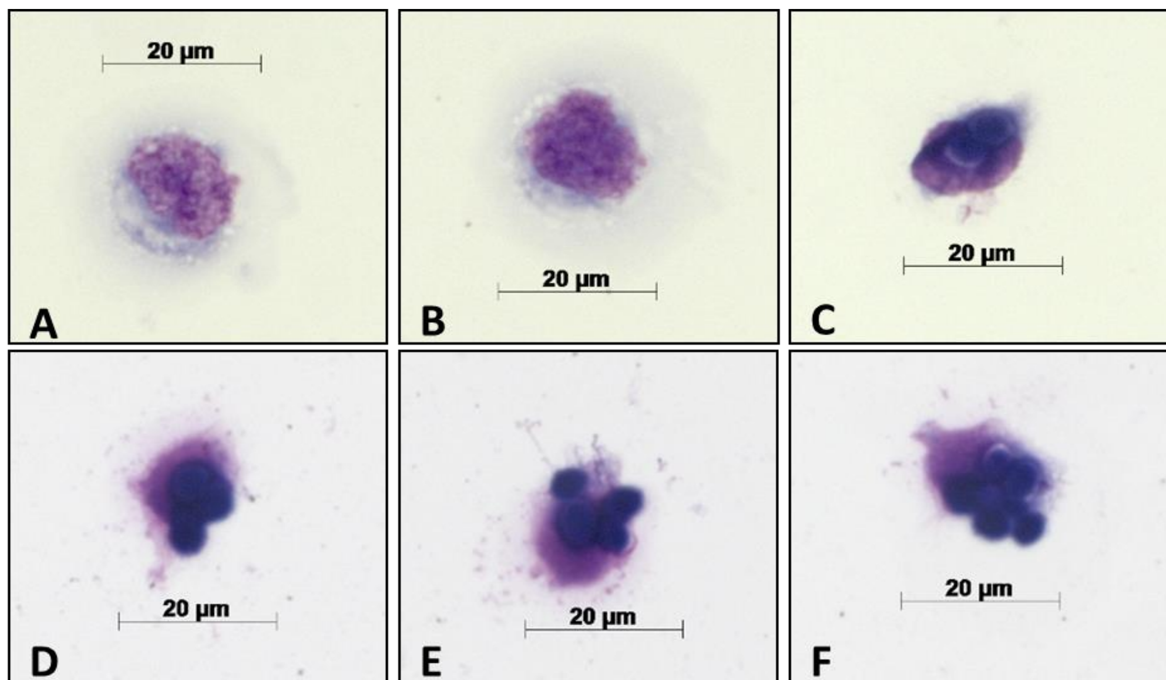

Figure 8 Head kidney macrophages from phagocytosis assays. A) Head kidney macrophage (HKM); B) HKM; C) HKM with 2 engulfed yeast cells; D) HKM with 4 engulfed yeast cells; E) HKM with 4 engulfed yeast cells; F) HKM with 7 engulfed yeast cells (× 1000 magnification).

2.4.2 Leucocyte counts and blood smears

A 1/1000 dilution of blood was used to estimate the total number of leucocytes $\times$ ml^{-1}of blood. The number of leucocytes within each blood dilution was achieved using a Neubauer chamber, by counting the number of leucocytes in the 4 sets of the 16 corner squares of one Neubauer grid. The total number of leucocytes within the 4 sets of squares was then divided by 4 and multiplied by the dilution factor giving the number of leucocytes $\times 10^4 \times$ ml^{-1}.Differential leucocyte counts were estimated using blood smears (Rowley, 1990). Briefly, a drop of heparinized blood was placed at one end of a clean dry microscope slide and spread uniformly across the slide using another microscope slide and then allowed before staining them with a rapid Romanowsky staining kit (Raymond A Lamb, Eastbourne, East Sussex). Each slide was immersed for 30 sec in each of the three stock solutions provided in the kit, and then washed with tap water. Two hundred leucocytes were counted and identified under a light microscope ($\times$ 100magnification) as lymphocytes, granulocytes, monocytes, or thrombocytes. Each leucocyte type was expressed initially as a percentage of total leucocytes and then each percentage was transformed into an actual number of cells based on the total leucocyte count. An example of the different types of leucocytes found in Atlantic salmon is presented in Fig. 9.

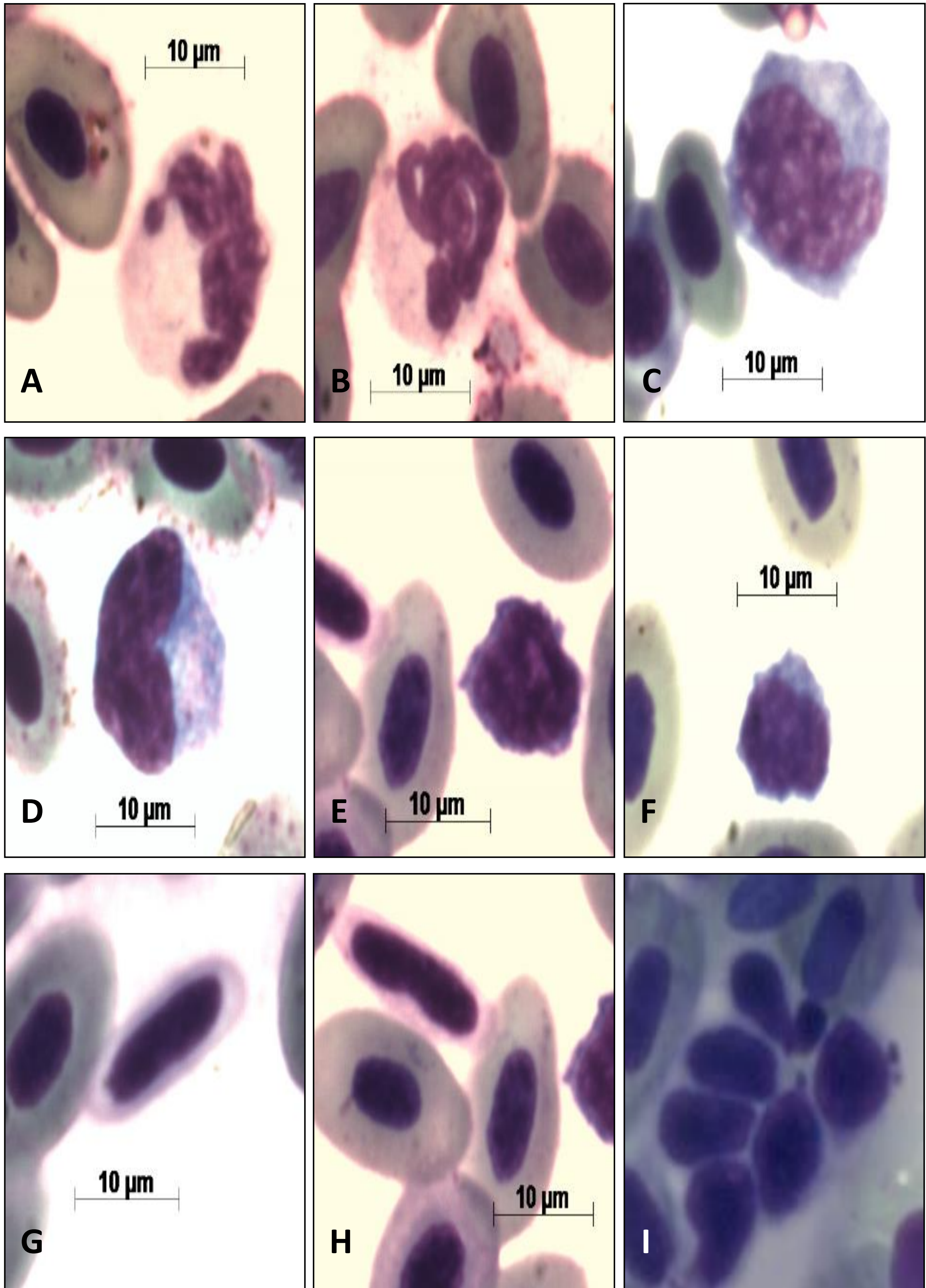

Figure 9 Head kidney macrophages from phagocytosis assays. A) Head kidney macrophage (HKM); B) HKM; C) HKM with 2 engulfed yeast cells; D) HKM with 4 engulfed yeast cells; E) HKM with 4 engulfed yeast cells; F) HKM with 7 engulfed yeast cells (× 1000 magnification).

2.4.3 Determination of lysozyme activity from serum

Serum lysozyme activity was based on the lysis of lysozyme sensitive *Micrococcus lysodeikticus* as described by Korkea-aho et al. (2011) with some modifications. Briefly, serum (10 µl × well^{-1}) was placed in quadriplicate wells of a 96-well plate (Sterilin, Newport, UK), and 190 µl of *M. lysodeikticus* (Sigma-Aldrich) solution (0.2 mg × ml^{-1}) in sodium 0.04 mol × 1^{-1} phosphate buffer (SPB, pH 5.8) then added. The absorbance was measured at 540 nm after 1 and 5 min. Two columns of the plate contained 200 µl of SPB and were used as negative control while another set of two columns contained 10 µl of SPB and 190 µl of *M. lysodeikticus* and represented the amount of bacteria at time 0 in the bacterial suspension prior to the addition of serum. A unit of lysozyme activity was defined as the amount of serum causing a decrease in absorbance of 0.001 × min^{-1}. Readings at 5 min were subtracted by the average O.D.$_{540}$ reading taken for *M. lysodeikticus* (Sigma-Aldrich) suspension, which represents the O.D.$_{540}$ at time zero.

2.4.4 Determination of total protein from serum

Protein content of serum 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). Serum samples were diluted at a level of 1:100 in phosphate buffered saline (PBS) and were measured in duplicates in 96-well plates using 25 µl of each serum dilution with 200 µl of the kit's working reagent. For the determination of total protein in serum samples, a standard curve was plotted, using fixed solutions of bovine serum albumin (BSA) (Sigma-Aldrich) in PBS as shown below (Table 1) and their estimated O.Ds.$_{562}$. Total protein concentration of plasma samples (in mg × ml^{-1}) was extrapolated from this graph after obtaining the O.D.$_{562}$ for each sampled plasma dilution.

Table 1 Detailed description of the protocol followed to obtain the standard curve for the protein assay.

Vial	Volume of PBS (µl)	Volume of BSA/stock solution (µl)	Volume of BSA/stock solution (µl)
A	0	300 stock sol.	2000
B	125	375 stock sol.	1500
C	325	325 stock sol.	1000
D	175	175 stock B	750
E	325	325 stock C	500
F	325	325 stock E	250
G	325	325 stock F	125
H	400	100 stock G	25
I	400	0	0

2.4.5 Estimation of complement activity (alternative complement and classical complement activity) and natural haemolytic activity

2.4.5.1 Preparation of sheep red blood cell suspension (SRBCs)

Defibrinated SRBCs in Alsever's solution (no more than 2 weeks old from the day of their collection) were washed three times with saline buffer (NaCl 0.85%) by centrifugation at 750 × g for 6 min and then suspended to 0.1% gelatin-complement fixation buffer (G-CFB) (0.1% gelatine, barbitone 0.575 µg × ml^{-1}, sodium chloride 8.5 µg × ml^{-1}, magnesium chloride 0.168 µg × ml^{-1}, calcium chloride 0.028 µg × ml^{-1}, barbitone soluble 0.185 µg × ml^{-1}, pH 7.2±2) to an approximately 5% suspension. Then the SRBC suspension is diluted 1:15 in 0.1% anhydrous Na_2CO_3 (v/v). The absorbance of the haemolysate was determined using distilled water as a blank reference at 540nm, determined using a spectrophotometer (CECIL CE 2021 2000 Series, Cambridge, UK). Since 5% suspension of SRBC has an absorbance of approximately 0.700 and about 10^9 SRBC × ml^{-1}, the volume was adjusted according to the following formula:

$$V\ final = V\ initial \times \frac{Absorbance\ 540\ of\ 5\%\ SRBC\ suspension\ in\ 0.1\%\ \mathrm{Na2CO3\ (100\%\ lysis)}}{0.700}$$

Then 1 volume of blood was diluted in three volumes of 0.1% G-CFB to give a final concentration of 2.5×10^8 cells $\times$ ml^{-1} of blood.

2.4.5.2 Preparation of 0.1% Gelatine Complement Fixation Buffer (G-CFB)

A complement fixation test diluent tablet (Oxoid, UK) and 0.1 g of gelatine (Sigma-Aldrich) were added in 100 ml of warm distilled water and stirred until the tablet was dissolved and gelatin created froth on the top of the mixture. The buffer was then poured into a 100 ml glass container, vigorously shaken and stored at 4°C.

2.4.5.3 Preparation of 20mM EDTA; 0.1% Gelatine Complement Fixation Buffer (G-CFB)

The buffer prepared in Section 2.4.5.2 was subsequently used to prepare this buffer. Prior to using the buffer, 20 ml of 0.1 M EDTA (Sigma-Aldrich) was added to obtain 0.1% G-CF Buffer 20 mM EDTA.

To prepare the 0.1M EDTA solution, 2.9224 g of EDTA (Sigma-Aldrich) was added to 100 ml of distilled water. The EDTA was solubilised in water by adding some drops of 10 M NaOH and the pH of the solution then adjusted to 8.5. The solution was shaken vigorously and stored at 4°C.

2.4.5.4 Preparation of 20mM EDTA 0.1% G-CFB

Twenty millilitres of 0.1 M EDTA was added to 80 ml of G-CFB to prepare a 20 mM EDTA 0.1% G-CFB solution.

2.4.5.5 Preparation of 0.1M EGTA-Mg

For the preparation of 0.1M EGTA-Mg, 38 g of EGTA was added together with 20.3 g of $MgCl_2.6H_2O$ to 700 ml of distilled water. EGTA was solubilised by adding 10 M NaOH drop-wise to the solution to adjust the pH to 7.2.

2.4.5.6 Preparation of 20mM EGTA-Mg /G-CFB

Twenty millilitres of 0.1 M EGTA-Mg was added to 80 ml of 0.1% G-CFB for the preparation of a 20mM EGTA-Mg 0.1% G-CFB solution.

2.4.5.7 Collection of salmon anti-SRBC serum

Salmon antiserum against sheep RBC was produced in ten 300g Atlantic salmon post-smolts held in saltwater tanks by injecting them intra-peritoneally with 10^9 SRBC in PBS (0.15 M, pH 7.2). Four weeks after priming, a booster injection of 10^9 SRBC was given to the fish. Two weeks later, fish were bled and the anti-SRBC serum collected. Control fish were injected with PBS. Endogenous complement activity of anti-SRBC salmon serum was inactivated by heating the serum for 30 min at 50°C before using it.

2.4.5.8 Dilution of Antiserum Required for Optimal Sensitization

Two-fold serial dilutions of heat inactivated antiserum as described in Section 2.4.5.7, anti-SRBC serum ranging from 1:400 to 1: 6400 in duplicate was prepared in 0.1% G-CFB in U-well plate (two 5-well replicate rows). Ten microliters of SRBCs (2.5×10^8 cells $\times$ ml^{-1} in 0.1% G-CFB) were added in two 5-well replicate rows of another U-well plate before transferring ten micro-litres of each anti-SRBC serum from the first plate. To each well 130 µl of 1: 4 dilution of pooled serum samples of the experimental fish described in Chapter 7, were added. The plates were incubated at 22℃ for 60 min with constant shaking. Two replicate wells of 100 % haemolysis (containing 10 of SRBCs and 140 µl of 0.1% anhydrous Na_2CO_3 -v/v-) and another two of 0 % haemolysis (containing 10 of SRBCs and 140 µl of G-CFB -v/v-) were used as controls. The plates were then centrifuged at 1500 × g for 5 min and 100 µl of the supernatant from each well was transferred to a new flat-bottomed 96-well non-absorbent micro-titre plate (Sterilin) before measuring the absorbance of the wells at 450 nm using a micro-plate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA) and the percentage lysis of SRBCs was then calculated. The absorbance values of samples

were corrected by subtracting the absorbance of the sample blank control (0% haemolysis). Then the percentage haemolysis (based on the O.D. values) obtained was plotted against the reciprocal of the anti-serum dilution to identify the point at which the rate of haemolysis reached a plateau. The dilution of the antiserum providing roughly twice the amount of antibody present at this point was used for optimal sensitization of SRBCs.

2.4.5.9 Preparation of sensitized SRBCs

Heat-inactivated anti-SRBC fish serum diluted with the addition of 0.1% G-CFB with 20mM EDTA, was added to an equal volume of SRBC suspension (2.5×10^8 cells $\times$ ml^{-1} 20mM EDTA 0.1% G-CFB) with constant mixing, and the cell suspension was incubated at 25ºC for 30 min. The sensitized cells were then washed by centrifugation at $500 \times g$ with 0.1% G-CFB and adjusted to 2.5×10^8 cells $\times$ ml^{-1}.

2.4.5.10 Measurement of natural haemolytic activity

Measurement of natural plasma haemolytic activity was based on a method described by Langston et al. (2001) with modifications. Plasma was diluted in double serial dilutions in 0.1% gelatine-complement fixation buffer (0.1% G-CFB) (1 complement fixation tablet (Oxoid, UK) and 0.1g of gelatine (Sigma-Aldrich) in 100 ml of warm distilled water) and 25 µl added to each well of a non-absorbent U-well micro-plate (Sterilin) in duplicate. Ten microliters of 2.5×10^8 cells $\times$ ml^{-1} SRBC suspension was added to each serum dilution. Controls on each plate comprised of 0.1% anhydrous Na_2CO_3 (v/v) (100% lysis) replacing serum and G-CFB replacing serum (0% lysis). The micro-titre plates were incubated at 22°C for 90 min with constant shaking and the reaction terminated by the addition of 140 µl G-CFB with 20mM EDTA, followed by centrifugation at $1500 \times g$ to spin down the remaining SRBC. After centrifugation, 100 µl of supernatant was transferred to the wells of a new flat-bottomed 96-well non-absorbent micro-titre plate. The absorbance of the wells was read at 450 nm using the micro-plate reader and the percentage lysis of SRBCs calculated. The

absorbance values of the complement samples and the 100% haemolysis controls were corrected by subtracting the absorbance of the sample blank control (0 % haemolysis). The estimation of the complement sample haemolysis percentage was done according to the following equation:

$$\text{Complement sample haemolysis \%} = \left(\frac{Absorbance\ of\ complement\ sample}{Absorbance\ of\ 100\%\ lysis\ control}\right) \times 100$$

A graph of log x (x = concentration of plasma) (ordinate axis) *vs* log y/ (1-y) (y = % SRBC haemolysis) (abscissa axis) was drawn and after estimating the volume of plasma giving 50 % haemolysis ($H_{50\%}$), and the haemolytic activity of plasma was calculated in Units $H_{50} \times ml^{-1}$ by dividing the dilution factor of plasma with the estimated plasma volume causing lysis to the 50% of the RBCs in the wells expressed in ml.

2.4.5.11 Performance of complement assays

Unsensitised SRBCs were used to determine lysis caused by the alternative complement pathway (ACP), while sensitized SRBCs (see Section 2.4.5.10), were used to determine total complement (TC) and classical complement pathway (CCP) activity. Buffer for the AC was 0.01 M EGTA-Mg-G-CFB and for determination of total and classical haemolytic activity G-CFB. Tests were performed in round-bottomed 96-well microtiter plates (Sterilin). Briefly, complement activity determination was based on methods described by (Yano et al., 1987, 1988) with modifications. Briefly, serum was diluted four times in double serial dilutions accordingly (starting from 1: 4 for the estimation of AC activity and 1:16 for the estimation of TC and CC activity) and 25 µl of each dilution was added to wells of a non-absorbent U-well micro-plate in duplicate. Ten microliters of 2.5×10^8 cells ml^{-1}SRBC suspension was added to each serum dilution. Controls on each plate comprised 0.1 % anhydrous Na_2CO_3 (v/v) (100% lysis) replacing serum. G-CFB replacing serum (0% lysis) and serum blanks (duplicate wells of serum dilutions with G-CFB replacing SRBC

suspension). The plates destined for the estimation of TC and CCP activity also included, a CC control sensitization of sheep RBC with non-immune pooled carp serum and a standard complement sample (serum pool) for correction of plate differences were included.Microtitre plates were incubated at 22°C for 90 min with constant shaking and the reaction terminated by the addition of 140µl G-CFB with 20mM EDTA, followed by centrifugation at 1500 × g to pellet the remaining SRBCs. After centrifugation, 100 µl of the supernatant from each well was transferred to a new flat-bottomed 96-well non-absorbent micro-titre plate. The absorbance of the wells was read at 450 nm using a micro-plate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA) and the percentage lysis of the SRBCs calculated. The absorbance values of samples were corrected by subtracting the absorbance of the sample blank control (0 % haemolysis). A graph of log x (x = concentration of plasma) (ordinate axis) *vs* log y/ (1-y) (y = % SRBC haemolysis) (abscissa axis) was drawn and after estimating the volume of plasma giving 50% haemolysis ($H_{50\%}$), and the units of serum haemolytic activity (AC from the plates using 0.01 M EGTA-Mg-G-CFB and TC from the plates using G-CFB as dilution buffer)(Units $H_{50}\times ml^{-1}$) were calculated by dividing the dilution factor of plasma with the estimated plasma volume causing lysis to the 50% of the SRBCs in the wells expressed in ml. CC was calculated by subtracting AC activity from TC activity.

2.4.6 Antiprotease Activity

The method to detect anti-protease activity in trout plasmawas based on the method described by Ellis (1990), modified for use in microtitre plates. A 100 µg × ml^{-1} trypsin solution was prepared by adding 1 ml of 25 mg × ml^{-1} of trypsin stock solution (Invitrogen, UK) in 249 ml 0.1 M Tris.HCl (pH 8.2). Plasma samples were diluted two-fold in the Tris.HCl buffer in round-bottomed 96 well plates (Sterilin); giving final plasma volumes of 2.5, 1.25, 0.625 and 0.313 µl. In a flat-bottomed 96 well plate, 5 µl of diluted samples were

added to 15µl trypsin and incubated for 5 min; duplicates were used where enough plasma was available. Finally, 200 µl of freshly made chromogen solution in distilled water (0.1% Na-Benzoyl- L -arginine 4-nitroanilide hydrochloride (Sigma-Aldrich) -BAPNA- 0.1 g of BAPNA were dissolved in distilled water at > 65°C and then cooled to 22°C prior to using it) was added to each well. Wells containing only BAPNA solution and Tris.HCl buffer without the addition of plasma samples served as a zero reference. The plates were then incubated for 30 min at 22ºC before centrifuging them for 6 min at 750 × g. One hundred µl from each well was transferred to wells of a flat bottom 96-well plate and the absorbance measured with a micro-plate reader (Biotek Synergy HT) set on a 5 min kinetic run, reading every 1 min at 410 nm. Tryptic activity was a measure of the difference in values at 5 min from the ones at time zero divided by 5 (units expressed as change of 0.001 units of absorbance at 410 nm × min^{-1}). The 75% inhibition value was calculated from the blank samples, which represent the 100% inhibition of tryptic activity and reference samples which represent the 0% inhibition of trypsin. The volume of plasma required to achieve 75% inhibition of trypsin activity was calculated from a graph of % trypsin inhibition against the volume of plasma used. The units of trypsin inhibited at a percentage of 75% per ml of plasma were obtained by multiplying the estimated value of tryptic activity by 1000; as a unit of trypsin activity was the amount of trypsin causing a decrease in absorbance of 0.001 and dividing this number by the volume of plasma required to inhibit the activity of trypsin at a percentage equal to 75%. The values obtained were then multiplied by 1000 to transform µl to ml so as to express 75% trypsin inhibition in Units × min^{-1} × ml^{-1}.

2.4.7 Total plasma Immunoglobulin M (IgM) assay

The level of plasma/serum IgM in experimental fish was determined using an indirect enzyme linked immunosorbent assay (ELISA) (Magnadottir and Gudmundsdottir, 1992), with modifications. Briefly, two replicate rows of a 96-well an Immulon™ 4HBX plate

(Thermo Scientific, Maine, USA) were coated with 100 µl × well^{-1} serial dilution of purified IgM (Aquatic Diagnostics Ltd, Stirling, Scotland) in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6 (starting from 0.32 mg × ml^{-1} – 0.00016 mg × ml^{-1}) to form a standard curve of IgM concentration *vs.* absorbance at 450 nm. To the remainder of the wells, 100 µl of a 1/500 and 1/1000 dilution of serum was added from experimental fish, diluted in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6, using two replicates wells for each dilution. The plates were then incubated overnight at 4°C and washed 5 times with low salt wash buffer (LSWB; 0.02 M Trizma base, 0.38 M NaCl, 0.05% (v/v) Tween 20, pH 7.2). Non-specific binding site on the ELISA plates were blocked with 250 µl of 3% w/v dried skimmed milk (Marvel, Dublin, Ireland) in water and the plates were incubated for 120 min at 21°C. The casein solution was removed before adding 100 µl of mouse anti-trout/salmon IgM (F11-monoclonal anti trout/salmon IgM - Aquatic Diagnostics Ltd, Stirling, Scotland) (1:66) solution in antibody buffer (1% BSA in LSWB) for 1 h at 21°C. Plates were then washed with 5 washes of high salt wash buffer (HSWB; 0.02 M Trizma base, 0.5 M NaCl, 0.01% (v/v) Tween 20, pH 7.4) and incubated for 5 min on last wash before adding 100 µl × well^{-1} goat anti-mouse immunoglobulin-G labelled with horseradish peroxidase (HRP) (Sigma-Aldrich) diluted 1/4000 in conjugate buffer-1% BSA in LSWB) incubating for 60 min at 21°C. Plates were washed with 5 washes of HSWB, incubating for 5 min on last wash and the reaction was developed by adding 100µl × well^{-1} of substrate/chromogen (i.e. 15 ml substrate buffer containing 5 µl hydrogen peroxide and 150µl trimethyl-benzidine (TMB) dihydrochloride) and incubating for 10 min at 22°C. The reaction was stopped with 50µl × well^{-1} of 2M H_2SO_4 and plate read at 450 nm after 5 seconds in a micro-plate reader (Biotek Synergy HT).

From the 2 first rows of each plate; a standard curve was obtained (known amounts of purified salmon IgM against $O.D._{450}$). A graph correlating $\log_{10}$ of $O.D._{450}$ and $\log_{10}$ of IgM

concentration was formed, which gave a better fit. The intercept and slope of the equation of the standard curve of each plate was used for the estimation of the total amount of IgM in mg × ml^{-1} for each dilution. Then obtained values were multiplied by the plasma dilution factor to obtain the amount of IgM mg × ml^{-1} of plasma sample.

2.4.8 Enzyme linked immunosorbent assay 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). Briefly, 96-well Immulon™ 4HBX plates (ThermoScientific, Maine, USA) were coated with 50µl of 0.05% w/v poly-L-lysine (Sigma-Aldrich) in 0.05M sodium carbonate/bicarbonate buffer, pH 9.6 and incubated for 60 min at 21°C. Plates were then washed twice with a LSWB. Bacteria were added to the wells at 100 µl $well^{-1}$ and plates incubated overnight at 4°C. The bacteria had been cultured at 22°C in tryptic soy broth (TSB) overnight, washed twice with PBS (10 min at 3000 × g) and the bacterial concentration adjusted to an absorbance of 1.0 at 610 nm. Fifty µl × $well^{-1}$ of 0.05% v/v glutaraldehyde in 0.02M PBS was added to the bacteria and the plate were incubated at 21°C for 30 min before washing three times with low salt wash buffer (LSWB; 0.02 M Trizma base, 0.38 M NaCl, 0.05% (v/v) Tween 20, pH 7.2). Non-specific binding sites were blocked by incubating plates with 3% w/v skimmed milk powder in water at 21°C for 120 min. After washing the plates three times with LSWB, 100 µl of serially diluted fish serum diluted in 1 % casein (from 1:32 to 1:1024) was transferred to the ELISA plate, which was then incubated overnight at 4°C. Both positive (monoclonal antibodies against *A. salmonicida*) and negative controls (serum blanks/ sham- injected controls and naïve fish sampled at the first time-point) were also added to each plate. Plates were washed five times with high salt wash buffer (HSWB; 0.02 M Trizma base, 0.5 M NaCl, 0.01% (v/v) Tween 20, pH 7.4) with a 5 min soak on the last wash. Anti-rainbow trout/Atlantic salmon MAb (F11-monoclonal anti

trout/salmon IgM - Aquatic Diagnostics Ltd, Stirling, Scotland) diluted (1:33) in 1% BSA in 0.02M PBS was then added and plates are incubated at 21°C for 60 min. The plates were washed with HSWB as previously described, before adding goat anti-mouse IgG labelled HRP (Sigma-Aldrich), diluted 1/4000 in conjugate buffer (1% w/v BSA in LSWB) at 100 µl × well^{-1} for 60 min at 21°C. Plates were once again washed with HSWB as above. The assays were developed with 100 µl × well^{-1} of substrate/chromogen (i.e. 15ml substrate buffer containing 5µl hydrogen peroxide and 150µl trimethyl-benzidine -TMB-dihydrochloride). After incubating for 10 min at 21°C, the reaction was stopped by the addition of 50 µl × well^{-1} 2 M H_2SO_4 and the absorbance measured at 450 nm on a micro-plate reader (Biotek Synergy HT). The antibody titre was defined as the reciprocal of the highest dilution (1/x dilution) showing an absorbance at least two times greater than the negative control.

2.5 DISEASE CHALLENGES

2.5.1 Pre Challenges

The challenge bacterium, *A. salmonicida* (Hooke) was passaged one time through Atlantic salmon prior to increase the virulence of the pathogen. Recovered bacteria from the head kidney of the dead fish on TSA plates were then cultured in TSB for 27 h at 22°C, and washed twice with sterile PBS solution. The OD of the bacterial suspension was adjusted to 1.0, which represented approximately 6×10^8 cfu×ml^{-1}, according to the standard curve for this particular strain of bacteria. Bacteria were then stored at -20°C in 15% sterile glycerol in PBS. Bacteria were used for pre-challenges after culturing the bacteria on TSA plates for 48 h, and then picking 6 discrete bacterial colonies and culturing them in TSB for 27 h as described above. The lethal dose 50% (LD_{50}) of *A. salmonicida* (low dose) was determined using non-vaccinated Atlantic salmon parr of an average weight of 25g. Forty-two fish were

anaesthetized with 0,005% benzocaine, divided into 7 groups using random sampling method and stocked into four 25 l fiberglass tanks supplied with a flow through water at a rate of 0.3 l $\times$ min^{-1}. Water temperature was maintained at 13.5°C. Each group contained six animals (2 per diet), which were injected intraperitoneally (i.p.) with 0.1 ml volumes of freshly prepared *A.salmonicida* (Hooke) bacterial suspensions in PBS. Fish groups were injected with different bacterial suspensions corresponding to 0.1×10^1, 0.3×10^1, 1×10^1, 2×10^1, 3×10^1, 6×10^2, 6×10^3, 6×10^4 and 6×10^5 colony forming units-cfu $\times$ ml^{-1}). Tanks were examined 4 times per day for mortalities. The dose giving mortalities of about 70% was chosen as the high lethal dose (LD_{70}) with which vaccinated fish and PBS-injected salmon were. injected with i.p. Dead fish were subjected to standard microbiological and pathological examinations and specific mortality was determined from kidney swabs cultured on TSA plates and TSA plates containing 0.01% (w/v) Coomassie brilliant blue (see Fig. 10).

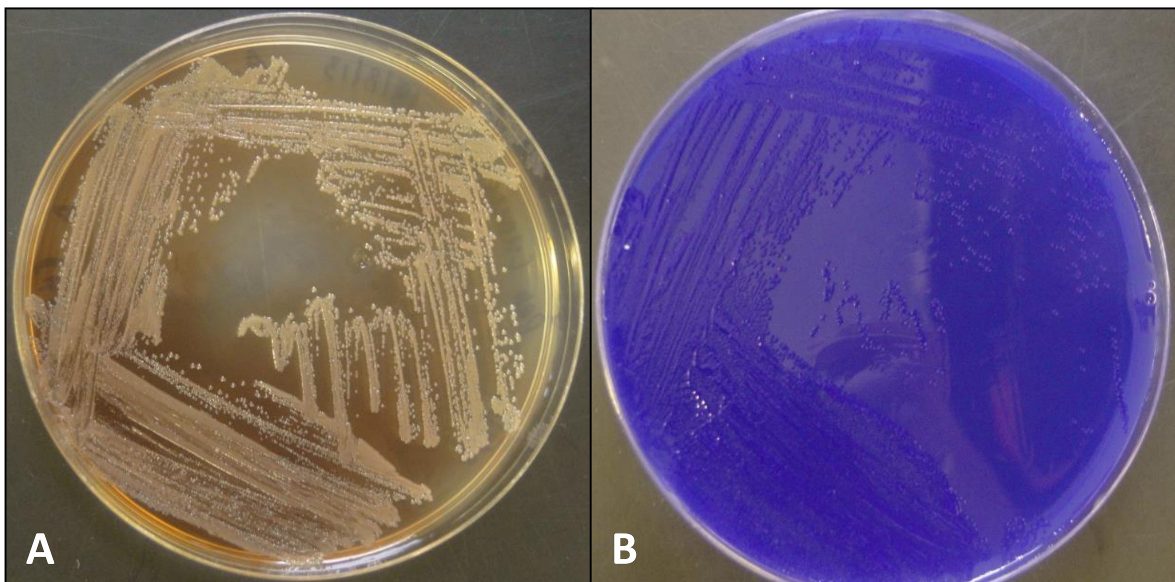

Figure 10 ***Aeromonas salmonicida*** **(Hooke strain) culture recovered from head kidney of moribund fish during pre- challenge.** A) TSA agar and (note the brown colouration diffused in the TSA agar and around the bacterial colonies is characteristic of *Aeromonas salmonicida*); B) TSA containing 0.01% (w/v) Coomassie brilliant blue (blue colourization of the bacterial colonies due to the presence of A-layer protein).

2.5.2 Infection trial

Twenty five Atlantic salmon from each one of the twelve tanks holding the vaccinated dietary salmon and the twelve tanks carrying the PBS-injected dietary groups were removed and stocked in another two sets of 12 replicate tanks in the Aquatic Research Facility (ARF) challenge aquarium at the Institute of Aquaculture, University of Stirling (see Section 6.4.2). The tanks used were circular fiberglass tanks supplied with flow-through fresh water. The fish were housed under a controlled photoperiod (12 h of light: 12 h of darkness) at a controlled temperature of 13-15℃. Seventy five hours before administering *A. Salmonicida*to the fish, a fresh culture of the bacterium was grown on blood agar. Twenty seven hours before starting the infection trial, seven bacterial colonies were cultured in tryptone soy broth (TSB) and prior to infecting the fish, bacteria were washed twice with sterile PBS by centrifuging at 3500×g, 10 min. The OD of the bacterial suspension was adjusted to 1.0 at 610 nm (equivalent to 8×10^8 cfu $\times$ ml^{-1}). The bacterial suspension was then serially diluted to 0.25×10^{-7} (corresponding to ~2×10^2 cfu $\times$ ml^{-1}), which was the dilution found to give almost 70 % mortalities. Cell densities were confirmed by distributing eight 25μl drops of each dilution (1.0×10^{-7},0.25×10^{-7},0.5×10^{-7}, 1.0×10^{-6}) onto plates TSA and counting colonies after 48 h. One-hundred microlitres of the 0.25×10^{-7} dilution (corresponding to 1.5×10^1 cfu×$fish^{-1}$) was injected i.p. into each fish after being anaesthetized with benzocaine (30 mg×l^{-1}). Tanks were examined four times per day, and the cause of death confirmed by culturing kidney swabs (after sterile dissection) onto TSA and checking colonial morphology. The challenge was terminated after 30 days at which time mortalities had ceased.

2.6 CHEMICAL ANALYSES OF DIETS AND TISSUES

Chemical composition of the diets, pooled body cross-sections and pooled defatted vertebral bones were analysed according to the following methods described by (AOAC, 1990).

2.6.1 Moisture

Moisture content in samples was determined by examining the weight lost after oven drying the samples. Samples were first weighed into pre-weighed aluminium dishes, then placed into the drying oven (Gallenkamp Oven 300) overnight at 110ºC and were subsequently allowed to cool in a desiccator before weighing the dishes with the dried samples.

$$Moisture\ (\%) = 100 - [(\frac{dry\ weight(g)}{wet\ weight(g)}) \times 100]$$

2.6.2 Crude Protein

The crude protein content of the samples was estimated by using the tecator Kjeltec system. Approximately 250 mg of each sample were weighed into a Kjeldahl digestion tube and 2 mercury Kjeltabs and 5ml conc. sulphuric acid were added. The tube was then placed into the digestion block at 420ºC for 1 h. The tubes were then removed from the block and allowed to cool inside the fume cupboard for at least 20 min. Then, 20ml de-ionised water were added to the digestion tube and mixed thoroughly. The tubes were subsequently distilled using the Kjeltec 2300 analyser. All samples were analysed in duplicates. Similarly, for each batch of samples, 2 glycine standard tubes and 3 blank tubes were prepared and analysed. The calculation for the percentage of protein was made by using the following equation:

$$Protein(\%) = \frac{(Sample\ titre - Blank\ titre) \times 1750.875}{Sample\ weight\ (mg)}$$

where 1750.875 is a multiplication factor to convert titre vol. to % protein based on a standardised protein factor.

2.6.3 Crude Fat

The fat content of the samples were analysed using a Soxhlet apparatus (NAS, Institute of Aquaculture, Stirling, U.K.). Approximately 1g of homogenised dried sample (feed/flesh

weight taken to 4 decimal places) was mixed thoroughly with an almost equal portion of Celite into an extraction thimble. Extraction cups (containing 5-10 glass balls) for each thimble were weighed and recorded to 4 decimal places. Cups were then placed into the cup holders of the Soxhlet apparatus and filled with 80 ml of chloroform methanol. The thimbles were placed into the pre-set and heated unit, and after adjusting the cup holder to the unit, being sure that the matching of the thimbles with the cups, samples were run. After the completion of this, the cups were removed and placed in the drying oven at 100 – 105ºC for an hour. The cups were then removed and allowed to cool within a chemical desiccator over fresh silica gel. The cups were re-weighed after 1 h. The fat content of finely ground diets was analysed in the Soxhlet apparatus after the diets were hydrolysed with 37% HCl. Briefly, approximately 1 g of diet sample was mixed with almost an equal portion of Celite into a 250 ml hydrolysis Foss tube. Subsequently, 100 ml of 37% HCl was added within the tubes and the contents thoroughly mixed, before being placed on the hot plate of a hydrolysing unit (Tecator, Soxtec system 1047) set at 75-80ºC for an hour. The solutions within the tubes were then filtered through the Foss glass thimbles, placed in the thimble supports of the hydrolysing unit, by opening the support taps. Each tube was washed with 5 × 50 ml hot deionised water (from the hot plate approximately 50ºC) using a spray gun and the solutions were then filtered through the thimbles as described above. The thimbles were removed from the hydrolysing unit and placed in an oven set at 80ºC overnight before being analysed for fat using the Soxhlet apparatus.

2.6.4 Ash content

Total ash content of the fish was determined by ashing 1 g of sample into pre-weighed porcelain crucibles at 600°C for 22 h in a muffle furnace (Carbolite, Parson Lane Hope, Hope Valley, S33 6RB, England). The samples were then removed and cooled to room

temperature in a desiccator and reweighed to determine the ash content using the following equation

$$Mean\ ash\ (\%) = \frac{Ash\ weight\ (g)}{Dried\ sample\ weight\ (g)} \times 100$$

2.6.5 Phosphorus and mineral analysis

One hundred micrograms of the feed, pooled body cross-section samples or 20-25 mg of pooled defatted vertebral bones were placed into Teflon digestion tubes with 5ml of nitric acid 69 % (v/v), which were then sealed and incubated within a microwave digester for 1 h. The relevant digestion method was selected from a menu on the microwave digester (Step 1: 21-190°C for 10 min at 800 W; Step 2: 190°C for 20 min at 800 W; Step 3: Cooling period from 190-21°C for 30 min). The samples were then poured carefully from the digestion tubes into 10 mL volumetric flasks, with the digestion tubes being rinsed with a small amount of distilled water and poured into a volumetric flask. The flask was filled up to 10 ml using distilled water and digested samples were then stored at 4°C until ICP-MS analysis). The volume of 10ml was used to calculate the final concentration of phosphorus or minerals. For ICP-MS analysis: 400 µL of digested sample was taken and added to a 10 ml sample tube and then the final volume was made to up to 10 ml with distilled water (adding 9.6ml distilled water). A dilution factor of 1:25 resulting in a final concentration of 2 % nitric acid was required before placing the samples into the ICP-MS. The calculation for the final elemental concentration in µg/mg was made by using the following equation:

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

2.6.6 Energy content by bomb calorimetry

One gram of feed sample was pelleted with a briquette press and weighed in a crucible. The pelleted sample was connected to the firing wire, which was fitted between the electrodes,

by a cotton thread. The electrode assembly was placed into the bomb and the bomb was tightened. The circuit was tested and the bomb was filled with oxygen to a pressure of 3000 Pa (30 bar). The calorimeter vessel was filled with water (total weight 2812.8 kg) at 20-21°C and the prepared bomb was placed inside the calorimeter vessel and then the calorimeter vessel was placed into the water jacket. The machine was switched on and left for a while (10-15 min) to warm up. Prior to firing, the initial temperature of the water was checked and recorded and 10-15 min after firing the final temperature was recorded. Benzoic acid was used as a standard. The energy value determined from such a standard of known energy was used to calibrate the system. Finally, the sample energy content was calculated according to the formula:

$$Gross\ Energy\ \left(\frac{kJ}{g}\right) = \frac{(Final\ Temp. - Initial\ Temp) \times 10.82.}{Sample\ weight\ (g)}$$

2.6.7 Crude fibre analysis

One gram of the defatted feed samples within a pre-weighed organic capsule was digested with 350ml of boiling sulphuric acid solution (1.25% v/v) for 35 min in a glass crucible attached to the extraction unit (Fibertec system 1020 hot Extractor). The process was then stopped and the acid was drained out and the samples were washed thrice with 350 ml of boiling distilled water. After this, samples were placed in 350 ml of boiling 1.25% sodium hydroxide solution and digested for 35 min. The alkali was then drained out and the sample was washed three times with 350 ml of boiling distilled water. Finally, the samples were defatted once more, the crucible was removed from the extraction unit and oven dried at 65°C overnight. The samples were cooled in a desiccator and weighed (W1). The samples were then ashed at 600°C in a muffle furnace (Gallenkamp Muffle Furnace) for 4 h, cooled in a desiccator and reweighed (W2). Extracted fibre was expressed as percentage of the original undefatted sample and calculated according to the formula:

$$Fibre(\%) = \frac{(W1 \times 1.0011) - Capsule\ weight - (W2 - 0.0025)}{Sample\ weight\ (g)} \times 100$$

Where:

W1= digested sample weight

W2=ashed sample weight

2.6.8 Total carbohydrate content analysis

Four portions of 3.0-5.0 mg sample (2 decimal places) were weighed and placed into pyrex test tubes. Subsequently, 2.5ml of deionised water were placed within each tube. The following tubes were analysed in duplicate to prepare a calibration curve: (1) 2.5 ml of deionised water only (blank); (2) 0.5 ml glucose standard and 2.0 ml deionised water (0.5 mg glucose); (3) 1.0 ml glucose standard and 1.5 ml deionised water (1.0 mg glucose); (4) 1.5 ml glucose standard and 1.0 ml deionised water (1.5 mg glucose); (5) 2.0 ml glucose standard and 0.5 ml deionised water (1.0 mg glucose); (6) 2.5 ml glucose standard only (2.5 mg glucose). The content of the tubes was then mixed vigorously and 1 ml of phenol solution was added to each tube. The content of the tubes was mixed thoroughly and the tubes were then placed into an ice bath for approximately 5 min, before adding 8 ml of concentrated H_2SO_4 to each tube. The solutions were then allowed to stabilise before reading the absorbance of each tube at 520 nm. Absorbance and glucose concentration were plotted to form a calibration curve, from which the amount of glucose within the samples was determined.

2.6.9 Phytate Analysis

For the determination of phytate content in the diets a Megazyme Phytate / Total Phosphorus Assay kit (Megazyme, Ireland) was used. The assay is based on the hydrolysis of phytate into myo-inositol (phosphate)$_n$ and inorganic phosphate (P_i) and a further hydrolysis of myo-inositol (phosphate)$_n$ by alkaline phosphatase into myo-inositol and P_i and the reaction

between P_i and ammonium molybdate to form 12-molybdophosphoric acid, which is reduced under acidic conditions to molybdenum blue. Approximately 1 g of finely ground (40 mesh) feed samples were weighed and dispensed into 250 ml glass tubes (Foss). Phytate was extracted in 100 ml of 0.66 M HCl overnight with mechanical shaking at room temperature. One millilitre of the extract was transferred in a 1.5 ml eppendorf tube and was centrifuged at 13000 × g for 10 minutes. Immediately after, 0.5 ml of the supernatant was transferred to a fresh 1.5ml eppendorf tube and was neutralised by the addition of 0.5 ml of 0.75M NaOH solution. Fifty microliters of the neutralised sample extract was transferred in two fresh eppendorf tubes for an enzymatic dephosphorylation reaction process described in Table2:

Table 2 Standard protocol for the enzymatic dephosphorylation reaction process of dietary samples

Solutions pipetted in 1.5ml eppendorf tube	Free Phosphorus	Total Phosphorus
Distilled water	0.62 ml	0.60 ml
Megazyme phytate/total phosphorus kit Buffer I (pH 5.5)	0.20 ml	0.20 ml
Sample extract	0.05 ml	0.05 ml
Megazyme phytate/total phosphorus kit Phytase suspension		0.02 ml
Content within microfuge tubes was vigorously mixed and incubated in a water bath set at 40°C for 10 min. After 10 min, the next reaction was initiated by the addition of:		
Distilled water	0.02 ml	-
Megazyme phytate/total phosphorus kit Buffer II (pH 10.4), plus $MgCl_2$, $ZnSO_4$	0.20 ml	0.20 ml
Megazyme phytate/total phosphorus kit alkaline phosphatase suspension	-	0.02 ml
Content within microfuge tubes was vigorously mixed and incubated in a water bath set at 40°C for 15 min. After 15 min, the next reaction was initiated by the addition of:		
Trichloroacetic acid (50% v/v)		

Content within microfuge tubes was centrifuged at 13000 rpm for 10 minutes. The supenatant was subsequently used for colorimetric determination of phosphorus.

Standard phosphorus solutions were prepared as described in Table 3 below and were then treated as samples for the colorimetric determination of phosphorus.

Table 3 Protocol for the preparation of standard phosphorus solutions

Solutions pipetted into a 1.5ml microfuge tube	STD0	STD1	STD2	STD3	STD4
Distilled water	5.00 ml	4.95 ml	4.75 ml	4.50 ml	4.25 ml
Phosphorus standard solution 5	-	0.05 ml	0.25 ml	0.50 ml	0.75 ml
Total volume	5.00 ml	5.00 ml	5.00 ml	5.00 ml	5.00 ml

The colorimetric determination of phosphorus for both samples and standard phosphorus solutions was achieved following the process described in Table 4:

Table 4 Preparation protocol for samples and standards prior to colorimetric determination

Solutions pipetted into a 1.5ml microfuge tube	Sample
Sample or phosphorus standard	1.00 ml
Colour reagent	0.50 ml
Contents were then mixed by vortex and incubated in a water bath set at 40ºC for an hour.	
After an hour, the contents were mixed again by vortex and then 1 ml was transferred to a semi-micro cuvette and absorbance was read at 655nm (A_{655}) immediately.	

After the determination of the absorbance (A_{655}) for both free phosphorus sample and the total phosphorus sample the difference between the absorbance values of the two samples was estimated obtaining $\Delta A_{PHOSPHORUS}$.

The concentration of phosphorus (**$C_{PHOSPHORUS}$**) was calculated as shown below:

$$Cphosphorus = \frac{mean\ M \times 100 \times F}{10000 \times 1.0 \times v}$$

Where:

Mean M = mean value of phosphorus standards (µg/ $\Delta A_{PHOSPHORUS}$)

100 = original sample extract volume (ml)

F = dilution factor

ΔA = absorbance change for the sample

10000 = conversion from µg/g to g/100g

1.0 = weight of original sample material (g)

v = sample volume (used in the colorimetric determination step)

2.8 CALCULATIONS

The following formulae were applied to the data:

Feed Conversion Ratio:

$$FCR = \frac{Feed\ Intake\ (g)}{Wet\ weight\ gain\ (g)}$$

Specific Growth Rate:

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

Thermal Growth Rate:

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

Weight gain:

$$WG\ \left(\frac{g}{fish}\right) = \frac{Final\ \ biomass(g) - Initial\ Biomass\ (g)}{Number\ of\ fish}$$

In the above formulae W is the weight of the sampled fish in grams; W0 and W1 are the initial and the final biomasses (per tank or per group) in grams.

2.9 STATISTICS

Initially for each one of the studies the sample size of the fish was estimated through the application of power testing. Prior to the commencement of the studies, fish from the same population of salmon that were used in the trials were sampled for the evaluation of several immune responses such as (lysozyme activity, leucocyte and differential leucocyte counts, total IgM and HKM respiratory burst activity as described previously). Moreover, data from the literature were collected, regarding the normal range of these values. For the power testing, R language was used without the application of any specific R package. Briefly the procedure applied followed the steps presented below:

1) Firstly the normal distributions for each one of the immune responses of interest according to literature data, was assumed for the performance of power test analysis.
2) The location and spread of the parameters (depending on the distribution above, for the normal distribution these are mean and standard deviation) for the control treatment was subsequently assumed.
3) Then the expected location (e.g. expressed as % effect in relation to the control) for the treatment (i.e. the responses of interest were assumed to be the same as for the control).
4) The number of replicate tanks and the number of fish sampled from each tank was then assumed.
5) Then the number of trials in which the power estimates were based on, was assumed (for the present study we used 1000 trials).
6) Subsequently expected outcomes were used (i.e. generated pseudo data) corresponding to the structure of the data (e.g. multiple fish sampled from each replicate tank), from the correct distribution, with the location and spread of the

parameters for both the control and treatment for the number of trials decided above by using random number generators specific for the distribution in question

7) Then the simulated results were estimated, using a hierarchical (multilevel) model which was the case in the reported studies of the present thesis (further details are given below).
8) The effect size estimates and standard errors were then saved for the assessment of all the experimental trials.
9) An operational curve with the data from the simulated trials was subsequently built, by using, e.g. $p < 0.05$ criteria (for the studies presented herein, a mean of +/- 2 standard errors was used to approximate the $p = 0.05$ threshold).

Power testing of all the studies focused on the immunological responses of salmon, in the present thesis indicated that the sampling of 6 individuals from each tank would give an 80 % chance of detecting a specific effect in any immune response whilst accepting a type 1 error of 0.05.

In all studies presented herein multiple observations from a single tank are available (several individuals, several pools or repeated measurements). For this reason a hierarchical (multilevel) statistical model was applied. Such models are also called mixed-effects models and statistical analyses using this method allow for a random tank effect when applicable (Crampton et al., 2010; Nanton et al., 2012; Hartviksen et al., 2014).

Any response that was a proportion and bound to 0-100% (or 0-1) could not be analysed according to a normal distribution assumption since at the extremities of the distribution range, values are compressed towards the brims (0-100). Because some of such responses in this study include zero values, a so-called arcsin transformation was applied to make the distributions normal. The arcsin transformation is defined as follows:

$$\hat{y} = \sin^{-1}\sqrt{P}$$

where $\hat{y}$ is the transformed value and p is the original proportion (in the case of percentages they need to be divided by 100 to get p). Absorbance response that has a lower bound at zero (or 0-1) is considered to follow the log-normal distribution and all such response values are transformed to logarithms before statistical modelling. The logarithmic transformation is defined as follows:

$$\hat{y} = \log_{10}(y) \text{ for } y > 0$$

where $\hat{y}$ is the transformed value and y is the original observation.

All other responses were assumed to follow a normal distribution (either without or with transformation) except counts that follow the Poisson distribution. However, microbe counts that are in millions can be safely assumed to be normally distributed since with large counts the Poisson and normal distributions become similar.

The statistical approach applied was model-based. This means that for finding if any specific effect is statistically significant two models were fitted to the data: (1) a model with the term of interest and (2) a model without the terms of interest. These two models were then nested together and compared with a likelihood ratio test (LRT) assessing if the improvement in the likelihood by using a more complex model was warranted or whether a simpler model could be considered as equally efficient. Subsequently the simplest possible model was adopted according to Occam's razor principle that Einstein formulated as “Everything should be made as simple as possible, but no simpler”.

Fish weight could be used as a covariate since many responses may be size-dependent. However, for the immunological responses in this study no such prior information, supporting the use of fish weight as a covariate is still present. Doing it blindly could be harmful, in particular when fish weight correlates with the treatments, as is the case in this study because SPC inclusion affected growth. Hence, fish weight was not included in the statistical models of immunological responses.

The adopted model is demonstrated by plotting and/or tabulating the expected mean response with 95% confidence intervals (C.I.). For a categorical effect these were represented as points with error bars (denoting 95% C.I.) and for a continuous effect as a curve with shaded confidence region. The expected mean and 95% C.I. were solved by a posterior simulation from the adopted statistical model (n=1500 random draws were used throughout this study). Sampling time in this study was considered as a categorical variable, i.e. discrete time points and no attempt to model any time-dependent effects were made.

Chapter 3.

The effects of increasing dietary levels of soy protein concentrate and constant dietary supplementation of phosphorus on growth and elemental composition of juvenile Atlantic salmon (Salmo salar L.) prior and post-vaccination

3.1 INTRODUCTION

Aquaculture is the most rapidly expanding sector of worldwide animal production, with Atlantic salmon (*Salmo salar*, Linnaeus, 1758) representing the most important cultured fish species in European and worldwide aquaculture. Atlantic salmon is typically raised in intensive aquaculture production systems and fed nutritionally complete formulated diets, which have traditionally been made with a high inclusion of FM as the main source of protein and indispensable amino acids (IAAs). As the demand for, and production of, farmed fish increases however, it is necessary to reduce FM inclusion since its supply is limited. Given that plant feedstuffs are readily available, they have received most attention over the past years as alternative protein sources to FM (Kaushik et al., 1990; Carter et al., 1994; Tacon et al., 1994; Carter and Hauler, 2000).

Alcohol extracted SPC is increasingly important as a protein alternative to FM. SPC in aquafeeds is produced by immobilising the soy globulin proteins while allowing the soluble carbohydrates, soy whey proteins, and salts to be leached from the defatted flakes or flour (Peisker, 2001). This process results to a final product with increased concentration of protein compared to other soybean products which are characterised by decreased amounts of non-digestible carbohydrates. A growing body of research indicates that SPC is a high quality protein product for use in aqua-feeds for Atlantic salmon post smolts (Olli et al., 1994; Refstie et al., 1998; Storebakken et al., 1998b, 2000). The main advantages of using SPC over other plant products include its high protein level, its favourable comparison to the EAA profile of FM with the exception of methionine and lysine, low levels of harmful indigestible components (such as trypsin inhibitors, lectins and soy antigens) and other anti-nutrients reported to cause soybean-induced enteritis, the fact that they are resistant to oxidation and spoilage, and that they are naturally free of organisms such as fungi, viruses and bacteria (NRC, 1993; Peisker, 2001). However, while SPC has been shown to be an

excellent ingredient for practical feeds for many commercially important salmonid species at the adult stage (Dersjant-Li, 2002), and is widely used in such feeds, further information is required regarding the effects of this product on the growth, proximate composition and especially phosphorus (P) and mineral compositions of Atlantic salmon parr. The main reason for that is the presence of a certain anti-nutritional factor (ANF) which is not removed through the aqueous ethanol process namely phytic acid and its corresponding salts. Phytic acid is largely bound on the protein fraction of soybeans resulting in the increased concentration of this substance in SPCs.

Much of the P in soybeans is found in the form of phytate, which is not digestible by the peptic enzymes of Atlantic salmon, thus decreasing the availability of P (Francis et al., 2001). Furthermore, phytate reduces the availability of many positively charged cations, thus rendering them unavailable for the fish (Francis et al., 2001) while it was also found to form complexes with proteins and amino acids and inhibit protein hydrolysis, thus reducing their availability (Riche and Garling, 2004). Clearly phytic acid and phytate levels also depend on the genetic background of the soy used for the production of SPCs. Therefore the presence phytate found in SPC, could potentially, affect salmon carcass proximate and bone elemental compositions, since the two aforementioned tissues consist the main protein and mineral reservoirs respectively. For this reason, and due to the fact that many of the internal organs were sampled for immunological evaluation, as the current study was designed to mainly focus on the immune performance of the fish (the results of the immune performance are not provided in the present manuscript), body cross-sections were preferred over whole body samples for the assessment of proximate and elemental composition (therefore reducing pressure at sample collection). In the present study the use of body cross-sections over whole body proximate and elemental composition analysis is explored, by comparison of the data obtained in the experimental trial herein, to previously reported whole body values

(Baeverfjord et al., 1998; Storebakken et al., 1998; Helland et al., 2007). Furthermore, the growth performance and body cross-section proximate and elemental composition of Atlantic salmon fed on four different diets with increasing levels of dietary protein from SPC is investigated.

The main objective of the present study was to monitor how growth and proximate composition of Atlantic salmon was affected after feeding them on four different diets with increasing levels of dietary protein from SPC, under intensive commercial rearing conditions prior to (Day 63) and post-vaccination with a commercial vaccine (Day 97/34 days post vaccination (dpv)). Vaccination is a common prophylactic measure in salmon culture, which can provide protective immunity against a wide range of diseases, but can also result influence negatively or positively vertebral bone mineral levels (Berg et al., 2005, 2006; Grini et al., 2011; Berg et al., 2012). On the other hand application of continuous light to prevent salmon smoltification and maturation (Stefansson et al., 1991; Handeland and Stefansson, 2001; Berrill et al., 2003; Stefansson et al., 2007) has been found to speed up salmon growth rate (Krakenes, 1990; Stefansson et al., 2008) demoting the mineralisation process in salmon skeletal tissues (Fjelldal et al., 2004, 2005).Atlantic salmon can still maintain fast growth while developing subclinical deficiencies of Ca, Mg, P and Zn, increasing their susceptibility to diseases (Åsgard and Shearer, 1997; Baeverfjord et al., 1998; Helland et al., 2006). Therefore P and mineral compositions were monitored using pooled body cross-sections of Atlantic salmon parr from the four different dietary groups of salmon prior to (Day 63 of the feeding trial) and 34 dpv (Day 97 of the feeding trial).

3.2 MATERIALS AND METHODS

3.2.1 Diets and growth trial

Four experimental diets were prepared. These were formulated so that SPC 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 5. 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's 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 l of water. Water temperature was maintained at 13 ± 1ºC, 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/tank) 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 4 experimental diets. Fish were fed with automatic belt feeders, continuously every 435 seconds for 20 seconds.

Table 5 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)	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Di-calcium phosphate[d] (g)	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
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)	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
$MgSO_4$ [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)	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Fish Oil [h] (g)	1130	120.0	127.0	132.26	113.0	12.0.0	127.0	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	471.0	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 prt: Crude fat ratio	2.9	3.0	3.1	3.3	2.9	3.1	3.2	3.4
Phytate levels (g)	11.87	13.24	14.12	15.33	11.54	13.14	14.00	15.16
Ash (g)	108.4	106.3	103.0	97.1	106.2	102.8	100.9	100.1
Phosphorus (P) (g)	16.56	16.03	15.33	14.58	16.4	15.98	15.45	14.65
Calcium (Ca) (g)	33.44	26.78	22.46	18.96	31.82	27.75	23.07	18.54
Ca: P ratio	2.0	1.7	1.5	1.3	1.9	1.7	1.5	1.3
Zn (mg)	295.41	285.3	273.43	265.87	286.99	285.49	275.39	268.43
Mg (mg)	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 (Imcopa, - Importação, Exportação e Indústria de Óleos Ltda., Araucária - Paraná, Brazil) with an apparent protein digestibility coefficient (ADC protein) of 90.8 %; [a] Fishmeal (Egersund Sildoljefabrikk, Norway) with an apparent protein digestibility coefficient (ADC protein) of 90.2 % ; [b] SPC (Imcopa, Brazil) with an apparent protein digestibility coefficient (ADC protein) of 90.8%; [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).

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). It should be mentioned that as the level of SPC was increased in the diets of Atlantic salmon parr, the food ration was decreased, however direct feed intake through waste feed collection was not determined due to the difficulty in dividing uneaten feed of such a small size from faecal material.

Fish were weighed after being anaesthetized (metacaine, 30 mg $\times$ l^{-1}). The growth trial was conducted for a total of 97 days, at which time all fish were removed and weighed. On Day 37 all fish in the tanks were bulk weighed. Fish were also weighed on Day 63 (pre-vaccination) before being intraperitoneally (i.p.) vaccinated with 100µl of a commercial vaccine (AquaVac™ Furovac, Intervet UK Ltd., Milton Keynes) against *A. salmonicida*, and on Day 97 (34 days post vaccination (dpv)). On Days 63 and 97, pools of body cross-sections between the end of the dorsal fin and the start of the anal fin were obtained from six fish per tank for proximate composition analyses.

3.2.2 Calculations

Estimated FCR values 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:

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

Specific Growth Rate:

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

Thermal Growth Rate:

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

3.2.3 Proximate composition analysis

Cross-section samples obtained as described at the end of the Section 3.2.1. 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 TecatorKjeltec System (AOAC, 1990) and crude fat was determined using Soxhlet extraction with chloroform: methanol (2:1 v/v) (Christie, 2003). The methodologies applied are described in more detail in Section 2.6. 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 digest 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 Equation 1.

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

Phytate levelsin the diets were estimated using a Megazyme Phytate/Total Phosphorus Assay kit (Megazyme, Ireland).

3.2.4 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 the proximate and mineral analysis of pooled body cross-sections 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., 2014). The statistical approach applied was model-based. This means that to find if 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 non-linear effects:

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

The three models were nested and compared with a likelihood ratio test (LRT) that evaluated if 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).

3.3 RESULTS

3.3.1 Growth performance

The mortalities among the four experimental groups of Atlantic salmon were negligible (<1%). Figure 11 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. 12 and 13). The mean expected values of the aforementioned growth parameters with their estimated 95% confidence intervals (C.I.) are demonstrated with different colour for each timepoint or period respectively. More specifically the black line (expected mean values) and its corresponding shaded region (estimated 95 % C.I.) represents the expected mean weight values for Day 36 and the credible mean weight gain 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 (34dpv). Overall, increasing levels of SPC resulted in lower mean weight and daily weight gain in Atlantic salmon juveniles at all timepoints of the study. Approximate reductions of 12 and 10% in expected mean weight were observed in SPC80 compared to SPC35 salmon at Day 36 and 63 of the feeding trial respectively, while an 8% reduction in weight was demonstrated at Day 97 (34dpv) between the two dietary groups (Fig. 12). Moreover, SPC65 and SPC80 salmon exhibited significantly lower mean weights compared to the SPC35 group at all timepoints. In a similar 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. 13). 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.

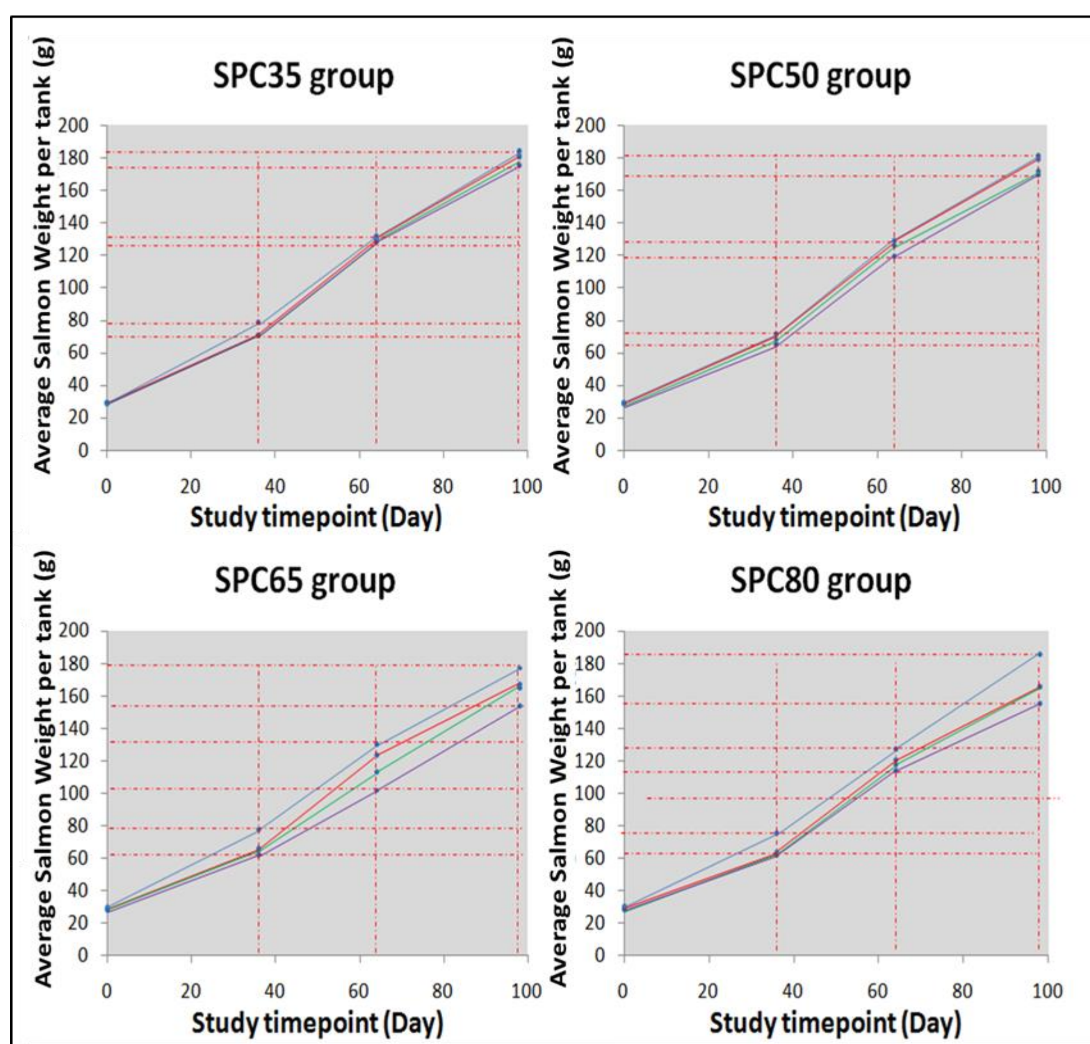

Figure 9 Observed weight development of the four dietary groups of Atlantic salmon juveniles. Dashed bold vertical lines separate different study periods: pre vaccination period, Day0-Day36; pre-vaccination period, Day36-Day63; post-vaccination period Day 63-Day 97. Broken lines with different colours within each treatment subplot connect the mean weight values of each replicate tank.

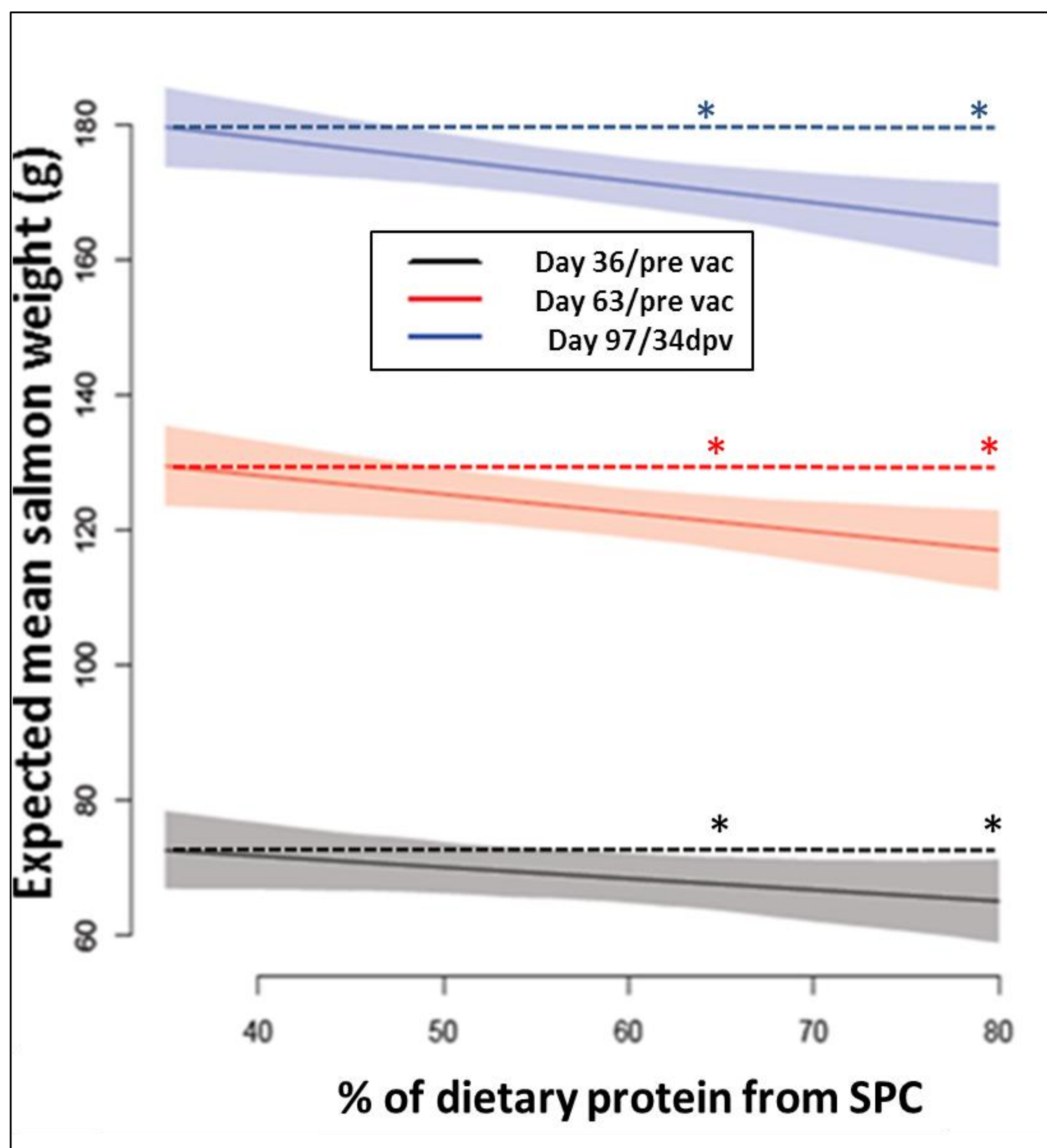

Figure 10 Modelled values of mean salmon weight for the four dietary groups at each timepoint. Statistical model showing a significant linear effect of the percentage of protein from SPC on salmon performance data with *P* values < 0.05; Expected mean weight (g) (with 95% C.I.) in relation to % SPC inclusion over the course of the study. Black, Red, and blue lines connect the expected mean weight gain for each one of the groups at different study periods: Black line refers to Day 36 of the feeding trial and prior to the vaccination; Red line represents the expected mean values from Day 63 of the feeding trial and prior to the vaccination and Blue line represents the expected mean weights from Day 97 of the feeding trial and at 34 days post vaccination. Shaded regions of the same colours indicate the 95% confidence interval regions for these measurements. 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.

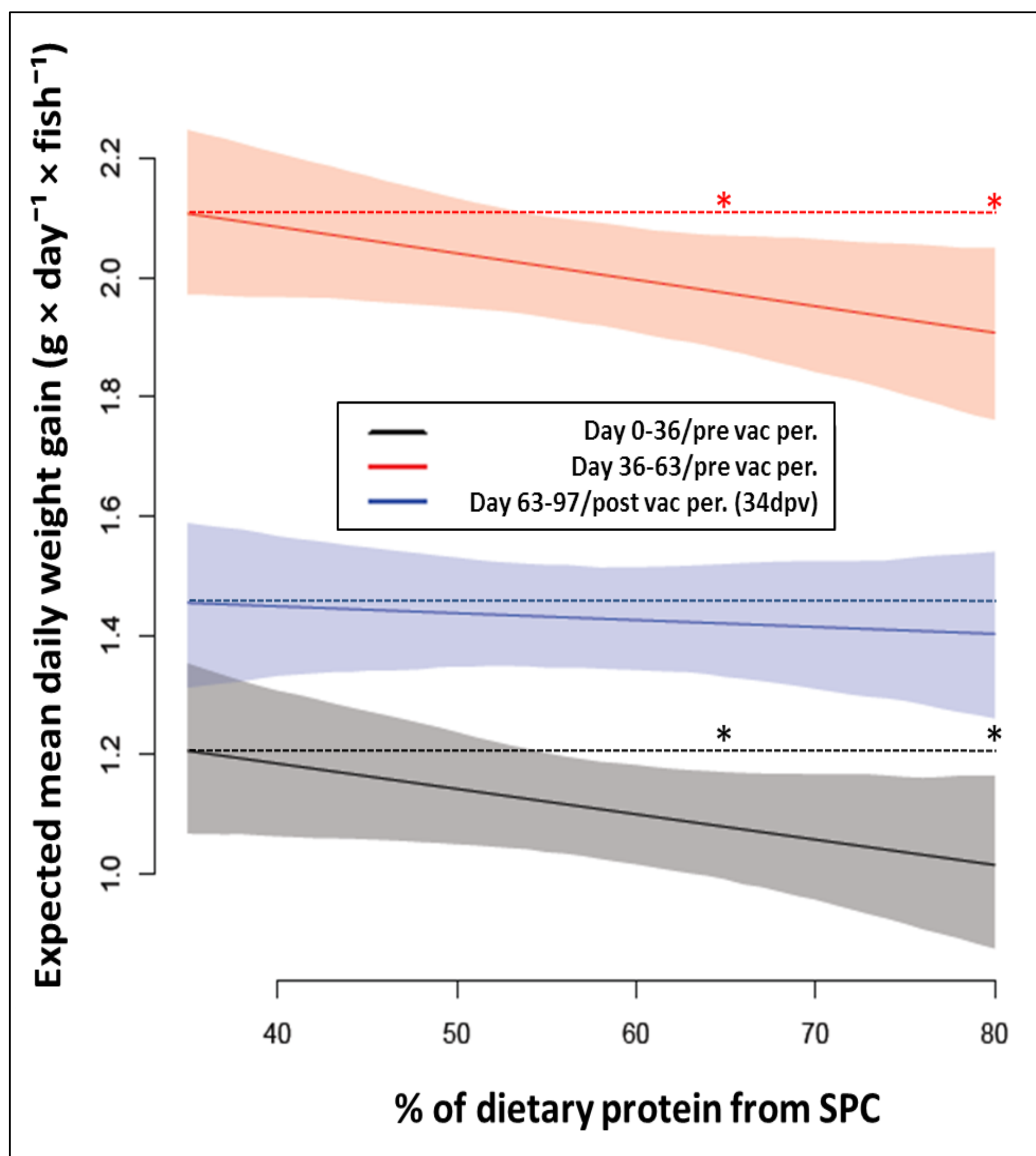

Figure 11 Modelled daily weight gain per study period. Statistical model showing a significant linear effect of the percentage of protein from SPC on salmon performance data with *P* values < 0.05; Expected mean daily weight gain per fish (g × day^{-1}) (with 95% C.I.) in relation to % SPC inclusion over the course of the study. Black, Red, and blue lines connect the expected mean weight gain for each one of the groups at different study periods: Black line refers to Day 0 - Day 36 of the feeding trial prior to the vaccination; Red linerepresents the expected mean weigth gains from Day36 - Day 63 of the feeding trial and prior to the vaccination and Blue line represents the expected mean weight gains from Day 63 - Day 97 of the feeding trial and the 34-day post vaccination. Shaded regions of the same colours indicate the 95% confidence interval regions for the values of these measurements. 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.

3.3.2 Proximate and mineral 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.14A). Overall, moisture, lipid and protein concentrations were only affected by time. A reduction of about 36% was observed in the vaccinated salmon body cross-section credible moisture levels at 34 dpv compared to pre vaccination levels. Furthermore, an increase of 18% and 4% was demonstrated in Atlantic salmon body cross-section credible lipid and protein levels respectively at 34 dpv in comparison to levels prior to vaccination. 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. Lower levels of ash were demonstrated at 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 while at this timepoint the SPC80 group exhibited a decrease of only 5% in expected ash concentration compared to the SPC35 group of fish.

The “raw data” for the pooled body cross-section elemental compositions are presented numerically in Table 6. Credible calcium to phosphorus (Ca:P) ratio, 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 to the other two models (Models selected are presented in Fig. 14B, 14C, 14D, 15B and15C). Therefore the dietary groups receiving feeds with higher levels of SPC inclusion exhibited lower amounts of the abovementioned 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. 15A). Overall, at Day 63, SPC35 salmon demonstrated significantly higher Ca: P ratio, Ca, P and Mg concentrations than the SPC50, SPC65 and SPC80 groups and higher amounts of Zn and Mn in comparison to the SPC65 and SPC80 groups.

In general, lower body cross-section elemental amounts were demonstrated for all dietary groups at Day 97 (34 dpv) compared to Day 63 (prior to vaccination). At this timepoint significantly lower Ca: P ratio, Ca, P and Zn concentrations were recorded for SPC65 and SPC80 salmon compared to 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, Ca, P, Mg and Mn was demonstrated from the modelled based statistical analysis in vaccinated salmon receiving increased dietary SPC. This was apparent from the slope connecting the expected mean values for the dietary groups at this timepoint (blue lines in Fig. 14B, 14C, 14D, 15A and 15C) 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 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).

Table 6 Ash and elemental composition of Atlantic salmon parr pooled body cross-sections.

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

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.

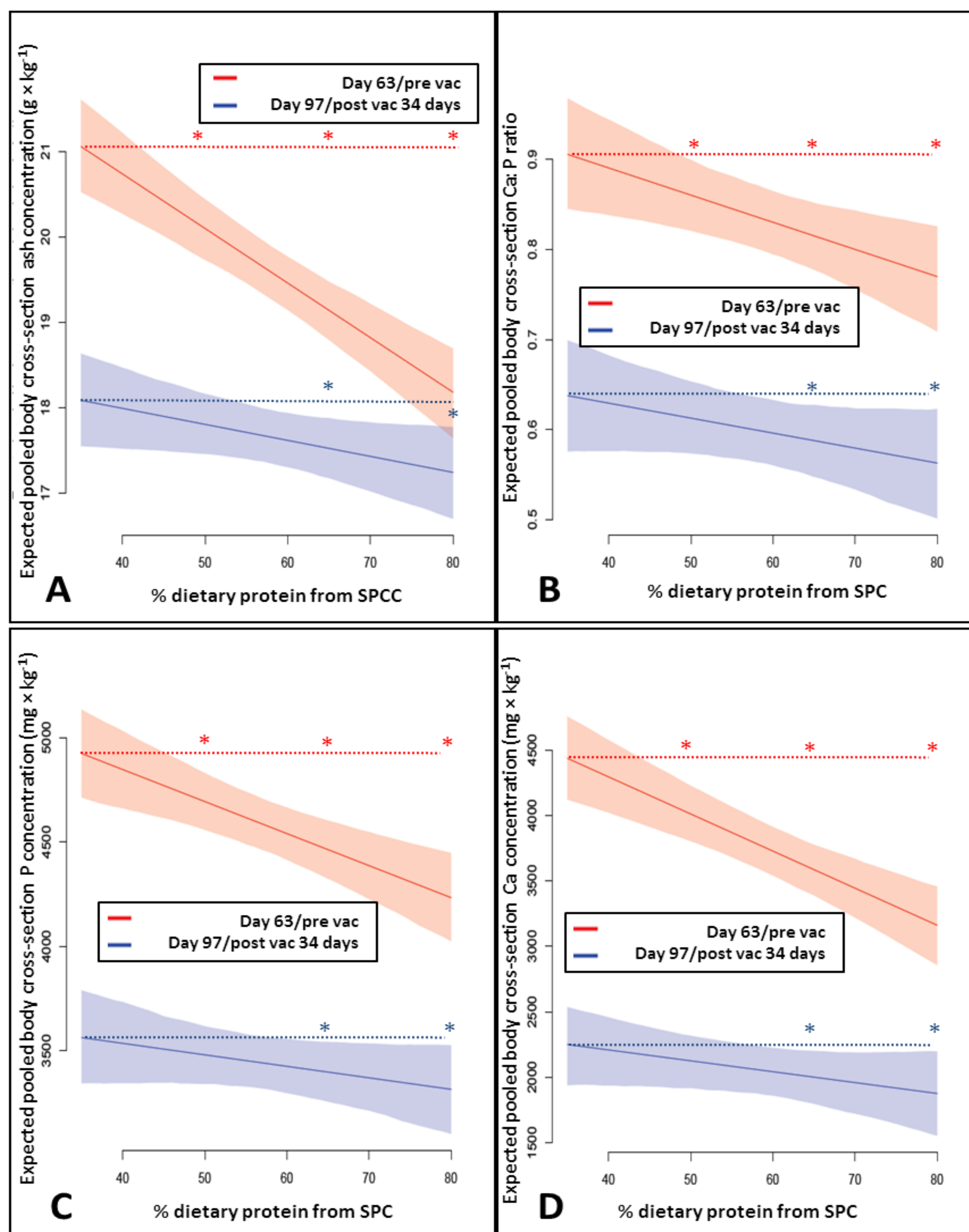

Figure 12 Modelled ash, Ca: P ratio, P and Ca pooled body cross-section levels. Statistical models showing a significant linear effect of the percentage of protein from SPC on pooled body cross-section ash and elemental levels with P values < 0.05; Expected **(A)** Ash (g × kg^{-1}); **(B)** Ca: P ratio; **(C)** Phosphorus (P) (mg × kg^{-1}); **(D)** Calcium (Ca) (mg × kg^{-1}) (with 95% 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 the values of these measurements. 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.

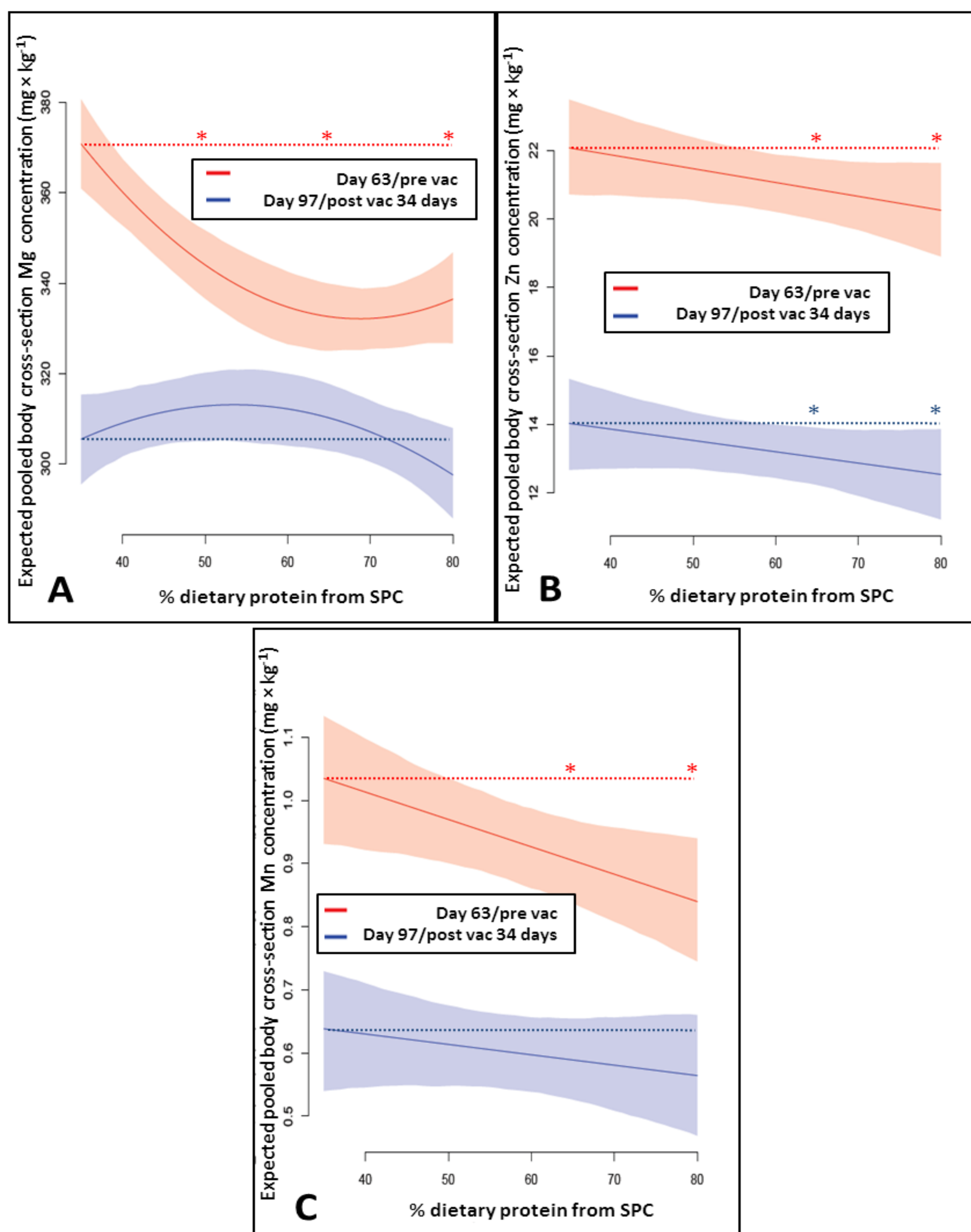

Figure 13 Modelled Mg, Zn and Mn pooled body cross-section levels. Statistical models showing a significant linear effect of the percentage of protein from SPC on pooled body cross-section elemental levels with *P* values < 0.05; Expected **(A)** Magnesium (Mg) (mg × kg^{-1}); **(B)** Zinc (Zn) (mg × kg^{-1}); **(C)** Manganese (Mn) (mg × 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 the values of these measurements. 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.

3.4 DISCUSSION

The results obtained in the current study indicate that an increase in dietary SPC inclusion can influence an overall reduction in the growth performance (mean weight and mean daily weight gain) of Atlantic salmon pre-smolts. The reduction in WG was much more obvious during the first (Day 0 - Day 36) and second (Day 36 – Day 63) period of the study prior to the vaccination of the fish. The lowered daily WG observed during this period, could be attributed to reduced feed intake; however the exact amount of feed intake (FI) by the experimental groups of fish was not determined in the present study. Decreased FI resulting in reduced growth, has been previously reported in several feeding trials testing increasing levels of dietary SPC in several farmed piscine species (Kissil et al., 2000; Arãgao et al., 2003; Deng et al., 2006), while Mambrini et al. (1999) demonstrated decreased feed intake and growth in rainbow trout receiving diets with more 80% of dietary protein from SPC. However, at the last period of the study and post vaccination, despite the observed reduction in salmon WG upon increased dietary SPC inclusion, no significant differences were obtained among the four dietary groups. The former finding indicates an improvement in the growth performance of fish receiving higher levels of SPC (SPC65 and SPC80) compared to the best performing SPC35 and SPC50 groups of the first trial period.

Reduced WGs were observed for the last part of the study, at post vaccination compared to the previous study period from Day 36 to Day 63 despite the larger size of the fish and the longer duration of the last part of the trial. This could be attributed to increased post-vaccination stress (Van Muiswinkel and Wiegertjes, 1997), reduced fish appetite (Melingen and Wergeland, 2000), stress-induced reduction of growth modulating hormones (Pickering, 1993) or reduced energy expenditure for growth due to immune induction (Wendelaar Bonga, 1997). The fact that no differences in WG were obtained among the four dietary groups of salmon at the last timepoint could have also been influenced by the

vaccination of the dietary groups. Nevertheless, an overall improvement in daily WG was observed at the previous period of the study for the SPC65 and SPC80 groups of fish, pointing at a gradual enhancement salmon mean daily WG. Traditionally used growth performance indicators including SGRs and TGCs demonstrated no differences among the four dietary groups of salmon at all timepoints, revealing minor changes in the overall growth performance of Atlantic salmon pre-smolts and that the use of WG for the aforementioned type of statistical analysis was much more useful.

Juvenile Atlantic salmon are much more sensitive to the inclusion of vegetal protein in their diets than smolts. Burr et al. (2012) demonstrated 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 obvious reduction in growth performance during the full period of the trial, an improvement in WG was witnessed for high SPC inclusion dietary salmon during the post-vaccination period. This was an indication that late stage Atlantic salmon of ~125 g 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. This is partly in accordance with the findings of Burr et al. (2012). The results of the present study are also in line with several other studies in which early stage Atlantic salmon post-smolts exhibited reduced growth when fed diets containing less than 30% FM and high levels of plant proteins, supplied as blends or as a single protein source (Refstie et al., 2001; Refstie and Tiekstra, 2003; Espe et al., 2006; Torstensen et al., 2008; Øverland et al., 2009). Previously it was proven that Atlantic salmon needs a longer adaptation period before fully accepting any diet with low levels of FM with alternative protein sources (Torstensen et al., 2008). Moreover,several studies have suggested that salmon are able to compensate growth after periods of restricted feed intake (Johansen et al.,

2001; Torstensen et al., 2008). This could explain the enhanced growth performance observed during the last period of the trial (from Day 63 prior to vaccination at Day 97 at 34 dpv) in fish fed increasing amounts of SPC.

Overall, despite the observed significant reduction in salmon growth upon increased dietary inclusion of SPC, numerically this decrease was not very large. Thus, a reduction in WG of 11 g (SPC80 vs SPC35 group of salmon) was only 8% of the WG in SPC35-fed fish during the study. It is also worth noting that the differences detected in the present study are much less than between feeds of different FM qualities (Anderson et al., 1997).

Phytate represents almost two thirds of the soybean phosphorus content and is not bioavailable to fish since it cannot be digested by the fish's gastrointestinal enzymes (Francis et al., 2001). Moreover analyses of the experimental dietary treatments demonstrated an increment in the amounts of phytate upon increased inclusion of SPC. Phosphorus was therefore supplemented in the diets of experimental fish by adding 30 $g \times kg^{-1}$ dicalcium phosphate (Storebakken et al., 1998b) to their diets, while the minimum reported dietary requirement for Atlantic salmon is around 10 $g \times kg^{-1}$ (Åsgard and Shearer, 1997). Proximate composition and mineral analysis of pooled body cross-section homogenates revealed a linear reduction of ash level, Ca:P ratio, Ca, P, Mn, and Zn content and an overall reduction in Mg in salmon parr fed on increasing dietary proportions of SPC prior to (Day 63) and 34 dpv (Day 97), which could be attributed to the increasing amounts of ANFs present in high SPC inclusion diets. The reason for this seems to be the reduced availability of P in high SPC inclusion diets, since most of the P in SPC is found in the form of phytic acid and phytate which are largely indigestible by salmon digestive enzymes, negatively influencing the concentrations of body minerals (Lall, 2003). Similarly to our findings Storebakken et al.(1998b) reported decreased levels of whole body ash, P, Ca Mg, Zn and Ca:P ratio in

salmon fed 75% of dietary protein from undephytinized SPC (i.e. non-treated with phytase) for 84 days compared to a FM fed salmon.

Expected phosphorus levels in the current study for the experimental groups, lay between the values for full body P levels reported by Shearer et al. (1994) and Baeverfjord et al. (1998) (~5000 mg $\times$ kg^{-1}); and those reported by Helland et al. (2006) (4000 - 4200 mg $\times$ kg^{-1}) for Atlantic salmon parr of a similar size to the fish in the present trial at Day 63 (Denstadli et al., 2006). However on Day 97 (34 dpv) of the feeding trial, credible P levels were estimated to be 3500 mg $\times$ kg^{-1} for salmon parr fed SPC35 and 3250 mg $\times$ kg^{-1} for salmon parr fed SPC80. These values are lower than the whole body P concentrations reported by Helland et al. (2006), possibly pointing at P deficiency in the experimental fish by the end of the trial. However, fish at this time point were at least 1.6 times larger (~165 g) than fish from the study of Helland et al. (2006) revealing far higher growth rates in the present trial, which could be attributed to the application of continuous light and intense feeding regime (Kråkenes et al., 1991; Fjelldal et al., 2005). Helland et al. (2006) reported that phytate did not promote any negative effects on either whole body or vertebral P levels; however P levels in the experimental diets were properly balanced, upon increased phytate inclusion which was not the case in the present study.

Expected calcium concentrations at Day 63 were lower compared to the whole body Ca levels stated by Shearer et al. (1994) for Atlantic salmon parr, who reported concentrations of up to 5000 mg $\times$ kg^{-1}. However Ca levels were found to be similar to those reported by Helland et al., (2006) for salmon parr of about the same size, who estimated that whole body Ca concentration for well mineralised salmon is between 3900 - 3600 mg $\times$ kg^{-1} and 2500 mg $\times$ kg^{-1} for Ca deficient individuals. At Day 97 the average Ca concentration in body cross-sections of Atlantic salmon parr with an average weight of 165 g was found to be within 2100-2200 mg $\times$ kg^{-1} for SPC35, SPC50 and SPC65 salmon parr and 1800 mg

$\times$ kg^{-1} for the SPC80 group of salmon. These values are lower than those described by Helland et al. (2006) for Ca deficiency in Atlantic salmon parr and could be pointing at a state of subclinical Ca deficiency in these fish. Normally the Ca:P ratio should be close to 1.0 in whole body, however similarly to the ratios reported by Helland et al. (2006) (~ 0.9 whole body Ca:P ratio for well mineralised Atlantic salmon parr) the credible values calculated in the current study at Day 63 presented a linear reduction with the expected Ca:P ratios ranging from 0.91 to 0.78 in salmon fed increasing levels of SPC. This is an indication of reduced calcification of the bony structures and therefore reduced bone mass in SPC80 salmon compared to the other groups, which could lead to an increased prevalence of vertebral deformities. Since Ca metabolism is closely related to that of P the above observation is more probably linked with the reduced availability of P from phytate which represents the main storage form of P in SPC. Moreover, the results of the analysis at Day 97 could be an indication of subclinical Ca deficiencies in all dietary groups of salmon parr since both Ca concentrations were below 2500 mg $\times$ kg^{-1} while Ca: P ratios ranged between 0.62-0.55, which is far below the levels reported before by Helland et al.(2005, 2006).

Shearer et al. (1994) reported that manganese (Mn) levels could range from 3.0 to 1.0 mg $\times$ kg^{-1} in the whole body of Atlantic salmon parr. Credible manganese concentrations in the present study were closer to the lower end of these values, either due to the larger size of the fish or the fact that body cross-sections instead of whole body samples (which contain the liver tissue; the main storage organ of Mn) were used for the determination of mineral levels. The linear reduction of this mineral with increasing SPC inclusion could be related to the combined effect of the increased dietary inclusion of phytate and other minerals such as iron (Fe), copper (Cu) and vanadium (V), which are found in higher quantities within SPC (Table 6 of Chapter 5) competing with Mn for the same binding sites for absorption (Andersen et al., 1996). Previously, Cheng et al. (2004) reported that supplementation with

500 FTU phytase $\times$ kg^{-1} of a soy-based diet, could improve Mn availability in rainbow trout, indicating that phytate could affect body concentration levels.

Whole body magnesium (Mg) concentration in well mineralised Atlantic salmon shown by Baeverfjord et al. (1998) was 426 mg $\times$ kg^{-1} while Shearer et al. (1994) reported similar whole body Mg levels for Atlantic salmon parr. On the other hand El-Mowafi and Maage (1998) found that whole body Mg concentrations in Atlantic salmon parr are around 300 mg $\times$ kg^{-1}, whereas Helland et al. (2006) documented values between 253 and 284 mg $\times$ kg^{-1}. The expected values obtained in the current study at Day 63 and Day 97 of the feeding trial were intermediate to those reported by El-Mowafi and Maage (1998) and Helland et al. (2006). Helland et al., (2006), reported that diets with high phytate levels can have a negative impact on whole body concentration of Ca, Mg, and the Ca: P ratio.

Expected Zn levels in the current study were lower than the whole body Zn values reported to be normal for Atlantic salmon parr (40-60 mg $\times$ kg^{-1}) by Shearer et al. (1994). Closer to the Zn values reported in the current study at Day 63 were the whole body Zn values described by Helland et al. (2006), which were between 21-33 mg $\times$ kg^{-1}. Helland et al. (2006) suggested that the reported Zn levels of 21-33 mg $\times$ kg^{-1} could be an indication of Zn deficient salmon. At Day 97 (34 dpv) Zn values were found to be lower than 15 mg $\times$ kg^{-1} for all dietary groups, indicating even higher Zn deficiencies (Helland et al., 2006). However, similarly to the current study no external signs of Zn deficiency including lens cataracts, fins and skin erosion, and short body dwarfism described in rainbow trout (Ogino and Yang, 1978; Satoh et al., 1983) were displayed in the Atlantic salmon parr fed increasing levels of phytate at both time points (Helland et al., 2006).

The reduction in expected pooled body cross-section ash, Ca: P ratio, 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 and could potentially point at a state of

sub-clinical Ca, P, Mg, Mn and Zn deficiency for salmon parr by the end of the study. Salmon parr in this study were reared under continuous light for the prevention of smoltification and fish maturation (Bromage et al., 2001; Stefansson et al., 2007; Stefansson et al., 2008). Stefansson et al. (2007; 2008) stated that Atlantic salmon parr reared under constant light photoperiod can develop into "pseudo-smolts" exhibiting all the external changes in appearance which characterise smoltifying salmon but not the physiological changes required for seawater adaptation. However, there is a lack of information regarding the way ion and osmo-regulation is affected in Atlantic salmon pseudo-smolts. It is thus possible that the reduced elemental levels at Day 97, could have been influenced by disturbances in the ion and osmo-regulation of the sampled individuals. Another possible hypothesis for the diminished elemental amounts obtained at this timepoint could be the enhancement of salmon growth through the application of constant light (Kråkenes et al., 1991; Fjelldal et al., 2005; Stefansson et al., 2008).

In the current study salmon parr with an initial weight of 29 g had achieved an average weight of 174 g in a period of 97 days, while salmon parr of an average 36.5 g used by Helland et al. (2006) achieved a weight of 100 g in a period of 80 days. In the current trial a daily WG of almost 1.5 $g \times day^{-1}$ was achieved in comparison to the overall 0.8 $g \times day^{-1}$ WG in the aforementioned study. Furthermore, it has also been reported that fast growing Atlantic salmon post-smolts after 6 months under continuous light (Fjelldal et al., 2006) demonstrated lower mineral content and mechanical strength in vertebral bones than slower growing salmon under natural light. Furthermore, it has been stated that during periods characterised by fast growth, the actual time required for bone matrix to be produced and mineralised could be reduced to a critical level (Hernandez et al., 2000), leading to under-mineralised and soft bony tissues. This was one of the proposed reasons 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).

Vaccination on the contrary, is known to delay growth due to stress induction, the reduction of circulating hormones promoting fish growth and the increased energy expenditure for immune induction (Pickering, 1993; van Muiswinkel and Wiegertjes, 1997; Wendelaar Bonga, 1997). It has also been demonstrated that i.p. injected oil-based vaccines can affect bone mineralisation depending on the experimental conditions (Berg et al., 2005, 2006, 2012). One hypothesis for the lower levels of ash and mineral seen at 34dpv is the combined effect of continuous light promoting fast growth in the salmon and the vaccination process promoting lower mineralisation of bone. Rungruangsak-Torrissen et al. (2009) reported that increased growth through increased lipid deposition by continuous light stimulation can be associated with reduced vertebral mineralisation and strength. Perhaps in order to retain higher mineralisation upon vaccination under constant light conditions, a diet with even higher available protein content should have been provided to the fish (Rungruangsak-Torrissen et al., 2009). This is crucial in terms of preventing deformities during the early growth period, as a high protein deposition is important for the normal growth and mineralisation of the skeleton (Rungruangsak-Torrissen et al., 2009).

Despite the large reduction in bone ash and minerals, revealing a subclinical state of mineral deficiency, most likely promoted by the interaction of fish vaccination and the application of continuous light, an improvement in these parameters was observed in vaccinated salmon maintained on diets with increased SPC levels. Fjelldal et al. (2010) reported that high inclusion levels of plant derived products could have a positive effect on bone health in fast growing Atlantic salmon post smolts by delaying salmon growth. This could be reflected in ash levels and explain the observed improvement in body cross-section

ash values of fish fed diets with more than 35% of dietary protein from SPC, which in general exhibited lower growth rates throughout the whole study period.

3.5 CONCLUSIONS

The present study, confirms the usefulness of SPC as a partial substitute for good quality FM in late stage salmon parr feeds, since the reduction in SPC80 salmon growth (mean weight and WG) was only 8% of that in SPC35 fed fish during the study, which was much less than the growth changes observed in salmon fed diets of different FM varieties (Anderson et al., 1997). Moreover, the reduction of P, Ca, Mg and Mn levels in body cross-sections due to the increasing dietary SPC inclusion, determined for the initial 63 day period seems to improve in the long term and upon vaccination. Whether this is an outcome of the long term provision of Atlantic salmon parr with high SPC inclusion in their diets or the interaction of the increased dietary SPC inclusion and fish vaccination, is still unknown.

Furthermore, it was shown that the exposure of Atlantic salmon to constant light as a means of delaying fish maturation and smoltification, promoting fast growth could compromise the elemental composition of the fish. Therefore, research on high FM replacement with plant products such as SPC, should also consider the conditions in which fish are exposed. Dietary levels of available P can influence the dietary uptake of many other important minerals including Ca and Zn which are integral for healthy bones. In the current trial it is clear that upon increased dietary SPC inclusion, the supplementation of P in salmon diets should be increased appropriately. This is pivotal for salmon normal growth and bone mineralisation. It is also possible that increasing dietary supplementation of Zn in salmon feeds with increasing SPC inclusion could improve growth performance, the uptake and body concentration of the specific mineral in Atlantic salmon parr. Furthermore, it was shown that the use of body cross-sections for elemental examination in Atlantic salmon,

gives similar patterns to the already reported whole body elemental concentrations. Therefore the use of body cross-sections could potentially be a very informative way for the evaluation of the mineral status of Atlantic salmon at various life stages.

Chapter 4.

The effects of increasing dietary levels of soy protein concentrate and constant dietary supplementation of phosphorus on the immune function of juvenile Atlantic salmon (Salmo salar L.)

4.1 INTRODUCTION

The culture of carnivorous piscine species such as Atlantic salmon (*Salmo salar* L) requires the production of compounded aqua-feeds that have traditionally been made with a high inclusion of fishmeal (FM) as the main source of protein and IAAs. However, the rapid growth of intensive aquaculture necessitates the use of alternative protein sources since there is a limited supply of FM. Besides its limited availability, FM is also a relatively expensive feedstuff for aqua-feed production. For this reason fish nutrition research has focused on alternative protein sources and plant-based ingredients, which are currently readily available (Kaushik, 1990; Tacon, 1994; Carter and Hauler, 2000). Among plant proteins, SBM is considered to be one of the most nutritious plant protein sources (Dersjant-Li, 2002). However, high amounts of antinutritional factors (ANFs), resulting in reduced nutrient digestibility, poor performance and hind gut enteritis, have limited its inclusion in Atlantic salmon aqua-feeds (Dersjant-Li, 2002).

On the other hand, in alcohol extracted SPC most of the ANFs are eliminated making this feedstuff an increasingly important protein alternative to FM (Peisker, 2001). Many studies have demonstrated the suitability of SPC as an alternative dietary protein source for Atlantic salmon post smolts (Refstie et al., 1998; Storebakken et al., 2000, 1998b) and the absence of soybean induced enteritis in salmonids receiving diets with even 100% substitution of FM with SPC (Escaffre et al., 2007; Krogdahl et al., 2000). However, while it has been shown that SPC is an excellent ingredient for practical feeds for many commercially important salmonid species and is widely used as a feed ingredient, further information is needed about the effects of SPC on Atlantic salmon health. To date only one published study by (Krogdahl et al., 2000) has investigated the effects of diets with 30% of dietary protein from SPC on the disease resistance and intestinal immunity of seawater

adapted Atlantic salmon. In this study increased lysozyme activity and total IgM levels were demonstrated in the intestinal mucosa of seawater adapted Atlantic salmon smolts maintained on feeds with 30% of dietary protein from soy products (SBM and SPC). Moreover, SPC-fed salmon demonstrated enhanced disease resistance when challenged with *Aeromonas salmonicida* compared to the groups fed a FM or a SBM diet. Nonetheless, a lack of information about the effects of increasing dietary levels of SPC on the general immune status of Atlantic salmon parr still exists. In addition, the majority of the reported studies observing the effects of diets with increasing dietary levels of vegetable proteins (VPs) on the immune responses of several farmed fish have focused on innate immune responses of fish that have not been stimulated immunologically. Thompson et al.(1996) proposed that measuring defence mechanisms prior to immune stimulation only represents resting levels. However, determining immune responses shortly or later after immune stimulation (via vaccination in the current study) may highlight dietary modifications that were not evident before. Vaccination is an integral prophylactic practice in large scale salmon culture, which usually takes place during the freshwater salmon parr stage, increasing salmon's resistance against several lethal bacterial diseases such as furunculosis (Poppe and Koppang, 2014).

The aim of the present trial was to monitor how increasing dietary SPC inclusion on the immunological responses of Atlantic salmon parr, prior to and post-vaccination (at 7 and 34 dpv) with a commercial anti-*A. salmonicida* vaccine.

4.2 MATERIALS AND METHODS

4.2.1 Diets and fish husbandry

The present study is the same to that presented in Chapter 3, giving however an insight on the immunological responses of Atlantic salmon pre-smolts fed on increasing amounts of

dietary levels SPC. Four diets were formulated so that SPC supplied 35, 50, 65 and 80 % of totaldietary protein (termed SPC35, SPC50, SPC65 and SPC80 respectively) with high quality FM providing the rest of the dietary protein in all cases. The diets were manufactured by EWOS Innovation, Dirdal, Norway and were formulated so that protein: fat ratios were constant, while lysine, methionine and threonine were supplemented to give the same AA: protein ratios among dietary treatments (Table 5 of the Chapter 3). The amount of supplemented dicalcium phosphate was constant between all four experimental diets at 30 g $\times$ kg^{-1} of feed, exceeding Atlantic salmon reported phosphorus requirements (Åsgard and Shearer, 1997; Lall, 2003) in order to mediate problems, which could be caused by increased amounts of phytate found in SPC (Storebakken et al., 1998b, 2000). The feeding trial, conducted at EWOS Innovation experimental facilities in Dirdal, Norway, was performed in sixteen circular tanks each containing 60 l of freshwater. Water temperature was maintained at 13 ± 1ºC, 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 unvaccinated Atlantic salmon parr (from a SalmoBreed strain selected for improved growth) of an average weight of 29 g (i.e. 120 fish/tank) were randomly allocated to the tanks. Fish were acclimatised to tanks for 28 days prior to the commencement of the trial, during which time they were provided a commercial EWOS freshwater diet for salmonids (EWOS micro). During the feeding study period quadruplicate tanks of fish were provided one of the 4 experimental diets. Fish were fed with automatic belt feeders, continuously every 435 seconds for 20 seconds. 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). It should be mentioned that as the level of SPC was increased in the diets of Atlantic salmon parr, the food ration was decreased, however direct feed intake through waste feed collection was not determined due to the difficulty in dividing uneaten feed of such a small size from faecal material.

Fish were weighed after being anaesthetized (metacaine, 30 mg $\times$ l^{-1}). The growth trial was conducted for a total of 97 days, at which time all fish were removed and weighed. On Day 37 all fish in the tanks were bulk weighed. Fish were also weighed on Day 63 (prior vaccination) before being intraperitoneally (i.p.) vaccinated with 100µl of a commercial vaccine (AquaVac™ Furovac, Intervet UK Ltd., Milton Keynes) against *A. salmonicida*, and on Day 97 (34 days post vaccination (dpv)). Fish were sampled for the evaluation of their immune responses on Days 63, 70 and 97 (termed pre vaccination, 7 and 34 dpv). Feed formulations and chemical analyses as well as growth performance, and pooled body cross-section proximate and elemental composition data from the same trial are reported in Chapter 3.

4.2.2 Sample collection

Blood was withdrawn from the caudal vein of 6 fish per tank per dietary group on Days 63, 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 $\times$ 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. These numbers were decided due to time and personnel limitations at the study site, since total leucocyte count observations and respiratory burst activity of HKMs had to be performed on the sampling day, in order not to

compromise the quality of the samples, undermining the validity of the observations. Measurement of the aforementioned blood parameters aimed the highest possible degree of information that could be extracted from the present study, using traditional immunological techniques. Three blood smears were prepared for each fish for the determination of differential leucocyte counts, which were air dried then 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 -70 ºC 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.

4.2.3 Head kidney macrophage isolation and respiratory burst by head kidney macrophages

For the isolation of head kidney macrophages (HKMs), the head kidney was teased through a 100-lm nylon mesh (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) into 2.5 ml Leibovitz medium (L-15; Sigma-Aldrich) containing 40 µl of heparin (10 IU × ml^{-1}). The mesh was rinsed with 2.5 ml of the medium and cells placed on ice. 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). A more detailed description about the methodologies applied is given in Section 2.4.1.

4.2.4 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). A more detailed description about the methodologies applied is given in Section 2.4.3 and 2.4.4.

4.2.5 Measurement of natural haemolytic activity

Plasma haemolytic activity used was based on a method described by (Langston et al., 2001) with modifications. Briefly defibrinated SRBCs (Oxoid, UK) were used as target cells at a final concentration of 2.5×10^8 cells $\times$ ml^{-1} of blood. Serum was diluted in double serial dilutions in 0.1 % gelatine-complement fixation buffer (0.1% G-CFB) (1 complement fixation tablet (Oxoid, UK) and 0.1 g of gelatine (Sigma-Aldrich) in 100 ml of warm distilled water) and 25 µl added to each well of a non-absorbent U-well micro-plate (Sterilin) in duplicate. Ten microlitres 0.5 % SRBC suspension was added to each serum dilution. Controls on each plate comprised 0.1 % anhydrous Na_2CO_3 (v/v) (100 % lysis) replacing serum. G-CFB replacing serum (0 % lysis) and serum blanks (duplicate wells of serum dilutions with G-CFB replacing SRBC suspension). The micro-titre plates were incubated at 22°C for 90 min with constant shaking and the reaction terminated by the addition of 140 µl G-CFB with 20 mM EDTA, followed by centrifugation to pellet the remaining SRBCs. After centrifugation 100 µl of the supernatant from each well was transferred to a new flat-

bottomed 96-well non-absorbent micro-titre plate (Sterilin). The absorbance of the wells was read at 450 nm using a micro-plate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA) and the percentage lysis of SRBCs calculated. More details about the estimation of plasma haemolytic activity are given in Section 2.4.5.10. Details regarding the preparation of SRBCs and the various buffers are presented in Sections2.4.5).

4.2.6 Total plasma Immunoglobulin M (IgM) assay

The level of IgM in sera of experimental fish was determined using indirect enzyme linked immunosorbent assay (ELISA) (Magnadottir and Gudmundsdottir, 1992), with modifications. A more detailed description of the methodology used is given in Section 2.4.7.

4.2.7 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). A more detailed description of the method used is given in Section 2.4.8.

4.2.8 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. The method is described in detail in Section 2.4.6.

4.2.9 Statistics

A hierarchical (multilevel) statistical model was used since multiple observations from a single tank were available (several individuals and/or several pools) (Espe et al., 2012; Nanton et al., 2012; Hartviksen et al., 2014). At first, data were normalised as described in Section 2.9. 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., 2014). The statistical approach applied was model-based. This means that to find if any specific effect was statistically significant data were fitted in three different models with increasing complexity where tank was included as random effect, sampling time as a categorical variable and the percentage of protein from SPC as a continuous factor possibly having non-linear effects:

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

The three models were nested and compared with a Likelihood ratio test (LRT) that evaluated if 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 in the majority of the cases. Adopted models are

demonstrated by plotting the expected mean response with 95% confidence intervals. The continuous effect of the increasing dietary SPC proportions on salmon immune responses are presented as curves 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).

4.3 RESULTS

The "raw data" for measured haematological and immune responses are shown in Table 7. Vaccination was found to affect all the health parameters measured post-vaccination. Haematocrit levels declined with increasing dietary proportions of SPC at all time-points (Modelled response is presented in Fig.16A). Significant differences were demonstrated between SPC80 compared to SPC35 salmon at 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 is much more noticeable at 7 dpv compared to the other sampling dates. Seven days after vaccination, haematocrit decreased significantly in the SPC50, SPC65 and SPC80 groups of salmon compared to the SPC35 control. At 34dpv despite the slight improvement in haematocrit values exhibited for the former groups of salmon, 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 two timepoints.

Table 7 Haematological and immunological responses of Atlantic salmon juveniles.

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

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
Total plasma protein (mg × ml^{-1} of plasma)	42.1±5.7	39.5±2.6	40.3±3.1	37.3±3.5
Antiprotease activity (Units TI_{75} × ml^{-1})**	653.3±98.6	677.9±138.2	647.1±98.0	667.8±97.6
Total plasma IgM (mg × ml^{-1} of plasma)	3.6±2.3	4.9±3.6	6.5±4.1	3.8±2.9
34 DAYS POST VACCINATION				
Haematocrit (%)	55.3±4.1	54.5±2.8	54.4±3.3	53.0±4.9
Leucocytes (× 10^7× ml^{-1})	8.7±1.5	8.6±1.5	9.4±2.9	9.3±2.9
Lymphocytes (×10^7× ml^{-1})	4.1±0.9	3.9±1.2	4.8±1.7	5.5±2.7
Thrombocytes (×10^7× ml^{-1})	4.2±1.1	4.5±1.2	4.8±2.2	3.8±1.2
Granulocytes (×10^7× ml^{-1})	0.4±0.2	0.2±0.1	0.3±0.2	0.2±0.1
Monocytes (×10^7× ml^{-1})	0.05±0.05	0.03±0.03	0.05±0.05	0.04±0.05
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
Haemolytic act. (units H_{50} × ml^{-1} of plasma)	1183.7±126.7	1344.6±242.2	1424.9±208.2	1072.4±126.9
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
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
Total plasma protein (mg × ml^{-1} of plasma)	46.4±7.7	8.6±1.5	9.4±2.9	9.3±2.9
Antiprotease activity (Units TI_{75} × ml^{-1})**	596.6±15.9	604.0±16.8	600.6±30.2	598.9±11.6
Total plasma IgM (mg × ml^{-1} of plasma)	3.6±3.8	6.4±2.6	5.6±2.8	2.9±2.2
Specific IgM (plasma titers)	0.02±0.01	0.02±0.01	0.02±0.01	0.02±0.01

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} × min^{-1} × ml^{-1} of plasma.

The same trend as in haematocrit was obtained for leucocyte numbers for the first two 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. 16B). No significant differences in total leucocyte levels were recorded among the three dietary groups of salmon at this timepoint. 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 numbers 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 lymphocytes presented a slight reduction. At this point significantly lower lymphocyte counts were demonstrated 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. 17A). Nonetheless, no differences on lymphocyte levels were recorded among the three dietary groups at 34dpv. On the other hand, thrombocytes were affected curvilinearly in salmon fed increasing amounts of SPC with SPC50 and SPC65 salmon demonstrating the highest levels of thrombocytes and the SPC35 group having slightly higher thrombocyte numbers than SPC80 salmon, at all time- points (Modelled response is presented in Fig.

17B). However, no significant differences were revealed prior to and 7 days post vaccination. Overall, thrombocytes increased upon vaccination with levels peaking at 34 dpv. At this point significantly higher amounts of thrombocytes were revealed for the SPC50 and SPC65 groups compared to SPC35 salmon. Increasing dietary SPC inclusion in the diets of Atlantic salmon parr had no significant effect circulating granulocyte and monocyte levels. Neutrophilic granulocytes increased after vaccination with the highest levels obtained 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.

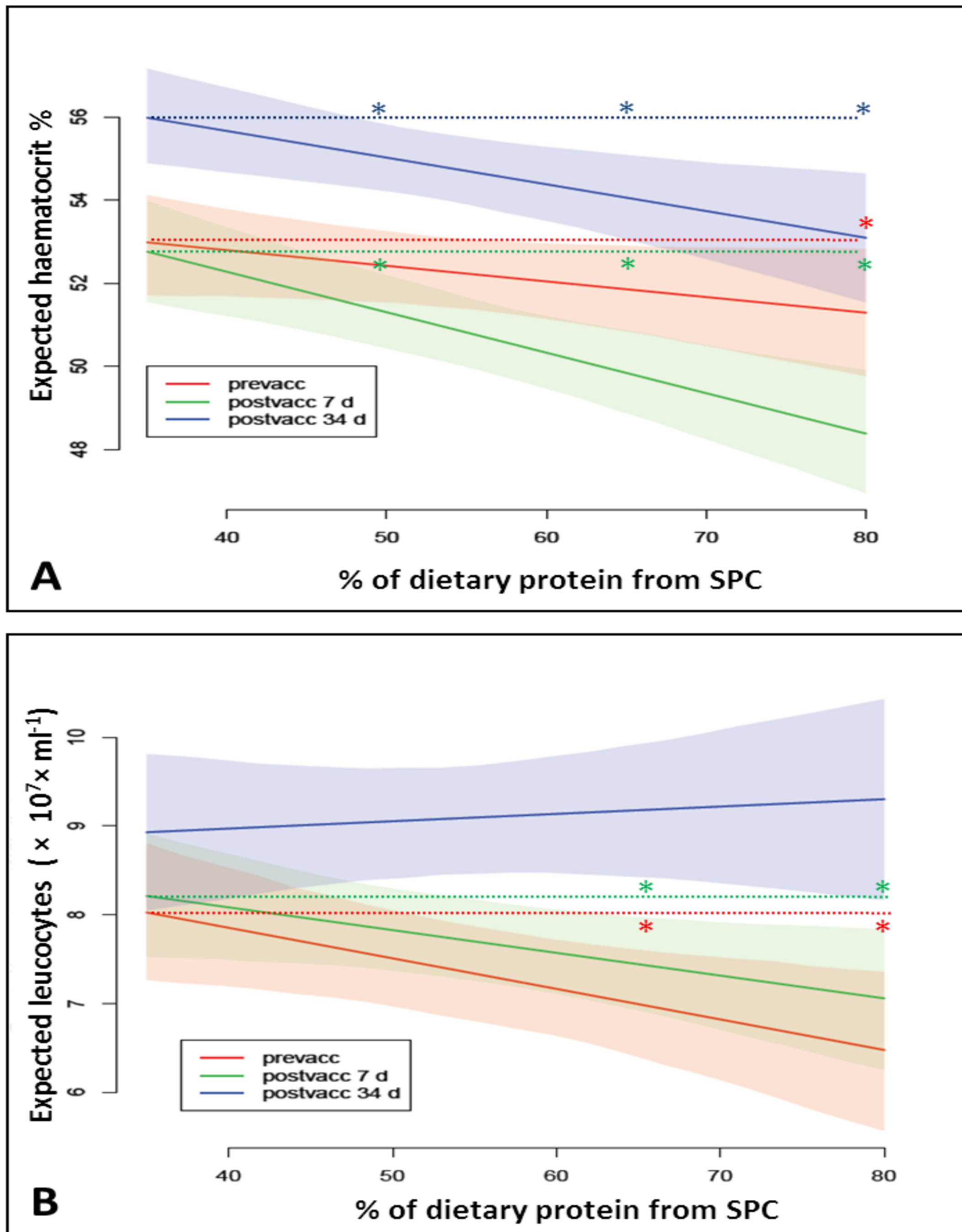

Figure 14 Modelled haematological responses (haematocrit and total leucocyte counts). Statistical models showing the linear (Models selected with P values < 0.05) effect of the percentage of dietary protein from SPC on: (A) haematocrit (%); (B) leucocytes ($\times 10^7 \times ml^{-1}$). Red, green and 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 the values of these measurements. 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.

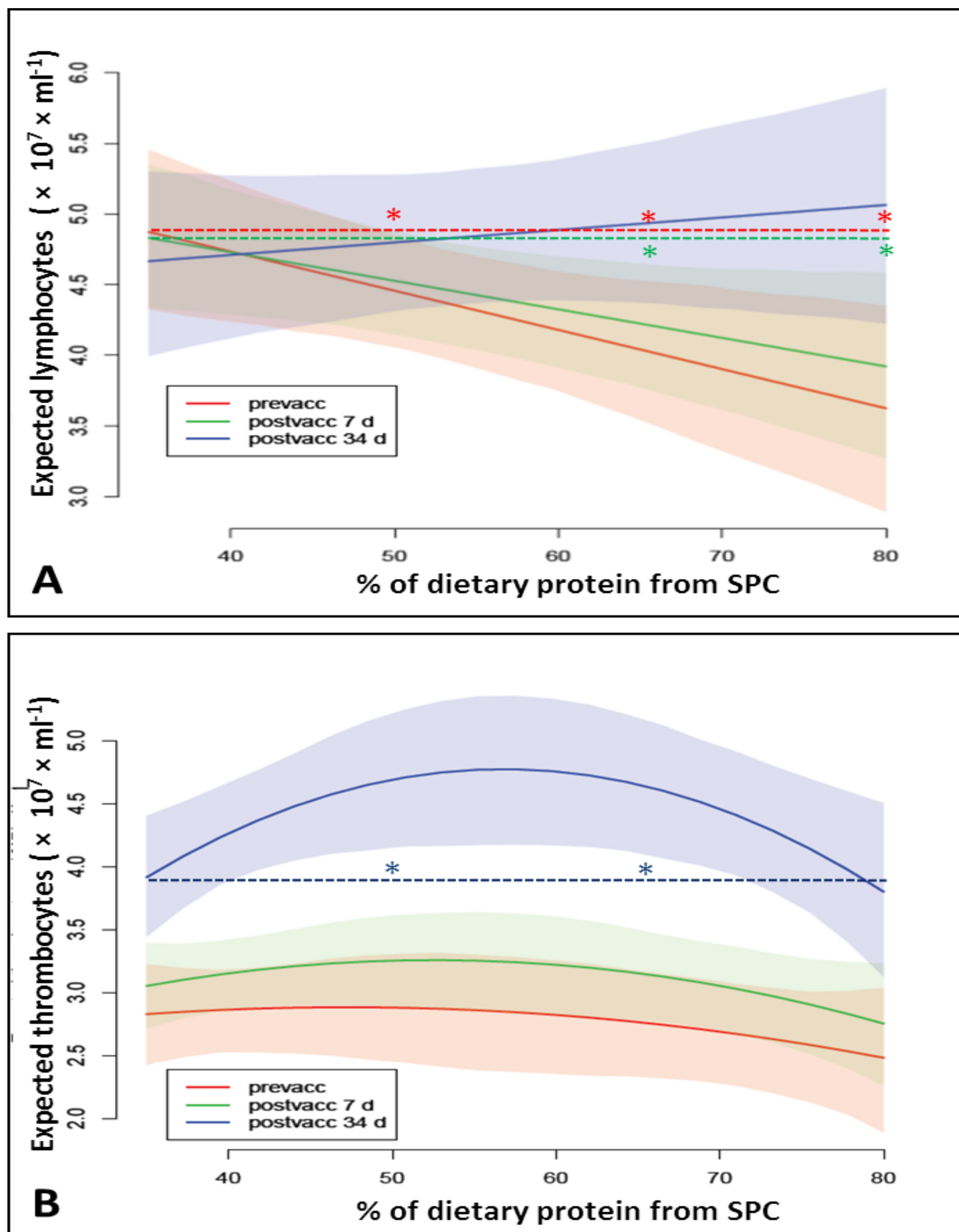

Figure 15 Modelled haematological responses (lymphocytes and thrombocyte counts). Statistical models showing the linear and curvilinear (Models selected with *P* values < 0.05) effect of the percentage of dietary protein from SPC on: (A) lymphocytes ($\times 10^7 \times ml^{-1}$); (B) thrombocytes ($\times 10^7 \times ml^{-1}$). 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 the values of these measurements. 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.

Statistical models revealed no effect of increasing dietary SPC inclusion on the respiratory burst activity of both PMA-stimulated and non-stimulated HKMs (the model with the time effect was selected with a $P < 0.05$). Head kidney macrophages in general demonstrated an increased respiratory burst 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 (the model with the time effect was selected with a $P < 0.05$). 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 periodcompared 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 haemolytic and lysozyme activity and total plasma IgM levels (Modelled responses are presented in Fig.18, 19A and 19B). 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 lower than prior to vaccination. Moreover, significantly higher activity was demonstrated in SPC50 and SPC65 salmon compared to the SPC35 group (Modelled response is shown in Fig. 18). Expected plasma IgM levels prior to vaccination demonstrated no differences among the four dietary groups of salmon. At both post vaccination points (7dpv, 34dpv), total plasma IgM concentrations were found to be significantly higher for SPC50 and SPC65 salmon compared to SPC35 salmon while similar levels were demonstrated for SPC35 and SPC80 salmon (Modelled response is illustrated in Fig.19A). Expected haemolytic activity demonstrated minor differences among the four experimental dietary groups of salmon prior to and at 7 dpv. However, at 34 dpv alternative complement activity presented a salient increase for 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 to SPC35 salmon at 34dpv (Modelled response is shown in Fig.19B).

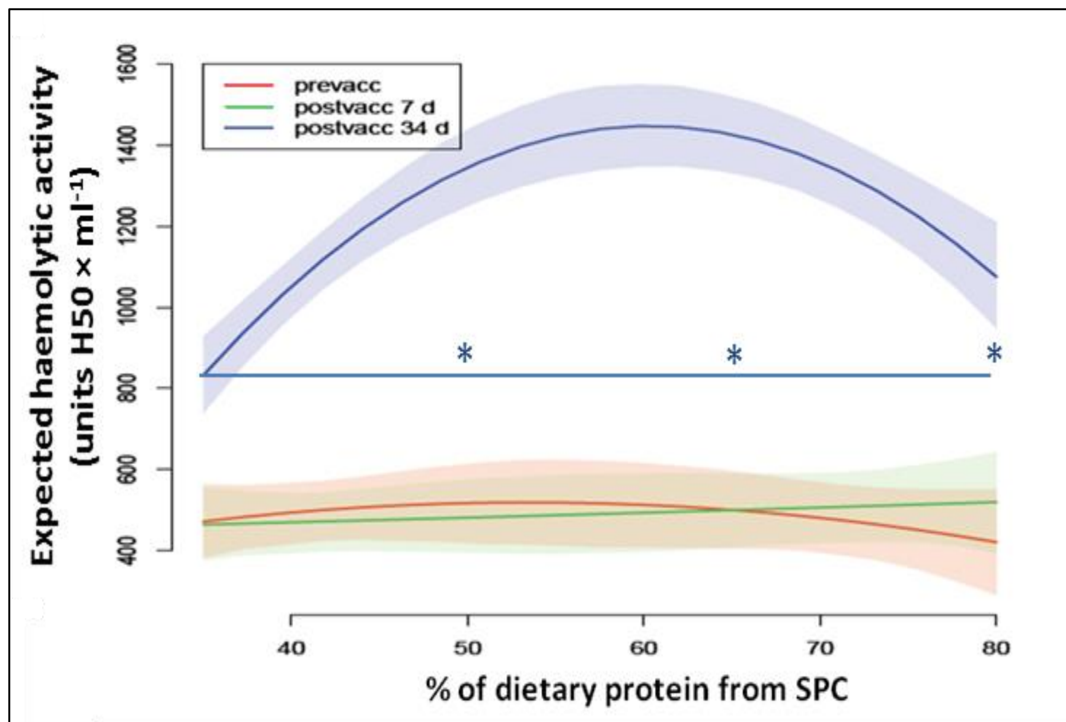

Figure 16 Modelled plasma haemolytic activity. Expected haemolytic activity (Units $H_{50} \times ml^{-1}$). The model for haemolytic activity was selected with a *P* value< 0.05. Red, green and blue lines connect the expected mean values for each measurement prior to vaccination –prevacc-(Day 63 post 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 the values of these measurements. 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.

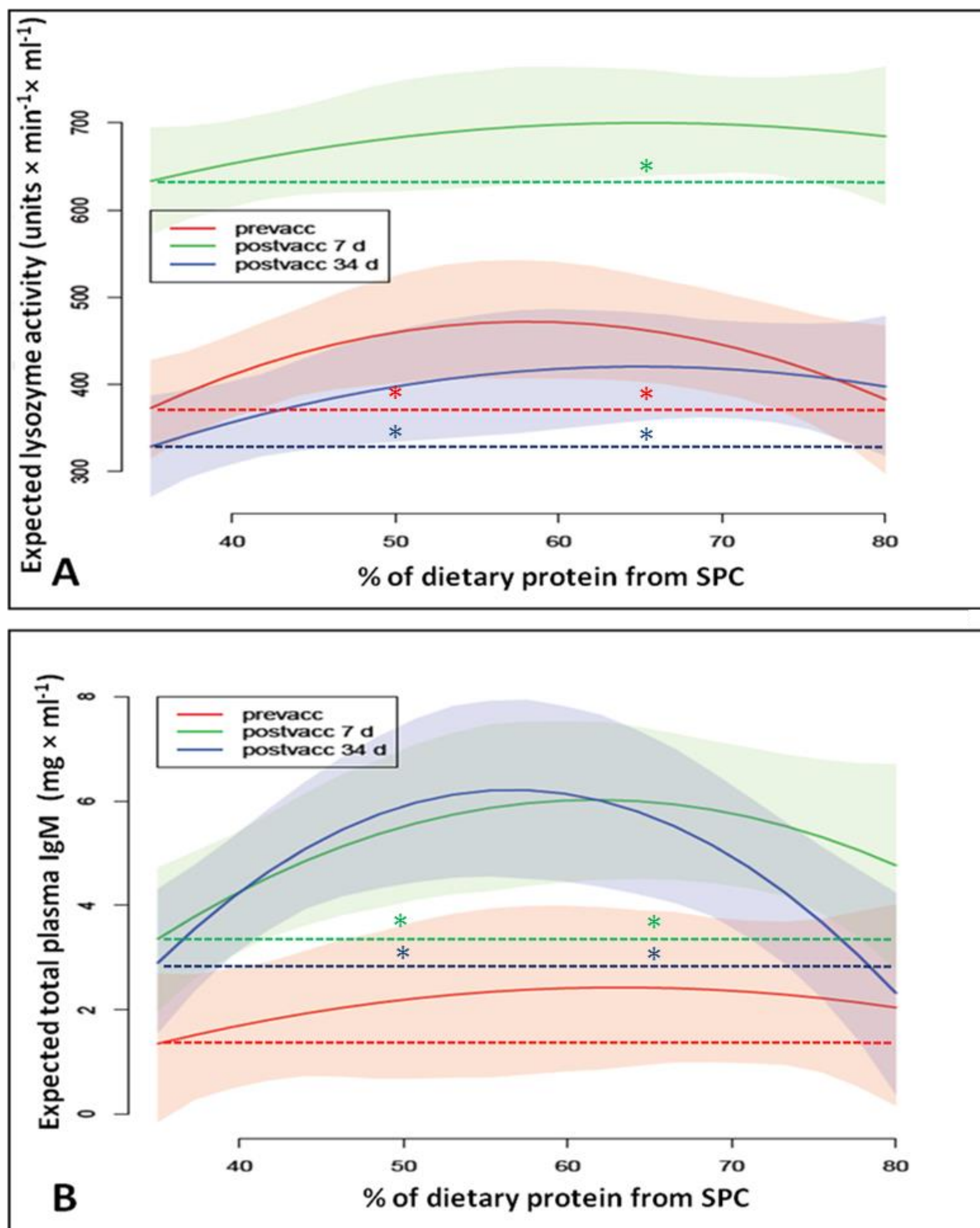

Figure 17 Modelled immunological responses. Statistical models showing significant linear or curvilinear (Models selected with P values < 0.05) effect of the percentage of protein from SPC on: (A) lysozyme activity (Units × min^{-1} × ml^{-1}); (B) total plasma IgM (mg × ml^{-1}). Red, green and blue 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 the values of these measurements. 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.

4.4 DISCUSSION

The immunological responses examined in the current study are considered essential components of salmon defence against pathogens, while the measured haematological indices can give an indication of the nutritional, immune or stress status of the fish. Changes in various blood parameters such as haematocrit, total serum protein, total leucocyte and differential leucocyte counts can be used to assess fish condition and health (Blaxhall, 1972; Waagbø et al., 1988). Regarding the estimated immune parameters, anti-protease activity is related to the inactivation of extracellular proteases released by invading pathogenic organisms (Ellis, 1990), lysozyme and phagocytes oxygen radical production are important bactericidal and parasiticidal agents (Rumsey et al., 1994; Secombes, 1990), whereas complement factors, lectins (participating in natural haemolysis), total and specific IgM are involved in the neutralization and opsonisation of a wide range of pathogenic agents (Nikoskelainen et al., 2002; Kiron, 2012).

For Atlantic salmon parr, haematocrit values, which represent the oxygen carrying capacity of blood, normally range from 35 to 60 % (Hardie et al., 1990; Thompson et al., 1996) and haematocrit levels were found to be within this range in the present study. However, expected mean haematocrit values, declined with increasing levels of SPC inclusion. Blaxhall (1972) reported that reduction in haematocrit values could be an indicatio of reduced feed intake, for which data were not collected. Furthermore the reduction in erythrocyte size, reported by Hemre et al. (2005) in Atlantic salmon fed on diets with increased FM substitution by soybean products, could explain the aforementioned observation. Another possible explanation could be the dilution of blood due to osmoregulatory changes caused by a reduction in Ca and Mg body levels as it was demonstrated in the previous chapter and possibly their circulating amounts, which was also

shown previously by Klinger et al.(1996) in channel catfish (*Ictalurus punctatus*). Ion and osmoregulatory changes could have also been promoted by the exposure of salmon parr to constant light promoting a state of "pseudo-smolts" for which not much information is still available (Stefansson et al., 2007). The lowest haematocrit values for the four dietary salmon groups were obtained at 7dpv. This could be attributed to increased stress induction arising from vaccination which could have reduced fish appetite, nutrient uptake and thus erythropoiesis (van Muiswinkel and Wiegertjes, 1997; Wendelaar Bonga, 1997; Melingen and Wergeland, 2000; Poppe and Koppang, 2014). This can also explain the observed reduction in plasma protein levels in all dietary salmon groups at 7 dpv. However, no differences were observed in plasma protein levels among different dietary groups.

Increased leucocyte levels due to immune stimulation are expected after vaccination (Nikoskelainen et al., 2007; Silva et al., 2009), which was the case in the present study. Similarly to haematocrit values, total leucocyte counts declined with increasing dietary SPC prior to and 7 dpv. Contrary to this, Rumsey et al. (1994) showed that naïve rainbow trout fed on diets containing soy proteins exhibited increased numbers of circulating leucocytes. The increase in leucocyte numbers at 34dpv in salmon fed diets with higher than 35% of dietary protein from SPC is an indication of immune and/or haemostatic stimulation due to vaccination (Nikoskelainen et al., 2007; Silva et al., 2009), since both lymphocyte and thrombocyte levels demonstrated an increase in the medium and high SPC inclusion groups. The former type of leucocytes presented a slight increase in salmon fed high levels of SPC compared to the SPC35 group, a week after vaccination. Nonetheless, the magnitude of this increase was even higher at 34 dpv, revealing a trend of linear increase in vaccinated salmon receiving increased dietary SPC at this point, however, in the absence of significant changes among the four treatment groups. Overall, the state of leucocyto- and lymphocytopenia prior

to vaccination in the high SPC inclusion groups could be an indication of increased stress (Maule and Schreck, 1990; Ainsworth et al., 1991) due to poor nutrition caused by the increased dietary levels of ANFs present in SPC (Fletcher, 2011). In this case measurement of cortisol activity from the various sampling points of the present trial could have been useful in order to assess that. Another possible reason for that is the fact that naive salmon fed on diets with moderate to high SPC inclusion levels exhibited a general suppression of various pathways including the ones involved in systemic immunity and possibly haematology, utilizing most of the dietary energy on growth (Tacchi et al., 2012). This trend however, was reversed upon immune stimulation through vaccination, with salmon fed on high SPC inclusion diets investing more energy for the stimulation of the immune responses. On the contrary to circulatory lymphocytres, thrombocytes were affected curvilinearly by the increased dietary SPC amounts, with SPC50 and SPC65 salmon possessing higher levels than the other two experimental groups. Overall, thrombocytes increased post-vaccination with the highest levels being measured at 34 dpv, when SPC50 and SPC65 salmon presented higher levels compared to the SPC35 group. Thrombocytes are mainly involved in haemostasis regulation, although they have been associated with cellular immunity (Meseguer et al., 2002). Thrombocyte levels were found to be well correlated with complement activity in the present study. Complement cascade and blood coagulation systems (which participate in wound healing in association with thrombocytes) are intimately related due to the presence of enzymes that are involved in both pathways (Amara et al., 2008). The exact relation between these two observations requires further investigation and could have been influenced by differences in ion absorption and regulation between the dietary groups of salmon, especially for Ca and Mg which are known cofactors for many of the enzymes in both cascades (Yano et al., 1988). Nonetheless, circulating neutrophilic

granulocytes, which represent the main innate immune cells determining salmon resistance against bacterial diseases (Øverland et al., 2009; Kumar et al., 2010), increased at 7 dpv. Neutrophils demonstrated a subsequent reduction at 34 dpv, however their numbers were still higher than pre-vaccination levels, exhibiting an extended stimulation of salmon immunity due to vaccination. Monocytes connect the innate immune system with the adaptive system by migrating to the sites of infection and differentiate into macrophages, where they partake in phagocytosis, antigen presentation and the production of cytokines that stimulate lymphocyte activity. These cells demonstrated numerical increases after vaccination, with the highest values obtained at 34 dpv, revealing their importance in both innate and acquired immune responses (Nikoskelainen et al., 2007). The increase in granulocyte and monocyte levels at post-vaccination in the current study were similar to earlier reports by Chin and Woo (2005) and Nikoskelainen et al. (2007), who demonstrated significant increases in circulating granulocyte and monocyte numbers in salmonids vaccinated against parasites or several different bacterial pathogens. The extent of change observed in these leucocyte populations at post-vaccination was the same in all dietary groups, indicating that increasing SPC levels did not compromise the production of these immunologically important cells.

Respiratory burst of both PMA-stimulated and naive HKMs did not appear to be affected by the percentage of SPC protein added to the diets. In accordance to that, neutrophil oxygen radical production has been shown to be unaffected in Atlantic salmon fed on dehulled lupin meal at 40% inclusion, compared to FM protein feeds Bransden et al. (2001a), and Burrells et al.(1999) found that dehulled solvent extracted SBM at inclusion levels of 10-50 % in rainbow trout diets, had no significant effect on macrophage oxidative radical formation, whereas inclusion levels of up to 80% caused a reduction in macrophage

respiratory burst activity. On the contrary, Sitjà-Sitja-Bobadilla et al. (2005) reported higher respiratory burst levels by HK leucocytes in juvenile sea bream fed diets with 75 and 100 % substitution of FM by a mixture of different plant protein sources (i.e. corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) balanced with essential amino acids. Moreover, rainbow trout fed on diets with different soy proteins, demonstrated increased peripheral leucocytic respiratory burst activity (Rumsey et al., 1994).

Plasma protein levels decreased upon vaccination. This could be attributed to the reduction in fish appetite after vaccination as a result of stress due to the handling and the injection procedure (Melingen and Wergeland, 2000). However, no differences were observed in plasma protein levels among different dietary groups. Similarly, no differences in anti-protease activity were exhibited between the groups. Anti-protease levels usually reduction in fish after vaccination, infection or intra-peritoneal injection (Secombes and Olivier, 1997), which agrees with the findings of the present study. Bransden et al. (2001) showed that Atlantic salmon could be fed on diets with 40% substitution of FM with dehulled lupin meal without any adverse effects on anti-protease activity. They also found, plasma lysozyme activity and total IgM levels to be unaffected, which is similar to Jalili et al.(2013), who reported no differences in serum lysozyme and total IgM in rainbow trout receiving diets with up to 100% substitution of FM with plant proteins. This is not in line with the current results, where higher lysozyme activity was recorded for SPC50 and SPC65 salmon at all time points 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. The increase in total IgM levels, obtained as a result of salmon vaccination at 7 and 34 dpv is expected (Melingen and Wergeland, 2000). Regardless, the increased levels of total IgM and

lysozyme activity observed for the SPC50 and SPC65 groups of salmon could point at better regulation of certain innate immune responses of salmon at post vaccination which could be a favourable trade upon an unexpected disease outbreak. Specific antibody titres against *A. salmonicida* were determined at 34 dpv and there appeared to be no effects of SPC inclusion levels on specific antibodies. Kiron et al.(1995; 1993) demonstrated that specific antibody production is not affected by the level of dietary protein in rainbow trout. Hence, it is possible that differences in protein intake or protein digestibility, affected by the presence of ANFs present in the diet, do not affect specific antibody production in vaccinated salmon

Haemolytic activity in the plasma of experimental fish, as a consequence of complement activation (mainly through the alternative and partially the classical pathway as a result of the presence of natural antibodies) and/or other substances such as lectins and haemolysins (Alexander and Ingram, 1992) did not seem to be affected linearly by increasing dietary SPC inclusion. Prior to and 7 dpv, salmon from the different dietary groups exhibited no differences in plasma haemolytic activity (overall complement activity). However at 34 dpv, there was a marked increase of haemolytic activity in fish from all the dietary treatments, with salmon fed diets with higher than 35% of protein from SPC demonstrating significantly higher activity than SPC35 salmon. Stimulation of alternative complement activity as a result of altering the dietary composition through the inclusion of plant proteins, has been previously reported by Jalili et al. (2013) and Sitjà-Bobadilla et al. (2005), who found similar or increased complement activity in rainbow trout and gilthead sea bream respectively, in fish fed 50% plant protein diets, while decreased activity was observed in fish fed on diets with higher levels of plant proteins. The present findings, clearly demonstrate an increase in the overall complement activity of salmon fed on the SPC50 and SPC65 dietary treatments.

4.5 CONCLUSIONS

The current study demonstrated that feeding juvenile Atlantic salmon on diets with up to 65% of protein from SPC, appropriate dietary supplementation with lysine, methionine and threonine and a constant supply of P at 30 g $\times$ kg^{-1}, could enhance some of the non-specific immune responses prior to and post-immunisation. Therefore, it is possible that juvenile salmon diets with such specifications could be applied without undermining the health status of the fish. On the contrary, SPC seems to contain certain amounts of immunogenic compounds which seem to exert their beneficial health effects only at the aforementioned level of dietary SPC supplementation (Krogdahl et al., 2000). Higher SPC inclusions in salmon diets are possibly resulting in additional dietary limitations, revealing the necessity for further nutritional supplementation of such diets. An alternative hypothesis could be that the immunological changes among the dietary groups of salmon could have been influenced by differences in the availability of P in the four dietary treatments. Despite the fact that inorganic phosphates in the dietary treatments were supplemented at levels higher than the known P requirements of Atlantic salmon according to NRC (1993), the availability of P in the diets can be reduced, especially in diets with very high SPC inclusion due to the increased presence of phytate found in SPC. Hence, it is possible that diets with intermediate levels of SPC (SPC50 and SPC65) were more optimally balanced in terms of available dietary P, compared to the other two treatments (SPC35 and SPC80), suggesting the requirement for lower supplementation of P (< 30 g $\times$ kg^{-1}) in the SPC35 diet and higher addition of P in the SPC80 treatment (> 30 g $\times$ kg^{-1}).

The involvement of P in the immune mechanisms of the fish is still not clear. However, P could affect fish immunity either directly through supplying the energy required for immunological stimulation (Kiron, 2012), or indirectly by influencing the digestion of other

dietary elements including Zn, Mg or Ca (Lall, 2003), modulating several important immune responses including the complement activity (Yano et al., 1987, 1988; Lim et al., 2001). Whether the level of immune stimulation provided by these diets would provide higher resistance to infectious disease is still unclear. It is more likely that a combined dietary increment of SPC levels with a concomitant increased addition of P and possibly other elements (e.g. Zn) or compounds in which SPC is deficient of, could promote better growth and immunity in Atlantic salmon parr. Nevertheless, the impact of increasing dietary SPC inclusion on the immune status of Atlantic salmon parr requires further investigation and the best way to validate the results derived from this study is through the performance of disease challenges.

Chapter 5.

Increased bone mineralisation in vaccinated and non-vaccinated early stage Atlantic salmon parr fed on increasing dietary levels of soy protein concentrate and phosphates (*Salmo salar* L.)

5.1 INTRODUCTION

Farmed salmon are bred for rapid growth, and the main aim of the salmon aquaculture industry is to achieve an optimal growth rate in their stock through optimization of both diets and environmental conditions accordingly. However, intensive rearing conditions are linked with an increased occurrence of skeletal-related pathologies (Silverstone and Hammell, 2002; Fjeldall et al., 2004, 2005, 2006). The growing need to replace FM in commercial fish feeds has also come into focus, together with the deformities related to feed ingredient replacements and unbalanced macro-nutrient supplementation leading to low nutrient availability (Baeverfjord et al., 1998; Storebakken et al., 1998b, 2000). Suboptimal supply of minerals (phosphorus and potentially zinc) and nutritional imbalances of fatty acids, vitamins and amino acids are considered the main challenges in regard to normal skeletal development (Lall and Lewis-McCrea, 2007). The challenges related to nutrient bioavailability are further amplified with the introduction of vegetable meals, some of which are rich in ANFs (e.g. phytic acid and polysaccharides) that may further impair the absorption of these nutrients (Storebakken et al., 1998b, 2000).

SPC, made from SBM through aqueous ethanol extraction, contain a reduced carbohydrate fraction and typically have lower concentrations in ANFs related to intestinal enteritis (Dersjant-Li, 2002). Most of the nutritional characteristics that a good plant-based alternative protein source needs to replace high quality FM in salmon diets can be met using SPC (Dersjant-Li, 2002; Naylor et al., 2009). SPC contains high levels of digestible protein, with a favourable amino acid profile (with the exception of lysine and methionine), high nutrient digestibility, and is reasonably acceptable by the fish. Hart and Brown (2007) showed that up to 50% of FM can be replaced with SPC (with supplemented methionine) in Atlantic salmon fingerling diets without adversely affecting growth performance. However,

the presence of several ANFs and the lack of certain bioactive compounds found in FM still limit the use of SPC in diets for early stage Atlantic salmon parr that have an undeveloped digestive system (Burr et al., 2012). High dietary levels of SPC have been linked with decreased feed intake and reduced growth in salmonids (Storebakken et al., 1998b; Mambrini et al., 1999), whereas phytate the main ANFs present in SPC were found to reduce intestinal enzyme activity, and protein and mineral bioavailability, with decreased levels of Ca, Mg and Ca:P ratio detected in the body of the fish, together with decreased skeletal Zn concentrations (Denstadli et al., 2006; Helland et al., 2006). Measurements of body and skeletal mineral composition are sensitive indicators of both mineralisation and health, which in turn, can reveal dietary deficiencies (Shearer et al., 1994; Helland et al., 2005, 2006). Low bone mineralisation in on-growing and apparently healthy salmon can be an indicator of subclinical deficiency for a range of elements, which in the long term could increase the prevalence of bone deformities (Fjelldal et al., 2007, 2009a, 2009b, 2012) or even worst, cause increased mortalities due to negative elemental balance (Baeverfjord et al., 1998).

Intraperitoneal injection of oil adjuvanted vaccines is a common prophylactic practice in salmon aquaculture. Some studies have shown that vaccination can induce vertebral deformities in Atlantic salmon, visible by the time of harvest (Berg et al., 2005, 2006). Moreover, Berg et al. (2012) demonstrated a correlation in the increased occurrence of bone deformities with reduced bone mineralisation. Contrarily, other studies have demonstrated increased bone mineralisation in vaccinated salmon under certain post-vaccination temperature regimes, such as 10˚C for 6 weeks in freshwater followed by 16˚C for 6 weeks in saltwater or 16˚C for 6 weeks in freshwater followed by 16˚C for 6 weeks in saltwater (Grini et al., 2011), while Berg et al. (2005) reported similar findings with increased levels

of bone mineralisation in salmon after vaccination. Additional studies have found no signs of vertebral deformities in Atlantic salmon post-vaccination, however (Gil Martens et al., 2010; Grini et al., 2011). It is apparent that the effect of vaccination on bone depends on vaccination strategy (Berg et al., 2006; Grini et al., 2011), especially associated with the size of fish during vaccination (Berg et al., 2006; Grini et al., 2011), the type of vaccine applied (Aunsmo et al., 2008) and environmental parameters (Grini et al., 2011). It would seem that low mineralisation as a result of vaccination, may contribute to an increased frequency of spinal deformities in smoltifying Atlantic salmon, especially during the early period of seawater transfer (Fjelldal et al., 2006). Salmon with bone mineral deficiencies are prone to developing spinal deformities (Fjelldal et al., 2007, 2012), do not swim efficiently, are less capable of acquiring food and are more susceptible to physiological imbalances, as well as being down-graded at slaughter (Silverstone and Hammell, 2002). In the study performed in Chapter 3, a decrease in body cross-section ash, Ca: P ratio, Ca, Mg, Mn, P and Zn levels were found in late stage Atlantic salmon parr when the fish were fed for 63 days with diets containing increasing levels of SPC and balanced levels of lysine and methionine, but constant amounts of supplemental di-calcium phosphate. It was suggested that this might have been a result of reduced bio-availability of P in the diets containing increased amounts of SPC, due to the presence of certain ANFs, such as phytate, oligosaccharides and non-starch polysaccharides, present in SPC (Francis et al., 2000). However, at 32 days post-vaccination (dpv), using a commercial vaccine (i.e. 95 days from the onset of feeding), both ash and mineral levels except Zn, presented an overall improvement in bone mineralisation of salmon fed increasing levels of dietary SPC, revealing a positive effect of vaccination on bone mineralisation in these dietary groups.

Meanwhile, the aqua-feed industry still relies on the application of inorganic salts to improve mineral retention in fish diets containing increased amounts of plant products. For this reason, the present study was undertaken to identify the effects of increasing dietary levels of SPC, lysine, methionine and mono-calcium phosphate in the diets of salmon parr and the interaction these diets have with vaccination on salmon parr growth and bone mineral levels, which are indicative of bone mineralisation in dietary fish. Skeletal mineral analysis was used as a tool to assess if there are any additional mineral requirements for Atlantic salmon parr fed with these SPC diets.

5.2 MATERIALS AND METHODS

5.2.1 Diets and growth trial

A dietary trial was conducted for 154 days (June 2013-December 2013) in the Aquatic Research Facility (ARF) at the Institute of Aquaculture, University of Stirling. A fresh water system consisting of twenty four 100-litre circular tanks supplied with flow-through water at a rate of $1.5 \text{ l} \times \text{min}^{-1}$ was used to hold the fish. The water temperature was maintained at an ambient temperature, whilst photoperiod was constant to prevent smoltification (12 hours of light: 12 hours of darkness). Dissolved oxygen, ammonia, nitrate, nitrite and pH were monitored and remained within limits recommended for Atlantic salmon. Before the start of the feeding trial, unvaccinated Atlantic salmon parr (S0) purchased from Scottish Seafarms Ltd (Hollywood, Dumfries) from AquaGen QTL (AquaGen Ltd, Kilmacolm, Scotland) eggs, selected for improved growth and resistance to Infectious Pancreatic Necrosis Virus (IPNV), previously maintained on a commercial BioMar (BioMar Ltd, Grangemouth, Scotland) diet (BioMar Inicio PLUS) were allowed to acclimate for one week in two stock tanks. During this period fish were maintained on a commercial EWOS (EWOS Ltd, Westfield, Near Bathgate, Scotland) diet (EWOS micro). One hundred and thirty fish were

then randomly allocated into each of the 12 tanks and allowed to acclimate for 7 days. At the beginning of the trial, the average weight of fish was 9.3g. The fish were starved for two days and then subsequently fed the trial diets they had been assigned. The experimental diets used contained different levels of SPC protein (35, 58 and 80% of protein from SPC). The diets were manufactured by EWOS Innovation, Dirdal, Norway and were formulated in a way to ensure that any observed differences among treatments would only be attributed to the protein source. Feed formulations are given in Table 8. Therefore protein/fat ratios were kept constant (~3.0) while methionine, lysine and phosphorus (P) supplementation increased concomitantly with increased dietary SPC inclusion to give similar amino acid/protein ratios and increase the availability of P in diets with higher levels of SPC. Four replicate tanks were used for each one of the 3 trial diets, and parr were acclimatised to the feeds for 3 days prior to the starting the trial. The feeding trial lasted for almost 6 months (154 days). On day 92 of the feeding trial, 8 fish per tank were sampled to monitor their immune status and vertebral mineral composition. The remainder of the parr (77 fish per tank) was divided between the original set of 12 tanks and a further set of 12 replicate tanks. The fish in the original tanks were intraperitoneally (i.p.) injected with 0.1ml of a commercial *A. salmonicida* /Infectious Pancreatic Necrosis Virus (IPNV) vaccine (Pharmaq, Alphaject 2.2), while the salmon within the replicate set of tanks were i.p. injected with 0.02 M phosphate buffer saline (PBS) (0.15M NaCl, pH = 7.2). Whole fish samples were taken prior to vaccination/PBS-injection (Day 92 of the feeding trial) and at 62 dpv/dpPBSinj (Day 154 of the feeding trial) for vertebral composition analysis. Fish were bulk weighed on Day 92 (prior to injection) and Day 154 of the feeding trial (62 dpv/PBS injection) to monitor their growth performance (Final weight, weight gain and specific growth rate).

Table 8 Formulation and chemical composition of experimental diets with varying soy protein concentrate levels.

Feed composition (× kg^{-1})	SPC35	SPC58	SPC80	SPC35	SPC58	SPC80
		2mm			3mm	
Fishmeal [a] (g)	449.2	269.8	114.6	449.2	269.8	114.6
SPC [b] (g)	288.3	453.8	598.6	288.3	453.8	598.6
Tapioca [c] (g)	110.0	100.0	90.0	110.0	100.0	90.0
MonoCalcium phosphate [d] (g)	20.0	30.0	40.0	20.0	30.0	40.0
Vitamin and mineral premixes[e] (g)	4.00	4.00	4.00	4.00	4.00	4.00
Vitamin C 35% [e] (g)	1.00	1.00	1.00	1.00	1.00	1.00
Methionine [f] (g)	2.10	4.00	5.50	2.10	4.00	5.50
Lysine 78% [f] (g)	1.80	2.60	3.30	1.80	2.60	3.30
L-Threonine [f] (g)	0.6	0.8	1.0	0.6	0.8	1.0
Nobacithin Powder [g] (g)	10.00	10.00	10.00	10.00	10.00	10.00
Fish Oil [h](g)	113.0	124.0	132.0	113.0	124.0	132.0
Chemical composition (× kg^{-1})						
Dry matter (g)	959.7	944.1	923.0	923.2	936.1	922.5
In dry matter basis						
Energy (KJ)	222.4	218.9	216.3	223.8	218.8	217.1
Crude protein (g)	531.2	515.2	494.1	532.4	511.8	489.8
Crude fat (g)	182.4	170.6	162.0	177.7	165.4	1621
Crude prt: Crude fat ratio	29.1	30.2	30.5	30.0	31.0	30.3
Ash (g)	95.5	92.5	89.0	96.1	91.1	87.6
Carbohydrate (g)	289.7	307.9	332.4	287.8	308.0	318.8
Crude fibre (g)	0.1	0.2	0.3	0.1	0.2	0.3
Phytate (g)	11.49	14.71	15.41	10.65	13.71	14.22
P (g)	16.01	16.08	14.98	15.74	16.13	15.38
Ca (g)	15.12	13.96	11.52	14.85	13.63	11.50
Ca: P ratio	0.94	0.87	0.77	0.94	0.84	0.75
Zn (mg)	295.32	295.3	266.54	280.63	285.88	267.58
Mg (mg)	2.27	2.44	2.43	2.11	2.33	2.42
Mn (mg)	83.98	89.58	84.4	82.38	87.78	85.51

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.

[a] Fishmeal (Egersund Sildoljefabrikk, Norway) with an apparent protein digestibility coefficient (ADC protein) of 90.2 % ; [b] SPC (Imcopa, - Importação, Exportação e Indústria de Óleos Ltda., Araucária - Paraná, Brazil) with an apparent protein digestibility coefficient (ADC protein) of 90.8%; [c] Tapioca (Hoff Norske Potetindustrier, Gjøvik, Norway); [d] Monocalcium 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] Nobacithin: De-oiled lecithin powder (Noba Vital Lipids, Netherlands); [h] Fish Oil (Egersund Sildoljefabrikk, Norway).

5.2.2 Calculations

The following formulae were applied to the data:

Weight gain:

$$WG\ (\frac{g}{day}) = \frac{Weight\ gain(g)}{Number\ of\ days}$$

Specific Growth Rate:

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

Thermal Growth Rate:

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

In the above formulae W0 and W1 is the initial and the final fish mean weights in grams.

5.2.3 Diet composition analysis

Dietary crude fat was determined following acid hydrolysis using a Soxtec System 1047 hydrolysing unit (Tecator Application note 92/87) followed by exhaustive Soxhlet extraction using petroleum ether (40–60°C boiling point) on a Soxtec System HT6 (Tecator application note 67/83)as described by (Bell et al., 2001). Dry weight and ash contents of diets were determined after oven-drying the samples to constant weight (at 100ºC) and by ashing dried samples in an oven at 550°C (AOAC, 1990). Dietary energy content was determined by performing bomb calorimetry (AOAC, 1990). For the determination of phytate content in the diets a Megazyme Phytate/Total Phosphorus Assay kit (Megazyme, Ireland) was used. Dietary energy content was determined through bomb calorimetry (AOAC, 1990). More details regarding the aforementioned methodologies are given in Section 2.6.

Dietary carbohydrate was determined following a modified Dubois phenol sulphuric method. Dietary fibre was determined after subjecting defatted dietary samples (3 washes

with petroleum ether) within pre-weighed organic capsules, to acid (with 1.25% sulphuric acid solution)and alkaline hydrolysis (with 1.25% sodium hydroxide solution) for 35 min each, using a Fibertec system 1020 hot Extractor. Following one last defatting step (3 washes with petroleum ether), the samples were ashed at 600°C in a muffle furnace (Gallenkamp Muffle Furnace) for 4 hours, cooled in a desiccator and reweighed (W2). Extracted fibre was expressed as percentage of the original undefatted sample and calculated according to the formula:

$$Fibre(\%) = \frac{(W1 \times 1.0011) - Capsule\ weight - (W2 - 0.0025)}{Sample\ weight\ (g)} \times 100$$

Where W1 is the initial weight of the unprocessed dietary sample and W2 is the weight after processing. More details regarding the determination of dietary carbohydrates and fibre levels are given in Sections 2.6.7 and 2.6.8.

Dietary minerals and phosphorus were determined using inductively coupled plasma mass spectroscopy (ICP-MS) with collision cell technology (CCT) (Thermo XSeries 2). A more thorough description of the method is given in Sections 2.6.1, 2.6.4, 2.6.5.

5.2.4 Bone examination and composition analysis

A total of 288 fish were examined for external signs of deformities and analyzed for bone moisture and mineral composition. Eight individuals were collected from each tank at each time point. The samples were divided into 3 groups of 96 fish each, consisting of naïve fish (prior to vaccination/PBS injection), vaccinated salmon and PBS-injected salmon from Day 154 (62 dpv/PBS injection). After undergoing an initial external gross examination, in which fish were examined for external signs of malformations, discoloration and other irregularities, fish were scored and then placed within trays and stored at -70 °C. Salmon were defrosted prior to the analysis. The thawed samples were placed on trays, weighed

individually, and transferred into an oven at 160°C for 30 min in order to soften the flesh. The soft tissues were carefully removed to display the spine and ribs as intact as possible. Then, bony structures were visually evaluated for anomalies and any lesions were examined using a dissection microscope according to Baeverfjord et al. (1998b). Subsequently, the bones of each individual were then defatted in isohexane for 20 h, placed on small trays and weighed. Dry matter and moisture were determined according to standard methods (AOAC, 1990) by oven drying for 16 h to constant weight. After drying, spine samples were weighed again, homogenised and used for ash and mineral determination. In order to determine the amount of ash, 4 pools of 2 individuals were made. Bone pools were then ashed in a muffle furnace (Gallenkamp Muffle Furnace) at 550ºC according to (AOAC, 1990). Minerals and phosphorus in homogenised dried bone pools were determined using inductively coupled plasma mass spectroscopy (ICP-MS) with collision cell technology (CCT) (Thermo XSeries 2). Twenty milligrams of bone homogenates were added in Teflon digestion tubes with 5 ml of 69% nitric acid. The same steps described in section 2.3 for determination of dietary minerals and P levels were followed. More details regarding the applied methodologies are given in Section 2.6.1, 2.6.4 and 2.6.5.

5.2.5 Statistics

Growth trajectories based on the mean weight estimates at Day 92/prior vaccination, Day and Day 154/ 62 days post vaccination were modelled as repeated measures (Crampton et al., 2010; Espe et al., 2012). Moreover a hierarchical (multilevel) statistical model was used for the mineral analysis of pooled body cross-sections 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). Firstly, data were normalised as described in Section 2.9. 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., 2014). The statistical approach applied was model-based. This means that to find if any specific effect was statistically significant, four nested models with increasing complexity were fitted to the data and compared with a likelihood ratio test (LRT). In the applied models tank was considered as a random effect, sampling time as a categorical variable and the percentage of protein from SPC as a continuous factor possibly having non-linear effects. The four nested models are presented below:

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

Models demonstrating probabilities (*P* values) < 0.05 were selected for the description of data. The simplest possible of the three latter models was adopted according to Occam's razor principle. The adopted model was demonstrated by plotting the expected mean response with 95% confidence intervals. The expected mean and 95 % confidence intervals were solved by a posterior simulation from the adopted statistical model (n=1500 random draws was used throughout this study) (Gelman and Hill, 2007). In the present study two differentially treated (vaccinated and PBS-injected) salmon populations were achieved by the end of trial, therefore data obtained from the vaccinated and PBS-injected groups of salmon were modelled separately, together with data obtained for pre-injected salmon as described above.

5.3 RESULTS

5.3.1 Growth performance

In general, negligible mortalities (< 1%) were observed in the 3 dietary groups of salmon prior and post vaccination/PBS-injection. Growth performance parameters (mean daily WG model plotted in Fig. 20, TGC model plotted in Fig. 22A, SGR model plotted in Fig. 22B) were generally adversely affected in a linear fashion by the inclusion of increased dietary SPC in vaccinated salmon. On the other hand, growth indices for the PBS injected group of fish were affected in a curvilinear fashion, with SPC58 salmon demonstrating similar performance to the SPC35 group and SPC80 salmon exhibiting a greater reduction in growth parameters compared to SPC35 salmon at 62 days post PBS injection compared to the previous timepoint, prior to the vaccination/PBS-injection of the fish (mean daily WG model presented in Fig. 23, TGC model plotted in Fig. 25A, SGR model plotted in Fig. 25B). An overall reduction of 6 and 12% in expected weight gain (WG) was observed for the initial 3-month feeding period in SPC58 and SPC80 groups of salmon parr respectively, compared to the SPC35 group (Fig 20). Similar trends were confirmed for the naive dietary groups of fish from the statistical models selected for the best description of the data in both vaccinated and PBS-injected groups, revealing a more or less linear trend in WG reduction. Adaptation of the linear model of decrease in mean WG for this timepoint, revealed significant decreases for both SPC58 and SPC80 salmon in comparison to SPC35 fish prior vaccination/PBS injection (since the expected 95% confidence intervals of the former group did not overlap with the expected mean value of the latter in all combinations of modelled growth responses). These reductions corresponded to approximate decreases of 5 and 10% in expected mean weight for the medium and high SPC inclusion dietary groups of fish compared to SPC35 salmon, with SPC80 salmon demonstrating a significantly lower mean weight than the SPC35 group (Fig. 21). Significant differences were also demonstrated in

TGC and SGR values for both SPC80 and SPC58 salmon compared to the SPC35 group at this timepoint.

At 62 days post vaccination, the reduction in daily WG for the SPC58 and SPC80 groups of salmon was 8.5 and 17%, corresponding to decreases of 7 and 14% in the mean salmon weight values when compared to SPC35 salmon (Fig. 20 and 21). Significantly higher WG was demonstrated for the SPC35 group compared to both SPC58 and SPC80 salmon. Moreover, significant decreases in salmon mean weight were revealed for the SPC58 and SPC80 groups of salmon in contrast to SPC35 salmon. Nonetheless, no differences in TGC and SGR values were demonstrated among the vaccinated dietary groups (Fig. 22A and 22B). At 62 dpPBSinj, the reduction in daily WG for the SPC58 and SPC80 groups of salmon was 6 and 25%, corresponding to decreases of 5 and 18% in the mean weight values when compared to SPC35 salmon (Fig. 23 and 24). Significant changes in WG, TGC and SGR were demonstrated for the SPC80 group in comparison to SPC35 salmon while no differences were detected between SPC58 and SPC35 salmon for the aforementioned growth performance indices (Fig. 25A and 25B). However, both SPC58 and SPC80 salmon exhibited significantly lower mean weight in contrast to the SPC35 group.

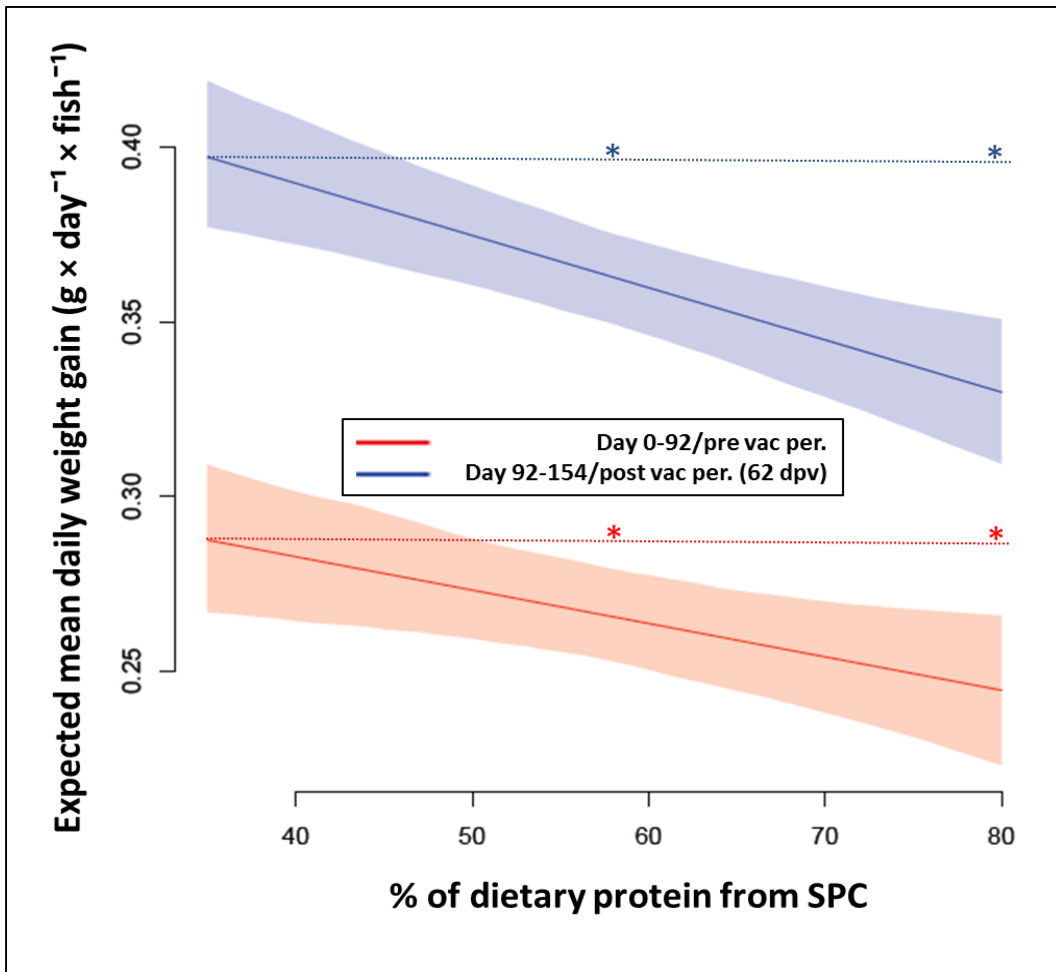

Figure 20 Modelled daily weight gain (WG) response for the vaccinated dietary groups of salmon plotted against values obtained for naive salmon groups. Expected mean daily weight gain (g × day^{-1}× $fish^{-1}$) (with 95% C.I.) in relation to dietary protein from SPC prior and post vaccination. Red and blue lines connect the expected mean values for each measurement prior to and 62 days post vaccination respectively, while shaded regions of the same colours indicate the 95% confidence regions for the values of these measurements. Red Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

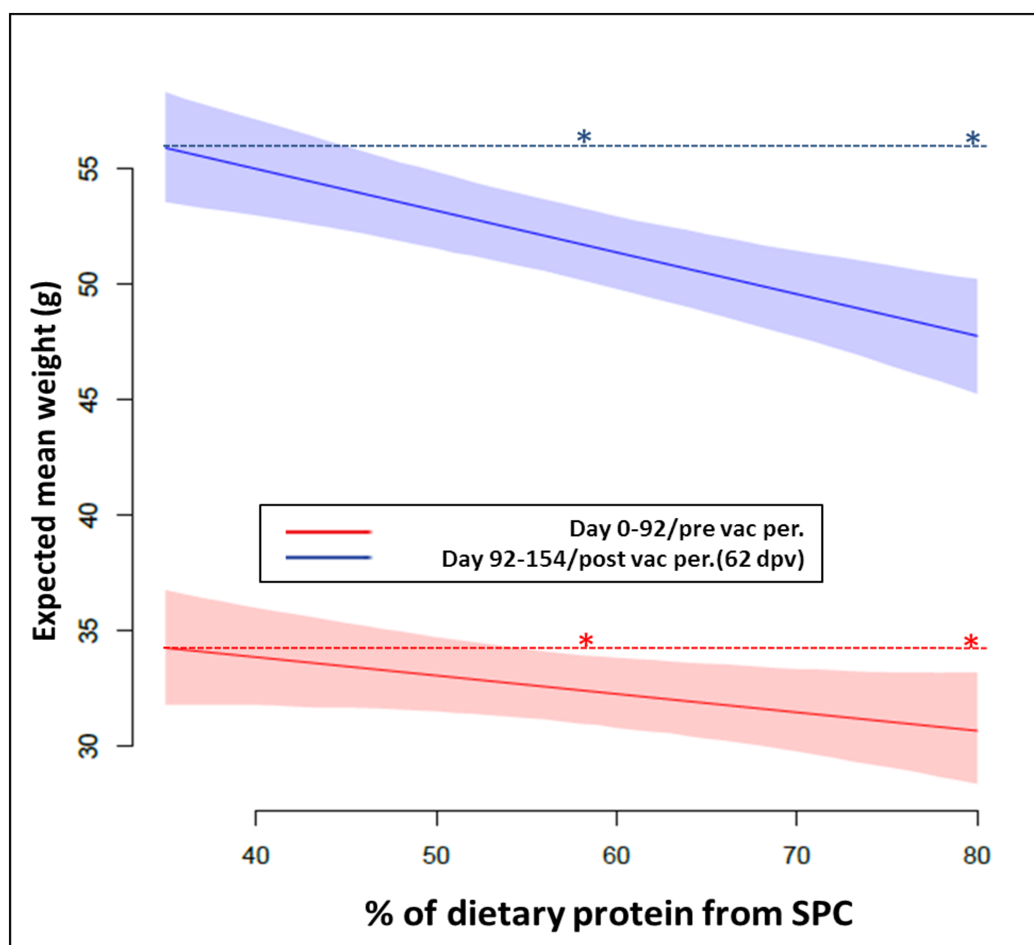

Figure 21 Modelled growth performance (mean weight) for the vaccinated dietary groups of salmon plotted against values obtained for naive salmon groups. Expected mean weight (g × fish^{-1}) (with 95% C.I.) in relation to dietary protein from SPC prior and post vaccination. Red and blue lines connect the expected mean values for each measurement prior to and 62 days post vaccination respectively, while shaded regions of the same colours indicate the 95% confidence regions for the values of these measurements. Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

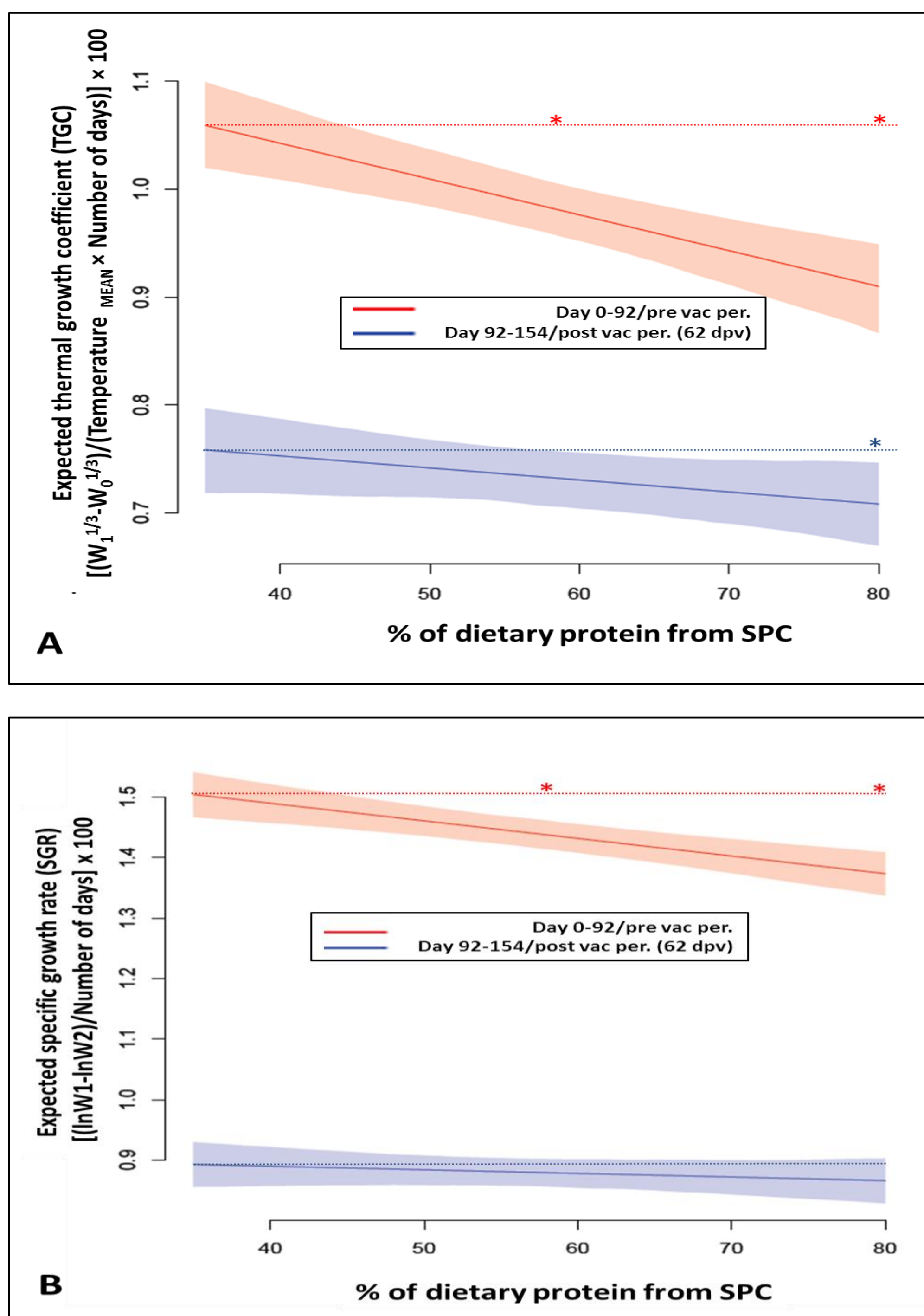

Figure 22 Modelled growth performance indices for the vaccinated dietary groups of salmon plotted against values obtained for naive salmon groups. (A) Expected TGC and (B) SGR values (with 95% C.I.) in relation to dietary protein from SPC prior and post vaccination. Red and blue lines connect the expected mean values for each measurement prior to and 62 days post vaccination respectively, while shaded regions of the same colours indicate the 95% confidence regions for the values of these measurements. Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned colours denote significant differences between salmon fed 35% of protein from SPC and salmon fed diets with higher percentage of dietary protein fromSPC at different timepoints.

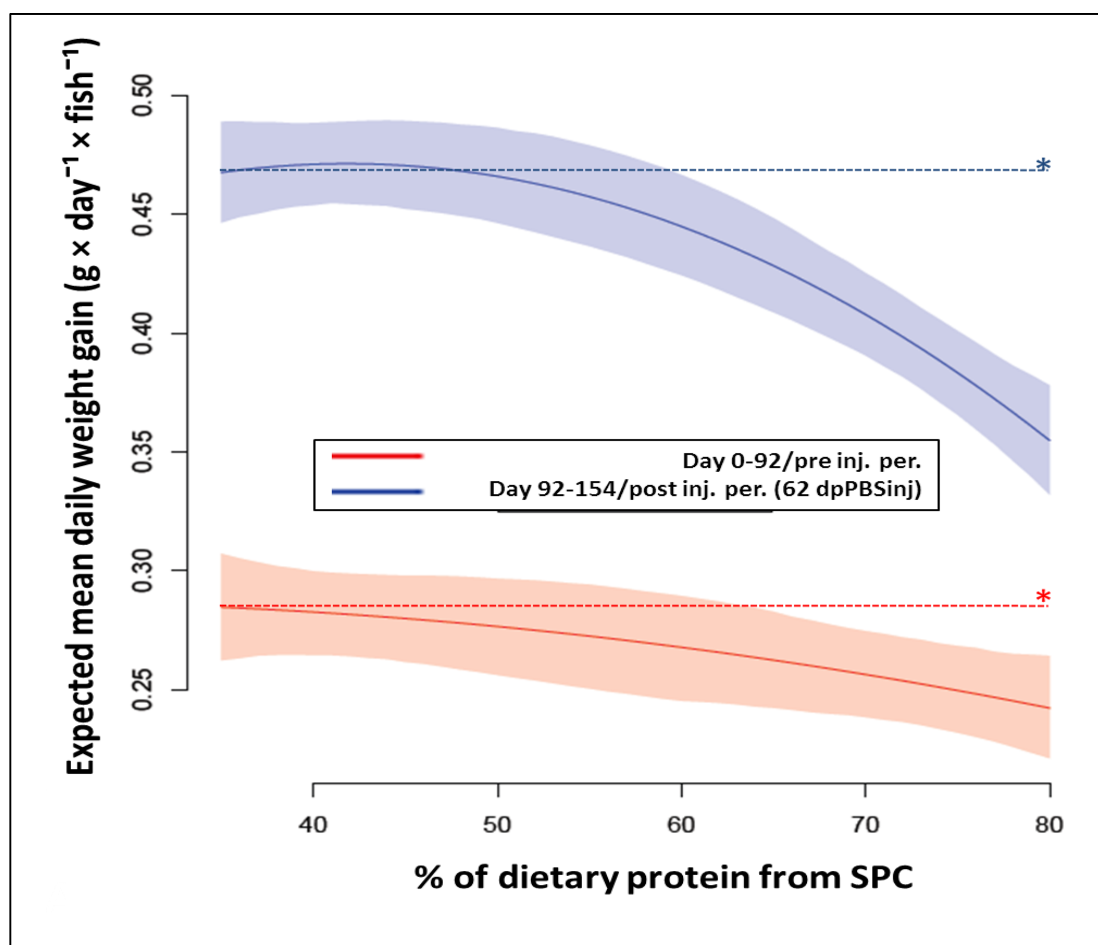

Figure 23 Modelled daily weight gain (WG) response for the PBS-injected dietary groups of salmon plotted against values obtained for naive salmon groups. Expected mean daily WG (g × day^{-1}) (with 95% C.I.) in relation to dietary protein from SPC prior and post vaccination. Red and blue lines connect the expected mean values for each measurement prior to and 62 days post vaccination respectively, while shaded regions of the same colours indicate the 95% confidence regions for the values of these measurements. Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

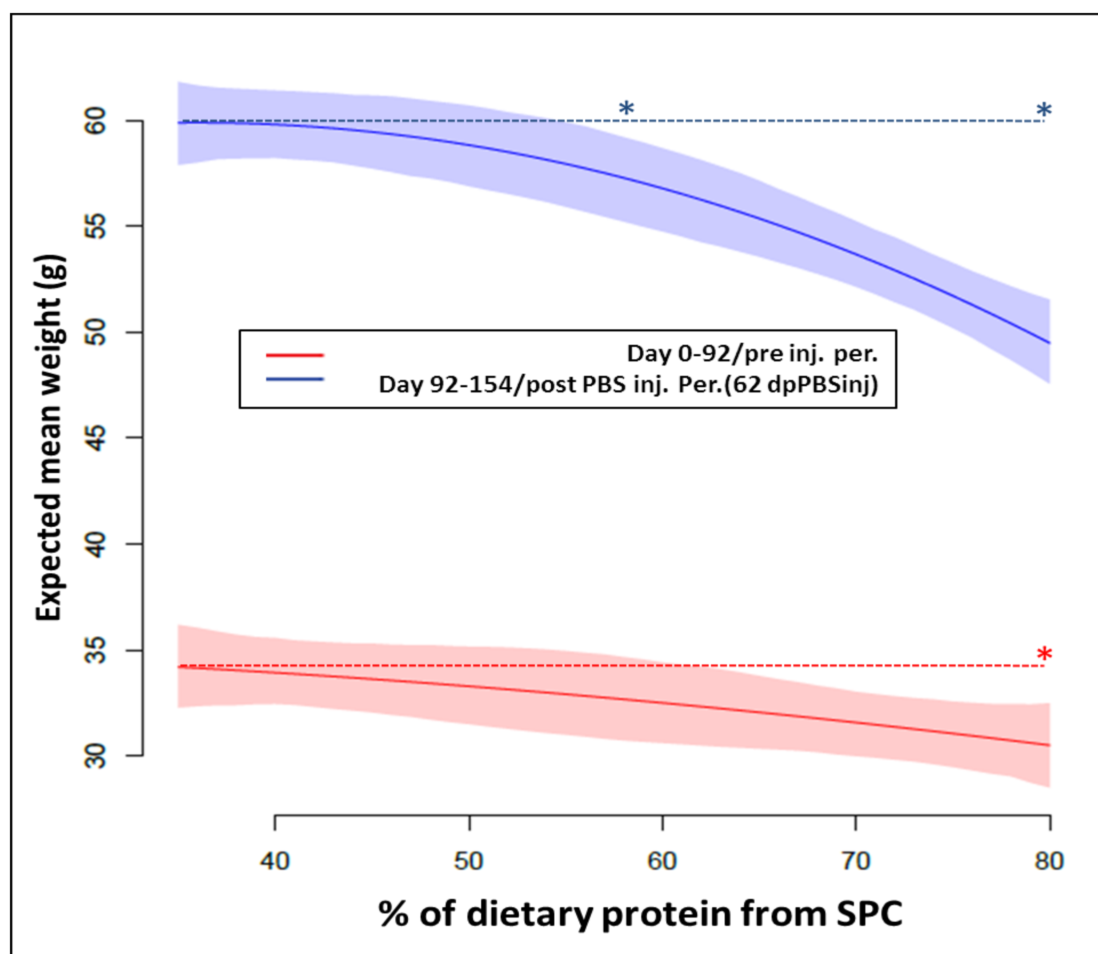

Figure 24 Modelled growth performance (mean weight) for the PBS-injected dietary groups of salmon plotted against values obtained for naive salmon groups. Expected mean weight (g × fish^{-1}) (with 95% C.I.) in relation to dietary protein from SPC prior and post PBS-injection. Red and blue lines connect the expected mean values for each measurement prior to and 62 days post PBS injection respectively, while shaded regions of the same colours indicate the 95% confidence regions for the values of these measurements. Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

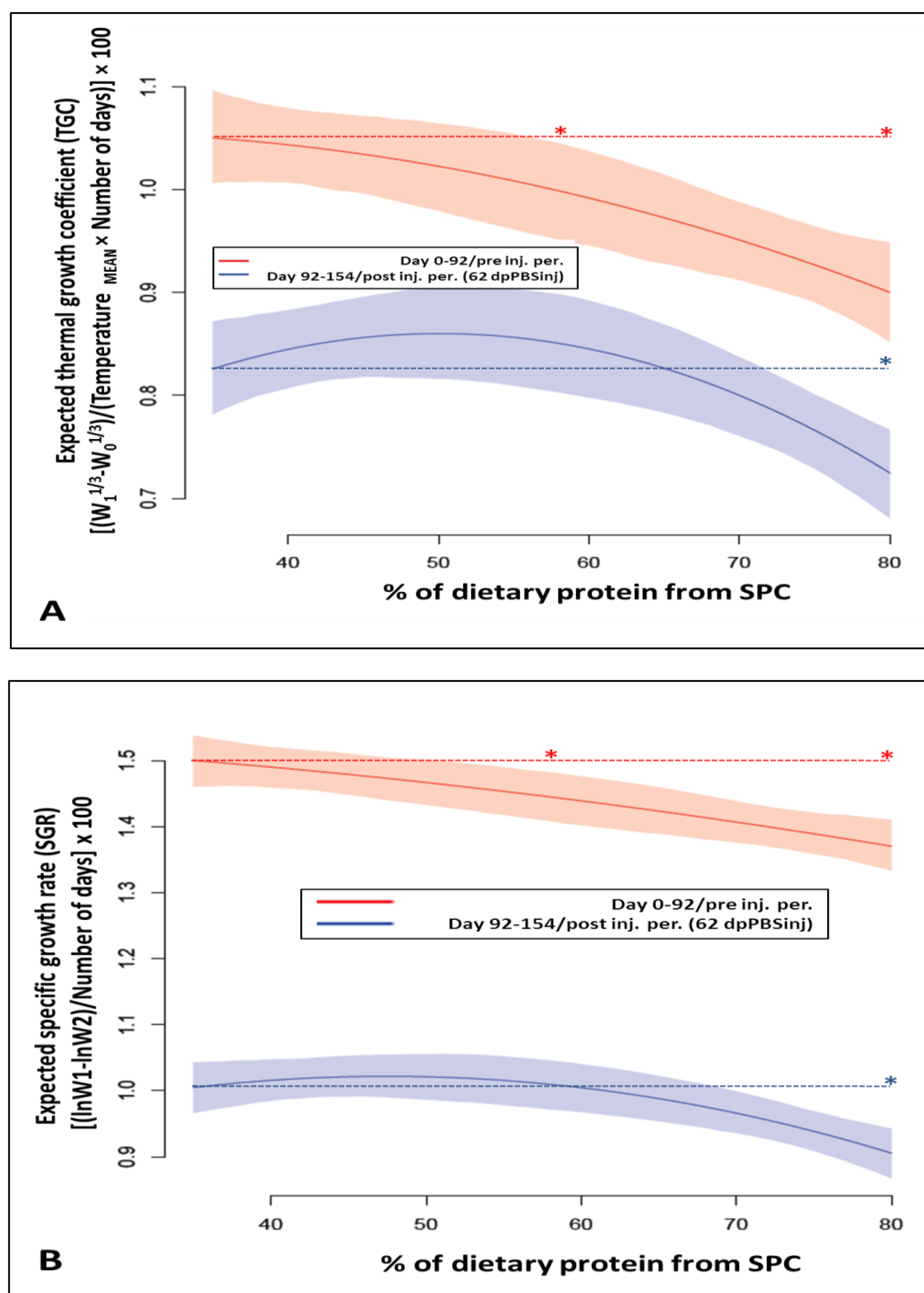

Figure 25 Modelled growth performance indices for the PBS-injected dietary groups of salmon plotted against values obtained for naive salmon groups. (A) Expected TGC and (B) SGR values (with 95% C.I.) in relation to dietary protein from SPC, prior and post vaccination. Red and blue lines connect the expected mean values for each measurement prior to and 62 days post vaccination respectively, while shaded regions of the same colours indicate the 95% confidence regions for the values of these measurements. Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

5.3.2 Bone gross pathology and composition

5.3.2.1 Skeletal pathology

None of the sampled fish from any of the groups at anyone of the two timepoints demonstrated external signs of malformations or discolorations. At both timepoints salmon exhibited dark colouration and presence of parr marks along the flanks revealing the absence of smoltification. Dorsal fin erosion was apparent for about 15% in all dietary groups. No signs of cataracts were observed in anyone of the sampled fish. The observations were consistent at both prior and post vaccination/PBS-injection. Moreover, upon necropsy operculae and gill arches were noted to be fairly rigid, presenting normal resistance to manipulation and cutting. Operculae of salmon parr were bent > 90° and samples were evaluated on their resistance to tortion. The results were also consistent among the different dietary groups of salmon at both timepoints and states of vaccination. Scales in their majority presented the same degree of rigidity and thickness under the dissection microscope. Again observations were consistent among groups. Upon ovening of the samples for 30 minutes, structural distortions of the ribs and spinal arches were only minor and regarded only a percentage of less than 2% from all sampled fish. Individuals presenting shortened and wrinkly ribs (throughout the full length of the rib) were accounted as carriers of rib deformities and recorded. Furthermore vertebral samples with crooked spinal arches were also recorded as carriers of malformations. Approximately 15% of the samples presented some form of anomaly in the ribs, while another 10% of the samples demonstrated distortions in spinal arches. Similar numbers were observed for all groups prior and post vaccination/PBS-injection. Approximately 2% of the sampled fish presented vertebral fusions in the mid and caudal section with the latter fusions being more prominent. Once

again even numbers were revealed for all dietary groups at both points and states. No fractures of the bone structures were seen.

5.3.2.2 Skeletal composition

The modelled proximate skeletal composition responses are plotted in Fig. 26 (naive against PBS-injected salmon bone composition data) and Fig. 27 (naive against vaccinated salmon bone composition data). Significant differences in the modelled graphs are denoted when the 95% confidence intervals for each one of the SPC58 and SPC80 groups, do not overlap with the expected mean value of salmon fed the diet with 35% of dietary protein from SPC. A linear decrease in expected bone ash levels of salmon receiving higher dietary SPC was detected prior to injection with PBS. Nonetheless, no significant differences were observed among the 3 dietary groups of fish. This trend was reversed at 62 days post-injection with PBS, in salmon parr fed increasing levels of dietary SPC exhibiting an increase in bone ash concentrations (Fig. 26A). Significant increases in credible skeletal ash were demonstrated for both PBS-injected SPC58 and SPC80 groups, compared to the SPC35 salmon at this point (since 95% confidence intervals from the modelled skeletal ash concentrations of the former groups did not overlap with the mean ash levels of the SPC35 batch). At 62 dpv, salmon fed increasing dietary SPC levels demonstrated a linear increase in expected skeletal ash concentrations (Fig. 27A). However, no significant changes were recorded among the vaccinated dietary groups. An increase in expected bone moisture levels was observed in salmon fed increasing levels of SPC prior to vaccination/PBS-injection, while a linear reduction was witnessed at 62 dpPBSinj (model plotted in Fig. 26B). Significant reductions were recorded for the PBS-injected SPC58 and SPC80 salmon in comparison to the corresponding SPC35 group. For vaccinated salmon, the model using a squared term of the percentage of protein from SPC resulted in an improvement of the data (model plotted in

Fig. 27B). Expected bone moisture levels in vaccinated salmon followed a curvilinear line at 62 dpv in fish fed increasing amounts of dietary SPC, with SPC35 and SPC80 salmon demonstrating similar moisture concentrations and SPC58 salmon exhibiting the lowest levels of credible bone moisture. No significant changes were observed among the dietary groups of fish.

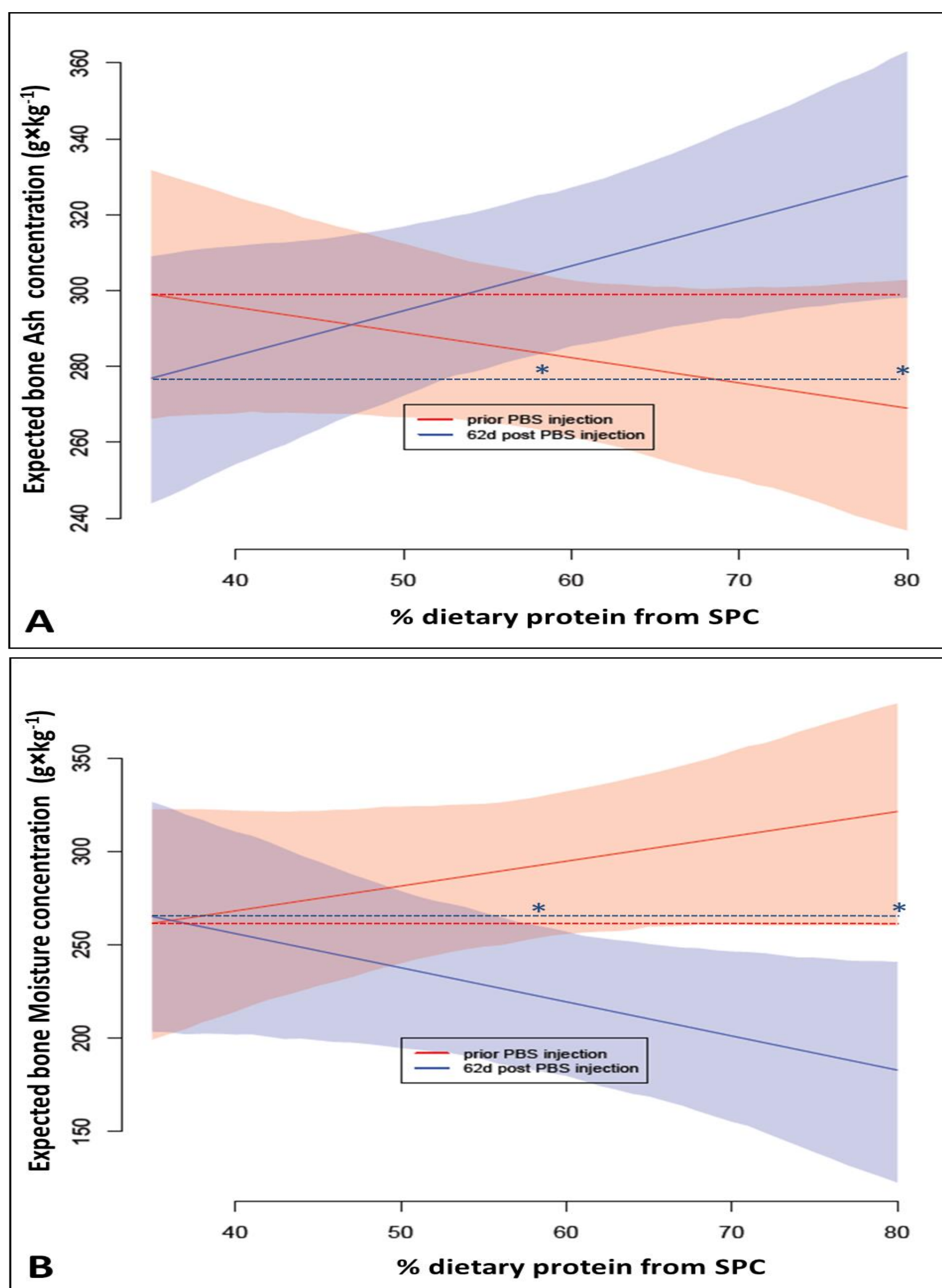

Figure 186 Modelled skeletal bone proximate analysis measurements (PBS-injected salmon). Expected mean bone composition (with 95% C.I.) from bone pools of 2 individuals per tank (4 pools × tank^{-1}) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Ash levels (g × kg^{-1} of defatted bone); (B) Moisture levels (g × kg^{-1} of defatted bone). Red line connects the expected mean values for each measurement at prior vaccination/PBS injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post PBS injection (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

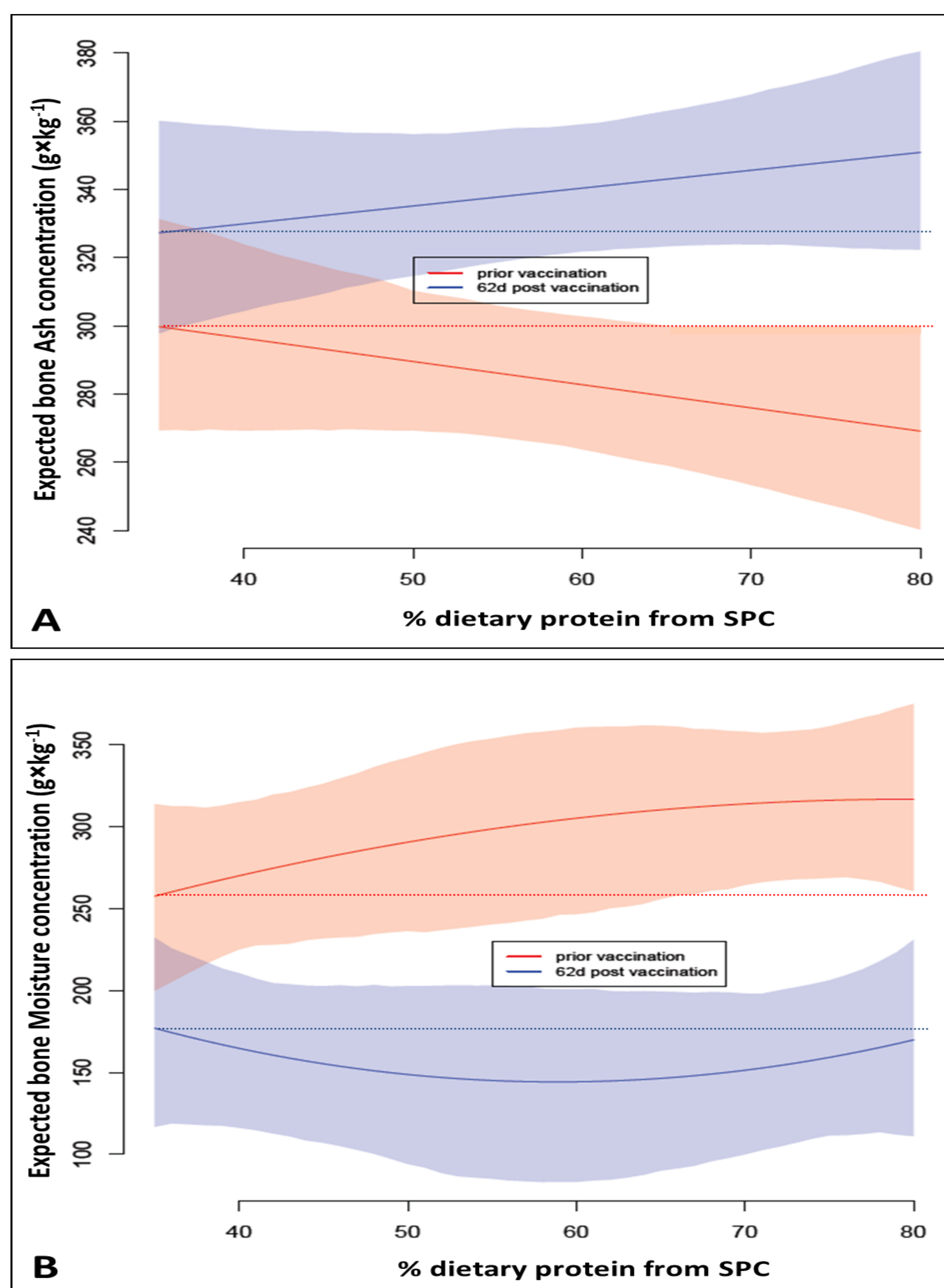

Figure 27 Modelled skeletal elemental levels (vaccinated salmon). Expected mean bone composition (with 95% C.I.) from bone pools of 2 individuals per tank (4 pools × tank^{-1}) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Ash levels (g × kg^{-1} of defatted bone); (B) Moisture levels (g × kg^{-1} of defatted bone).Red line connects the expected mean values for each measurement at prior vaccination/PBS injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post vaccination (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

The selected modelled responses of bone elemental analysis are plotted in Fig. 28, 29 and 30 (naive against PBS-injected salmon bone composition data) and Fig. 31, 32 and 33 (naive against vaccinated salmon bone composition data). Overall, naive salmon (pre-vaccinated/PBS-injected fish) showed a linear decrease in Ca, Mg, P and Zn levels, a linear increase in Mn and almost constant Ca:P ratios with increasing dietary SPC inclusion. At this timepoint, significant differences were detected in expected skeletal Ca, P, Mg and Zn concentrations between the SPC80 group and SPC35 salmon while a significant decrease in skeletal Mg and Zn was also apparent for the SPC58 salmon compared to the SPC35 group of fish. At 62 days post PBS-injection, expected Ca:P ratio and Mg levels increased linearly as dietary SPC levels increased, with SPC35 salmon showing lower values than seen prior to injection. On the contrary, concentrations for the SPC58 and SPC80 groups were higher at post PBS-injection (model plotted in Fig. 29A and 29B). Moreover, significantly higher Mg concentrations and Ca:P ratios were demonstrated for the SPC58 and SPC80 group compared to SPC35 salmon. Expected bone Zn levels followed a similar trend, however, a 25 and an approximate 13% decrease in credible bone Zn was observed in SPC35 and SPC58 salmon respectively, with the SPC80 group being the only group exhibiting an increase at post PBS-injection (model plotted in Fig. 30B). No significant differences in skeletal Zn were obtained among the PBS-injected dietary groups at 62 days post injection. On the other hand, credible bone Ca, P and Mn levels increased curvilinearly with SPC58 and SPC80 salmon values being higher than in naïve salmon (models plotted in Fig 28A, 28B and 30A respectively). On the contrary, expected concentrations for the aforementioned elements were found to be lower in PBS-injected SPC35 salmon in contrast to their naive counterparts. Credible bone Ca and P concentrations were similar in the SPC58 and SPC80 groups of PBS-injected salmon. SPC80 salmon was the group exhibiting the highest expected levels

in bone Mn. Significantly higher levels of Ca, P and Mn were demonstrated in the PBS-injected SPC58 and SPC80 groups of salmon in comparison to the SPC35 group at the end of the study.

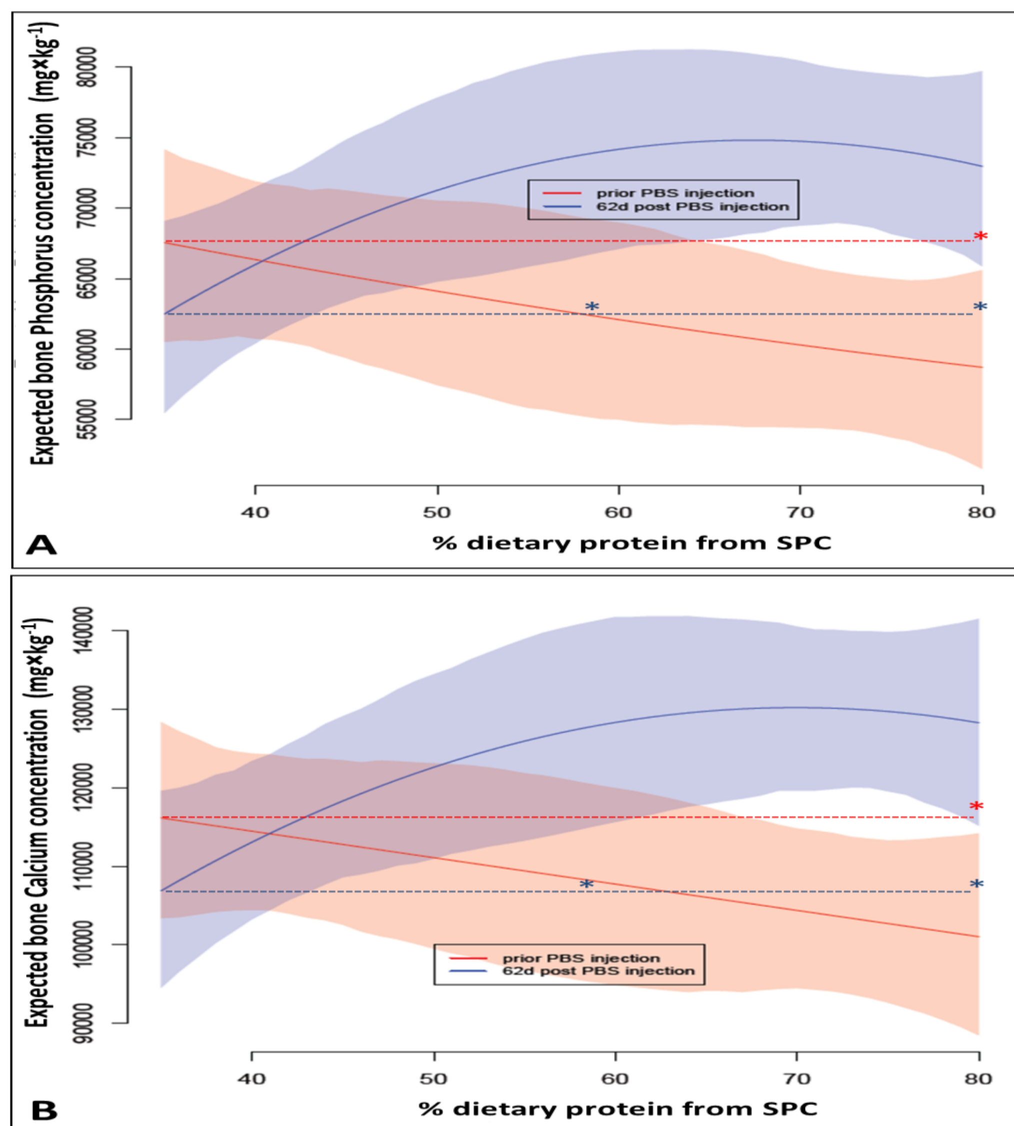

Figure 198 Modelled skeletal elemental levels (PBS-injected salmon). Expected mean bone composition (with 95% C.I.) from bone pools of 2 individuals per tank (4 pools × tank^{-1}) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Phosphorus levels (mg × kg^{-1} of defatted bone); (B) Calcium levels (mg × kg^{-1} of defatted bone). Red line connects the expected mean values for each measurement at prior vaccination/PBS injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post PBS injection (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned colours denote significant differences between salmon fed 35% of protein from SPC and salmon fed diets with higher percentage of dietary protein frm SPC at different timepoints.

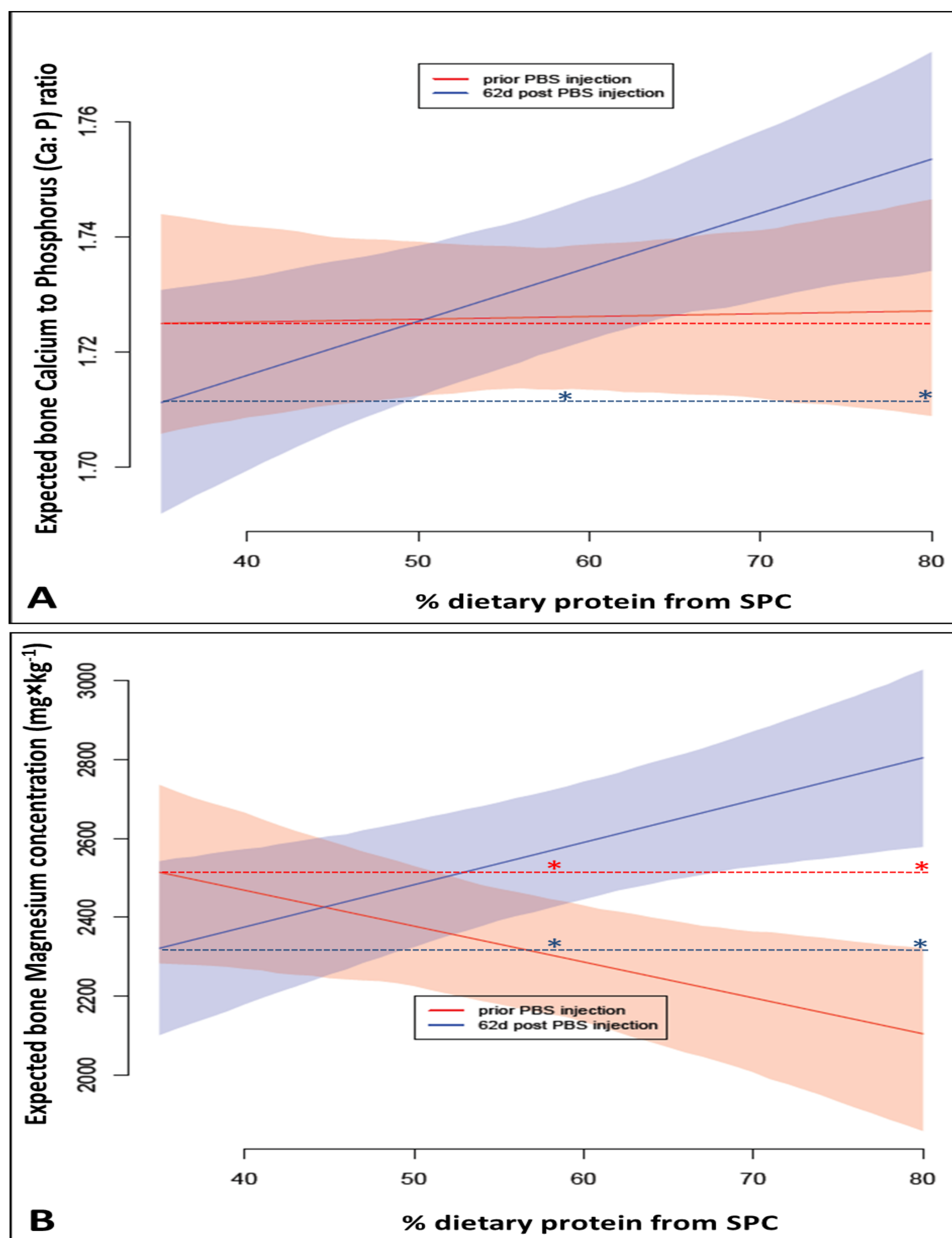

Figure 209 Modelled skeletal elemental levels (PBS-injected salmon). Expected mean bone composition (with 95% C.I.from bone pools of 2 individuals per tank (4 pools × tank^{-1}) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Ca: P ratio; (B) Magnesium levels (mg × kg^{-1} of defatted bone). Red line connects the expected mean values for each measurement at prior vaccination/PBS injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post PBS injection (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

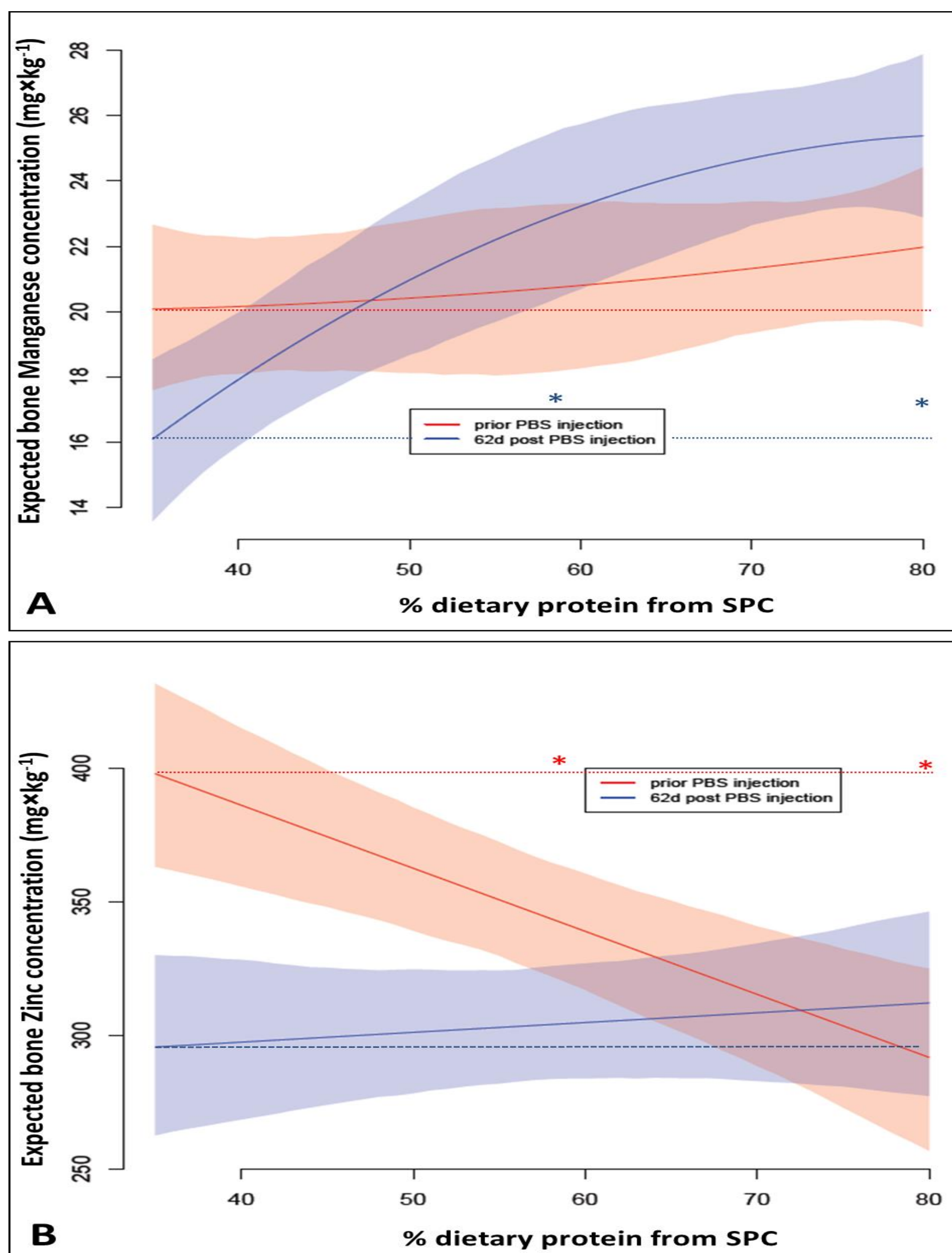

Figure 30 Modelled skeletal elemental levels (PBS-injected salmon). Expected mean bone composition (with 95% C.I.) from bone pools of 2 individuals per tank (4 pools × $tank^{-1}$) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Manganese levels ($mg \times kg^{-1}$ of defatted bone); (B) Zinc levels ($mg \times kg^{-1}$ of defatted bone). Red line connects the expected mean values for each measurement at prior vaccination/PBS/injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post PBS injection (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

In vaccinated salmon, expected bone Ca, Ca:P ratios, Mg, Mn and Zn were affected in a linear fashion by increasing dietary SPC inclusion (models plotted in Fig. 31B, 32A, 32B, 33A and 33B). Most of these elements presented a linear increase with increasing dietary SPC supplementation except Zn, which exhibited a slight decrease at 62 dpv. Significantly higher expected bone Ca: P ratio and Mn levels were obtained for both vaccinated SPC58 and SPC80 salmon in comparison to the SPC35 group. Moreover, significantly higher expected bone Ca and Mg were obtained for the SPC80 group in contrast to SPC35 salmon. Credible bone P concentrations presented a curvilinear mode of increase at 62 dpv, with SPC58 and SPC80 salmon demonstrating the highest levels (model plotted in Fig 31A). However, no significant differences in bone P concentrations were observed among the dietary groups of fish.

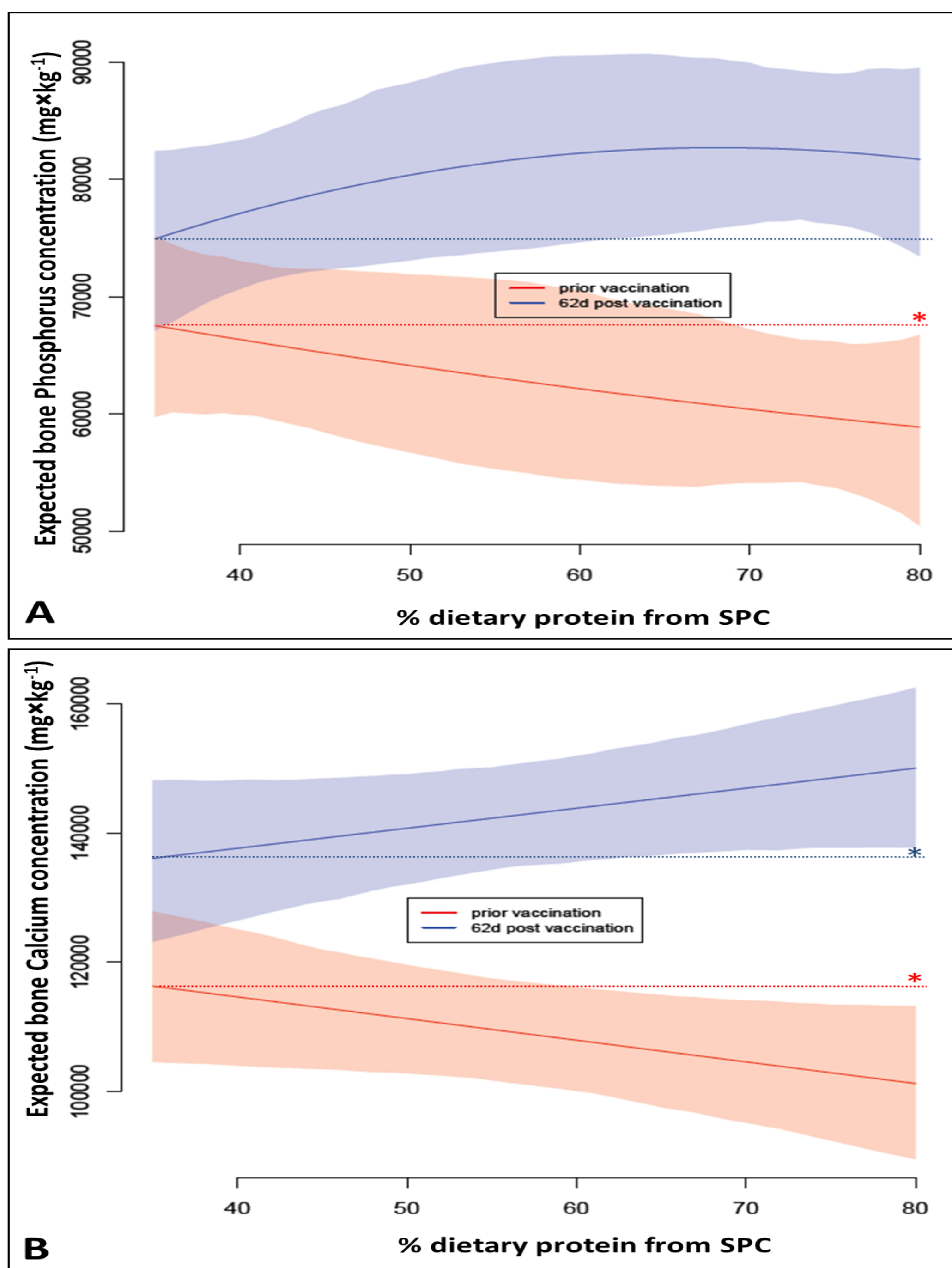

Figure 31 Modelled skeletal elemental levels (vaccinated salmon). Expected mean bone composition (with 95% C.I.) from bone pools of 2 individuals per tank (4 pools × tank^{-1}) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Phosphorus levels (mg × kg^{-1} of defatted bone); (B) Calcium levels (mg × kg^{-1} of defatted bone). Red line connects the expected mean values for each measurement at prior vaccination/PBS injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post vaccination (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

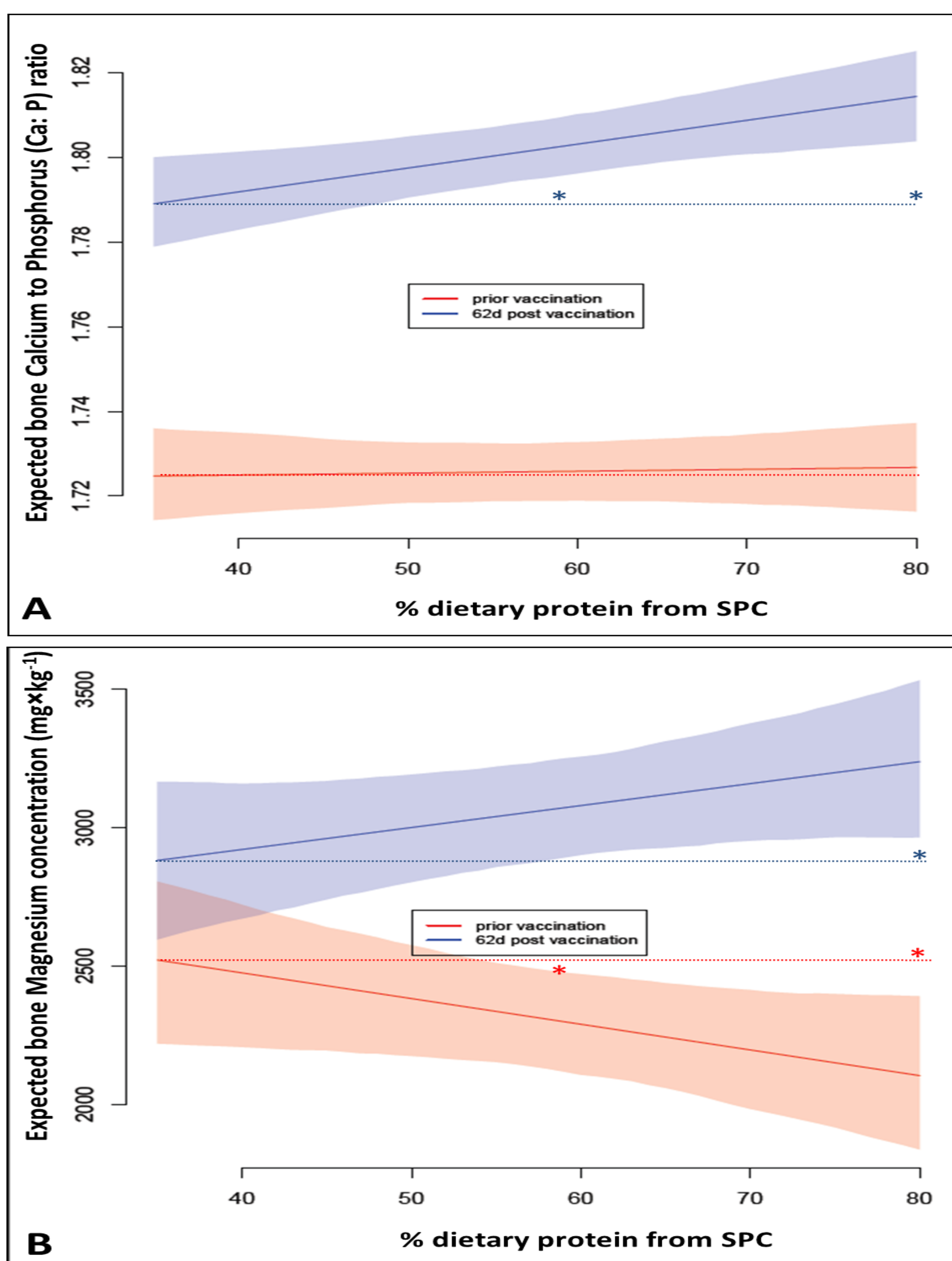

Figure 32 Modelled skeletal elemental levels (vaccinated salmon). Expected mean bone composition (with 95% C.I.) from bone pools of 2 individuals per tank (4 pools × tank^{-1}) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Ca:P ratio; (B) Magnesium levels (mg × kg^{-1} of defatted bone). Red line connects the expected mean values for each measurement at prior vaccination/PBS injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post vaccination (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

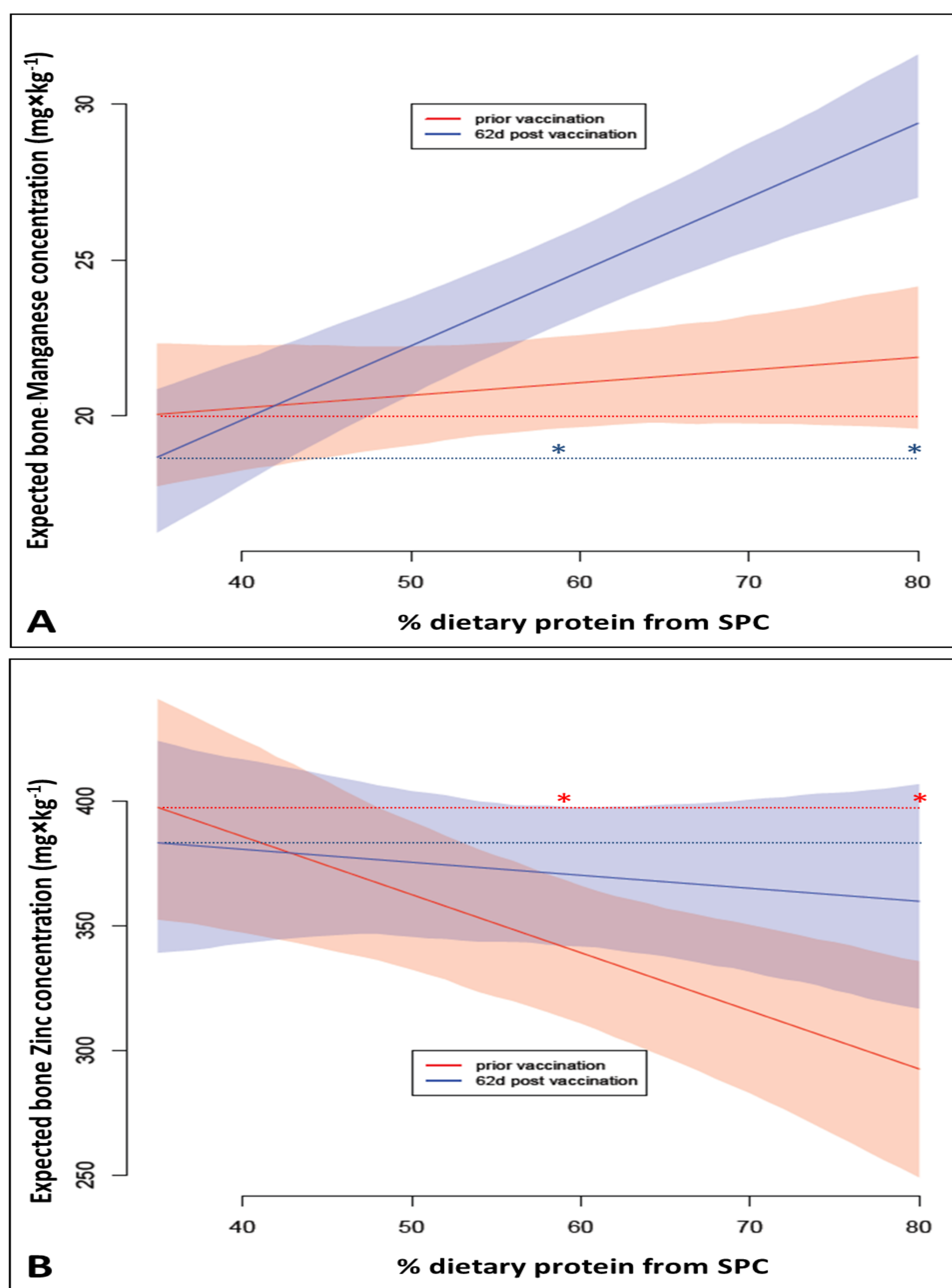

Figure 33 Modelled skeletal elemental levels (vaccinated salmon). Expected mean bone composition (with 95% C.I.) from bone pools of 2 individuals per tank (4 pools × tank^{-1}) (y axis) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC (x axis): (A) Manganese levels (mg × kg^{-1} of defatted bone); (B) Zinc levels (mg × kg^{-1} of defatted bone). Red line connects the expected mean values for each measurement at prior vaccination/PBS injection (Day 92 of the feeding trial) while red shaded region indicates the 95% confidence region. Similarly, blue lines and its corresponding shaded region represent the respective values at 62 days post vaccination (Day 154 of the feeding trial). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

5.4 DISCUSSION

5.4.1 Growth performance

In the present trial, salmon expected growth performance indices (mean weight, daily WG, TGC and SGR) were adversely affected, upon feeding with diets with 58 and 80% of dietary protein from SPC compared to salmon fed diets with only 35% of protein from SPC, for an initial period of 92 days. While significantly lower mean weights were also demonstrated for both SPC58 and SPC80 salmon compared to the SPC35 group at post vaccination/PBS-injection, no differences in WG, TGC and SGR values were exhibited in both vaccinated and PBS-injected SPC58 in contrast to SPC35 fish. This suggests an improvement in the overall performance of salmon and the fact that larger size parr (30-35 g), with a more developed digestive tract, can make a more efficient use of the nutrients within the SPC58 diet compared to smaller size parr (9g initial size of the fish) (Denstadli et al., 2006; Helland et al., 2006). Contrary to this, no improvement in anyone of the growth performance indices was observed in the SPC80 group in comparison to SPC35 salmon during the second period of the study in both vaccinated and PBS-injected fish. In particular, PBS-injected SPC80 salmon presented significant reductions in daily WG, TGC and SGR compared to the corresponding SPC35 group which far exceeded the ones observed during the first study period. Vaccination in general, is a stressful process, especially when administered by injection, resulting in reduced growth (Poppe and Koppang, 2014), which could explain the differences in growth performance between vaccinated and PBS-injected salmon. The reason is that vaccination increases the use of energy for stress and immune induction (Wendelaar Bonga, 1997) and reduces energy expenditure for growth. Furthermore, vaccination-induced stress inhibits growth by affecting the levels of certain hormones such as the growth and

thyroid hormones and the insulin-like growth factor (IGF-1), regulating positive growth (Brown et al., 1991; Pickering, 1993; Plisetskaya and Duan, 1994).

Overall, 75 % reduction of high quality FM (since the lowest FM inclusion was one quarter of the highest) caused a reduction of 17% in daily WG in vaccinated SPC80 salmon and 25% in their PBS-injected SPC80 counterparts. This suggests that this replacement is beyond the optimal level for early stage Atlantic salmon parr. In Experiment I described in Chapter 3 an overall decrease of only 8% in the WG of bigger size juvenile Atlantic salmon (~30g) fed with the SPC80 was demonstrated by the end of a 97-day trial in fish that had been vaccinated on Day 63 of the study. Previously, it was stated that Atlantic salmon needs long adaptation periods before fully accepting diets containing very low levels of FM and/or FO substituted with alternative protein or oil sources (Torstensen et al., 2008). Moreover, Burr et al. (2012) demonstrated that the growth performance of Atlantic salmon parr fed diets in which protein was supplied from alternative protein blends improved with the age and size of the fish, with late stage parr fed on these diets, presenting improved growth compared to the FM-fed control group of salmon. In general, the reduction of growth in fish fed diets with higher levels of SPC could be attributed to the increased amounts of phytate reducing nutrient digestibility and availability (Storebakken et al., 1998b; Mambrini et al., 1999; Storebakken et al., 2000; Denstadli et al., 2006), or the lower concentrations of several key nutritional components (macro-minerals, trace minerals, sterols and non-nitrogen compounds), which are abundant in FM (Espe and Lied, 1999; Liaset and Espe, 2008) promoting feed acceptability and thus growth in fish.

5.4.2 Bone assessments

Expected bone moisture levels increased in salmon parr fed increasing levels of SPC during the initial 92 day feeding period, prior to vaccination, in the absence significant changes

among the three dietary groups of fish. This trend was reversed at the post-vaccination period, with vaccinated fish demonstrating a curvilinear decrease at 62 dpv, with lower levels detected in SPC58 salmon, while the decrease observed in bone moisture concentration followed a linear trend. Since lipid and moisture levels are inversely related (Shearer, 1994), the decrease in moisture suggests that higher levels of lipid were stored in salmon bones at 62 dpi, which seems to be related to the growth retardation induced by the induction of stress following vaccination. In general, higher expected bone moisture levels were obtained in PBS-injected salmon than in vaccinated salmon, with concentrations ranging from 270 g $\times$ kg^{-1} for SPC35 salmon to 180 g $\times$ kg^{-1} for SPC80 salmon. For vaccinated salmon, credible bone moisture values ranged from 175 g $\times$ kg^{-1} to 150 g $\times$ kg^{-1} with SPC35 and SPC80 salmon having similar values and SPC58 salmon demonstrating the lowest bone moisture concentrations. Fast growing juvenile salmon generally tend to have higher body moisture levels at the expense of lipid (Shearer, 1994). Therefore, low moisture levels are related to growth retardation due to vaccination (Melingen and Wergeland, 2000), which seems to be the case in vaccinated fish. Moreover, decreased bone moisture could be linked to the higher levels of dietary SPC inclusion, which seems to have a detrimental effect on early salmon parr growth performance as discussed above.

Credible bone ash concentrations of naïve Atlantic salmon parr at 92 days post feeding with the diets ranged from about 270 g $\times$ kg^{-1} for the SPC80 fed salmon to 300 g $\times$ kg^{-1} for the SPC35 group, while the SPC58 group of salmon presented intermediate ash values of about 280 g $\times$ kg^{-1}. No significant differences were detected among the 3 dietary groups of fish. The expected bone ash content of PBS-injected salmon spanned from 280 to 330 g $\times$ kg^{-1} moving from the SPC35 to the SPC80 group, with both SPC58 and SPC80 salmon exhibiting significantly higher ash than the SPC35 group, while bone ash for vaccinated

salmon parr varied from 330 to 350 g $\times$ kg^{-1} progressing from the SPC35 to SPC80 salmon and no significant differences among the treatment groups. Recorded ash levels from different time points were similar to those reported by Gil Martens et al. (2006) (~320 g $\times$ kg^{-1}) for smoltifying salmon parr ready for seawater transfer, pointing at well mineralised bones free from malformations.

For the initial 3-month feeding period and prior to vaccination/PBS-injection significantly lower expected bone Ca, P, Mg and Zn concentrations were demonstrated for the SCP80 group compared to SPC35 salmon, while SPC58 salmon exhibited lower Mg and Zn values in contrast to the SPC35 group. Despite the observed reductions in SPC58 and SPC80 salmon compared to the SPC35 group, most skeletal elements in all the dietary groups were found to be close to the normal range reported by Gil Martens et al. (2006). Expected bone Zn levels were the only exception, demonstrating 13 and 25% lower concentrations in the SPC58 and SPC80 groups compared to SPC35 salmon. In line with this, Storebakken et al. (1998b) reported decreased levels of whole body ash, P, Ca, Mg and Zn in salmon post-smolts fed 75% of dietary protein from undephytinized SPC after 84 days of feeding, compared to a fish maintained on a full FM diet. Moreover, Helland et al. (2006) reported decreased bone Zn levels in salmon fed increased levels of dietary phytate, which is the main ANF in SPC. Most of the bone elemental values determined in the current study were approximately 3 times higher than the ones reported by Helland et al. (2005, 2006). This is attributed to the fact that bone elemental concentrations in the current trial were determined from defatted skeletal samples. Moreover, previously it was reported that 20-30% of normal salmon bone is composed of lipids (Toppe et al., 2007), which could explain the differences between the two studies. Baeverfjord et al. (1998b) reported that reductions in bone ash and mineral content of Atlantic salmon parr fed on low levels of P for 84 days

contributed to abnormally soft vertebral structures, wrinkly ribs and scoliotic spines. In this study, no obvious external signs of mineral depletion were observed in salmon parr, while gross examination of dissected vertebral bone revealed negligible differences in terms of deformities (rib and arch distortions and various spinal changes) among different dietary groups of salmon parr at all time points. Furthermore, bony structures appeared to be fairly rigid and hard for all groups.

In addition, constant skeletal Mn and Ca:P ratios were reported for salmon fed on increasing levels of SPC and monocalcium phosphate for the initial period of the current study. The observed increment in bone Mn levels could be explained by the higher concentrations of these elements in SPC compared to FM (Table 13), increasing their availability for Atlantic salmon. Credible Ca:P ratios in naïve salmon parr were found to be 1.7 and were similar to the lower end of the values reported by Helland et al. (2006) and close to the ones determined by Gil Martens et al. (2006). However, contrary to Helland et al. (2006), who reported a reduction in bone Ca:P ratios, the results of the present trial revealed constant Ca:P ratios, which could be related to the increased supplementation of inorganic phosphorus in the high SPC inclusion diets (Åsgard and Shearer, 1997). In Chapter 3 a linear decrease in expected body cross-section ash, Ca:P ratio, Ca, Mg, P and Zn, in late stage salmon parr fed on diets containing increasing levels of SPC and constant addition of di-calcium phosphate after 63 days of feeding, was demonstrated. This reduction was combined with significant differences between the SPC35 group and salmon fed diets with higher than 35% of dietary protein from SPC. Increased amounts of phytate as proven from the dietary analysis in Table 6 were previously reported to reduce mineral availability and digestibility in fish (reviewed by Francis et al. 2001). One hypothesis is that the possible reduction in mineral digestibility due to the higher levels of phytate in diets with high SPC

levels combined with the under-developed gastro-intestinal tract of the fish (Denstadli et al., 2006; Burr et al., 2012) during the first period of the trial, might have led to temporary mineral deficiencies, in farmed Atlantic salmon parr, which were subsequently compensated in the second part of the study, over a longer period.

At 62 dpv, higher skeletal ash and elemental levels were measured in vaccinated fish compared to PBS-injected salmon, revealing greater retention of minerals in the bones of vaccinated salmon. Expected elemental concentrations were higher than the ones reported by Gil Martens et al. (2006) for well mineralised Atlantic salmon smolts. In addition, Ca:P ratios in the PBS-injected salmon were found to be similar to the values measured for naïve individuals, while values for vaccinated salmon were around 1.8 and closer to the higher end of the ratios reported by Helland et al. (2006), further supporting higher retention of minerals in the bone tissues. Similar ratios were also reported by Gil Martens et al. (2006) for salmon smolts. These observations are supported by previous studies revealing an increment in bone mineral rate and strength in Atlantic salmon at the post-vaccination period (Berg et al., 2005; Grini et al., 2011). One hypothesis for the higher levels of bone ash and elemental levels in vaccinated salmon compared to the non-vaccinated group is the fact that vaccination in general reduces fish growth for all the reasons proposed previously. Another striking fact was that lower levels of expected bone ash, Ca:P ratios and elemental concentrations were demonstrated in the PBS-injected SPC35 group, while higher values were observed in SPC58 and SPC80 salmon compared to their pre-PBS-injection values. Moreover, the initial trend of linear reduction in expected bone ash, Ca:P ratios, Ca, Mg, Mn and P in salmon fed increasing dietary SPC inclusion, prior to vaccination, was reversed at 62 dpv in both PBS-injected and vaccinated groups of salmon. Fjelldal et al. (2010) suggested that high inclusion levels of vegetable ingredients in salmon diets could have a positive effect on bone health in

Atlantic salmon post-smolts by delaying salmon growth. This may also reflect the differences in bone ash and elemental concentrations seen here and explain the improved bone elemental concentrations observed in SPC58 and SPC80 salmon, which in general exhibited lower growth rates at 63 dpv compared to the faster growing SPC35 group. Moreover, it has been suggested that during periods of rapid growth the time required for bone matrix to be produced and mineralised to occur can be reduced to a critical level (Hernandez et al., 2000), resulting in soft bones. This has been proposed as the cause for the higher incidence of vertebral deformities in fast growing under-yearling Atlantic salmon smolts compared to slower growing smolts (Fjelldal et al., 2006).

Expected bone Zn concentrations demonstrated no significant differences among the three dietary groups in both vaccinated and PBS-injected salmon. Moreover, an improvement in Zn concentrations was revealed in the vaccinated group compared to pre-vaccination levels with expected Zn levels being closer to the values reported by Gil Martens et al. (2006) for a normal healthy skeleton. On the other hand, credible bone Zn levels for the PBS-injected salmon groups were found to be lower than the ones reported by Gil Martens et al. (2006), especially for the fast growing SPC35 group of salmon; possibly indicating a state of subclinical Zn deficiency or a trend towards it. Overall, an effect of the growth rate on bone Zn levels during the second period of the feeding trial was demonstrated, with slow growing vaccinated fish showing higher skeletal Zn values. It is suggested that despite the high level of Zn present in the diets of the salmon (an average of (250 mg $\times$ kg^{-1}), and considering that Atlantic salmon requirement for Zn is 37-67 mg $\times$ kg^{-1} (Maage and Julshamn, 1993), Zn availability from both protein sources used for the formulation of the diets (FM and SPC) seems to be low, especially for fast growing salmon parr of ~30g. It is thus possible that higher supplementation of these diets with inorganic Zn salts ($ZnSO_4$ and

$ZnNO_3$) could compensate for the reduced dietary Zn availability (Lall, 2003). Therefore, a combined increment in both P and Zn supplementation is suggested for diets containing increasing levels of dietary SPC, to balance the availabilities of these elements and their incorporation into bones.

Estimated vertebral Mn levels for fast growing SPC35 salmon were found to be lower than the values reported by Gil Martens et al. (2006) at 62 dpv, while SPC58 and SPC80 salmon revealed similar or higher levels. This could be an indication of decreased availability of Mn in the SPC35 diet and the requirement for increased Mn supplementation. Storebakken et al. (1998b) suggested that the uptake of Mn in Atlantic salmon could be entirely controlled by the gut, which could explain the increasing amounts of this element in salmon fed increasing amounts of SPC (containing higher Mn Table 8) at all sampling time points. In Chapter 3 an improvement in body cross-section expected ash, Ca: P ratio, Ca, Mg, Mn and P levels in salmon juveniles receiving increased dietary SPC for a total of 97 days (and 34 dpv) was recorded, compared to the levels detected at Day 63, just prior to vaccination, despite an overall trend of linear reduction over the full course of the trial. However, the main difference was that dietary levels of supplemental phosphates were kept constant among dietary treatments ($30\ g \times kg^{-1}$) in the aforementioned trial. Therefore, diets with increasing SPC levels contained lower amounts of bioavailable P (Storebakken et al., 1998b, 2000). In addition, the dietary source of P was di-calcium instead of mono-calcium phosphate, which was used as an alternative P source in the present study. Since phosphates with an adverse Ca:P ratio are preferred for salmonid nutrition due to their higher digestibility and increased availability of P (Davies and Serwata, 2004), the use of mono-calcium phosphate as a source of inorganic P in salmon feeds could have influenced a better P and mineral concentration in the long term. Another discrepancy between the previous and

the current study was that salmon in the previous trial were maintained under continuous light, which acts as a growth promoter (Kråkenes et al., 1991), thus reducing mineral levels in salmon (Fjelldal et al., 2004, 2005; Wargelius et al., 2009). Lastly, the developmental stage of Atlantic salmon parr could have been an additional factor for the observed differences in elemental levels prior and post-vaccination seen between the two trials (Poppe and Koppang, 2014).

5.5 CONCLUSIONS

Replacement of 80% of high quality FM protein with SPC in the diets of early stage Atlantic salmon parr is not recommended and the penalty in growth is quite severe with 17 and 23% loss in WG in vaccinated and PBS injected-salmon, respectively. Diets for early stage Atlantic salmon parr with 58% of protein from SPC can provide similar growth to parr fed on a commercial diet containing 35% of protein from SPC without compromising skeletal mineralisation. Furthermore, it was shown that the decrease in bone mineral levels observed during the initial 3 month period in early stage salmon parr fed with high SPC inclusion diets when combined with increased mono-calcium phosphate could be reversed over time, with vaccinated fish exhibiting even higher levels of vertebral mineralisation than unvaccinated fish. Therefore, the interaction of dietary SPC and fish vaccination does not negatively affect bone mineral concentrations. In addition, it was shown that long term provision of high SPC inclusion diets could eliminate the initial reductions in bone minerals observed in the initial 3 month feeding period. Thus, increasing dietary SPC levels balanced with increased phosphate and amino acid inclusion do not seem to adversely affect bone mineralisation when fish are held under 12h light:12h dark conditions.

Chapter 6.

The effects of increasing dietary levels of soy protein concentrate and phosphates on the disease resistance (furunculosis) of vaccinated and non-vaccinated early stage Atlantic salmon (*Salmo salar*L.) parr

6.1 INTRODUCTION

Farmed Atlantic salmon are typically raised in intensive aquaculture production systems and fed nutritionally complete formulated diets. Historically, fish meal (FM) has been the source of protein and essential amino acids for salmon feeds (Rumsey, 1993). In 2009, aquaculture's use of global FM production was estimated to be 68%, with salmonid aqua-feeds consuming 13.7% (FAO, 2012). Unless alternative sources are used, the reliance on FM for aquafeed diets may reduce the potential for salmon culture growth, since the worldwide demand for FM is rapidly exceeding supply, and new sources of protein are needed for the sustainable production of Atlantic salmon. Given that plant feedstuffs are readily available, these have received most attention as an alternative to FM (Kaushik, 1990; Tacon, 1994; Olli et al., 1994; Carter and Hauler, 2000).

Among plant protein ingredients, SPC manufactured through aqueous alcohol extraction is a very promising protein source for Atlantic salmon. Alcohol extracted SPC has a protein content, which is very similar to that of FM (NRC, 1993), while its EAA content compares favourably with FM, with the exception of methionine and potentially lysine (Masumoto et al., 1996). Furthermore, lectins, saponins soy antigens and trypsin inhibitors concentrations, which are ANFs, are found at lower the concentrations than those found in conventional SBM (Peisker, 2001; Russett, 2002; Hart et al., 2007). Several studies have demonstrated the suitability of SPC as an alternative to FM in Atlantic salmon post smolt diets (Refstie et al., 1998; Storebakken et al., 1998b, 2000). Moreover, a few studies reported the absence of soybean-induced intestinal inflammation in salmonids receiving diets with even 100% substitution of FM with SPC (Krogdahl et al., 2000; Escaffre et al., 2007). However, the tolerance of salmon for plant feedstuffs depends on salmon size and stage. Burr et al. (2012) demonstrated that early stage Atlantic salmon parr are much more sensitive to dietary vegetal protein inclusion than late stage Atlantic salmon parr. While studies on the

use of soybean meals in salmonid diets and their subsequent effects on immune function have been widely undertaken, only one reported study by Krogdahl et al. (2000) has investigated the effects of dietary SPC on the immunity of Atlantic salmon. Krogdahl et al. (2000) demonstrated an enhancement of lysozyme activity and total IgM levels in the intestinal mucosa of Atlantic salmon smolts, maintained on feeds with 30% of dietary protein from soy products (SBM and SPC), and in turn, enhanced the resistance of SPC fed salmon to infection by *A. salmonicida* the causative agent of furunculosis. Furunculosis is a highly infectious disease, causing serious fish losses, such as those observed during the epidemic of 1991-1992, which led to the loss of approximately 25% of the total Scottish salmon production (Austin, 1997). Successful vaccination has enabled the disease to be brought under control and currently the majority of farmed Scottish and Norwegian Atlantic salmon are vaccinated against fununculosis. Thus, vaccination with a commercial *A. salmonicida* vaccine and subsequent infection challenge of the experimental Atlantic salmon parr in this study was used to describe the effects of increasing dietary SPC levels on vaccination and resistance of Atlantic salmon parr against furunculosis. Since the site(s) of pathogen uptake into fish, is a subject of conjecture and seems likely to include gills, mouth, anus and/or surface injury (Klontz, 1968; McCarthy, 1980; Hodgkinson et al., 1987) an i.p. injection of *A. salmonicida* was used for the infection of Atlantic salmon parr in this study.

Generally, soy products and several other vegetable derived products used as FM replacements have been shown to stimulate several innate immune responses in fishes and these have been interpreted as inflammatory/hypersensitivity or immunostimulatory effects (Rumsey et al., 1994; krogdahl et al., 2000; Sitjà-Bobadilla et al., 2005). The aim of the current study was to monitor how increased dietary SPC, methionine, lysine and phosphate inclusion (to give similar amino acid/protein ratios and increase the availability of P in diets with higher levels of SPC) affects the immunological responses of naïve and vaccinated

(against *A. salmonicida)* Atlantic salmon parr and their protection against furunculosis after i.p. infection with *A. salmonicida*.

6.2 MATERIALS AND METHODS

6.2.1 Diets and fish husbandry

The dietary trial was carried out at the Aquatic Research Facility (ARF), Institute of Aquaculture, University of Stirling and lasted 177 days. The feeding trial started in June, 2013 and ended in December 2013. The fresh water system consisted of twelve 100 l circular tanks supplied with flow-through water at a rate of 1.5 l × min^{-1}. Water temperature was maintained at 12 ± 1ºC (ambient temperature of 12 ± ºC for the first 3 months of the study and application of heating later on Day 115 of the feeding trial to maintain the temperature constant throughout the study), whilst photoperiod was constant to prevent smoltification (12 hours of light: 12 hours of darkness). Dissolved oxygen, ammonia, nitrate, nitrite and pH were monitored and remained within limits recommended for Atlantic salmon. Prior to the trial unvaccinated S1 Atlantic salmon parr (AquaGen QTL eggs - AquaGen Ltd, Kilmacolm, Scotland - selected for improved growth and resistance to IPNV) purchased from Scottish Seafarms Ltd (Dumfries, Scotland, UK) previously maintained on a commercial BioMar (BioMar Fishes Ltd, Grangemouth, Scotland) diet (BioMar Inicio PLUS) were allowed to acclimate for a week within two stock tanks, during which time they were maintained on a commercial EWOS (EWOS Ltd, Westfield, Near Bathgate, Scotland) diet (EWOS micro). The fish were then randomly allocated into the twelve trial tanks, each tank containing 130 individuals in which they were allowed to acclimatise for 7 days. The fish had an average weight of 9.3 g at the start of the trial. The fish were then starved for two days and were subsequently fed a mixture of the commercial feed and the trial diets they were assigned to. The experimental diets contained different levels of protein from SPC (35,

58 and 80% of protein from SPC) replacing FM and were manufactured by EWOS Innovation, Dirdal, Norway. Protein/fat ratios were kept constant (~3.0), while methionine, lysine and phosphorus (P) supplementation increased concomitantly with increased dietary SPC inclusion. Each dietary treatment included four replicate tanks. Dietary formulations are presented in Table 8 of Chapter 5. Parr were acclimatised to the trial feeds for 3 days prior to the start of the trial. After the initiation of the trial the fish were maintained on the trial feeds for about 6 months (177 days). Eight fish per tank were sampled on Day 92 of the feeding trial prior to vaccination with a commercial anti *A. salmonicida* and infectious pancreatic necrosis virus vaccine (Alpha-Ject 2-2, Pharmaq) to monitor their immune status (2 serum and 2 head kidney pools from 4 fish per tank were sampled for the performance of several different immunoassays) and vertebral mineral composition (8 fish per tank for vertebral mineral analysis). After the first sampling approximately 80 fish per tank were divided between the original set of tanks and another set of 12 replicate tanks with the ones kept in the original set of tanks being vaccinated with the above vaccine and the salmon parr transferred to the replicate set of tanks being injected with 0.02 M phosphate buffer saline (PBS) (0.15M NaCl, pH 7.2). The remaining salmon parr (~40 fish distributed in a third set of 3 tanks -1 tank per diet-) were to establish the lethal dose of bacteria giving 70% mortalities when fish were experimentally infected with *A. salmonicida*. In order, to trigger specific antibody production in vaccinated fish at post vaccination water temperature in the tanks was increased at 10ºC with the use of heaters. Pools of serum samples (2 serum and 2 head kidney pools from 4 fish per tank) were taken at 2 days post vaccination (2 dpv) (serum samples from only vaccinated individuals/head kidney samples from both vaccinated and PBS-injected salmon) (Day 94 of the feeding trial) and at 62 dpv (Day 154 of the feeding trial-sampling of vaccinated and PBS-injected salmon).

6.2.2 Disease resistance

On Day 154 of the feeding trial (62 dpv), twenty five salmon from each tank of vaccinated and PBS-injected fish were removed and stocked in another two sets of 12 replicate tanks in the ARF. The tanks used were also circular fiberglass tanks supplied with flow-through fresh water as described above. The fish were housed under a controlled photoperiod (12 h of light: 12 h of darkness) at a controlled temperature of 13-15ºC. Seventy five hours before *A. salmonicida* was administered; a fresh culture of the passaged bacterium was prepared on a blood agar plate. Twenty seven hours before the commencement of the challenge, seven bacterial colonies were cultured in tryptone soy broth (15ºC for 18 h). Subsequently the bacteria were washed twice with sterile PBS with intermediate centrifugation (3500 × g, 10 min). The OD of the bacterial suspension was then adjusted to 1.0 at 610 nm (6×10^8 cfu × ml^{-1}), and serially diluted to 0.25×10^{-7} (corresponding to ~ 2×10^2 cfu × ml^{-1}), which was the dilution found to give approximately 70% mortalities in salmon parr in a pre challenge trial. Cell densities were confirmed by distributing eight 25 µl drops of each one of the obtained serial bacterial suspensions (1.0×10^{-7}, 0.5×10^{-7}, 0.25×10^{-7}, 1.0×10^{-6}) onto tryptone soy agar plates (TSA) (Sigma-Aldrich) and colonies counted after 48 h. One-hundred microlitres of the 0.25×10^{-7} bacterial suspension (corresponding to and 2×10^1 cfu × $fish^{-1}$) was i.p. injected into each Atlantic salmon after anaesthetizing them (benzocaine, 30 mg × l^{-1}). Specific mortalities were confirmed by culturing kidney swabs onto TSA and checking colonial morphology. The challenge was terminated after 30 days, at which time mortalities had ceased.

6.2.3 Head kidney macrophage isolation, respiratory burst and phagocytic activity by head kidney macrophages

For the isolation of head kidney macrophages (HKMs), the head kidney was teased through a 100 µm nylon mesh (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) into 2.5 ml L-15 containing 40 µl of heparin (10 IU $\times$ ml^{-1}). The mesh was rinsed with 2.5 ml of the medium and the cell suspension placed on ice. Levels of O^{-2} production by head kidney macrophage suspensions were measured by the conversion of nitroblue tetrazolium (NBT; Sigma-Aldrich) to formazan, following the method described by Secombes (1990), with some modifications described by Korkea-aho et al. (2011). The head kidney cell suspension was also used to determine phagocytosis activity by head kidney macrophages as described by Korkea-aho et al. (2011). A more thorough description of the methodologies is given in Section 2.4.1.

6.2.4 Determination of serum glucose, protein and lysozyme activity

Serum glucose was determined using a CONTOUR blood glucose monitoring system (Bayer HealthCare LLC) according to manufacturer's instructions. Briefly a CONTOUR strip was inserted accordingly into the Contour blood glucose monitor and then 5µl of serum were pipetted onto the blood receiving end of the CONTOUR strip and held for 5 sec until the test result was displayed on the screen of the monitor. Protein content of serum was determined using a Pierce BCA (bicinchoninic acid) protein determination kit (Thermo Scientific, IL, USA) using bovine serum albumin (BSA) as a standard. Serum lysozyme activity was estimated according to the protocol described by Korkea-aho et al. (2011), based on the lysis of lysozyme sensitive *Micrococcus lysodeikticus*. A thorough description of the latter two methodologies is given in Sections 2.4.4 and 2.4.3 respectively.

6.2.5 Determination of serum total IgM

The level of IgM in sera of experimental fish was determined using an indirect enzyme linked immunosorbent assay (ELISA) (Magnadottir and Gudmundsdottir, 1992), with modifications. A more detailed description of the applied method is illustrated in Section 2.4.7.

6.2.6 Determination of specific antibody against *Aeromonas 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). Briefly, 96-well Immulon™ 4HBX plates (ThermoScientific, Maine, USA) were coated with 50 µl of 0.05 % w/v poly-L-lysine (Sigma-Aldrich) in 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6 and incubated for 60 min at 21°C. Plates were then washed twice with a LSWB. *A.salmonicida* (Hooke) in 0.1M PBS was added to the wells at 100 µl × well^{-1} and plates incubated overnight at 4°C. Fifty microliters per well of 0.05 % v/v glutaraldehyde in PBS was added to the bacteria and the plate incubated at 21°C for 30 min before washing three times with LSWB. Non-specific binding sites were blocked by incubating plates with 3% w/v skimmed milk powder in water at 21°C for 120 min. After washing the plates three times with LSWB, 100 µl of serially diluted fish serum diluted in 1 % casein (from 1: 50, 1: 200 and 1: 1000) was transferred to the ELISA plate, which was then incubated overnight at 4°C. Both positive (serum pools from challenged salmon survivors which have been vaccinated prior to challenge) and negative controls (serum blanks/ pooled serum from naïve salmon) were also added to each plate. Plates were washed five times with HSWB with a 5 min soak on the last wash. Anti-rainbow trout/Atlantic salmon IgMmonoclonal antibody (F11-monoclonal anti trout/salmon IgM - Aquatic Diagnostics Ltd, Stirling, Scotland) was then added and plates were incubated at 21°C for

60 min. The steps followed until the development of the plates were the same with those described above. The percentage of specific antibody production was estimated by the comparison of positive (pooled serum from vaccinated and challenged salmon, 100 % antibody production) and negative controls (pooled naïve salmon serum, 0% antibody production) and was expressed as percentage of specific antibody production.

6.2.7 Measurement of alternative, classical and overall complement activity

Salmon antiserum against sheep RBC was produced by immunising fish i.p. with 10^9 sheep red blood cells (SRBC) in PBS (0.15 M phosphate-buffered saline, pH 7.2). Four weeks after priming a booster injection (10^9) was given, and two weeks later, fish were bled. Control fish were injected with PBS. Endogenous complement activity of anti-SRBC salmon serum was inactivated by heating at 50°C for 30 min and the anti-SRBC serum was diluted with 0.1% Gelatin-Complement Fixation Buffer (G-CFB) (1 tablet of Oxoid complement fixation tablets in 100 ml of warm water and 0.1 g of gelatin from Sigma-Aldrich) with 20mM EDTA. Diluted anti-SRBCs were then stored at – 20°C. Sheep blood (Oxoid) was stored at 4°C in Alsever’s solution (1: 1) for 1 week before use. The SRBCs were used to determine lysis by the alternative complement pathway (ACP), while SRBCs, sensitized (60 minutes, 37°C) with pooled and diluted (1: 400) salmon anti-SRBC serum, was used for determination of total (TC) and classical complement pathway (CCP) activity. Buffer for the AC was 0.01 M EGTA-Mg-G-CFB and for determination of the total and classical haemolytic activity G-CFB. Tests were done in round-bottomed 96-well microtiter plates (Sterilin).Briefly complement activity determination was based on methods described by (Yano et al., 1987, 1988) with modifications. Briefly serum was diluted four times in double serial dilutions accordingly (starting from 1:4 for the estimation of AC activity and 1: 16 for the estimation of TC and CC activity) and 25 μl of each dilution was added to each well of a non-absorbent

U-well micro-plate (Sterilin) in duplicate. Ten microliters of 0.5 % SRBC suspension was added to each serum dilution. Controls on each plate comprised 0.1 % anhydrous Na_2CO_3 (v/v) (100 % lysis) replacing serum. G-CFB replacing serum (0 % lysis) and serum blanks (duplicate wells of serum dilutions with G-CFB replacing SRBC suspension). The plates destined for the estimation of TC and CCP activity also included, a CC control sensitization of SRBC with non-immune pooled carp serum and a standard complement sample (serum pool) for correction of plate differences were included. Microtitre plates were incubated at 22°C for 90 min with constant shaking and the reaction terminated by the addition of 140 µl G-CFB with 20mM EDTA, followed by centrifugation at 1500 × g to spin down the remaining SRBCs. After centrifugation 100 µl of the supernatant from each well was transferred to a new flat-bottomed 96-well non-absorbent micro-titre plate (Sterilin). The absorbance of the wells was read at 450 nm using a micro-plate reader (Synergy HT; BioTek Instruments, Winooski, VT, USA) and the percentage lysis of SRBCs calculated. The absorbance values of samples were corrected by subtracting the absorbance of the sample blank control (0 % haemolysis). Details regarding the estimation of serum haemolytic activity by the different complement pathways are given in Section 2.4.5.11, whereas descriptions about the preparation of SRBCs, buffers, collection of salmon anti-SRBC serum, dilution of serum required for optimal sensitization and preparation of sensitized SRBCs are given in Section 2.4.5.

6.2.8 Diet composition analysis

Dietary crude fat was determined following acid hydrolysis using a Soxtec System 1047 hydrolysing unit (Tecator Application note 92/87) followed by exhaustive Soxhlet extraction using petroleum ether (40–60°C boiling point) on a Soxtec System HT6 (Tecator application note 67/83) as described by (Bell et al., 2001). Dry weight and ash contents of diets were determined after oven-drying the samples to constant weight (at 100 ºC) and by ashing dried

samples in an oven at 550°C (AOAC, 1990). Dietary energy content was determined through bomb calorimetry (AOAC, 1990). For the determination of phytate content in the diets a Megazyme Phytate/Total Phosphorus Assay kit (Megazyme, Ireland) was used. Dietary energy content was determined through bomb calorimetry (AOAC, 1990). More details regarding the aforementioned methodologies are given in Section 2.6.

Dietary carbohydrate was determined following a modified Dubois phenol sulphuric method. Dietary fibre was determined after subjecting defatted dietary samples (3 washes with petroleum ether) within pre-weighed organic capsules, to acid (with 1.25% sulphuric acid solution)and alkaline hydrolysis (with 1.25% sodium hydroxide solution) for 35 min each, using a Fibertec system 1020 hot Extractor. Following one last defatting step (3 washes with petroleum ether), the samples were ashed at 600°C in a muffle furnace (Gallenkamp Muffle Furnace) for 4 hours, cooled in a desiccator and reweighed (W2). Extracted fibre was expressed as percentage of the original undefatted sample and calculated according to the formula:

$$Fibre(\%) = \frac{(W1 \times 1.0011) - Capsule\ weight - (W2 - 0.0025)}{Sample\ weight\ (g)} \times 100$$

Where W1 is the initial weight of the unprocessed dietary sample and W2 is the weight after processing. More details regarding the determination of dietary carbohydrates and fibre levels are given in Sections 2.6.7 and 2.6.8.

Dietary minerals and phosphorus were determined using inductively coupled plasma mass spectroscopy (ICP-MS) with collision cell technology (CCT) (Thermo X Series 2). A more thorough description of the method is given in Section 2.6.5.

6.2.9 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). Moreover a hierarchical (multilevel) statistical model was used for the mineral analysis of pooled body cross-sections 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). Firstly, data were normalised as described in Section 2.9. 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., 2014). The statistical approach applied was model-based. This means that to find if 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 non-linear effects:

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

The three models were nested and compared with a likelihood ratio test (LRT) that evaluated if 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). In the present study two differentially treated (vaccinated and PBS-injected) salmon populations were achieved by the end of trial, therefore data obtained from the vaccinated and PBS-injected groups of salmon were modelled separately, together with data obtained for pre-injected salmon as described above.

Further statistical modelling was applied using R language and its corresponding packages (reshape, xtable and coxme) for the analysis of the disease challenge trial results. The three SPC levels were modelled with the help of polynomial models. Nested models with increasing complexity up to a second-degree polynomial of the SPC inclusion were fitted and compared by a LRT, based on a multilevel Cox proportional hazard model.

6.3 RESULTS

6.3.1 Immune Responses

Credible levels of serum proteins were only affected by time and not by dietary levels of SPC in both vaccinated and PBS-injected groups of salmon, when modelled together with pre-injected naïve salmon (the model with only the sampling time was selected with a *P<0.05*). A sharp decrease in serum protein levels was exhibited in vaccinated salmon at 2 dpv while at 62 dpv serum protein increased compared to the levels at 2 dpv. Nevertheless, serum protein concentrations were found to be lower than initial pre-vaccination levels. On contrast, serum protein levels in PBS-injected fish doubled at 62 days post PBS injection (62 dpPBSinj) when compared to naïve salmon.

Expected concentrations of serum total IgM did not exhibit any differences among salmon dietary groups (the models with only the sampling time were selected with *P values<0.05* in both cases). While no differences were observed in total IgM levels between naïve and PBS-injected salmon serum, in vaccinated salmon total IgM levels demonstrated

a decrease at 2 dpv compared to their initial pre-vaccination values, whereas at 62 dpv total IgM increased 4-fold compared to the concentration in the serum of naïve salmon. Specific IgM was measured at 62 dpv and no differences were detected among the three dietary groups of salmon.

Lysozyme activity was another immune response exhibiting no changes among the dietary groups of salmon. Both naïve and PBS-injected salmon at 62 dpPBSinj exhibited no changes in expected lysozyme activity levels, however an increment was obtained at 2dpv compared to naïve parr values, followed by a further increase at 62dpv (the model with only the sampling time was selected with a $P<0.05$). Credible serum glucose concentrations did not show any differences among experimental dietary groups. Moreover, no changes were detected in naïve and PBS-injected salmon serum glucose. However at 2 dpv, glucose levels were found to be higher than pre-vaccinated salmon, while at 62 dpv glucose levels in vaccinated groups were lower than in naïve salmon (the model with only the sampling time was selected with a $P<0.05$).

While the amount of SRBC haemolysis due to total complement activity did not show any differences between the dietary groups, the contribution made by the different complement pathways differed in fish receiving increasing levels of SPC. Statistical analysis has shown that serum expected ACP activity decreased linearly upon increased dietary SPC protein uptake at 62 dpPBSinj compared to naïve salmon, in which AC activity increased linearly (model plotted in Fig. 34A). No significant differences were observed prior vaccination/PBS-injection whereas both SPC58 and SPC80 salmon demonstrated significant reductions in AC activity at post PBS-injection. Similarly, levels of serum AC activity presented a linear reduction at both time points post-vaccination when salmon were fed diets with increasing SPC levels (model plotted in Fig. 35A). Significantly lower expected AC activity was observed for both SPC58 and SPC80 salmon at both timepoints post vaccination

compared to SPC35 salmon (Mean values lie outside the 95% C.I. of the SPC35 group). Furthermore, salmon parr fed increasing dietary levels of SPC presented a linear increase for expected classical complement activity (CCP) at both timepoints post vaccination, demonstrating an inverse trend in comparison to pre-vaccinated/PBS-injected salmon (model plotted in Fig. 35B). Significantly higher CC activity was observed at the last timepoint (62dpv) for both SPC58 and SPC80 salmon in contrast to SPC35 salmon. No differences on expected CC activity prior to or post-PBS injection, were demonstrated. Furthermore, credible levels of TC activity were unchanged among the different dietary groups of vaccinated and PBS-injected salmon, while no differences were noted between naïve and vaccinated salmon. However, an increase of almost 18 % in TC activity was obtained for all dietary groups at 62 days after their injection with PBS.

No changes were detected in expected PMA-stimulated and non-stimulated HKM respiratory burst and HKM phagocytic activity among dietary groups of vaccinated or PBS-injected salmon parr. For the vaccinated salmon parr, the trend obtained was similar for all the HKM responses measured between the dietary groups, with non- and PMA-stimulated respiratory burst activity, phagocytic index levels and the percentage of phagocytosis increasing sharply at 2 dpv compared to pretreated values and decreasing at 62 dpv below naïve salmon levels (the models with only the sampling time were selected with *P values* <0.05 in all cases). For PBS-injected salmon, respiratory burst and phagocytic index levels were similar to the ones obtained for vaccinated salmon at 2 and 62 dpv exhibiting an almost equal increase at 2 dpPBSinj, followed by a subsequent reduction at levels lower than the ones determined for naïve salmon except for PMA-stimulated HKM, which presented a higher activity at 62 days post injection compared to naïve salmon (the models with only the sampling time were selected with *P values* <0.05 for all the aforementioned cases). Lastly expected phagocytosis percentage was affected in a curvilinear fashion in PBS-injected

salmon. Salmon at prior and 62 dpPBSinj presented similar trends with the SPC58 group demonstrating higher levels than the other two groups. Nonetheless, significant differences were detected only at 62 dpPBSinj, when both SPC58 and SPC80 salmon exhibited a higher percentage for HKM phagocytosis. A trend of an almost linear increment of HKM phagocytosis was detected at 2dpPBSinj (model plotted in Fig. 34B), however, no significant changes were detected among the dietary groups of fish.

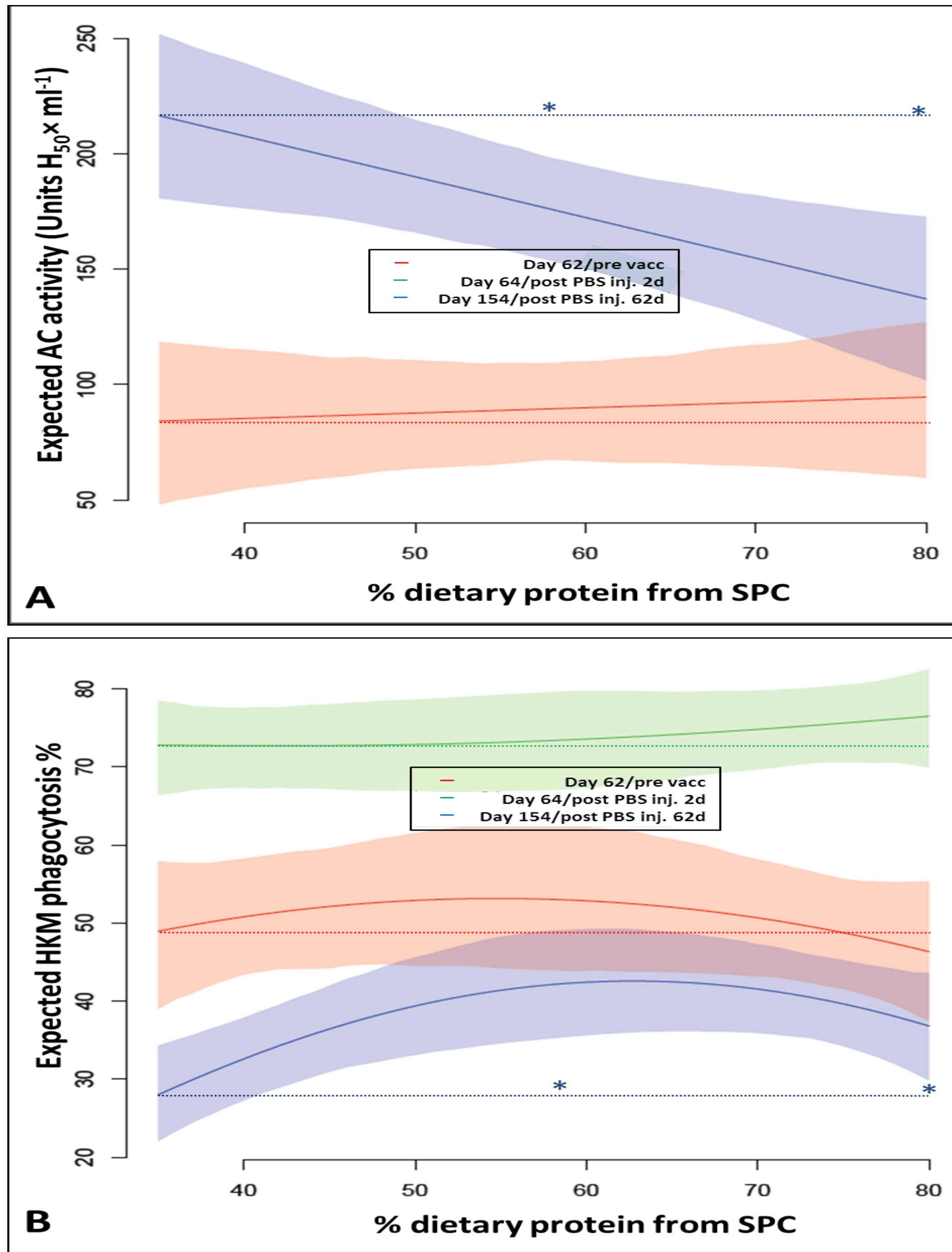

Figure 34 Modelled Immune responses (PBS-injected salmon). Expected mean immune responses (with 95% C.I.) from serum pools (4 fish × tank^{-1}) of naïve and vaccinated Atlantic salmon parr in relation to dietary protein from SPC: (A) alternative complement activity (haemolytic units H_{50}× ml^{-1}); (B) Percentage (%) of head kidney macrophages performing phagocytosis.Red line connects the expected mean values for each measurement at prior vaccination (prevacc) (Day 92) while red shaded region indicates the 95% confidence region. Similarly, green and blue lines and their corresponding shaded regions represent the respective values for each one of the measurements above at 2 and 62 days post vaccination (2 and 62d post vacc) (Days 92 and 154 of the feeding trial respectively). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

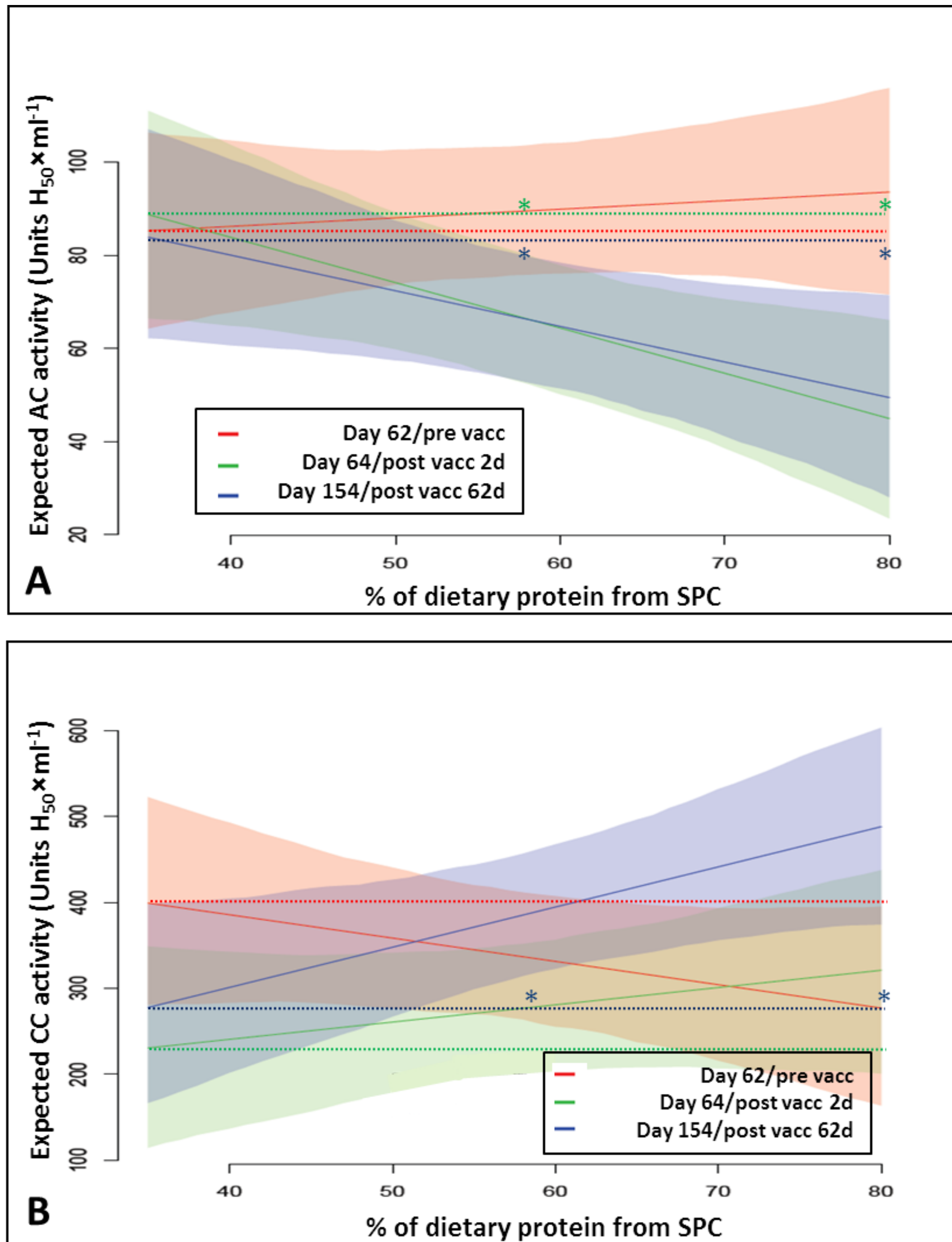

Figure 35 Modelled Immune responses (vaccinated salmon). Expected mean immune responses (with 95% C.I.) from serum and head kidney macrophage pools (4 fish × $tank^{-1}$) of naïve and PBS-injected Atlantic salmon parr in relation to dietary protein from SPC: (A) alternative complement activity (haemolytic units H_{50} × ml^{-1}); (B) classical complement activity (haemolytic units H_{50} × ml^{-1}).Red line connects the expected mean values for each measurement at prior PBS-injection (Day 92) while the red shaded region indicates the 95% confidence region. Similarly, green and blue lines and their corresponding shaded regions represent the respective values for each one of the measurements above at 2 and 62 days post PBS-injection (Days 92 and 154 of the feeding trial respectively). Dashed horizontal lines of the aforementioned colours denote the mean values for salmon fed diets with 35% of dietary protein from SPC for each timepoint. 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 aforementioned 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.

6.3.2 Disease resistance trial

No mortalities were observed for any of the dietary groups vaccinated against *A. salmonicida* after challenging them with the bacterium. Therefore, no further statistics for the establishment of survival differences among different dietary groups were performed. The raw data of PBS-injected salmon cumulative survival during the experimental infection are presented in Fig. 36. However, mortalities were obtained from all dietary groups in PBS-injected and subsequently challenged dietary groups of Atlantic salmon parr. Mortalities from these fish first started to occur at 6 days post challenge, but only for the SPC35 salmon group. On Day 7 post-challenge the first mortalities from SPC58 salmon were observed, while on Day 8, mortalities from the SPC80 group of salmon also started to occur. Mortalities ceased on Day 19 post-challenge. At this time, the survivors for the SPC35 group from all tanks were 38 fish corresponding to 56% survival. Fourty five surviving salmon were recorded for the SPC58 group which was equivalent to 66% survival while 48 survivors revealing a 71% survival were reported for the SPC80 group of salmon. Statistical analysis revealed no significant differences among different dietary groups (*P value* < 0.05).

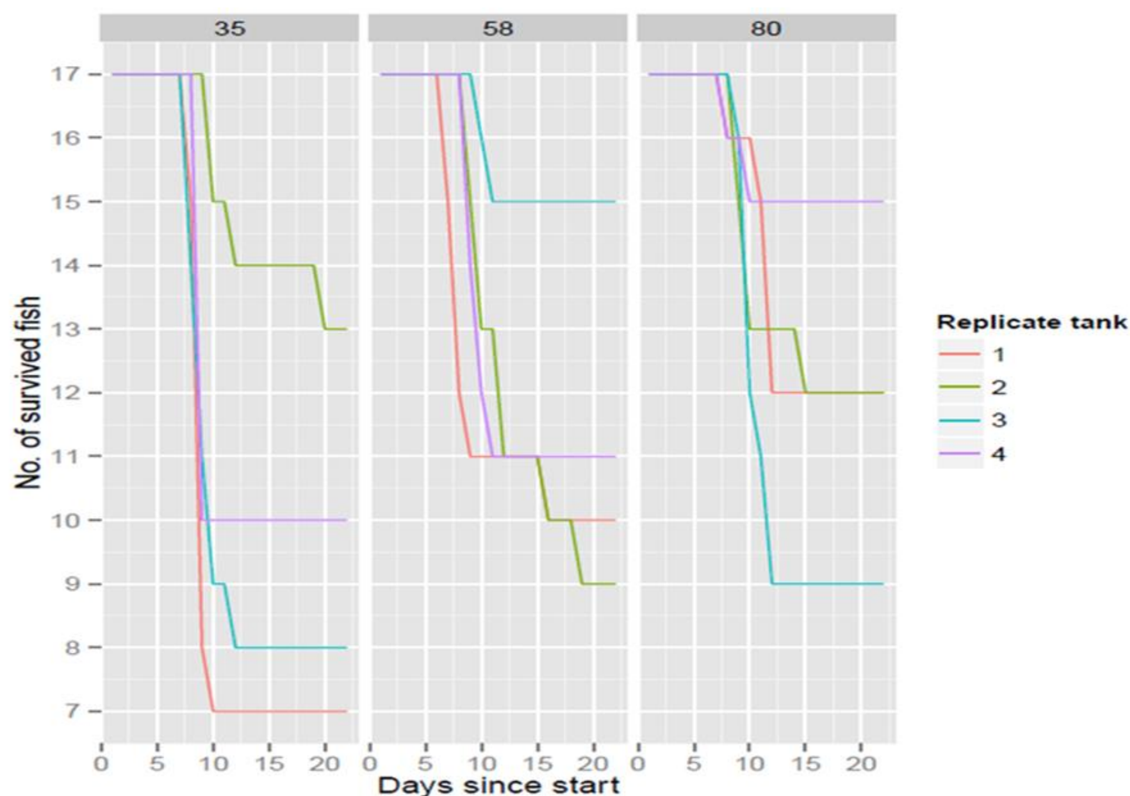

Figure 36 Observed survival in the quadruplicate tanks of the experimental dietary groups of intra-peritoneally challenged against *A. salmonicida* Atlantic salmon parr. Stripped text above each panel gives the percentage of protein from SPC. Broken lines with different colouration give the percentage of daily survival in each one of the quadruplicate tanks in each treatment for 23 days.

6.4 DISCUSSION

In the present study, determination of serum protein levels was used to evaluate the general condition of experimental salmon (Waagbø et al., 1988), whereas serum glucose was measured for the evaluation of the general stress status of fish from different dietary groups (Iwama, 1998). Furthermore the immune parameters measured in the present study are salient components of salmon defence mechanisms against diseases. Lysozyme and macrophage phagocytosis followed by oxygen radical production, are important bactericidal and parasiticidal agents (Secombes, 1990; Rumsey et al., 1994), while complement, and total and specific IgM are linked with the neutralisation and opsonisation of various pathogens (Nikoskelainen et al., 2002; Kiron, 2012). Furthermore, Thompson et al. (1996) suggested

that measuring defence mechanisms prior to challenge only represents resting levels. However, measuring immune function after immune stimulation (either through challenge or vaccination) may highlight dietary modifications that were not evident before. This is further highlighted by mammalian research, which has demonstrated that nutrients are preferentially directed towards the immune system, rather than growth, during times of infection (Beisel, 1977). This includes the distribution of amino acids towards the liver for the synthesis of acute phase proteins (Wannemacher, 1977) and suggests suboptimal nutrient intake prior to infection may result in a diminished immune response. It therefore seems a logical step to evaluate the immune function of the fish after its dietary protein source has been altered. Therefore, salmon parr receiving the experimental diets in the present trial were initially vaccinated and subsequently challenged with *A. salmonicida*.

While most of these immunological parameters (serum lysozyme, protein, total IgM, head kidney macrophage phagocytic activity and oxygen radical production) have been previously shown to be modulated by dietary change in various teleosts (summarized by Kiron, 2012), no modification relative to the control SPC35 diet occurred prior or post-vaccination in the present study. Similar to the current trial, Bransden et al. (2001) reported no differences in lysozyme activity, plasma concentrations of total IgM, total protein or glucose levels of non-immunized salmon parr fed on diets where dehulled lupin meal replaced 40% of FM. Furthermore, in line with the present study, Jalili et al. (2013) demonstrated no differences in serum lysozyme levels of non-immunized rainbow trout (*Oncorhynchus mykiss*) fed on diets fully based on mixed plant proteins. Rumsey et al. (1994) reported increased lysozyme activity in rainbow trout fed SBM diets. Contrary to this, Farhangi and Carter (2001) revealed decreased serum protein levels in rainbow trout receiving 30, 40 and 50 % of dehulled lupin meal, compared to trout fed 10% dehulled lupin meal, whereas no changes were detected in serum glucose levels. Vaccination is a stressful

process, which could explain the sharp increase in serum glucose at 2 dpv (van Muiswinkel and Wiegertjes, 1997; Siwicki et al., 2002). Furthermore, the stress-related reduction in fish appetite due to vaccination could explain the concomitant reduction in serum protein and total IgM levels observed at 2 dpv in vaccinated salmon (Melingen and Wergeland, 2000). The observed initial reduction in serum total IgM could also be related to the formation of antibody-antigen complexes, reducing the number of free circulating natural antibodies at 2 dpv (Bricknell et al., 1999; Villumsen et al., 2012).

The reduction in serum glucose levels below those of pre-vaccinated or PBS-injected salmon at 62 days post injection demonstrates the reduced responsiveness to acute stressors (e.g. sampling processes such as netting and exposure to anaesthetic) of salmon subjected to higher stress for a prolonged time period (e.g. vaccinated salmon vs PBS-injected salmon due to immune stimulation) (Espelid et al., 1996; Kvamme et al., 2013). However, the 4-fold increase in total IgM for all dietary groups at 62 dpv is an indication of the efficacy of vaccination (Melingen and Wergeland, 2000), and the absence of polyetiological stress during the study, which could have compromised this response (Siwicki et al., 2002). In the study performed in Chapter 4 increased total plasma IgM and lysozyme activity in SPC50 and SPC65 salmon at all time-points was demonstrated, whereas SPC80 fish presented lower levels by comparison with the aforementioned groups; and similar levels to the SPC35 group. However, the overall pattern of serum lysozyme activity post-vaccination showed that lysozyme increased sharply at 7 dpv, while a reduction below pre-vaccination levels were observed at 34 dpv, whereas total IgM increased rapidly by 7 dpv to a level which was more or less maintained until 34 dpv. The trends in the levels of measured immune responses post-vaccination were not similar between this and the previous study. However, the differences obtained could be attributed to experimental disparities, such as the use of different commercial adjuvanted vaccines (i.e. liquid paraffin vs montanide) (Anderson,

1992), the timing of sampling (2 and 62 dpv vs 7 and 34 dpv), the photoperiod applied (12 h dark: 12 h light vs constant light) (Uribe et al., 2011), the developmental stage of salmon parr used (early vs late stage salmon) (summarised by Gannam and Schrock, 1999) and the dietary P balance (increasing vs constant dietary P addition) (Kiron, 2012). No effects of the SPC inclusion levels on specific antibody production were detected, which is in line with the data from the study presented in Chapter 4, on increasing dietary SPC levels on late salmon parr by our group. Kiron et al. (1993, 1995) demonstrated that the adaptive response (in terms of humoral antibody levels) in rainbow trout fed on different dietary protein levels does not seem to depend on protein quantity in the diet. This could explain why in the present trial, differences in protein intake attributed to the presence of ANFs disrupting normal feeding activity or reduction of protein digestibility, did not affect the production of specific antibodies. Moreover, many studies on the effects of dietary or injectable immuno-stimulants have found no effect on specific antibody production in fish (summarised by (Anderson, 1992; Gannam and Schrock, 1999), which could be an indication that increasing dietary levels of plant proteins with potential immunostimulatory effects, do not promote specific antibody production.

Complement signals the presence of potential pathogens to the host, and engages in destroying and eliminating them through phagocytosis, cytolysis of the pathogens, initiation of the inflammatory response, promoting humoral immunity and the coordination of adaptive immunity development (Boshra et al., 2006). Complement is triggered by one or a combination of three pathways, the alternative, lectin and classical (Boshra et al., 2006). The alternative complement (AC) pathway is a major humoral component of the innate immune response. The classical complement (CC) pathway is an integral part of the specific humoral immunity of the fish, initiated by a complex between an antigen and an antibody, which activates the enzymatic cascade associated with this pathway. Finally, the lectin pathway is

triggered by the formation of complexes between lectins including mannose binding lectin and ficolins, with sugar moieties located on the surface of invading microorganisms (Fujita, 2002; Turner, 2003). The results of the present study indicated that AC activity in early salmon parr could be adversely affected by long term feeding with increased dietary levels of SPC solely or in combination with vaccination, used to assess immuno-stimulation. Furthermore, it was shown that upon vaccination CC activity increased in salmon parr receiving increased dietary levels of SPC, whereas for SPC35 salmon CC activity was found to be reduced. Nevertheless, no differences were detected in TC activity among dietary treatments indicating that overall complement activity (the sum AC, CC and lectin activity) remained constant by maintaining a balance between the different pathways of this integral immunological component, linking both innate and specific immunity. The present study is the first one giving an insight on both complement pathways upon increased dietary plant protein inclusion. In similar studies, increased or unaffected AC activity were reported in gilthead sea bream (*Sparus aurata*) and rainbow trout respectively, in fish fed up to 50% of protein from plant ingredients, while decreased activity was observed in fish fed on diets with higher levels of plant proteins (Sitjà-Bobadilla et al., 2005; Jalili et al., 2013).

While most of the immune related responses measured for HKMs did not show any differences among the different dietary groups, a quadratic effect of dietary SPC on the percentage of HKM phagocytosis was detected in salmon injected with PBS at 62 dPBSinj, when plotted against pre-injection values. This curvilinear effect was much more obvious at 62dpPBSinj, when the percentage of HKMs performing phagocytosis presented higher values in both SPC58 and SPC80 salmon compared to the SPC35 group. This could be an indication of immunostimulation by increasing SPC levels in non-vaccinated salmon. Similarly, Rumsey et al. (1994) demonstrated increased phagocytosis and respiratory burst activity by circulating leucocytes in rainbow trout fed on SBM. However, those findings

were attributed to inflammatory and hypersensitivity processes, since SBM ANFs are linked with intestinal inflammation (Krogdahl et al., 2000). Previously, Burrells et al.(1999) reported that HKM respiratory burst in rainbow trout fed on diets containing 10-50 % of SBM remained unaffected, whereas inclusion levels of up to 80 % caused a reduction of this immune response. Sitjà-Bobadilla et al.(2005), on the other hand, reported higher respiratory burst levels by HK leucocytes in juvenile sea bream fed on balanced diets in which 75 and 100% of FM was substituted with a mixture of different plant protein sources.

Challenge with *A.salmonicida* in the present trial resulted in lower mortality levels (44 %), than expected (~70%) according to the pre-challenge. Reduced virulence for this strain of *A.salmonicida* has been previously observed after long-term storage (6-7 months) of these bacteria in beads at -70ºC or in glycerol at -20ºC (Herath,T., personal communication; Chalmers, L., personal communication). Nevertheless, the data presented suggest that the increasing dietary SPC levels were not detrimental to disease resistance. Similar to the present findings, Bransden et al.(2001) reported no differences in the resistance of salmon parr fed diets with 0 or 40% of FM substitution with dehulled lupin meal when experimentally infected with *Vibrio anguillarum*, whereas Jalili et al. (2013) found no differences in the mortality of rainbow trout fed diets with 0, 40, 70 and 100% replacement of FM when challenged against *Yersinia ruckerii.* Furthermore, Krogdahl et al. (2000) reported increased survival in SPC-fed salmon compared to FM-fed fish challenged through cohabitation with *A. salmonicida*. The proposed reason for the observed reduction in mortalities in SPC fed salmon was the observed increase in IgM levels in the intestinal mucosa of these fish compared to the FM-fed salmon.

6.5 CONCLUSIONS

Overall careful replacement of high quality FM protein with SPC did not promote any differences in the immune parameters or disease resistance of naïve and/or vaccinated salmon against *A. salmonicida,* suggesting that nutritionally balanced diets do not compromise salmon immunity. The current study shows that replacement of 80% of dietary protein with SPC combined with an increased addition of lysine, methionine and P could be used in feeds for early stage juvenile salmon without compromising their immunity or disease resistance against *A. salmonicida*. Further challenge experiments against other bacterial, viral and parasitic diseases are required to further evaluate the effects of high SPC inclusion diets on the disease resistance of Atlantic salmon parr.

Chapter 7.

Effects of marine protein-, marine oil- and marine-free diets on growth and innate immune responses of Atlantic salmon (Salmo salar L.) post-smolts

7.1 INTRODUCTION

The production of aqua-feeds, especially for carnivorous fish such as Atlantic salmon, is currently dependent on the use of FMs and FOs. In the last two decades the increasing demand, for FM and FO, allied to their volatile price and stable demand, has emphasized the need to find alternative protein and lipid sources for salmon aqua-feeds. Numerous protein and lipid sources have the potential to be used as substitutes for FM and FO, respectively, in aquafeeds, including animal by-products (ABPs), vegetable proteins (VPs) and oils (VOs) and products from lower trophic levels such as algal meals and oils. However, due to their high availability, competitive prices compared to FM and FO and their high nutritional properties, VPs and VOs have been the main focus of aquaculture nutrition, as sustainable alternatives to marine derived feedstuffs. On the contrary, the use of other protein sources, such as animal by-products (ABPs) from terrestrial slaughterhouses, although of great potential has not been explored to a similar extend in Atlantic salmon feeds, despite the fact that they have been used in many salmon producing countries including Australia, Canada and Chile. The main reason for that were the legal restrictions established by the European Union (EU), regarding the use of the greater majority of animal derived products, aiming the eradication of transmissible spongiform encephalopathies (TSE) (EU, 2001, 2003). However, these rules have now changed for aqua-feeds (van Dyck, 2012).

Using vegetable-based proteins in aqua-feeds requires that the applied feedstuffs possess certain nutritional characteristics, such as the low levels of non-starch polysaccharides (fibre), oligosaccharides and ANFs (Naylor et al., 2009). Vegetable proteins should also contain a relatively high protein content, favourable amino acid profile, high nutrient digestibility, and reasonable acceptability by the fish. Some of these protein sources such as SPC, wheat and corn gluten possess most of the aforementioned desirable characteristics (Naylor et al., 2009). On the contrary, ABPs are free from ANFs and therefore

their application in salmon feeds could be more desirable compared to their plant protein counterparts. To date several studies using various salmonid species (Higgs et (Higgs et al., 1979; Steffens, 1994; Twibell et al., 2012; Hatlen et al., 2013, 2014) have evaluated the potential of some of these ABPs with promising results. Moreover, recent studies have proven that close to 100% FM replacement with good quality plant and animal- proteins is possible in diets for Atlantic salmon with no negative effect on growth and feed intake when the amino acid profile of the diet is well balanced (Espe et al., 2006; Torstensen et al., 2008). While a few studies have investigated the effects of moderate FM substitution with plant feedstuffs on Atlantic salmon immune responses (Krogdahl et al., 2000; Bransden et al., 2001) the current knowledge about the effects of the complete dietary FM substitution on Atlantic salmon immune responses is non-existent. Moderate levels of dietary SPC in salmon diets have been shown to promote gut immune responses such as lysozyme and total IgM levels and resistance to *A. salmonicida* (Krogdahl et al., 2000). However, Bransden et al.(2001) demonstrated that moderate FM replacement with dehulled lupin meal (DLM) or a blend of DLM with hydrolysed poultry feather meal (HPFM) is possible in Atlantic salmon diets without compromising salmon growth, immune responses and disease resistance. On the other hand, no changes in disease resistance against *Yersinia ruckerii* despite the reduced serum total IgM and alternative complement activity, have been reported in rainbow trout (Jalili et al., 2013).

Similarly to FM, several feeding trials have revealed that FO can be completely substituted by selected single or mixed VOs in Atlantic salmon diets, leaving the FM lipid as the only source of long chain polyunsaturated fatty acids (LC PUFAs) (Bell et al., 2001, 2002; Torstensen et al., 2005, 2008). In fact, Atlantic salmon fed on VO diets demonstrated significantly improved growth and protein utilisation (Torstensen et al., 2005). In salmonids, the dietary inclusion of VO affects membrane composition while alterations on the ratios of

n-6/n-3 C_{20} PUFAs, could have pronounced effects on haematological indices and immune responses of the fish (Waagbø et al., 1993; Thompson et al., 1996; Kiron et al., 2004; Balfry et al., 2006; Petropoulos et al., 2009). In the present trial, diets with complete replacement of FO were supplemented with algal oil as a source of n-3 PUFAs while the n-3/n-6 C_{20} PUFA ratio was relatively constant among FO and FO-free diets (dietary n-3/n-6 C_{20} PUFA ratio ~ 1). Carter et al.(2003) reported no changes on the growth performance and the immune responses of Atlantic salmon fed on diets containing a mixture of canola oil and FO or canola oil and algal (traustochytrids) oil. Today, aquaculture nutrition requires knowledge on the effects of the concomitant complete replacement of FM and FO on both growth performance and immune responses, and similar studies have not yet been reported.

Currently FM inclusion levels in commercial salmon feed formulations range from 15% to 55%, while dietary FO concentrations span from 3% to 40%. These variations largely depend on the country feeds are manufactured, and could partly reflect discrepancies in the employed production systems, local regulations or legal restrictions as well as differences among the cultured salmon breeds (DeSilva et al., 2012). The aim of the present study was to compare the performance of Atlantic salmon post-smolts fed on commercially applied marine protein and oil feed formulations, either according to the European standards of 2011-12 or non-EU standards for salmon producing countries, to salmon fed on fully VP or VO diets or two feeds with complete substitution of both FM and FO with vegetal products (VP and VO) or a combination of VP and ABPs and VO. The evaluation of salmon performance was done by determining the growth, feed consumption and efficiency, carcass proximate composition and lastly the immune function of the tested Atlantic salmon groups. For the present trial, the FM used was tested along with many other FM sources and was found to be consistently the highest performing source. Protein and lipid sources utilised in the present trial were selected on the basis that they are of high quality. To ensure that any

of the observed dietary differences would only be attributed to the selected protein and oil sources, protein/oil ratios were constant in the six diets whereas macro- and micro-nutrients were supplemented to give similar patterns and ratios among the commercial and the experimental dietary treatments.

7.2 MATERIALS AND METHODS

7.2.1 Diets and growth trial

Six different feeds with different levels of FM and FO substitution (Table 9) were tested for their effects on growth performance and health status of Atlantic salmon post-smolts.

Table 9 Feed formulations of the six diets.

FEED FORMULATION	**DIETS**					
Ingredient composition (g×kg^{-1})	**MBE**	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
LT Fishmeal[a]	300.0	200.0	-	-	300.0	-
Plant Protein Concentrates[b]	255.3	207.6	386.8	528.1	255.3	528.1
Starch Binder	87.9	85.4	88.6	92.8	87.9	92.8
Animal By-Products[c]	-	155.0	160.0	-	-	-
Amino Acids[d]	9.1	7.4	15.2	1.7	9.1	1.7
Vitamin/Mineral & Pigment Mixes[e]	35.9	35.9	41.9	41.9	35.9	41.9
Fish Oil[f]	148.0	157.6	-	177.0	-	-
Plant Lipids[g]	163.8	150.9	220.3	138.4	239.0	228.2
Algal Lipids[h]	-	-	87.2	-	72.8	87.2

*Diet abbreviations: LT Fishmeal, fishmeal of superior quality due to the fact that is treated at lower drying temperatures allowing fish meal to keep a great essential amino acid profile and low biogenic amines; MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, marine protein- (fishery-) free with inclusion of animal-by-product proteins diet; [a] LT Fishmeal (low temperature fishmeal from Egersund Sildeoljefabrikk AS, Egersund, Norway); [b] Vegetable proteins: includes protein concentrates from soy (Imcopa, - Importação, Exportação e Indústria de Óleos Ltda., Araucária - Paraná, Brazil) and pea (AgriMarin, Stavanger, Norway) and wheat gluten (Henan Tianguan, Nanyang City, China); [c] Animal by-products: includes Poultry by-product meal (Poultry by-product meal, GePro Geflügel-Protein Vertriebs- GmbH & Co, Diepholz, Germany) and porcine blood meal (Daka Proteins, Løsning, Denmark); [d] Amino acids from Evonik Degussa International AG, Hanau, Germany; [e] proprietary of EWOS Innovation; Plant lipids: includes mainly rapeseed oil (Cargill PLC, Lincoln, UK); [f] Fish Oil: Capelin oil (Egersund Sildeoljefabrikk AS, Egersund), Norway; [h] Algal lipids: includes oil from heterotrophically grown algal species (Origin Unknown).

Two commercially applied feed formulations by EWOS were used as controls. Both of the control diets contained FM and FO. However, partial inclusion of vegetable protein concentrates (VPCs) and vegetable oils (VOs) according to the EU standards of 2011-12

were used in the first control diet namely European marine based (MBE) diet, while partial addition of a mixture of VPCs, proteins from land animal by-products (ABPs) and VOs according to non-EU standards were utilised in the second control denoted as marine based with inclusion animal by-products diet (MBABP). In the VP diet the FM was completely substituted by VPCs, while complete replacement of FO by VOs and algal lipids (the latter used as a source of long chain n-3 PUFAs) was applied in VO diet. Despite the full substitution of FO with VOs, the elimination of FO from such diets containing FM as the main protein source is impossible, since notable amounts of FO are present within the FM fraction. Complete substitution of marine proteins and lipids was tested using two different diets, a fully vegetarian one with complete replacement of FM and FO with VPCs and a combination of VOs and algal oils denoted as VP/VO diet and a second one combining also the inclusion of land animal by-product proteins (poultry and porcine blood meal) namely MFABP diet. Diets with partial or total substitution of FM with alternative protein sources were supplemented with synthetic amino acids and premixes with a starch binder in order to balance nutrients. Diets were pelleted through extrusion (EWOS Innovation, Dirdal, Norway). All diets were formulated to meet and exceed the nutrient requirements of Atlantic salmon according to the (NRC, 1993).

Growth trials were conducted at EWOS Innovation facilities at Dirdal, Norway. Before the acclimation period, unvaccinated Atlantic salmon post-smolts (S0 smolts) of a mean body weight of about 250g, from a commercial SalmoBreed AS (Bergen, Norway) strain selected for improved growth performance, were sampled for the evaluation of their immunity status. Atlantic salmon post-smolts were randomly allocated in two sets of 12 seawater supplied tanks, located in the same block, until a total of 55 fish were in each tank. Fish were acclimatised to the experimental tanks for 84 days prior to starting the experiment, during which time they were fed a commercial diet (EWOS OPAL 500) and later allocated

their experimental diet. Fish were weighed before the acclimation period (16th of December 2010), at the start of the trial (day 0) (10th of March 2011), at days 104-105(22nd and 23rd of June 2011) and at the end of the study (days 175-176) (2nd and 3rd of September 2011). Fish were reared in cylindrical fibreglass tanks with a water volume of 3.0 m^3. Each tank was supplied by running seawater pumped from the nearby fjord at 50m depth (salinity range of 29 ± 1.3 g $\times$ l^{-1} and temperature ranging from 6.3 ºC in March (lowest temperature recorded) to 9.1 ºC in August (25th) (highest temperature recorded) (Average water temperature 7.7 ± 1.4 ºC) at a flow rate of 0.8 l $\times$ kg $\times$ biomass^{-1}$\times$ min^{-1}.

A continuous lighting regime was used during the acclimation and feeding trial period. During the study period quadruplicate tanks of fish were fed one of the 6 experimental diets. Salmon were fed with pellets of 5mm during the full period of the trial. Two different batches of the feeds were used during the full duration of the study. The first batch was given to the fish during the first period of the study from the 10th of March until the 21st of June whereas the second lot was used for the second part of the trial from the 24th of June until the 31st of August. Fish were fed four times daily (feed intervals 01:00, 07:00, 19:00, and 22:00) using an automatic feeding system (Exact; Storvik Aqua, SV, Sundalsøra, Norway). The daily amount of feed was equally distributed within these four feed intervals. Feeding period for each feed interval was about 30-60 min depending on the total feed amount per day. Feed doses were delivered every 60 sec to the tanks for each feeding period whilst the total amount of feed delivered at each feeding time of the feeding period was 3-7 g. Feeding time and period within feed intervals were the same for acclimation and trial period. The level of daily feed ratio was about 0.5-1.5 of total biomass. To ensure that all fish (or at least as many fish as possible) received adequate feed each day, daily feed ration was adjusted accordingly so that the amount of uneaten feed laid between 15-30% of the total feed amount offered. An average of 25% "overfeeding" was obtained for the full trial period. Uneaten feed was

collected using waste feed collectors. Therefore, estimates of the actual feed consumption and thereby FCRs were available. The growth trial was conducted for a total of 196 days, at which time all fish were removed from the tanks and weighed 2 times (days 125-126 and days 194-197) after being anaesthetised (MS222, $2g \times l^{-1}$). On days 125-126 twenty fish from each tank were euthanised with an overdose of MS222 ($7\ g \times l^{-1}$) to keep biomass densities below 90 $kg \times m^{-3}$ in the tanks resulting in a final number of 35 fish $\times$ $tank^{-1}$ (starting mean biomass density of 52 $kg \times m^{-3}$ (55 post-smolts); intermediate mean biomass density of 67 $kg \times m^{-3}$ after the removal of 20 salmon (35 salmon); final mean biomass density of 87 $kg \times m^{-3}$ (35 salmon)).

7.2.2 Sample collection

For immunological analyses, blood was withdrawn from the caudal vein of 6 fish from each tank on days 194 and 195 from start of the experiment, using 1 ml syringes rinsed with heparin (10 IU $\times$ ml^{-1}); Sigma-Aldrich, Dorset, UK) while pools of Norwegian quality cut (NQC) fillet samples from 4 fish per tank were obtained for carcass proximate composition analyses. A heparinised capillary tube per sampled fish was filled with blood from the syringe for haematocrit observations. Haematocrit determination was performed for 6 fish per tank. Haematocrit values were measured after centrifugation at 6000 revolution per minute (rpm) for 25 min. The remaining blood from three first blood samples was used to determine the total leucocyte and differential leucocyte numbers from 3 fish per tank. A 10^{-3} dilution of blood in L-15 was used to determine total leucocyte counts. The cells were counted in four squares of a haemocytometer per sample and expressed as:

$$Number\ of cells \times ml^{-1} = N \times DilutionFactor \times 10^{4}$$

where N is the average number of counted blood cells.

For the determination of differential leucocyte counts 3 blood smears were obtained (for the determination of the percentages of the different leucocyte types in each blood sample initially and their transformation into numbers of cells × ml^{-1} of blood according to total leucocyte numbers). The cells on the blood smears were left to air dry and were stained with Rapid Romanowsky's stain (Raymond A lamb, Eastbourne, UK) in the Institute of Aquaculture, University of Stirling. The slides were later examined at × 400 magnification for the determination of differential leucocyte proportions. After sampling the blood from individual fish for haematocrit, blood smears and red blood cell counts (using blood samples diluted 1: 1000), blood samples from 3 individuals from the same tank were pooled together in one eppendorf tube (3 × 400 µl of blood × sampled $fish^{-1}$× $tube^{-1}$). The blood was left to clot overnight at 4°C and the next day the pooled plasma was aliquoted into 7 eppendorf tubes (about 40-50 µl) and stored at -80°C. Head kidney samples (approximately 5 mm) from three individuals (derived from the same tank), were aseptically removed according to Secombes (1990), pooled in plastic bjoux vials containing 5ml ice-cold L-15 medium containing 40 µl heparin (10 IU × ml^{-1}) and used for respiratory burst assays. Two pools per tank were used to determine the level of superoxide anion (O^{-2}) produced by head kidney macrophages.

7.2.3 Calculations

Feed intake is the calculated amount of food ingested by fish per treatment expressed in g

Feed Conversion Ratio (FCR):

$$FCR = \frac{Feed\ Intake\ (FI)\ (g)}{Wet\ weight\ gain\ (g)}$$

Specific Growth Rate:

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

Thermal Growth Rate:

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

Weight gain (*WG*):

$$WG\ (\frac{g}{day}) = \frac{Wet\ weight\ gain(g)}{Number\ of\ days}$$

Condition Factor (*K*):

$$K = \frac{Fish\ weight\ (g)}{Fish\ length\ (cm)^3}$$

In the above formulae W is the weight of the sampled fish in grams; W0 and W1 are the initial and the final fish mean weights in grams.

7.2.4 Isolation of head kidney macrophages and respiratory burst of head kidney macrophages

For the isolation of head kidney macrophages, the head kidney was teased through a 100µm nylon mesh (BD Falcon; BD Biosciences, Franklin Lakes, NJ, USA) into 2.5 ml Leibovitz medium (L-15; Sigma-Aldrich) containing 40 µl of heparin (10 IU $\times$ ml^{-1}). The mesh was rinsed with 2.5 ml of the medium and placed on ice. The O^{-2} production by head kidney macrophages was measured by the conversion of NBT (Sigma-Aldrich) to formazan, following the method of Secombes (1990) with some modifications described by Korkea-aho et al. (2011). A more detailed description of the methology is presented in Section 2.4.1.

7.2.5 Determination of plasma protein and lysozyme activity

Protein content of plasma was determined using 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 BSA as standard. Serum lysozyme activity was

based on the lysis of lysozyme sensitive *Micrococcus lysodeikticus*as described by Korkea-aho et al. (2011). More details about the methods used are given in Section 2.4.3.

7.2.6 Measurement of natural haemolytic activity

The assay used was based on a method described by Langston et al. (2001) with modifications. A more detailed description of the methodology is given in Section 2.4.5.10 (details about the preparation of SRBCs and the various buffers are presented in Section 2.4.5).

7.2.7 Total plasma Immunoglobulin M (IgM)

The level of IgM in sera of experimental fish was determined using indirect enzyme linked immunosorbent assay (ELISA) (Magnadottir and Gudmundsdottir, 1992), with modifications. The methodology is thoroughly described in Section 2.4.7.

7.2.8 Plasma 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 et al. (1990), modified for use in microtitre plates. The method is described in more detail in Section 2.4.6.

7.2.9 Carcass and dietary composition analysis

The assessment of dietary lipid and FA composition was conducted by The Nutrition Analytical Services (NAS) of the Institute of Aquaculture, University of Stirling and were run in duplicates. Dietary crude fat was determined using two different methodologies. Firstly dietary lipid content was determined following acid hydrolysis using a Soxtec System 1047 hydrolysing unit (Tecator Application note 92/87) followed by exhaustive Soxhlet extraction using petroleum ether (40–60°C boiling point) on a Soxtec System HT6 (Tecator application note 67/83)as described by Bell et al.(2001). In addition, dietary lipid fraction

was determined according to the Folch method (Folch et al., 1957) with non-lipid impurities removed by washing with 0.88% (w/v) KCl. The lipid weight was determined gravimetrically after evaporation of solvent under nitrogen and desiccation under vacuum for at least 16 h. Dietary Fatty acid methyl esters (FAME) were prepared from total lipid by acid catalyzed transesterification as described by Christie (2003) and FAMEs extracted and purified as described by Tocher and Harvie (1988). FAMEs were separated and quantified by Gas Liquid Chromatography (GLC) (Carlo Erba Vega 8160, Milan, Italy) using a 30 m × 0.32 mm capillary column (CP Wax 52CB, Chrompak, London, UK). Hydrogen was used as carrier gas and temperature programming was from 50 to 150°C at 40°C × min^{-1} and then to 230°C at 2.0°C × min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980). Peak data was processed using Chromcard for Windows (version 1.19) computer package (Thermoquest Italia S.P.A., Milan, Italy). Dry weight and ash contents of diets just like NQC fillet samples were determined after oven-drying the samples to constant weight and by ashing dried samples in an oven at 550ºC (AOAC, 1990). Dietary nitrogen was determined by Eurofins Scientific (Norway) after total combustion using a Nitrogen-Analyser (Perkin Elmer, 2410 Ser. II, Norwalk, CT, USA), crude protein content calculated assuming that proteins contain 16% N. Amino acid composition of the feed raw materials was analysed by near infrared reflectance (Fontaine et al., 2001) and was also performed by Eurofins Scientific (Norway). Amino acid composition of compound feed was analysed according to (Llames and Fontaine, 1994).

Pools of homogenised NQC fillet carcass samples (1 pool of 4 individuals per tank) for chemical analysis were frozen and then thawed before blending (whole). Dry matter, moisture, ash and crude protein levels were determined according to standard methods (AOAC, 1990) by oven drying to constant weight. Crude protein from dry carcass samples

was estimated by Kjeldahl's method using the TecatorKjeltec System. Lastly, crude fat from dried NQC carcass was determined using petroleum ether (40–60 °C boiling point) on a Soxtec System HT6 (Tecator application note 67/83) (Christie, 2003). More thorough description of the techniques can be found in Section 2.6.

7.2.10 Statistics

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., 2014). Similar statistical analyses are presented by Espe et al. (2012) and Hartviksen et al. (2014). To investigate the effect of the diets on the haematological and immunological responses, the data were fitted in two different models without (only the tank effect was added) and with the feed variable (tank effect nested within the dietary effect), which were then nested and compared with a likelihood ratio test (LRT).

Feed intake (FI) and feed conversion ratio (FCR) over the full trial period and during first and second study period were modelled as an ordinary linear model since there is no multilevel structure (only one observation per tank). TGC and SGR were also modelled as ordinary linear models since there no multilevel structure was used (only one observation per tank was used for the analysis). Gutted weights are available from a subsample of fish at the end of the trial. Daily WG for the same period was modelled with the help of splines to allow the identification of non-linear responses of the diets in time. Since there were three weight points available, the degree of freedom for the spline was constrained to 2. Two models were then fitted without and with the dietary effect, and compared with LRT.

The modelling of the condition factor was conducted by fitting a length-weight relationship and adding the treatment as a covariate to the model. Since round weights were available at the end of the trial, these were used as a predictor, in order for the model to

adjust for an average-sized sampled fish and for a direct comparison to be possible. Lastly, two nested models were fitted, without and with the dietary effect, and compared with a LRT as above.

Composition percentages were modelled with an ordinary linear model (only one observation per tank) using an arcsin transformation to the responses which were expressed as a percentage of wet weight and adding the mean weight of the sample as a covariate. Sample mean weights were mean-centred before the analysis so that the results are easy to interpret as for the average-sized sampled fish.

Models demonstrating possibilities (*P* values) of 0.1 were selected for the description of data. For the modelled immune responses affected by the dietary treatments, the results are summarised as graphs with the mean response and 95% confidence interval. Confidence intervals were solved by a posterior simulation from the statistical model with 1500 random draws (Gelman and Hill, 2007). Differences among dietary treatments were revealed when the 95% confidence intervals for a certain response of a dietary group did not overlap with the mean values of the same response from another group.

7.3 RESULTS

7.3.1 Diets

The dietary amino acid profiles of the six experimental diets differed as a consequence of plant protein substituting for fishmeal (Tables 10 and 11) reflecting the amino acid composition of the different protein ingredients used.

Table 10 Chemical composition of the first lot of the six diets expressed in wet weight (w.w.) basis.

Chemical composition (w.w.)	DIETS					
	MBE	MBABP	MFABP	VP	VO	VP/VO
Moisture ($g \times kg^{-1}$)	72.3	60.7	81.3	69.4	76.5	85.9
Protein N*6.25 ($g \times kg^{-1}$)	394.0	384.0	385.0	380.0	393.0	384.0
Lipid ($g \times kg^{-1}$)	371.3	359.2	345.9	361.3	374.9	332.7
Crude fibre ($g \times kg^{-1}$)	9.1	9.7	10.7	10.6	8.1	10.6
Ash ($g \times kg^{-1}$)	47.6	59.7	30.2	19.9	41.2	15.5
P ($g \times kg^{-1}$)	12.00	15.0	10.00	7.7	12.0	9.1
Ca ($g \times kg^{-1}$)	14.00	20.00	11.00	8.1	14.00	9.4
Mg ($g \times kg^{-1}$)	1.6	1.5	0.8	0.9	1.5	1.0
Zn ($mg \times kg^{-1}$)	265.00	288.00	255.00	233.00	259.00	284.00
AMINO ACID COMPOSITION						
Methionine ($g \times kg^{-1}$)*	9.40	8.60	8.20	8.70	9.50	9.20
Cysteine ($g \times kg^{-1}$)	4.30	4.60	4.50	5.00	4.50	5.40
Lysine ($g \times kg^{-1}$)*	30.60	29.40	28.10	30.30	30.90	30.50
Threonine ($g \times kg^{-1}$)*	15.70	15.70	14.00	14.80	16.00	15.50
Isoleucine ($g \times kg^{-1}$)*	18.40	16.90	15.20	18.10	17.60	17.60
Histidine ($g \times kg^{-1}$)*	8.90	9.40	7.90	8.50	8.90	8.60
Valine ($g \times kg^{-1}$)*	20.20	19.20	16.40	18.80	19.50	18.20
Leucine ($g \times kg^{-1}$)*	29.50	32.20	42.20	44.90	29.50	46.60
Arginine ($g \times kg^{-1}$)*	24.80	23.60	22.30	22.30	25.00	22.90
Phenylalanine($g \times kg^{-1}$)*	16.60	17.20	18.90	20.50	17.20	21.40
Tyrosine ($g \times kg^{-1}$)	13.10	13.00	14.30	15.80	13.60	15.80
Alanine ($g \times kg^{-1}$)	20.40	23.00	23.30	19.90	20.50	20.40
Glycine ($g \times kg^{-1}$)	20.00	21.80	21.30	13.50	29.70	13.60
Hydroxyproline($g \times kg^{-1}$)	1.90	2.80	5.30	0.1	1.20	0.1
Ornithine ($g \times kg^{-1}$)	0.1	0.1	0.2	0.1	0.1	0.1
Proline ($g \times kg^{-1}$)	20.50	20.90	24.00	25.30	20.30	27.00
Serine ($g \times kg^{-1}$)	17.10	18.00	18.50	18.00	18.50	20.40
Tryptophane ($g \times kg^{-1}$)*	4.30	4.50	3.30	4.10	4.40	3.80
IAA: DAA ratio	1.7	1.6	1.5	1.9	1.6	1.8

* Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (*P* values < 0.05) between dietary groups are denoted by different letters. ([a] Vegetable proteins: includes protein concentrates from soy, pea, corn and wheat; [b] Animal by-products: includes Poultry by-product meal and porcine blood meal; [c] Plant lipids: includes rapeseed oil and other lipid sources).

Table 11 Chemical composition of the second lot of the six diets expressed in wet weight (w.w.) basis.

Chemical composition (w.w.)	DIETS					
	MBE	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
Moisture ($g \times kg^{-1}$)	72.3	60.7	81.3	69.4	76.5	85.9
Protein N*6.25 ($g \times kg^{-1}$)	394.0	384.0	385.0	380.0	393.0	384.0
Lipid ($g \times kg^{-1}$)	371.3	359.2	345.9	361.3	374.9	332.7
Crude fibre ($g \times kg^{-1}$)	9.1	9.7	10.7	10.6	8.1	10.6
Ash ($g \times kg^{-1}$)	47.6	59.7	30.2	19.9	41.2	15.5
P ($g \times kg^{-1}$)	12.00	15.0	10.00	7.7	12.0	9.1
Ca ($g \times kg^{-1}$)	14.00	20.00	11.00	8.1	14.00	9.4
Mg ($g \times kg^{-1}$)	1.6	1.5	0.8	0.9	1.5	1.0
Zn ($mg \times kg^{-1}$)	265.00	288.00	255.00	233.00	259.00	284.00
AMINO ACID COMPOSITION						
Methionine ($g \times kg^{-1}$)*	9.40	8.60	8.20	8.70	9.50	9.20
Cysteine ($g \times kg^{-1}$)	4.30	4.60	4.50	5.00	4.50	5.40
Lysine ($g \times kg^{-1}$)*	30.60	29.40	28.10	30.30	30.90	30.50
Threonine ($g \times kg^{-1}$)*	15.70	15.70	14.00	14.80	16.00	15.50
Isoleucine ($g \times kg^{-1}$)*	18.40	16.90	15.20	18.10	17.60	17.60
Histidine ($g \times kg^{-1}$)*	8.90	9.40	7.90	8.50	8.90	8.60
Valine ($g \times kg^{-1}$)*	20.20	19.20	16.40	18.80	19.50	18.20
Leucine ($g \times kg^{-1}$)*	29.50	32.20	42.20	44.90	29.50	46.60
Arginine ($g \times kg^{-1}$)*	24.80	23.60	22.30	22.30	25.00	22.90
Phenylalanine($g \times kg^{-1}$)*	16.60	17.20	18.90	20.50	17.20	21.40
Tyrosine ($g \times kg^{-1}$)	13.10	13.00	14.30	15.80	13.60	15.80
Alanine ($g \times kg^{-1}$)	20.40	23.00	23.30	19.90	20.50	20.40
Glycine ($g \times kg^{-1}$)	20.00	21.80	21.30	13.50	29.70	13.60
Hydroxyproline($g \times kg^{-1}$)	1.90	2.80	5.30	0.1	1.20	0.1
Ornithine ($g \times kg^{-1}$)	0.1	0.1	0.2	0.1	0.1	0.1
Proline ($g \times kg^{-1}$)	20.50	20.90	24.00	25.30	20.30	27.00
Serine ($g \times kg^{-1}$)	17.10	18.00	18.50	18.00	18.50	20.40
Tryptophane ($g \times kg^{-1}$)*	4.30	4.50	3.30	4.10	4.40	3.80
IAA: DAA ratio	1.7	1.6	1.5	1.9	1.6	1.8

* Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (*P* values < 0.05) between dietary groups are denoted by different letters. ([a] Vegetable proteins: includes protein concentrates from soy, pea, corn and wheat; [b] Animal by-products: includes Poultry by-product meal and porcine blood meal; [c] Plant lipids: includes rapeseed oil and other lipid sources).

The ratio of IAAs to DAAs fluctuated in the experimental diets ranging from 1.6 to 1.9. Supplementation of the fully VP-based treatments (VP and VP/VO) with lysine, methionine and threonine in which VP are known to be devoid of, improved the dietary levels of these amino acids. However, it seems that an overestimation of the aforementioned amino acid concentrations in the ABP proteins applied resulted in lower supplementation of the MBABP and the MFABP diet with these amino acids. As a result decreased methionine was recorded

for these diets. Furthermore, reduced lysine and threonine levels were demonstrated in the MFABP diet in comparison to the control diet. Furthermore, reduced levels of isoleucine, histidine were recorded for the MFABP diet compared to the MB one. Reduced dietary levels of valine and arginine (~10% decrease) were observed in the diets containing the highest levels of VPs (VP, VP/VO, MFABP treatments) compared to the MB control with valine exhibiting a 19% reduction in MFABP diet. Higher levels of cysteine, leucine, phenylalanine and tyrosine were demonstrated for diets containing the highest levels of VPs compared to the control.

The dietary FA concentrations of the two lots of the experimental diets used in the present study are presented in Tables 12 and 13. Lower amounts of total saturated fatty acids and higher levels of total PUFAs were observed for the VO-based diets. However, LC-PUFAs and monounsaturated FAs were found to be constant among all dietary treatments. Complete substitution of FO with the VO blend (which included algal oils) resulted in increased proportions of 18:3n−3, 18:2n−6 and 18:1n−9, with concomitant decreased proportions of 20:5n-3 (EPA) and long chain monoenoic FAs (LC-MUFAs) including 20:1, 22:1 and 24:1. The level of 22:6n-3 was found to be higher for the VO-based diets due to the inclusion of algal oils, characterised by increased levels of this FA (Carter et al. 2003; Miller et al. 2007). Furthermore, the ratio of n-3 to n-6 PUFAs demonstrated a gradual decrease in the diets in the following order: MB =MBABP>VP>VO> VP/VO=MBABP.

For the second batch of feeds, amino acids presented similar levels to the initial dietary batch. However, FO based diets demonstrated a decrease in the proportions of n-3 PUFAs with 22:6n-3 exhibiting the most striking reduction. In addition, higher amounts of 16:1, 18:1, 20:1 and 22:1 monoenoic FAs were observed, resulting in a general increase in total monounsaturated FAs. Furthermore, the total amount of unsaturated FAs presented a reduction with VO-based diets exhibiting higher levels than their FO-based counterparts. No

striking variations were observed in the total concentrations of n-6 PUFAs among the two batches of trial feeds.

Table 12 Fatty acid composition of the first lot of the six diets expressed in wet weight (w.w.) basis.

Fatty acid composition ($g \times kg^{-1}$)(w.w.)	DIETS					
	MBE	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
14:0	30.00	32.00	16.00	33.00	17.00	14.00
15:0	3.00	3.00	1.00	4.00	2.00	1.00
16:0	127.0	141.0	103.0	139.0	94.0	91.0
18:0	29.0	33.0	28.0	36.0	25.0	27.0
20:0	4.00	4.00	5.00	4.00	5.00	5.00
22:0	2.00	2.00	3.00	2.00	3.00	3.00
Sum saturated	165.00	183.00	140.00	185.00	129.00	127.00
16:1n-7	30.00	33.00	5.00	31.00	7.00	2.00
16:1n-9	3.00	3.00	2.00	3.00	2.00	1.00
18:1n-7	26.00	25.00	23.00	24.00	24.00	22.00
18:1n-9	359.00	349.0	448.00	355.00	458.00	453.00
20:1n-7	1.00	1.00	<1.00	1.00	1.00	<1.00
20:1n-9	34.00	29.00	11.00	27.00	17.00	10.00
20:1n-11	5.00	4.00	<1.00	4.00	1.00	<1.00
22:1n-9	6.00	5.00	4.00	5.00	5.00	4.00
22:1n-11	52.00	49.0	3.00	44.00	13.00	1.00
24:1n-9	6.00	6.00	2.00	6.00	4.00	2.00
Sum MUFAs	522.00	504.00	499.00	500.00	532.00	496.00
18:2n-6	109.0	108.0	157.0	119.0	146.0	164.0
18:3n-6	1.00	1.00	1.00	<1.00	<1.00	1.00
20:2n-6	2.00	2.00	1.00	2.00	1.00	1.00
20:3n-6	<1.00	<1.00	1.00	<1.00	1.00	1.00
20:4n-6	3.00	3.00	3.00	3.00	2.00	2.00
Sum n-6 PUFAs	115.5	114.5	163.00	125.00	150.5	169.00
18:3n-3	47.00	43.00	56.00	44.00	59.00	60.00
18:4n-3	13.00	12.00	1.00	12.00	2.00	1.00
20:3n-3	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
20:4n-3	3.00	3.00	2.00	3.00	2.00	2.00
20:5n-3	38.00	39.00	4.00	36.00	8.00	3.00
22:5n-3	4.00	4.00	1.00	3.00	1.00	1.00
22:6n-3	56.0	60.0	84.0	53.0	75.0	89.0
Sum n-3 PUFAs	161.5	161.5	148.5	151.5	147.5	156.5
n-3/n-6	1.4	1.4	0.9	1.2	1.0	0.9

* Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (P values < 0.05) between dietary groups are denoted by different letters. ([a] Vegetable proteins: includes protein concentrates from soy, pea, corn and wheat; [b] Animal by-products: includes Poultry by-product meal and porcine blood meal; [c] Plant lipids: includes rapeseed oil and other lipid sources).

Table 13 Fatty acid composition of the second lot of the six diets expressed in wet weight (w.w.) basis.

Fatty acid composition (g×kg^{-1}) (w.w.)	DIETS					
	MBE	MBABP	MFABP	VP	VO	VP/VO
14:0	33.00	31.00	18.00	31.00	19.00	17.00
15:0	2.00	2.00	1.00	2.00	1.00	1.00
16:0	94.00	93.00	97.00	97.00	85.00	86.00
18:0	22.00	24.00	25.00	24.00	23.00	21.00
20:0	3.00	4.00	4.00	4.00	4.00	4.00
22:0	<1.00	2.00	3.00	2.00	3.00	3.00
Sum saturated	121.5	125.00	130.00	129.00	116.00	115.00
16:1n-7	44.00	41.00	6.00	41.00	9.00	3.00
16:1n-9	2.00	1.00	1.00	2.00	1.00	1.00
18:1n-7	32.00	32.00	22.00	32.00	24.00	21.00
18:1n-9	325.00	331.00	421.00	303.00	419.00	420.00
20:1n-7	3.00	3.00	<1.00	4.00	1.00	<1.00
20:1n-9	111.00	108.00	11.00	113.00	23.00	10.00
20:1n-11	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
22:1n-9	11.00	11.00	2.00	12.00	3.00	2.00
22:1n-11	84.00	79.00	3.00	81.00	15.00	1.00
24:1n-9	6.00	7.00	2.00	6.00	3.00	2.00
Sum MUFAs	618.5	613.5	469.00	594.5	498.5	461.00
18:2n-6	108.00	112.00	173.00	133.00	146.00	181.00
18:3n-6	<1.00	1.00	<1.00	1.00	<1.00	<1.00
20:2n-6	2.00	2.00	1.00	2.00	1.00	1.00
20:3n-6	<1.00	1.00	1.00	1.00	1.00	1.00
20:4n-6	2.00	2.00	2.00	2.00	2.00	2.00
Sum n-6 PUFAs	113.00	118.00	177.5	139.00	150.5	185.5
18:3n-3	43.00	42.00	59.00	39.00	60.00	63.00
18:4n-3	11.00	10.00	1.00	10.00	3.00	1.00
20:3n-3	<1.00	<1.00	<1.00	<1.00	<1.00	2.00
20:4n-3	2.00	2.00	2.00	2.00	2.00	2.00
20:5n-3	28.00	26.00	2.00	28.00	10.00	2.00
22:5n-3	2.00	2.00	1.00	4.00	2.00	1.00
22:6n-3	27.00	25.00	102.00	21.00	103.00	109.00
Sum n-3 PUFAs	113.5	107.5	167.5	104.5	180.5	180.00
n-3/n-6	1.0	0.9	0.9	0.8	1.2	1.0

* Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (*P* values < 0.05) between dietary groups are denoted by different letters. ([a] Vegetable proteins: includes protein concentrates from soy, pea, corn and wheat; [b] Animal by-products: includes Poultry by-product meal and porcine blood meal; [c] Plant lipids: includes rapeseed oil and other lipid sources).

7.3.2 Growth and chemical composition of NQC carcass samples

The raw performance data for the six dietary groups of Atlantic salmon post-smolts are presented in Table 14.The results of this study demonstrated negligible mortality (<1%) in all dietary groups during the experimental period. Salmon fed the control MBE diet had

higher FI levels than the rest of the experimental groups (Fig. 37A) (since the expected mean FI values of the MBE group did not overlap with the 95% C.I. of the other dietary groups). Furthermore, VP-fed salmon exhibited higher FI than the MBABP, MFABP and VP/VO groups, while the latter group demonstrated lower FI than the VO-fed group. For the same period the MBE control group exhibited higher weight gain (WG) than the majority of the dietary groups except for the VO-fed salmon (Fig. 37B). The majority of the other dietary groups exhibited no differences in WG. The only exception was the VP/VO salmon which exhibited lower WG in contrast to the VO-fed fish. Higher FCR values were obtained for the MBE and VP-fed salmon compared to all the other dietary groups, during the first period of the feeding trial (Fig. 37C). No differences were detected between the two former groups. Specific growth rates demonstrated significant differences among all the experimental dietary groups of salmon for the first part of the study, with MBE salmon exhibiting the highest levels followed in order of highest to lowest by the VO>MBABP>VP>VP/VO>MFABP groups of fish. At the same time, thermal growth rates presented significant differences in almost all dietary groups of salmon. MB salmon demonstrated the highest TGC followed by VO>VP>MBABP>VP/VO=MFABP salmon (Fig. 38A and 38B). Both FI and WG demonstrated no differences among the six dietary groups of salmon for the second period of the study. Nevertheless, a significant increment in FI was observed for the MBABP, MFABP and VP/VO groups, while a rather substantial but not significant increase was also witnessed for the VO group during this period compared to the initial phase of the study. Furthermore, a significant reduction in WG was observed for the MBE at the second period in comparison to the initial one while no significant differences were witnessed for all other groups. Significantly higher TGC and SGR were demonstrated for the VP/VO group of fish in comparison to the other dietary groups at this timepoint. VP salmon was the group with the second best growth performance indices

followed in by the VO, MBABP and MFABP groups which exhibited similar growth ratios and coefficients and lastly the MBE group which demonstrated the lowest values for this period (Fig. 38A and 38B).

Overall, salmon post-smolts maintained on the MBE diet had a higher FI at the end of the trial compared to most of the other dietary groups, except VP-fed salmon (Fig. 37A). In addition, higher FI was observed for VP salmon in comparison to the MBABP salmon and the marine-free (MFABP and VP/VO) groups of salmon. Furthermore, higher WG was observed for the MBE salmon compared to the MBABP, MFABP and VP/VO fed salmon (Fig. 37B). FCR values for the full duration of the trial were found to be higher for the MBE and VP salmon in contrast to all other dietary groups while no differences were observed between the former groups (Fig. 37C).

The final *K* values were found to be higher for the VP group, while salmon fed the VO diet exhibited lower *K* values compared to the rest of the groups. Moreover MBABP, salmon presented higher *K* values than the MBE group and salmon maintained on the marine free diets (MFABP and VP/VO). Furthermore, higher *K* was obtained for VP/VO salmon in comparison to the MBE salmon, while no difference was noticed between the latter group and MFABP salmon (Fig. 39).

The raw proximate analysis data of the NQC carcass samples are presented in Table 14. Statistical analysis of NQC carcass samples proximate analysis data, revealed no differences in moisture fat, protein and ash levels among the 6 dietary groups of salmon.

Table 14 Performance data and NQC proximate composition of Atlantic salmon post-smolts.

Growth and survival	DIETS					
	MBE	**MBABP**	**MFABP**	**VP**	**VO**	**VP/VO**
Initial weight (g)	992.30±119.80	901.78±58.14	924.80±32.25	940.70±127.20	983.90±66.80	892.93±29.86
Intermediate weight (g)	2041.30±224.60	1858.40±111.90	1856.70±113.10	1927.10±301.20	2014.70±142.40	1795.70±39.34
Final weight (g)	2608.70±268.60	2417.50±171.10	2415.60±197.10	2528.90±417.00	2626.90±286.80	2381.70±11.20
*Final Condition Factor *(K)*	1.52±0.11	1.54±0.12	1.51±0.11	1.61±0.12	1.46±0.10	1.52±0.10
**Feed Intake 1st Period (g)	48668±5461	34930±3244	33551±5475	41960±9074	39515±4007	33084±2803
Feed Intake 2nd Period (g)	20019±3384	19111±2996	18616±3260	20425±3518	20707±4049	20040±1910
Feed Intake full Period (g)	68687±6772	54042±1461[B]	52168±1418	62386±12277	60222±7390	53124±3386
Mortalities (%)	0.25±0.5	0	0	0	0.25±0.5	0
NQC composition						
Moisture (%)	65.07±0.23	65.19±1.17	65.67±1.10	64.27±0.57	65.75±1.08	65.65±0.29
Protein (%)	18.46±0.23	18.87±0.27	18.69±0.51	18.82±0.81	18.89±0.70	18.99±0.28
Crude Lipid (%)	13.56±0.21	12.57±0.58	12.64±0.82	13.47±1.03	12.07±0.32	12.92±0.48
Ash (%)	1.37±0.04	1.34±0.05	1.27±0.06	1.38±0.08	1.33±0.09	1.38±0.11

Data for performance factors are referred as means ± SD of 4 replicate tanks. Data for the proximate composition of Norwegian quality cut (NQC) carcass samples are referred as means ± SD of 4 pooled samples per diet (1 pool per tank).
Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, marine protein- (fishery-) free with inclusion of animal-by-product proteins diet.

**Condition factor (K) = 100 × fish weight (g) ×* $(\text{length (cm)})^{-3}$

***Feed intake = Amount of food ingested by fish per treatment (g);*

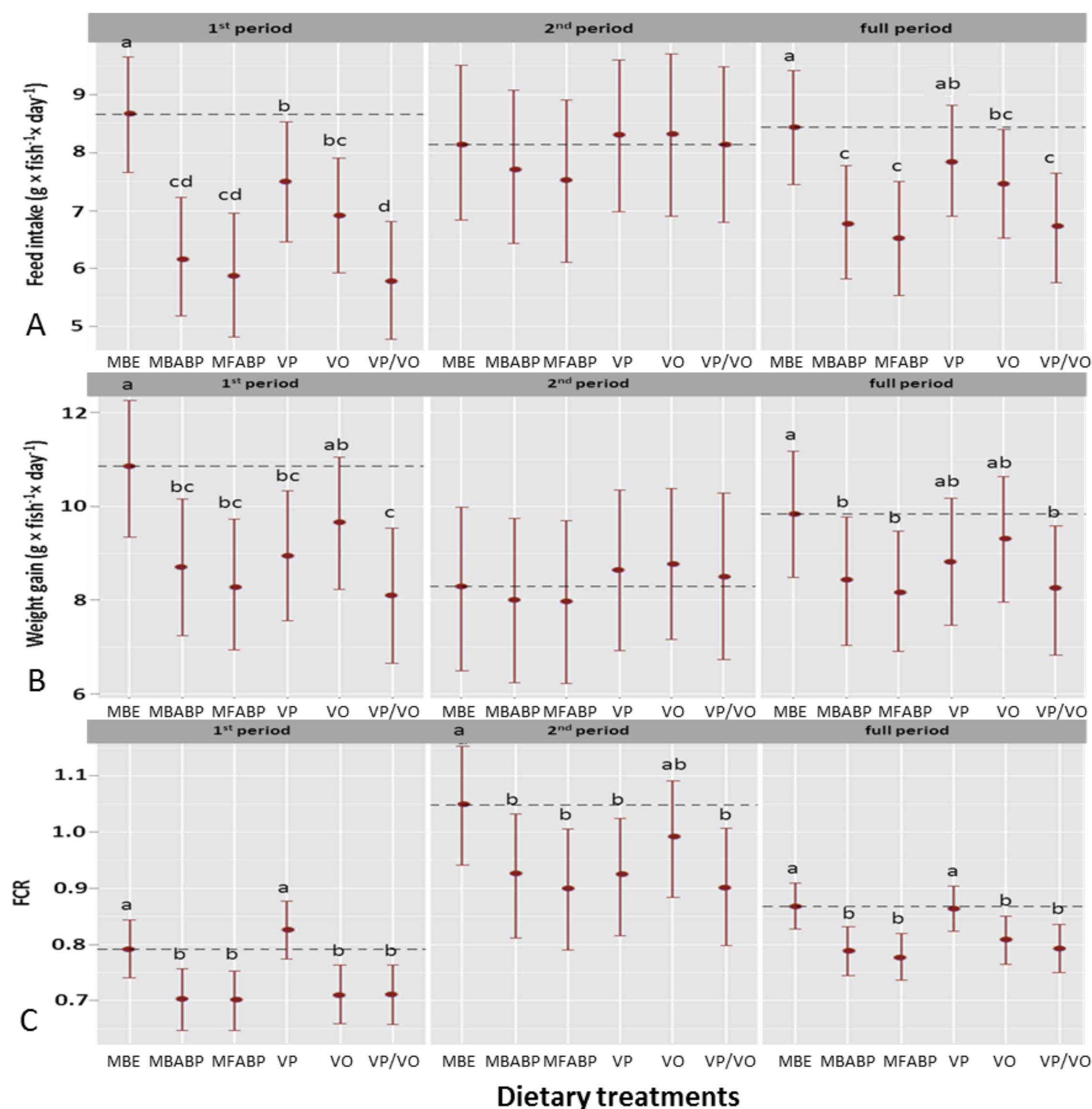

Figure 37 Expected performance indices at different study points. (A) Feed Intake (FI); (B) Daily weight gain (WG); (C) Feed Conversion Ratio (FCR) of the Atlantic salmon groups over the periods 1 and 2 and the full duration of the trial and (D) condition factor (*K)* of the salmon groups for the full period of the trial. These parameters were affected by the diets and the results are summarised as graphs with the mean response and 95% confidence interval. Confidence intervals were solved by a posterior simulation from the statistical model with 1500 random draws. Significant differences among dietary groups are revealed when the confidence intervals bars for a certain response of a dietary group do not overlap with the mean values of the same response from another group. The effect of feeds on the above growth performance indices was confirmed by fitting a model without the dietary effect (only tank effect) and with it (tank effect nested within it) and comparing the models with a likelihood ratio tests (LRT). FI and FCR are modelled as ordinary linear models since there is no multilevel structure (only one observation per tank) whereas WG modelled with the help of splines to allow non-linear response in time. FI and WG are expressed as $g \times fish^{-1} \times day^{-1}$ to adjust for the different duration of the periods. Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (*P* values < 0.05) between dietary groups are denoted by different letters.

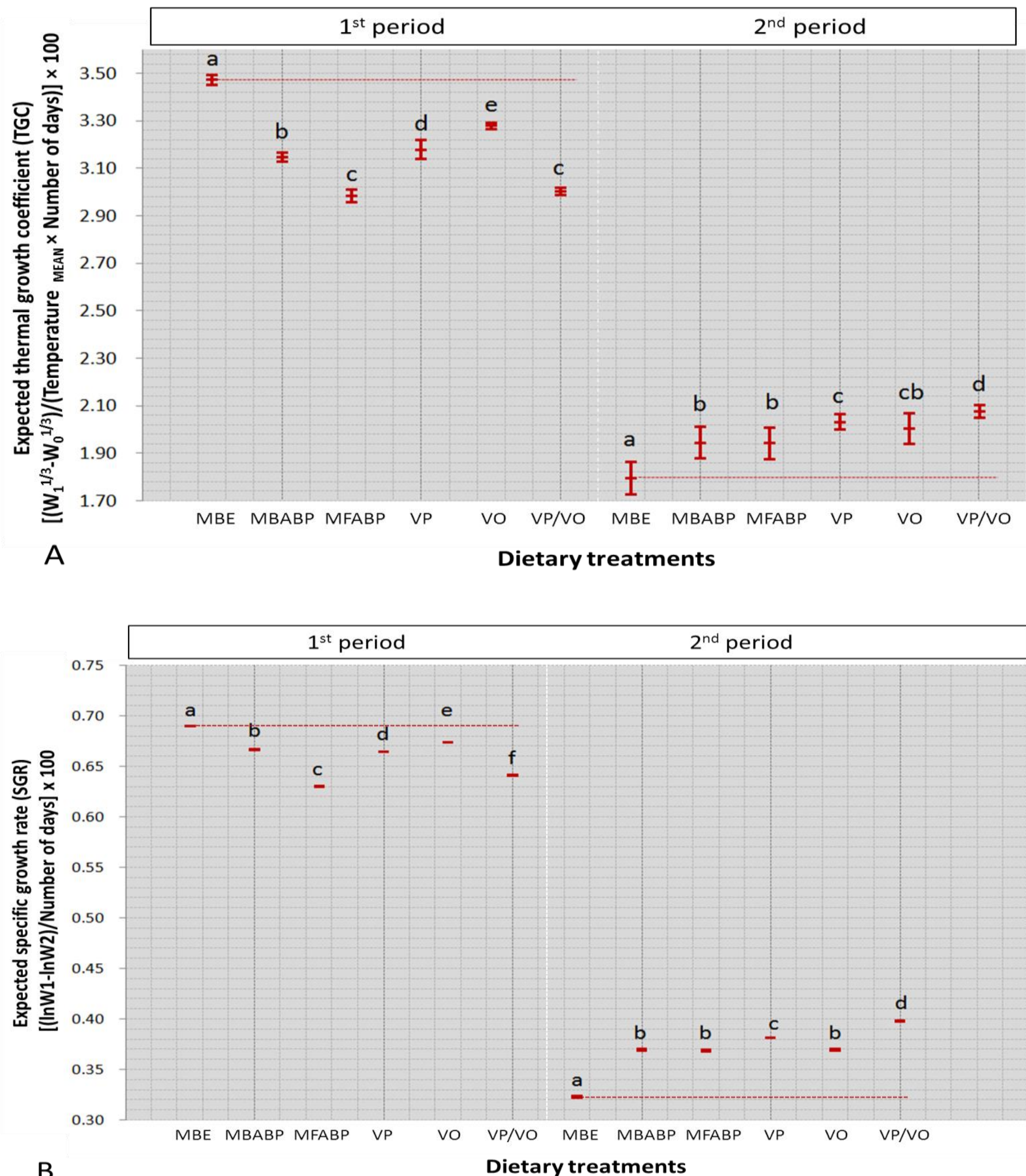

Figure 38 Expected growth performance indices at different study periods. (A) TGC and (B) SGR, of the Atlantic salmon groups over the periods 1 and 2 of the feeding trial. These parameters were affected by the diets and the results are summarised as graphs with the mean response and 95% confidence interval. Confidence intervals were solved by a posterior simulation from the statistical model with 1500 random draws. Significant differences among dietary groups are revealed when the confidence intervals bars for a certain response of a dietary group do not overlap with the mean values of the same response from another group. The effect of feeds on the above growth performance indices was confirmed by fitting a model without the dietary effect (only tank effect) and with it (tank effect nested within it) and comparing the models with a likelihood ratio tests (LRT). TGC and SGR are modelled as ordinary linear models since there is no multilevel structure (only one observation per tank). Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (P values < 0.05) between dietary groups are denoted by different letters.

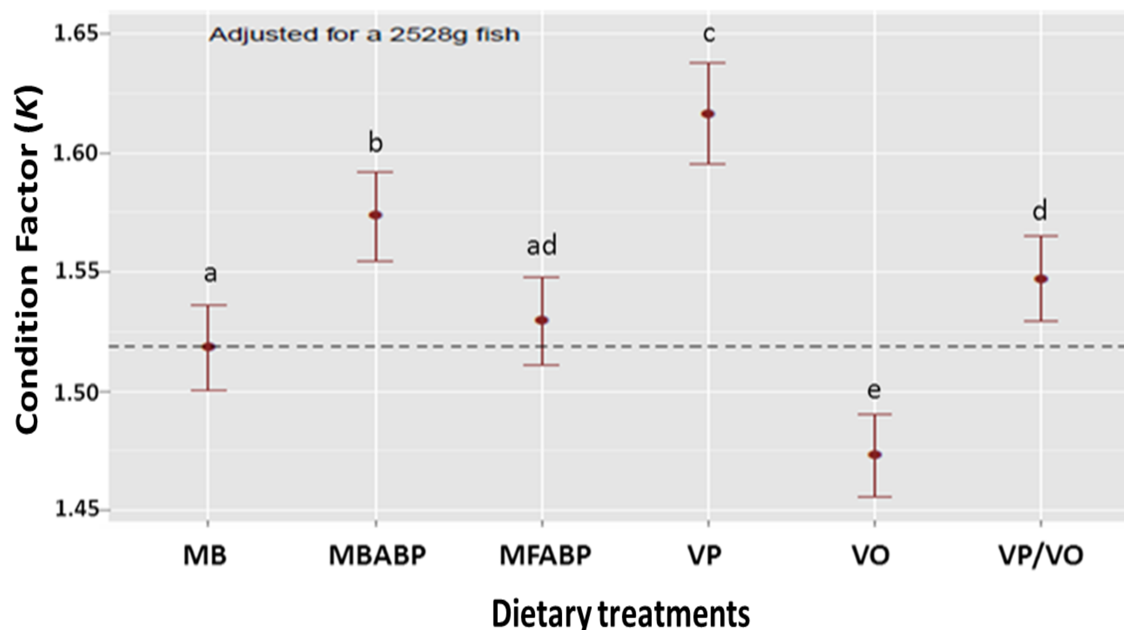

Figure 39 Expected condition factor for the different groups of salmon at the end of the study. Condition factor (*K*) of the salmon groups for the full period of the trial. *K* values were affected by the diets and the results are summarised as graphs with the mean response and 95% confidence interval. Confidence intervals were solved by a posterior simulation from the statistical model with 1500 random draws. Significant differences among dietary groups are revealed when the confidence intervals bars for a certain response of a dietary group do not overlap with the mean values of the same response from another group. The effect of feeds on the above growth performance indices was confirmed by fitting a model without the dietary effect (only tank effect) and with it (tank effect nested within it) and comparing the models with a likelihood ratio tests (LRT). The modelling of the *K* values was conducted by fitting the values and adding the treatment as a covariate to the model. Mean-centred round weights were used as the predictor. Since round weights were available at the end of the trial, these were used as a predictor, in order for the model to adjust for an average-sized sampled fish and for a direct comparison to be possible. Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, fish free animal-by-product diet. Significant differences (P values < 0.05) between dietary groups are denoted by different letters.

7.3.3 Haematology and innate immune responses

The raw haematological and immunological data collected from the current study are presented in Table 15. No significant differences were revealed for the majority of the estimated haematological parameters. However, lower haematocrit values were obtained for the MBE group compared to the MBABP, MFABP and VP/VO groups, while the latter two groups of salmon presented higher values in comparison to the VO-fed group (Fig. 40A). No differences in total and differential leucocyte numbers were detected among the six dietary groups of Atlantic salmon post-smolts.

No differences were observed among the six dietary salmon groups, regarding plasma haemolytic activity, plasma protein and total IgM levels, stimulated and non-stimulated head kidney macrophage respiratory burst activity. Nonetheless higher lysozyme activity was demonstrated for the MBE group of salmon compared to all other dietary groups of salmon (Fig. 40B). No further differences regarding lysozyme activity were witnessed amongst the other dietary groups. Furthermore, higher levels of anti-protease activity were exhibited for the MFABP salmon compared to the salmon fed the marine based (MBE and MBABP) and VO diets while higher anti-protease activity was observed for the VP and VP/VO dietary groups in contrast to the MBABP group (Fig. 40C).

Table 15 Haematological and immunological responses of Atlantic salmon post-smolts.

Haematological & Immune parameters	MBE	MBABP	MFABP	VP	VO	VP/VO
Haematocrit (%)	47.9±5.3	51.4±5.7	52.7±6.5	50.9±3.7	48.6±3.7	53.5±6.3
Leucocytes ($\times 10^7 \times ml^{-1}$)	10.3±4.0	10.2±2.9	10.2±2.8	11.7±3.7	10.4±3.9	8.3±1.9
Lymphocytes ($\times 10^7 \times ml^{-1}$)	5.2±2.1	5.6±1.4	4.8±1.4	5.8±1.6	4.9±1.4	4.3±1.3
Thrombocytes ($\times 10^7 \times ml^{-1}$)	4.4±1.7	4.2±1.7	4.80±1.60	5.3±1.9	4.9±2.8	3.6±1.4
Granulocytes ($\times 10^7 \times ml^{-1}$)	0.6±0.4	0.4±0.15	0.50±0.30	0.6±0.3	0.51±0.46	0.5±0.2
Monocytes ($\times 10^7 \times ml^{-1}$)	0.05±0.08	0.03±0.04	0.04±0.05	0.05±0.06	0.06±0.06	0.03±0.03
Lysozyme activity (units $\times min^{-1} \times ml^{-1}$)	1259.8±252.1	913.7±319.5	794.7±372.2	791.9±289.6	912.4±185.1	830.1±317.0
Plasma haemolytic activity (units $SH_{50} \times ml^{-1}$)	928±298.2	954.6±139.1	1043.1±378.3	928.5±260.1	986.8±444.2	906.8±271.4
HKMs respiratory burst (NBT) (O.D.$_{610}$ for 10^5 nuclei)	0.40±0.15	0.5±0.3	0.4±0.2	0.4±0.2	0.4±0.2	0.4±0.2
Stimulated HKMs respiratory burst (O.D.$_{610}$ for 10^5 nuclei)	0.55±0.22	0.7±0.3	0.5±0.2	0.5±0.3	0.5±0.2	0.5±0.3
Total plasma protein ($mg \times ml^{-1}$)	66.4±6.3	66.1±8.3	65.3±6.7	66.5±6.3	67.9±4.3	67.9±5.9
Plasma antiprotease act. (Units $TI_{75} \times min^{-1} \times ml^{-1}$)	713.6±15.9	665.4±66.0	757.9±32.1	740.2±12.0	701.0±57.3	727.5±28.2
Total plasma IgM ($mg \times ml^{-1}$)	6.4±2.8	5.8±2.6	3.7±2.8	5.1±3.1	7.9±2.6	3.9±2.8

Data for immune responses are means ± SD from 8 pools of 2 fish per diet; for haematocrit values are means ± SD from 24 individual fish per diet; and for leucocyte and differential leucocyte counts values are means ± SD from 12 individual fish per diet. Within a row, means with different superscript letters differ significantly.

Diet abbreviations: MBE, European commercial marine based diet (2011-12); VP, vegetable protein diet; VO, vegetable oil diet; VO/VP, vegetable protein/vegetable oil diet; MBABP, Non-EU commercial marine based diet with inclusion of animal-by-product (2011-12); MFABP, marine protein- (fishery-) free with inclusion of animal-by-product proteins diet.

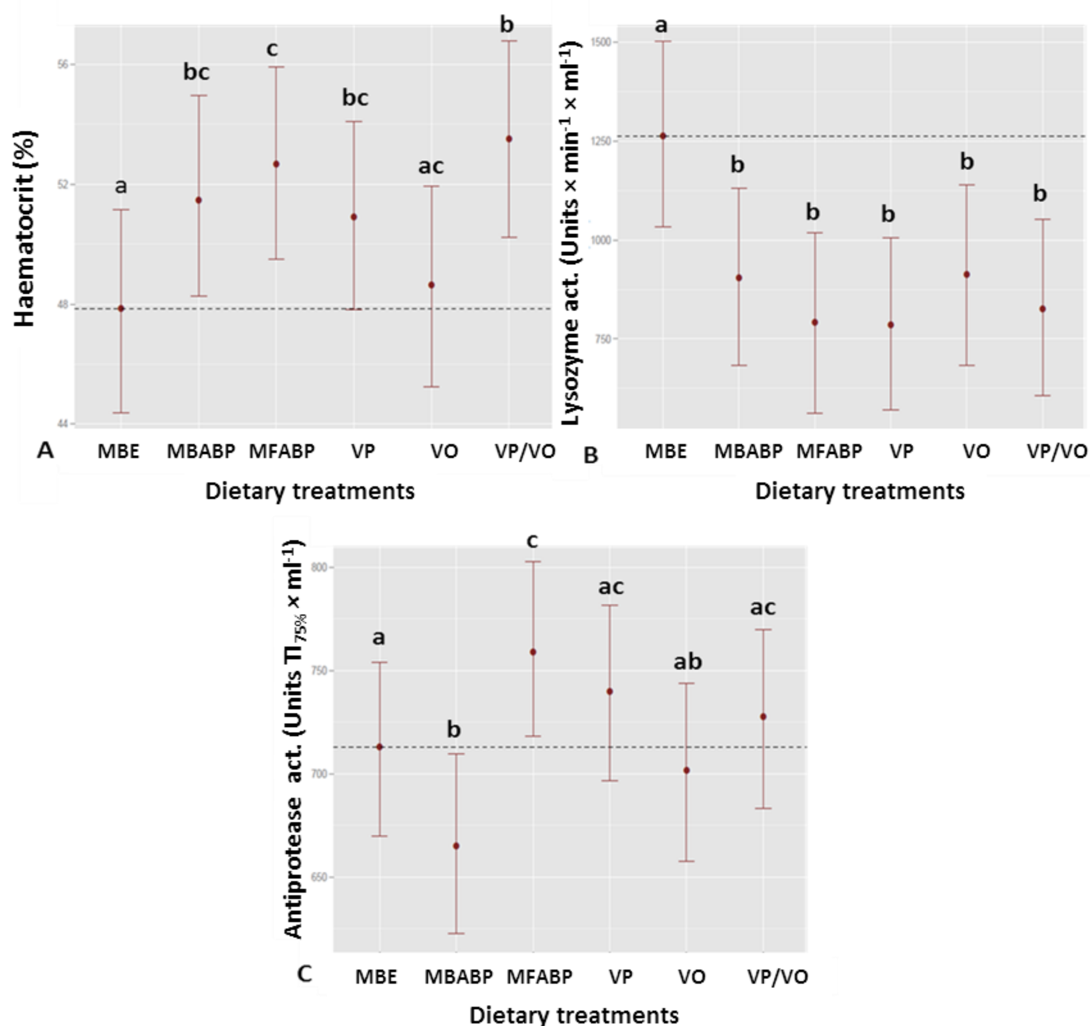

Figure 40 Expected levels of the affected haematological and immunological responses.(A) Haematocrit (%); (B) Lysozyme (Units × min^{-1} × ml^{-1}); Plasma anti-protease activity (Units TI_{75} × ml^{-1}); (%); of the dietary groups of Atlantic salmon post-smolts for the full period of the trial. These parameters were affected by the diets and the results are summarised as graphs with the mean response and 95% confidence interval. Confidence intervals were solved by a posterior simulation from the statistical model with 1500 random draws. Significant differences among dietary groups are revealed when the confidence intervals bars for a certain response of a dietary group do not overlap with the mean values of the same response from another group. The dietary effect on the health responses was confirmed by fitting a model without the dietary effect (only tank effect) and with it (tank effect nested within it) and comparing the models with a likelihood ratio tests (LRT).The modelling for all above parameters Diet abbreviations: MB, marine based diet; VP, vegetable protein diet; VO, vegetable oil diet; VP/VO, vegetable protein/vegetable oil diet; MBABP, marine based animal-by-product diet; MFABP, marine free animal-by-product diet.

7.4 DISCUSSION

7.4.1 Growth and carcass chemical composition

Total replacement of FM or FO and/or both marine ingredients in the experimental feeds was done in order not to compromise salmon known nutrient requirements (NRC, 1993).

The corn gluten, wheat gluten, soy and pea protein concentrate were selected based on previous studies reporting adequate growth in fish (de Francesco et al., 2004; Kaushik et al., 2004; Espe et al., 2006; Øverland et al., 2009). Nonetheless, the results of feed composition analysis from the two lots of feeds used in the present study reveal inconsistencies in the quality of the raw materials demonstrating the requirement for nutritional assessment of feedstuffs prior to the production of the feeds. This could allow for adjustments in dietary formulations and consistencies in the quality of aquafeed supplies by feed manufacturers.

Firstly it should be mentioned that the growth performance in terms of WG and FI for the first and full period of the study were largely influenced by the initial size differences of the fish assigned to the different diets. Redistribution salmon populations in the tanks prior to the initiation of the feeding trial, could have given even more sound and clear results regarding the overall performance of the experimental groups of salmon. However, comparable to the present study, were also the differences in salmon starting weights before the commencement of a similar commercial feeding study conducted by EWOS and reported by Crampton et al. (2011), utilising a commercial EWOS marine based and a low FM diet in Atlantic salmon post-smolts reared in sea cages. All the above, highlight the difficulties when conducting large scale scientific studies involving salmon of large size like the present one. However, this should not detract from the significance of this investigation as important conclusions could still be drawn from it.

MBE salmon demonstrated a significantly higher FI than the rest of the experimental dietary groups, during the initial period of the feeding trial. This finding however, could have been influenced by the bigger size of the fish assigned to the MBE diet by the end of the acclimation period and before the initiation of the feeding trial. Contrary to this the MBABP group of fish presented lower levels of FI than the group of salmon receiving the VP diet which also demonstrated greater size at the start of the trial, while no differences were

observed between the former group and salmon receiving both of the marine-free diets (MFABP and VP/VO). Previous studies, have reported that total (de Francesco et al., 2004; Espe et al., 2006) or partial replacement of FM (Gomes et al., 1995; Kaushik et al., 2004) in salmonid diets can negatively affect the FI in fish. Moreover, Liland et al. (2012), proposed that dietary FA composition might be a regulating component of Atlantic salmon appetite and that VOs characterised by increased levels of 18:2n-6 and decreased n-3/n-6 ratio which is the case in the VO-based diets of the present study could decrease FI in Atlantic salmon. This could only explain the numerically lower FI in VO-fed fish in comparison to the VP group and the absence of differences between MBABP salmon in contrast to the former group, despite the size difference at the start of the study. In addition to that, previous trials investigating self-selecting feeding demonstrated that rainbow trout prefer diets containing FO over those with VO, suggesting that some fish do actively select feeds based on marine lipids (Geurden et al., 2005, 2007). In accordance to this, the present trial demonstrated that the elimination of FO from salmon diets seems to affect salmon FI to a higher degree, than FM withdrawal. Moreover, low amounts of FO are always present in FM which could explain the absence of differences among the VO, MBABP and VP-fed salmon groups. In contrast to our findings, Carter et al. (2003) and Miller et al. (2007) reported unaffected FI in Atlantic salmon fed diets containing just algal oil, a combination of algal and palm oil or just palm oil compared to salmon fed a full FO based diet. However, these studies also demonstrated unaffected growth performance in salmon fed a FM based diet containing algal oils, which is in agreement with our results.

In addition to the higher FI values higher growth performance indices (WG, SGR and TGC values) were observed for MBE salmon during the first period. No significant changes in daily WG were observed between VO and MBE salmon which is in line with previous FO replacement studies (Bell et al., 2001, 2002; Torstensen et al., 2005). VO salmon was

the second best performing dietary group exhibiting higher SGR and TGC values than the rest of the groups. On the contrary, MFABP and VP/VO salmon presented the lowest growth while MBABP and VP-fed salmon demonstrated intermediate growth to the VO and latter groups. The initial size discrepancies promoting contrasting FIs and thus further size differences among the latter groups of salmon seem to be the main reason for the last observation. Despite the higher levels of FI and WG for the MBE control group, FCR values in MBE salmon for the first period of the study were found to similar to that of VP salmon and significantly higher from that of the other dietary groups, revealing a more efficient dietary nutrient utilisation in all other groups.

Increased feed consumption and thus growth (defined as "compensatory growth") after periods of restricted FI have been demonstrated in Atlantic salmon (Johansen et al., 2001; Torstensen et al., 2008). This growth can be greater or equal compared to normal growing fish, although incomplete or impartial gain has also been known to occur (Jobling and Johansen, 1999; Johansen et al., 2001). During the second study period, a significant increase in FI was observed for the MBABP-, MFABP-, VP/VO- and VO-fed salmon, while numerically higher levels were also noticed for the VP group of fish in comparison to their initial values, revealing an initiation of the compensatory growth process. Further signs of compensatory growth, for all the aforementioned groups, were the increased SGR and TGC values which were found to be higher than the ones obtained for MBE group. The above findings demonstrate that Atlantic salmon requires long adaptation period before accepting any diet with high levels of alternative protein and lipid sources as previously reported by Torstensen et al. (2008). Furthermore, a significant reduction in WG for MBE salmon was witnessed, in comparison to the first period. The latter observation coincided with the provision of the second lot of the diets which was in general characterised by the poor nutritional profile of the applied FO source. Among the changes observed in the two lots of

the aforementioned diets, mentioned in Section 7.3.1, the most striking change was the reduced n-3/n-6 ratio in the second batch of FO-based feeds (MBE, MBABP and VP) as a result of the low levels of n-3 PUFAs, with the 22:6n-3 (DHA) demonstrating the greatest reduction in this FA group, while reduced levels of 20:5n-3 (EPA), were also presented for these diets. The aforementioned group of FAs is required for the maintenance of membrane integrity and as precursors for the synthesis of eicosanoids which are important signalling molecules made from the C_{20} EFAs controlling many bodily functions including inflammation and immune regulation (De Pablo et al., 2000; Tocher, 2003). No further changes on the physical characteristics of the pellets (floatability, sinking rate, colour or durability) were recorded. Furthermore, MBE presented significantly higher FCR than most of the experimental groups, excluding VO-fed salmon while no differences were detected among the rest of the salmon, indicating higher efficiency for these diets.

Overall, higher FI for the full period of the feeding trial was revealed in MBE and VP salmon compared to the other groups. This could highlight the important role of the FO fraction on the acceptability of aquafeeds in Atlantic salmon (Liland et al. 2012), as lower FI was obtained for the VO group regardless the greater initial size of these fish compared to VP salmon. Significant WG differences however, were only revealed among the MBE and salmon from the MBABP and the two marine free dietary groups. These were most likely caused by size discrepancies among these groups at the start as previously mentioned. On the contrary, significantly higher FCR values were obtained in MBE and VP salmon in contrast to the other groups, during the full trial period. Therefore, among the two control groups, MBE salmon exhibited lower feed efficiency while MBABP salmon with intermediate growth performance values demonstrated better efficiency in the utilisation of dietary nutrients. Excluding VP salmon, the low FCR values demonstrated for the majority of the experimental groups during the full study period, indicate that judicious selection of

alternatives to FM and FO and careful formulation of salmon feeds in order to satisfy their nutrient requirements could promote adequate growth even when both marine-derived proteins and lipids are fully excluded. Espe et al. (2006) reported equal FCR values in Atlantic salmon fed a FM-based compared to FM-free diets, which in the case of the present study was true only for the MBABP compared to the VP and the two marine-free dietary groups. However, in the latter study marine based by-products were included in the experimental diets in order to improve their acceptance by the fish. Contrary to our findings, were also the increased FCR values reported in Atlantic salmon post-smolts fed on diets where marine and plant derived ingredients from commercial salmon diets were partially substituted by terrestrial ABPs (Hatlen et al., 2013, 2014). Similar to our results, unaffected FCRs were also reported in Atlantic salmon fed low marine ingredient diets compared to a fully marine dietary group (Torstensen et al., 2008). Moreover, most FO replacement studies for Atlantic salmon diets demonstrated unaffected FCRs for VO- in comparison to FO-fed salmon which are partially in agreement with the present findings (Bell et al., 2002; Torstensen et al., 2005; Karalazos et al., 2007).

Condition factor (*K)* values at the end of the trial ranged between 1.25 and 1.75 for the majority of the fish, describing salmon with fairly good to excellent quality (Barnham and Baxter, 1998). The VP group possessed the highest *K* values, while the VO-fed salmon exhibited the lowest values compared to the rest of the groups. Since *K* factor describes the relationship between the full weight and salmon length, the high values obtained for VP-fed salmon is an indication of “stocky” salmon whereas the low *K* values observed for the VO-fed fish show that these salmon were leaner than all the other dietary groups. In general, Atlantic salmon post-smolts of 2.5 kg (mean final salmon weight for all groups) are characterised by increased carcass growth, hepatic and visceral fat deposition (Shearer, 1994). Therefore, the increased *K* values exhibited for VP salmon could actually be an

indirect indication of higher hepatic and visceral fat accumulation. The low *K* values of VO-fed salmon, on the contrary, could point at lower adiposity, however, a more thorough investigation is required. Nonetheless, data regarding the hepato-somatic and viscero-somatic indices were not obtained, therefore the above comment consists a speculation and reveals one more flaw of the experimental design. Despite the fact that the opposite trend was illustrated for the VO-fed group, the assumption of lower adiposity in these fish requires a more thorough investigation. Ruyter et al. (2006) and Jordal et al. (2007) demonstrated that diets with high levels of soybean oil or complete substitution of FO with VO blends could induce visceral and/or hepatic adiposity. Furthermore, Torstensen et al. (2011) reported increased visceral adiposity in salmon fed diets with high levels of FM and FO substitution with VP and VO respectively. Since fat levels in the liver, intestine and pancreas were not estimated in the current study no further comments could be made on this matter. In contrast to our findings, Espe et al. (2006) demonstrated that *K* values were not significantly altered by feeding Atlantic salmon with diets in which FM had been completely replaced with VPs. Furthermore, Torstensen et al. (2008) reported lower *K* values in Atlantic salmon maintained on diets with high levels of VPs and moderate or high supplementation with VOs compared to fish fed a marine-based diet or a diet containing a moderate inclusion of VPs and high inclusion of VOs.

The results of NQC carcass proximate analysis revealed no differences in moisture, crude protein, lipid and ash levels among the six dietary groups, suggesting similar levels of nutrient accumulation in the carcass for all the salmon groups. Similarly, previous studies have demonstrated that complete substitution of FO with VO in feeds for salmonids does not have an effect on their carcass composition (Karalazos et al., 2007; Turchini and Francis, 2009). Contrary to our findings, a body of literature has demonstrated concomitant

reductions in carcass lipid and increases in carcass protein in salmonids fed diets in which FO was replaced with VOs (Jokumsen and Alsted, 1990; Bell et al., 2001, 2002).

7.4.2 Haematological parameters and immune responses

To our knowledge reports on the effects of a concomitant dietary substitution of FM and FO with alternative ingredients on Atlantic salmon haematology have not yet been investigated. In the present study it is apparent that the elimination of the FM fraction from the diets resulted in increased haematocrit values, while the elimination of both fractions promoted even higher haematocrits. Most of the existent reports of FM and FO substitution with alternative feed ingredients are contradictory to the present findings. Twibell et al. (2012) reported lower haematocrit levels in coho salmon and rainbow trout (*Oncorhynchus mykiss*) fed on VP and ABP in combination with VO diets compared to salmon fed MB diets. Furthermore, complete replacement of dietary FM with ABPs did not significantly affect haematocrit levels in previous feeding trials with coho salmon (Higgs et al., 1979) or rainbow trout (Steffens, 1994) compared with fish fed a FM control diet. On the other hand Hemre et al. (1995; 2005) reported decreased haematocrit levels in Atlantic salmon fed on diets with increased substitution of FM with soybean products or increased dietary inclusion of crude fibre which is the case at high levels of FM replacement with most plant derived feed proteins. In addition, Thompson et al. (1996) reported unaffected haematocrit in Atlantic salmon fed on diets with complete substitution of FO with sunflower oil (SO). The observed changes in haematocrit seem to have a multifactorial cause, which could be quite complex to put into context. The most promising finding however, was that haematocrit values for all dietary salmon groups were found to be within normal ranges varying from 43-60%, indicating healthy fish (Hardie et al., 1990; Waagbø et al., 1994; Thompson et al., 1996) without compromised blood oxygen carrying capacity, since there was lack of anaemia which could be related to iron or other mineral deficiencies. Total and differential

leucocyte numbers did not demonstrate any significant changes among the six dietary groups of salmon which is an indication that all of the diets are modulating similar levels and patterns of leucocyte production. In line, Thompson et al. (1996) reported that Atlantic salmon fed on diets with complete replacement of FO with sunflower oil (SO) did not exhibit differences in total and differential circulating leucocyte levels. On the contrary Rumsey et al. (1994), showed that rainbow trout fed on soy proteins presented increased numbers of circulating leucocytes.

The only two innate immune responses demonstrating differences among diets were the plasma lysozyme and anti-protease activity. No differences were observed among the six dietary groups of salmon regarding plasma haemolytic activity, plasma protein and total IgM levels, stimulated and non-stimulated HKM burst activity.On the contrary, (Jalili et al., 2013) demonstrated no notable negative effects on lysozyme levels in rainbow trout fed on diets with full replacement of FM with VPs. In contrast to the present results, were the reduced levels of total serum IgM reported by Jalili et al. (2013) in rainbow trout fed on diets with total substitution of FM with VPs. Moreover, Jalili et al. (2013) and Sitjà-Bobadilla et al. (2005) reported decreased alternative complement activity in rainbow trout and gilthead sea bream fed diets with 100% substitution of FM with VPs.

In line with the present study, no differences in HKM respiratory burst activity were observed in feeding trials where Atlantic salmon and rainbow trout were fed on FM-based diets supplemented only with soybean oil or linseed oil (Kiron et al., 2004; Seierstad et al., 2009). Furthermore, Carter et al. (2003) demonstrated no changes in serum total immunoglobulin and protein levels, serum anti-protease activity and circulating leucocytes respiratory burst activity for Atlantic salmon fed diets with complete replacement of FO with canola oil (CO) or 2 blends of CO and FO or CO and algal oil which are in line with the present findings. Similar results were also obtained by (Thompson et al., 1996) who

demonstrated no differences in serum complement, anti-protease and HKM respiratory burst activities of Atlantic salmon fed full soybean oil diets compared to FO fed salmon. However, in both aforementioned studies unaffected plasma lysozyme was also reported which is in contrast to the present study. The present findings demonstrated higher lysozyme activity from for MBE salmon which could be a favourable trade against bacterial or viral diseases (Saurabh and Sahoo, 2008). However, it should be stressed that several disease resistance selection trials have observed a negative correlation between survival rate and lysozyme activity in Atlantic salmon challenged against furunculosis, bacterial kidney disease (BKD) and vibriosis (Røed et al., 1993; Fevolden et al., 1994; Lund et al., 1995), demonstrating that disease resistance in salmon might be more dependent on other immune responses or the efficiency in detoxifying from the by-products of immune activation. Moreover, Fevolden et al. (1994) suggested that for salmonids, lysozyme activity following a disease challenge is not a reflection of a superior immune mobilisation of the fish, but an indication of stress induction which could increase the susceptibility of these fish under challenging conditions. In the present study, the second period of the trial coincided with a great growth reduction (which could also be related to increased stress) (Pickering, 1993; Plisetskaya and Duan, 1994) in MBE salmon could indicate increased stress for this dietary group.

In the present study, it was also shown that diets containing only plant proteins (VP and VP/VO) or high levels of plant proteins (MFABP) promoted plasma anti-protease activity, which could be a favourable feature for combating several bacterial infections (Ellis, 1990). Several plant extracts used as additives in aqua-feeds in previous studies demonstrated an increase in serum anti-protease activity (Rao and Chakrabarti, 2004; Kaleeswaran et al., 2011) It is possible that high levels of plant derived ingredients and more specifically plant protein concentrates even after processing manipulations targeting the improvement of their nutritional quality, (through protein concentration primarily and the

removal of ANFs secondarily) might contain certain levels of bioactive compounds exerting an immunostimulatory activity to the fish. However, the identification of such compounds in plant derived products and the positive effect of marine-free diets on antiprotease activity requires further investigation.

7.5 CONCLUSIONS

The findings of the current study suggest that marine protein-, marine oil- and marine-free diets can be utilised satisfactorily by Atlantic salmon post-smolts in comparison to commercial feed formulations, stimulating both adequate growth and innate immune responses. However, longer adaptation periods might be required for the fish to fully accept these diets. Therefore complete substitution of FM or FO or both marine derived ingredients is possible without undermining the general condition of the fish, however future application of such feeds will depend on the prices of the applied raw materials, which currently do not consist a cost-efficient solution compared to the already applied feedstuffs found in commercial feed formulations. Nevertheless, future studies on similar levels of FM and FO replacement in salmon feeds should evaluate these feed formulations under the stressful commercial conditions or assess the resistance of salmon fed on these diets, against industrially important diseases.

Chapter 8.

General Discussion, Conclusions and Future Perspectives

8.1 GENERAL DISCUSSION AND CONCLUSIONS

Farming of fish and especially the culture of carnivorous fish species, including salmonids relies on high protein feeds. Atlantic salmon (*Salmo salar*) in general requires 55-60% of dietary protein in juvenile stages, thereafter the protein requirement declines (NRC, 1993). Historically the protein source in salmon diets has been FM, produced from wild fisheries, while the lipid source has been FO from the same source. For the last 30 years worldwide production of farmed Atlantic salmon has increased steadily whereas the production from wild caught fish has been stable for the same period (Tacon and Metian, 2008; FAO, 2013). Therefore, if a continued increase of farmed fish is to occur, both alternative protein and lipid sources need to be evaluated as feedstuffs for commercial aquafeeds. FM and FO replacement should be done in the basis of supporting good growth without compromising the health and welfare of farmed fish. Currently, dietary FM inclusion percentage in Atlantic salmon feeds ranges from 15% to 55% while FO level spans from 3% to 40% and these variations depend on the country diets are manufactured, partially reflecting differences in the production systems employed and differences among the farmed salmon breeds applied commercially in these countries (DeSilva et al., 2012).

SPC based diets supplemented with lysine, methionine and threonine (the main IAA found below requirement levels for salmon in SPC) are very promising since they can support good growth in both juvenile and adult Atlantic salmon stages, in the absence of enteritis while also promoting several local immune responses in the GIT, thus increasing the resistance of salmon to *Aeromonas salmonicida* (causative agent of furunculosis). Despite the benefits of using SPC, the high levels of phytate (the main source of P in soybean) found in this product could limit the uptake of P from the diets since this substance cannot be digested by salmon digestive enzymes (due to the lack of phytase), influencing

also the absorbance of several other elements. Furthermore, phytate was found to form non-digestible complexes with several positively charged dietary ions, rendering them unavailable for absorption in the gut of monogastric animals such as fish, resulting in elemental deficiencies. These, could potentially result in bone deformities or clinical conditions which could increase the susceptibility of farmed Atlantic salmon to environmental stressors. Moreover, modern salmon farming is also characterised by the use of several procedures aiming the increase of its productivity, including vaccination of the fish in order to improve their survival and resistance against commercially important diseases such as furunculosis (Austin, 1997; Poppe and Koppang, 2014) and the use of continuous light to prevent salmon smoltification and maturation (Taranger et al., 1991; Sigholt et al., 1995; Duncan and Bromage, 1998; Duncan et al., 1998; Taranger et al., 1998; Bromage et al., 2001; Handeland et al., 2001), directing salmon energy on growth. In the second case, improved growth performance of Atlantic salmon has been reported. However, the application of different production techniques (such as the use of elevated water temperatures) targeting the improvement of salmon growth performance and vaccination have been linked with the increased prevalence of bone deformities in Atlantic salmon post-smolts (Ytteborg et al., 2009; Grini et al., 2011). Often, the presence of bone deformities in Atlantic salmon post-smolts, coincides with elemental deficiencies in earlier juvenile stages (Grini et al., 2011).

For all the aforementioned reasons, the primary objective of the present thesis was to assess the effects of increasing dietary SPC, lysine, methionine and threonine levels and the constant (1st experimental study, Chapter 3 and 4) or increasing (2nd experimental study, Chapter 5 and 6) supplementation of P (above recommended Atlantic salmon requirements according to NRC, 1993) in juvenile Atlantic salmon diets, on growth and the overall health status and welfare of vaccinated individuals exposed to different light regimes in the two

experimental studies. In the present thesis, juvenile Atlantic salmon from different developmental stages fed on increasing levels of dietary protein from SPC for extended periods (62 days in the first study and 92 days in the second study), were assessed for a wide range of immunological responses prior to and after vaccination, using traditional immunological techniques (immunity before and after vaccination was assessed in both the first and second experimental studies). In the second study a second group of non-vaccinated Atlantic salmon was used as a control for the vaccinated dietary groups of salmon, while both vaccinated and PBS-injected fish were then challenged against *A.salmonicida* at the end of the study and 62 days post vaccination/ PBS injection for the evaluation of their overall resistance against furunculosis. Nevertheless, in both experimental studies growth performance and elemental concentrations in body cross-sections (in the 1st experiment) and whole vertebral tissues (in the 2nd experiment) were used for the assessment of normal growth performance and elemental deficiencies which could potentially result in pathological conditions (bone deformities and other pathologies increasing their susceptibility to environmental stressors). An additional aim of the present thesis was to assess the effects of FM-, FO- and marine-free diets on growth, carcass proximate composition and the immunological responses of Atlantic salmon post-smolts on-grown under a continuous light regime during the full period of the study.

From the two experimental trials using diets with increasing SPC levels, we were able to detect increasing amounts of phytate in diets with higher SPC inclusions. As mentioned phytate could be a major limiting factor in such diets and as such, it should be monitored in a regular basis as it could promote several issues in the production of farmed fish. Therefore, the increasing levels of dietary phytate could have reduced the nutrient digestibility and thus the growth performance of Atlantic salmon fed on increasing dietary SPC levels (Kumar et al., 2012). In addition, it was shown that salmon pre-smolts receiving higher dietary SPC

levels exhibited better growth performance compared to early stage parr. In line with this, Burr et al. (2012) demonstrated 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 showing improved growth. Moreover, Denstadli et al. (2006) reported that large Atlantic salmon parr can adapt more easily to diets with increased phytate levels due to a more developed gastrointestinal tract, making a more efficient use of the dietary nutrients. Due to experimental limitations the calculation of actual FI and thus FCR was not possible in both of these trials. Therefore we were not able to prove if the actual reason for the observed growth reduction was a decrease in FI or the increasing amounts of phytate. However, it should be stressed that several studies have reported reduced FI and thus compromised growth in a wide variety of fish species receiving increased dietary SPC with or without AA supplementation (Kissil et al., 2000; Arãgao et al., 2003; Deng et al., 2006). Mambrini et al. (1999) on the other hand reported reduced FI in rainbow trout, only when fish fed diets with 75 and 100% of dietary protein from SPC with or without supplementation of the diets with methionine, resulting in inadequate growth of the fish.

From the existing literature, the best approach for the evaluation of the mineral status in fish is generally the use of full body samples (Shearer, 1994). The first trial of the present thesis, was primarily designed to focus on the effects of the diets on the immunity of the fish. Therefore, various samples were obtained for immunological evaluation (head kidney and plasma for the performance of immunoassays) while gill, intestine, liver and spleen samples were also collected for immune gene expression. In an attempt to make a more efficient use of the sampled fish and to reduce the pressure at sample collection, body cross-sections were preferred over whole body samples for the assessment of proximate and elemental composition in salmon. Due to the novelty of this technique and the fact that whole body samples were not taken at anyone of the chosen timepoints, data taken for body cross-

sections were compared with literature data on full body samples, which does not consist an orthodox and direct comparison of datasets. Albeit, important conclusions could be drawn from the study, on the potential use of body cross-sections for elemental assessments which could apply even for bigger size salmon, the processing of which consists a challenge in order to perform mineral analyses. Furthermore, we were still able to elucidate changes in several key elements upon feeding juvenile salmon with increasing amounts of dietary protein from SPC. These were related with reduced amounts of ash and several elements such as P, Ca, Zn, Mg, Mn as well as reduced Ca: P ratios in Atlantic salmon juveniles maintained on diets with increased dietary levels of SPC and constant supplementation of P at 30 g $\times$ kg^{-1}of feed, under continuous light. Phosphorus and calcium concentrations presented significant reductions throughout the full study period (prior and post vaccination) in most of the dietary groups receiving higher than 35% of dietary protein from SPC compared to SPC35 salmon (SPC65 and SPC80 for both timepoints and SPC50 only for the first timepoint). Zinc demonstrated significant reductions only in the SPC65 and SPC80 groups of fish compared to SPC35 salmon at both timepoints, whereas Mg and Mn demonstrated reductions only during the first part of the study, prior to the vaccination of the fish. At this point SPC65 and SPC80 salmon exhibited significant Mn reductions compared to the SPC35 group, while all groups fed on diets with higher than 35% of dietary protein from SPC, presented significantly lower Mg levels in contrast to SPC35 salmon. In general, improved body cross-section elemental amounts were revealed in salmon fed increasing dietary SPC levels at 32dpv compared to the SPC35 group. It is suggested that this could be an effect of the long term provision of salmon with high SPC diets or an interaction of this with fish vaccination (Storebakken et al., 1998b; Storebakken et al., 2000; Grini et al., 2011). Both of these processes have been shown to delay salmon growth having a beneficial effect in bone mineral deposition. Overall, lower elemental amounts were

detected for all dietary groups at the end of the study. The most prominent reason for that seems to be the exposure of salmon to constant lighting which could have promoted salmon growth, reducing the time for the developing bone matrix to mineralise adequately. More experiments are required to further strengthen the abeove hypothesis. Notably Ca, P and Zn levels at the end of the study were much lower from already reported whole body levels of these elements in juvenile salmon and could possibly be an indication of subclinical elemental deficiencies which could potentially trigger salmon welfare issues including bone deformities and increased susceptibility to diseases. It was also suggested that increased dietary inclusion of SPC in salmon diets should be combined with increased supplementation of P (since most of the P in SPC is in the form of phytate and is largely unavailable) and this seems to lie between 30 and 40 $g \times kg^{-1}$ for salmon diets with higher than 50% and up to 80% of dietary protein from SPC containing about 15-16 $g \times kg^{-1}$ phytate. The adequate provision of P in salmon diets could promote the dietary uptake of minerals such as Ca and Zn which are integral for healthy bones and thus robust fish (Lall and McCrea, 2007).

Measured haematological parameters of naive salmon juveniles were adversely affected by increased dietary SPC levels, in the first study. Significant reductions in haematocrit, leucocyte and lymphocyte counts were demonstrated in SPC65 and SPC80 salmon compared to the SPC35 group. While haematocrit values demonstrated significant reductions in salmon receiving higher than 35% of dietary protein from SPC throughout the full study period, both leucocyte and lymphocyte levels presented a substantial increase in these fish at 34 dpv, eliminating the differences observed at the two first timepoints, between the dietary groups and possibly revealing an increased capacity for cellular immune modulation. Moreover, increased thrombocyte levels were observed for the SPC50 and SPC65 groups of salmon. These were significantly higher at 34 dpv compared to the SPC35 group and were well correlated with the changes observed in complement activity at this

timepoint. Complement and blood coagulation systems are closely linked via the use of the same enzymes (Amara et al., 2008). It is believed that the two pathways could have been influenced by changes and differences in ion absorption, osmo- and ion regulation between the four dietary groups, especially for Ca and Mg which are cofactors for many enzymes involved in both pathways. In addition, to the increased complement activity, groups with intermediate levels of SPC inclusion demonstrated increased lysozyme activity and circulating amounts of total IgM in their plasma which shows that these diets can stimulate a range of integral innate immunological responses, potentially influencing beneficially the health status of juvenile salmon. The reason for that seems to be the presence of specific immunogenic compounds in SPC, which at these levels of FM substitution do exert an immunostimulatory effect in treated salmon (Krogdahl et al., 2000). On the contrary, higher supplementation of FM with SPC seems to result in nutritional limitations which have to be addressed before implementing higher levels of SPC. Moreover, differences in the availability of P in the four dietary groups of salmon could have been an alternative cause for the observed changes in the aforementioned humoral immune responses. It is suggested that diets with intermediate levels of SPC had a more optimum level of available dietary P in comparison to the low and high SPC inclusion groups Therefore, lower (< 30 g $\times$ kg^{-1}) and higher (> 30 g $\times$ kg^{-1}) P supplementation is proposed for the SPC35 and SPC80 treatment respectively.

Having the first experiment as a driver, in the second trial increasing dietary SPC levels were combined with increasing P supplementation from 20 g $\times$ kg^{-1} to 40 g $\times$ kg^{-1} moving from SPC35 to SPC80 (20 g $\times$ kg^{-1} for SPC35, 30 g $\times$ kg^{-1} for SPC58 and 40 g $\times$ kg^{-1} for SPC80 diet). In the second trial, early stage salmon parr were used and fish were exposed to a more conventional lighting regime of 12h L: 12h D. Salmon after an initial feeding period of three months, were vaccinated against furunculosis or PBS-injected with

the feeding trial extending for an additional period of 2 months. A linear decrease in bone minerals was revealed for the initial 3-month feeding period, moving from SPC35 to SPC80 salmon. Significant differences in P, Ca, Mg and Zn were demonstrated between SPC80 and SPC35 salmon for this period while significantly lower bone concentrations of Mg and Zn were demonstrated for the SPC58 group in comparison to SPC35 salmon. However, the observed skeletal elemental reduction was reversed by the end of the feeding trial in both vaccinated and PBS-injected groups of salmon with the SPC80 group exhibiting higher levels for the majority of the analysed bone elements in contrast to SPC35 salmon. In addition, it was shown that vertebral mineralisation in vaccinated salmon was higher than in unvaccinated fish. Combining the observations from both vaccination studies it could be said that the interaction of dietary SPC and fish vaccination does not negatively affect bone mineralisation. However, commonly applied culture techniques having direct (feeding regimes, thermoperiod) or indirect (photoperiod) effects on salmon growth (Jørgensen and Jobling, 1992; Stefansson et al., 2008; Grini et al., 2011), should be taken into account when monitoring the concentrations of elements in salmon bones. In this case a lighting regime of 12h L:12h D, resulted in improved bone elemental concentrations compared to the continuous lighting regime of the first study, which was shown to demote body cross-section elemental levels.

In the second study, it was shown that replacement of 80% of dietary protein with SPC combined with an increased addition of lysine, methionine, threonine and P did not compromise the overall immune capacity of salmon and their resistance against *A. salmonicida*. Nonetheless, differential regulation of the different complement pathways was exhibited among the three dietary groups of salmon. More specifically, the second experimental study demonstrated that the AC activity in early salmon parr was adversely affected by the long term feeding of the fish with medium or high SPC inclusion feeds solely

or in combination with vaccination which was applied to assess their capacity for immunostimulation. Furthermore, it was shown that upon vaccination CC activity increased in salmon parr receiving increased dietary levels of SPC, whereas for SPC35 salmon CC activity was found to be reduced, while no differences were detected in TC activity among the dietary treatments. The latter indicates that the overall complement activity (the sum AC, CC and lectin activity) remained constant, and this was achieved by maintaining a balance between the different pathways of complement system, which seems to operate on the crossroads of both innate and specific immunity. The combined findings from both studies investigating the effects of increasing SPC levels on immunity, show that complement and its components is a target system for the immunological evaluation of salmon or other fish receiving diets with increased inclusion of SPC and other soy protein aqua-feed products, since it was found to be modulated in both studies (Jalili et al., 2013).

The last objective of the present thesis was to assess the effects of FM-, FO- and fishery- free diets on the growth performance and innate immune responses of salmon post-smolts. In the corresponding study, MBE (EWOS commercial dietary formulation for EU countries) salmon demonstrated the highest FI and WG in contrast to the other dietary groups for the first and the full period of the study. These results were however, largely influenced by differences in the size of the fish assigned to the diets and the greater size of salmon assigned to the MBE diet, revealing an important fault in the experimental plan. A more even distribution of the fish in the tanks by the end of the acclimation period in order to eliminate size differences between the tanks at the start of the study, would have been a major refinement for the experimental design, allowing for better assessment of the feed efficiency and the growth performance of the different groups. This fact also highlights the difficulties encountered when conducting large scale scientific studies like the present one. The reduction in FI and growth in salmon fed FM- and FO-free diets is in line with many

other previous studies showing that complete substitution of FM or FO can negatively affect the acceptability of such feeds by the fish, their FI and eventually their growth (Espe et al., 2006; Kousoulaki et al., 2012; Liland et al., 2012). Overall, the treatment demonstrating the best performance during the first period of the study was the VO diet, since lower FCR was exhibited for VO salmon compared to the MBE and VP groups, in conjunction with higher growth rates (SGR, TGC) compared to the other four experimental groups excluding MBE fish. In agreement with these results, unaffected FCRs were previously reported in rainbow trout fed diets with complete replacement of FM compared to fish fed a FM based diet, while no changes in FCR were also recorded for Atlantic salmon post-smolts fed diets with low marine ingredients compared to a fully marine based dietary group. Diets with complete substitution of both fishery-based feedstuffs together with the MBABP (EWOS commercial diet for non EU countries) feed exhibited similar FCR to the VO group and significantly lower values than MB and VP salmon, also pointing at better efficiency in dietary nutrient utilisation in contrast to the latter groups. Lower FCRs were also demonstrated, during the second period of the study, for the majority of the experimental dietary groups of salmon in contrast to the MBE group, revealing better nutrient utilisation. At this timepoint, lower FCRs were combined with improved FI and growth performance indices (TGC and SGR). Both TGC and SGR values for all diets with low levels of marine derived ingredients, including the MBABP group were found to be higher than in MB salmon, with VP and VP/VO salmon presenting the highest SGR and TGC values. The results are in agreement with previous studies demonstrating increased FI and compensatory growth after periods of restricted FI due to dietary modifications, in Atlantic salmon post-smolts (Torstensen et al., 2008). It is thus shown in one more trial that Atlantic salmon requires longer adaptation periods to diets with low levels of marine feedstuffs or complete absence of them.

Moreover, at the end of the study VP salmon demonstrated the highest condition factor while VO salmon, the lowest. The latter seems to be an indication of higher fat accumulation in the viscero-hepatic tissues of VP salmon, however, neither data of the hepato- or viscerosomatic indices were taken, nor analyses on the lipid composition of the aforementioned tissues was performed revealing an additional flaw in the experimental design. Torstensen et al. (2008) reported lower *K* values for Atlantic salmon post smolts fed diets with high levels of VPs and moderate to high levels of VOs in comparison to salmon fed a full marine based diet and a diet with intermediate levels of VPs for a year. Further analysis of the whole body lipid storage pattern, revealed increased levels of whole body and visceral fat deposition in salmon fed high VP and VO diets (Torstensen et al., 2011). Proximate analyses on salmon carcasses demonstrated no differences in moisture, lipid, protein or ash concentrations. As mentioned before similar analysis on the viscero-hepatic tissues or the whole bodies could have been much more informative regarding the assessment of salmon adiposity in the different dietary groups of fish. In fact several studies, reported increased visceral and hepatic adiposity in Atlantic salmon fed on diets with plant derived products (Ruyter et al., 2006; Jordal et al., 2007; Torstensen et al., 2011)

Overall, in the present study it is demonstrated that complete replacement of FM or FO and simultaneous substitution of both FM and FO are possible in salmon diets, without undermining the growth performance of the fish, in comparison to currently applied commercial feed formulations. The promotion of adequate growth rates in Atlantic salmon with the provision of marine-free diets is an important novel finding, revealing that salmon diets can be potentially, completely free from FM and FO, two of the integral feedstuffs, supporting the growth of the industry for the past 40 years. Nonetheless, still the commercial application of such diets is far from being possible since many of the feed ingredients applied

in the marine free diets are characterised by high production costs which make their current use unaffordable.

In the last study, the majority of measured haematological and immune responses did not exhibit significant differences among the dietary groups of salmon, revealing adequate stimulation of salmon innate immune responses. Haematocrit was the only one of the measured haematological responses exhibiting changes among the dietary groups of salmon. Briefly, it was shown that diets with complete replacement of FM with both VPs or VPs and ABPs, resulted in higher haematocrit values, while total substitution of both FM and FO influenced even higher haematocrit levels which contradicts previous studies reporting unaffected or reduced haematocrits in salmonids receiving diets with high levels of ABPs or VPs or a combination of ABPs and VOs (Higgs et al., 1979; Steffens et al., 1994; Hemre et al., 1995; Hemre et al., 2005; Twibell et al., 2012). The observed differences between the dietary groups seem to have a multifactorial cause and could be quite complex to explain. Most importantly, haematocrit values were found within normal reported ranges for the specie (Waagbø et al., 1988; Hardie et al, 1990; Waagbø et al., 1994; Thompson et al., 1996) indicating normal levels of blood oxygen carrying capacity and the general condition of the different salmon groups since there was lack of anaemia which could be related to iron or other mineral deficiencies. Of the immune responses demonstrating changes was lysozyme activity, which was found to be higher for the MB group by the end of the study in comparison to all other salmon which exhibited similar levels of this immune parameter. The use of lysozyme activity as an indicator of improved immune response and thus survival however, has been argued in several studies which have demonstrated that increased lysozyme activity upon challenge with several bacterial diseases including furunculosis, vibriosis and BKD, was inversely correlated with survival (review by Saurabh and Sahoo, 2008). For this reason it was proposed that lysozyme activity is not a good indicator for

improved immunomodulation, but a reflection of stress induction (Fevolden et al., 2004). In this case, the assessment of plasma cortisol, glucose and lactate levels could have been ideal for the assessment of the stress status of salmon from different groups, especially since a significant reduction in the overall performance of MBE fish was witnessed during the second part of the study, which could point at increased stress levels during this period. It is suggested that changes in the nutritional profile of the second lot of feeds and suboptimal nutrient requirements could have acted as stress promoters in MBE salmon. Moreover, it was shown that diets with total substitution of FM by VPs (VP and VP/VO diets) or high levels of VPs (MFABP), promoted plasma antiprotease activity which could be a favourable trait especially against bacterial diseases (Ellis , 1990, 1991). Similar results regarding the enhancement of antiprotease activity in fish fed diets with complete replacement of FM with VPs have never been reported before in the literature, simply because most FM and FO replacement studies are mainly focused on the growth rather the immunological performance of fish. Nonetheless, various plant extracts are known to contain bioactive compounds, which can enhance anti-protease activity in fish (Rao and Chakrabarti, 2004; Kaleeswaran et al., 2011).

8.2 FUTURE PERSPECTIVES

In general it is accepted that performing experimental disease challenges is the best way for the evaluation of the overall health status of an animal. Therefore, experimental infection against various industrially important diseases, with mortality or morbidity, as the end point would have been ideal for the assessment of FM-, FO-, fishery-free diets or feeds with increasing levels of SPC. Unfortunately, the lack of disease challenge facilities at EWOS, where two out of the three experimental trials took place, did not allow this to be undertaken. Instead, vaccination as a mode of immunostimulation was used in both studies using diets with increasing SPC inclusion to test the immunological status of the dietary fish. However,

it is likely that the vaccination of the fish is not enough to illustrate the differences among dietary treatments, since fish are not under the same levels of stress as when they are infected and which could alter the magnitude of the observed responses. Another useful method for the immunological evaluation of the above diets would be the performance of stress challenges (applying one or more stressors which are commonly experienced by the fish under open sea culture conditions) with post-exposure stress mapping. One of the main objectives of this thesis was to assess the resistance of Atlantic salmon fed increasing levels of dietary SPC, against *A. salmonicida*. Despite the results showing no effects of increasing dietary SPC inclusion on salmon immune responses or resistance against furunculosis, the study is not representative to how salmon would respond when infected against different diseases involving other bacteria, parasites or viruses. Nonetheless, the study could be roughly appointed as a bacterial model.

Regarding the effects of increasing dietary SPC on salmon bone health the current thesis has generated some additional information regarding the skeletal mineralisation of salmon parr fed on diets with constant or increasing supplementation of inorganic phosphates before and after vaccination, under different photoperiodic regimes. This thesis clearly presents the necessity for supplementing diets with high levels of SPC with elements such as P and Zn. Elemental supplementation of high SPC inclusion diets for Atlantic salmon parr reared in inland facilities were emissions can be controlled (through the application of recirculation systems or the use of special filters) is possible and could potentially be beneficial for both salmon's health and normal vertebral development (Fjelldal et al., 2010). The use of high SPC inclusion diets supplemented on high levels of P for Atlantic salmon growers reared in sea cages or pens could increase the levels of P emissions in the open sea, giving rise to environmental concerns. Therefore, environmentally friendlier solutions are required rather than increasing the levels of inorganic P in high SPC inclusion diets for the

on-growing of Atlantic salmon smolts and post-smolts, including the use of microbial enzymes such as phytases and/or carbohydrases or fermentation. However, further processing of the SPC could dramatically increase the cost of the SPC-based aquafeeds, while the use of such enzymes cannot currently guarantee the elimination of polysaccharides and phytates, as these enzymes have a requirement for certain temperatures and pH to demonstrate activity on their substrates (as discussed previously in Section 1.4.4). Fermentation of primary soy products, such as soy flour or SBM (Teng et al., 2012), could potentially be a more cost efficient solution to these problems, while experiments using fermented products in piglet, broiler and fish diets demonstrated similar or improved growth and enhanced immune responses in animals fed these diets when compared with groups fed commercial diets (Shimeno et al., 1993; Wang et al., 2011; Zhou et al., 2011; Azarm and Lee, 2014).

Nevertheless, further testing of high SPC inclusion in diets for Atlantic salmon is recommended, over the course of their production cycle. For these experiments, mineral analyses from sampled fish at different points of the production cycle should be combined with full radiographs to reveal any effects of increasing dietary SPC inclusion on the prevalence of deformities and how these are related with bone mineralisation. Nonetheless, the present study shows that the use of certain commonly apllied salmon production strategies including photoperiodic regimes and vaccination can influence the mineralisation status of salmon parr, with the latter having a positive effect on body mineralisation at least when planned and/or applied properly.

While the extent to which dietary SPC, VP and VO inclusion can be used without affecting the growth of Atlantic salmon at various life stages is well investigated and largely known; the use of fishery-free diets on salmon growth is unexplored and the current thesis includes one of the first studies regarding this. However, the extent to which all of the above

diets affect Atlantic salmon health and the welfare is largely unknown and should be further investigated. The successful application of low marine or marine free diets for salmon in the future seems to be possible. Meanwhile, the main salmon feed producers rely on the dietary nutritional recommendations provided by the National Research Council (NRC, 1993). Nevertheless, many studies have repeatedly addressed the necessity to update salmon's nutritional requirements according to their growth rate and life stage (Espe et al. 2006, 2007; Torstensen et al. 2008), since most of these recommendations are based on the performance of juvenile freshwater salmon parr. Low marine ingredient diets can result in poor uptake of essential nutrients (including essential amino and fatty acids and minerals) when diets are high in alternative protein and lipid sources and mainly plant derived proteins (which have lower levels of certain indispensable amino acids, minerals and lacking of EPA and DHA compared to FM). Further research is required to fill the knowledge gaps regarding Atlantic salmon's amino acid and mineral requirements, especially for smolt and post-smolt stages. Once this is achieved, the necessary information relating to dietary formulation should be comprehensive enough to satisfy all the nutritional requirements for Atlantic salmon, and their manufacture by feed companies should be methodised to ensure a standardised quality for these diets. Ultimately, the future utilisation of low marine ingredient diets in salmon feeds will depend on the price of the available raw materials.

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