December 2012

Use of functional feeding strategies to protect Atlantic salmon from virally-induced inflammatory diseases- mechanistic insights revealed by transcriptomic analysis

A thesis submitted for the degree of doctor of philosophy

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DECLARATION

This thesis has been composed in its entirety by the candidate. Except where specifically indicated and acknowledged, the work described in this thesis has been conducted independently and has not been submitted for any other degree.

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Abstract

Over the past few years one of the major concerns in the Atlantic salmon (*Salmo salar*) farming industry has been the increasing incidence and severity of inflammatory viral diseases. Heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS) are currently two of the most prevalent viral diseases in commercial Atlantic salmon farms in Norway. Mortality levels in both diseases are generally low but morbidity can be very high with the associated chronic inflammatory response lasting for several months. The consequent reduced growth performance is causing considerable financial impact as HSMI has become increasingly widespread in recent years. The impact of CMS is further exacerbated as it generally affects large fish close to harvest. HSMI lesions occur in the atrium and ventricle in the heart including inflammation and necrosis in epi- endo- and myocardium along with myositis of red skeletal muscle. CMS lesions are commonly observed in the spongy myocardium in the atrium and ventricle of the heart with severe mononuclear inflammation and necrosis. Furthermore, circulatory disturbances associated with reduced cardiac function cause multifocal liver steatosis and necrosis in both diseases. Currently there are no vaccines or any other effective treatments for these diseases and so alternative therapies that could potentially modulate the intensity of the inflammatory response could be crucial to improve the clinical manifestation of the diseases. Therefore, the overall aim of the present study was to evaluate the concept of “clinical nutrition” to improve the clinical symptoms of both viral diseases, HSMI and CMS, through the use of functional feeds formulated with reduced lipid content and increased proportions of anti-

inflammatory fatty acids to moderate the apparently uncontrolled inflammatory response in the heart tissue associated with both diseases and also alleviate the secondary hepatic lesions. The experimental work consisted of three major dietary trials in Atlantic salmon in seawater. Two large trials investigated the effects of functional feeds in Atlantic salmon challenged with Atlantic salmon piscine reovirus (ASRV) and piscine myocarditis virus (PMCV), the causal agents of HSMI and CMS, respectively. In both trials, heart transcriptome, heart and liver histopathology and tissue lipid and fatty acid compositions and metabolism were determined post-infection in fish fed with the functional feeds in comparison with fish fed with a standard commercial feed formulation considered as a reference diet. All the functional feeds were formulated to have reduced digestible energy through lower dietary lipid and higher protein contents, and increased levels and proportions of anti-inflammatory long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (EPA) compared with the reference diets. Histopathology, fatty acid composition and gene expression of heart were assessed over a long time-period of 16 weeks and 14 weeks post-challenge with ASRV and PMCV, respectively. Viral load in heart tissue, hepatic histopathology and fatty acid composition of liver and head kidney along with expression of the genes involved in the eicosanoid and LC-PUFA and eicosanoid biosynthesis pathways were also determined in the HSMI trial. The third trial was a nutritional trial evaluating the effects of dietary digestible energy content on lipid and fatty acid metabolism in salmon fed diets containing graded amounts of lipid. Fatty acid composition of liver and heart were assessed over 12 weeks, along with the hepatic expression of genes of lipid and fatty acid metabolism. The results of this research are presented in four chapters (Chapters 2-5) as four paper manuscripts. The manuscripts/Papers are either

published (Chapter 2), in review (Chapter 3 and 4) or drafted for submission (Chapter 5) in appropriate peer-reviewed international journals. Chapter 2 and 3 correspond to the HSMI trial, Chapter 4 to the nutritional trial, and Chapter 5 to the CMS trial.

Chapter 2 showed that viral load and histopathology scores were lower in fish fed the functional feeds, especially diet FF1, which displayed better performance. Diet strongly influenced the expression of genes related with the immune and inflammatory responses, with delayed expression in fish fed the functional feeds. Up-regulation of pro-inflammatory genes was correlated with the higher viral load observed at early-mid stages of the disease in fish fed the reference diet (ST). Expression of genes related with the immune response at 16-weeks post challenge reflected the differences in immunomodulation between the functional feeds, with fish fed diet FF1 showing lower expression. Therefore, severity of the heart lesions was correlated with the intensity of the immune response and could be associated with tissue anti-inflammatory LC-PUFA levels. Chapter 3 was focused on liver histopathology, fatty acid composition and LC-PUFA biosynthesis, along with phospholipid fatty acid composition and eicosanoid production in head kidney and heart tissue at early and late stages of ASRV infection. Liver was severely affected by the virus at the beginning of the infection in fish fed the reference ST diet, but the level of lesions were similar in all dietary groups at the end of the trial. Hepatic expression of fatty acyl desaturases was significantly depressed in fish fed the ST diet compare with fish fed the functional feeds despite the lower levels of dietary LC-PUFA in that feed. Thus endogenous production and bioavailability of anti-inflammatory LC-PUFA was potentially enhanced in fish fed the functional feeds. Changes in tissue lipid content, mobilization of fatty acids involved in inflammatory

responses and changes in expression of transcription factors and genes involved in eicosanoid biosynthesis were more prominent in head kidney, confirming the important role of this organ in dietary immunomodulation after viral infection. To a lesser extent similar changes were observed in heart tissue, suggesting i*n situ* production of eicosanoids could also be important. The unexpected effects of diet on expression of genes of LC-PUFA biosynthesis were specifically investigated in the trial described in Chapter 4. One aim of this study was to clarify whether dietary lipid content or viral infection was the cause of altered expression of desaturase genes between the different diets. Hepatic expression of other genes of lipid and fatty acid metabolism were also determined to evaluate metabolic changes associated with dietary lipid/energy level. In general, reduction of dietary energy and lipid contents while maintaining similar proportions of dietary fatty acids, led to a general up-regulation of genes involved in lipid biosynthetic pathways. Thus salmon fed lower energy diet showed increased liver expression of fatty acyl desaturases in comparison with fish fed higher energy levels. Heart transcriptomic data in Chapter 5 showed a similar delay in the inflammatory response in fish fed the functional feeds after PCMV infection as observed in the HSMI study. Modulation of inflammatory responses, similar to that previously described after ASRV infection, was also observed in fish fed the functional feeds. However, the differences in the expression of immune related genes and the level of heart lesions were not as prominent at mid-late stages of the disease as in fish fed FF1 in the HSMI trial. The present study demonstrated the beneficial effects of a clinical nutrition approach via functional feeds in two viral inflammatory diseases, HSMI and CMS, currently affecting farmed Atlantic salmon. Dietary immunomodulation increased the availability of anti-inflammatory LC-PUFA and significantly influenced the expression of the genes

related with the immune/inflammatory response reducing the level and severity of cardiac and liver lesions and therefore improving the performance of fish suffering the diseases.

Acknowledments

First of all I would like to express my sincere thanks to my first supervisor, Professor Douglas Tocher, for his valuable guidance, encouragement and support. Thank you for believing in me from the very beginning, for being always available every time I needed you, for listening to all of my opinions and supporting all of my decisions. It would have been really much harder to deal with the pressure of working in this big project without your help. I would also like to thank my second supervisor, Professor Gordon Bell, for also being always there for me, even getting "nicely" wet and cold in one of the coldest winters in Scotland just to help me with my samplings. I really appreciate it.

Thanks to my sponsor EWOS Innovation, especially to Simon Wadsworth and Jose González Vecino, for giving me the opportunity to be involved in this big and challenging commercial project, allowing me to work with modern molecular techniques. Thanks as well for their support.

I would also like to sincerely thank Dr Øystein Evensen, for the quality and rapidity of his comments, his enthusiasm and encouragement from the first trial of this thesis.

My gratitude also goes to all the staff of the "Nutrition Analytical Service" for being always up to give me a hand and cheer me up on the long days working in the lab. I am especially grateful to James Dick, because there was not a problem in the lipid lab that he could not solve and that really made things easier. Thanks as well for being so nice with me from the beginning. I would also like to especially thank Fiona Strachan for all

the help in the lab and during the winter samplings; I really appreciate you always being available to give me a hand.

I would really like to thank Dr. John Taggart for all of his invaluable support and help in doing the microarrays experiments. Thanks for being always available regardless of how busy you were. Thanks as well to Jacquie Ireland, for being so helpful while I was working in the biomolecular lab.

To all my dearest friends in the Institute of Aquaculture, “my little worldwide family” who were always there cheering me up, giving me all their support and the affection that I’ve needed, being far from home, thanks to Amy, Greta, Poly, Xell, Sara, Pedro, Eric, Olga, Sean, Chris, Beatrix, Stephen, Silvia, Monica, Farah and the list could go on and on...thank you all. I will never forget you.

Especial thanks to my “unofficial supervisor” and best friend Dr. Sofia Morais, for patiently teaching me all that I know about molecular biology and for showing me the way to be a successful scientist. You will be indeed the one to blame if I manage to have a little bit of success in my career. But foremost I would like to thank you for your endless support, encouragement, full availability and special frienship...you are the “big sister” that I’ve never had. Muito Obrigada!

Last but not least, I would like to thank my family: Gracias papa, mama, hermano y hermana por creer ciegamente en mí y por el apoyo tan grande que me disteis cuando decidí irme tan lejos aunque eso fuera un gran sacrificio para vosotros. Por todo ese cariño que me dais, que me llena el alma y me empuja a seguir adelante. No sabéis lo afortunada que me siento con vosotros…

List of Abbreviations

ACO	Acyl CoA oxidase
ALOX5	Lipooxygenase 5
ANF	Anti-nutritional factor
ANOVA	Analysis of variance
ARA	Arachidonic acid
aRNA	Amplified antisense ribonucleic acid
ASRV	Atlantic salmon Reovirus
Ca^{2+}	Calcium
cDNA	Complementary deoxyribonucleic acid
CMS	Cardiomyopathy syndrome
COX2	Cyclooxygenase 2
CPT1	Carnitine palmitoyl transferase-1
D5FAD	Δ5 fatty acyl desaturase
D6FAD	Δ6 fatty acyl desaturase
DE	Digestible energy
DHA	Docosahexaenoic acid
DNA	Deoxyribonucleic acid
EFA	Essential fatty acids
ELOVL2	Elongase 2
ELOVL5	Elongase 5
EPA	Eicosapentaenoic acid
FAME	Fatty acid methyl esters
FAS	Fatty acid synthase
FF	Functional feed

FLAP	5-lipoxygenase-activating protein
FM	Fish meal
FO	Fish oil
HPTLC	High-performance thin-layer chromatography
HSMI	Heart and skeletal muscle inflammation
IFN	Interferon
IHN	Infectious hematopoietic necrosis
IL	Interleukin
ILAB	Industrial and Aquatic Laboratory
IPN	Infectious pancreatic necrosis
ISA	Infectious salmon anaemia
ISAV	Infectious salmon anemia virus
LA	Linoleic acid
LC-PUFA	Long-chain polyunsaturated fatty acids
LNA	α-linolenic acid
LTB	Leukotrienes
LTB4	Leukotriene B_4
LXR	Liver X receptor
MHC	Major histocompatibility complex
mRNA	Messenger ribonucleic acid
MUFA	Monounsaturated fatty acids
NK	Natural cell killer
PC	Phosphatidylcholine
PCMV	Piscine myocarditis virus
PD	Pancreas Disease
PE	Phosphatidylethanolamine
PG	Prostaglandins

PGE2	Prostaglandin E_2
PI	Phosphatidylinositol
PLA2G1 sPLA2	Secretory phospholipase A2
PLA2G4 cPLA2	Cytosolic calcium-dependent phospholipase A2
PLA2G6 iPLA2	Cytosolic calcium-independent phospholipase A2
PPAR	Peroxisome proliferator-activated receptor
PRR	Pathogenic recognition receptors
PS	Phosphatidylserine
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
RT-qPCR	Real-time polymerase chain reaction
SFA	Saturated fatty acids
SRBP	Sterol-responsive element-binding protein
TAG	Triacylglycerol
TLC	One-dimensional thin-layer chromatography
TNF	Tumour necrosis factor
TX	Thromboxanes
VHSV	Viral hemmorrhagic septicemia virus
VO	Vegetable oil
VRG	Virus responsive genes

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Chapter 1

General Introduction

Global production of farmed fish has been growing rapidly over the past decade reaching 60 million tonnes in 2010 (FAO/NACA, 2012). The estimated data for 2012 indicates that more than 50% of global food fish production and consumption will be from aquaculture (Figure 1.1).

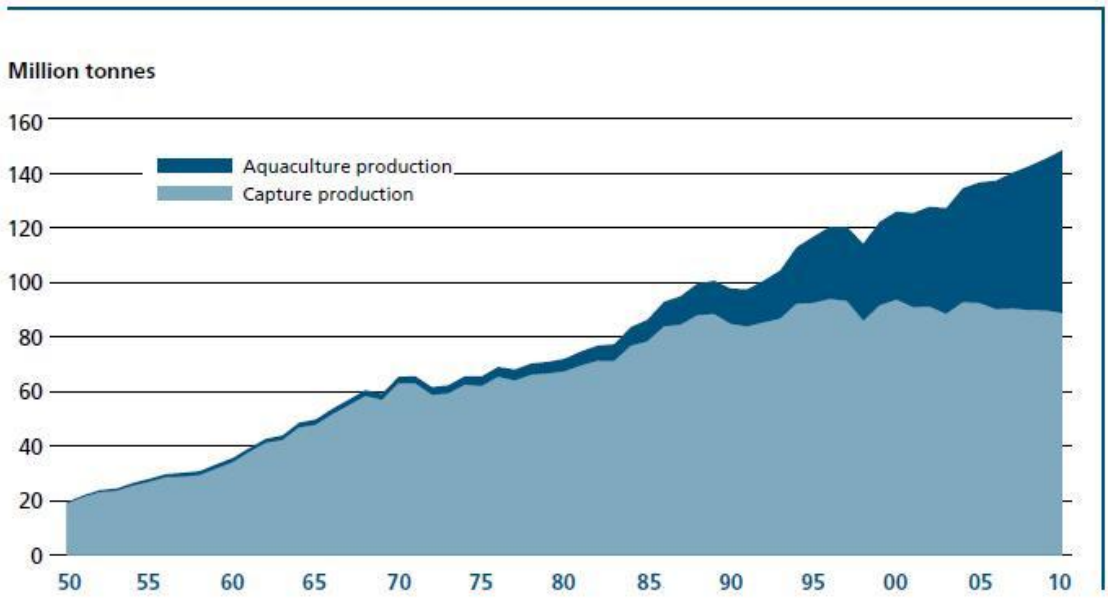

Figure 1.1. World capture fisheries and aquaculture production (FAO, 2012)

Even with an increase in production of Atlantic salmon (*Salmo salar*) of more than 600% (1.25 million tons) between 1990 and 2008, total global supply of salmonids is still marginal compared to most other seafood categories. However, Atlantic salmon is still at the top of the list of major cultured species with production of 1.43 million tonnes in 2010 (FAO 2010). Salmon is also the most important farmed finfish species in

terms of value with a production valued at USD 7.8 billion (FAO, 2010). The leading producers of farmed salmon are Norway with 33 % of global production, followed by Chile with 31 % and the United Kingdom, specifically Scotland, with 19% of global production.

The salmon farming production cycle can be largely divided into two phases. During the first phase, which occurs over 12 to 18 months, the salmon are hatched from eggs and the parr raised in freshwater (tanks or freshwater lakes/lochs) up to smoltification. The second phase starts with the transfer of the salmon smolts to seawater net pens or cages where they will be on grown over a period ranging from 12 to 24 months until they reach market size (2.5 - 4+ kg). The seawater stage takes place in a more or less uncontrolled environment in which infectious diseases are one of the major concerns. Indeed, the incidence of various diseases has increased in the past decades and these lead to economic losses that can dramatically impact the salmon farming industry. The recent drastic reduction in Chilean production was the most dramatic example of the impacts of a generally uncontrolled disease outbreak, albeit this has now been reversed.

Viral infections remain the most significant diseases in Salmon aquaculture, as highlighted by the most recent Fish Health Report from the Norwegian Veterinary Institute (Alarcon et al., 2012). In addition to direct mortality losses due to viral disease, reduced growth and secondary infections in fish showing lower disease resistance are major problems associated with these viral infections (Alarcon et al., 2012). The diseases showing the highest incidence of outbreaks in the past year in Norway were infectious pancreatic necrosis (IPN), pancreas disease (PD), heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS). Infectious salmon anaemia (ISA) was previously the disease with very high incidence in the last decade, not only in Norway, but also in other salmon farming countries and was primarily

responsible for the recent problems in Chile. However, ISA is now under control after successful vaccination programs and biosecurity counter-measures against this virus. Similarly, since IPN and PD were first diagnosed in 1979 and 1981, respectively, the incidences of both diseases have had important impacts in the European salmon farming industry (see McCoy et al., 1994; Roberts and Pearson, 2005). However, several studies and management strategies, including vaccination and selective breeding programs appear to be working successfully to reduce the number of outbreaks and the consequential impact of these diseases (McLoughlin et al., 2007; Gomez-Casado et al., 2011). In contrast, the situation with HSMI and CMS is currently a more pressing concern as the number and severity of outbreaks are increasing. Therefore, incidence of HSMI in the Norwegian sector increased 20 % from 2010 to 2011 alone (Alarcon et al., 2012). The severity of these inflammatory diseases is also appearing to increase with higher morbidity and, importantly, the fish suffering from these diseases, especially CMS, are those close to commercial size just prior to harvest. Thus, the impact of both these cardiac inflammatory diseases, leading to financial losses, combined with the lack of effective treatments is currently one of the major concerns for both farmers and authorities involved in salmon production industries in Europe.

Heart and Skeletal Muscle Inflammation was first reported in the mid-coast of Norway in 1999 and generally affects salmon after 5-9 months following seawater transfer (Kongtorp et al., 2004a). In recent years, however, it has become widespread along the entire Norwegian coast, affecting 162 sites in 2011 (Alarcon et al., 2012). However, although the incidence of the disease has been mainly focused in Norway, outbreaks in Scotland have also been reported (Ferguson et al., 2005). The mortality associated with HSMI varies from 0 to 20 % at affected sites although morbidity in most cases is close to 100 %. Fish affected by the disease do not present obvious

external signs, although they can be anorexic and display abnormal swimming behaviour (Kongtorp et al., 2004b). Autopsy reveals pale heart, yellowish liver, ascites, swollen spleen and petechiae in perivisceral fat, along with general circulatory disturbance caused by the inflammation of the heart tissue (Figure 1.2).

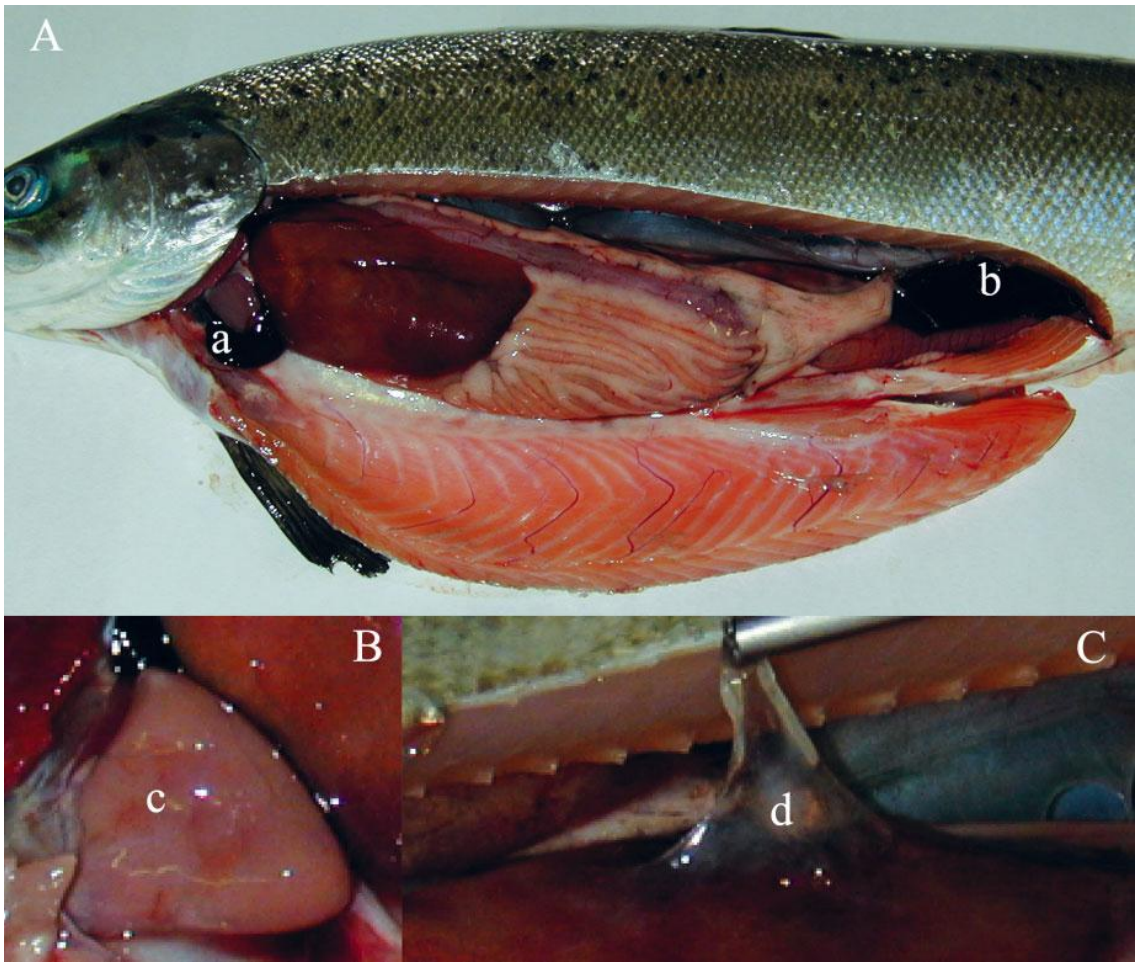

Fig. 1.2 by Kongtorp et al., 2004b *Salmo salar*. Macroscopical findings in heart and skeletal muscle inflammation (HSMI). (A) In this salmon, the heart is pale and there is haemopericardium. The spleen appears swollen. (B) Closer picture of a pale heart. (C) Fibrinous layer on the liver. a: haemopericardium, b: spleen, c: heart, d: fibrinous layer

Histopathologically, major changes occur in the myocardium and red skeletal muscle, where extensive inflammation by infiltration of lymphocytic cells and degeneration in spongy and compact layers of the ventricle are evident (Figure 1.3) (Kongtorp et al., 2004b). Atlantic salmon Reovirus (ASRV), a double-stranded RNA virus, has been described recently as the causal agent of HSMI (Mikalsen et al., 2012). In this study, cytotoxic CD8 T-cells were identified as the main cells infiltrating in the heart tissue playing a major role in the immune and inflammatory responses against this virus, although the presence of CD3 markers on those T-cells in the heart tissue during the inflammatory process has also been reported recently (Yousaf et al., 2012).

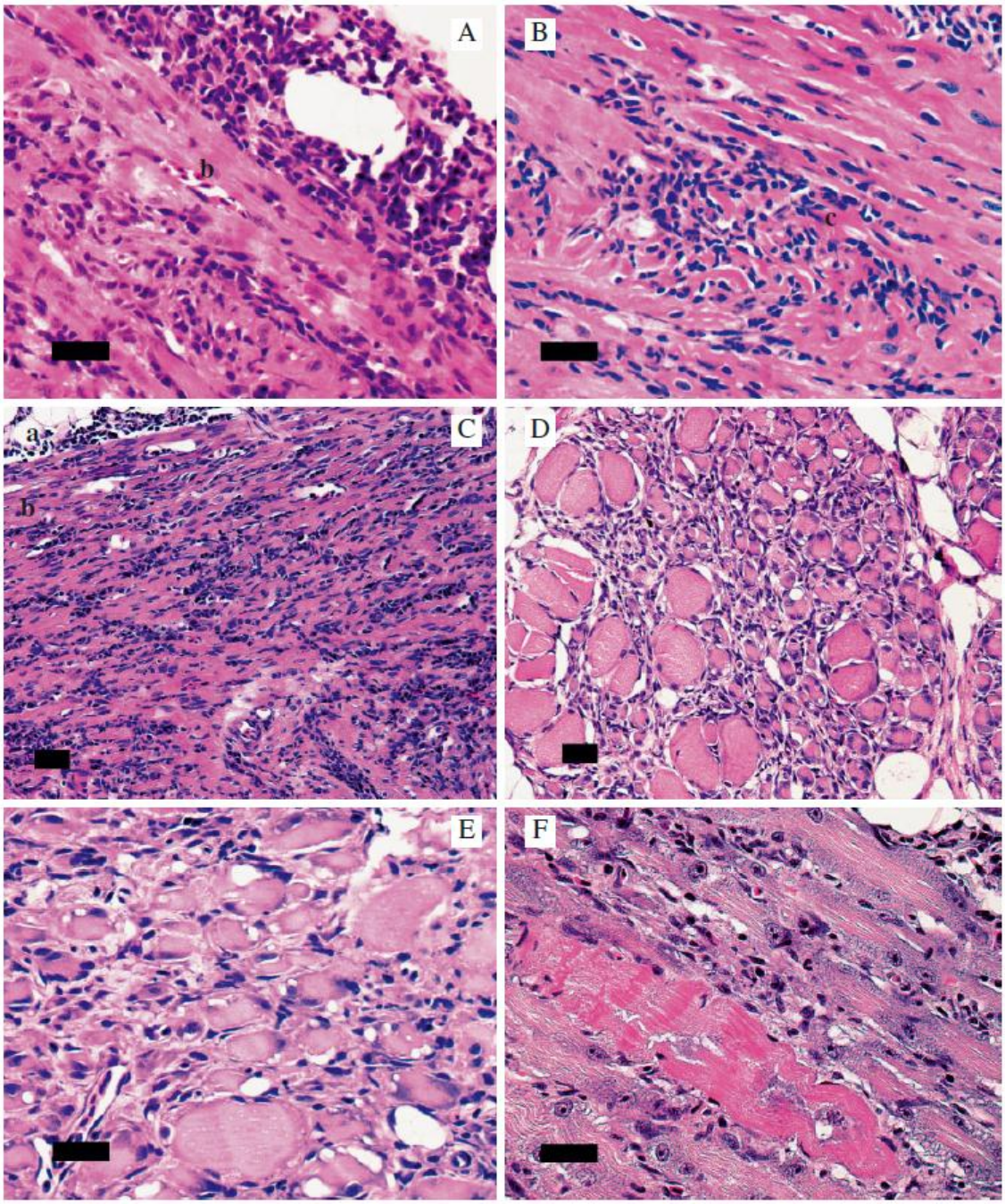

Figure. 1.3 by Kongtorp et al., 2004b. *Salmo salar*. Micrographs of sections from affected fish (H&E). (A) Ventricle showing severe epicarditis and myocarditis in the underlying compact myocardium. (B) Focal cellular infiltration in the compact myocardium. (C) Severe, diffuse myocarditis in the compact myocardium. (D) Cross section of red skeletal muscle, featuring severe myositis. (E) Vacuolisation and degeneration of red skeletal muscle fibres. Infiltration of mononuclear cells. (F) Longitudinal section of red skeletal muscle. The muscle fibres show signs of degeneration. a: epicardium, b: compact myocardium, c: focal cellular infiltration. Scale bars = 20 μm

Cardiomyopathy syndrome is also a cardiac disease with a viral aetiology, with a double-stranded RNA virus of the totiviridae family named piscine myocarditis virus (PCMV) recently reported as the causal agent (Haugland et al., 2011). This disease primarily affects adult Atlantic salmon during the second year after seawater transfer, when it is close to being harvested. CMS was firstly described in Norwegian farmed salmon in 1985 (Ferguson et al., 1990) and has since been diagnosed in Scotland (Rodger and Turnbull, 2000), the Faroe Islands, Denmark and Canada (Bruno and Noguera, 2009). In 2011, the Norwegian Veterinary Institute diagnosed the presence of CMS at 74 salmon farming sites mainly located in the mid-coast of Norway (Alarcon et al., 2012). Although the number of fish affected by CMS and hence the sites in where CMS outbreaks were reported, are generally lower compared with HSMI or PD, as the fish are large fish close to harvest, the economic impact can be considerable (Brun et al., 2003). Indeed, Brun et al. (2003) estimated the direct annual economic losses to the industry due to CMS were between €4.5 to 8.8 million. Similar to HSMI, fish suffering CMS do not show any specific obvious symptoms apart from abnormal swimming behaviour and anorexia. However, sudden deaths may also occur due to rupture of the atrium or sinus venosus resulting in cardiac tamponade (Bruno and Noguera, 2009). At autopsy, fish with CMS can show skin haemorrhages, ascites, blood in the pericardial cavity, and the atrium and sinus venosus are usually enlarged (Bruno and Pope, 1996). Histopathologically, the diagnosis of CMS is characterized by severe inflammation, degeneration and necrosis of the spongious part of the myocardium of the atrium and ventricle. Inflammatory infiltrates consist of mononuclear cells, probably lymphocytes and macrophages (Ferguson et al., 1990). As CMS can last several months, it is also regarded as a chronic disease, and circulatory disturbances with multifocal liver necrosis may also occur.

Figure. 1.4 as in Alarcon et al., 2012. Salmon with cardiomyopathy syndrome (CMS). The atrium is distended to the point of rupture and there is a layer of fibrin on the liver. Photo: Trygve T. Poppe, Norwegian School of Veterinary Science.

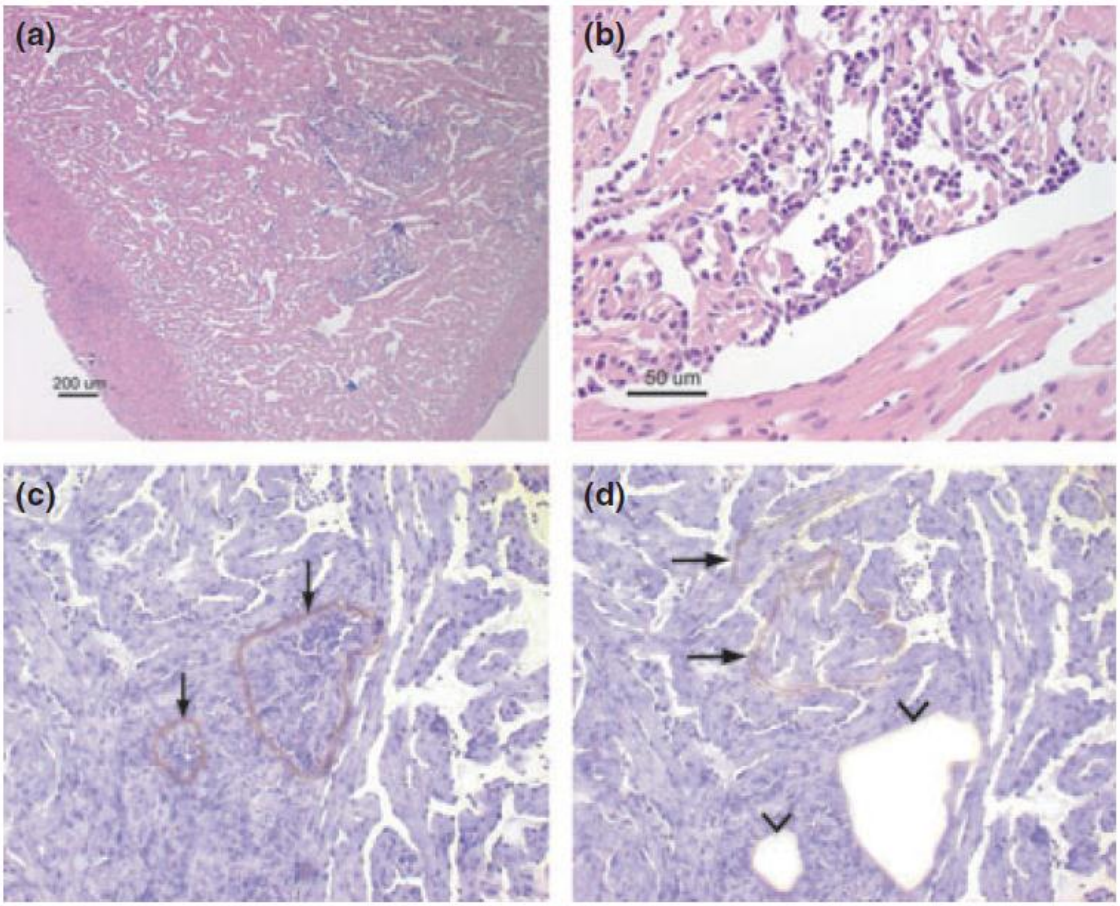

Figure. 1.5 as in Wiik-Nielsen et al., 2012. (a) Micrograph of a heart section with typical, focal cardiomyopathy syndrome (CMS) lesions in the spongy layers of the ventricle (H&E). (b) Micrograph of a myocardial lesion with mononuclear cell infiltration (H&E). (c) Micrograph of the spongy layers of a ventricle with typical myocardial lesions obtained for laser capture microdissection (Hx) showing the two lesions to be captured (brown line, vertical arrows). (d) Following removal of the lesion (arrowheads), an adjacent apparently normal area is outlined to serve as normal control tissue (light brown line, horizontal arrows; Hx).

Thus, both viral diseases, HSMI and CMS, have in common lower mortality but high morbidity, and both show chronic, severe inflammation of the heart tissue, Therefore in

both diseases, external factors related to the inflammatory process could be highly relevant in potential therapies to moderate the impact of the diseases. For example, environment and stressor factors like temperature and vaccination or handling of farmed fish, respectively, could affect the general health status of the fish. However, in addition, the unavoidable changes in the formulation of aquafeeds, with the increasing necessary use of terrestrial plant-derived ingredients could potentially be detrimental to the control and consequences of the inflammatory responses to these infectious diseases.

As a carnivorous fish, the nutritional requirements of salmon have traditionally been effectively covered by the use of fishmeal (FM) and fish oil (FO) from wild capture fisheries as the primary diet ingredients (Tacon and Metian, 2008). FM had been chosen as the main protein source in those aquafeeds for several scientifically sound nutritional reasons, which were mainly based on its high protein content, excellent amino acid profile, high digestibility and the general lack of antinutrients (Gatlin et al., 2007). The fat/lipid content of the diet is the main source of metabolic energy in feeds for carnivorous fish in addition to being the source of essential lipids, specifically the supply of essential polyunsaturated fatty acids (PUFA) (Sargent et al., 2002). In vertebrates, essential PUFA are, theoretically, the short chain PUFA 18:3n-3 (α-linolenic acid; LNA) and 18:2n-6 (linoleic acid; LA), precursors for the biosynthesis of the biologically active long-chain polyunsaturated fatty acids (LC-PUFA) of 20:5n-3 (eicosapentaenoic acid; EPA) and 22:6n-3 (docosahexaenoic acid; DHA), and 20:4n-6 (arachidonic acid; ARA), the main members of the n-3 and n-6 PUFA series, respectively.

Synthesis of ARA is achieved by Δ6 desaturation of LA to produce 18:3n-6, which is then elongated to 20:3n-6 followed by Δ5 desaturation (Cook, 1996). The pathway

for EPA synthesis from LNA is essentially similar, but DHA synthesis requires two further elongation steps, a second Δ6 desaturation and a chain shortening step (Sprecher, 2000) (Figure 1.6).

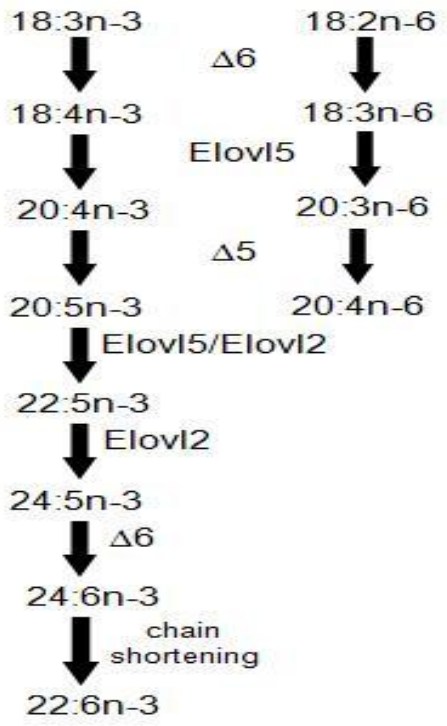

Figure 1.6. The LC-PUFA biosynthesis pathways for n-3 and n-6 fatty acids. Δ5, Δ6, fatty acyl desaturases. Elovl, elongases of very long-chain fatty acids.

Apart from being the precursors of LC-PUFA, LNA and LA have no physiological role themselves in fish. However, LC-PUFA are major components of biological membranes, with particularly important specific roles in neural tissues and immune cells, regulating a multitude of metabolic and immune pathways through their roles as secondary messengers and transcription factor ligands, and being precursors of the immune modulators signaling molecules termed eicosanoids that can have both pro- or anti-inflammatory properties (Tocher, 2003). Although, Atlantic salmon are able to biosynthesise LC-PUFA from their C_{18} PUFA precursors, it is now well accepted that the conversion is not particularly efficient and may also decline when the fish are older

and in seawater (Sargent et al., 1999). Thus, LC-PUFA, especially those of the n-3 PUFA series and ARA, are considered essential for salmon.

The aforementioned global expansion of the aquaculture industry, with an average growth rate of 12.6 percent in the 1990s and 7.5 percent in the 2000s (FAO, 2012) coupled with the static or even diminishing global fisheries, including those utilised for the production of FM and FO, has led to the search for alternatives to these marine ingredients in aquafeeds. Therefore, in the last decade or so, aquafeed manufacturers have been increasingly replacing marine ingredients with cheaper and more sustainable sources of protein and lipid (Figure 1.7), (Tacon and Metian, 2008).

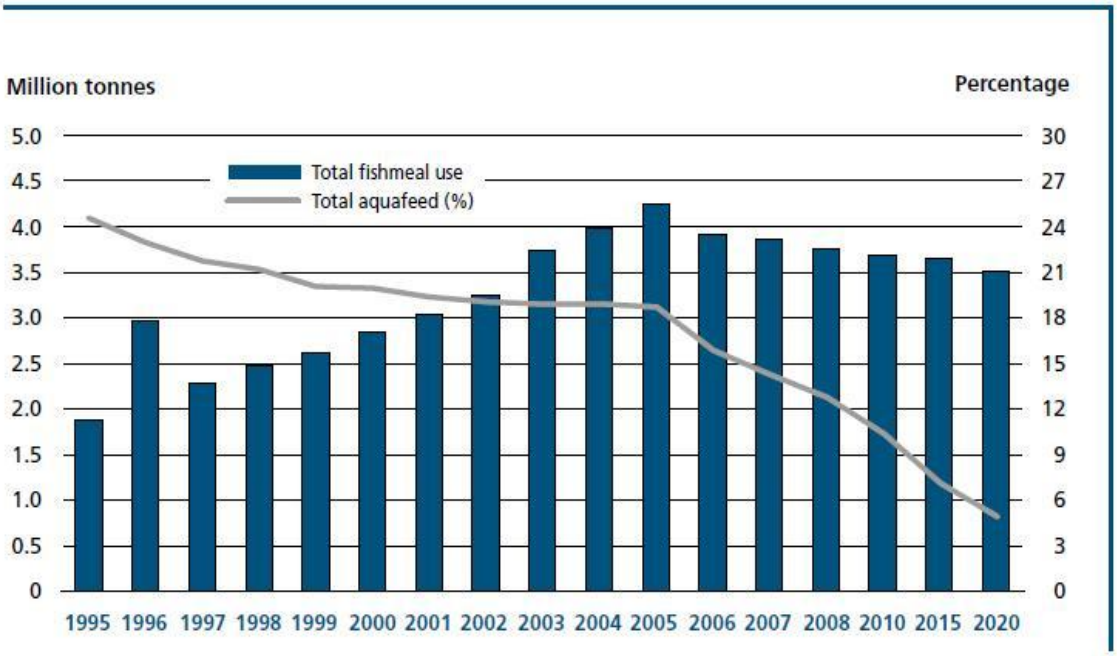

Figure 1.7. Actual and predicted fishmeal use relative to the global production of compound aquafeed

Among the alternative sources available, plant-derived raw materials have been widely studied as they are produced in large volumes, are renewable and can be less expensive than marine-sourced ingredients (Gatlin et al., 2007; Turchini et al., 2009; Miller et al., 2008) (Figure 1.8).

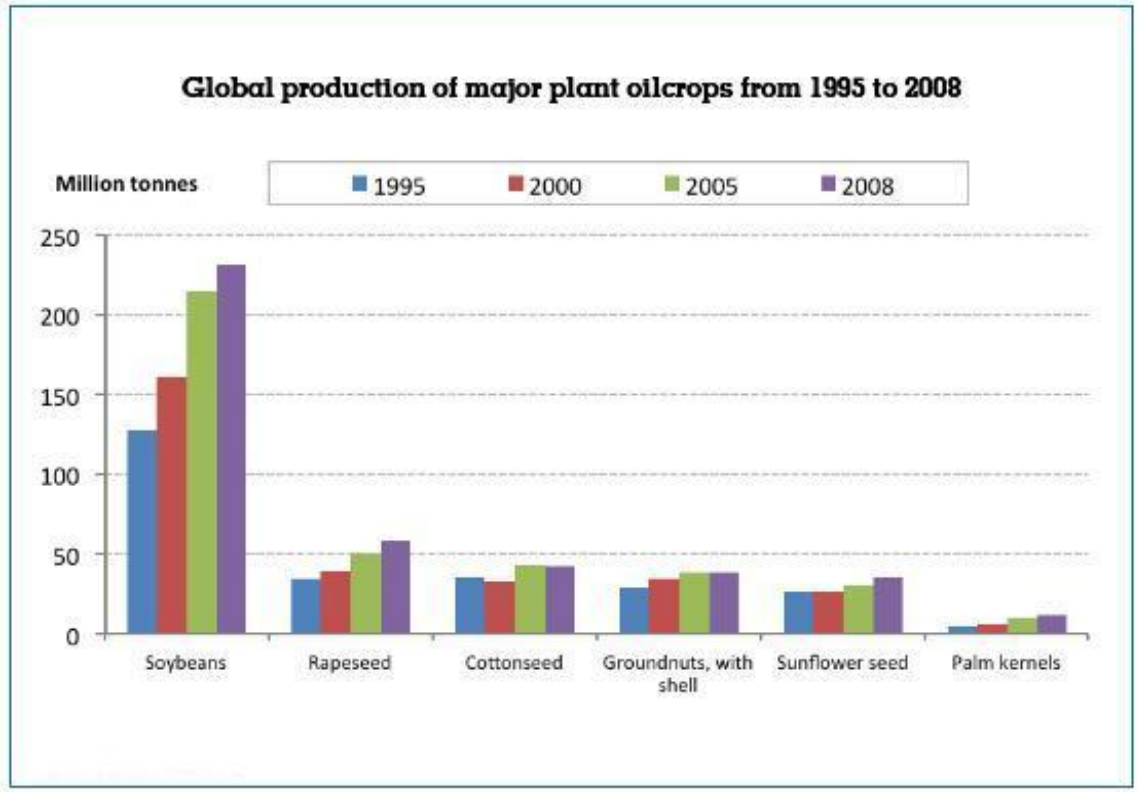

Figure 1.8. Global production of major oilseed plants (FAO/NACA 2012)

In general, high levels of FO and FM substitution with plant-derived ingredients does not affect growth of salmonids (Bell et al., 1993, 2001, 2002), including Atlantic salmon, but detrimental effects on fish health have been reported (Krogdahl et al., 2003, 2010). Inclusion of soybean meal has been extensively studied as this plant meal has reasonably high crude protein content and a relatively well-balanced amino acid profile, although high inclusions can cause enteritis characterized by shortening of the primary and secondary mucosal folds and infiltration of inflammatory cells in the intestine (Krogdahl et al., 2003). Several other plant protein sources have been also studied as potential candidates for FM substitution including peas, wheat, rapeseed, corn, cottonseed and faba bean (Gatlin et al., 2007; Adamidou et al., 2009). Generally, no major pathological changes have been described with low levels of FM substitution. However, potential problems with medium to higher levels of inclusion of plant protein

sources have been associated primarily with their relatively high contents of potential anti-nutritional factors (ANFs) that include proteinase inhibitors, oligosaccharides, saponins and lectins (Krogdahl et al., 2010). These components can also affect the function of the fish gastrointestinal tract and gut health by altering digestibility and the efficiency of the gut as a barrier to pathogens. In the past few years, improved industrial processing technologies for plant meals and, in particular protein concentration methods, have reduced the contents of ANF compounds such that the use of plant protein concentrates has become increasingly common. Indeed, dietary plant protein concentrates have been able to sustain and even improve feed conversion ratios and growth at relatively high inclusion levels (Carter and Hauler, 2000; Storebakken et al., 2000). However, further investigation of these concentrates is required as some potential pathology associated with the use of certain protein concentrates have also been reported (Penn et al., 2011; Kortner et al., 2012).

Lipids are the major part of aquafeeds constituting more than 30-35 % of Atlantic salmon commercial feeds (Turchini et al., 2009). The traditional use of FO, which is particularly rich in n-3 LC-PUFA, EPA and DHA, has until now ensured that salmon feeds contain sufficient essential fatty acids. As mentioned above, sustainability, wide production and lower price are crucial factors that make VOs currently one of the best alternatives for the substitution of FO in aquafeeds (Figure 1.9). Replacement of FO by different vegetable oils (VO) has been well documented and recently reviewed by Turchini et al. (2009). Most VO sources are relatively poor in n-3 LC-PUFA but instead contain high levels of short-chain PUFA, LA and LNA, monounsaturated (MUFA) and saturated (SFA) fatty acids (Table 1.1)

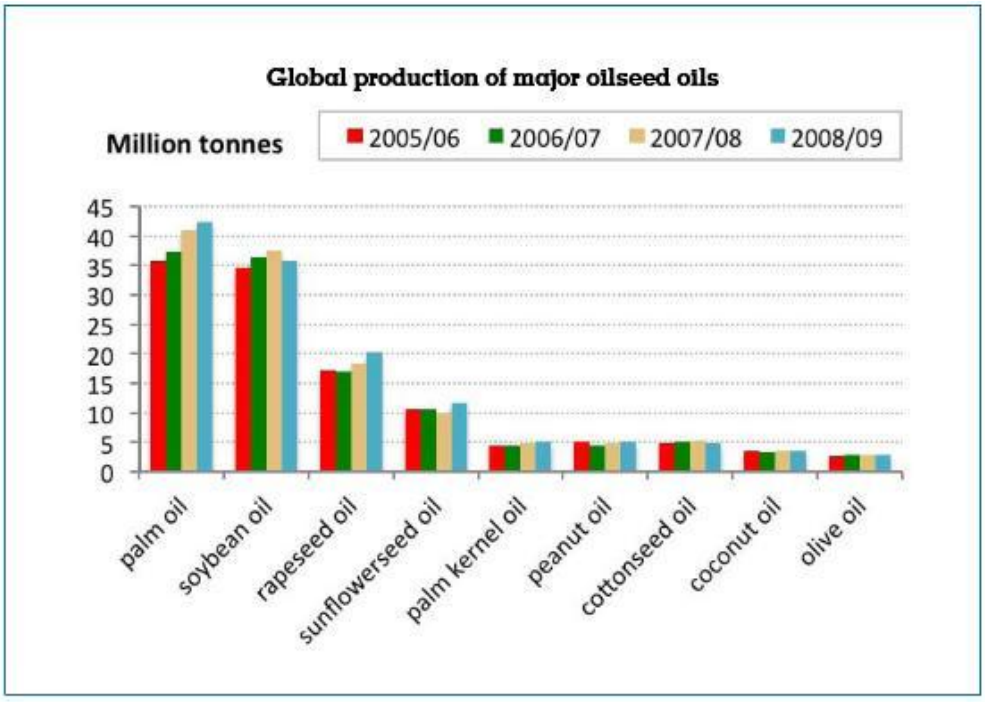

Figure 1.9. Global production of major oilseed oils (FAO/NACA 2012)

Table 1.1. Fatty acid composition (% total fatty acids) of fish oils, vegetable oils used in fish feed formulations (Adapted from Turchini et al., 2009)

Oils/fats	SFA	MUFA	LA	ARA	LNA	EPA	DHA	n-6 PUFA	n-3 PUFA	n-3/n-6
Fish oils										
Anchovy oil	28.8	24.9	1.2	0.1	0.8	17.0	8.8	1.3	31.2	24.0
Capelin oil	20.0	61.7	1.7	0.1	0.4	4.6	3.0	1.8	12.2	6.8
Menhaden oil	30.5	24.8	1.3	0.2	0.3	11.0	9.1	1.5	25.1	16.7
Herring oil	20.0	56.4	1.1	0.3	0.6	8.4	4.9	1.4	17.8	12.7
Cod liver oil	19.4	46.0	1.4	1.6	0.6	11.2	12.6	3.0	27.0	9.0
Vegetable oils										
Crude palm oil	48.8	37.0	9.1	--	0.2	-	-	9.1	0.2	0.0
Soybean oil	14.2	23.2	51.0	-	6.8	-	-	51.0	6.8	0.1
Canola/rapeseed oil	4.6	62.3	20.2	-	12.0	-	-	20.2	12.0	0.6
Sunflower oil	10.4	19.5	65.7	-	-	-	-	65.7	0.0	0.0
Cottonseed oil	45.3	17.8	51.5	-	0.2	-	-	51.5	0.2	0.0
Groundnut oil	11.8	46.2	32.0	-	-	-	-	32.0	0.0	0.0
Corn oil	12.7	24.2	58.0	-	0.7	-	-	58.0	0.7	0.0
Linseed oil	9.4	20.2	12.7	-	53.3	-	-	12.7	53.3	4.2

AA, arachidonic acid, 20:4 n-6; ALA, α-linolenic acid, 18:3 n-3; DHA, docosahexaenoic acid, 22:6 n-3; EPA, eicosapentaenoic acid, 20:5 n-3; LA, linoleic acid, 18:2 n-6; MUFA, monounsaturated fatty acids; n-3 PUFA, polyunsaturated fatty acids with the first double bond at the 3rd carbon atom; n-6 PUFA, polyunsaturated fatty acids with the first double bond at the 6th carbon atom; SFA, saturated fatty acids.

Although digestion and absorption of SFA and MUFA are known to be inferior to PUFA in fish (Torstensen et al., 2000), the VO best suited as a substitute for FO should contain high SFA and MUFA as energy sources and low amounts of LA because this fatty acid may be relatively poorly oxidized (Turchini et al., 2009). Furthermore, as it will be mentioned further in this text n-3 LC-PUFA are involved in several pathways of the immune response mostly displaying anti-inflammatory roles, in contrast n-6 LC-PUFA, ARA, stimulate the inflammatory response. Therefore, even with an inefficient LC-PUFA biosynthesis in carnivorous fish, VOs containing the precursor of n-3 LC-PUFA, LNA could be potentially more desirable for FO substitution.

Partial level of substitutions with different VOs such as sunflower oil (Bell et al., 1993; Tocher et al., 1997), rapeseed oil (Bell et al., 2001), soybean oil (Rosenlund et al., 2001) and palm oil (Bell et al., 2002) did not compromise the growth of different species of farmed fish, although morphology of the gastrointestinal tract and, hence, digestion and nutrient uptake (Caballero et al., 2002, 2003; Jutfel et al., 2007) as well as liver morphology (Figuereido-Silva et al., 2005; Ruyter et al., 2006) have been reported to be negatively affected by high levels of VO inclusion. Nevertheless, the major concern with the inclusion of dietary VO is the influence of specific PUFA (EPA, DHA, and ARA) on the different pathways of the fish immune response, recently reviewed by Montero and Izquierdo (2010). According to this publication, studies over the last few years have yielded contradictory results that have shown the effects of VO substitution on fish health are dependent upon species, VO used, levels of substitution, other dietary ingredients, antioxidants and dietary lipid levels, although some general trends can be observed. As described in mammals (Calder, 2007) changes in fatty acid profiles of the membranes of fish immune cells are highly dependent on the fatty acid composition of the diet. Therefore inclusion of VO in aquafeeds results in a reduction

of n-3 LC-PUFA, especially EPA and DHA, and increased incorporation of LNA and especially LA, and lower n-3/n-6 PUFA ratios in the phospholipids of immune cells such as peripheral blood leukocytes (Thompson et al., 1996; Petropoulos et al., 2009) and macrophages (Waagbø et al., 1995; Montero et al., 2003), compared with diets containing FO.

LC-PUFA are involved in many physiological processes that provide several mechanisms whereby they may affect different pathways of the immune response (Stulnig and Zeyda, 2004). Thus, changing the fatty acid composition of the membranes of human immune cells affected phagocytosis, production of eicosanoids, T-cell signalling pathways, antigen presentation capability and expression of transcription factors (Shaik and Edidin, 2006; Calder, 2008). Phagocytosis is the first stage of the elimination of many invading pathogens. The influence of n-3 PUFA on phagocytic activity in human studies appears to be controversial and highly dependant on the methodology used to assess that activity, although it has been reported that phagocytosis was negatively correlated with the n-3/n-6 PUFA ratio (Calder, 2007). However, macrophage phagocytic activity against bacterial infections and respiratory burst activity was reduced in fish fed dietary VO, which increased the levels of dietary n-6 PUFA (Sheldon and Blazer, 1991; Montero et al., 2003).

Eicosanoids, highly metabolically active derivatives of LC-PUFA, are a direct link between the fatty acid compositions of phospholipids of the immune cell membrane and the inflammatory response (Calder, 2001). Eicosanoids are synthesised mainly from ARA and EPA, those fatty acids being released from the membranes by the action of phospholipases A_2 that is activated in response to particular stimuli. Once released from the membrane phospholipids, the LC-PUFA are converted through the action of the enzymes cyclooxygenase 2 (COX2) and lipooxygenase 5 (ALOX5), to give rise to

prostaglandins (PG) and leukotrienes (LT). Specifically, metabolism of ARA gives rise to the 2-series PG, including PGE_2, and the 4-series LT including LTB_4. In comparison, EPA gives rise to 3-series PG, including PGE_3, and the 5-series LT, including LTB_5 (Figure 1.6). The ARA-derived eicosanoids have a largely pro-inflammatory action, whereas the EPA derivatives are generally less or anti-inflammatory (Calder, 2001). As the enzymes related with the eicosanoid pathway have higher affinity for ARA, this n-6 LC-PUFA is the primary substrate for the eicosanoid forming pathways, even in fish (Bell et al., 1996; Tocher et al., 1997). The actions of the eicosanoids derived from ARA have been well documented in humans. PGE_2 increases vascular permeability and vasodilatation contributing to the mobilization of the immune cells to the sites of the infection and thus enhancing the inflammatory process (Calder, 2001). However, PGE_2 can also suppress pro-inflammatory cytokines like tumour necrosis factor (TNF) - α and interleukin (IL) -1 during the recovery process after an inflammatory episode (Calder, 2009a). LTB_4 is a potent chemotactic agent for leukocytes and enhances the production of pro-inflammatory cytokines such as TNF- α, IL-1, IL-6, IL-2, Interferon (IFN) -γ and Natural killer activity. In contrast, eicosanoids produced from EPA are less potent and pro-inflammatory than those derived from ARA, although it is believed that the competition between these two fatty acids for the eicosanoid biosynthetic enzymes is also a key factor in regulating and controlling the release of the pro-inflammatory eicosanoids (Figure 1.6). Thus, incorporation of increasing levels of EPA in the membrane phospholipids through dietary supplementation has been reported to decrease the production of ARA-derived eicosanoids in humans (Calder, 2008).

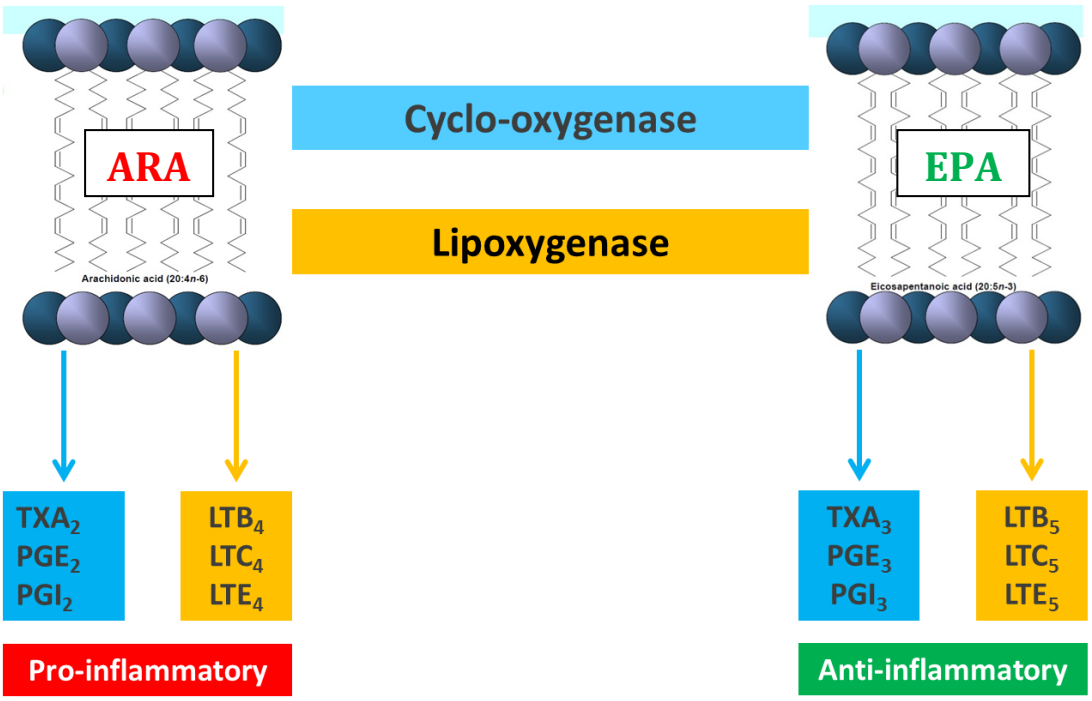

Figure 1.10. Biosynthesis of eicosanoids. LT, leukotriene; PG, prostaglandin; TX, thromboxane;ARA arachidonic acid; EPA eicosapentaenoic acid

Studies in fish are in agreement with those in humans described above albeit that the dietary interventions are in the opposite direction. That is, the normal situation in fish is high dietary EPA and the "intervention" is increased dietary n-6 PUFA. Therefore, the inclusion of VO rich in n-6 PUFA such as LA in aquafeeds increased the production of PGE_2 (Bell et al., 1993; Gjøen et al., 2004) and LTB_4 (Bell et al., 1996) and decreased the release of eicosanoids derived from EPA. As most of these were *in vitro* cell studies, further investigation would be required to fully elucidate the function, relevance and importance of the eicosanoids in fish after an infection. However, the positive correlation between heart lesions and the production of PGE_2 reported in Atlantic salmon fed VO (Bell et al., 1993) and the higher expression of LTB_4 described after a viral hemmorrhagic septicemia virus (VHSV) infection in leukocyte-derived supernatants of turbot (*Scophthalmus maximus*) (Tafalla et al., 2002) suggests similar roles of fish eicosanoids to those reported in humans.

Resolvins are a new family of n-3 LC-PUFA-derived mediators that have been recently identified in humans (Levy, 2010). These eicosanoid-like compounds are produced from EPA and DHA during the resolution phase of acute inflammation by a series of reactions involving COX2 and ALOX 15. The resolvins have potent anti-inflammatory actions by reducing natural killer cells migration, and decreasing production of TNF-α, LTB_4 and IL-1 and other inflammatory cytokines (Ji et al., 2011). This new pathway provides evidence of the important role of DHA in the control of the inflammatory process, as well as reinforcing the anti-inflammatory role of EPA. The roles of resolvins in fish have not been fully characterized (Montero and Izquierdo, 2010), probably due to the complexity of accurate identification and quantification, based on the fact that these mediators, like eicosanoids, have a short half-life *in vivo*.

In recent years, the potential immunomodulatory roles of LC-PUFA have been reported to go beyond the aforementioned production of eicosanoids and resolvins. As LC-PUFA constitute an important fraction of the phospholipids of the immune cell membranes, their roles controlling signalling pathways related to T-cell activation through antigen presentation and the production of cytosolic calcium (Ca^{2+}) are becoming of increasing interest in human clinical studies. One of the key events in the activation of T-cells is the production of cytosolic Ca^{2+} through the phosphorylation of the phospholipase C, which liberates inositol-1, 4, 5-triphosphate from phosphatidylinositol (the PI-cycle), which in turn, stimulates the increase of intracellular calcium (Stulning and Zeyda, 2004). That event is mediated by the signalling molecule linker of activated T cells (LAT) present on specific regions of the membranes called lipid rafts. The incorporation of n-3 LC-PUFA in T cell membranes has been reported to displace LAT from the lipid rafts inhibiting the membrane proximal signalling of those cells (Calder, 2007).

Antigen presentation through major histocompatibility complex (MHC) class I and MHC II is an additional crucial event for the activation of cytotoxic T cells and helper T cells after infection by intra- or extracellular pathogens (Manning & Nakanishi, 1997). Considerable data are available on LC-PUFA modulation of the expression of both MHC I and MHC II and it seems that incorporation of DHA and EPA may alter the conformation of both antigen presentation molecules (Shaikh and Edidin, 2006). Thus, FO supplementation in rats and addition of DHA and EPA in different *in vitro* studies has been reported to decrease the production of both MHC-I and MHC-II. The T cells have an important role in viral clearance in emerging virus diseases in Atlantic salmon such as CMS (Timmerhaus et al., 2011) and HSMI (Mikalsen et al., 2012). However, it appears that there are no published studies evaluating the interaction of LC-PUFA in the activation of T-cells in fish.

The recent appreciation of the ligand-activated transcription factors, peroxisome proliferator-activated receptors (PPARs), in modulation of the immune response has implicated a new inmmunomodulatory role to LC-PUFA (Varga et al., 2011). As transcription factors, PPARs bind to DNA recognition sites and regulate gene expression. The role of PPARs in lipid and fatty acid metabolism has been extensively reported in humans (Xiao-Feng and Hui, 2006) and fish (Kennedy et al., 2006; Kjaer et al., 2008). In addition, PPARs are important regulators of the development of adipose tissue, fatty acid synthesis and peroxisomal and mitochondrial β-oxidation of fatty acids. PPARs are activated by wide range of ligands of which LC-PUFA and eicosanoids are among the most important. Recent studies of the expression of PPARs, especially PPARγ and PPARα, have reported that they are, in general, involved in the suppression of the inflammatory response (Varga et al., 2011), specifically controlling the production of inflammatory cytokines through the inhibition of NF-κB and activator

protein 1 (AP-1) family members, which are critical transcription factors involved in pro-inflammatory responses (Choi and Bothwell, 2012). PPARs are expressed not only on the adipose tissue but also in macrophages, dendritic cells and T cells. PPAR expression changes with T cell activation and proliferation following T-cell receptor (TCR) stimulation controlling T-cell survival and differentiation (Choi and Bothwell, 2012).

Whether the recently appreciated immune modulatory role of PPARs in mammals has a similar role in the fish immune response is something that requires further investigation. The recent study by Sundvol et al. (2010), reporting higher expression of PPARγ in fish resistant to *Aeromonas salmonicida*, has contributed to this area and has pointing to a similar relationship between PPARs and inflammatory response in fish.

Despite the potential detrimental consequences for fish health described above and the associated implications for the incidence of emerging inflammatory diseases following the inclusion of plant-derived meals and VO in aquafeeds, the aquaculture industry still has to rely on these substitutes to FM and FO given the lack of suitable alternative products.

Recently, based on quite widely documented experiments investigating "clinical nutrition" in humans (Olson, 1978; Roberfroid, 2000), the concept of functional feeds has emerged as a potential solution to counteract the harmful effects of plant-derived ingredients on fish health (Kiron, 2012). In general, functional feeds are defined as food that, beyond simple nutritional (nutrient) effects, can provide additional benefits for health and well-being (Tacchi et al., 2011). There are different strategies regarding the formulation of functional feeds in aquaculture. They can contain additives from synthetic or natural sources that could potentially be used, in some cases, on a regular

basis as, in general, only the increase in price would limit step their use. Alternatively, a functional feed could have a high quality macronutrient composition derived from marine sources which in this case, due to the aforementioned sustainability problems, would limit their use to certain specific periods of stress and to prevent/mitigate the inflammatory episodes associated with disease outbreaks. However, vitamin and mineral supplementation also seems to be important as, even though the raw materials used in the aquafeeds generally meet the nutrient requirements for fish, antagonistic interactions with other dietary ingredients could lower their natural bioavailability, leading to immune status suppression that could further affect disease resistance (Lim et al., 2001).

Many recent studies taking a functional feeding approach in aquaculture have used additives or micronutrients in the feeds, particularly focusing on probiotics, prebiotics, nucleotides and immunomodulatory polysaccharides (Kiron, 2012). Probiotics and prebiotics have, in general, been used to improve growth, feed conversion and health status of the gastrointestinal tract of fish while also conferring resistance to certain diseases. Probiotics are live microorganisms that can compete with pathogenic bacteria for attachment to the gut wall and improving the digestion, while prebiotics are fermented ingredients with a low molecular weight that cannot be digested by the fish, but influence the composition and activity of the endogenous gastrointestinal microflora (Tacchi et al., 2012). Immunomodulation of phagocytic activity, respiratory burst, lysozyme and complement activity with the inclusion of both these types of additives in fish feeds have been reviewed previously (Merrifield et al. 2010). However, their effects on immune responses vary between species and different studies and thus future research is required to plan feeding strategies on a practical industrial scale using pre- and probiotics for improved preventive health care. Even so, these dietary components

may offer potential strategies for mitigating certain possibly detrimental effects on the gastrointestinal tract when plant proteins are included in aquaculture feeds.

Immunomodulatory polysaccharides and nucleotides have also been included in aquafeeds (Kiron, 2012). These are specific factors that, when added to the diet, can modulate the immune system of fish increasing disease resistance by enhancing the host immune response.

Dietary nucleotides would support their physiologically required levels in tissues with limited capacity of their novo synthesis like lymphoid tissues, preventing an inadequate energetic expenditure and modulating gene expression patterns and immunoendocrine interactions. (Li and Gatlin, 2006). Immunomodulatory polysaccharides act directly on pathogenic recognition receptors (PRR) stimulating them to release and produce pro-inflammatory cytokines that could potentially lead to more rapid development of the immune response after an infection (Tacchi et al., 2011). Both components have been reported to increase disease resistant towards various bacterial infections. However, immunomodulatory polysaccharides are not particularly effective against intracellular pathogens like viruses, although nucleotide supplementation was reported to increase resistance to IPN in juvenile rainbow trout (Leonardi et al., 2003). Even though the use of both additives in functional feeds has been intensively studied in the past few years, further investigations are required to fully understand all the possible interactions of these components on different physiological responses and expression of immunogenes, as well as appropriate doses and timing of administration (Li and Gatlin, 2006; Kiron, 2012).

As the main source of lipid for aquafeeds was traditionally n-3 LC-PUFA-rich FO, the concept of using sources of anti-inflammatory fatty acids such as EPA and DHA in

functional feeds is very new for the aquaculture industry. The use of increasing levels of n-6 PUFA-rich VO as replacement for traditional FO has meant that the concept of n-3 LC-PUFA as an ingredient in functional feeds is now completely logical. In humans, n-3 LC-PUFA have been widely documented as an alternative therapy in chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel diseases and asthma (Calder and Yaqoob, 2009; Miles and Calder, 2012). Among these chronic inflammatory diseases, the effects of FO supplementation in patients suffering rheumatoid arthritis has shown the most consistently positive effects, with most trials reported significant clinical benefits (Stamp et al., 2005). As one of the primary symptoms of some of the aforementioned emerging diseases in farmed salmon such as HSMI and CMS is a chronic inflammatory process, it is expected that novel formulations of functional feeds including sources of n-3 LC-PUFA including alternative marine sources such as unicellular algae, pelagic organisms or benthic invertebrates containing high amounts of n-3 LC-PUFA could also mitigate the incidence of these inflammatory diseases in fish.

In addition to traditional measures of the effects of dietary formulations on fish performance such as growth and feed efficiency, tissue composition, nutritional quality and histopathology, recent advances in functional genomics, essentially the study of gene expression, has opened up new possibilities for understanding the mechanisms involved in the use of functional feeds. Determining patterns of gene expression through study of tissue transcriptomes (mRNA expression) can provide large amounts of information on individual molecular responses that, with detailed bioinformatic analyses, can provide great insights into the physiological and immunological responses being studied (Taggart et al., 2008; Panserat et al., 2009). The use of transcriptomic analyses to provide a global overview of cellular and molecular events has become

increasingly popular in salmonid studies including the description of metabolic changes associated with different sources and levels of dietary lipids (Kolditz et al., 2008a; Morais et al., 2011). In addition, characterization of the immune response after viral infections such as infectious hematopoietic necrosis (IHN) virus (Miller et al., 2007), ISA (Jørgensen et al., 2008) and CMS (Timmerhaus et al., 2011) has helped to achieve a better understanding of antiviral and disease resistance mechanisms and to indicate possible new ways of treatment.

Aim and outline of the thesis

The current situation regarding the increased incidence of chronic inflammatory disease outbreaks associated with a viral aetiology, such as HSMI and CMS, in Atlantic salmon aquaculture, is resulting in severe financial losses for producers. Although this problem is currently particularly manifest in the Norwegian salmon aquaculture sector, it is highly likely that it has the potential to also severely affect the UK sector. The lack of vaccines to date requires a review of the use of alternative therapies that are crucial to mitigate the detrimental impacts of both diseases on growth and health of farmed salmon. In both diseases, although mortality rates are generally relatively low, morbidity can be very significant. Lesions are mainly focussed in the heart tissue and are characterized by severe inflammation and necrosis while lesions in the liver are also a commonly found, partly as a consequence of circulatory disturbances. Hence, any dietary factor modulating or attenuating chronic inflammatory processes would be a prime candidate for improving the performance, and subsequent recovery, of fish suffering from these two cardiac diseases.

The concept of clinical nutrition, widely studied and applied in humans, is becoming increasingly popular in salmonid aquaculture. This concept is based on the use of functional feeds enriched with active components, in this case anti-inflammatory

agents, to improve disease resistant and restore the health status of the fish through dietary immunomodulation.

Dietary lipid composition has been extensively studied in fish regarding multiple implications of effects of n-3 LC-PUFA in the inflammatory responses. In addition, reduction of dietary lipid content has also been used in commercial scale trials as a strategy/therapy to treat fish infected with pancreas disease (PD) having promising results with decreased mortality (EWOS internal communication). Although the precise mechanism is unclear, reduction in dietary lipid has been previously used to treat dogs with different pancreas pathologies related with lipid digestion (Westermack et al., 1995, Suzuki et al., 1999). Apart from pancreas, liver is one of the main organs in charge of the lipid digestion (Bogevik et al., 2009) and as aforementioned, is affected by both HSMI and CMS viral infections. Therefore reduction on the dietary lipid content could potentially reduce the digestive pressure in this organ alleviating the level of pathology associated with both viral diseases. Thus, both increasing the level of anti-inflammatory agents and reducing total lipid load could potentially contribute to the control of chronic inflammatory processes related to emerging viral diseases in Atlantic salmon.

Within the context of the above, the overall aim of the present thesis was to investigate the application of a clinical nutrition-based strategy in the therapy for two viral diseases, heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS), currently causing a significant financial impact in salmonid aquaculture. Functional feeds were specifically formulated to attenuate the chronic inflammatory processes in affected fish and thereby reduce the level of lesions in heart tissue. Thus, the functional feeds contained reduced dietary lipid content and elevated levels of n-3 LC-PUFA, specifically eicosapentaenoic acid (EPA; 20:5n-3).

To this end, three dietary trials were performed in Atlantic salmon. The first trial investigated the effects of two functional feed formulations in comparison to a standard, commercial reference feed on Atlantic salmon experimentally infected with ASRV, the causal agent of HSMI. The effects on tissue lipid and fatty acid compositions, heart inflammation, and heart tissue transcriptome were determined. As the mechanism of reduced dietary lipid in functional feeds was unclear, a second trial specifically investigated the effects of decreased dietary digestible energy through reduced dietary lipid on fish performance, tissue lipid and fatty acid metabolism and gene expression. Based on the results of trials one and two, a third and final trial investigated the effects of two revised functional feed formulations, again in comparison to a standard, commercial reference feed, on Atlantic salmon experimentally infected with piscine myocarditis virus (PMCV), the causal agent of CMS. The effects on tissue lipid and fatty acid compositions, heart and liver inflammation, and heart tissue transcriptome were determined.

The specific objectives of the four primary manuscripts produced from the dietary trials were:

1. To evaluate transcriptomic responses of fish fed with functional feeds having reduced lipid content and increased levels of EPA, by the inclusion of krill-based products following an infection with ASRV.
2. To investigate the correlation of the moderate immune response observed after the use of functional feeding in fish suffering HSMI with the eicosanoid and LC-PUFA biosynthesis pathways.
3. To assess the effects of dietary energy on the hepatic expression of genes related to lipid and fatty acid metabolism in Atlantic salmon fed diets containing graded amounts of lipid.

4. To evaluate the dietary immunomodulation of functional feeds with reduced lipid content increased levels of EPA and histidine supplementation after infection with PCMV through the use of oligomicroarray technology.

Chapter 2

Functional feeds reduce heart inflammation and pathology in Atlantic salmon (*Salmo salar* L.) following experimental challenge with Atlantic salmon reovirus (ASRV)

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Published Plos One journal: November 2012, doi:10.1371/journal.pone.0040266

Abstract

Heart and Skeletal Muscle Inflammation (HSMI), recently associated with a novel Atlantic salmon reovirus (ASRV), is currently one of the most prevalent inflammatory diseases in commercial Atlantic salmon farms in Norway. Mortality varies from low to 20%, but morbidity can be very high, reducing growth performance and causing considerable financial impact. Clinical symptoms, including myocarditis, myocardial and red skeletal muscle necrosis, correlate with the intensity of the inflammatory response. In the present study, the effects of two functional feeds (FF1 and FF2) were compared to a standard commercial reference feed (ST) in Atlantic salmon subjected to an ASRV challenge. The functional feeds had reduced levels of total lipid and digestible energy, and different levels and proportions of long-chain polyunsaturated fatty acids (LC-PUFA). The objective was to determine whether these feeds could provide effective protection by decreasing the inflammatory response associated with HSMI. Histopathology, viral load, fatty acid composition and gene expression of heart tissue were assessed over a period of 16 weeks post-infection with ASRV. The viral load and histopathology scores in heart tissue in response to ASRV infection were reduced in fish fed both functional feeds, with FF1 showing the greatest effect. Microarray hierarchical cluster analysis showed that the functional feeds greatly affected expression of inflammation/immune related genes over the course of the ASRV infection. Viral load correlated with up-regulation of pro-inflammatory genes at the early-mid stages of infection in fish fed the ST diet. Expression of inflammatory genes 16-weeks after ASRV challenge reflected the difference in efficacy between the functional feeds, with fish fed FF1 showing lower expression. Thus, severity of the lesions in heart tissue correlated with the intensity of the innate immune response and was associated with tissue fatty acid compositions. The present study demonstrated

that dietary modulation through clinical nutrition had major influences on the development and severity of the response to ASRV infection in salmon. Thus, HSMI was reduced in fish fed the functional feeds, particularly FF1. The modulation of gene expression between fish fed the different feeds provided further insight into the molecular mechanisms and progression of the inflammatory and immune responses to ASRV infection in salmon.

2.1 Introduction

Heart and Skeletal Muscle Inflammation (HSMI) was first diagnosed in 1999 (Kongtorp et al., 2004a) and has been associated with a novel piscine reovirus (Palacios et al., 2010) recently defined as Atlantic salmon reovirus (ASRV) (Mikalsen et al., 2012). It is currently one of the most prevalent inflammatory diseases in commercial Atlantic salmon farms in Norway, with over 600 sites affected since 2003. Isolated cases have also been detected in the United Kingdom (Ferguson et al., 2005). Mortality associated with HSMI varies between outbreaks; from insignificant to 20% of stock (Kongtorp et al., 2004a), but morbidity can be very high, significantly reducing growth performance, increasing feed conversion ratio, and causing considerable financial impact. Clinical symptoms include aberrant swimming behaviour (lethargic fish) believed to be associated with reduced heart function and poor circulation. Histopathologically, heart and red skeletal muscle appear to be the main organs affected showing severe inflammation. Epi-, endo-, and myocarditis and myocardial necrosis, as well as necrosis of red skeletal muscle are characteristic of the disease, although liver damage is also found (Kongtorp et al., 2004b). These clinical symptoms are believed to correlate with the intensity of the inflammatory response, so factors modulating inflammation might influence the clinical manifestation of this disease.

One strategy to potentially control outbreaks of HSMI, or reduce the severity of the disease, is through the application of clinical nutrition and functional feeds. The concept of clinical nutrition and functional foods is well known in human nutrition. Functional foods are defined as foods that contain a component (whether a nutrient or not) that could be beneficial for the state of well-being and health, or reduce the risk of a disease, beyond the basic nutritional requirement (Bellisle et al., 1980). Therefore, dietary supplementation is now a common complementary therapy, not only to improve the general health status of the population, but also for the management of common inflammatory diseases such as

rheumatoid arthritis (Stamp et al., 2005) or coronary diseases (Erkkilä et al., 2008). This type of approach is also becoming increasingly popular in aquaculture as it could potentially result in great economical savings in terms of increased productivity and lower costs of disease treatment/management (Raynard et al., 1991; McCoy et al., 1994; Tacchi et al., 2011). At the current time HSMI vaccines are still under development and efficacy of other commercially available viral vaccines may be variable (Sommerset et al., 2005). Clinical nutrition offers a valuable additional tool to reduce the impact of viral diseases.

There are many additives used in aquaculture diets including probiotics, prebiotics, immunostimulants, vitamins and nucleotides, which are included in commercial feeds to increase growth and feed conversion efficiency, as well as having positive effects on the fish immune system (Tacchi et al., 2011). The replacement of fishmeal with plant-derived protein sources can lead to changes in gut flora and physiology, probably due to higher contents of non-digestible fibrous components, and increased levels of anti-nutritional factors, whose negative effects can be ameliorated by the additives mentioned above (Dimitroglou et al., 2010). Another concern in recent years has been the replacement of dietary fish oil (FO) by vegetable oils (VO). This is a consequence of generally decreasing wild fisheries worldwide that, not only supply a declining proportion of fish for direct human consumption, but also, paradoxically, could constrain the growth of aquaculture due to reduced availability of fishmeal and FO, required for the manufacture of aqua feeds (Tacon and Metian, 2008; FAO, 2010).

It is well known that dietary polyunsaturated fatty acids (PUFA) can influence the immune response. Arachidonic acid (ARA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), derived from cell membrane phospholipids, are precursors of eicosanoids, resolvins and protectins, which modulate leukocyte function and thereby influence production of inflammatory cytokines and adhesion molecules (Calder, 2007). The eicosanoids derived from ARA promote pro-inflammatory responses,

whereas those derived from EPA and DHA either produce a reduced inflammatory response or actually resolve (terminate) the inflammatory response (Calder et al., 2009b). Dietary supplementation with n-3 long-chain (LC) PUFA, EPA and DHA, protects against cardiovascular diseases, and may also be beneficial for some common inflammatory disorders in humans (Calder and Yaqoob, 2009a). Studies have demonstrated that these LC-PUFA have the same role in immunomodulation in fish, and this has been an important concern particularly in recent years with increased energy (lipid) content of commercial diets and the aforementioned replacement of dietary FO by VO, which are devoid of LC-PUFA, but rich in n-6 PUFA (Balfry and Higgs, 2001; Ganga et al., 2005). Such dietary replacement decreases the n-3/n-6 LC-PUFA ratio in tissues, potentially promoting the synthesis of pro-inflammatory eicosanoids, and several studies have examined whether this might affect disease resistance in farmed animals (Bell et al., 1996; Montero et al., 2008a). In general, fish fed diets with a high level of VO inclusion may be less resistant to infection and prone to increased severity of inflammatory responses due to the reduced n-3/n-6 PUFA ratio. Influence of dietary lipid content has also been investigated. It was reported that diets with higher lipid content may be associated with increased mortality and muscle lesions in Atlantic salmon suffering from pancreas disease, a common viral infection in salmon farming (McCoy et al., 1994).

Oligonucleotide microarray analysis of tissue transcriptomes has become an increasingly popular tool in fish nutritional studies but, in addition, has also provided a global overview of the cellular and molecular events activated upon viral infections, and genome-wide changes after vaccination (Zhu et al., 2010) or infection (Kawada et al., 2006). Host genes and immune responses induced after infection with different viral diseases of salmon such as infectious salmon anaemia (ISA) (Jørgensen et al., 2008; Workenhe et al., 2009) and infectious hematopoietic necrosis (IHN) virus (Miller et al., 2007) have been characterized using transcriptomic analysis. Krasnov et al. (2011)

identified virus responsive genes (VRG) in different viral diseases including HSMI, but there are no further descriptions of the immune response specifically related with this disease, such as the expression of host genes after infection with ASRV.

The aim of the present study was to investigate the application of a clinical nutrition strategy in Atlantic salmon subjected to experimental ASRV challenge, through the use of two potential functional feed formulations in comparison to a standard commercial feed. The standard reference diet (ST diet) contained 31% lipid derived from marine (FO and fishmeal) and terrestrial (rapeseed oil) sources. The functional feeds had reduced energy levels through lower lipid (18%), and increased levels of EPA, and increased n-3/n-6 PUFA and EPA/ARA ratios, which were achieved by increased protein (fishmeal and krill meal) (FF1 diet), or krill meal plus krill oil (FF2 diet). Krill products, especially krill oil, are good sources of EPA and DHA in a phospholipid form (Storebakken, 1988). Dietary krill oil increased incorporation of EPA and DHA into heart phospholipids in rats (Reeves et al., 1993), and decreased the inflammatory process associated with chronic diseases such as rheumatoid arthritis in humans (Ierna et al., 2010). The incorporation of anti-inflammatory LC-PUFA into heart, the main organ affected by the disease, and head kidney (the main immune organ) was assessed. In addition to histological evaluation, the inflammatory profile in heart tissue was monitored by microarray analysis of gene expression over a period of 16 weeks after infection with ASRV, and viral load assessed over the later collections times (>10 weeks post challenge). Therefore, this was a nutritional study specifically designed to determine whether functional feeds could ameliorate the pathological effects of the ASRV infection, and to identify pathways/mechanisms underpinning the dietary effects in order to improve feed formulations.

2.2 Materials and Methods

2.2.1 Fish and experimental feeds

Three fishmeal-based diets were formulated and manufactured by EWOS Innovation (Dirdal, Norway) (Table 2.1). The reference diet (ST) was essentially a standard, commercial formulation with 31 % lipid with the added oil being a blend of FO and rapeseed oil. The two functional feeds (FF) both contained a lower level of lipid that was balanced by increased protein, provided by fishmeal and krill meal. Both also contained increased proportions of EPA, but with different levels of n-6 PUFA and DHA, resulting in the n-3/n-6 PUFA ratio in the feeds varying from 1.5 to 3.6, through the inclusion of krill oil in one of the feeds replacing rapeseed oil (Table 2.2). The precise formulations were based on small-scale commercial screening trials (unpublished). Therefore, as one major factor being investigated was dietary lipid level, the diets were not isolipidic or isoproteic. The diets were termed ST (standard reference diet), FF1 and FF2.

A total of 450 Atlantic salmon (*Salmo salar* L.), unvaccinated AquaGen strain, were distributed into three tanks at the EWOS facility, Lønningdal, Norway and fed one of the three feeds for a period of 8 weeks. After this pre-challenge feeding phase, 390 (130 fish per treatment) fish (non-vaccinated) with an initial average weight 220g (±3.2g, standard error), were transferred to the challenge facility at the Industrial and Aquatic Laboratory (ILAB), Bergen, Norway. Fish were distributed into two independent experimental rooms supplied with filtered sea water (of approximately 30‰) each containing 9 tanks (3 tanks per dietary treatment in each system). Water delivery was flow-through, with a supply to maintain oxygen concentration in outlet water > 8 mg/L. Water temperature was maintained at 10 ± 1°C, water flow rate was 0.8 L / kg fish / min, and a 12:12 h light/dark

regime was followed. The fish were acclimated for 2 weeks prior to challenge and were fed with the same diets during the acclimation period and throughout the period of the challenge (16 weeks) that they were fed prior to transfer. No previous diseases were described. Ten fish from each feeding group (n = 30 in total) were sampled prior to challenge (0 time-point controls) for histological examination to confirm they were disease-free.

Table 2. 1 Formulation of the experimental diets.

Component %[1]	**STD**	**FF1**	**FF2**
Fish meal and hydrolysates	42.1	53	53
Fish oil	13.7	7	5.2
Vegetable protein concentrates[2]	21	18	18
Vegetable oil	11.2	3	0
Carbohydrate-based binders[3]	11.2	12.1	12.1
Micro premixes[4]	0.8	1.9	1.7
Krill meal[5]	0	5	5
Krill oil[5]	0	0	5
Total	100	100	100
Proximate composition			
Moisture	6.5	6.5	6.5
Fat	31	18	18
Protein	42.2	53.4	53.4

1) All ingredients sourced from EWOS stocks unless otherwise stated.

2) Includes soy protein concentrate, pea protein concentrate, wheat gluten and sunflower meal

3) Includes wheat grain

4) Includes vitamins, minerals, crystalline amino acids, ammonium phosphate

5) Aker Biomarine A

Table 2.2 Total lipid fatty acid composition (percentage of total fatty acids) and lipid class composition (percentage of total lipid) of the experimental diets

Fatty acid	**ST**	**FF1**	**FF2**
Saturated	23.97	36.48	31.86
18:1n-9	32.30	18.65	16.31
Monounsaturated	45.73	33.10	37.05
18:2n-6	10.73	7.11	5.30
20:3n-6	0.06	0.11	0.10
20:4n-6	0.36	0.48	0.54
n-6 PUFA	11.63	8.09	6.50
18:3n-3	4.62	2.35	1.55
20:5n-3	4.85	8.76	9.53
22:6n-3	6.09	5.98	9.03
n-3 PUFA	17.83	19.83	23.32
PUFA	30.31	30.42	31.09
EPA/ARA	13.67	18.16	17.75
N-3/N-6	1.53	2.45	3.59
Phospholipids	8.94	10.77	17.30
Triacylglycerols	65.39	53.47	52.45
% Lipid	24.52	13.71	13.35

ARA, Arachidonic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acids.

2.2.2 Virus isolation and preparation of inoculum

Heart tissue, collected from a clinical outbreak of HSMI, was homogenized and centrifuged 3500 x g for 20 min 4ºC to remove cellular debris and then filtered (0.45 μm). The homogenate was inoculated onto GF-1 cells derived from fin tissue of orange-spotted grouper, *Epinephelus coioides*. This was done in collaboration with PHARMAQ AS, Oslo, Norway who supplied the cell line. The cells were grown at 15°C in Leibovitz L-15 medium (Invitrogen AS, Oslo, Norway) supplemented with 1 % L-glutamine, 0.1 % gentamicin sulphate (Sigma Aldrich Norway AS, Oslo, Norway) and 10 % fetal bovine serum (Invitrogen). At 2 weeks after inoculation the supernatant and cell lysate were harvested and cleared by centrifugation at 3500 x g for 20 min at 4ºC.

2.2.3 ASRV Challenge

As this was a nutritional trial designed to test the effects of different feeds, all 360 remaining fish were challenged with pathogen. The fish were sedated using Aqui-S at final concentration of 5 mg/L of isoeugenol, followed by anaesthesia in Benzoak using a final concentration of 30 ml/L of water. Fish were challenged by intramuscular injection (0.1 ml on each side close to the lateral line) of ASRV collected from cell culture supernatant of piscine reovirus (as above). There were no mortalities during the trial, which was consistent with previous experimental studies of HSMI (Kongtorp et al., 2004a; Kongtorp et al., 2004b).

2.2.4 Sampling

Ten fish, chosen at random, from each dietary treatment group (5 fish from each of the two independent systems) were sampled at 8-, 10-, 12-, and 16-weeks post-challenge for histology, lipid and biomolecular analysis (data presented). However, in addition a further 10 fish per treatment were sampled at 14 weeks post-challenge for histology (data not shown) and all remaining fish were culled at week 16 post-challenge for possible further histological analysis. Fish were anaesthetized and killed by a blow to the head, and heart and head kidney collected for analyses. Part of each heart sample was transferred to 10% buffered formalin for histological analyses and another part was immediately frozen in liquid N_2 and stored at -80 ^{o}C prior to lipid and molecular analyses. Head kidney was immediately frozen in liquid N_2 and stored at -80 ^{o}C.

2.2.5 Histological examinations

Inflammatory response in ASRV challenged fish was assessed by histological changes in heart tissue (epicardium and myocardium) as assessed by light microscopy. The scoring

system marked samples on a visual analog scale on the basis of the criteria given in Supplementary Table 2.3 (Mikalsen et al., 2012). A maximum score of 6 can be achieved for combined epicardial and myocardial changes. All evaluations were carried out blind. Further, details of the different feeds were not revealed to the histological examiner (ØE), i.e. the histological examination was carried out as a double-blind study.

Micrographs showing examples of the correspondence between different levels of heart lesions and histoscores assigned are shown in Figure 2.1

2.2.6 Lipid analyses

Lipid and fatty acid analyses were performed on heart and head kidney tissue samples from five fish per treatment at 8- and 16-weeks post-challenge. Total lipid from approximately 1g of heart and head kidney tissue was extracted by homogenization in chloroform/methanol (2:1, by volume) according to Folch et al. (1957), and determined gravimetrically. Fatty acid methyl esters (FAME) of total lipid were prepared by acid-catalyzed transmethylation (Christie, 2003), and FAME separated and quantified by gas chromatography as described in detail previously (Bell et al., 1993). Tissue and diet lipid class compositions were determined by single-dimension double-development high-performance thin-layer chromatography (HPTLC) and densitometry as described previously (Bell et al., 1993). Significance of differences due to diet and time were determined by two-way ANOVA ($p<0.05$) using the SPSS 19.0 statistical package (SPSS Inc., Chicago IL, USA).

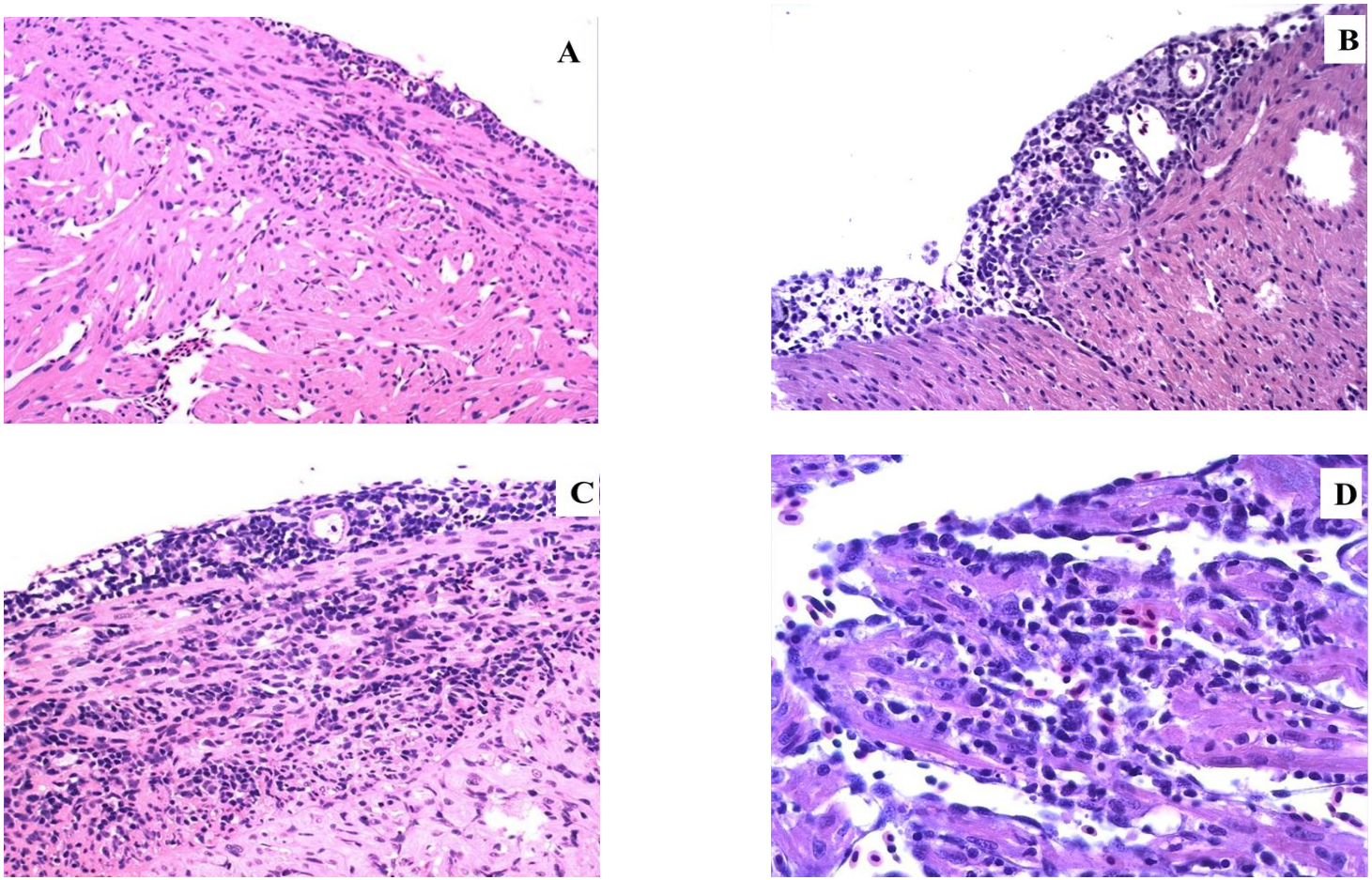

Figure 2.1 Micrographs showing different levels of pathology on the heart tissue. A, epicarditis (score 1); B, epicarditis (score 2); C, epicarditis with inflammation in compactum of the ventricle (score 1.5 epicard and 2 of compactum); D, Spongious part of ventricle, Infiltration of inflammatory cells and degeneration/necrosis of myocytes. (score 2).

2.2.7 RNA extraction and purification

Heart tissue from six individuals per dietary treatment was rapidly homogenized in TRI Reagent (Ambion, Applied Biosystems, Warrington, U.K.) using an Ultra-Turrax homogenizer (Fisher Scientific, Loughborough, U.K.). Total RNA was isolated following manufacturer's instructions, and RNA quality and quantity assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.), respectively. One hundred micrograms of total RNA from each individual sample was further purified by mini spin-column purification (RNeasy Mini Kit, Qiagen, Crawly, UK), and quantified and quality assessed as above.

2.2.8 Microarray hybridizations and analysis

The transcriptomic experiment used an Atlantic salmon custom-made oligoarray with 44k features per array on a four-array-per-slide format, with each feature printed singly (Agilent Technologies UK Ltd., Wokingham, UK). The probes were co-designed by researchers at the Institute of Aquaculture, University of Stirling, U.K. and The Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima, Tromsø, Norway), and array design is available on request. A dual-labelled experimental design was employed for the microarray hybridizations. Each experimental sample was competitively hybridized against a common pooled-reference sample, which comprised equal amounts of each of the replicates used in the study. This design permits valid statistical comparisons across all treatments to be made. The entire experiment comprised 72 hybridizations; 4 time points (8, 10, 12 and 16 weeks) × 3 diets (ST, FF1 and FF2) × 6 biological replicates.

Indirect labelling methodology was employed in preparing the microarray targets. Amplified antisense RNA (aRNA) was produced from each RNA sample using the Amino Allyl MessageAmpTM II aRNA Amplification Kit (Ambion, Applied Biosystems, Warrington, UK), following the manufacturer's methodology, followed by Cy3 or Cy5

fluor incorporation through a dye-coupling reaction. Briefly, 500 ng of total RNA per sample were amplified and column-purified according to manufacturer's instructions including a 17 h transcription step. Resultant aRNA was quantified and quality assessed as above. Subsequently, Cy dye suspensions (Cy3 & Cy5) in sufficient quantity for all labelling reactions were prepared by adding 36 µL high purity dimethyl sulphoxide (Stratagene, Agilent Technologies UK Ltd.) to each tube of Cy dye (PA23001 or PA25001, GE HealthCare, Chalfont St. Giles, UK). To attach the Cy dyes, 3 µg each aRNA sample was suspended in 10 µL nuclease-free H_2O and heated to 70 ºC for 2 min. When cooled to room temperature, 3 µL of coupling buffer (0.5 M $NaHCO_3$; pH 9.2) and 2µL of Cy3 dye suspension stock was added and then incubated for 1 h at 25 ºC in the dark. To label the common pooled reference sample with Cy5, a scaled-up batch reaction was similarly performed. Unincorporated dye was removed by column purification (Illustra AutoSeq G-50 spin columns; GE Healthcare). Dye incorporation and aRNA yield were quantified by spectrophotometry (NanoDrop) and further quality controlled by separating 0.4 µL of the sample on a thin mini-agarose gel and visualising products on a fluorescence scanner (Typhoon Trio, GE Healthcare).

Hybridization of a total of 6 slides (24 arrays) was performed in a single day, with sample order semi-randomized, using SureHyb hybridization chambers in a DNA Microarray Hybridization Oven (Agilent Technologies). For each hybridization, 825 ng of Cy3-labelled experimental biological replicate and Cy5-labelled reference pool were combined and total volume made up to 38 µl with nuclease-free water. A fragmentation master mix was prepared containing, per reaction, 11 µl 10× blocking agent, 2.2 µl 25× fragmentation buffer and 6.8 µl nuclease-free water, and 20 µl was dispensed into the Cy-dyes mix. After incubating in the dark at 60 ºC for 30 min, 57 µl 2×GE Hybridization buffer (pre-heated to 37 ºC) was added, contents gently mixed, spun at 16,000 g for 1 min and kept on ice until loaded onto the microarray slides as per the manufacturers protocol.

Hybridization was carried out in the oven rotator (Agilent Technologies) at 65 ºC and 10 rpm for 17 h. Post-hybridization washes were carried out in EasyDip™ Slide staining containers (Canemco Inc., Quebec, Canada). After disassembling the array-gasket sandwiches submersed in wash buffer 1 at room temperature, the microarray slides were transferred to an EasyDip™ container and incubated in wash buffer 1 for 1 min at 31 ºC in an orbital incubator rotating at 150 rpm, and then a further 1 min at 31 ºC at 150 rpm in wash buffer 2. A final dip in wash buffer 2 at room temperature was performed, after which the slides were dried by centrifugation (500 x g for 6 min) and kept in a desiccator in the dark until scanned the same day. Unless otherwise stated, all reagents were from Agilent Technologies.

Scanning was performed at 5 μm resolution using an Axon GenePix 4200AL Scanner (MDS Analytical Technologies, Wokingham, Berkshire, U.K.). Laser power was kept constant (80 %) and the "auto PMT" function within the acquisition software (v.4) was enabled to adjust PMT for each channel such that less than 0.1% of features were saturated and that the mean intensity ratio of the Cy3 and Cy5 signals was close to one. Agilent Feature Extraction Software (v 9.5) was used to identify features and extract fluorescence intensity values from the resultant TIF images. The remaining analysis was then performed in the GeneSpring GX version 10.0.2 analysis platform (Agilent Technologies). All intensity values < 0.1 were set to 0.1 followed by a block Lowess normalisation. After removing control features, a series of four quality filtering steps was carried out sequentially using a range of quality control metrics produced by the Agilent Feature Extraction software to remove features that were saturated, non-uniform, population out layers and spots non-significantly different from background.

Hybridization data were analyzed by two-way ANOVA, which examined the explanatory power of the variables 'diet' and 'time' and the interaction between the two, at

a significance level of 0.05. In the present study we focussed on immune-related genes whose expression was differentially affected by diet at 16 weeks post-challenge and thus only data from the significant interaction list was analyzed. That list contained 2584 genes, including repeated probes. Due to the large size of the list, hierarchical cluster analysis and 5x5 Self-Organizing Maps cluster analysis (SOM) were used to map the common temporal expression profiles of the genes from the list using the GeneSpring GX version 10.0.2 analysis platform (Agilent Technologies).

2.2.9 RT-qPCR

Expression of 12 selected genes showing a significant diet × time interaction in the microarray analysis and related to relevant immune-related pathways was studied by reverse transcription quantitative real-time PCR (qPCR). The qPCR primer sequences, annealing temperature (Tm) and size of amplicon are given in Supplementary Table 2.1 (end of the chapter). The sequences were obtained either by literature searches or designed from EST sequences corresponding to microarray clones or candidate genes of interest using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). In addition, amplification of three potential reference genes, *cofilin-2*, elongation factor-1α (*elf-1α*) and *β-actin*, was performed but only *cofilin-2* and *elf-1α* expression was sufficiently stable across treatments for normalization. *Cofilin-2* and *elf-1α* had been identified in previous salmon cDNA microarray and qPCR studies as suitable reference genes on the basis of constant expression between different feeds and time points (Jørgensen et al., 2006; Morais et al., 2011).

Samples used for the microarrays hybridizations from 12 and 16 weeks post challenge, were selected for the qPCR validation (6 samples x 3 treatments x 2 time points). For qPCR, 2 µg of column-purified total RNA per sample was reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Paisley, U.K.), following

manufacturer's instructions, but using a mixture of the random primers (1.5µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/ µl, Eurofins MWG Operon, Ebersberg, Germany). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. cDNA was then diluted 20-fold with water, after a similar amount of cDNA was pooled from all samples. qPCR analysis used relative quantification with the amplification efficiency of the primer pairs being assessed by serial dilutions of the cDNA pool. qPCR amplifications were carried out in duplicate (Quantica, Techne, Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL (for most genes) or 2µL (for the reference genes and other highly expressed genes) diluted (1/20) cDNA, 0.5µM of each primer and 10 µL AbsoluteTM QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a systematic negative control (NTC-non template control, containing no cDNA). The qPCR profiles contained an initial activation step at 95°C for 15 min, followed by 30 to 40 cycles: 15 s at 95 °C, 15 s at the specific primer pair annealing Tm and 15 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C increments from 75 ºC to 90 °C was performed, enabling confirmation of amplification of single products, and sizes were checked by agarose gel electrophoresis and identities confirmed by sequencing. Non-occurrence of primer-dimer formation in the NTC was also verified. Data were analyzed using the relative expression software tool (REST 2009, http://www.gene-quantification.info/), which employs a pair wise fixed reallocation randomization test (10,000 randomizations) with efficiency correction, to determine the statistical significance of expression ratios (or gene expression fold-changes) between two treatments (Pfaffl et al., 2002)

2.2.20 Assessment of viral load

Relative quantification of ASRV by qPCR was used to determine differences in the viral load between the different dietary treatments at the two critical points in the time-course of

the infection (12- and 16- weeks post-challenge) when differential expression of genes related with the inflammatory process was greatest between fish fed the ST diet and fish fed the functional feeds. The qPCR primer sequences (Supplementary Table 2.4) previously described by Mikalsen et al. (2012) were employed following the same procedure and from the same RNA samples used for the validation of the microarray (as described above).

2.2.11 Statistical analysis

Histological changes in epicardium and myocardium were ranked according to a non-continuous score grade from 0 to 4 (0 indicates no pathology, normal tissue; 4 intense inflammation). A description of each of the scores is included in Supplementary Table 2.3.

All data preparation and simulation output analysis was conducted with the R language (R:Languaje). The model was a mixed-effects linear model estimated with the lmer function in the lme4 package. The treatment estimates were based on posterior simulation (n = 2500) with 95 % credible intervals as absolute and proportional to the reference level (control diet).

Histopathological scores were analysed by using a multilevel ordered categorical logistic regression because the data are multinomial. The model was written in BUGS (Lunn et al., 2000) language and fitted with JAGS (Plummer, 2009). Vague non-informative uniform priors [0,100] were given for the variance parameters and vague non-informative normal priors N (0, 1.0E + 4) for all other parameters. 25000 “burn-in" simulation runs were used to adapt the Markov Chain Monte Carlo (MCMC) before subsequent 2500 runs that were used for inference. Three chains were run in parallel, i.e. there were a total of 7500 simulations for inference. These were thinned so that only every 10th simulation was saved to reduce the size of saved objects and to reduce the effects of autocorrelation. In effect, the posterior density is based on 750 draws from the posterior probability distribution.

Convergence of the MCMC simulation was judged by the so-called Gelman-Rubin convergence diagnostic (Gelman and Rubin, 1992).

2.3 Results

2.3.1 The viral load and histopathology scores in the heart tissue in response to ASRV infection were reduced in fish fed both functional feeds, with the FF1 feed showing the significantly greatest effect.

We first used RT-qPCR to compare the heart virus load between the different dietary groups at later stages of the infection, when the inflammatory response typical of HSMI appeared in heart tissue in challenged fish, i.e. 12- to 16-weeks post-challenge (Kongtorp et al., 2004a; Kongtorp et al., 2004b). We found there was considerably higher viral load at 12-weeks post-challenge in fish consuming the ST diet compared with fish fed the two functional feeds (Figure 2.2). Fish fed the ST diet showed 260-fold greater viral load ($p<0.05$) than fish fed the FF1 diet. Compared to the FF2 diet, relative levels were 25-fold higher in ST fish but, due to the high variability between individuals, this difference was not significant. The pathophysiological response to the infection characterised histologically by infiltration of lymphocytes and macrophages in heart tissue, reflected the pattern of virus load as had been shown earlier (Mikalsen et al., 2012). There was no infiltration of inflammatory or immune cells in heart tissue in any dietary group at 8-weeks post-challenge (Figure 2.3). However, from 10-weeks onwards, the histopathology scores showed that the levels of cardiac inflammation were much higher at all-time points in fish consuming the ST diet compared to fish fed the functional feeds (FF1 and FF2 groups). In addition, the fish fed diet FF1 showed the significantly lowest level of cardiac inflammation when the data were combined across all time points (Figure 2.3).

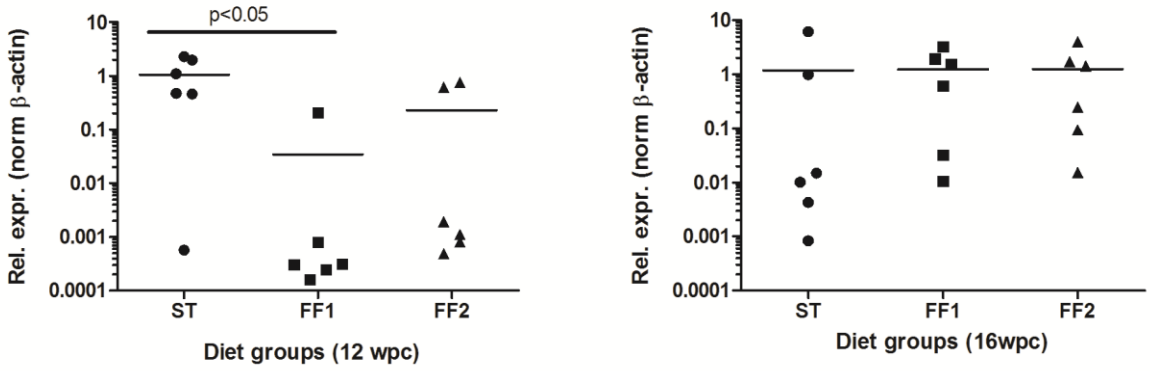

Figure 2.2 Viral load in heart tissue of the different dietary groups at 12- and 16-weeks post-challenge. Viral load was determined by quantitative real-time PCR analysis of Atlantic salmon reovirus OH-2010 strain ALV726 inner capsid protein lambda-1/VP3 gene (HM453201). Results are presented as normalised expression data (n=6) relative to β-actin on a log scale (log_{10}) with statistical analysis by Kruskal-Wallis test (non-parametric).

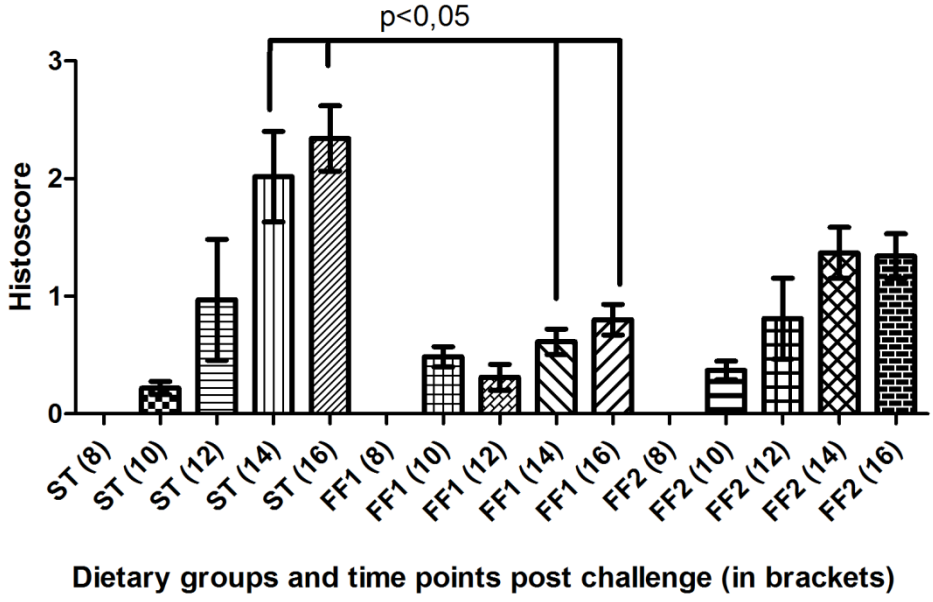

Figure 2.3. Average histoscores (± SEM) in the different dietary treatment groups at 8-, 10-, 12-, 14- and 16-weeks post-challenge. Significant differences between feed groups are indicated. N = 10 to 20 per group. Samples were scored by marking on a visual analog scale on the basis of criteria described previously by Mikalsen et al. (3) (see Supplementary Table 2.3). A maximum score of 6 can be achieved for combined epicardial and myocardial changes. Scoring was carried out with a double-blind format.

2.3.2 Fatty acid compositions of heart and head kidney tissues were associated with the severity of the inflammatory response/disease

The fact that diets modulated the viral load and inflammatory scores to the extent observed was surprising and, based on the difference in fatty acid composition between diets, we first examined the fatty acid profiles of heart and head kidney tissues (Tables 2.2 and 2.3). Generally, the composition of these organs reflected the diets fed to the fish (Table 2.4) in a similar manner to that observed in previous studies that investigated effects of diet on fatty acid profiles of heart (Bell et al., 1992) and head kidney (Gjoen et al., 2007). The proportion of EPA and the EPA/ARA ratio were significantly higher in heart tissue of fish consuming the functional feeds (Table 2.3). In general, the overall fatty acid profile of heart tissue changed relatively little during the course of the infection. In contrast, we found that the fatty acid profile of head kidney tissue showed more significant differences between the three dietary groups, and the proportions of most of the LC-PUFA related with the immune response changed during the course of the infection (Table 2.4). For instance, the proportions of total n-3PUFA and DHA increased, and those of ARA and monoenes decreased, over the course of the infection. The proportion of EPA also decreased in fish fed the ST diet over the course of the infection but, in contrast, EPA increased in fish fed the functional feeds between weeks 8 and 16 (Table 2.4). Previous studies have shown that levels of EPA, ARA and DHA could determine the intensity of inflammatory response, with EPA and DHA being anti-inflammatory fatty acids and ARA pro-inflammatory (Calder, 2005). In concert with this finding, our results showed that tissue levels of EPA and DHA and the EPA/ARA ratios were significantly higher in fish fed the functional feeds than in fish fed the ST diet, which was consistent with the reduced inflammatory response in heart tissue in these groups (Figure 2.3).

Table 2.3 Total lipid fatty acid composition (percentage of total fatty acids) of heart from Atlantic salmon fed the different diets at 8- and 16- weeks post-infection with ASRV

	8 weeks						16 weeks						TWO WAY ANOVA *P-value*		
Fatty acid	ST		FF1		FF2		ST		FF1		FF2		Diet	Week	Diet*Week
Saturated	25.48	± 4.17	27.22	± 1.92	28.45	± 1.45	22.33	± 0.90	25.16	± 1.05	27.07	± 1.01	0.001	0.008	ns
Mono	26.48	± 8.25	21.69	± 4.19	26.74	± 9.68	35.32	± 7.35	26.58	± 7.44	24.97	± 6.90	ns	ns	ns
20:3n-6	0.32	± 0.07	0.38	± 0.06	0.27	± 0.06	0.28	± 0.02	0.35	± 0.05	0.30	± 0.03	0.005	ns	ns
20:4n-6	1.87	± 0.76	1.95	± 0.44	1.40	± 0.56	1.10	± 0.37	1.45	± 0.42	1.53	± 0.39	ns	ns	ns
N-6 PUFA	8.36	± 1.11	6.82	± 0.31	6.00	± 0.43	9.34	± 0.88	7.30	± 0.65	5.66	± 0.38	0.000	ns	ns
20:5n-3	6.21	± 1.96	8.67	± 1.31	6.98	± 2.19	4.53	± 1.27	7.86	± 1.39	7.19	± 1.21	0.002	ns	ns
22:6n-3	27.69	± 7.03	30.08	± 3.40	26.67	± 9.10	22.19	± 6.52	27.23	± 7.34	30.26	± 7.29	ns	ns	ns
N-3 PUFA	38.64	± 8.39	43.44	± 4.54	38.30	± 11.09	32.45	± 7.20	40.28	± 8.20	41.62	± 8.08	ns	ns	ns
PUFA	48.04	± 8.05	51.08	± 4.22	44.81	± 11.12	42.35	± 6.48	48.22	± 7.19	47.95	± 7.70	ns	ns	ns
EPA/ARA	3.49	± 0.65	4.57	± 0.77	5.21	± 0.86	4.19	± 0.40	5.62	± 0.98	4.83	± 0.76	0.000	ns	ns
N-3/N-6	4.73	± 1.36	6.40	± 0.89	6.38	± 1.83	3.56	± 1.13	5.62	± 1.54	7.46	± 1.84	0.000	ns	ns

Results are means ± SD (n = 5). P-values of two-way ANOVA are presented for factors 'diet', 'time' and interaction between both factors.
ARA, arachidonic acid; EPA, eicosapentaenoic acid; ns, not significant ($p > 0.05$); PUFA, polyunsaturated fatty acids.

Table 2.4 Total lipid fatty acid composition (percentage of total fatty acids) of head kidney of Atlantic salmon 8- and 16-weeks post-infection with ASRV

	8 weeks			16 weeks			TWO WAY ANOVA *P-value*		
Fatty acid	ST	FF1	FF2	ST	FF1	FF2	Diet	Week	Diet*Week
Saturated	22.01 ± 0.72	25.13 ± 1.26	27.41 ± 0.58	21.84 ± 0.50	25.03 ± 0.41	28.15 ± 0.67	0.000	ns	ns
Monounsaturated	41.76 ± 2.01	40.24 ± 4.18	38.85 ± 3.44	40.44 ± 3.63	35.15 ± 1.97	30.38 ± 3.19	0.000	0.000	ns
20:3n-6	0.24 ± 0.02	0.25 ± 0.03	0.22 ± 0.03	0.26 ± 0.03	0.30 ± 0.01	0.24 ± 0.03	0.002	0.002	ns
20:4n-6	1.31 ± 0.21	1.22 ± 0.44	1.25 ± 0.35	0.66 ± 0.14	0.82 ± 0.08	0.88 ± 0.12	ns	0.000	ns
N-6 PUFA	9.88 ± 0.56	7.91 ± 0.33	6.56 ± 0.26	9.82 ± 0.60	7.83 ± 0.46	5.51 ± 0.21	0.000	0.000	0.007
20:5n-3	4.73 ± 0.57	5.78 ± 1.33	5.81 ± 1.18	3.72 ± 0.67	6.63 ± 0.44	6.71 ± 0.80	0.000	ns	0.027
22:6n-3	15.02 ± 1.53	14.39 ± 2.68	15.77 ± 2.26	17.47 ± 3.46	18.26 ± 2.68	23.69 ± 2.75	0.014	0.000	ns
N-3 PUFA	25.79 ± 1.81	25.79 ± 3.93	26.52 ± 3.30	27.42 ± 3.76	30.87 ± 2.60	35.37 ± 3.06	0.022	0.000	ns
PUFA	36.23 ± 1.78	34.63 ± 4.04	33.74 ± 3.44	37.72 ± 3.15	39.82 ± 2.07	41.47 ± 2.88	ns	0.000	ns
EPA/ARA	3.65 ± 0.44	4.91 ± 0.67	4.75 ± 0.46	5.69 ± 0.78	8.12 ± 0.91	7.69 ± 0.40	0.000	0.000	ns
N-3/N-6	2.62 ± 0.26	3.25 ± 0.41	4.03 ± 0.38	2.82 ± 0.52	3.97 ± 0.56	6.44 ± 0.76	0.000	0.000	0.001

Results are means ± SD (n = 5). P-values of two-way ANOVA are presented for factors 'diet', 'time' and interaction between both factors.
ARA, arachidonic acid; EPA, eicosapentaenoic acid; ns, not significant ($p > 0.05$); PUFA, polyunsaturated fatty acids.

2.3.3 Microarray hierarchical cluster analysis showed that the functional feeds greatly affected expression of immune related genes over the course of the ASRV infection

We then went on to profile the transcriptomes of the heart tissue over time post challenge in an effort to fingerprint the contrasting inflammatory responses observed in the different diet groups. A hierarchical cluster analysis of heart tissue transcriptomes was performed and the results indicated clearly contrasting patterns of gene expression over the course of the infection between different dietary treatments (Figure 2.4). There was a strong up-regulation (shown in red colour) of a group of genes at 12-weeks post-challenge in fish consuming the ST diet. The same group of genes was similarly strongly up-regulated at 16-weeks post-challenge in fish consuming the FF2 diet (Figure 2.4, outmost lane to the right). In both cases there was concomitant down-regulation of another distinct group of genes. On this basis we prepared two lists containing all up- and down-regulated genes from the cluster groups (identified by Self-Organizing Maps cluster analysis) showing these patterns of expression. The list of down-regulated features contained a set of 378 genes related with various cellular, biological and metabolic pathways including cell survival processes, regulation of apoptosis, regulation of transcription and general lipid metabolic processes (Supplementary Table 2.1). In contrast, the list of up-regulated features contained a set of 732 genes primarily related to various aspects and stages of the immune response, including both innate and adaptive immune system responses (Supplementary Table 2.2). To assist the interpretation of these data, the genes of the up-regulated list were sorted by fold-change comparing 8 weeks versus 16 weeks in fish fed the FF2 diet. All the genes showing an increase in expression from 8 to 12 weeks greater than or equal to 5-fold, along with a small number of other highly relevant genes with a fold-change lower than 5, were considered for more detailed discussion of the data. The

179 genes in this list (Supplementary Table 2.2) were categorized according to the specific pathways/processes to which they are commonly associated. The effects of the dietary treatments on these pathways were analyzed to provide both an overview of the major impacts of the functional feeds and, in particular, to discriminate differences in gene expression between the two functional feeds underpinning their differential effects on HSMI. The major findings are outlined in the ensuing paragraphs.

2.3.4 Virus load correlated with up-regulation of pro-inflammatory genes at the early-mid stages of the infection in fish fed the ST diet

The heat map in Figure 2.4 clearly indicated that timing of the immune response was affected by the functional feeds and that the ST diet at 12 weeks stands out compared to the other groups/time points. As mentioned above, viral load was highest in heart tissue of the ST diet (256x higher than FF1) at 12-weeks post-challenge (Figure 2.2), which was reflected in the strong up-regulation of genes associated with viral entry and infection in ST-fed fish at that time, (shown as down-regulated in functional diets relative to ST diet in Table 2.5, Figure 2.5A). As would be expected, the expression of genes associated with the different pathways of the innate immune response (see below) was considerably up-regulated at 12-weeks post-challenge in fish fed the ST diet (Table 2.6 and 2.7; Figure 2.5B and C), particularly those involved in the interferon-inducible response (Table 2.7; Figure 2.5C), the natural killer cells response and apoptosis (Table 2.8). Responses that fingerprint the cellular adaptive immune response, were in general of lower magnitude, but also markedly up regulated in the ST compared to the FF1 diet group, while lesser so compared to the FF2 diet group (Table 2.9). Fish in the ST diet group mount a strong, early response biased towards pro-inflammatory and early adaptive responses.

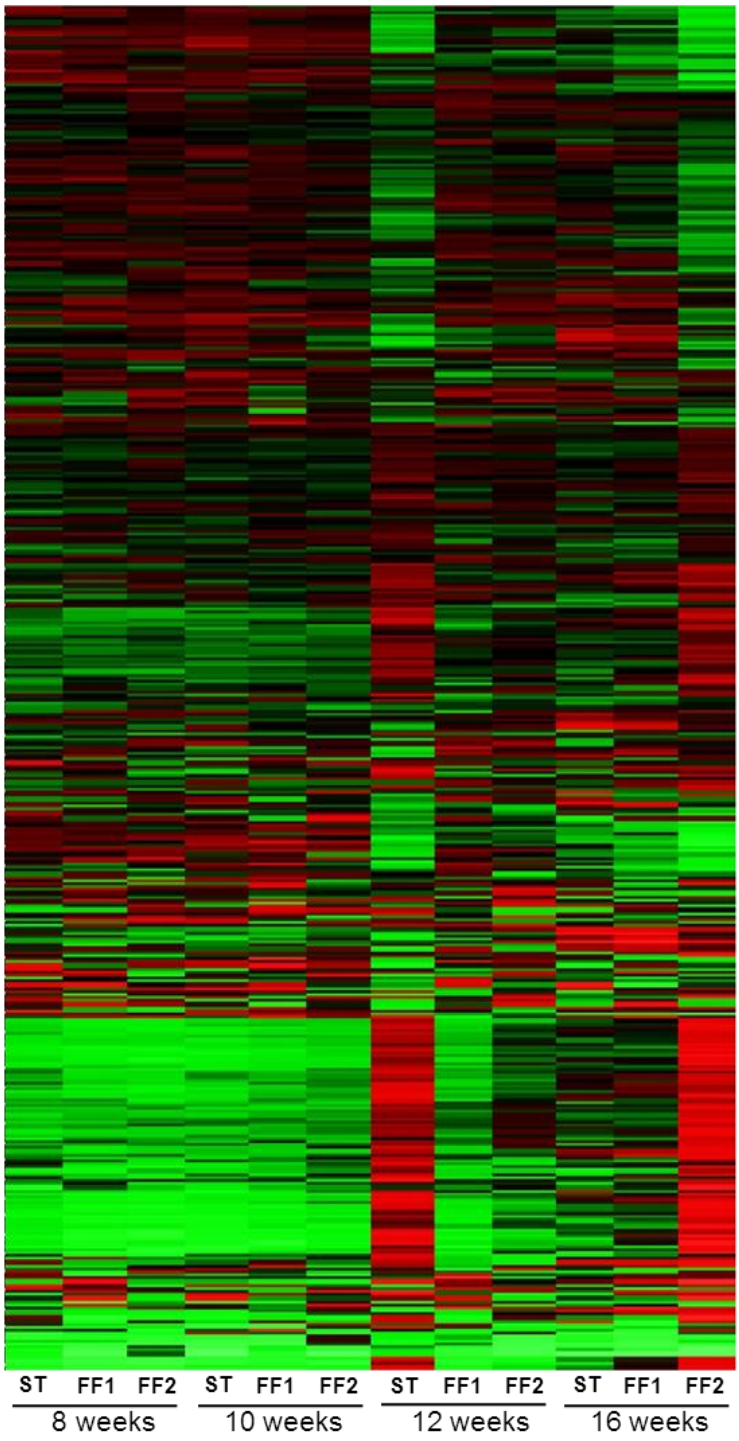

Figure 2.4. Hierarchical clustering of expression profiles for 2581 genes from the significant interaction list of the 2-way ANOVA analysis across the different dietary treatments over the time course of the infection. Individual gene expression profiles are plotted horizontally against vertical columns of each dietary group over the different time-points. Up- and down-regulation of gene expression with respect to a common reference (a pool of all the samples) are represented in red and green, respectively. Colour intensity depends on the value of the expression ratio.

Table 2.5 Viral infection-related genes differentially expressed in heart of fish fed the functional feeds (FF1 and FF2) relative to the standard diet during the course of the infection with ASRV

	Accession or	8 weeks		10 weeks		12 weeks		16 weeks	
BlastxHit	**probe number**	**FF1**	**FF2**	**FF1**	**FF2**	**FF1**	**FF2**	**FF1**	**FF2**
Viral entry and Cellular transport									
CD209 antigen-like protein d	Ssa#STIR16806	- 1,4	- 1,6	- 1,2	+ 1,0	- 3,3	- 2,7	+ 1,3	+ 3,3
C-type lectin domain family 4 member e	Ssa#STIR09786	- 1,4	- 1,2	- 1,4	+ 1,2	- 4,1	- 3,2	+ 1,5	+ 4,0
Kelch-like protein 6	DW579982	+ 1,2	+ 1,3	+ 1,3	+ 1,2	- 2,6	- 1,6	- 1,1	+ 3,1
CD209 antigen-like protein A	Ssa#KSSb2272	- 1,4	- 1,5	+ 1,0	+ 1,3	- 3,3	- 2,7	+ 1,1	+ 2,7
Ecotropic viral integration site 2a	Ssa#STIR14026	- 1,0	- 1,1	+ 1,2	+ 1,1	- 3,3	- 2,1	- 1,0	+ 2,4
Macrosialin precursor	Ssa#KSS4521	- 1,3	- 1,5	+ 1,2	+ 1,2	- 4,8	- 3,0	+ 1,1	+ 1,1
General viral induced proteins									
Proteasome subunit beta type-6 precursor	Ssa#STIR04422	- 1,4	- 1,4	+ 1,0	+ 1,1	- 3,3	- 2,4	+ 1,1	+ 2,3
Proteasome subunit beta type-6 precursor	Ssa#STIR02309	- 1,3	- 1,4	+ 1,0	+ 1,0	- 3,2	- 2,4	+ 1,2	+ 2,5
14 kda transmembrane protein	Ssa#STIR00068_4	- 1,5	- 1,4	+ 1,2	+ 1,8	- 3,1	- 1,6	+ 1,3	+ 3,1
Proteasome subunit beta type-6 precursor	Ssa#STIR09223	- 1,4	- 1,4	+ 1,0	+ 1,1	- 3,2	- 2,4	+ 1,1	+ 2,4
Anti-viral protein									
Ferritin, lower subunit putative mRNA	Ssa#STIR07882	+ 1,3	+ 1,1	+ 1,2	+ 1,5	- 5,0	- 2,9	- 1,1	+ 4,1
Retroviral DNA integration									
Barrier-to-autointegration factor	Ssa#CL95Contig1	- 1,4	- 2,6	+ 1,6	+ 3,1	- 12,4	- 11,0	+ 1,9	+ 3,5
Prob. ATP-dependent RNA helicase DHX58	DW582483	- 1,2	- 2,9	+ 1,9	+ 1,6	- 4,3	- 5,3	+ 4,1	+ 5,5
Prob. ATP-dependent RNA helicase DHX58	DW567942	- 1,5	- 2,2	+ 1,1	+ 1,6	- 6,3	- 6,1	+ 1,7	+ 2,5

Transcripts, with probe names or accession number (when possible), are arranged by functional categories and the data are presented as the expression ratio between the functional diet group and the standard group (ST/FF1 and ST/FF2), for each time point.

Table 2.6 Innate immune system-related genes differentially expressed in heart of fish fed the functional feeds (FF1 and FF2) relative to the standard diet during the course of the infection with ASRV

BlastxHit	Accession or probe number	8 weeks		10 weeks		12 weeks		16 weeks	
		FF1	FF2	FF1	FF2	FF1	FF2	FF1	FF2
Innate Immune system									
Platelet basic protein precursor	EG823365	- 2,5	- 5,0	+ 1,1	- 1,3	- 17,3	- 7,0	- 1,2	+ 3,9
Platelet basic protein	EG761718	- 1,9	- 3,0	- 1,5	- 1,2	- 14,7	- 7,0	+ 1,2	+ 4,0
c-c motif chemokine 19 precursor	Ssa#STIR10724	- 1,7	- 1,5	- 1,1	+ 1,0	- 5,2	- 2,7	- 1,3	+ 6,2
Ubiquitin-like protein	BG936428	- 1,2	- 2,0	+ 1,7	+ 1,8	- 11,1	- 5,3	+ 1,3	+ 4,1
Skin mucus antibact. l-amino acid oxidase	Omy#CA377250	- 4,1	- 1,8	+ 1,1	+ 1,3	- 27,8	- 23,4	+ 2,1	+ 3,1
C-C motif chemokine 19 precursor	DW571080	- 3,3	- 4,0	+ 1,1	+ 1,2	- 12,3	- 7,2	+ 1,2	+ 3,5
Skin mucus antibact. l-amino acid oxidase	DY698830	- 2,1	- 2,0	+ 1,2	+ 1,4	- 15,1	- 9,1	+ 1,9	+ 2,7
Chemokine (c-c motif) ligand 19	Ssa#STIR04367	- 2,5	- 2,6	+ 1,3	+ 1,4	- 5,9	- 3,5	+ 1,5	+ 3,7
Chemokine (c-c motif) ligand 19	Ssa#STIR21382	- 2,1	- 1,9	+ 1,2	+ 1,3	- 6,2	- 3,4	+ 1,5	+ 3,6
Interferon regulatory factor 7	BX307182	- 1,5	- 1,7	+ 1,1	+ 1,2	- 5,0	- 3,7	+ 1,7	+ 3,3
Neutrophil cytosolic factor 2	Ssa#KSS461	- 1,1	- 2,8	+ 1,4	+ 2,0	- 2,6	- 2,0	- 1,2	+ 1,9
Grn protein	Ssa#STIR07488	- 1,3	- 1,4	+ 1,2	+ 1,4	- 3,7	- 2,4	+ 1,2	+ 2,6
Engulfment adaptor ptb domain contain. 1	Ssa#STIR11362	+ 1,7	- 1,9	- 4,9	- 5,2	- 2,5	+ 1,3	+ 1,3	+ 4,4
Granulocyte colony-stimulating factor R prec	Ssa#STIR20316	+ 1,2	+ 1,3	- 1,0	- 1,6	- 1,4	- 1,2	- 1,2	+ 4,7
Interferon regulatory factor 7	DY725939	- 1,5	- 2,0	+ 1,0	+ 1,1	- 5,2	- 4,0	+ 1,5	+ 2,2
Interferon regulatory factor 7	Ssa#STIR00062_4	- 1,8	- 2,3	+ 1,2	+ 1,4	- 5,0	- 4,0	+ 1,4	+ 2,2
Interferon regulatory factor 7	Ssa#TC107831	- 1,6	- 1,6	+ 1,1	+ 1,3	- 5,9	- 4,1	+ 1,4	+ 2,3
Interferon regulatory factor 7	Ssa#STIR00062_3	- 1,6	- 1,9	+ 1,1	+ 1,3	- 5,1	- 3,8	+ 1,4	+ 2,1
Bloodthirsty	Ssa#DY699301	- 2,0	+ 1,1	- 1,2	+ 1,0	- 13,4	- 5,7	+ 1,5	+ 2,4
Interferon regulatory factor 7	Ssa#STIR00062_2	- 1,4	- 1,7	+ 1,1	+ 1,4	- 4,6	- 3,8	+ 1,4	+ 2,2
Immune-related, lectin-like receptor 4	CB511660	+ 1,1	+ 1,0	+ 1,3	+ 1,2	- 2,8	- 1,7	- 1,1	+ 3,0
Cysteine dioxygenase	Ssa#STIR21327	+ 1,1	- 1,0	- 1,2	+ 1,0	- 3,1	- 2,3	- 1,3	+ 2,7
Sub-fam. B ATP-binding cassette transport 2	DW563628	- 1,2	- 1,2	- 1,1	- 1,2	- 4,2	- 2,9	+ 1,5	+ 1,9
Tripartite motif-containing protein 35	EG878686	- 1,4	- 1,3	- 1,1	+ 1,2	- 3,6	- 2,8	+ 1,4	+ 2,0
Rho-related GTP-binding protein RhoG	Ssa#KSS4662	- 1,2	- 1,4	+ 1,1	+ 1,2	- 2,9	- 1,9	- 1,1	+ 1,9
RAB26, member RAS oncogene family	Omy#BX082318	- 1,9	- 2,3	+ 1,3	+ 1,5	- 2,7	- 1,7	- 1,0	+ 2,0
fMet-Leu-Phe receptor	Ssa#STIR17576	- 1,2	- 1,2	- 1,1	+ 1,1	- 2,6	- 1,7	+ 1,1	+ 2,8
Bactericidal permeability-increasing protein	DW540097	- 1,2	- 1,6	+ 1,2	+ 1,6	- 3,2	- 2,2	- 1,0	+ 2,0
TNFα-induced protein 8-like protein 2	Ssa#STIR00030_4	+ 1,1	- 1,1	+ 1,1	+ 1,2	- 2,3	- 1,5	- 1,1	+ 2,0

Transcripts, with probe names or accession number (when possible), are arranged by functional categories and the data are presented as the expression ratio between the functional diet group and the standard group (ST/FF1 and ST/FF2), for each time point.

Table 2.7 IFN I related-genes differentially expressed in heart of fish fed the functional feeds (FF1 and FF2) relative to the standard diet during the course of the infection with ASRV

BlastxHit	Accession or probe number	8 weeks		10 weeks		12 weeks		16 weeks	
		FF1	FF2	FF1	FF2	FF1	FF2	FF1	FF2
JAK-STAT signal transduction pathway									
Suppressor of cytokine signaling 1	Ssa#STIR25114	- 2,1	- 2,1	- 1,1	+ 1,4	- 7,0	- 5,1	+ 1,6	+ 2,5
IFN-inducible protein Gig2-like	Ssa#EG876062	- 1,3	- 1,6	- 1,1	+ 1,4	- 5,4	- 3,3	+ 1,9	+ 2,3
Suppressor of cytokine signaling 1	DW563373	- 1,6	- 1,4	- 1,1	+ 1,2	- 6,8	- 3,8	+ 1,5	+ 2,6
N-myc (and stat) interactor	Ssa#STIR09454	- 1,3	- 1,5	+ 1,1	+ 1,1	- 3,1	- 2,3	+ 1,2	+ 2,1
IFN-inducible protein Gig2-like	Ssa#EG871163	- 1,3	- 1,4	- 1,1	+ 1,1	- 3,2	- 2,5	+ 1,4	+ 2,1
JAK3 tyrosine kinase	Ssa#DY728848	+ 1,3	+ 1,1	+ 1,1	- 1,2	- 2,7	- 1,7	- 1,1	+ 2,0
STAT 1	Ssa#STIR06397	- 1,3	- 1,4	+ 1,0	+ 1,0	- 3,3	- 2,5	+ 1,4	+ 1,6
STAT 1α	EU016199	- 1,2	- 1,3	+ 1,1	+ 1,1	- 3,0	- 2,4	+ 1,5	+ 2,0
JAK1	Ssa#DW006200	- 1,2	- 1,3	+ 1,5	+ 1,3	- 3,0	- 2,4	+ 1,2	+ 2,0
Interferon inducible									
IFN inducible mx protein	U66475	- 1,8	- 2,3	+ 1,0	+ 1,2	- 13,9	- 8,9	+ 2,1	+ 3,7
IFN inducible mx protein	Ssa#STIR00154_2	- 1,8	- 2,2	+ 1,0	+ 1,2	- 14,0	- 8,3	+ 2,1	+ 3,7
Vig-2 protein	Ssa#STIR08402	- 1,4	- 2,5	+ 1,1	+ 1,7	- 15,2	- 9,2	+ 1,7	+ 2,6
Mx2 protein mRNA complete cds	Con_CANDS_07	- 1,7	- 1,9	+ 1,0	+ 1,1	- 11,8	- 7,6	+ 2,4	+ 4,0
14 kda transmembrane protein	Ssa#STIR17386	- 1,7	- 3,1	+ 4,9	+ 4,3	- 1,2	+ 1,5	- 1,2	+ 4,1
Myxovirus resistance 2	U66476	- 1,5	- 1,7	+ 1,1	+ 1,2	- 11,3	- 7,4	+ 2,3	+ 3,7
IFN inducible mx protein	Ssa#STIR00154_3	- 1,6	- 1,8	+ 1,0	+ 1,2	- 8,3	- 5,9	+ 1,7	+ 2,9
Mx1 protein mRNA complete cds	Con_CANDS_06	- 1,5	- 1,8	+ 1,0	+ 1,1	- 8,6	- 5,9	+ 1,7	+ 2,8
IFN inducible mx protein	Ssa#STIR00154_4	- 1,8	- 1,9	+ 1,0	+ 1,2	- 8,3	- 6,0	+ 1,8	+ 2,7
IFN-induced GTP-binding protein Mx	Ssa#STIR21272	- 1,9	- 2,5	+ 1,1	+ 1,3	- 8,6	- 6,9	+ 1,6	+ 2,1
vig-2 protein	Ssa#STIR10385	- 1,3	- 1,7	+ 1,1	- 1,0	- 7,1	- 4,5	+ 1,4	+ 2,4
Mx3 protein	U66477	- 1,5	- 1,7	+ 1,0	+ 1,1	- 7,6	- 5,0	+ 1,8	+ 2,5
IFN inducible mx protein	Ssa#STIR00067_3	- 1,5	- 1,6	+ 1,0	+ 1,1	- 7,6	- 5,0	+ 1,8	+ 2,3
Cholesterol 25-hydroxylase-like protein A	DW536322	- 2,1	+ 1,0	+ 2,1	+ 2,4	- 6,5	- 1,7	+ 1,3	+ 2,4
IFN-induced protein with tetratricopeptide	Ssa#STIR00073_2	- 2,3	- 1,8	- 1,0	+ 1,1	- 4,5	- 3,9	+ 1,4	+ 1,6
IFN-induced guanylate-binding protein 1	Ssa#STIR18623	- 1,0	- 1,1	+ 1,2	+ 1,2	- 4,6	- 3,0	+ 1,0	+ 2,9
IFN-induced guanylate-binding protein 1	DY713986	- 1,0	- 1,4	+ 1,3	+ 1,3	- 4,2	- 3,6	+ 1,3	+ 2,4
14 kda transmembrane protein	Ssa#STIR22465	- 1,4	- 1,4	+ 1,2	+ 1,6	- 2,9	- 1,6	+ 1,2	+ 2,7
IFNγ induc. lysosomal thiol reductase precur	Ssa#STIR04691	- 1,1	- 1,3	- 1,1	+ 1,0	- 2,1	- 1,5	- 1,3	+ 2,1
IFN-induced transmembrane protein 3	CA387902	- 1,4	- 1,5	+ 1,2	+ 1,5	- 2,4	- 1,3	+ 1,3	+ 2,7
Guanylate binding protein 3	Ssa#STIR19322	- 1,2	- 1,2	+ 1,3	+ 1,7	- 3,8	- 3,3	+ 1,3	+ 2,3

Transcripts, with probe names or accession number (when possible), are arranged by functional categories and the data are presented as the expression ratio between the functional diet group and the standard group (ST/FF1 and ST/FF2), for each time point.

Table 2.8 Antiviral host responses related-genes differentially expressed in heart of fish fed the functional feeds (FF1 and FF2) relative to the standard diet during the course of the infection with ASRV

	Accession or	8 weeks		10 weeks		12 weeks		16 weeks	
BlastxHit	**probe number**	**FF1**	**FF2**	**FF1**	**FF2**	**FF1**	**FF2**	**FF1**	**FF2**
Acute phase									
Serum amyloid A-5 protein	Ssa#KSS3451	- 1,7	- 2,2	+ 1,3	- 1,3	- 5,3	- 3,0	+ 2,1	+ 4,3
Serum amyloid a-5 protein	Ssa#CK882427	- 1,7	- 2,3	+ 1,4	- 1,3	- 5,1	- 2,9	+ 2,2	+ 4,0
CCAAT/enhancer-binding protein beta	Ssa#CL285Ctg1	- 1,2	- 1,2	+ 1,5	+ 2,0	- 2,1	+ 1,0	+ 1,4	+ 2,4
LBP (LPS binding protein)/BPI like-2	Ssa#KSS5230	- 1,2	- 1,1	+ 1,1	+ 1,3	- 3,3	- 2,1	+ 1,0	+ 2,8
Cathelicidin	EG840650	+ 1,2	+ 1,3	- 1,4	+ 1,1	- 3,7	- 2,0	+ 1,9	+ 5,4
Natural killer cells									
Granzyme A precursor	EG853597	+ 1,1	+ 1,7	+ 1,2	+ 1,6	- 26,6	- 9,1	- 3,2	+ 5,5
Interleukin 18 form a, IL-18A	AJ556990	- 1,0	+ 1,1	+ 1,2	+ 1,1	- 1,2	+ 1,1	- 1,3	+ 2,1
Immune-lectin-like receptor 3	Ssa#STIR08687	+ 1,0	- 1,0	+ 1,2	+ 1,0	- 1,8	- 1,2	- 1,0	+ 1,9
Interleukin 12b	Ssa#STIR00077_3	- 1,2	- 1,6	+ 1,3	+ 1,2	- 1,4	- 1,2	+ 1,3	+ 1,6
Perforin-1 precursor	CB515603	- 1,2	- 1,1	+ 1,1	- 1,2	- 1,4	- 1,3	+ 1,3	+ 1,9
Apoptosis									
Mgc81823 transcript variant 2	Omy#TC159807	- 1,0	- 1,2	+ 1,1	+ 1,1	- 7,8	- 4,1	- 1,2	+ 4,1
Cathepsin l precursor	Omy#CX262263	- 1,3	- 1,2	+ 1,1	+ 1,0	- 8,2	- 4,1	- 1,1	+ 4,0
Cathepsin l	Ssa#STIR23260	+ 1,0	- 1,0	+ 1,2	+ 1,1	- 7,9	- 4,5	- 1,2	+ 3,8
E3 ubiquitin-protein ligase LINCR	DY697357	+ 2,2	- 1,0	- 2,0	+ 1,2	- 10,9	- 2,1	- 1,4	+ 4,5
Caspase-14 precursor	EG868944	- 1,3	- 1,5	+ 1,0	+ 1,4	- 6,2	- 4,5	- 1,2	+ 2,4
Caspase 14	Ssa#STIR02208	- 1,4	+ 2,1	+ 1,4	- 3,6	- 2,0	+ 1,2	- 4,5	+ 1,8
E3 ubiquitin-protein ligase RNF144A-A	EG829820	- 1,7	- 1,5	+ 1,1	+ 1,4	- 6,0	- 4,2	+ 1,0	+ 2,0
E3 ubiquitin-protein ligase RNF144A-A	EG841469	- 1,3	- 1,7	+ 1,2	+ 1,2	- 5,9	- 3,3	+ 1,0	+ 2,5
AN1, ubiquitin-like, homolog	Omy#CU065924	- 1,2	- 3,4	+ 1,1	- 1,5	- 1,9	+ 1,5	- 1,4	- 1,3
Ubiquitin carboxyl-terminal hydrolase iso L5	Ssa#S35587724_S	- 1,9	- 2,1	+ 1,0	+ 1,4	- 1,5	- 1,1	- 1,8	+ 2,7
Ubiquitin specific protease 18	EG868088	- 1,3	- 2,1	- 1,1	+ 1,1	- 4,2	- 3,5	+ 1,5	+ 1,9
Tripartite motif-containing protein 39	DW545632	- 1,5	- 1,8	+ 1,3	+ 1,4	- 4,1	- 4,0	+ 1,1	+ 2,1
Tyrosine-protein kinase Lyn	Omy#CX255862	- 1,1	- 1,5	+ 1,2	+ 1,2	- 2,4	- 1,8	- 1,1	+ 2,0

Transcripts, with probe names or accession number (when possible), are arranged by functional categories and the data are presented as the expression ratio between the functional diet group and the standard group (ST/FF1 and ST/FF2), for each time point.

Table 2.9 IFN II-related genes differentially expressed in heart of fish fed the functional feeds (FF1 and FF2) relative to the standard diet during the course of the infection

BlastxHit	Accession or probe number	8 weeks		10 weeks		12 weeks		16 weeks	
		FF1	FF2	FF1	FF2	FF1	FF2	FF1	FF2
Induction proliferation/maduration of T-cells									
T-cell receptor α chain V region 2B4 precur.	EG773958	- 1,3	- 5,2	- 3,9	- 2,0	- 2,7	- 1,4	- 2,4	+ 3,1
T-cell receptor β chain	AJ517930	- 1,1	- 2,0	+ 2,1	+ 1,5	- 4,8	- 1,8	- 1,2	+ 3,9
Differentially expressed in FDCP 6 homolog	Ssa#S48397573	- 1,7	- 1,8	- 1,1	+ 1,1	- 6,2	- 3,2	+ 1,9	+ 2,2
Immediate early response gene 2 protein	DW562838	- 1,5	- 1,6	+ 3,6	+ 5,8	+ 1,1	+ 2,3	+ 1,2	- 1,0
CD9 antigen	CB515563	- 1,4	- 1,2	- 1,1	+ 1,2	- 4,2	- 3,1	+ 1,4	+ 2,5
T-cell receptor α chain V region 2B4 precur.	EG890703	- 2,2	- 1,6	- 1,7	- 1,4	- 1,7	- 1,4	+ 1,5	+ 3,2
Core-binding factor beta subunit	Ssa#TC107774	- 1,5	- 2,2	- 1,2	+ 1,1	- 2,9	- 1,6	+ 1,0	+ 3,1
CD97 antigen	EG935955	- 1,3	- 1,3	+ 1,3	+ 1,2	- 3,1	- 2,3	- 1,0	+ 2,4
Rho-related GTP-binding protein RhoG	Ssa#KSS4662	- 1,2	- 1,4	+ 1,1	+ 1,2	- 2,9	- 1,9	- 1,1	+ 1,9
Ras homolog genemember g	Ssa#STIR25495	- 1,3	- 1,5	+ 1,1	+ 1,2	- 2,4	- 1,7	- 1,0	+ 1,9
Ras homolog genemember g	Ssa#STIR05697	+ 1,1	- 1,2	- 1,6	+ 1,2	- 2,9	- 1,5	+ 1,3	+ 2,2
Hemopoietic cell kinase partial cds	AF321110	- 1,0	- 1,1	+ 1,2	+ 1,2	- 2,6	- 1,7	+ 1,1	+ 2,2
Tyrosine-protein kinase SRK2	Ssa#STIR23204	- 1,8	- 1,8	+ 1,2	+ 1,4	- 3,9	- 4,0	+ 1,3	+ 2,0
Galectin-9 putative mRNA	Ssa#STIR13309	- 1,2	- 1,3	- 1,1	+ 1,1	- 4,3	- 2,5	+ 1,1	+ 1,9
Galectin like protein	Ssa#STIR12085	- 1,2	- 1,3	- 1,1	+ 1,1	- 4,3	- 2,6	+ 1,1	+ 1,8
CD80-like protein	EG933501	+ 1,2	+ 1,1	+ 1,1	+ 1,2	- 1,7	- 1,3	+ 1,0	+ 2,3
C-C motif chemokine 28 precursor	Ssa#STIR03768	- 1,5	- 2,0	- 1,7	+ 9,3	+ 2,1	- 3,7	+ 1,5	+ 2,7
Cellular immunity									
Interferon gamma	AJ841811	- 1,8	- 2,1	+ 1,0	- 1,2	- 10,1	- 4,4	- 1,1	+ 6,4
Interferon gamma	Ssa#STIR00056_4	- 1,7	- 1,6	- 1,4	- 1,2	- 11,1	- 4,7	- 1,0	+ 4,5
Interferon gamma 2	Omy#gi238231582	- 1,3	- 2,4	+ 1,2	+ 1,4	- 5,8	- 4,1	- 1,0	+ 4,6
Interferon gamma	Ssa#STIR00056_3	+ 1,0	- 1,1	+ 1,0	- 2,0	- 9,4	- 5,7	- 1,3	+ 3,0
Tripartite motif-containing protein 47	Ssa#DY714453	- 1,1	- 1,1	- 1,4	- 1,2	- 5,4	- 1,9	+ 2,2	+ 5,6
TRIM 16 protein	EG875618	- 1,6	- 1,7	- 1,0	+ 1,3	- 3,7	- 3,3	+ 1,6	+ 2,4
Beta-2 microglobulin	AF180490	- 1,3	- 1,3	+ 1,0	+ 1,0	- 2,6	- 2,1	+ 1,1	+ 1,8
Tapasin-related protein	DW567893	- 1,2	- 1,1	- 1,1	- 1,1	- 2,4	- 1,9	+ 1,1	+ 1,4
CD83 antigen precursor	Ssa#KSS3110	- 1,1	- 1,0	+ 1,6	+ 1,6	- 1,9	- 1,6	- 1,2	+ 1,4
CD80-like protein	EG933501	+ 1,2	+ 1,1	+ 1,1	+ 1,2	- 1,7	- 1,3	+ 1,0	+ 2,3
Tapasin	DQ451008	- 1,3	- 1,5	+ 1,1	+ 1,1	- 2,6	- 2,1	- 1,0	+ 1,4
TAP2 protein	DW580644	- 1,4	- 1,6	+ 2,2	+ 2,7	- 1,7	- 2,3	- 1,4	- 1,1

Transcripts, with probe names or accession number (when possible), are arranged by functional categories and the data are presented as the expression ratio between the functional diet group and the standard group (ST/FF1 and ST/FF2), for each time point.

Table 2.10 Adaptive immune system related-genes differentially expressed in heart of fish fed the functional feeds (FF1 and FF2) relative to the standard diet during the course of the infection with ASRV

BlastxHit	Accession or probe number	8 weeks		10 weeks		12 weeks		16 weeks	
		FF1	FF2	FF1	FF2	FF1	FF2	FF1	FF2
Innate to adaptive									
MHC class II antigen beta chain	Ssa#STIR00007_3	- 1,2	- 4,5	- 16,5	- 4,2	- 3,1	+ 1,2	+ 41,0	+ 15,0
MHC class II antigen beta chain	Ssa#STIR00007_4	+ 1,3	- 2,4	- 36,5	- 7,4	- 2,1	- 1,2	+ 70,6	+ 27,4
IFNγ-inducible lysosomal thiolreductase prec.	Ssa#STIR09719	- 1,1	- 1,3	- 1,0	+ 1,0	- 2,1	- 1,5	- 1,3	+ 2,1
IFNγ-inducible lysosomal thiolreductase prec.	Ssa#STIR04691	- 1,1	- 1,3	- 1,1	+ 1,0	- 2,1	- 1,5	- 1,3	+ 2,1
Chemokine (c-x-c motif) ligand 13	Ssa#STIR03818	- 2,7	- 1,6	- 1,1	+ 1,0	- 4,8	- 2,3	+ 3,8	+ 13,1
CC chemokine	Ssa#STIR23541	- 1,5	- 1,4	+ 1,5	- 1,4	- 5,0	- 3,4	+ 1,6	+ 5,2
CD226 antigen	DW553192	- 2,2	- 2,2	+ 1,6	+ 1,2	- 3,5	- 1,4	+ 1,9	+ 6,5
TNF superfamily member 14	Ssa#S31975856_S	- 5,9	- 3,2	+ 1,4	- 1,0	- 5,2	- 1,5	- 1,2	+ 4,4
Chemokine cxcl-c1c	Ssa#STIR11364	- 1,6	- 1,6	+ 1,1	+ 1,2	- 5,1	- 3,0	+ 1,7	+ 3,5
High affinity IgG fc receptor i precursor	Ssa#STIR11789	- 2,6	- 1,1	- 1,9	+ 1,4	- 2,4	- 3,0	+ 1,3	+ 3,2
High affinity IgE receptor subunit γ precur.	Ssa#STIR03935	- 1,1	- 1,2	+ 1,0	+ 1,2	- 2,8	- 2,0	- 1,0	+ 2,4
High affinity IgG fc receptor i precursor	Ssa#STIR24788	- 1,3	- 1,5	+ 1,1	+ 1,2	- 2,8	- 1,8	+ 1,0	+ 2,2
High affinity IgE receptor subunit γ precur.	Ssa#STIR08428	- 1,1	- 1,3	+ 1,0	+ 1,2	- 2,8	- 2,0	- 1,0	+ 2,4
High affinity IgE receptor subunit γ precur.	Ssa#STIR09946	- 1,1	- 1,3	+ 1,0	+ 1,2	- 2,7	- 2,0	+ 1,0	+ 2,4
High affinity IgE receptor subunit γ precur.	Ssa#STIR05473	- 1,1	- 1,2	+ 1,1	+ 1,2	- 2,7	- 1,9	- 1,0	+ 2,3
Chemokine (c-x-c motif) receptor 3	Ssa#STIR10673	- 1,1	- 1,2	+ 1,5	+ 1,2	- 2,7	- 2,0	+ 1,1	+ 2,3
Chemokine (c-x-c motif) ligand 10	Ssa#STIR02663	- 1,1	- 1,1	+ 1,1	+ 1,1	- 2,6	- 2,3	+ 1,2	+ 2,2
C-X-C chemokine receptor type 3A, variant 1	AJ888878	+ 1,1	+ 1,0	+ 1,1	+ 1,2	- 2,3	- 1,7	- 1,0	+ 2,4
Humoral immunity									
Interleukin 20alpha	Ssa#STIR13743	+ 1,0	- 1,7	+ 1,3	+ 2,2	- 10,6	- 10,6	- 2,2	+ 1,9
PREDICTED: similar to CD22 molecule	CU073328	+ 2,2	- 1,2	+ 1,3	- 1,8	- 5,2	- 1,4	- 4,0	+ 1,3
Interleukin 20alpha	Ssa#STIR18005	+ 1,0	- 1,2	+ 1,2	+ 1,1	- 3,0	- 2,0	- 1,0	+ 2,2
Interleukin 10beta	Ssa#STIR00087_3	- 1,7	- 1,7	+ 1,1	+ 1,3	- 4,1	- 3,2	+ 1,5	+ 1,9
Leukocyte surface antigen CD53	EG836748	- 1,0	- 1,1	+ 1,2	+ 1,3	- 2,3	- 1,6	- 1,1	+ 2,2
TNFR superfamily member 5	Ssa#CL366Ctg1	- 1,1	- 1,1	+ 1,2	+ 1,4	- 2,1	- 1,6	+ 1,2	+ 2,2
B-cell linker protein	Ssa#KSS4516	+ 1,2	+ 1,3	- 1,0	+ 1,2	- 1,9	- 1,2	+ 1,4	+ 2,5

Transcripts, with probe names or accession number (when possible), are arranged by functional categories and the data are presented as the expression ratio between the functional diet group and the standard group (ST/FF1 and ST/FF2), for each time point.

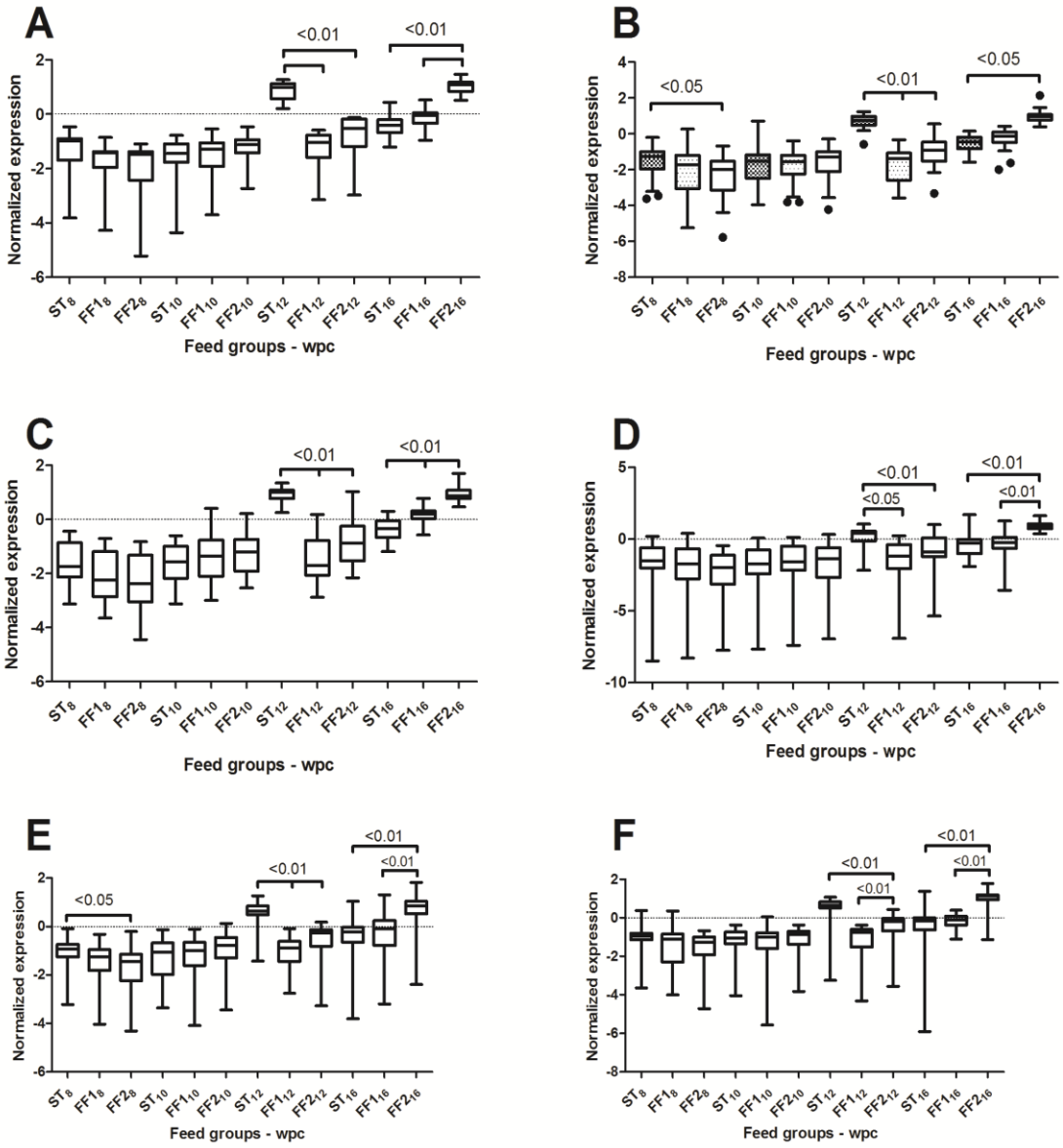

Figure 2.5A-F Normalized gene expression levels in heart for different gene groups. The genes listed in Tables 2.5-2.10 are included in Figures A-F, showing the average gene expression levels (as whiskers), with maximum and minimum range for each gene group. Outliers are depicted as black dots. A) Viral infection-related genes; B) Innate immune system-related genes; C) IFN I related-genes; D) Antiviral host responses related-genes; E) IFN II-related genes; and F) Adaptive immune system related-genes. P values are depicted (One-way ANOVA) and n is the same as in corresponding Tables 2.5-2.10.

2.3.5 Contrasting inflammatory gene expression in fish fed the two functional feeds at 16-weeks after ASRV challenge

Sixteen weeks after challenge, the replication of the viral load had increased in fish consuming both functional feeds (Figure 2.2). At this time they also had a similar pattern of expression of immune response genes2. (Tables 2.6-2.10), although the up-regulation was greater in fish fed the FF2 diet compared with those fed the FF1 diet (Figure 2.5B-F). Genes related with both the innate (Table 2.6; Figure 2.5B) and adaptive immune responses (Tables 2.9 and 10; Figures 2.5E and F) were particularly highly expressed in fish fed the FF2 diet compared with fish fed the FF1 diet. The expression of the MHC class II gene was, however, higher in fish fed the FF1 diet than in fish fed the FF2 diet and, in both groups, the expression of this gene was much higher than in the fish fed the ST diet (Table 2.10). Leukotriene B_4 receptor and arachidonate 5-lipoxygenase (ALOX) are directly related with the eicosanoid pathway and therefore the expression of their genes is likely related to levels of ARA and EPA. We found that both were up-regulated at 16-weeks in fish fed both functional feeds and interestingly the fold-changes comparing the expression of both genes between 8- and 16-weeks were twice as high in fish fed the FF2 diet compared to fish fed the FF1 diet (Table 2.11), which again concurs with the observed difference in the inflammatory profile between the two diets.

2.3.6 Severity of the lesions observed in heart tissue correlated with the intensity of the innate immune response

The microarray data showed that increased expression of genes related to the innate immune response correlated with higher histopathology scores. In general, this correlation was most obvious in fish fed the ST diet at 12 weeks post-challenge, with higher expression of genes involved in pathways of the virus-induced innate immune response.

These included platelet basic protein, IRF7 and barrier to auto integration factor (Table2.6), interferon-inducible Mx proteins, Vig-2, gig-2 and cholesterol 25-hydrolase (Table 2.7), granzyme A, serum amyloid A5, interleukins 12 and 18 and genes related with apoptosis (Table 2.8), and chemokines (Table 2.6 and 2.10) among others. Again, there was a lower intensity of this immune response in fish fed both the functional feeds, particularly FF1, which correlated with the lower histopathology scores in this group.

2.3.7 Innate and adaptive immune responses

Activation of the innate immune response in fish leads to the stimulation of different components of the adaptive immune response (Dixon and Stet, 2001; Magnadottir, 2006). Consistent with this, the data obtained in the present study show that differential upregulation of innate responses in the ST diet group at 12 weeks (Table 2.6, Figure 2.5B) coincide with high expression of adaptive immune response genes at 12 weeks in the ST group (Table 2.10; Figure 2.5F). Similarly FF1 and particularly FF2 have higher up regulation of innate and adaptive immune genes by 16 weeks (Table 2.6 and 2.10; Figure 2.5B and F).

2.3.8 The microarray analyses data were fully validated by RT-qPCR of selected genes

Validation of the microarray data was performed by comparing the expression of 12 key genes showing both up- and down-regulation with a range of fold-changes from 1.1 to 13.9, across all three feed comparisons, and at two time points (12- and 16-weeks), providing a matrix of 72 comparisons. There was extremely good agreement between the microarray results and the RT-qPCR quantification with 93 % of comparisons (67 out of 72) showing identical regulation with very similar fold differences (Table 2.12). This analysis indicates that the gene expression data obtained using the oligoarray were reliable

and robust, which enabled interpretations to be made with some confidence, at least with regards to immune response-related genes.

Table 2.11 Lipid-related inflammatory pathway genes differentially expressed in heart of fish fed the functional feeds (FF1 and FF2) relative to the standard diet during the course of the infection with ASRV

	Accession or	8 weeks		10 weeks		12 weeks		16 weeks	
BlastxHit	**probe number**	**FF1**	**FF2**	**FF1**	**FF2**	**FF1**	**FF2**	**FF1**	**FF2**
Inflamatory pathways									
Leukotriene B4 receptor 1 (LTB4-R 1)	DW558098	- 1,0	+ 1,1	+ 1,2	+ 1,1	- 2,6	- 1,5	+ 1,4	+ 3,4
Arachidonate 5-lipoxygenase	Ssa#DW575444	+ 1,2	+ 1,2	+ 1,0	+ 1,1	- 1,6	- 1,2	- 1,1	+ 1,9

Transcripts, with probe names or accession number (when possible), are arranged by functional categories and the data are presented as the expression ratio between the functional diet group and the standard group (ST/FF1 and ST/FF2), for each time point.

Table 2.12 RT-qPCR validation of microarray results

	12 weeks				16 weeks			
	FF1		FF2		FF1		FF2	
	Microarray	**qPCR**	**Microarray**	**qPCR**	**Microarray**	**qPCR**	**Microarray**	**qPCR**
IL10	- 3.89	- 3.73	- 3.31	- 2.87	+ 1.50	+ 1.32	+ 1.85	+ 1.13
Casp14	- 6.19	- 4.82	- 4.50	- 2.47	- 1.15	- 1.33	+ 2.39	+ 1.57
TCRa	- 2.70	- 2.34	- 1.39	- 1.32	- 2.37	- 1.58	+ 3.07	+ 1.26
IRF1	- 2.45	- 2.56	- 1.90	- 1.57	- 1.10	- 1.10	+ 1.82	+ 1.40
MX1	- 13.88	- 13.57	- 8.87	- 8.44	+ 2.08	+ 2.06	+ 3.66	+ 2.48
INFII	- 9.36	- 8.48	- 5.74	- 4.58	- 1.28	- 1.91	+ 3.01	+ 1.25
GIG2	- 5.41	- 5.59	- 3.34	- 4.41	+ 1.95	+ 2.06	+ 2.34	+ 1.69
SAA	- 5.07	- 3.95	- 2.90	- 2.43	+ 2.20	+ 1.74	+ 4.01	+ 2.00
FLAP	- 1.50	- 1.26	- 1.23	+ 1.34	+ 1.12	+ 1.01	+ 2.04	+ 1.42
BAF	- 12.38	- 11.99	- 11.02	- 11.78	+ 1.88	+ 1.82	+ 3.47	+ 2.03
B2M	- 2.59	- 1.83	- 2.11	- 1.68	+ 1.11	- 1.54	+ 1.79	- 1.49
IgER	- 2.23	- 2.18	- 1.68	- 1.35	- 1.04	- 1.22	+ 2.03	+ 1.22

Values represent the expression ratios for the selected genes between the functional diet group and the standard group at 12- and 16-weeks post-infection with ASRV obtained by microarray analysis or RTqPCR.

2.4 Discussion

This study demonstrated that a clinical nutrition approach through the use of functional feeds significantly reduced viral load in the heart tissue at early stages of the infection and resulted in significantly lower heart pathology over the course of the experiment. There was also a corresponding lower expression of inflammatory and immune markers in heart tissue of fish fed the functional feeds.

There are several studies evaluating the modulation of inflammatory responses to bacterial or viral infections in fish fed diets formulated with different lipid sources (Bell et al., 1993; Thompson et al., 1997; Montero et al., 2003; Alne et al., 2009). In general, diets containing high inclusion of VO, particularly n-6 PUFA-rich oils such as soybean (Montero et al., 2003) or sunflower (Bell et al., 1993), have lower n-3/n-6 PUFA and EPA/ARA ratios, which can lead to increased pro-inflammatory responses in fish. This could be a consequence of the production of pro-inflammatory eicosanoids (e.g. LTB_4, TXA_2 and PGE_2) derived from ARA released from cell membrane phospholipids of, especially, immune-related cells (Bell et al., 1992). In the present study, a high quality feed with a commercial formulation served as a standard reference diet (ST), with protein supplied by a mixture of fishmeal and plant meals, and FO and rapeseed oil as the main lipid sources. Functional feeds were designed with reduced lipid content and relatively small changes in fatty acid composition focussed on increasing supply of EPA in the form of phospholipid. The hypothesis was that these changes in dietary formulation would increase the potential bioavailability of EPA by increasing its relative proportion in tissue phospholipids, and thus mitigate the inflammatory response to ASRV infection in fish fed the functional feeds. One analytical tool was the oligoarray, and the present trial clearly demonstrated the utility of the Atlantic salmon 44k microarray for transcriptomic analysis and the evaluation of a large number of genes involved in many metabolic pathways. The

oligoarray results provided an overview of when and how the immune response developed after an infection with ASRV and, especially, how changes in diet (feed formulation) influenced the magnitude and progression of these processes.

In addition, a further hypothesis was that the mechanism whereby increased EPA availability in fish fed the functional feeds could affect immune response would involve eicosanoid pathways. Although heart is the main organ affected in fish suffering HSMI, head kidney is an important immune-related tissue in fish producing macrophages, monocytes, B cells and other immune-related cells (Balfry and Higgs, 2001). Thus, as macrophages are a major source of eicosanoids, the fatty acid profiles of both organs were determined to define the precise effects of the different feeds on key tissue compositions. Although the fatty acid profile of the heart tissue was more conserved during the course of the infection, the higher levels of EPA in the functional feeds was reflected in this organ, with the proportion of EPA and the EPA/ARA ratio being significantly higher in both groups of fish fed the FF diets. However, head kidney fatty acid profile showed more extensive differences in fatty acid composition, not only between fish fed the ST diet and fish fed the FF feeds, but also during the time course of the infection, including decreased ARA. These data confirm that salmon consuming the functional feeds did indeed display higher absolute and relative levels of EPA, which could increase its potential bioavailability for eicosanoid synthesis. The data also suggested that head kidney showed signs of a mobilization of key fatty acids, especially ARA, during the course of the infection.

The results from the histological examinations showed that fish fed the ST diet had relatively high scores 12-weeks after ASRV inoculation, as reported in previous studies describing the aetiology of HSMI (Kongtorp et al., 2004a; Kongtorp et al., 2004b). Histopathology scores from the ST-fed group showed that half of the fish sampled had major inflammation of the heart tissue at 12-weeks, and that the inflammatory changes

were even greater at the end of the experiment. In contrast, fish fed the functional feeds were relatively unaffected by the disease at 12-weeks. Furthermore, at later time points, when inflammation was observed, it was less severe than in fish fed the ST diet. These data are consistent with our overall hypothesis that increasing dietary EPA and reducing dietary energy content would be beneficial in reducing the severity of the disease through the salmon experiencing a milder inflammatory response. However, the results also showed that the fish fed the FF1 diet showed the lowest inflammatory scores and, therefore, supplementation of EPA in the form of krill oil, as in the FF2 diet, was not more effective than simply supplying it as FO. Although the EPA/ARA ratio was higher in the FF1 diet than in the FF2 diet, the similarity in lipid content and fatty acid profiles of the two functional feeds, and the tissues of salmon fed the diets, suggests that another dietary factor could underlie the differences in immune response (Lee et al., 2008). Therefore, the precise reason for the difference between the two functional feeds is not clear at present.

Transcriptomic analyses have been used recently to assess changes in the expression of immune-related genes in viral infections (Miller et al., 2007; Jørgensen et al., 2008; Schiøtz et al., 2008), but these studies have focussed on the early stages of the infection as, in some cases, the high virulence of the virus caused mortalities early in the infection. In the present study, the slow replication of ASRV and the low mortality previously recorded during HSMI outbreaks (Kongtorp et al., 2004a; Kongtorp et al., 2004b) enabled us to evaluate the immune response over a much longer term. In most microarray studies, clustering methods are commonly used where a wide range of genes require organization (Miller et al., 2007; Lin and Chien 2009). The graphical display of the gene categories, and their pattern of expression during the course of the infection, enabled clear discrimination of when, and in which dietary treatment, the major responses occurred. The cluster analysis showed that, at early stages of the infection, 8- to 10-weeks post-challenge, there were only relatively minor differences in gene expression between fish fed the different

dietary treatments, whereas there was strong up-regulation of immune-related genes 12-weeks post-challenge in fish fed the ST diet compared to fish fed the FF1 and FF2 diets. Similar up-regulation of the same gene set was observed 16-weeks post-infection in fish fed the FF2 diet whereas fish fed the FF1 diet did not show a similar up-regulation of this gene set during the time-course of the experiment. Although the modification of expression of some immune related genes by functional feeds has been reported previously (Tacchi et al., 2011), clear differential effects of feeds on gene expression patterns, as displayed in the cluster analysis in the present study, are largely unprecedented and not previously reported in fish. The markedly different modulation of gene expression confirms that changes in dietary composition, including lipid content and source, and relatively small changes in fatty acid profiles, may influence the severity of the immune response.

The transcriptome analysis revealed a clear correlation between the severity of histopathological lesions and activation of the innate immune response in heart tissue. A similar correlation was described in previous studies on viral infections in salmon, specifically ISAV (Jørgensen et al., 2008) and PMCV (Timmerhaus et al., 2011), the latter being the causal agent of cardiomyopathy syndrome (CMS). Furthermore, the transcriptome analysis uncovered important players in the immune response, aiding its characterization. Up-regulation of genes related to antiviral host responses was observed when the heart lesions were higher, and this also correlated with the higher viral load (12-weeks post-challenge for fish fed ST diet and 16-weeks post-challenge for fish fed the other diets, particularly FF2). Therefore, genes like barrier-to-autointegration factor, chemokine 19, platelet basic protein, gig-2, galectin-9 among others, and those genes related with apoptosis, were strongly up-regulated. Although up-regulation of similar suites of genes was reported in earlier studies on common salmon viral diseases (Miller et al., 2007; Krasnov et al., 2011; Timmerhaus et al., 2011), fold-changes as high as those obtained in the present study have not been reported previously. This could be related to

improvements in oligomicroarray technology but, most likely, also to the longer duration of the present experimental design.

When virus loads are high, interferon regulatory factors (IRF) are induced to activate the transcription of type I interferon (IFN) (Børre, 2006). In the present study, the expression of IRF7 was up-regulated. Although type I IFNs were not present in the up-regulated list, the activation of genes for proteins of the JAK-STAT signalling pathway, which may result in increased expression of Mx proteins, and of Vig-2 and cholesterol 25-hydroxylase, the latter being an enzyme of steroid biosynthesis activated with type I IFNs in mammalian macrophages (Park and Scott, 2010), indicate molecular mechanisms inducing immune pathways in ASRV infection. Consequences of activation of this pathway could include stimulation of natural killer cells (NK), confirmed in our list by strong up-regulation of granzyme A, and receptors found in NK cells such as CD229, and T cell receptor α. Activation of IFN I could lead to increased expression of major histocompatibility complex (MHC) class I to promote antigen presentation activation. However, although genes related to MHC I such as beta-2 microglobulin and TAP proteins were up-regulated (see Table 2.7), it appears that activation of this pathway may not be as important in ASRV infection as it is in other viral infections such as ISAV (Jørgensen et al., 2007). The previous ISAV study was performed at early stages of the infection in comparison to the much longer time scale of the present study, and so this may indicate that the importance or role of different pathways varies during the progression of the infection, as would be expected. The activation of NK cells, the prominent up-regulation of genes associated with cytotoxic T cells (T-cell receptor α and β chains, CD97 and CD9), and up-regulation of interleukins 12 and 18 will likely result in increased IFN II (IFN-γ), which was also shown to be up-regulated. This up-regulation is in agreement with the induction of the IFN II responses reported previously in viral infections of salmon, specifically ISAV (Jørgensen et al., 2007), IPNV (Skjesol et al., 2011) and PMCV

(Timmerhaus et al., 2011). As was reported previously, the activation of the cytotoxic T cells after infection with PMCV seems to be a prominent feature of the immune system for clearance of the viral infection (Timmerhaus et al., 2011). As LC-PUFA have been shown to modulate the production of IFN-γ, (Hara et al., 2003), the differences in the levels of EPA and in the EPA/ARA ratio between the diets may contribute to the differential immune responses observed although, as described above, the differences in LC-PUFA between the two functional feeds were relatively small.

The present study also showed up-regulation of MHC class II that may indicate an important humoral immune response to ASRV. In contrast, up-regulation of this gene was not observed in salmon in response ISAV at early stage infection (Jørgensen et al., 2007). The 70-fold higher expression of MHC class II in fish fed the FF1 diet at 16-weeks post-infection, when viral load was high, compared with those fed the ST diet, could be indicative of a more efficient response against ASRV at that time in fish fed FF1.

In general, the expression of all genes characteristic of the immune response was different between the dietary treatments, not only in terms of the time-courses of ASRV infection and response, but also when comparing gene expression in fish presenting high viral load, with consistently lower expression in fish fed the FF1 diet. The less extreme effects on immune gene expression, especially those of the innate immune response, may be indicative of a milder/controlled response in fish fed the FF1 diet, and the reason why fish on this treatment presented fewer and less severe heart lesions. This is consistent with previous data on salmon challenged with ISAV that showed fish with the highest expression of genes involved in the innate immune response were those with low resistance to the disease (Jørgensen et al., 2008). Therefore, it appears that in viral diseases like HSMI, with lower mortality but high morbidity, the control of over-expression of genes involved in antiviral host responses could be key to better performance of the fish.

Leukotriene LTB_4 plays important roles in the immune response, enhancing NK cell activity, stimulating lymphocyte production of IFN-γ, and modulating immune cell function promoting the proliferation of T cells and stimulating the release of cytokines from those cells (Chang et al., 1991¸Montero et al., 2008a). As LTB_4 is synthesized from ARA derived from cell membrane phospholipids, it represents a link between tissue LC-PUFA profiles and the expression of the immune related genes mentioned above. Two genes in the up-regulated list related with LTB_4 metabolism, arachidonate 5-lipoxygenase (LTB_4 biosynthesis) and leukotriene B_4 receptor, were up-regulated two-fold when the viral load was high in fish fed the ST and FF2 diets compared with fish fed the FF1 diet. This is one example of how, early in the cascade of events of the inflammatory response, differences can be found between fish fed the FF1 diet and fish fed the other diets. Differential expression of both these genes was also reported after infection with ISAV (Jørgensen et al., 2008), thus the eicosanoid pathways appear to be important in salmon viral diseases, determining the magnitude of the inflammatory response.

The present study has clearly demonstrated that application of clinical nutrition and the use of functional feeds can have a major influence on the development and severity of the response to ASRV infection in salmon. Furthermore, the differences in gene expression between fish fed the different diets have provided further insight into the molecular mechanisms and progression of the immune responses to ASRV infection in salmon. However, the precise mechanisms underpinning both the delayed replication of the virus in fish fed the functional feeds and the differential effects of these two formulations require further investigation.

2.5 Conclusion

The present study is the first to describe the effects of functional feeds on the expression of genes related with the immune response after infection with ASRV. The long duration of the experiment allowed us to evaluate the host-pathogen interaction, increasing knowledge of HSMI, an important emerging disease in Atlantic salmon aquaculture. Clear differences on viral load and the immune response were found in fish fed the different dietary treatments, highlighting the immune modulatory role of dietary lipid content and composition in viral infections. Reduction in dietary lipid along with increased EPA can lead to a milder inflammatory response and consequently less severity of the heart lesions caused by ASRV infection indicating that dietary immunomodulation could reduce the morbidity of the disease improving the performance of fish suffering HSMI.

Acknowledgements

We wish to thank MSc Ragnhild Hanche-Olsen for sampling salmon heart for isolation of ASRV, MSc Tove Hansen for assistance with salmon tissue sampling, and MSc Even G. Nordstrøm at ILAB for assistance during the same. We also thank Dr John Taggart and Dr James Bron for advice and direction with the microarray and bioinformatic analyses, and Jacquie Ireland for technical assistance with microarray analyses.

Author Contributions

Conceived and designed the functional feeds and experiments: SW JLGV KR. Performed the experiments: LM OE SM. Analyzed the data: LM OE SM KR. Wrote the paper: LM DRT. Discussed data and edited the manuscript: LM DRT OE SW JLGV SM JGB.

Supplementary Table 2.1. Primers used for the RT-qPCR

Transcript	Primer name	Primer sequence	Fragment	Tm	Accession No.	Source
IL10	IL10-F1	5'- AAGGCAGTGTTCGAGAGCAT -3'	162 bp	60º	BT047745 [1]	New design
	IL10-R1	5'- CTTCCCCATCCACTTCAAGA -3'				
Casp14	CASP14-F1	5'- TGGAGAGATCACAGGAGAGGA -3'	166 bp	60º	BT048360 [1]	New design
	CASP14-R1	5'- GGAAGCAAAATGGAACGAAA -3'				
TCRa	TCRa-F1	5'- GCCTGGCTACAGATTTCAGC -3'	220 bp	60ºC	BT050114 [1]	New design
	TCRa-R1	5'- CCAGAATGGTCAGGGATAGG -3'				
IRF1	IRF1-F2	5'- CGGTCACCAAGAAACCCTTA -3'	386 bp	60ºC	EF067841 [1]	New design
	IRF1-R2	5'- CGCAGCTCTATTTCCGTTTC -3'				
MX1	MX1-F2	5'- CTGCAGAACAAGCTCAAACG -3'	171 bp	60ºC	U66475 [2]	New design
	MX1-R2	5'- TCCTCTGGGTCCACATTGTA -3'				
INFII	INFII-F2	5'- TTCAGGAGACCCAGAAACACTAC -3'	125 bp	60ºC	AJ841811 [1]	S.M. Jørgensen et al. 2007
	INFII-R2	5'- TAATGAACTCGGACAGAGCCTTC-3'				
GIG2	GIG2-F	5'- CCCCTGAGGACAGCCACGTCT -3'	114 bp	60ºC	EG815123[1]	New design
	GIG2-R	5'- CACCGGCACCAAGCATGCCA -3'				
SAA	SAA-F	5'- GGGAGATGATTCAGGGTTCCA -3'	79 bp	60ºC	X99387 [2]	M.K. Raida, K. Buchmann 2009
	SAA-R	5'- TTACGTCCCCAGTGGTTAGC -3'				
FLAP	FLAP-F	5'- TCTGAGTCATGCTGTCCGTAGTGGT -3'	111 bp	60ºC	CA369467 [1]	Jørgensen et al. (2008)
	FLAP-R	5'- CCTCCCTCTCTACCTTCGTTGCAAA -3'				

Supplementary Table 2.1 (Continuation). Primers used for the RT-qPCR

Transcript	Primer name	Primer sequence	Fragment	Tm	Accession No.	Source
BAF	BAF-F2	5'- GCAGACAGTCACCTTCTCTCC -3'	187 bp	60ºC	BT049316 [1]	New design
	BAF-R2	5'- GGGTACAAGCAGGGGTCTTA -3'				
B2M	B2M-F	5'- TCCCAGACGCCAAGCAG -3'	138 bp	55ºC	AF180487 [1]	N.D. Young et al. (2008)
	B2M-R	5'- TGTAGGTCTTCAGATTCTTCAGG -3'				
IgER	IgER-F1	5'- GGGAAGTTGAGCTGTGGGTA -3'	201 bp	60ºC	BT048133 [1]	New design
	IgER-R1	5'- AGCGCCATAGAAGCTTTGAA -3'				
ASRV	ASRV-F1	5'- CGTACCGCTTCTAACCAAGC -3'	215 bp	58ºC	HM453201 [1]	Mikalsen et al.(2012)
	ASRV-R1	5'- ACATGACGACGGACTCCAAT -3'				
Reference genes:						
elf-1α	ELF-1A jbt2	5'-CTGCCCCTCCAGGACGTTTACAA-3'	175 bp	60ºC	AF321836 [1]	Morais et al. (2011)
	ELF-1A jbt2	5'-CACCGGGCATAGCCGATTCC-3'				
β-actin	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141 bp	56ºC	AF012125 [1]	Morais et al. (2011)
	BACT-R	5'-GACAACGGAACCTCTCGTTA-3'				
Cofilin-2	B2F	5'-AGCCTATGACCAACCCACTG-3'	224 bp	60ºC	TC63899 [2]	Morais et al. (2011)
	B2R	5'-TGTTCACAGCTCGTTTACCG-3'				

[1] GenBank (http://www.ncbi.nlm.nih.gov/)
[2] Atlantic salmon Gene Index (http://compbio.dfci.harvard.edu/tgi/)

Supplementary Table 2.3 Criteria used to score the histological changes in the heart (epicard, ventricle and atrium). Max score within each category is given in the left-most column. Scoring was done on a visual analogue scale.

Inflammation score for HSMI infected fish	**Pathological description - epicard**	**Pathological description - myocard**
0	No pathological changes observed.	No pathological changes observed.
0-1.8	**Score 0.1-0.9**: Focal / multifocal (2-4 foci) of inflammatory cells lifting the epicardial layer from the surface of the heart, typically 2-3 cell layers thick. Limited number (countable) of mononuclear inflammatory cells infiltrating the epicardium. If there is only involvement of epicard with minor or very little compact layer involvement; max 1.5 score (diffuse and >5 cell layer thick for most of the inflamed area).	**Score 0.1-0.9**. Vascular changes in the small vessels of the compact layer characterized by enlarged endothelial cells, typically stretching out. Minor inflammatory changes of the compact layer without significant involvement of the spongious layer.
2-3.8	**Score 1-1.9**: Diffuse infiltration of inflammatory cells (mononuclear) >5 cell layers thick in most of the epicard present. The infiltration of cells is multifocal to diffuse and can involve parts of or the entire epicardium available for assessment.	**Score 1-1.9:** Focal to multifocal inflammatory foci (2-5 foci) of the compact layer and/or in the spongious part (2-5 foci). Extension typically seen along small vessels and perivascular infiltration.
4-5.8	**Score 2.0-2.9**: Diffuse infiltration of inflammatory cells (mononuclear) >10 cell layers thick in most of the epicard present. Moderate pathological changes consisting of high number (uncountable) of inflammatory cells in the epicardium	**Score 2.0-2.9:** The changes in the compact layer are multifocal or diffuse in areas and typically concentrate along small blood vessels. Combined with focal or multifocal changes in the spongious layer.
6	**Score 3**: Diffusely thickened (>15 cell layers) epicard in more than ¾ of the layer present. Severe pathological changes characterized by intense infiltration of inflammatory cells in the epicardium,.	**Score 3:** Widespread to diffuse infiltration of inflammatory cells in the compact layer and involving the spongius layer in a multifocal pattern. Degeneration and or necrosis of muscle fibers may be/are seen. Atrium can also be involved with inflammatory changes

Chapter 3

Effect of functional feeds on expression of genes related to LC-PUFA biosynthesis and eicosanoid metabolism in liver and head kidney of Atlantic salmon (*Salmo salar* L.) with experimentally induced Heart and Skeletal Muscle Inflammation

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Abstract

Heart and Skeletal Muscle Inflammation (HSMI) is an emerging viral disease caused by a novel Atlantic salmon reovirus (ASRV) affecting farmed fish. Primary symptoms associated with HSMI include myocardial and skeletal muscle necrosis indicating a severe inflammatory process. Recently, we applied the concept of clinical nutrition to moderate the long-term inflammatory process associated with HSMI in salmon subjected to experimental ASRV challenge. The use of functional feeds with lower energy through reduced lipid content reduced inflammatory responses to ASRV infection and severity of associated heart lesions. The aim of the present study was to elucidate possible mechanisms underpinning the observed effects of the functional feeds, focussing on liver and head kidney lipid and fatty acid metabolism. Here, we confirmed that liver was also a site for histopathological lesions in HSMI showing steatosis reflecting impaired lipid metabolism. This study is also the first to evaluate the expression of a suite of key genes involved in pathways relating diet and membrane phospholipid fatty acid compositions, and the inflammatory response after ASRV infection. The expression of hepatic Δ6 and Δ5 desaturases was higher in fish fed the functional feeds, potentially increasing their capacity for endogenous production and availability of anti-inflammatory EPA. Effects on mobilisation of lipids and changes in the LC-PUFA composition of membrane phospholipids, along with the changes in the expression of the genes related to eicosanoid pathways, showed the important role of the head kidney in inflammatory diseases caused by viral infections. The effects on gene expression suggests that clinical nutrition through functional feeding could be an effective complementary therapy for emerging salmon viral diseases associated with long-term inflammation.

3.1 Introduction

Heart and Skeletal Muscle Inflammation (HSMI) is an emerging viral disease caused by a novel Atlantic salmon reovirus (ASRV) (Palacios et al., 2010) affecting farmed salmon in Norway (Rimstad, 2011). Mortality varies between outbreaks from 0 to 20% (Kongtorp et al., 2004b), and the morbidity of the lesions can reach 100%, affecting almost all the fish in a farm, leading to significant growth reduction and great financial impact. The main symptoms associated with HSMI are epi-, endo-, and myocarditis, myocardial and red skeletal muscle necrosis indicating these tissues experience a severe inflammatory process. Early stages of the disease have been reported around five months after transfer to sea water (Kongtorp et al., 2006) and the cardiac lesions persist for several months until the fish are able to control the infection and reduce tissue damage. Thus, factors modulating and dampening the inflammatory process might be key to mitigating the clinical symptoms and improving performance, including growth, of affected fish.

Polyunsaturated fatty acids (PUFA), in particular long-chain PUFA (LC-PUFA), are well known to have essential roles in the development and control of the inflammatory response. They are important components of the plasma membrane that are integral in controlling membrane signalling pathways. A key link between fatty acid composition of immune cell membranes and the inflammatory process are the eicosanoids, lipid mediators including prostaglandins (PG), leukotrienes (LTB) and thromboxanes (TX), which modulate the intensity and duration of inflammatory responses in humans (Calder, 2007) and fish (Rowley et al., 1995). In mammals, arachidonic acid (ARA, 20:4n-6) is the major LC-PUFA precursor of eicosanoids including leukotriene B_4 (LTB_4) and prostaglandin E_2 (PGE_2), which are two of the main eicosanoids studied in relation to several immune-related pathways (Calder, 2005;

Yedgar et al., 2006). LTB_4 is an important chemotactic agent for leukocytes, enhancing the proliferation of T and B cells, increasing the production of tumour necrosis factor α (TNFα) and interleukins, IL-1 and IL-6, and inducing NK cell activity and thus being highly relevant in viral infections (Tafalla et al., 2002). PGE_2 is generally related with pro-inflammatory, although it has also been associated with anti-inflammatory actions at the resolution of the inflammatory process (Calder et al., 2009c). In contrast, the n-3 LC-PUFA, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are considered as anti-inflammatory fatty acids, with EPA particularly relevant as an antagonist of ARA, acting as a competitive substrate for the main enzymes of the eicosanoid pathways, cyclooxygenase (COX) and lipoxygenase (LOX), giving rise to anti-inflammatory eicosanoids and inflammation-resolving E-series resolvins (Calder, 2009; Calder et al., 2009). Although fish cell membranes have higher levels of EPA than ARA, previous studies indicated that ARA was the preferred substrate for the enzymes involved in the eicosanoid pathway in fish, as in mammals (Bell et al., 1994). Similarly, altering the ratio of ARA and EPA in cell membranes alters the production of eicosanoids and modulates the inflammatory response in fish as it does in mammals (Bell et al., 1995). In the Western-style diet, prevalent in many human societies, the intake of n-3 LC-PUFA is very low compared to the habitual high intake of n-6 PUFA, and so there has been a strong focus on clinical nutrition involving dietary supplementation with EPA, particularly in a range of pathologies and diseases involving acute or chronic inflammation, such as rheumatoid arthritis, acute cardiovascular events or inflammatory bowel diseases (Calder, 2009).

The aquaculture industry has traditionally relied on the use of the marine resources, fish meal (FM) and fish oil (FO), as major feed ingredients and also to provide n-3 LC-PUFA and so maintain the high levels of these fatty acids in the farmed fish and seafood products. There

is a finite source of fish meal (6 million tonnes) and fish oil (1 million tonnes), which has been generally stable since 1975. However aquaculture output has increased at around 9% per annum. To sustain this continued increase in aquaculture production, from a fixed supply of marine raw materials, alternative sources of protein and oil have been successfully developed. Although VO are generally rich in n-6 PUFA and do not contain n-3 LC-PUFA, the replacement of high levels of FO by VO does not appear to negatively affect growth performance in salmonids (Turchini et al., 2010). However, the reduced dietary n-3 LC-PUFA and increased n-6 PUFA are directly reflected in the fish tissue fatty acid compositions, not only potentially impacting on the nutritional quality for the human consumer, but also raising concerns regarding the health of the fish themselves (Tocher, 2009c; Montero and Izquierdo, 2010a). Thus, the fatty profile of aquaculture diets, especially total n-3 LC-PUFA levels as well as the n-3/n-6 PUFA ratio are becoming of increasing interest in the modulation of disease resistance in fish (Thompson et al., 1996; Montero et al., 2003). Dietary modulation for the treatment of pancreas disease has been used in human and animal medicine for many years (Westermarck et al., 1996; Suzuki et al., 1999). These diets have now been adapted for salmon operations, with a focus on reducing overall digestible energy, adapting the lipid and fatty acid profile and increasing digestibility of nutrients.

In this context, we recently applied the concept of clinical nutrition to moderate the long-term inflammatory process associated with HSMI in salmon (Martinez-Rubio et al., 2012). In this study we investigated the effects of functional feed formulations, in comparison to a standard commercial feed, on inflammatory responses in the heart of Atlantic salmon subjected to experimental ASRV challenge. The standard reference diet (ST diet) contained 31% lipid and a digestible energy content of 22 MJ/Kg. The functional feeds had reduced DE levels (18 MJ/Kg) through lower lipid (18%), and increased levels of EPA, and increased

n-3/n-6 PUFA and EPA/ARA ratios (FF1 and FF2 diets). A milder (dampened) inflammatory response to ASRV infection, and reduced severity of heart lesions was found in fish fed the functional feeds, particularly FF1 and transcriptome (microarray) analysis of heart showed that expression of inflammation/immune related genes was greatly affected by the functional feeds. This study demonstrated that dietary modulation through clinical nutrition had major influences on the development and severity of the response to ASRV infection in salmon and the modulation of gene expression between fish fed the different feeds provided insight into the molecular mechanisms and progression of the inflammatory and immune responses to ASRV infection in salmon.

The primary objective of the present study was to elucidate possible mechanisms underpinning the observed effects of the functional feeds, particularly focussing on lipid metabolism, membrane fatty acid compositions and the fatty acid metabolic pathways involved in LC-PUFA biochemistry and inflammatory processes, and that would be affected by changes in dietary fatty acid composition. Thus, head kidney, heart and liver from the same fish sampled in the previous study were analysed and fatty acid compositions of the cell membrane phospholipids determined. In addition, as one of the tissues affected in HSMI, liver inflammation was measured and the expression of genes, including transcription factors controlling aspects of lipid metabolism, and those involved of LC-PUFA biosynthesis and eicosanoid metabolism, were determined in liver and head kidney.

3.2 Material and methods

3.2.1 Fish and feeds

Three fishmeal-based diets were formulated and manufactured by EWOS Innovation (Dirdal, Norway). Details of the formulation and proximate composition of the feeds were

reported previously (Martinez-Rubio et al. 2012) (Table 3.1). In short, the reference diet (ST) was essentially a standard, commercial formulation with 31% lipid with the added oil being a blend of FO and rapeseed oil. Both functional feeds had lower lipid content (18%) and increased proportions of EPA, but differed in n-3/n-6 PUFA ratio, which varied from 1.5 (ST) to 2.5 (FF1) and 3.6 (FF2) (Table 3.2). A total of 450 Atlantic salmon (*Salmo salar* L.), AquaGen strain, were distributed into three tanks at the EWOS facility, Lønningdal, Norway and fed one of the experimental feeds for a period of 8 weeks prior to being transferred to the challenge facility.

Table 3.1 Formulation and proximate compositions of the feeds.

Component %[1]	**ST**	**FF1**	**FF2**
Fish meal and hydrolysates	42.1	53	53
Fish oil	13.7	7	5.2
Vegetable protein concentrates[2]	21	18	18
Vegetable oil	11.2	3	0
Carbohydrate-based binders[3]	11.2	12.1	12.1
Micro premixes[4]	0.8	1.9	1.7
Krill meal[5]	0	5	5
Krill oil[5]	0	0	5
Total	100	100	100
Proximate composition			
Moisture	6.5	6.5	6.5
Fat	31	18	18
Protein	42.2	53.4	53.4

1) All ingredients sourced from EWOS stocks unless otherwise stated.

2) Includes soy protein concentrate, pea protein concentrate, wheat gluten and sunflower meal

3) Includes wheat grain

4) Includes vitamins, minerals, crystalline amino acids, ammonium phosphate

5) Aker Biomarine A

Table 3.2 Total lipid fatty acid composition (percentage of total fatty acids) and lipid class composition (percentage of total lipid) of the experimental diets. ARA, Arachidonic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acids.

Fatty acid	**ST**	**FF1**	**FF2**
Saturated	23.97	36.48	31.86
18:1n-9	32.30	18.65	16.31
Monounsaturated	45.73	33.10	37.05
18:2n-6	10.73	7.11	5.30
20:3n-6	0.06	0.11	0.10
20:4n-6	0.36	0.48	0.54
n-6 PUFA	11.63	8.09	6.50
18:3n-3	4.62	2.35	1.55
20:5n-3	4.85	8.76	9.53
22:6n-3	6.09	5.98	9.03
n-3 PUFA	17.83	19.83	23.32
PUFA	30.31	30.42	31.09
EPA/ARA	13.67	18.16	17.75
N-3/N-6	1.53	2.45	3.59
Total PL	8.94	10.77	17.30
TAG	65.39	53.47	52.45
% Lipid	24.52	13.71	13.35

3.2.2 ASRV Challenge

After the pre-feeding period, the fish (non-vaccinated) were transferred to the challenge facility at the Industrial and Aquatic Laboratory (ILAB), Bergen, Norway. A total of 390 fish (130 fish per treatment), initial average weight 220g (±3.2g, standard error), were distributed into two independent experimental rooms each containing 9 flow-through tanks (3 tanks per dietary treatment in each system). Filtered seawater (approximately 30‰) with a constant temperature of 10 ± 1°C was supplied with a flow sufficient to maintain oxygen concentration in the outlet water at > 8 mg/L (48 L / kg fish / h). The light regime was set at 12h:12h light/dark. The fish were acclimated for 2 weeks prior to challenge. Fish were fed with the same diets during the acclimation period, pre-challenge (8 weeks) and post-challenge periods

(16 weeks) No previous diseases were described. The fish were sedated using Aqui-S (at final concentration of 5 mg/L of isoeugenol) followed by anaesthesia in benzocaine (Benzoak) using a final concentration of 30 ml/L of water. After an initial sampling of 10 fish per dietary treatment, a total of 360 fish (20 per tank; 60 fish per diet per treatment room) were challenged by intramuscular injection (0.1 ml on each side close to the lateral line) of inoculum collected from cell culture supernatant of ASRV (Martinez-Rubio et al. 2012).

3.2.3 Sampling

Ten fish from each dietary treatment group (5 fish from each of the two independent systems) were sampled randomly at 8- and 16-weeks post-challenge. Fish were anaesthetised as above, killed by a blow to the head, and heart, liver and head kidney collected for analyses. Part of each liver sample was transferred to 10% buffered formalin for histological analyses and another part was immediately frozen in liquid N_2 and stored at -80^{o}C prior to lipid and molecular analyses. Heart and head kidney were immediately frozen in liquid N_2 and stored at -80^{o}C.

3.2.4 Liver histology

Liver histology was assessed for micro- and macrovesicular steatosis by light microscopy of hematoxylin and eosin-stained sections. At least five different fields at 20x original magnification where examined and scored for presence of formation of vesicles of individual hepatocytes. A subjective assessment of the proportion of hepatocytes that showed these changes was also made. The changes were scored based a pre-determined system (see below) and all sections were scored double-blinded. A selected proportion of liver samples were stained with Periodic-Acid Schiff stain (PAS) according to standard methods with the purpose

to rule out vacuole formation as a cause of glycogen storage. Positive samples were included and also treated pre-treated with diastase/amylase (Bancroft and Stevens, 1980).

Liver steatosis scores were ranked according to a non-continuous score grade from 0 to 5 (Table 3.3).

Table 3.3 Scoring of liver steatosis in individual sections was based on the following system.

0	formation of vacuoles in the cytoplasm, involving less than 10% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
1	formation of vacuoles in the cytoplasm, involving less than 25% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
2	formation of vacuoles in the cytoplasm, involving less than 50% of the hepatocytes and including less than 50% of the area of the individual hepatocytes
3	formation of vacuoles in the cytoplasm, involving less than 75% of the hepatocytes and including less than 75% of the area of the individual hepatocytes
4	formation of vacuoles in the cytoplasm, involving less than 90% of the hepatocytes and including less than 80% of the area of the individual hepatocytes
5	formation of vacuoles in the cytoplasm, involving more than 90% of the hepatocytes and including more than 80% of the area of the individual hepatocytes

Briefly, a score of 0 indicated the formation of vacuoles in the cytoplasm, involving less than 10% of the hepatocytes and including less than 25% of the area of the individual hepatocytes. A score of 5 indicated formation of vacuoles in the cytoplasm, involving more than 90% of the hepatocytes and including more than 80% of the area of the individual hepatocytes. All data preparation and simulation output analysis was conducted with the R language (R Development Core Team, 2009). The model was a mixed-effects linear model estimated with the lmer function in the lme4 package. The treatment estimates were based on posterior simulation (n=2500) with 95% credible intervals as absolute and proportional to the reference level (control diet). Histopathological scores were analysed by using a multilevel ordered categorical logistic regression because the data are multinomial. The model was written in BUGS (Lunn et al., 2000) language and fitted with JAGS (Plummer, 2009). Vague non-informative uniform priors (0,100) were given for the variance parameters and vague non-

informative normal priors N (0, 1.0E + 4) for all other parameters. 25000 “burn-in" simulation runs were used to adapt the Markov Chain Monte Carlo (MCMC) before subsequent 2500 runs that were used for inference. Three chains were run in parallel, i.e. there were a total of 7500 simulations for inference. These were thinned so that only every 10th simulation was saved to reduce the size of saved objects and to reduce the effects of autocorrelation. In effect, the posterior density is based on 750 draws from the posterior probability distribution. Convergence of the MCMC simulation was judged by the Gelman-Rubin convergence diagnostic.

3.2.5 Lipid analyses

Total lipid from approximately 1g of liver, 0.5 g of head kidney and 0.2g of heart was extracted by homogenisation in chloroform/methanol (2:1, by volume) according to Folch et al. (1957), and determined gravimetrically. Total lipid from head kidney and heart samples was separated by one-dimensional thin-layer chromatography (TLC) and the major phospholipid classes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were isolated as described previously (Bell et al., 1991). Fatty acid methyl esters (FAME) of heart and head kidney phospholipid classes and liver total lipid were prepared by acid-catalysed transmethylation (Christie, 2003). FAME were separated and quantified by gas chromatography (Tocher and Harvie, 1988). Tissue and diet lipid class compositions were determined by single-dimension double-development high-performance thin-layer chromatography (HPTLC) and densitometry (Henderson and Tocher, 1992). Significance of differences due to diet and time were determined by two-way ANOVA ($p<0.05$) using the SPSS 19.0 statistical package (SPSS Inc., Chicago IL, USA) of arcsin-transformed data.

3.2.6 Real-time quantitative PCR

Expression of 13 selected genes were studied by reverse transcription real time quantitative PCR (qPCR). Six genes are related to the eicosanoid biosynthesis pathway, secretory phospholipase A_2 or sPLA2 (PLA2G1), cytosolic calcium-dependent phospholipase A_2 or cPLA2 (PLA2G4), cytosolic calcium-independent phospholipase A_2 or iPLA2 (PLA2G6), cyclooxygenase 2 (COX2), 5-lipoxygenase-activating protein (FLAP) and arachidonate 5-lipoxygenase (ALOX5). Four genes are involved in the LC-PUFA biosynthesis pathway, Δ5 and Δ6 fatty acyl desaturases (D5FAD, D6FAD) and elongases 5 and 2 (ELOVL5, ELOVL2). The remaining genes are transcription factors related to regulation of storage and catabolism of dietary fats, and recently associated with the immune response, peroxisome proliferator-activated receptors α, β and γ (PPAR-A, PPAR-B and PPAR-G). The qPCR primer sequences, including the annealing temperature (Tm) and size of amplicon, are shown in Table 3.4. The primer sequences were obtained either by literature searches or designed from EST sequences corresponding to candidate genes of interest using Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). In addition, amplification of two reference genes, *cofilin-2* and elongation factor-1α (*elf-1α*) was performed as their expression was stable across treatments, and they had been identified in previous salmon qPCR studies as suitable reference genes on the basis of constant expression between different feeds and time points (Morais et al. 2011; Martinez-Rubio et al., 2012(Chapter 2)).

Six samples per treatment per time point (6 x 3 x 2) were randomly selected for qPCR analysis in each one of the 3 tissues collected.

For qPCR, 2 µg of column-purified total RNA per sample was reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Paisley, U.K.), following manufacturer's instructions, but using a mixture of the random primers (1.5µl as supplied) and

anchored oligo-dT (0.5 µl at 400 ng/ µl, Eurofins MWG Operon, Ebersberg, Germany). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. cDNA was then diluted 20-fold with water, after a similar amount of cDNA was pooled from all samples. RT-qPCR analysis used relative quantification with the amplification efficiency of the primer pairs being assessed by serial dilutions of the cDNA pool. qPCR amplifications were carried out in duplicate (Quantica, Techne, Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL (for most genes) or 2 µL (for the reference

Table 3.4 Primers used for RT-qPCR.

Transcript	Primer name	Primer sequence	Fragment	Tm	Accession No.	Source
Δ5fad	D5DES-F	5'-GTGAATGGGGATCCATAGCA-3'	192 bp	56º	AF478472 [1]	Morais et al. (2009)
	D5DES-R	5'-AAACGAACGGACAACCAGA-3'				
Δ6fad_a	D6DES-F	5'-CCCCAGACGTTTGTGTCAG-3'	181 bp	56º	AY458652 [1]	Morais et al. (2009)
	D6DES-R	5'-CCTGGATTGTTGCTTTGGAT-3'				
elovl5a	Elo1UTR-SM-1F	5'-ACAAGACAGGAATCTCTTTCAGATTAA-3'	137 bp	60ºC	AY170327 [1]	Morais et al. (2009)
	Elo1UTR-SM-1R	5'-TCTGGGGTTACTGTGCTATAGTGTAC-3'				
elovl2	Elo2-SM-1F	5'-CGGGTACAAAATGTGCTGGT-3'	145 bp	60ºC	TC91192 [2]	Morais et al. (2009)
	Elo2-SM-1R	5'-TCTGTTTGCCGATAGCCATT-3'				
COX2	COX2-F3	5'- TGTCCTGACATCTCGCTCAC -3'	217 bp	60ºC	AY848944 [1]	(New design)
	COX2-R3	5'- AAACCGCTTCCTCAACAAAA -3'				
PLA2G1	PLA2G1-F7	5'- AGGCCCTGTGGCAGTTCAGA -3'	97 bp	66ºC	DY716300 [1]	(New design)
	PLA2G1-R7	5'- CCCTTGCCACAGTAGCAGCCG -3'				
PLA2G4	PLA2G4-F2	5'- GTCGCTGGCTGGAGCTGTGG -3'	138 bp	65ºC	NM_001141333 [2]	(New design)
	PLA2G4-R2	5'- AGCCCTATGGGCCCTGGTCA -3'				
PLA2G6	PLA2G6-F4	5- AGGCCCATCAAGGAACTCTT -3'	72 bp	64ºC	DQ294237 [1]	(New design)
	PLA2G6-R4	5'- GATGATAGCCAGGGCCAGTA -3'				
FLAP	FLAP-F	5'- TCTGAGTCATGCTGTCCGTAGTGGT -3'	bp	60ºC	CA369467 [1]	Jørgensen et al. (2008)
	FLAP-R	5'- CCTCCCTCTCTACCTTCGTTGCAAA -3'				

Table 3.4 (Continuation)

Transcript	Primer name	Primer sequence	Fragment	Tm	Accession No.	Source
ALOX5	ALOX5-F1	5'- TATCTCCCTCTCCCTCAGTCC-3'	155 bp	56ºC	CX727592 [1]	Ø. Haugland et al. 2005
	ALOX5-R1	5'- GGTCAGCAGTGCCATCA-3'				
PPARα	SsPPAR-A-F1	5- TCCTGGTGGCCTACGGATC-3'	111 bp	60ºC	DQ294237 [1]	Kleveland et al. (2006)
	SsPPAR-A-R1	5'-CGTTGAATTTCATGGCGAACT-3'				
PPARβ	SsPPAR-B-F1	5'-GAGACGGTCAGGGAGCTCAC-3'	151 bp	60ºC	AJ416953 [1]	Kleveland et al. (2006)
	SsPPAR-B-R1	5'-CCAGCAACCCGTCCTTGTT-3'				
PPARγ	SsPPAR-G-F1	5'-CATTGTCAGCCTGTCCAGAC-3'	144 bp	60ºC	AJ416951 [1]	Kleveland et al. (2006)
	SsPPAR-G-R1	5'-TTGCAGCCCTCACAGACATG-3'				
Reference genes:						
elf-1α	ELF-1A jbt2	5'-CTGCCCCTCCAGGACGTTTACAA-3'	175 bp	60ºC	AF321836 [1]	Morais et al. (2009)
	ELF-1A jbt2	5'-CACCGGGCATAGCCGATTCC-3'				
β-actin	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141 bp	56ºC	AF012125 [1]	Morais et al. (2009)
	BACT-R	5'-GACAACGGAACCTCTCGTTA-3'				
Cofilin-2	B2F	5'-AGCCTATGACCAACCCACTG-3'	224 bp	60ºC	TC63899 [2]	Morais et al. (2009)
	B2R	5'-TGTTCACAGCTCGTTTACCG-3'				

[1] GenBank (http://www.ncbi.nlm.nih.gov/)
[2] Atlantic salmon Gene Index (http://compbio.dfci.harvard.edu/tgi/)

genes and other highly expressed genes) diluted (1/20) cDNA, 0.5 µM of each primer and 10µL AbsoluteTM QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a systematic negative control (NTC-non template control, containing no cDNA). The qPCR profiles contained an initial activation step at 95°C for 15 min, followed by 30 to 40 cycles: 15s at 95°C, 15 s at the specific primer pair annealing Tm (Table 3.4) and 15 s at 72°C. After the amplification phase, a melt curve of 0.5°C increments from 75ºC to 90°C was performed, enabling confirmation of the amplification of a single product in each reaction. qPCR product sizes were checked by agarose gel electrophoresis and identities confirmed by sequencing. Non-occurrence of primer-dimer formation in the NTC was also verified. Results were analysed using the relative expression software tool (REST 2009, http://www.gene-quantification.info/), which employs a pair wise fixed reallocation randomisation test (10,000 randomisations) with efficiency correction to determine the statistical significance of expression ratios (or gene expression fold-changes) between two treatments (Pfaffl et al., 2002).

3.3 Results

3.3.1 Liver histology

The liver histology scores, based on the degree of steatosis was read as vacuole-formation in hepatocyte cytoplasm characterised by both micro- and macro-vesicular lesions (Figure 3.1). There were clear differences between the fish fed the functional feeds and fish fed the ST diet 8-weeks post-challenge, with the latter group presenting higher micro- and macro-vesicular steatosis. Quantification of the liver histopathology thus showed that both the

incidence (percentage of fish sampled) and severity (based on the scoring system in Table 3.3) of steatosis at 8-weeks post challenge was clearly greater in salmon fed the ST diet compared to fish fed the two functional feeds (Figure 3.2). At 16 weeks post-challenge, there were no differences in liver histology between the three dietary treatments.

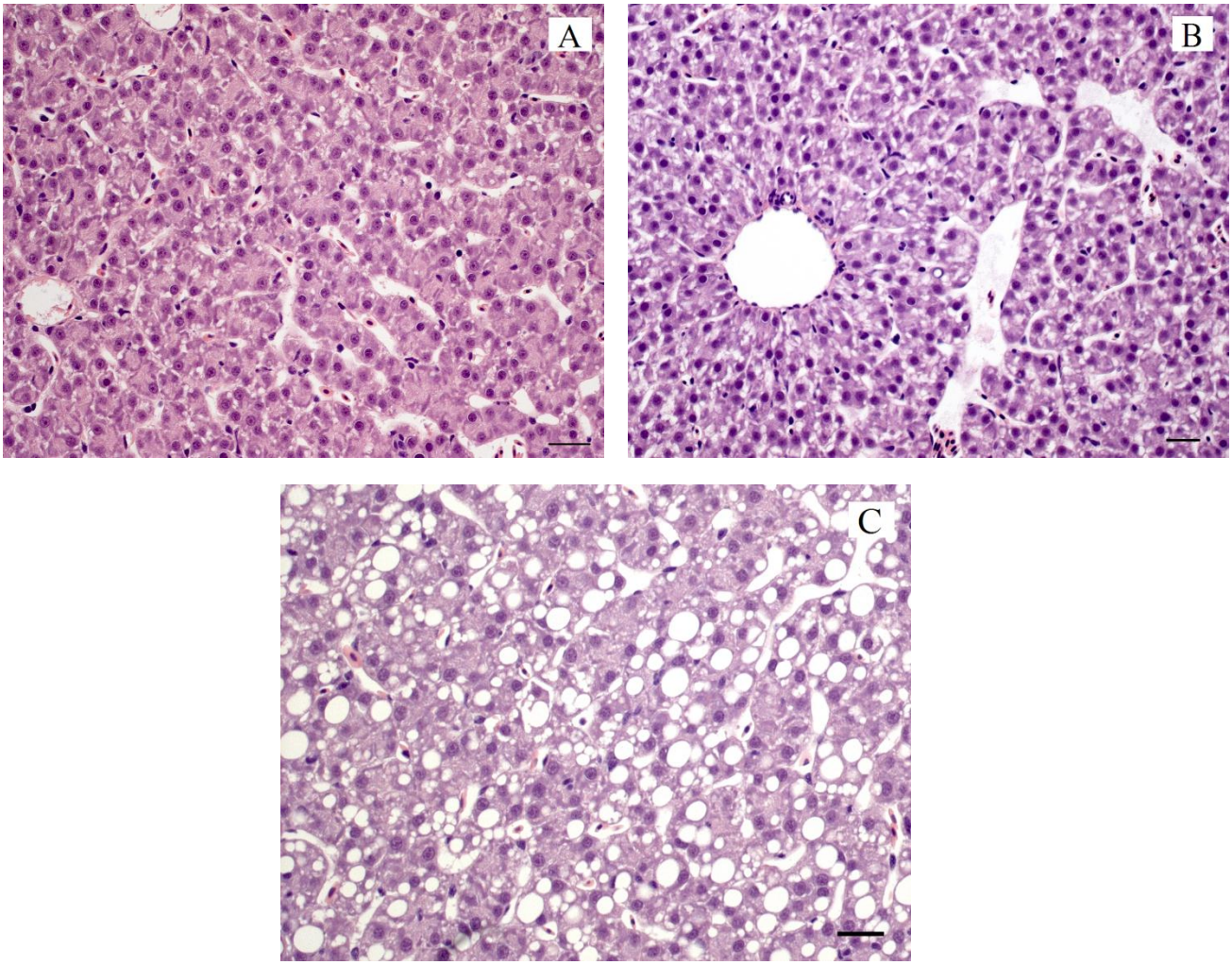

Figure 3.1 Liver histology. Micrographs showing the degrees of steatosis. A, Microvesicular steatosis, 0.5 score; B, Moderate steatosis with a mix of microvesicular and some macrovesicular, 2.0 score; C, Pronounced steatosis dominated by macrovesicular, 3.5 score.

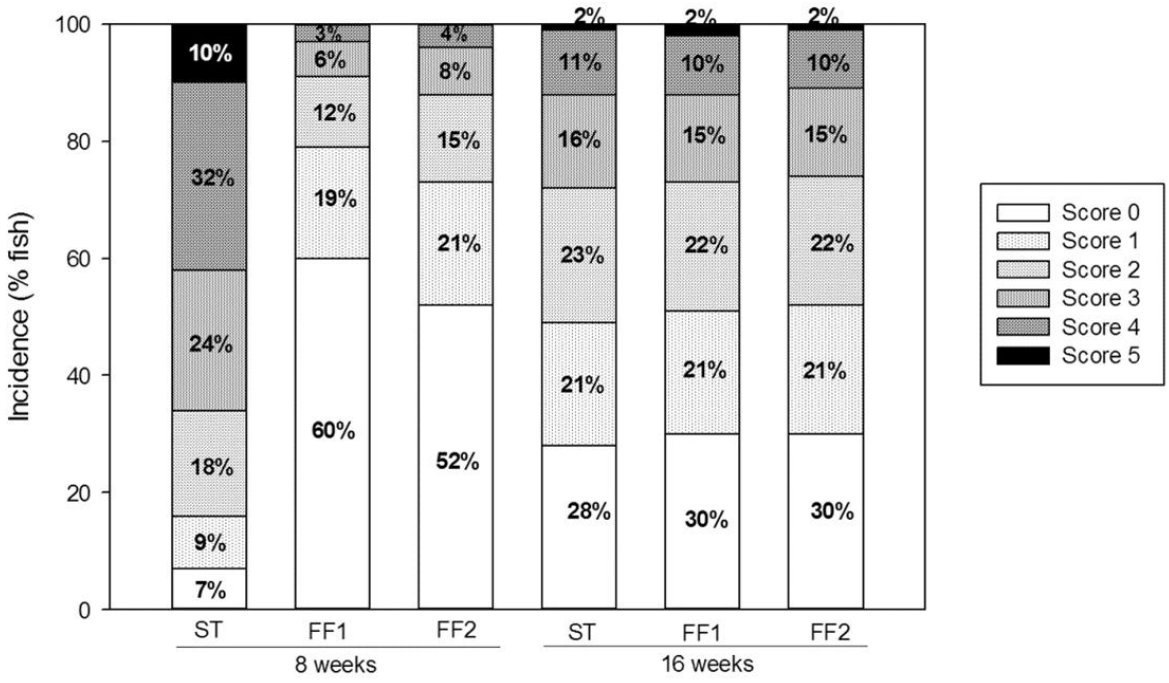

Figure 3.2 Incidence (percentage of fish sampled) and severity of histopathology (based on the steatosis scoring system in Table 3.3) in liver at 8-weeks and 16-weeks post challenge with Atlantic salmon reovirus in fish fed the Standard (ST) reference feed and the two functional feeds (FF1 and FF2).

3.3.2 Lipid content and composition of tissues

The total lipid and triacylglycerol (TAG) contents of the liver were lower in fish fed the functional feeds compared to fish fed the ST diet with higher lipid (Table 3.5).

Table 3.5 Lipid content, total Phospholipids (TPL) and Triacylglycerol (TAG) of liver, head kidney and heart from Atlantic salmon fed the different diets at 8- and 16- weeks post-infection with PRV. Results are means ± SD (n = 5).P-values of two-way ANOVA are presented for factors 'diet', 'time' and interaction between factors

	ST		FF1		FF2		TWO WAY ANOVA *P-value*		
Liver	**8w**	**16w**	**8w**	**16w**	**8w**	**16w**	**Diet**	**Week**	**Diet*Week**
Lipid content	5.7 ± 0.8	4.3 ± 0.5	4.5 ± 0.4	4.0 ± 0.3	4.3 ± 0.3	3.5 ± 0.2	0.000	0.000	ns
TPL	37.7 ± 2.4	44.7 ± 2.7	54.0 ± 4.6	50.3 ± 6.4	54.9 ± 3.9	53.5 ± 0.9	0.000	ns	0.007
TAG	32.4 ± 3.8	14.8 ± 4.0	13.8 ± 6.3	14.9 ± 7.8	12.0 ± 5.1	11.7 ± 2.6	0.000	0.025	0.001
Head kidney									
Lipid content	6.4 ± 0.5	2.9 ± 1.4	8.1 ± 4.4	2.1 ± 0.3	7.1 ± 2.4	1.6 ± 0.4	ns	0.000	ns
TPL	34.9 ± 4.1	25.2 ± 4.0	32.4 ± 5.2	37.3 ± 5.0	36.5 ± 5.2	38.1 ± 6.8	0.012	ns	0.009
TAG	42.8 ± 4.2	49.1 ± 8.8	49.6 ± 7.9	35.0 ± 7.0	44.6 ± 7.6	35.8 ± 9.0	ns	0.047	0.016
Heart									
Lipid content	6.5 ± 1.4	5.9 ± 1.8	5.7 ± 1.4	4.0 ± 1.4	7.6 ± 1.4	4.2 ± 2.1	ns	0.003	ns
TPL	48.7 ± 8.4	30.8 ± 7.1	48.8 ± 7.1	41.5 ± 9.6	45.1 ± 6.6	45.7 ± 7.5	ns	0.008	0.043
TAG	25.0 ± 14.3	46.5 ± 11.3	25.9 ± 11.7	34.7 ± 13.1	32.1 ± 13.0	26.1 ± 6.1	ns	ns	ns

In contrast, diet had no effect on the total lipid or TAG contents of heart and head kidney. Total lipid levels in all three tissues decreased during the time course of the infection irrespective of diet. In liver, this decrease was greatest in fish fed diet ST, which also showed a large decline in TAG. In contrast, the decline in liver lipid was associated with lower proportions of polar lipids in fish fed the functional feeds. The decline in tissue lipid content over the course of the infection was greatest in head kidney. This was associated with decreased percentages of TAG in fish fed the functional feeds but, conversely, with reduced proportions of polar lipids in fish fed diet ST (Table 3.5). In heart, the lower lipid content at 16-weeks post-challenge was associated with lower proportions of polar lipids in fish fed diet ST but lower TAG in fish fed FF2.

3.3.3 Fatty acid compositions of tissues

The fatty acid compositions of the functional feeds were characterised by increased proportions of saturated and n-3 PUFA, decreased proportions of monoenes and n-6 PUFA, and increased n-3/n-6 PUFA and EPA/ARA ratios (Table 3.1). The differences in saturated and monounsaturated fatty acid levels were greatest between diets ST and FF1, whereas the differences in PUFA levels were greatest between diet ST and FF2. The two functional feeds also varied in the relative proportions of ARA, EPA and DHA. The fatty acid profile of total lipid of liver generally reflected the composition of the diets, and so showed the same differences between the ST diet and functional feeds, and between the two functional feeds (Table 3.6). The overall liver profile changed relatively little during the course of the infection, especially in the groups of fish eating both functional diets, however there was increased proportions of DHA and n-3 PUFA, a decreased proportions of monoenes in fish fed the ST diet.

Table 3.6 Total lipid fatty acid composition (percentage of total fatty acids) of liver from Atlantic salmon fed the different diets at 8- and 16- weeks post-infection with PRV. Results are means ± SD (n = 5).P-values of two-way ANOVA are presented for factors 'diet', 'time' and interaction between factors.ARA, arachidonic acid; EPA, eicosapentaenoic acid; ns, not significant (p > 0.05); PUFA, polyunsaturated fatty acids.

	8 weeks			16 weeks			TWO WAY ANOVA *P-value*		
Fatty acid	**ST**	**FF1**	**FF2**	**ST**	**FF1**	**FF2**	**Diet**	**Week**	**Diet*Week**
Saturated	19.1 ± 1.2	26.8 ± 1.4	27.3 ± 1.0	22.5 ± 0.9	25.4 ± 2.0	25.6 ± 0.9	0.000	ns	0.000
Monounsaturated	42.9 ± 4.2	27.5 ± 5.0	25.7 ± 3.3	31.5 ± 6.2	28.0 ± 3.5	26.6 ± 3.3	0.000	ns	0.008
18:2n-6	6.5 ± 0.3	3.0 ± 0.2	2.2 ± 0.2	5.5 ± 0.5	3.0 ± 0.5	2.1 ± 0.22	0.000	0.028	0.038
20:3n-6	0.5 ± 0.1	0.8 ± 0.0	0.6 ± 0.2	0.6 ± 0.1	1.0 ± 0.1	0.8 ± 0.2	0.000	0.009	ns
20:4n-6	1.8 ± 0.4	2.5 ± 0.5	2.5 ± 0.5	2.3 ± 0.6	2.6 ± 0.4	2.6 ± 0.3	0.020	ns	ns
N-6 PUFA	11.0 ± 0.6	7.4 ± 0.6	6.3 ± 0.7	10.0 ± 0.4	7.7 ± 0.4	6.5 ± 0.5	0.000	ns	0.035
18:3n-3	2.0 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	1.6 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	0.000	0.031	ns
20:5n-3	3.8 ± 0.5	7.0 ± 1.1	7.5 ± 1.4	4.9 ± 0.8	7.0 ± 0.6	7.2 ± 0.4	0.000	ns	ns
22:6n-3	18.2 ± 3.6	27.3 ± 4.4	30.0 ± 2.5	26.6 ± 4.5	27.9 ± 2.8	30.2 ± 2.2	0.000	0.018	0.017
N-3 PUFA	26.7 ± 3.4	37.9 ± 5.4	40.3 ± 2.7	35.6 ± 5.2	38.5 ± 3.1	40.7 ± 2.7	0.000	0.026	0.029
PUFA	38.0 ± 3.7	45.7 ± 6.0	47.0 ± 3.5	46.0 ± 5.4	46.6 ± 2.8	47.8 ± 3.0	0.022	0.049	ns
EPA/ARA	2.1 ± 0.4	2.9 ± 0.5	3.1 ± 0.5	2.2 ± 0.3	2.7 ± 0.4	2.8 ± 0.2	0.000	ns	ns
n-3/n-6	2.4 ± 0.3	5.1 ± 0.4	6.5 ± 0.3	3.6 ± 0.5	5.0 ± 0.6	6.2 ± 0.5	0.000	0.027	0.001

The focus in head kidney and heart was on eicosanoid metabolism, and so fatty acid compositions were determined in the lipid sources of precursor LC-PUFA, the major membrane phospholipid classes phosphatidylcholine (PC) and phosphatidylethanolamine (PE). The phospholipid fatty acid profiles of head kidney showed some significant effects of diet, including increased proportions of saturated fatty acids and decreased proportions of n-6 PUFA, but the differences were not as pronounced as in the diet of liver total lipid (Table 3.7). Irrespective of diet, the proportions of the major eicosanoid precursors, ARA and EPA and the EPA/ARA ratio, declined in head kidney phospholipids, especially PE, over the course of the infection, being significantly reduced in fish at 16-weeks compared to 8-weeks post-challenge (Table 3.7). The percentage of DHA in both phospholipid classes was similar in all dietary groups and decreased in PC, but not PE, over the time-course of the infection.

As with head kidney, the phospholipid fatty acid composition of heart also showed effects of diet but they were not so pronounced and the profiles only changed slightly, and mainly in PC, over the course of the infection (Table 3.8). The changes in the proportions of the eicosanoid precursor LC-PUFA showed a different pattern in heart compared to head kidney. Whereas, the proportions of ARA decreased in PC, they increased in PE, during the course of the infection. It was noteworthy that, although EPA declined in both PC and PE of heart over the course of the infection, the decline was much lower in fish fed the functional feeds (Table 3.8). Similarly, although the EPA/ARA ratio in heart phospholipids decreased over the course of the infection, this was much less pronounced in fish fed the functional feeds. In contrast the n-3/n-6 PUFA ratio did not change over the course of the infection.

Table 3.7 Phospholipid fatty acid composition (percentage of total fatty acids) of head kidney from Atlantic salmon fed the different diets at 8- and 16- weeks post-infection with PRV. Results are means ± SD (n = 5).P-values of two-way ANOVA are presented for factors 'diet', 'time' and interaction between factors. ARA, arachidonic acid; EPA, eicosapentaenoic acid; ns, not significant ($p > 0.05$); PUFA, polyunsaturated fatty acids.

	PE								
	ST		FF1		FF2		TWO WAY ANOVA *P-value*		
	8w	16w	8w	16w	8w	16w	Diet	Week	Diet*Week
Saturated	14.5 ± 0.6	14.7 ± 0.6	15.6 ± 0.9	18.2 ± 3.1	16.1 ± 0.6	17.4 ± 1.9	0.007	0.030	ns
Monounsaturated	17.5 ± 0.6	17.2 ± 1.3	19.9 ± 4.7	18.6 ± 2.9	17.6 ± 2.0	17.5 ± 1.6	ns	ns	ns
18:2n-6	4.3 ± 0.1	3.4 ± 0.2	3.6 ± 0.6	2.7 ± 0.3	2.5 ± 0.3	2.0 ± 0.3	0.000	0.000	ns
20:3n-6	0.3 ± 0.0	0.4 ± 0.1	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.000	0.000	0.019
20:4n-6	3.5 ± 0.3	2.5 ± 0.4	2.8 ± 0.4	2.1 ± 0.2	2.8 ± 0.3	1.6 ± 0.4	0.000	0.000	ns
Total n-6 PUFA	9.5 ± 0.4	7.6 ± 0.5	7.9 ± 0.3	6.4 ± 0.3	6.5 ± 0.1	4.7 ± 0.7	0.000	0.000	ns
18:3n-3	0.8 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.000	0.000	ns
20:5n-3	11.3 ± 1.0	5.8 ± 0.8	10.4 ± 1.8	6.2 ± 0.7	12.1 ± 0.8	5.8 ± 0.3	ns	0.000	ns
22:6n-3	35.8 ± 0.6	38.8 ± 1.5	33.9 ± 4.1	35.8 ± 4.1	36.3 ± 1.6	38.9 ± 2.7	ns	0.030	ns
Total n-3 PUFA	50.0 ± 1.3	47.1 ± 1.1	47.3 ± 5.6	45.0 ± 5.0	51.0 ± 1.9	47.1 ± 2.7	ns	0.027	ns
Total PUFA	59.5 ± 1.0	54.7 ± 1.2	55.2 ± 5.4	51.3 ± 5.0	57.5 ± 1.9	51.8 ± 2.4	ns	0.001	ns
EPA/ARA	3.3 ± 0.6	2.3 ± 0.5	3.7 ± 0.4	3.0 ± 0.5	4.3 ± 0.4	3.8 ± 0.7	0.000	0.001	ns
n-3/n-6	5.3 ± 0.3	6.2 ± 0.4	6.0 ± 0.9	7.1 ± 0.8	7.8 ± 0.4	10.2 ± 1.7	0.000	0.000	ns

	PC								
	ST		FF1		FF2		TWO WAY ANOVA *P-value*		
	8w	16w	8w	16w	8w	16w	Diet	Week	Diet*Week
Saturated	30.6 ± 2.0	38.1 ± 0.7	35.0 ± 1.2	38.8 ± 1.0	35.1 ± 0.2	38.4 ± 1.2	0.000	0.000	0.001
Monounsaturated	23.2 ± 2.7	26.2 ± 1.6	19.5 ± 0.5	25.0 ± 1.0	17.7 ± 0.8	23.9 ± 0.5	0.000	0.000	0.023
18:2n-6	3.1 ± 1.0	2.8 ± 0.0	1.9 ± 0.1	2.1 ± 0.1	1.3 ± 0.1	1.6 ± 0.3	0.000	ns	ns
20:3n-6	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.4 ± 0.0	0.010	0.017	ns
20:4n-6	1.7 ± 0.2	1.5 ± 0.1	1.7 ± 0.1	1.5 ± 0.2	1.6 ± 0.1	1.3 ± 0.3	ns	0.002	ns
Total n-6 PUFA	6.0 ± 1.1	5.6 ± 0.2	4.8 ± 0.3	4.9 ± 0.1	3.8 ± 0.1	3.9 ± 0.5	0.000	ns	ns
18:3n-3	1.0 ± 0.2	0.4 ± 0.3	0.5 ± 0.0	0.3 ± 0.2	0.3 ± 0.0	0.2 ± 0.1	0.000	0.000	ns
20:5n-3	9.5 ± 0.7	6.5 ± 0.5	11.2 ± 1.1	8.7 ± 1.0	12.7 ± 1.6	9.6 ± 0.5	0.000	0.000	ns
22:6n-3	24.4 ± 2.9	17.8 ± 1.9	23.6 ± 1.8	16.5 ± 0.9	25.2 ± 1.6	18.2 ± 0.9	ns	0.000	ns
Total n-3 PUFA	37.0 ± 2.4	26.2 ± 2.0	37.3 ± 2.2	27.3 ± 1.3	40.2 ± 0.8	29.7 ± 1.0	0.000	0.000	ns
Total PUFA	43.0 ± 1.7	31.8 ± 2.1	42.1 ± 2.1	32.2 ± 1.2	44.1 ± 0.8	33.7 ± 1.1	0.049	0.000	ns
EPA/ARA	5.6 ± 0.9	4.3 ± 0.7	6.8 ± 1.1	5.9 ± 1.2	8.0 ± 1.3	7.8 ± 1.3	0.000	0.040	ns
n-3/n-6	6.3 ± 1.3	4.6 ± 0.3	7.9 ± 0.8	5.6 ± 0.4	10.6 ± 0.4	7.6 ± 0.9	0.000	0.000	ns

Table 3.8 Phospholipid fatty acid composition (percentage of total fatty acids) of heart from Atlantic salmon fed the different diets at 8- and 16- weeks post-infection with PRV. Results are means ± SD (n = 5).P-values of two-way ANOVA are presented for factors 'diet', 'time' and interaction between factors. ARA, arachidonic acid; EPA, eicosapentaenoic acid; ns, not significant (p > 0.05); PUFA, polyunsaturated fatty acids.

	PE								
	ST		FF1		FF2		TWO WAY ANOVA *P-value*		
	8w	16w	8w	16w	8w	16w	Diet	Week	Diet*Week
Saturated	16.7 ± 0.8	20.3 ± 0.5	16.8 ± 1.0	17.9 ± 0.8	18.6 ± 0.6	17.4 ± 1.1	0.049	0.003	0.000
Monounsaturated	11.3 ± 0.8	11.6 ± 0.8	10.7 ± 0.5	11.1 ± 2.0	15.0 ± 1.1	11.7 ± 2.2	0.019	ns	ns
18:2n-6	2.5 ± 0.2	2.3 ± 0.3	1.9 ± 0.2	2.4 ± 0.6	1.7 ± 0.3	1.7 ± 0.5	0.016	ns	ns
20:3n-6	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.2	ns	ns	ns
20:4n-6	1.5 ± 0.0	1.6 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.2 ± 0.0	1.4 ± 0.1	0.001	0.028	ns
Total n-6 PUFA	5.8 ± 0.4	5.5 ± 0.3	5.0 ± 0.3	5.3 ± 0.5	4.1 ± 0.4	4.5 ± 0.7	0.000	ns	ns
18:3n-3	0.7 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	0.7 ± 0.3	0.3 ± 0.1	0.4 ± 0.2	0.002	ns	ns
20:5n-3	4.6 ± 0.5	4.0 ± 0.3	6.0 ± 0.4	5.6 ± 0.6	4.8 ± 0.4	5.5 ± 0.1	0.000	ns	0.010
22:6n-3	49.1 ± 1.1	47.1 ± 1.9	48.4 ± 0.4	48.2 ± 3.0	45.5 ± 1.8	49.5 ± 2.3	ns	ns	0.039
Total n-3 PUFA	58.4 ± 0.7	54.8 ± 2.0	60.4 ± 0.8	59.1 ± 3.3	54.5 ± 2.5	59.3 ± 2.5	0.032	ns	0.008
PUFA	64.2 ± 1.0	60.3 ± 2.0	65.4 ± 0.9	64.5 ± 3.0	58.6 ± 2.8	63.8 ± 2.0	0.011	ns	0.003
EPA/ARA	3.2 ± 0.3	2.6 ± 0.3	4.3 ± 0.5	4.0 ± 0.3	3.9 ± 0.3	4.0 ± 0.3	0.000	0.044	ns
N-3/N6	10.1 ± 0.6	10.1 ± 0.6	12.1 ± 0.8	11.2 ± 1.6	13.5 ± 0.8	13.5 ± 2.5	0.001	ns	ns

	PC								
	ST		FF1		FF2		TWO WAY ANOVA *P-value*		
	8w	16w	8w	16w	8w	16w	Diet	Week	Diet*Week
Saturated	33.0 ± 0.8	37.5 ± 2.0	35.1 ± 0.5	37.4 ± 0.7	35.6 ± 2.4	36.4 ± 1.4	ns	0.000	ns
Monounsaturated	16.2 ± 1.9	17.9 ± 2.2	13.7 ± 0.7	13.4 ± 0.7	14.3 ± 2.6	13.1 ± 1.8	0.001	ns	ns
18:2n-6	1.8 ± 0.3	1.7 ± 0.4	1.2 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.8 ± 0.1	0.000	0.048	ns
20:3n-6	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	ns	ns	ns
20:4n-6	1.9 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.5 ± 0.0	ns	0.000	0.003
Total n-6 PUFA	4.8 ± 0.4	4.2 ± 0.4	4.0 ± 0.3	3.7 ± 0.2	3.8 ± 0.1	3.3 ± 0.2	0.000	0.001	ns
18:3n-3	0.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.000	0.007	ns
20:5n-3	9.5 ± 1.4	6.0 ± 1.0	12.7 ± 1.2	11.0 ± 0.4	12.8 ± 2.6	11.1 ± 0.4	0.000	0.000	0.067
22:6n-3	31.2 ± 3.8	29.2 ± 1.4	29.1 ± 1.0	29.8 ± 1.1	29.3 ± 2.9	32.1 ± 2.2	ns	ns	ns
Total n-3 PUFA	42.9 ± 2.8	37.1 ± 1.8	44.3 ± 0.9	43.0 ± 0.9	44.2 ± 0.3	45.2 ± 2.5	0.000	0.016	0.007
PUFA	47.8 ± 2.6	41.4 ± 1.5	48.3 ± 0.8	46.7 ± 0.9	48.0 ± 0.4	48.4 ± 2.4	0.001	0.002	0.003
EPA/ARA	5.1 ± 0.5	4.2 ± 0.6	7.4 ± 0.6	6.9 ± 0.5	7.9 ± 2.0	7.2 ± 0.4	0.000	0.023	ns
N-3/N6	8.9 ± 1.2	8.8 ± 1.2	11.1 ± 0.9	11.7 ± 0.6	11.7 ± 0.1	13.9 ± 1.4	0.000	ns	ns

3.3.3 Expression of genes of LC- PUFA biosynthesis pathway in liver

The expression of both Δ6 and Δ5 fatty acyl desaturases in liver was significantly up-regulated at both 8- and 16-weeks post-challenge in fish fed the functional feeds compared to fish fed the ST diet (Figure 3.3).

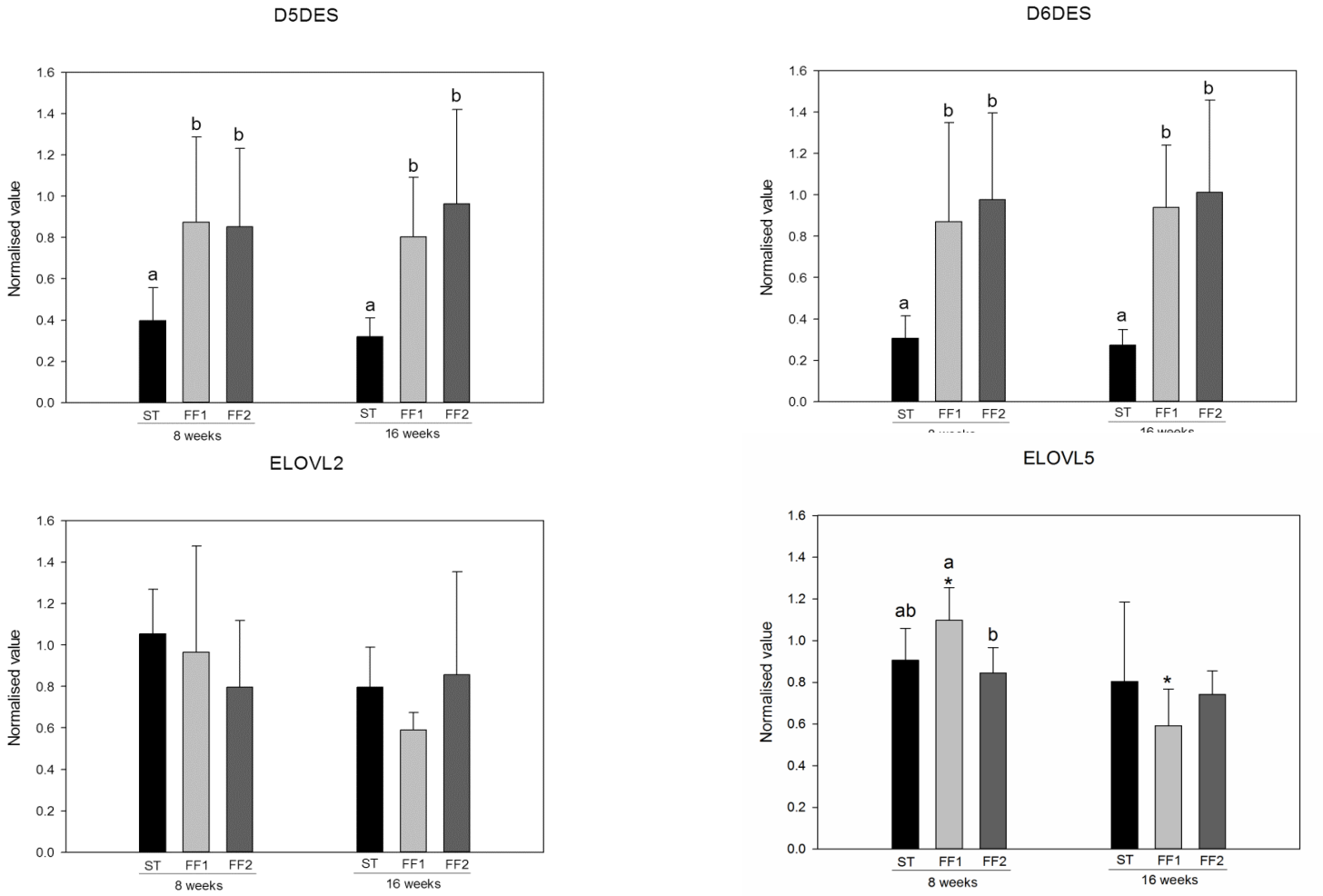

Figure 3.3 Expression of genes of LC-PUFA biosynthesis in liver. Expression of genes involved in long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis in liver was determined by real-time quantitative PCR. Values were normalised by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets within time points and symbols represents differences between time-points within diets (two way ANOVA, $p<0.05$).

There was no difference in expression of these genes between fish fed diets FF1 and FF2, or between 8- and 16-weeks. In contrast, diet had no significant effects on the expression of fatty acyl elongases in the liver other than ELOVL5 expression was higher in fish fed diet FF1 compared to fish fed diet FF2 at 8-weeks post-challenge (Figure 3.3). There appeared to be a trend for elongase expression to be lower at 16-weeks compared to 8-weeks post-challenge, but this was only significant for ELOVL5 in fish fed diet FF1.

3.3.4 Expression of PPARs

There were no significant effects of diet on the expression of PPARs in liver (Figure 3.4). There was a trend for the expression of all PPAR subtypes to be lower at 16-weeks compared to 8-weeks post-infection in fish fed the ST diet, but this was not statistically significant. Similarly, the expression of PPARγ was lower at 16-weeks compared to 8-weeks post-challenge in all fish, although it was only significant in fish fed FF1 (Figure 3.4).

The expression of PPARs in head kidney was also investigated due to the considerable decrease in lipid content observed in this tissue during the course of the infection, and the recently reported potential immune modulatory role of these transcription factors (Varga et al. 2011). All three PPAR subtypes showed higher expression at 8-weeks compared to 16-weeks post-challenge, with this being significant in fish being fed the functional feeds (Figure 3.5). The only other significant effect of diet was increased expression of PPARβ in fish fed the ST diet compared to fish fed diet FF2 at16-weeks post-challenge.

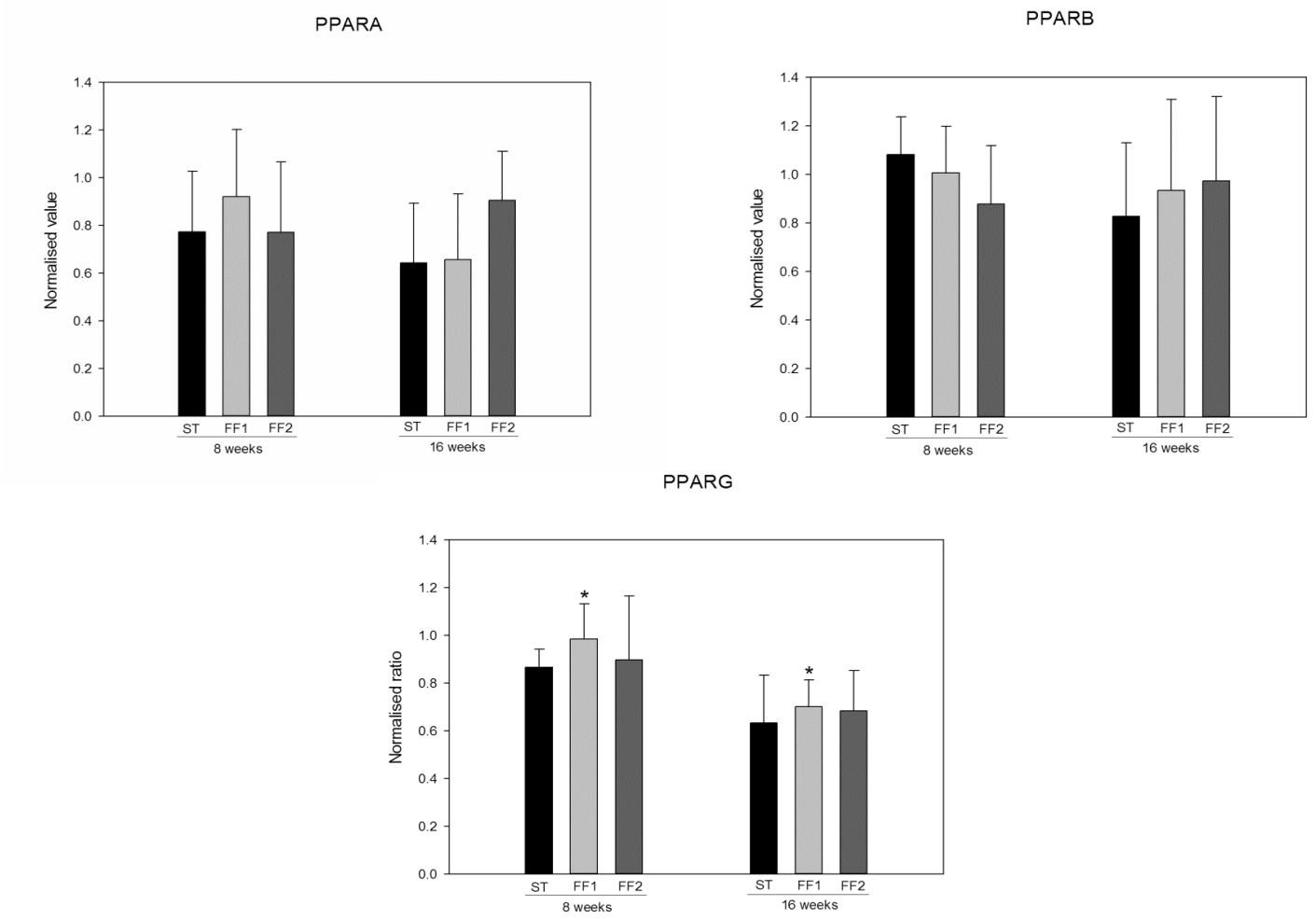

Figure 3.4. Expression of PPAR genes in liver. Expression of peroxisome proliferator-activated receptor (PPAR) genes in liver was determined by real-time quantitative PCR. Values were normalised by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets within time points and symbols represents differences between time-points within diets(two-way ANOVA, $p<0.05$).

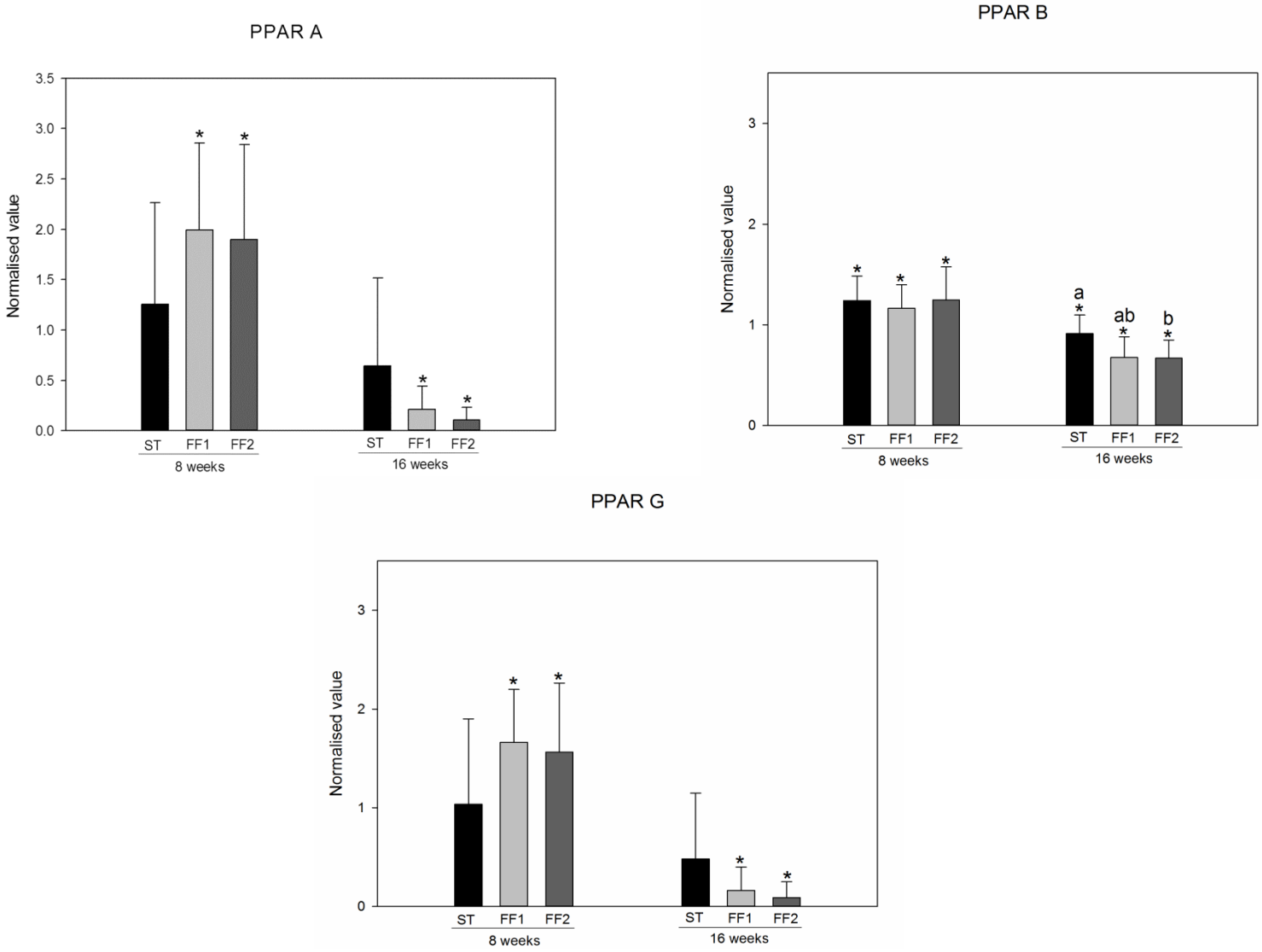

Figure 3.5 Expression of PPAR genes in head kidney. Expression of peroxisome proliferator-activated receptor (PPAR) genes in head kidney was determined by real-time quantitative PCR. Values were normalised by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets within time points and symbols represents differences between time-points within diets (two-way ANOVA, $p<0.05$).

3.5. Expression of genes related to eicosanoid biosynthesis

In head kidney, the expression of three phospholipases reported to be involved in the release of inflammatory fatty acids from cell membranes (Yedgar et al. 2006) was higher at 8-weeks compared to 16-weeks post-challenge in fish fed all diets, significantly so in fish fed the functional feeds (Figure 3.6). At 8-weeks, the expression of sPLA2 (PLA2G1) was higher in fish fed the functional feeds compared to fish fed the ST diet, albeit only significant with fish fed diet FF2. The expression of COX2 (prostaglandin synthesis), ALOX5 and FLAP, both involved in the biosynthesis of LTs, showed generally few differences between dietary treatments or over the course of the infection. However, the expression of ALOX5 was lower in fish fed diet FF1 compared to fish fed diet FF2, and expression of COX2 was significantly higher at 16-weeks compared to 8-weeks post-challenge in fish fed diet FF1 (Figure 3.6).

In heart, phospholipases showed a different pattern of expression compared to head kidney. Thus, expression of sPLA2 (PLA2G1) was negligible in heart (Figure 3.7). There were no major differences in expression of phospholipases over the course of the infection, other than higher expression of cPLA2 (PLA2G4) at 8- compared to 16-weeks post-challenge in fish fed diet FF2. Expression of cPLA2 at 16-weeks and iPLA2 (PLA2G6) at 8-weeks was significantly lower in fish fed diet FF2 compared to fish fed the other diets. Expression of ALOX5 and FLAP was not different between the three dietary groups but there was a trend for FLAP expression to be higher at 16- compared to 8-weeks post-challenge, although this was only significant in fish fed diet FF2 (Figure 3.7). In general, the expression pattern of COX2 in heart was similar to that observed in head kidney, with the expression significantly lower at 8-weeks post-challenge in fish fed the FF1 diet compared to fish fed the other diets, and with a general up-regulation of this gene in all dietary groups, significant in diet FF1, at 16- compared to 8-weeks post-challenge (Figure 3.7).

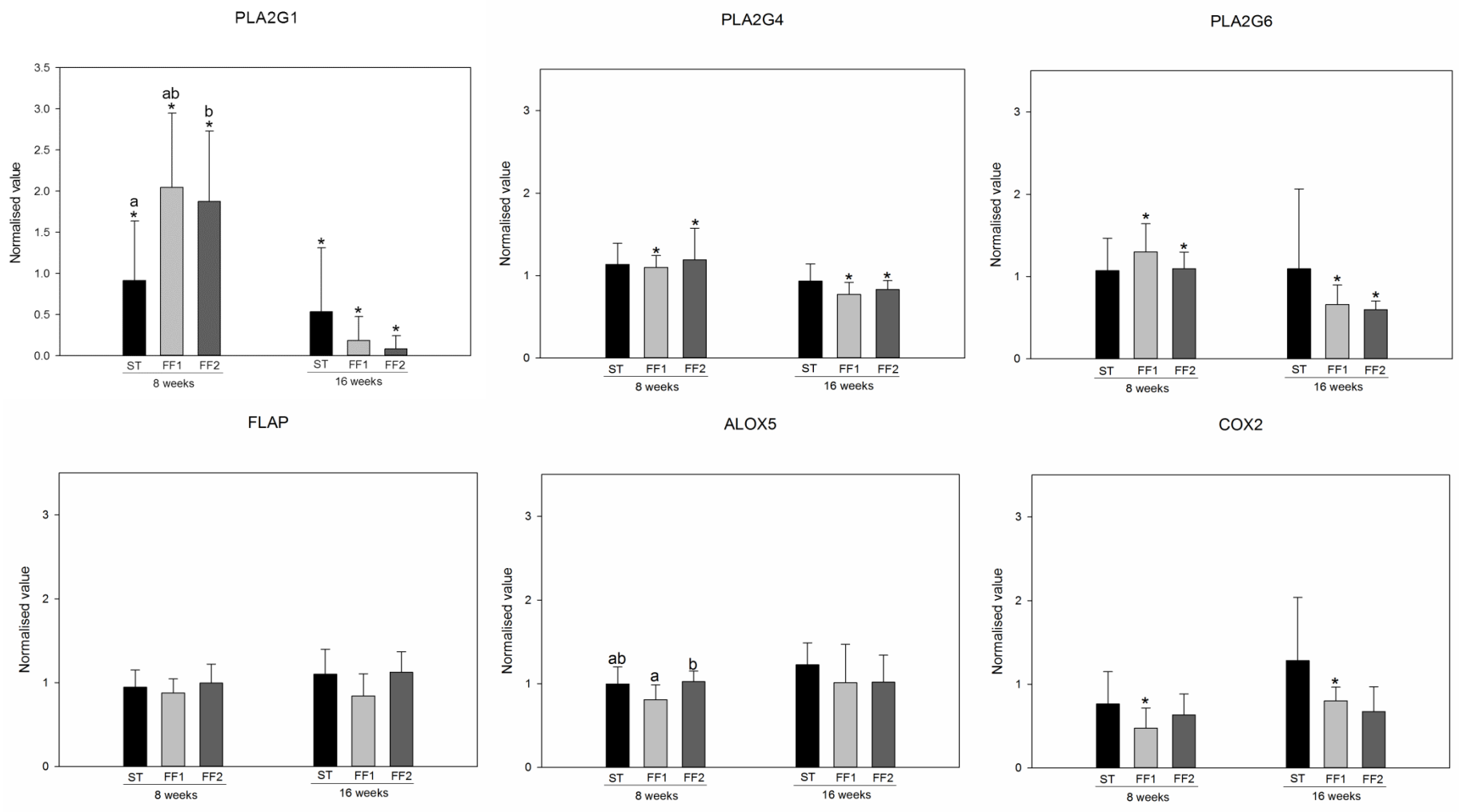

Figure 3.6 Expression of genes related to eicosanoid pathways in head kidney. Expression of genes involved in eicosanoid metabolism pathways was determined in head kidney by real-time quantitative PCR. Values were normalised by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1 α). Different letters represent significant differences between diets within time points and symbols represents differences between time-points within diets (two-way ANOVA, $p<0.05$).

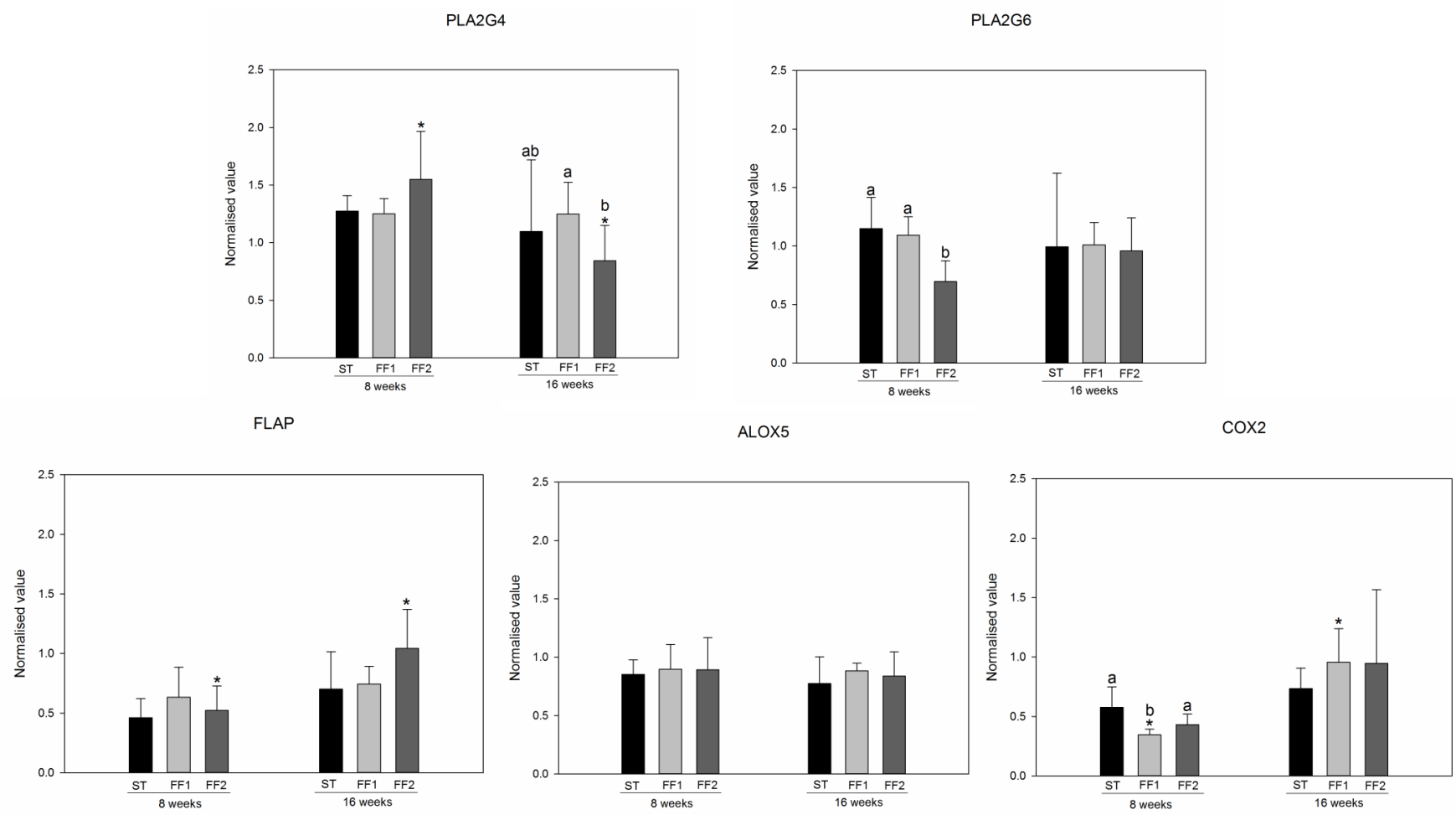

Figure 3.7.Expression of genes related to eicosanoid pathways in heart. Expression of genes involved in eicosanoid metabolism pathways was determined in heart by real-time quantitative PCR. Values were normalised by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets within time points and symbols represents differences between time-points within diets (two-way ANOVA, $p<0.05$).

4. Discussion

As previously described, reduced dietary lipid along with increased dietary EPA and EPA/ARA ratio can modulate the inflammatory response and consequently reduce the severity of the heart lesions caused by ASRV infection (Martinez-Rubio et al., 2012). The present study investigated the metabolic pathways that closely relate lipids and fatty acids, especially LC-PUFA, with the inflammatory response, correlating the previous findings on dietary immuno-modulation during ASRV infection, with availability of the LC-PUFA and eicosanoid metabolism in the main organs involved in those processes.

Fatty acids can impact the immune response in a variety of ways apart from simply being an energy source for the immune cells, including being integral components of cell membrane phospholipids, influencing the physical and functional properties of those membranes, affecting signalling pathways and regulating the activation of different transcription factors and, thus, gene expression (Calder, 2007; Yaqoob, 2003). It is well-known that different levels and proportions of the key LC-PUFA, ARA, EPA and DHA, can determine the intensity of the inflammatory response (Calder, 2005), with a major link between LC-PUFA and inflammation being their role as precursors of eicosanoids, critical inflammatory mediators. The levels of LC-PUFA in immune cell membrane phospholipids in both mammals and fish are determined by both dietary input and hepatic fatty acyl desaturase and elongase activities, and delivery via plasma lipoproteins (Calder, 2001; Tocher, 2003). In addition to liver being a major organ involved in LC-PUFA biosynthesis, liver lesions were reported in fish affected by HSMI, and so liver was also investigated in the present study to determine how the different dietary levels of lipid and EPA (and C18 PUFA), as well as how the disease progression, could affect the

expression of the genes involved in the LC-PUFA biosynthesis pathway. Eight weeks post-challenge there was higher micro- and macro-vesicular steatosis in the fish fed the ST diet compared with fish fed the functional feeds, which is consistent with the higher levels of TAG and monounsaturated fatty acids, and lower levels of n-3 PUFA, especially DHA, observed at this time point in ST-fed fish. Although the primary cause of the increased steatosis index in the ST-fed fish was not identified in detail, the observed differences could possibly relate to this fact. There is however a potential interaction with the ongoing viral infection although previous studies have found no correlation between the severity of heart and liver lesions during different stages of HSMI disease (Kongtorp et al., 2006). However, in the current study, liver steatosis was usually more frequent when the severity of the heart lesions was high, and this was found in fish from all three dietary groups at the end of the present trial.

The most notable effect of diet upon the expression of genes of LC-PUFA biosynthesis was the higher expression of both fatty acyl desaturases in fish fed with the functional feeds compared with fish fed the standard diet. As this effect largely correlates with viral load it is possible that this effect on desaturase expression could be related to the viral infection and, indeed, it could be seen as an effective strategy for a virus to block the production of LC-PUFA key to the inflammatory response (Martinez-Rubio et al., 2012). Supporting this, *in vitro* studies with human cells have reported inhibition of Δ6 fatty acyl desaturase activity after infection with several different viruses (Horrobin, 1990). However, if the effect on the expression of fatty acyl desaturase genes was related to the viral infection it is more likely to be due to a more general down-regulation of host cell metabolism rather than a specific effect on desaturase genes per se. Furthermore, the differences in the feed formulations also offer a possible explanation for the observed effect on desaturase expression; with the higher lipid (and energy) content of the ST diet being the reason for lower expression of desaturases in

salmon fed this diet. Previously, it was shown that fatty acyl desaturase activity was lower in rainbow trout (*Oncorhynchus mykiss*) consuming high-energy diets (Kolditz et al., 2008a). In addition, the levels of hepatic mRNA for both Δ6 and Δ5 desaturases were 4-fold higher in rats fed a fat-free diet in comparison to animals fed diets containing vegetable or fish oils (Cho et al., 1999a,b). The precise mechanism underpinning the increased desaturase expression in animals fed diets of lower energy (fat/oil) content is unknown. However, the higher expression of Δ6 and Δ5 fatty acyl desaturases in fish fed the FF diets would increase their capacity for the endogenous production and, possibly availability, of EPA. The general lack of major effects, whether related to nutrition and feed composition or to the course of the viral infection, on the expression of elongases was perhaps not unexpected as they do not appear to be under the same level of transcriptional control as desaturases (Zheng et al., 2005a; Leaver et al., 2008b; Torstensen and Tocher, 2010). Further investigation of the relationship between dietary lipid and energy contents and the expression of genes of the LC-PUFA biosynthesis pathway in Atlantic salmon without a viral infection is required. However, the data indicate that, irrespective of the precise molecular mechanism, the LC-PUFA biosynthesis activity in fish fed the ST diet was low further limiting the availability of anti-inflammatory n-3 LC-PUFA in these fish in comparison to fish fed the functional feeds with higher proportions of these fatty acids, highlighting, therefore, the crucial role of diet to maintain the availability of n-3 LC-PUFA in membrane phospholipids.

PPARs are a group of nuclear transcription factors activated by dietary fatty acids and their metabolic derivatives that have been associated traditionally with lipid metabolism regulation in mammals and fish (Feige et al., 2006; Leaver et al., 2008b). Therefore, as differences in lipid and energy contents of the feeds were expected to affect the pathways of lipid metabolism, the expression of PPARs were investigated. However, few effects of diet

were observed, with only PPARβ in head kidney showing a dietary effect with reduced expression in fish fed the functional feeds. In contrast there were substantial effects of the progression of the viral infection or HSMI with the expression of all PPARs, particularly PPARα and γ. This was a trend in liver but was highly significant in head kidney. This was noteworthy as, recently, there has been interest in the involvement of PPARs in the inflammatory process as they are expressed in the immune cells and activated by different components of the eicosanoid pathways (Varga et al., 2011). Although, all three isoforms of PPAR, PPARα, PPARβ and PPARγ, appear to be involved in the immune response, most of the studies in humans focus on the immunomodulatory role of PPARα (Becker et al., 2007) and PPARγ (Varga and Nagy, 2008). The expression of PPAR genes was reported to decrease during acute inflammatory process in lung (Becker et al., 2007), adipose tissue (Khovidhunkit et al., 2004) and activated lymphocytes (Jones et al., 2002). In mammals, *in vitro* studies generally suggest that PPARs play roles in the resolution of inflammation, modulating the expression of inflammatory genes such as iNOS2, p38mitogen activated protein (MAP) kinase and STAT1 in the case of PPARγ and inflammatory mediators such as LTB_4 in the case of PPARα (Varga et al., 2011). The results from the present study on ASRV/HSMI are in general agreement with those studies suggesting that PPARs may have similar roles in regulating or modulating inflammatory processes in Atlantic salmon. The considerable reduction in lipid content observed in head kidney between 8- and 16-weeks post-challenge has not previously been reported in fish studies and may be related to the differences in PPAR expression in head kidney discussed above. However, it is possible that it may also reflect mobilisation of immune cells from this organ towards the tissue sites of infection and inflammation.

The fatty acid compositions of the major membrane phospholipids in head kidney and heart gave some insight into the availability of LC-PUFA for the production of the inflammatory

mediators, eicosanoids (Rowley et al., 1995). The tissue phospholipid fatty acid compositions at 8-weeks post-challenge, when no signs of tissue damage were found in hearts irrespective of diet (Martinez-Rubio et al., 2012) and therefore before the major inflammatory response to ASRV, showed that the proportions of the main LC-PUFA involved in inflammatory response generally reflected the characteristic compositions of PC (higher saturated fatty acid and EPA) and PE (higher DHA and total PUFA), as reported in previous studies in salmon (Bell et al., 1993; 1996). The effects of dietary fatty acid composition affected the phospholipid compositions although this varied between the different LC-PUFA. Thus the proportions of ARA in PE of the head kidney was not correlated with the levels of ARA in the feeds, but reflected the levels of precursor 18:2n-6 in the feeds suggesting in vivo biosynthesis of ARA as previously reported (Bell et al., 1993). In contrast, the levels of EPA in PC, but not PE, reflected the dietary EPA intakes at 8-weeks. These results are in general agreement with previous studies in humans and salmon where increased dietary EPA lead to increased proportions of this fatty acid in immune cells and heart (Calder, 2001; Bell et al., 1993). However, the levels of LC-PUFA in PC and PE were significantly impacted by the course of the infection with ARA and EPA both being considerably reduced at 16-weeks compared to 8-weeks post-challenge. Importantly, these losses of ARA and, especially, EPA were somewhat mitigated by the functional feeds possibly contributing to the better outcome in fish fed these diets. In contrast, the levels of DHA were relatively unaffected by diet. It is difficult to predict whether these changes in LC-PUFA composition are cause or effect, or if they would lead to pro- or anti-inflammatory responses, but it appears clear that EPA and ARA are released from the membrane to a greater extent than DHA. Further investigation is therefore required to elucidate if the production of resolvins from DHA are as relevant to the resolution of the inflammatory process in fish as they are in humans (Calder, 2009).

The initial step in eicosanoid metabolism (biosynthesis) is the release of LC-PUFA precursors from phospholipids, mediated by phospholipase A_2 (PLA_2) that hydrolyse fatty acids from the sn-2 position of phospholipids. The above phospholipid fatty acid composition data suggested that there had been mobilisation of ARA and EPA during the course of the infection. The generally higher expression, particularly in head kidney, of phospholipases at 8-weeks post-challenge is consistent with the important role of these enzymes in the initiation of inflammatory processes at early stages of infection. PLA_2 is a family of enzymes with at least 15 isoforms described in mammals with the most accepted classification system based on cellular disposition and calcium dependence, including calcium-dependent ($cPLA_2$) and calcium-independent ($iPLA_2$), both cytosolic, and secretory PLA_2 ($sPLA_2$) (Gilroy et al., 2004). To date, most studies in fish have investigated $cPLA_2$ to evaluate a possible activation of the eicosanoid pathway (Bell et al., 1993; Zhou et al., 2008), but all three classes of PLA_2 could be potentially involved in the inflammatory response. However, research in this field has usually focussed on the immunomodulatory role of $cPLA_2$ and $sPLA_2$, as $iPLA_2$ is considered more important for the remodelling of cell membrane phospholipids (Yedgar et al., 2006).

The present study is the first to report the effects of viral infection on the expression of PLA_2 genes in fish. All three main classes of PLA_2 were expressed in head kidney, although expression of $sPLA_2$ was negligible in heart. The higher expression of the three PLA_2 at the early (8-week) stage of the disease in head kidney could indicate an activation of the eicosanoid pathway in macrophages and other immune related cells present in this tissue highlighting the importance of the fatty acid composition of the cell membrane of those cells for the synthesis of either pro- or anti-inflammatory eicosanoids. In heart, this pattern of expression was only observed for $cPLA_2$ in fish the FF2 diet. Diet also affected PLA_2

expression with $sPLA_2$ being up-regulated in head kidney of fish fed the functional feeds, and $iPLA_2$ down-regulated in fish fed diet FF2, but the mechanism or significance of these effects are not clear. Obviously, the present results only represent snapshots of gene expression and so can similarly only provide an indication of the possible inflammatory processes at specific time-points particularly in a largely non-fatal disease such as HSMI that presents as a long on-going infection. The choice of time points in the present study was informed by histological data on the progression of inflammatory disease in heart and liver (Martinez-Rubio et al., 2012), and may not represent ideal times for assessing all parts of the inflammatory response.

Therefore, in order to broaden scope of the study, the expression of enzymes further downstream in the production of eicosanoids was assessed. FLAP and ALOX5 are both enzymes involved in the biosynthesis of leukotrienes, with ARA-derived leukotriene B_4 (LTB_4) being one of the most critical in the inflammatory response. LTB_4 has an important role during the inflammatory process stimulating aggregation of neutrophils and promoting leukocyte adherence to the endothelium (Bates, 1995). Regarding viral immunity, LTB_4 enhances the activity of NK cells and IFNγ (Calder, 2001) both elements of the immune system that play important roles in the immune response following ASRV infection (Martinez-Rubio et al., 2012). Expression of ALOX5 and FLAP were reported after infection with infectious salmon anaemia virus (ISAV) and infectious pancreatic necrosis virus (IPNV) and, in both cases, fold changes of expression were not very high when the inflammatory process was more evident, suggesting that either the expression of these genes occurs in short periods (pulses) or small changes in expression are functionally effective (Jørgensen et al., 2008; Skjesol et al., 2011). In the present study, there was a clear trend for lower expression, significant at 8-weeks, of ALOX5 and FLAP in head kidney of fish fed the FF1 diet, which

may indicate lower production of LTB_4 that would correlate with the milder inflammatory response in this dietary group (Martinez-Rubio et al., 2012).

In general, viral infections stimulate the production of COX2 in humans and there is evidence that PGE_2 plays important roles in controlling viral replication and modulating the inflammatory responses to virus (Steer and Corbett, 2003). This prostanoid has also been recently related to anti-inflammatory responses, inhibiting the production of LTB_4 and some other inflammatory cytokines during resolution of the inflammatory process (Calder, 2009). However, PGE_2 has generally been regarded as pro-inflammatory and, in the present study, there was a clear trend of higher expression of COX2 at 16-weeks post-challenge in both head kidney and heart and in all diets, significant in fish fed the FF1 diet. This was in agreement with previous studies in both humans (Steer and Corbett, 2003) and fish (Chettri et al., 2011) in which an enhanced expression of COX2 was reported along with cytokines involved in the inflammatory response. Furthermore, the present study also showed a trend of lower expression of COX2 in fish fed the functional feeds and that could indicate lower production of PGE_2 in these fish, consistent with results from *in vitro* studies in which increased levels of n-3 PUFA decreased production of PGE_2 (Calder, 2001).

As aforementioned EPA-derived eicosanoids are also produced through the action of ALOX5 and COX2 (Calder, 2007) so biosynthesis of anti-inflammatory eicosanoids, LTB_5 and PGE_3 could be also happening during the time-course of ASRV infection. However, changes on the expression of both enzymes in this study were correlated with the enhancement of the inflammatory response and therefore production of pro-inflammatory eicosanoids derived from ARA, was more likely to be happening at those later stages of the viral infection. In line with this, no changes on the EPA-derived eicosanoids were reported in turbot despite different levels of this FA on the phospholipids of the cell membranes of different tissues (Bell

et al.,1998a) however production of LTB4 and PGE2 was inversely correlated with the levels of EPA/ARA on that polar fraction of the cell membranes. Furthermore, based on the results obtained on the latter study and the findings of a previous study using primary cultures of turbot astrocytes where supplementing EPA suppressed the production of PGE_2 (Bell et al., 1994) they concluded that the role of EPA in attenuating the effects of AA-derived eicosanoids largely involves interfering with the production of the latter rather than forming eicosanoids in its own right.

In conclusion, this study on HSMI (ASRV infection) in salmon is the first to evaluate the expression of a suite of important genes involved in several pathways relating diet and membrane phospholipid LC-PUFA compositions, and the inflammatory response after a viral infection. Previously, we showed the potential benefits of using diets with reduced dietary lipid and increased EPA on disease outcomes (Martinez-Rubio et al., 2012), and the present study has supported the earlier results with data on the LC-PUFA status of membrane phospholipids during the course of the infection. In addition, however, the present study has demonstrated that liver was also a site for histopathological lesions in HSMI. The observed steatosis in liver likely reflects a general impairment of lipid metabolism. However, the higher expression of hepatic Δ6 and Δ5 desaturases in fish fed the FF diets would increase their capacity for the endogenous production and availability of anti-inflammatory EPA. Thus, mobilisation of lipids, changes in the LC-PUFA composition of membrane PC and PE, along with the changes in the expression of the genes related to eicosanoid pathways, showed the important role of the head kidney in inflammatory diseases caused by viral infections. Further studies will be required to elucidate the crucial role of the production of eicosanoids at different stages of the inflammatory process, especially at the resolution of inflammation. However, the effects on gene expression reported in the present study further suggests that, as

in humans, clinical nutrition through functional feeding could be an effective complementary therapy for emerging salmon viral diseases associated with long-term inflammation.

Acknowledgements

We would like to thank the technical staff in EWOS Innovation at Lønningdal and the Technology Centre in Dirdal for fish husbandry pre-challenge and producing the feeds respectively. We also thank Ragnhild Hanche-Olsen for sampling salmon heart for isolation of PRV, Tove Hansen for assistance with salmon tissue sampling, and Even G. Nordstrøm at ILAB for assistance during the same.

Chapter 4

Effect of dietary lipid content on expression of genes of lipid metabolism and LC-PUFA biosynthesis in liver of Atlantic salmon (*Salmo salar* L.)

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Abstract

The relationship between lipid and digestible energy content of the feed and growth performance has been exploited with great effect in Atlantic salmon (*Salmo salar*). The precise metabolic consequences of so-called "high-energy" feeds have not been fully defined, but increased and altered tissue lipid deposition patterns impacting on carcass and product quality have been reported. Recent studies on global gene expression have shown that dietary lipid and digestible energy content can have significant effects on gene expression in salmonids. In addition, we recently showed that functional feeds with reduced digestible energy significantly improved outcomes in response to inflammatory disease in salmon. The present study aimed to elucidate and clarify the effects of dietary digestible energy content (22, 20 and 18 MJ/Kg; HE, ME and LE diets, respectively) on lipid and fatty acid metabolism in salmon fed diets containing graded amounts of lipid. Specifically the effects on tissue lipid and fatty acid composition and on the hepatic expression of genes of lipid and fatty acid metabolism were determined. Final weight and weight gain were significantly higher, and FCR lower, in fish fed the HE diet. Crude lipid content was significantly lower in fish fed the LE diet compared to fish fed the two higher energy contents. Significantly lower total lipid and triacylglycerol levels were recorded in liver and heart of fish fed the LE diet compared to fish fed the higher energy diets. Tissue lipids in salmon fed the LE diet had generally significantly higher proportions of saturated fatty acids and long-chain polyunsaturated fatty acids (LC-PUFA), and lower monounsaturated fatty acids, C18 and n-6 PUFA. Consistent with this, salmon fed the LE diet showed increased liver expression of both Δ6 and Δ5 fatty acyl desaturases in comparison to fish fed the diets with higher energy levels. Fatty acid synthase expression showed a clear upward trend as dietary energy decreased, and sterol regulatory element binding protein 2 and liver X

receptor showed reciprocal trends that were consistent with the level of dietary cholesterol that reflects digestible energy content. Although not statistically significant, these trends were biologically logical, significant and relevant. Expression of genes of fatty acid oxidation was less consistent. Overall, reduced dietary digestible energy/lipid content alone, without major changes in dietary fatty acid composition, altered the expression of key genes of lipid and fatty acid metabolism resulting in general up-regulation of biosynthetic pathways. The results suggest that the beneficial effects of a clinical nutrition approach to improving inflammatory disease outcomes, through the use of functional feeds with reduced digestible energy, include alterations in lipid and fatty acid metabolism that may underpin effects on inflammatory responses.

4.1 Introduction

Lipid is required in the diet of fish to supply both metabolic energy and some essential nutrients such as specific (essential) fatty acids (EFA) and, in early life stages, possibly cholesterol and intact phospholipid (Sargent et al., 2002). Consequently, it is not possible to define a single dietary lipid requirement for any species as this will vary depending upon the other dietary components supplying energy, such as protein and carbohydrate (NRC, 2011). Although an optimum level of dietary lipid cannot be defined, there are lower and upper limits within which dietary lipid should be supplied. The lower limit is the level required to supply the requirements for essential components like EFA, and will depend upon the precise lipid class and fatty acid compositions of the dietary lipid sources. Increasing dietary lipid above this minimum level will support higher growth rates due to the simple fact that lipid supplies twice as much energy per unit mass compared to other dietary energy sources, and so more energy can be supplied in the feed per unit mass, a phenomenon often referred to as "protein sparing" (Hemre et al., 1995; Bendiksen et al., 2003). However, an upper limit will be reached where the biochemical and physiological capacities of the animal to digest and/or metabolise dietary lipid is exceeded leading to reduced digestibility (undigested lipid in faeces) and/or unwanted deposition of lipid in the peritoneal cavity, liver, or other tissues (Company et al., 1999; Craig et al., 1999; Gaylord and Gatlin, 2000).

This relationship between lipid and digestible energy (DE) content of the feed and growth performance has been particularly exploited in species such as Atlantic salmon (*Salmo salar*), which deposit significant amounts of lipid in the flesh and thus are able to tolerate and utilize high lipid, such that the dietary levels have increased steadily over the years (Sargent et al., 2002). Although excess deposition and altered tissue lipid

deposition patterns are known to cause various problems in terms of carcass and product quality in farmed fish like salmon (Sargent et al., 2002), the precise metabolic consequences of so-called "high-energy" feeds have not been fully defined. However, recent studies looking at global gene expression using transcriptomic (microarray) and proteomic approaches have shown that dietary lipid and DE content can have significant effects on gene expression in salmonids (Kolditz et al., 2008a,b; Panserat et al., 2008; Higgs et al., 2009).

Recently, we investigated the effects of functional feeds, which included alteration of dietary lipid and DE content, in the control of Heart and Skeletal Muscle Inflammatory (HSMI) disease in Atlantic salmon experimentally infected with the causative agent, Atlantic salmon reovirus (ASRV) (Martinez-Rubio et al., 2012). The concept of clinical nutrition and functional foods is well known in human nutrition. These are defined as foods that contain a component (whether a nutrient or not) that could be beneficial for the state of well-being and health, or reduce the risk of a disease, beyond the basic nutritional requirement (Bellisle et al., 1980). This approach is also becoming increasingly used in aquaculture, as it could potentially lead to economic savings in terms of increased productivity and lower costs of disease treatment/management (Raynard et al., 1991; McCoy et al., 1994; Tacchi et al., 2011). In the HSMI study, we investigated the effects of functional feed formulations that contained reduced energy levels through lower lipid contents (18%) and altered levels of long-chain polyunsaturated fatty acids (LC-PUFA), including increased eicosapentaenoic acid (EPA, 20:5n-3) in comparison to a standard commercial feed that contained 31% lipid. A much reduced inflammatory response to ASRV infection, and reduced severity of heart lesions were found in fish fed the functional feeds, and transcriptome (microarray) analysis of heart showed that expression of

inflammation/immune related genes was greatly affected. However, in addition to effects on immune genes, it was clear that the feeds were also having significant effects on the expression of metabolic genes in the heart, including those of lipid and fatty acid metabolism (Martinez-Rubio et al., 2012). However, it was not clear whether dietary DE and lipid content or fatty acid composition were primarily responsible for the alterations in metabolic gene expression.

The primary objective of the present study was to elucidate and clarify the effects of dietary DE on lipid and fatty acid metabolism in Atlantic salmon fed diets containing graded amounts of lipid. Specifically the effects on tissue lipid and fatty acid composition, and on the hepatic expression of genes of lipid and fatty acid metabolism were determined. Lipid compositions of liver, heart and flesh, representing the main tissue of intermediary metabolism, a lipid-utilising tissue, and the farmed product, were determined. The expression of key genes involved in the major lipid metabolic pathways including lipogenesis (fatty acid synthase, FAS), fatty acid β-oxidation (carnitine palmitoyl transferase-1, CPT1; acyl CoA oxidase, ACO), and LC-PUFA biosyntheis (fatty acyl desaturases, *Δ6fad_a* and *Δ5fad*; fatty acid elongases, *elovl2*, e*lovl5a* and *elovl5b*), and the major transcription factors (liver X receptor, LXR; peroxisome proliferator-activated receptors, PPAR) and nuclear receptors (sterol-responsive element-binding protein, SREBP) controlling and regulating their expression, were investigated by quantitative real-time PCR (qPCR).

4.2 Materials and Methods

4.2.1 Fish and feeds

Three hundred and sixty pit-tagged Atlantic salmon (*Salmo salar* L.) post-smolts were distributed equally into 9 tanks of 1.5m diameter, 1.5m^3 volume (40 fish /tank) at the

University of Stirling, Marine Environmental Research Laboratory, (Machrihanish, Argyll, Scotland, U.K.). The tanks were supplied with flow-through seawater at ambient temperature, average 12 ^{o}C (± 1 ^{o}C). After a 3-week acclimatisation period, the fish in triplicate tanks were fed in excess (i.e. feed was not limiting) for 12 weeks with one of three feeds supplied by automatic feeders every 30 min 23 h per day. The three fishmeal-based diets were manufactured by EWOS Innovation (Dirdal, Norway), and were formulated to be isoproteic (40 % crude protein), but deliver three levels of DE being 22 (high, HE), 20 (medium, ME) and 18 (low, LE) MJ/Kg by replacing dietary oil (a 50:50 mix of fish and rapeseed oils) with starch (Table 4.1). The fatty acid compositions of the feeds reflected the formulations with trends of increased saturated fatty acids, 20:1n-9, 22:1n-11 and n-3 LC-PUFA, and decreased 18:1n-9, as DE decreased, reflecting the lower level of rapeseed oil and increased proportion of lipid derived from marine sources (Table 4.2). All the fish in each tank were individually weighed at the initiation of the experiment (415g average weight) and the mid-point and the feed ration adjusted to 0.9%. Lights and feeders were on 24h/day and waste feed was collected using an airlift system. Feed fed, waste feed, fish behaviour, and general health and mortalities were monitored daily as were water temperature and quality measurements.

4.2.2 Sampling

Fish were sampled at the end of the feeding period (12 weeks) with body weight and length, and liver and viscera weights recorded for all fish culled. A total of 15 fish/tank were anaesthetised (MS222) and killed by a blow to the head with three whole fish frozen immediately for analysis of proximate composition. Tissues for lipid and biomolecular analysis were collected from the remaining 12 fish. Thus, samples of flesh (Norwegian Quality Cut) were collected and frozen immediately on dry ice before

storage at –20 ^{o}C prior to lipid and fatty acid analyses. Similarly, samples of liver (for fatty acid and molecular analyses) and heart (lipid and fatty acid analyses) were collected and frozen immediately in liquid nitrogen and stored at -80 ^{o}C prior to analysis. Further samples of liver, specifically for lipid class analysis, were collected in 5ml glass vials containing 4 ml of chloroform/methanol (2:1, by volume).

Table 4.1. Formulation (g/Kg) proximate composition (percentage of wet weight) and digestible energy (MJ/Kg) of experimental feeds with high (HE), medium (ME) and low (LE) levels of digestible energy

	HE	ME	LE
Fish meal	350.0	350.0	350.0
Wheat grain	93.1	93.1	93.1
Wheat gluten	80.0	80.0	80.0
Soy protein conc.	80.0	80.0	80.0
Pea protein conc.	70.0	70.0	70.0
EWOS premix[1]	26.9	26.9	26.9
Soy lecithin	15.0	15.0	15.0
Starch (tapioca)	10.0	80.0	150.0
Fish oil	137.5	102.5	67.5
Rapeseed oil	137.5	102.5	67.5
Proximate composition			
Moisture	4.9	5.7	6.5
Dry matter	95.1	94.3	93.5
Crude protein	40.4	40.5	40.6
Crude lipid	33.9	27.0	20.0
Digestible energy	21.9	20.1	18.2

[1]EWOS premix including minerals, vitamins, inorganic phosphorus, lysine, methionine and astaxanthin to satisfy nutritional requirements (NRC, 2011)

Table 4.2 Fatty acid composition (percentage of total fatty acids) of total lipids of feeds containing high (HE), medium (ME) and low (LE) levels of digestible energy

	HE	ME	LE
14:0	3.3	3.5	3.7
16:0	10.0	10.2	11.4
18:0	1.9	1.8	1.9
Total saturated[1]	16.0	16.3	17.7
16:1n-7	3.2	3.5	3.8
18:1n-9	32.1	30.1	24.0
20:1n-9	7.0	7.3	7.8
22:1n-11	8.0	8.3	9.0
Total monoenes[2]	52.0	51.0	48.2
18:2n-6	12.3	12.1	12.6
20:4n-6	0.2	0.2	0.2
Total n-6 PUFA[3]	13.0	12.6	13.2
18:3n-3	5.0	4.8	4.2
18:4n-3	2.4	2.6	2.7
20:5n-3	4.4	4.8	5.2
22:6n-3	5.4	5.9	6.6
Total n-3 PUFA[4]	18.0	19.0	19.8
Total PUFA	32.0	32.7	34.1

Data are means of duplicate analyses. [1]Totals include 15:0, 20:0 and 22:0 at up to 0.3%; [2]Totals include 20:1n-7, 22:1n-9 and 24:1n-9 at up to 0.8%; [3]Totals include 18:3n-6, 20:2n-6, 20:3n-6, 22:4n-6 and 22:5n-6 at up to 0.2%; [4]Totals include 20:3n-3, 20:4n-3 and 22.5n-3 at up to 0.6%. PUFA, polyunsaturated fatty acid.

4.2.3 Growth performance and feed utilization

The effects of feeds on growth performance, biometry and feed utilization efficiency were calculated according to the following formulae. Weight gain (g/fish) = final weight – initial weight. Specific growth rate (SGR, % day) = 100 × [ln (final mean

weight) − ln (initial mean weight)] × days^{-1}. Feed consumption (g/day) = feed intake (g) × [number of fish × days]$^{-1}$, and Feed conversion ratio (FCR) = feed intake (g) × [final biomass − initial biomass + dead fish]$^{-1}$. Hepato-somatic index (HSI, %) = 100 × [weight of liver (g)] × [weight of fish (g)]$^{-1}$. Viscero-somatic index (VSI, %) = 100 × [weight of viscera (g)] × [weight of fish (g)]$^{-1}$

4.2.4 Proximate composition of feeds and fish

The proximate compositions of feeds and whole fish at the end of the trial were determined by standard procedures (AOAC, 2000). For fish, the three fish per tank were pooled and minced prior to analysis (n = 3 per dietary treatment). Moisture content was determined after drying to constant weight in an oven at 105 °C for 24 h. The samples were then rigorously blended into a homogeneous crumb and used for determination of feed or whole body lipid, protein and ash contents. Lipid content of dried crumb was determined using the Soxhlet method with extraction using petroleum ether at 120 °C (Avanti Soxtec 2050 Auto Extraction apparatus; Foss, Warrington, UK). Crude protein content (N × 6.25) was determined using the automated Kjeldahl method (Tecator Kjeltec Auto 1030 Analyser; Foss, Warrington, UK). Ash contents were determined after heating at 600 °C for 24 h. The gross energy content of the feeds was determined by Bomb Calorimetry (Gallenkamp Autobomb System).

4.2.5 Lipid content, lipid class and fatty acid compositions

Lipid content, lipid class and fatty acid compositions of total lipid were determined in liver, heart, and flesh. Tissue samples were pooled according to tank (n = 3). Total lipid from approximately 1g of pooled heart, liver and muscle (flesh) tissue was extracted by homogenization in chloroform/methanol (2:1, by volume) according to Folch et al. (1957), and determined gravimetrically. Tissue lipid class compositions

were determined by single-dimension double-development high-performance thin-layer chromatography (HPTLC) and densitometry (Henderson and Tocher, 1992). Fatty acid methyl esters (FAME) of total lipid were prepared by acid-catalyzed transmethylation (Christie, 2003), and separated and quantified by gas-liquid chromatography (Carlo Erba Vega 8160, Milan, Italy) using a 30m x 0.32 mm i.d. capillary column (CP Wax 52CB, Chrompak, London, U.K.) and on-column injection at 50^{o}C. Hydrogen was used as carrier gas and temperature programming was from 50 ^{o}C to 150 ^{o}C at 40 ^{o}C min^{-1} and then to 230 ^{o}C at 2.0 ^{o}C min^{-1}. Individual methyl esters were identified by comparison with known standards and by reference to published data (Ackman, 1980; Tocher and Harvie, 1988). Data were collected and processed using Chromcard for Windows (version 1.19).

4.2.6 Determination of gene expression by quantitative real-time PCR

Reverse transcription quantitative real-time PCR (qPCR) analysis was performed to evaluate the relative expression of genes involved in major lipid metabolism pathways including fatty acid synthesis (fatty acid synthase, FAS), LC-PUFA biosynthesis (fatty acyl desaturases, *Δ6fad_a* and *Δ5fad*; fatty acid elongases, *elovl2*, e*lovl5a* and *elovl5b*), and β-oxidation (carnitine palmitoyl transferase-1, CPT1; acyl CoA oxidase, ACO), and their control and regulation (sterol-responsive element-binding protein 2, SREBP2; liver X receptor, LXR; peroxisome proliferator-activated receptors, PPARα, PPARβ and PPARγ). The qPCR primer sequences (obtained by literature searches), annealing temperature (Tm) and size of amplicon are given in Table 4.3. In addition, amplification of cofilin-2 and elongation factor-1α Elf-1α) was performed and their expression was confirmed as sufficiently stable across treatments for normalization. These genes had been identified as suitable reference genes in previous qPCR studies in salmon (Morais et al., 2011).

Table 4.3 Sequences, annealing temperatures (Tm) and fragment sizes produced by the primer pairs used for real-time quantitative PCR (qPCR)

Transcript	Primer name	Primer sequence	Fragment (bp)	Tm (°C)	Accession No.	Source
Δ5fad	D5DES-F	5'-GTGAATGGGGATCCATAGCA-3'	192	56	AF478472 [1]	Hastings et al. (2005)
	D5DES-R	5'-AAACGAACGGACAACCAGA-3'				
Δ6fad_a	D6DES-F	5'-CCCCAGACGTTTGTGTCAG-3'	181	56	AY458652 [1]	Zheng et al. (2005a)
	D6DES-R	5'-CCTGGATTGTTGCTTTGGAT-3'				
elovl5a	Elo1UTR-SM-1F	5'-ACAAGACAGGAATCTCTTTCAGATTAA-3'	137	60	AY170327 [1]	Morais et al. (2009)
	Elo1UTR-SM-1R	5'-TCTGGGGTTACTGTGCTATAGTGTAC-3'				
elovl5b	Elo2UTR-5F	5'-ACAAAAAGCCATGTTTATCTGAAAGA-3'	141	60	DW546112 [1]	Morais et al. (2009)
	Elo2UTR-5R	5'-AAGTGGGTCTCTGGGGCTGTG-3'				
elovl2	Elo2-SM-1F	5'-CGGGTACAAAATGTGCTGGT-3'	145	60	TC91192 [2]	Morais et al. (2009)
	Elo2-SM-1R	5'-TCTGTTTGCCGATAGCCATT-3'				
SREBP2	SREBP2-1F	5'-GACAGGCACAACACAAGGTG-3'	215	60	DY733476 [1]	Leaver et al. (2008b)
	SREBP2-1R	5'-CAGCAGGGGTAAGGGTAGGT-3'				
cpt1	CPT1-1F	5'-CCTGTACCGTGGAGACCTGT-3'	212	60	AM230810 [1]	Leaver et al. (2008b)
	CPT1-1R	5'-CAGCACCTCTTTGAGGAAGG-3'				
aco	ACO-2F	5'-AAAGCCTTCACCACATGGAC-3'	230	60	TC49531 [2]	Leaver et al. (2008b)
	ACO-2R	5'-TAGGACACGATGCCACTCAG-3'				
PPARα	SsPPAR-A-F1	5- TCCTGGTGGCCTACGGATC-3'	111	60	DQ294237 [1]	Kleveland et al. (2006)
	SsPPAR-A-R1	5'-CGTTGAATTTCATGGCGAACT-3'				
PPARβ	SsPPAR-B-F1	5'-GAGACGGTCAGGGAGCTCAC-3'	151	60	AJ416953 [1]	Kleveland et al. (2006)
	SsPPAR-B-R1	5'-CCAGCAACCCGTCCTTGTT-3'				

Table 4.3 (Continuation)

Transcript	Primer name	Primer sequence	Fragment (bp)	Tm (°C)	Accession No.	Source
PPARγ	SsPPAR-G-F1	5'-CATTGTCAGCCTGTCCAGAC-3'	144	60	AJ416951 [1]	Kleveland et al. (2006)
	SsPPAR-G-R1	5'-TTGCAGCCCTCACAGACATG-3'				
FAS	SsFAS-F4	5'-GTGCCCACTGAATACCATCC-3'	212	60	CK876943 [1]	Morais et al.(2011)
	SsFAS-R4	5'-ATGAACCATTAGGCGGACAG-3'				
LXR	SsLXR-F	5'-GCCGCTGCTATCTGAAATCTG-3'	210	58	FJ470290	Cruz-Garcia et al. (2009)
	SsLXR-R	5'-CCATCCGGCAACCAATCTGTAGG-3'				
Reference genes:						
elf-1α	ELF-1A jbt2	5'-CTGCCCCTCCAGGACGTTTACAA-3'	175	60	AF321836 [1]	Morais et al. (2009)
	ELF-1A jbt2	5'-CACCGGGCATAGCCGATTCC-3'				
β-actin	BACT-F	5'-ACATCAAGGAGAAGCTGTGC-3'	141	56	AF012125 [1]	Morais et al. (2009)
	BACT-R	5'-GACAACGGAACCTCTCGTTA-3'				
Cofilin-2	B2F	5'-AGCCTATGACCAACCCACTG-3'	224	60	TC63899 [2]	Morais et al. (2009)
	B2R	5'-TGTTCACAGCTCGTTTACCG-3'				

[1] GenBank (http://www.ncbi.nlm.nih.gov/)
[2] Atlantic salmon Gene Index (http://compbio.dfci.harvard.edu/tgi/)

For qPCR, 2 µg of column-purified total RNA per sample was reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Paisley, U.K.), following manufacturer's instructions, but using a mixture of the random primers (1.5µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/ µl, Eurofins MWG Operon, Ebersberg, Germany). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination. cDNA was then diluted 20-fold with water, after a similar amount of cDNA was pooled from all samples. qPCR analysis used relative quantification with the amplification efficiency of the primer pairs being assessed by serial dilutions of the cDNA pool. qPCR amplifications were carried out in duplicate (Quantica, Techne, Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL (for most genes) or 2 µL (for the reference genes and other highly expressed genes) diluted (1/20) cDNA, 0.5 µM of each primer and 10 µL AbsoluteTM QPCR SYBR® Green mix (ABgene). Amplifications were carried out with a systematic negative control (NTC-non template control, containing no cDNA). The qPCR profiles contained an initial activation step at 95 °C for 15 min, followed by 30 to 40 cycles: 15s at 95 °C, 15 s at the specific primer pair annealing Tm and 15 s at 72 °C. After the amplification phase, a melt curve of 0.5 °C increments from 75 ºC to 90 °C was performed, enabling confirmation of amplification of single products, and sizes were checked by agarose gel electrophoresis and identities confirmed by sequencing. Non-occurrence of primer-dimer formation in the NTC was also verified. Data were analyzed using the relative expression software tool (REST 2009, http://www.gene-quantification.info/), which employs a pair wise fixed reallocation randomization test (10,000 randomizations) with efficiency correction, to determine the statistical significance of expression ratios (or gene expression fold-changes) between two treatments (Pfaffl et al., 2002).

4.2.7 Statistical analyses

The effects of dietary treatments on fish growth performance, feed efficiency, biometry, tissue lipid contents, class and fatty acid compositions were analysed by Analysis of Covariance (ANCOVA) with fish weight (or pooled fish weights) as the covariate. Briefly, the weight gain based on repeated weights on individual fish was modelled by fitting individual growth trajectories with the help of a multilevel model using the fish tag ID, tank and treatments (DE levels) as the levels of variation. Time dimension was added to the model by the day of weighing since the start of the trial and its effect was modelled with the help of cubic splines. FCR was calculated by dividing the observed feed intake by the observed weight gain and log-transformed before conducting stats. Differences in body length, liver and visceral weights between diets were analysed including fish body weight as a covariate to account for fish size differences at the end of the trial. Afterwards the condition factor, hepatosomatic and visceral indices were calculated based on the model estimates. Differences in proximal composition analyses of the fish, fatty acid profiles and lipid class composition in the flesh, heart and liver were also analysed statistically including a covariate. Since these analyses were conducted on pool samples the average weight of each fish pool was used as a covariate in order to account for size differences. Multilevel models were fitted with the lme4 package of the R language (R Development Core Team 2008). All treatment effects were based on posterior simulation (n = 2,500) with 95 % credible intervals. Ninety-five per cent credible intervals were interpreted as statistical significant at p = 0.05 % level when the interval did not overlap the reference value in question.

4.3 Results

4.3.1 Growth performance and body composition

The salmon more than doubled their weight over the period of the trial with final weights and weight gain showing clear effects of DE content and so both were significantly higher in fish fed the HE diet compared to fish fed the diets with lower DE (Table 4.4). Similarly, FCR showed an increasing trend as DE decreased and so was significantly lower in fish fed the HE diet compared to fish fed the diets with lower DE. Both HSI and VSI tended to be lower in fish fed the LE diet with the lowest DE, but the differences were not statistically significant (Table 4.4). Crude lipid of whole fish showed a clear trend with DE content with the content being significantly lower in fish fed diet LE compared to fish fed the ME and HE diets (Table 4.4).

4.3.2 *Tissue lipid and fatty acid compositions*

The lipid contents of liver and heart showed clear relationships with dietary DE content, increasing as DE increased, with tissue of fish fed HE having significantly higher lipid contents (Tables 4.5 & 4.6). Although this association between dietary DE content and tissue lipid content was not observed in muscle (flesh), there was a clear significant increase in storage lipid, triacylglycerol (TAG), in muscle tissue as dietary DE increased (Table 4.7). Similarly, the lower lipid contents in liver and heart of fish fed the diets with lower DE were reflected in lower levels of TAG, with significantly lower levels in fish fed diet LE (Tables 4.5 & 4.6). Consistent with this, tissue polar lipids (phospholipids) and cholesterol, reflecting membrane lipids, were generally significantly higher in salmon fed the LE diet (Tables 4.5-4.7). The effects of the different feeds on fatty acid compositions were similar in all three tissues investigated with salmon fed diet LE showing generally increased saturated fatty acids and LC-

PUFA, and decreased monounsaturated fatty acids, C18 PUFA and n-6 PUFA (Tables 4.8-4.10). Thus, 16:0, 18:0, EPA and DHA were all generally increased, whereas, 18:1n-9, 18:2n-6 and 18:3n-3 were generally decreased in liver, heart and muscle of salmon fed diet LE compared to the tissues in fish fed the HE diet. Due to the variation observed in the data not all of these effects were statistically significant in each case, but the clear consistent pattern observed in all tissues supported the overall conclusion.

4.3.3 Liver gene expression

Salmon fed the LE diet showed significantly increased liver expression of both Δ6 and Δ5 fatty acyl desaturases (Fad) in comparison to fish fed the diets with higher energy levels (Figure 4.1). In contrast, there were no significant effects of diet on the expression of fatty acyl elongases (Elovl2 or Elovl5 transcripts) (Figure 4.2). There was a clear effect of dietary energy upon fatty acid synthase (FAS) expression, which increased with reducing DE albeit that the relatively large standard deviations rendered the effect non-significant statistically (Figure 4.3A). There was no effect of dietary energy on carnitine palmitoyl transferase 1 (CPT-1), a marker of mitochondrial fatty acid β-oxidation, and although it appeared as though the expression of acyl-CoA oxidase (ACO), a marker of peroxisomal fatty acid oxidation, was higher in fish fed the LE diet, this was not significant (Figure 4.3B & C). Although statistically non-significant, reciprocal trends in the expression of sterol regulatory element binding protein 2 (SREBP2) and liver X receptor (LXR) with DE were observed (Figure 4.4). Similarly, the liver expression of all three peroxisome proliferator-activated receptor subtypes (α, β and γ) tended to show increasing expression as dietary DE decreased, but none of the differences were statistically significant (Figure 4.5).

Table 4.4 Growth performance, feed efficiency, biometry and proximate composition of salmon fed diets containing high (HE), medium (ME) or low (LE) levels of digestible energy. Values between brackets indicate 95%CI.

	HE	**ME**	**LE**
Initial weight (g)	420.3	420.1	407.3
	(405.9, 435.0)	(405.8, 435.3)	(392.9, 421.7)
Final weight (g)	952.5	900.4	877.4
	(910.2, 993.4)	(860.4, 945.1)	(835.5, 921.0)
Weight gain (g)	532.3	480.3	470.2
	(492.6, 569.6)	(443.9, 520.3)	(431.2, 511.4)
FCR	0.67	0.71	0.82
	(0.63, 0.70)	(0.68, 0.74)	(0.78, 0.86)
HSI	1.27	1.30	1.25
	(1.13, 1.41)	(1.19, 1.40)	(1.12, 1.39)
VSI	10.89	11.22	10.60
	(10.50, 11.31)	(10.91, 11.54)	(10.21, 10.98)
Condition factor	1.43	1.34	1.35
	(1.53, 1.33)	(1.42, 1.26)	(1.45, 1.26)
Proximate composition (percentage of wet weight) [1]			
Moisture	65.4	66.4	66.8
	(64.2, 66.6)	(65.2, 67.5)	(65.7, 67.9)
Dry matter	34.6	33.6	33.2
	(33.4, 35.8)	(32.5, 34.7)	(32.0, 34.4)
Crude protein	17.1	17.2	17.3
	(16.4, 17.7)	(16.5, 17.8)	(16.7, 18.0)
Crude lipid	14.2	12.7	10.9
	(12.3, 16.0)	(10.9, 14.5)	(9.0, 12.6)
Ash	1.9	1.7	2.0

Data are means (n=3). Upper and lower limits for 95% credible intervals (CI) are in parenthesis. Ninety-five percent CI were interpreted as statistically significant at P=0.05% level when the interval did not overlap the reference value in question. [1]Proximate compositions adjusted for fish weight at the end of the trial.

Table 4.5 Lipids content **(**percentage of wet weight) and lipid class composition (percentage of total lipid) of liver of salmon fed diets containing high (HE), medium (ME) or low (LE) levels of digestible energy. Values between brackets indicate 95%CI.

	Diet		
	HE	ME	LE
Lipid content	7.0	6.3	5.5
	(5.6, 8.4)	(5.7, 6.9)	(4.7, 6.3)
Lipid class			
PC	18.8	19.5	21.4
	(17.8, 19.9)	(18.6, 20.3)	(20.5, 22.4)
PE	10.2	10.9	12.0
	(9.7, 10.6)	(10.5, 11.3)	(11.6, 12.5)
PI	3.4	3.2	3.6
	(2.9, 3.9)	(2.8, 3.6)	(3.2, 4.0)
PS	2.1	1.9	2.5
	(1.9, 2.4)	(1.7, 2.1)	(2.28, 2.7)
CL/PG	2.1	2.0	2.4
	(1.8, 2.4)	(1.7, 2.3)	(2.1, 2.8)
Sphingomyelin	1.6	1.8	2.1
	(1.3, 1.9)	(1.5, 2.1)	(1.8, 2.4)
LPC	0.1	0.1	0.2
	(0.0, 0.4)	(0.0, 0.3)	(0.0, 0.5)
Total polar lipid	38.3	39.4	44.3
	(36.3, 40.3)	(37.7, 41.1)	(42.6, 46.2)
Total neutral lipid	61.7	60.7	55.7
	(59.6, 63.7)	(59.0, 62.4)	(54.0, 57.5)
Triacylglycerol	42.7	42.6	35.4
	(39.8, 45.5)	(40.1, 45.2)	(32.8, 38.1)
Cholesterol	11.8	11.6	12.5
	(11.0, 12.5)	(11.0, 12.3)	(11.8, 13.2)
Free fatty acid	0.4	0.3	0.6
	(0.0, 0.7)	(0.0, 0.6)	(0.4, 1.0)
Steryl ester	6.8	6.1	7.0
	(5.1, 8.4)	(4.6, 7.6)	(5.4, 8.6)

Data are means (n=3). Upper and lower limits for 95% credible intervals (CI) are in parenthesis. Ninety-five percent CI were interpreted as statistically significant at P=0.05% level when the interval did not overlap the reference value in question. All effects adjusted for fish weight at the end of the trial. CL, cardiolipin; LPC, lyso-PC; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 4.6 Lipids content **(**percentage of wet weight) and lipid class composition (percentage of total lipid) of heart of salmon fed diets containing high (HE), medium (ME) or low (LE) levels of digestible energy. Values between brackets indicate 95%CI.

	Diet		
	HE	ME	LE
Lipid content	4.7	3.9	3.3
	(3.7, 5.6)	(3.4, 4.3)	(2.7, 3.9)
Lipid class			
PC	13.5	12.3	13.5
	(12.6, 14.4)	(11.4, 13.1)	(12.7, 14.3)
PE	13.8	12.9	14.7
	(13.0, 14.7)	(12.2, 13.4)	(14.0, 15.4)
PI	3.4	3.3	3.8
	(3.0, 3.9)	(2.9, 3.8)	(3.3, 4.2)
PS	2.1	2.0	2.3
	(2.0, .2.2)	(2.0, 2.3)	(2.0, 2.4)
CL/PG	5.8	6.3	6.6
	(4.6, 7.5)	(5.1, 7.8)	(5.4, 8.2)
Sphingomyelin	1.5	1.6	1.7
	(1.3, 1.8)	(1.4, 1.8)	(1.5, 1.9)
LPC	0.1	0.1	0.1
	(0.0, 0.3)	(0.0, 0.3)	(0.0, 0.3)
Total polar lipid	40.6	38.6	42.9
	(37.6, 43.6)	(35.7, 41.4)	(40.0, 45.8)
Total neutral lipid	59.4	61.4	57.1
	(56.3, 62.3)	(58.6, 64.1)	(54.3, 60.0)
Triacylglycerol	39.2	41.5	37.3
	(35.8, 42.5)	(38.5, 44.4)	(34.4, 40.2)
Cholesterol	12.4	11.8	12.6
	(12.0, 12.8)	(11.5, 12.1)	(12.2, 12.9)
Free fatty acid	5.8	5.8	5.8
	(3.9, 7.8)	(3.9, 7.7)	(3.9, 7.6)
Steryl ester	2.2	2.2	1.5
	(0.2, 4.2)	(0.4, 4.0)	(0.0, 3.3)

Data are means (n=3). Upper and lower limits for 95% credible intervals (CI) are in parenthesis. Ninety-five percent CI were interpreted as statistically significant at P=0.05% level when the interval did not overlap the reference value in question. All effects adjusted for fish weight at the end of the trial. CL, cardiolipin; LPC, lyso-PC; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 4.7 Lipids content **(**percentage of wet weight) and lipid class composition (percentage of total lipid) of flesh of salmon fed diets containing high (HE), medium (ME) or low (LE) levels of digestible energy. Values between brackets indicate 95%CI.

	Diet		
	HE	ME	LE
Lipid content	3.7 (0.8, 6.6)	5.5 (4.1, 6.8)	5.3 (3.4, 7.1)
Lipid class			
PC	6.8 (6.0, 7.6)	7.5 (6.8, 8.1)	8.0 (7.3, 8.7)
PE	5.3 (4.7, 5.9)	5.9 (5.3, 6.4)	6.4 (5.9, 7.0)
PI	1.7 (1.5, 2.0)	1.8 (1.6, 2.1)	2.2 (1.9, 2.4)
PS	0.9 (0.4, 1.3)	1.1 (0.7, 1.5)	1.1 (0.7, 1.5)
CL/PG	1.1 (0.9, 1.4)	1.2 (1.0, 1.5)	1.4 (1.2, 1.7)
Sphingomyelin	0.7 (0.6, 0.8)	0.8 (0.7, 0.9)	0.6 (0.5, 0.7)
LPC	0.3 (0.0, 0.7)	0.4 (0.1, 0.8)	1.0 (0.6, 1.4)
Total polar lipid	16.9 (15.0, 18.9)	18.7 (17.0, 20.4)	20.8 (19.1, 22.5)
Total neutral lipid	83.1 (81.3, 85.0)	81.3 (79.7, 83.1)	79.2 (77.6, 81.0)
Triacylglycerol	69.1 (67.0, 71.1)	66.0 (64.2, 67.7)	62.8 (61.1, 64.5)
Cholesterol	7.5 (6.6, 8.4)	8.4 (7.5, 9.3)	8.7 (7.8, 9.6)
Free fatty acid	6.7 (5.6, 7.8)	7.0 (6.0, 7.9)	7.8 (6.8, 8.8)
Steryl ester	0.0 (0, 0)	0.0 (0, 0)	0.0 (0, 0)

Data are means (n=3). Upper and lower limits for 95% credible intervals (CI) are in parenthesis. Ninety-five percent CI were interpreted as statistically significant at P=0.05% level when the interval did not overlap the reference value in question. All effects adjusted for fish weight at the end of the trial. CL, cardiolipin; LPC, lyso-PC; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine.

Table 4.8 Fatty acid composition (percentage of total fatty acids) of total lipid of liver of salmon fed diets with high (HE), medium (ME) and low (LE) levels of digestible energy. Values between brackets indicate 95%CI.

	Diet		
	HE	ME	LE
14:0	1.5	1.7	1.5
	(1.1, 1.9)	(1.6, 1.9)	(1.2, 1.7)
16:0	9.9	11.2	13.6
	(8.2, 11.5)	(10.5, 11.9)	(12.6, 14.6)
18:0	3.7	4.2	5.3
	(3.2, 4.3)	(4.0, 4.5)	(5.0, 5.6)
Total saturated[1]	15.5	17.5	20.6
	(13.6, 17.4)	(16.7, 18.4)	(19.5, 21.8)
16:1n-7	2.9	3.2	4.0
	(2.1, 3.5)	(2.8, 3.5)	(3.5, 4.4)
18:1n-9	32.1	30.7	30.2
	(27.5, 36.7)	(28.6, 32.7)	(27.4, 32.9)
18:1n-7	2.6	2.7	2.4
	(1.9, 3.4)	(2.36, 3.0)	(1.9, 2.8)
20:1n-9	6.7	6.4	4.8
	(5.9, 7.6)	(6.0, 6.8)	(4.2, 5.3)
22:1n-11	1.9	2.0	1.5
	(1.6, 2.3)	(1.9, 2.2)	(1.3, 1.7)
Total monounsaturated[2]	47.8	46.6	44.5
	(42.0, 53.5)	(44.1, 49.2)	(40.9, 48.0)
18:2n-6	7.7	6.6	5.0
	(6.9, 8.6)	(6.2, 7.0)	(4.5, 5.5)
20:2n-6	1.7	1.5	1.1
	(1.5, 1.9)	(1.4, 1.6)	(1.0, 1.2)
20:3n-6	0.4	0.5	0.7
	(0.0, 0.7)	(0.4, 0.6)	(0.5, 0.8)
20:4n-6	0.7	0.7	0.8
	(0.5, 0.9)	(0.7, 0.8)	(0.7, 0.9)
Total n-6 PUFA[3]	10.9	9.7	7.8
	(9.8, 12.1)	(9.2, 10.2)	(7.0, 8.4)
18:3n-3	2.4	1.9	1.1
	(2.1, 2.7)	(1.8, 2.0)	(0.9, 1.2)
20:4n-3	1.6	1.5	1.0
	(1.4, 1.8)	(1.4, 1.6)	(0.9, 1.1)
20:5n-3	4.8	5.0	5.0
	(3.7, 5.9)	(4.5, 5.5)	(4.4, 5.7)
22:5n-3	1.2	1.5	1.6
	(1.0, 1.5)	(1.4, 1.6)	(1.4, 1.8)
22:6n-3	14.6	15.2	17.6
	(10.5, 18.8)	(13.4, 16.8)	(15.2, 20.1)
Total n-3 PUFA[4]	25.4	25.8	26.8
	(20.5, 30.4)	(23.6, 28.1)	(23.9, 29.8)
Total PUFA	36.9	35.9	34.9
	(32.2, 41.6)	(33.9, 38.0)	(32.0, 37.6)
n-3/n-6	2.3	2.7	3.4
	(1.8, 3.0)	(2.4, 3.0)	(3.0, 4.0)

Data are means (n=3). Upper and lower limits for 95% credible intervals (CI) are in parenthesis. Ninety-five percent CI were interpreted as statistically significant at P=0.05% level when the interval did not overlap the reference value in question. effects adjusted for fish weight at the end of the trial. [1]Totals include 15:0, 20:0 and 22:0 at up to 0.2%; [2]Totals include 16:1n-9, 20-1n-7, 22:1n-9 and 24:1n-9 at up to 0.8%; 3Totals include 18:3n-6, 22:4n-6 and 22:5n-6 at up to 0.2%; 4Totals include 18:4n-3 and 20:3n-3 at up to 0.5%. PUFA, polyunsaturated fatty acid.

Table 4.9 Fatty acid composition (percentage of total fatty acids) of total lipid of heart of salmon fed diets with high (HE), medium (ME) and low (LE) levels of digestible energy. Values between brackets indicate 95%CI.

	Diet		
	HE	ME	LE
14:0	2.4	2.2	2.2
	(1.9, 3.0)	(2.0, 2.5)	(1.9, 2.5)
16:0	15.4	16.1	17.6
	(14.1, 16.7)	(15.6, 16.7)	(16.8, 18.4)
18:0	3.5	3.8	4.0
	(3.2, 4.0)	(3.6, 3.9)	(3.7, 4.2)
Total saturated[1]	21.9	22.6	24.3
	(20.3, 23.5)	(21.9, 23.3)	(23.3, 25.2)
16:1n-7	2.7	2.5	2.7
	(2.0, 3.5)	(2.2, 2.9)	(2.3, 3.2)
18:1n-9	20.6	17.8	15.1
	(18.1, 23.0)	(16.7, 18.9)	(13.7, 16.6)
18:1n-7	2.6	2.5	2.5
	(2.4, 2.8)	(2.42, 2.6)	(2.4, 2.64)
20:1n-9	4.7	4.3	3.7
	(4.1, 5.3)	(4.0, 4.6)	(3.3, 4.1)
22:1n-11	3.6	3.3	2.8
	(3.0, 4.3)	(3.0, 3.6)	(2.4, 3.1)
Total monounsaturated[2]	35.8	32.0	28.2
	(31.4, 40.2)	(30.1, 34.0)	(25.5, 30.7)
18:2n-6	7.2	6.5	5.5
	(6.5, 7.9)	(6.2, 6.8)	(5.1, 5.9)
20:2n-6	0.6	0.6	0.7
	(0.5, 0.8)	(0.6, 0.7)	(0.6, 0.8)
20:3n-6	0.3	0.3	0.4
	(0.2, 0.3)	(0.3, 0.3)	(0.4, 0.4)
20:4n-6	0.7	0.8	0.9
	(0.5, 0.8)	(0.8, 0.9)	(0.8, 1.0)
Total n-6 PUFA[3]	9.2	8.7	8.0
	(8.5, 9.8)	(8.4, 9.0)	(7.6, 8.4)
18:3n-3	2.6	2.3	1.8
	(2.3, 2.8)	(2.2, 2.4)	(1.6, 1.9)
18:4n-3	0.9	0.8	0.7
	(0.6, 1.1)	(0.7, 0.9)	(0.5, 0.8)
20:4n-3	1.0	1.1	1.1
	(0.9, 1.1)	(1.1, 1.1)	(1.0, 1.1)
20:5n-3	5.8	6.9	7.6
	(4.9, 6.8)	(6.5, 7.3)	(7.0, 8.1)
22:5n-3	1.7	2.0	2.3
	(1.4, 1.9)	(1.9, 2.1)	(2.2, 2.5)
22:6n-3	20.5	22.9	25.5
	(15.5, 25.3)	(20.8, 25.0)	(22.5, 28.5)
Total n-3 PUFA[4]	32.7	36.2	39.1
	(26.9, 38.5)	(33.7, 38.7)	(35.8, 42.5)
Total PUFA	42.3	45.4	47.5
	(37.3, 47.5)	(43.2, 47.7)	(44.4, 50.6)
n-3/n-6	3.6	4.2	4.9
	(2.7, 4.5)	(3.8, 4.6)	(4.3, 5.4)

Data are means (n=3). Upper and lower limits for 95% credible intervals (CI) are in parenthesis. Ninety-five percent CI were interpreted as statistically significant at P=0.05% level when the interval did not overlap the reference value in question. effects adjusted for fish weight at the end of the trial. [1]Totals include 15:0, 20:0 and 22:0 at up to 0.2%; [2]Totals include 16:1n-9, 20-1n-7, 22:1n-9 and 24:1n-9 at up to 0.8%; 3Totals include 18:3n-6, 22:4n-6 and 22:5n-6 at up to 0.2%; 4Totals include 18:4n-3 and 20:3n-3 at up to 0.5%. PUFA, polyunsaturated fatty acid.

Table 4.10 Fatty acid composition (percentage of total fatty acids) of total lipid of flesh of salmon fed diets with high (HE), medium (ME) and low (LE) levels of digestible energy. Values between brackets indicate 95%CI.

	Diet		
	HE	ME	LE
14:0	3.1	3.4	3.7
	(2.3, 3.9)	(3.1, 3.8)	(3.2, 4.2)
16:0	12.2	13.0	14.9
	(11.4, 12.9)	(12.7, 13.4)	(14.4, 15.3)
18:0	2.6	2.8	3.3
	(2.4, 2.8)	(2.8, 2.9)	(3.2, 3.4)
Total saturated[1]	18.4	19.8	22.6
	(17.0, 19.6)	(19.2, 20.3)	(21.8, 23.4)
16:1n-7	3.7	4.3	5.1
	(3.2, 4.3)	(4.0, 4.5)	(4.8, 5.4)
18:1n-9	27.5	26.2	24.2
	(25.8, 29.2)	(25.5, 26.9)	(23.2, 25.1)
18:1n-7	2.7	2.3	2.9
	(1.9, 3.5)	(2.0, 2.7)	(2.7, 3.0)
20:1n-9	6.1	6.1	5.7
	(5.7, 6.6)	(5.9, 6.3)	(5.5, 6.0)
22:1n-11	5.3	5.0	4.8
	(3.7, 6.9)	(4.3, 5.7)	(3.9, 5.8)
Total monounsaturated[2]	46.6	46.0	44.1
	(44.1, 48.8)	(45.0, 47.0)	(42.8, 45.4)
18:2n-6	9.8	9.1	8.1
	(9.3, 10.3)	(8.9, 9.3)	(7.8, 8.4)
20:2n-6	0.8	0.8	0.8
	(0.8, 0.9)	(0.7, 0.8)	(0.8, 0.8)
20:3n-6	0.2	0.2	0.3
	(0.1, 0.4)	(0.1, 0.3)	(0.2, 0.4)
20:4n-6	0.4	0.4	0.4
	(0.3, 0.4)	(0.4, 0.4)	(0.4, 0.5)
Total n-6 PUFA[3]	11.6	10.7	10.0
	(11.1, 12.1)	(10.5, 11.0)	(9.6, 10.3)
18:3n-3	3.6	3.2	2.4
	(3.4, 3.8)	(3.1, 3.3)	(2.3, 2.5)
18:4n-3	1.4	1.5	1.4
	(1.3, 1.5)	(1.4, 1.5)	(1.3, 1.4)
20:4n-3	1.2	1.3	1.2
	(1.1, 1.3)	(1.21, 1.3)	(1.1, 1.2)
20:5n-3	4.4	4.9	5.3
	(3.8, 5.1)	(4.6, 5.2)	(5.0, 5.6)
22:5n-3	1.6	1.8	2.1
	(1.1, 2.2)	(1.6, 2.1)	(1.8, 2.4)
22:6n-3	10.0	9.5	9.9
	(7.8, 12.1)	(8.6, 10.5)	(8.6, 11.2)
Total n-3 PUFA[4]	22.7	22.5	22.2
	(20.1, 25.2)	(21.5, 23.6)	(20.8, 23.6)
Total PUFA	35.0	34.3	33.2
	(32.3, 37.6)	(33.1, 35.4)	(31.8, 34.7)
n-3/n-6	2.0	2.1	2.2
	(1.7, 2.2)	(2.0, 2.2)	(2.1, 2.4)

Data are means (n=3). Upper and lower limits for 95% credible intervals (CI) are in parenthesis. Ninety-five percent CI were interpreted as statistically significant at P=0.05% level when the interval did not overlap the reference value in question. Effects adjusted for fish weight at the end of the trial. [1]Totals include 15:0, 20:0 and 22:0 at up to 0.2%; [2]Totals include 16:1n-9, 20-1n-7, 22:1n-9 and 24:1n-9 at up to 0.8%; 3Totals include 18:3n-6, 22:4n-6 and 22:5n-6 at up to 0.2%; 4Totals include 18:4n-3 and 20:3n-3 at up to 0.5%. PUFA, polyunsaturated fatty acid.

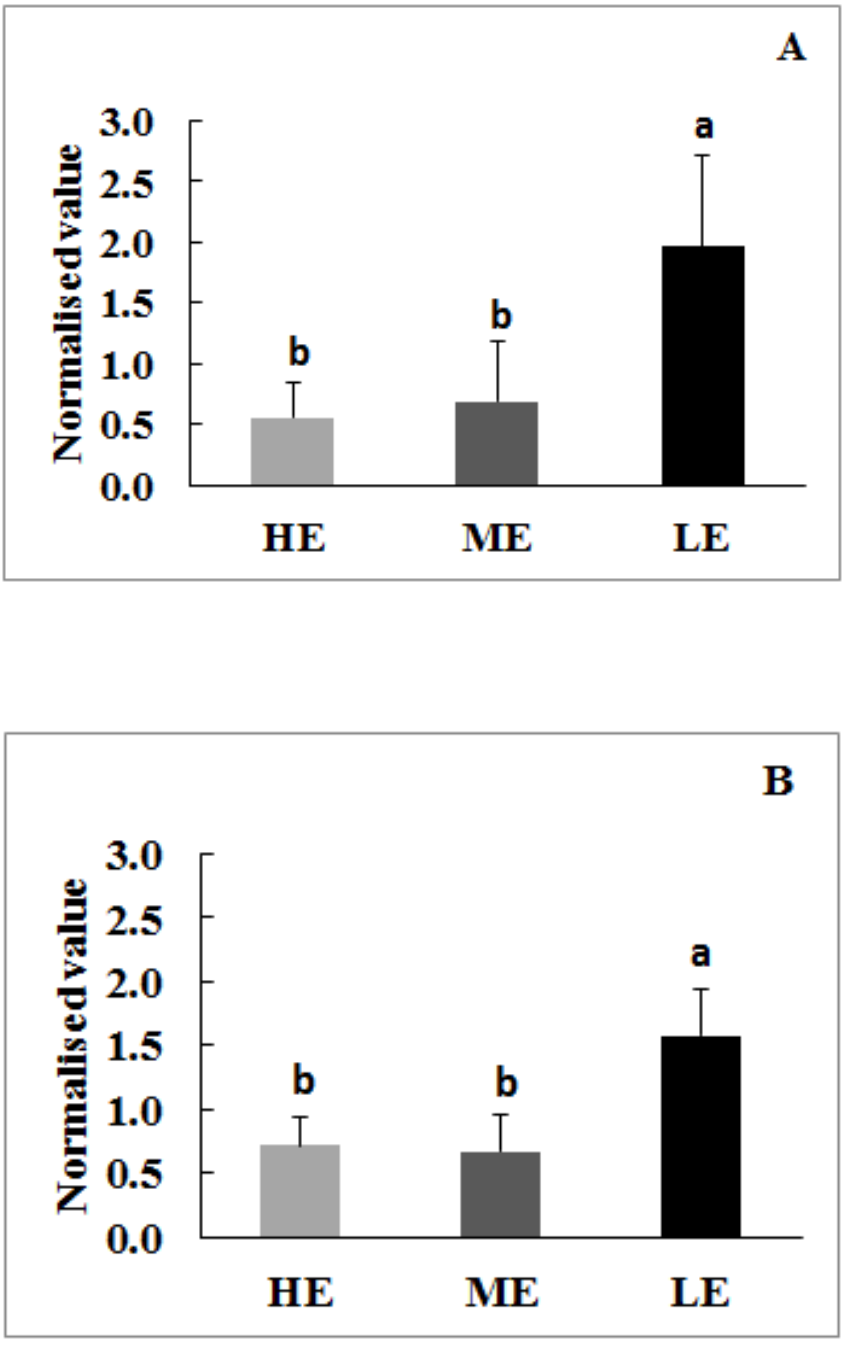

Figure 4.1 Expression of fatty acyl desaturase (Fad) genes involved in long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis. Expression of Δ6 Fad (A) and Δ5 Fad (B) genes in liver was determined by real-time quantitative PCR. Values were normalized by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets (ANOVA, $p<0.05$)

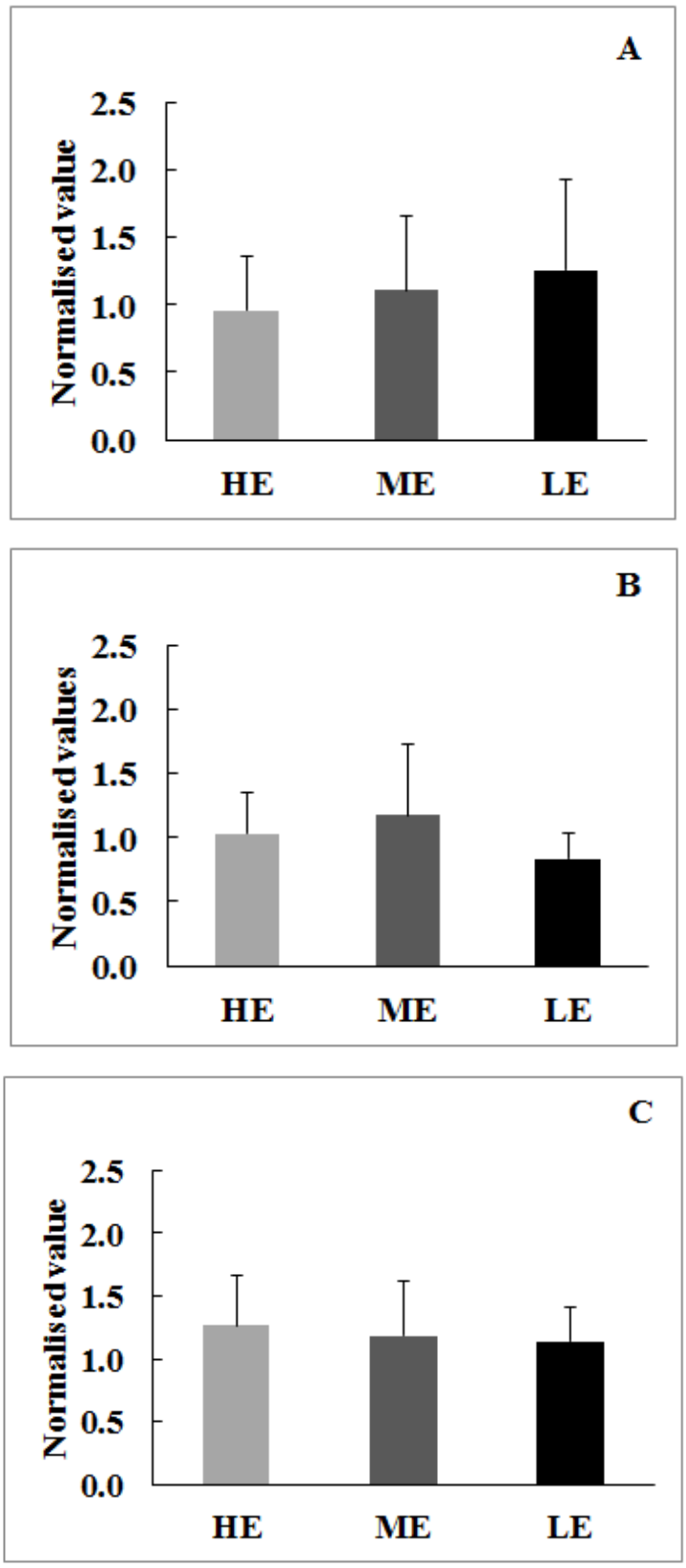

Figure 4.2 Expression of fatty acid elongase (Elovl) genes involved in long-chain polyunsaturated fatty acid (LC-PUFA) biosynthesis. Expression of Elovl2 (A), Elovl5a (B) and Elovl5b (C) genes in liver was determined by real-time quantitative PCR. Values were normalized by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets (ANOVA, $p<0.05$).

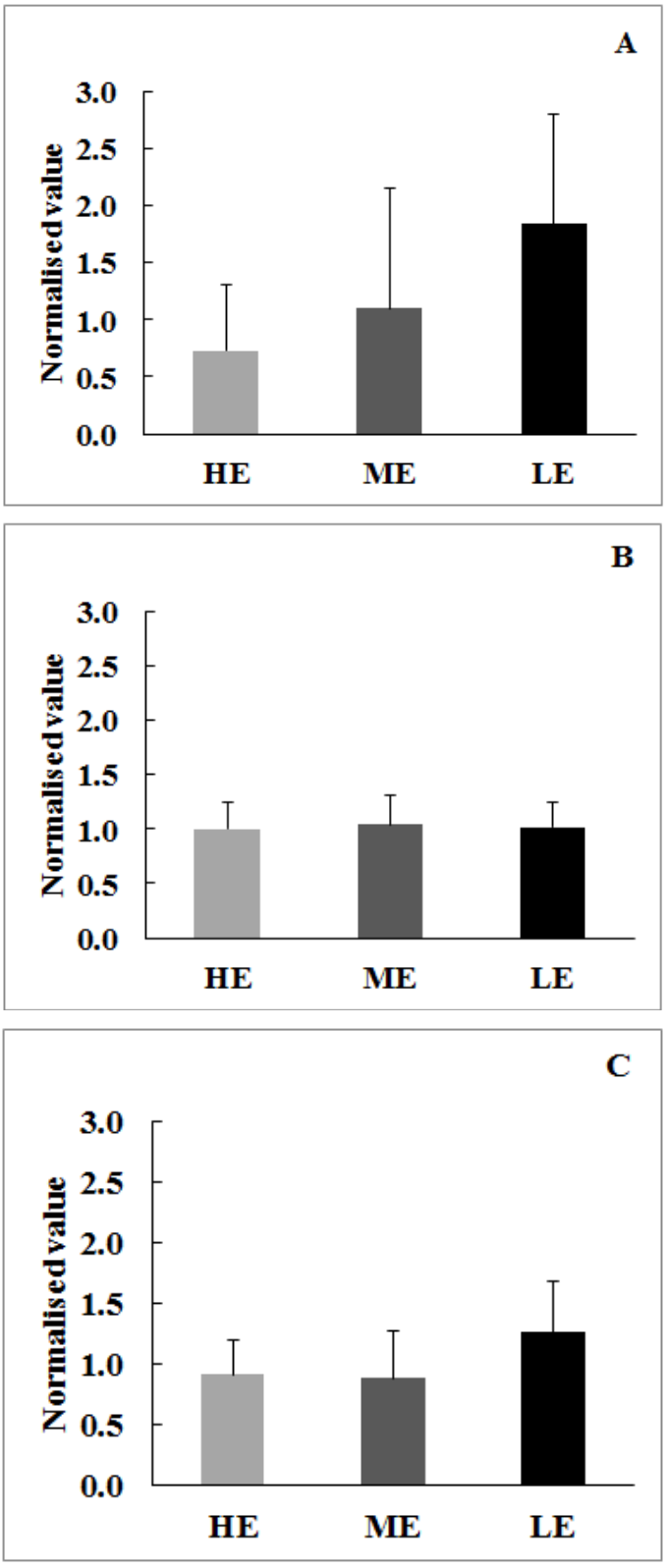

Figure 4.3 Expression of genes involved in fatty acid biosynthesis (lipogenesis) and oxidation. Expression of fatty acid synthase (A), carnitine palmitoyl tranferase-1 (B) and acyl coA oxidase (C) genes in liver was determined by real-time quantitative PCR. Values were normalized by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets (ANOVA, $p<0.05$).

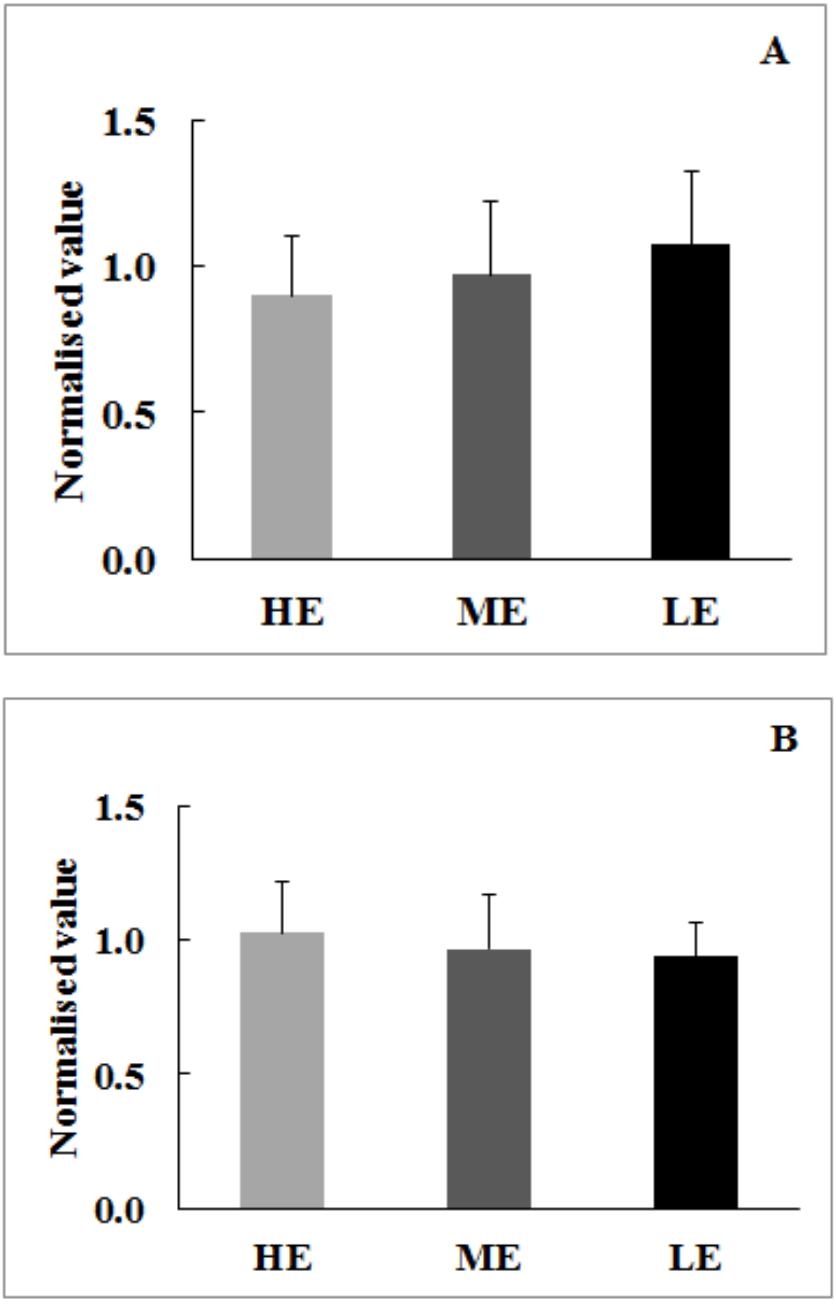

Figure 4.4 Expression of genes involved in the regulation of cholesterol biosynthesis and catabolism. Expression of sterol regulatory element binding protein 2 (A) and liver X receptor (B) genes in liver was determined by real-time quantitative PCR. Values were normalized by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets (ANOVA, $p<0.05$).

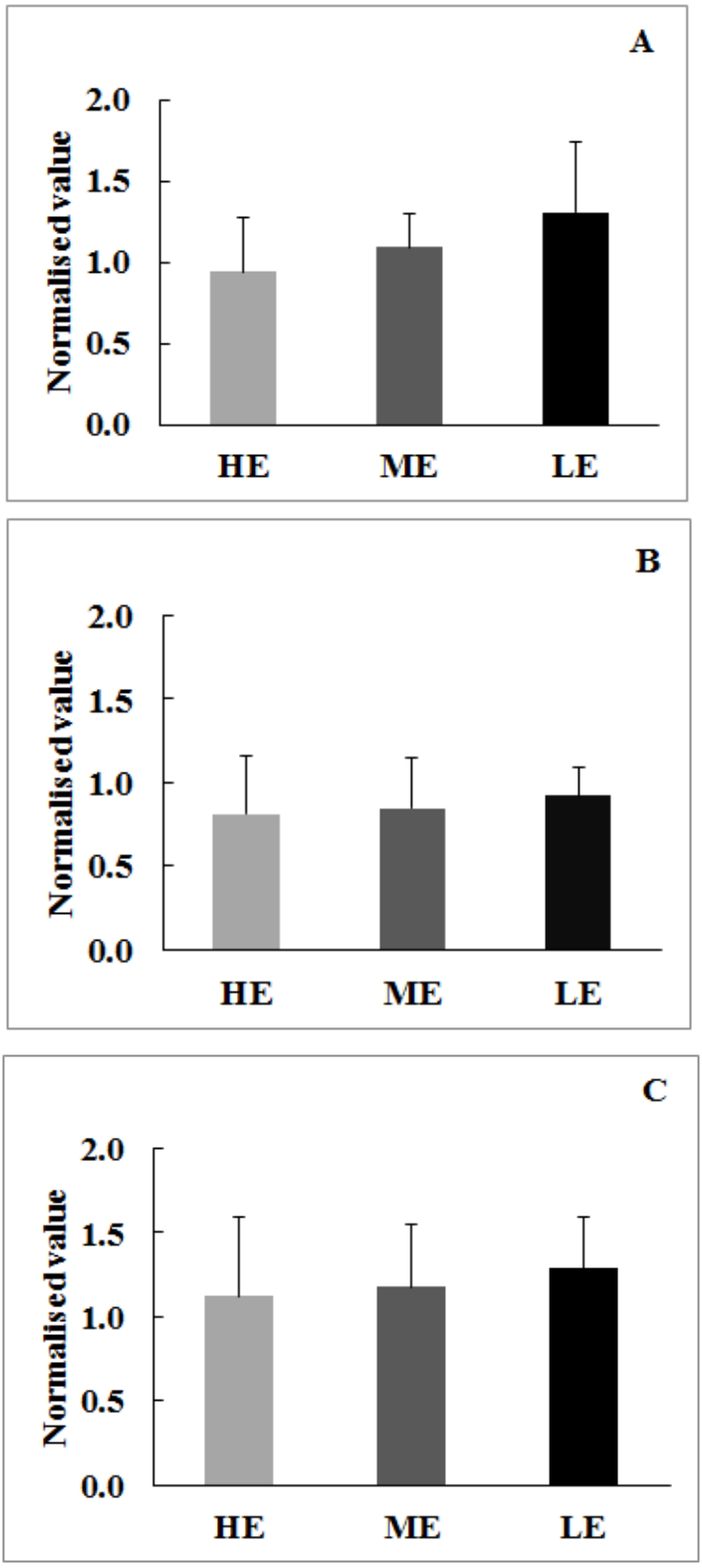

Figure 4.5 Expression of genes involved in the regulation of fatty acid metabolism. Expression of peroxisome proliferator –activated receptors (PPAR), PPARα (A), PPARβ (B) and PPARγ (C), genes in liver was determined by real-time quantitative PCR. Values were normalized by dividing the number of copies of the target genes by the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters represent significant differences between diets (ANOVA, $p<0.05$).

Discussion

Although the present study was designed to investigate effects of dietary DE content, the differences in DE were achieved by varying the level of dietary lipid such that the LE diet was formulated with half the amount of added oil compared to the HE diet, and so it is not possible to distinguish effects of energy content per se from effects of lipid content. Therefore, as lipid content is used here as a proxy for energy content, we focussed on lipid metabolism and so the results are discussed in these terms.

The use of high energy feeds in aquaculture is based on their ability to promote growth and feed efficiency and this was observed in the present study with increased final weights and weight gain, and lower FCR, in fish fed diet HE. It is important to note that this was observed despite the fish not being limited by ration. That is, the fish were fed in excess so that fish had the opportunity to vary feed intake to compensate for the different dietary DE. Increased weight gain in response to increased dietary lipid content has been consistently shown in salmonids including brown (*Salmo trutta*) and rainbow (*Oncorhynchus mykiss*) trout, and Atlantic salmon (Arzel et al., 1993; Luzzana et al., 1994; Hemre and Sandnes, 1999). As a result, dietary lipid in commercial feeds for salmon doubled in a twenty-year period reaching around 35% of total diet by the mid-1990s (Einen and Roem, 1997).

However, in addition to increasing growth, increased dietary DE through higher dietary lipid can also have negative impacts on other aspects of fish performance. Chief among these is based on the well-known positive correlation between dietary lipid levels and tissue/body lipid levels of fish (Sargent et al., 2002). Although the lipid data in the present study showed quite large variation, due to the use of an essentially ungraded stock, chosen to eliminate bias towards fast or slow growers, tissue lipid contents were generally reduced, significantly in liver and heart, with decreasing dietary

DE content. This was confirmed by the significantly lower total neutral (storage) lipid and/or TAG in all tissues in fish fed diet LE compared to fish fed HE. These consistent effects in all tissues supported the assertion that the differences are biologically significant. In this respect it is noteworthy that previous studies have reported that the level of lipid in flesh of salmon can vary considerably within a population, but the extent to which the observed biological variation is determined by environmental or genetic factors is not known (Bell et al., 1998b). Notwithstanding the above, previous studies support the present data, as high dietary lipid increased flesh lipid levels in salmonids including rainbow trout and Atlantic salmon (Bell et al., 1998b; Dias et al., 1999; Hemre and Sandnes, 1999). Increased tissue lipid in response to increased dietary lipid was also shown in marine fish including sea bass (*Dicentrarchus labrax*) and turbot (*Psetta maximus*) (Catacutan and Coloso, 1995; Saether and Jobling, 2001). Increased tissue lipid deposition may have unwanted physiological effects such as promoting early sexual maturation in male salmon (Shearer and Swanson, 2000), or the development of fatty liver pathology in marine fish (Caballero et al., 1999), possibly through mechanisms involving altered gene expression, and hence the focus on this aspect in the present study.

In relation to the above, a highly significant effect of dietary DE content in terms of altered gene expression was the higher expression of both Δ6- and Δ5-Fads in liver of salmon consuming the LE diet compared with fish consuming the higher energy diets. Liver transcript expression in fish fed LE was around 3-fold higher than that in fish fed HE, and was highly significant (p-value = 0.002). In contrast, dietary DE did not appear to affect the expression of fatty acid elongases, consistent with the fact that these enzymes do not show the same level of nutritional regulation as desaturases (Leaver et al., 2008a; Morais et al., 2009; Tocher, 2010). In addition to being the clearest effect of

DE on gene expression, this was also an important result as the effects of DE and/or lipid content on the expression of genes of LC-PUFA biosynthesis had not been reported previously in salmon. Previously, microarray analysis had shown that Δ6-desaturase transcript expression was down-regulated in trout fed a high energy feed compared to fish fed a lower energy feed (Kolditz et al., 2008b). Nutritional regulation of fatty acid desaturase gene expression was first reported in mammals. The levels of liver mRNA for both Δ6- and Δ5-desaturases were approximately 3-fold higher in rats fed a fat-free diet compared to animals fed either safflower oil (18:2n-6) or fish oil (n-3LC-PUFA) (Cho et al., 1999a). However, rats fed a diet containing triolein (a symmetrical triglyceride derived from glycerol and three units of the unsaturated fatty acid oleic acid) showed a similar high expression of both desaturases as observed in rats fed the fat-free diet (Cho et al., 1999a). Therefore, this suggested that it was the fatty acid composition of the diet, specifically a lack of PUFA that was responsible for the higher expression rather than the lipid content of the diets. Consistent with this, hepatic expression of Δ6-desaturase in mice fed an essential fatty acid (EFA)-deficient diet (triolein) was double that in mice fed a corn oil diet rich in 18:2n-6 (Cho et al., 1999b).

Previous work investigating the nutritional regulation of LC-PUFA biosynthesis in fish has focussed on dietary fatty acid composition (Leaver et al., 2008a; Tocher, 2010). The activity of the LC-PUFA biosynthesis pathway in freshwater carp cells was increased by EFA-deficiency (Tocher and Dick, 1999). *In vivo* dietary trials showed that the activity of the LC-PUFA biosynthetic pathway was increased in freshwater and salmonid fish fed vegetable oils rich in C_{18} PUFA compared to fish fed fish oil, rich in the n-3 LC-PUFA, EPA and DHA (Tocher et al., 1997, 2002, 2003a,b). Consistent with this, expression of Δ6 Fad mRNA was increased in salmon fed diets lacking LC-PUFA (vegetable oil), compared to fish fed diets containing EPA and DHA (fish oil) (Zheng et

al., 2004b, 2005a,b; Leaver et al., 2008b; Taggart et al., 2008). Therefore, Fad expression was increased when diets contain lower levels of the pathway end-products such as EPA and DHA. In the present study, the fatty acid compositions of the feeds were similar and, indeed the levels of the LC-PUFA, EPA and DHA were slightly higher in the LE diet, which resulted in generally higher levels of these fatty acids in fish fed LE. Therefore, the higher expression of the Fad genes in liver of fish fed the LE diet is not consistent with the previous data, supporting the view that dietary lipid content itself underpins the differences in expression observed in the present study. The only previous study in salmon that reported LC-PUFA biosynthesis in fish fed different levels of dietary lipid gave inconclusive results. Consistent with the data in the present study, LC-PUFA synthesis in liver was higher in fish fed a low lipid diet compared to a high lipid diet when supplied as fish oil (Tocher et al., 2003a, b). However, when the dietary lipid was supplied as vegetable oil, hepatic LC-PUFA synthesis was higher than both low and high fish oil, but there was no difference between low and high vegetable oil. This perhaps suggests a more complex interaction between lipid content and fatty acid composition, but LC-PUFA biosynthesis could be generally increased by low dietary lipid, perhaps associated with increased lipid biosynthesis in general (see below). The finding that dietary lipid content can affect the expression of the genes of LC-PUFA biosynthesis may be related to early work that suggested that the quantitative requirement for EFA may vary with dietary lipid level. Thus, the dietary requirement for n-3 LC-PUFA appeared to increase with increased dietary lipid in fingerlings of red sea bream (*Pagrus major*) and yellowtail (*Seriola quinqueradiata*) (Takeuchi et al., 1992a,b), although this was not apparent in larval gilthead sea bream (Salhi et al., 1994).

Other than the above effect on Fad genes, there were few statistically significant effects of dietary DE (lipid content) on the expression of the other genes of lipid metabolism investigated. However, several showed clear trends with the DE regression that were scientifically logical and, as argued above, in combination with each other and the growth and lipid compositional data discussed above, support the contention that some have biological significance. For instance, the effect of DE on FAS, although not statistically significant, was highly likely biologically significant as it is well established that lipogenesis and FAS, as the rate-limiting step of lipid biosynthesis pathway, is regulated by dietary lipid and is up-regulated by diets with lower lipid (DE) contents (Sargent et al., 2002). Consistent with this, early studies showed increased dietary lipid depressed lipogenesis in common carp (Shimeno et al., 1995), and high lipid diets decreased the activities of lipogenic enzymes in juvenile yellowtail (Shimeno et al., 1996). Furthermore FAS activity and gene expression was lower in trout fed a high energy diet compared to fish fed a low energy diet (Kolditz et al., 2008a).

A further example of data reinforcing each other was observed in the reciprocal responses observed in the liver expression of LXR and SREBP2, which are key regulators controlling cholesterol homeostasis. The transcription factor, LXR, regulates cholesterol catabolism, storage, absorption and transport through the transcriptional regulation of key target genes involved in these processes (Aranda and Pascual, 2001). A single LXR cDNA was recently isolated and characterised from Atlantic salmon and shown to be similar to mammalian LXRα (Cruz-Garcia et al., 2009). The nuclear receptor, SREBP2, is activated by reduced cholesterol and is a key regulator in the biosynthesis of cholesterol (Horton et al., 2002) and, recently, SREBP2 was isolated and characterised from Atlantic salmon (Minghetti et al., 2011). In the present study, although relatively small, there seems to be an effect of DE on these factors with LXR

showing a trend of decreased expression, and SREBP2 showing a trend of increased expression, in fish fed feeds with lower DE. This is consistent with the level of dietary cholesterol supplied by the feeds, which reflects level of dietary fish oil (Leaver et al., 2008b; Taggart et al., 2008; Tocher et al., 2008). Thus, even not significant, it seems that the lower level of dietary cholesterol in the LE feed compared to the HE feed, resulted in a slightly increased expression of SREBP2, promoting cholesterol biosynthesis, and lower expression of LXR, reducing cholesterol catabolism. Previously, lower expression of LXR was observed in liver of Atlantic salmon fed vegetable oil (lower cholesterol) compared to fish fed fish oil (Cruz-Garcia et al., 2009).

Peroxisome proliferator-activated receptors are ligand-activated transcription factors that have key roles in regulating lipid and fatty acid metabolism including fatty acid oxidation (esp. PPARα) and tissue lipid deposition (esp. PPARγ) in mammals (Desvergne et al., 2006). Their natural ligands include unsaturated fatty acids and their derivatives, which has led to the view that PPARs are general fatty acid sensors responding to changes in nutritional status and energy metabolism (Michalik et al., 2006). Although, compared to mammals, there is considerably less known, but available data suggests that PPARs have similar roles in the control of metabolism in fish as in mammals (Leaver et al., 2005, 2008a). It is therefore likely that PPARs would be involved in the metabolic response to dietary DE content. In the present study, although not significant, there was a clear trend for the hepatic expression of all PPAR subtypes, but especially PPARα, to increase with decreasing DE, and this was accompanied by increased expression of liver ACO, but CPT-1 expression was unaffected. Similarly, possible association of PPAR and ACO expression was observed in sea bream (*Sparus aurata*), with expression of all PPAR subtypes and ACO reduced in liver of fish fed conjugated linoleic acid (CLA) (Diez et al., 2007). In contrast, feeding Atlantic salmon

with CLA increased PPARα expression in liver and this was associated with increased CPT-1 expression and β-oxidation (Leaver et al., 2006). In salmon fed the thia fatty acid, tetradecylthioacetic acid (TTA), PPARα expression in liver was decreased, but expression of ACO was unaffected (Kleveland et al., 2006). In trout fed a high energy diet, CPT-1 and ACO expression, and β-oxidation activity in liver were all increased in comparison to fish fed a low energy diet, but PPARα expression was unaffected (Kolditz et al., 2008a). All of these data highlight the inconsistency of results obtained on expression of PPARs and genes of fatty acid oxidation. Clearly, as PPAR function is dependent upon activation by ligands, the relationship between PPAR expression, its functionality, and thus its role in controlling expression of target genes, is complicated and unclear at present (Leaver et al., 2008a,b).

In conclusion, the present study determined the effects of dietary DE on the hepatic expression of key genes of lipid and fatty acid metabolism in Atlantic salmon fed diets containing graded amounts of lipid. Dietary DE and/or lipid content had important effects on the expression of genes involved in LC-PUFA biosynthesis pathway (Δ6 Fad, Δ5 Fad) and could possibly play a role on major lipid pathways including lipogenesis (FAS), and cholesterol metabolism (LXR and SREBP2) as the trend of the expression of the genes involved in those pathways was biologically significant and consistent with current understanding. In contrast, the effects on fatty acid β-oxidation (CPT1, ACO and PPARs) were more inconclusive. Overall though, it was clear that changes in dietary DE alone, without major changes in dietary fatty acid composition, could result in altered expression of key genes of lipid and fatty acid metabolism. Combined, these changes resulted in an overall up-regulation of lipid biosynthetic pathways.

Acknowledgements

We acknowledge EWOS Innovation Technology Centre for their skilful feed production. The authors would also like to thank Fiona Strachan, Dr. Sofia Morais and Meritxell Diez Padrisa for their help during the collection of the samples and all the staff in the Lipid group from the Institute of Aquaculture at the University of Stirling for the technical support.

Chapter 5

Effects of functional feeds on the transcriptomic responses and heart pathology in Atlantic salmon (*Salmo salar L.*) following experimental challenge with Piscine Myocarditis Virus (PMCV)

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Abstract

Cardiomyopathy syndrome (CMS) is a severe cardiac disease of farmed Atlantic salmon (*Salmo salar*) recently associated with a double-stranded RNA virus, Piscine Myocarditis Virus (PMCV). CMS has affected 75-85 per cent of Norwegian salmon farms each year over the last decade resulting in annual economic losses estimated at up to €9 million. Recently, we showed that functional feeds lead to a milder inflammatory response and consequently less severity of heart lesions in salmon experimentally infected by Atlantic salmon reovirus (ASRV), the causal agent of heart and skeletal muscle inflammation (HSMI). In the present study we employed the same strategy to investigate the effects of similar functional feeds, with reduced lipid content and increased eicosapentaenoic acid (EPA) levels, in controlling CMS in salmon after experimental infection with PMCV. Hepatic steatosis associated with CMS was significantly reduced over the time course of the infection in the fish fed the functional feeds. Significant differences in immune and inflammatory responses and pathology in heart tissue were found in fish fed the different dietary treatments over the course of infection. Specifically, fish fed the functional feeds with reduced dietary lipid content and increased EPA showed a milder and delayed inflammatory response and, consequently, less severity of heart lesions at earlier and later stages after infection with PMCV. Decreasing levels of phosphatidylinositol in cell membranes combined with the increased expression of genes related with T-cell signalling pathways revealed new interactions between dietary lipid composition and the immune response in fish after a viral infection. Dietary histidine supplementation did not significantly affect immune responses or levels of heart lesions. Combined with the previous data on ASRV/HSMI, the present results highlight the potential role of clinical nutrition in controlling inflammatory diseases in Atlantic salmon. In particular, dietary lipid content and fatty

acid composition can have important immune-modulatory roles of potential benefit in Atlantic salmon viral infections.

5.1 Introduction

Cardiomyopathy syndrome (CMS) is a severe cardiac disease of farmed Atlantic salmon (*Salmo salar*) recently associated with a double-stranded RNA virus termed piscine myocarditis virus (PMCV) (Haugland et al., 2011). The disease was first described and diagnosed in Norway in 1985 (Amin and Trasti, 1988), and has since also been diagnosed in Scotland (Rodger and Turnbull, 2000), the Faroe Islands (Poppe et al., 1994) and, possibly, Canada (Brocklebank and Raverty, 2002). Annual economic losses due to CMS have been estimated from €4.5 - 8.8 million as this disease has affected 75 to 85 per cent of Norwegian salmon farms each year over the last decade (Brun et al., 2003). Histopathologically, CMS is characterized by severe inflammation and necrosis of the spongy myocardium of the atrium and ventricle (Ferguson et al., 2009), but liver may also be affected due to the circulatory disturbance associated with the heart lesions. Early stages of the disease have been reported in adult Atlantic salmon around 14 to 18 months after transfer to seawater (Fritsvold et al., 2009). Mortality is usually moderate although, as this is a chronic disease developing over a period of several months, cumulative mortality can be relevant.

Presently, the lack of commercial vaccines to CMS makes the use of alternative therapies crucial. Factors that modulate the inflammatory process might be key to mitigate the clinical symptoms and improve the performance of affected fish. The concept of clinical nutrition and functional feeds is well known in humans (Roberfroid, 2002), and is becoming of increasing interest in fish farming. Functional feeds are defined as high-quality feeds that, beyond their nutritional composition, are formulated with health promoting features that could be beneficial in supporting disease resistance and mitigation of clinical disease symptoms. Thus clinical nutrition enables a shift away

from chemotherapeutic and antibiotic treatments, lowering the costs of disease treatment and management (Tacchi et al., 2011).

There are several studies in fish linking nutrition and immunology, recently reviewed by Kiron (2012). The inclusion in aquaculture feeds of additives such as probiotics, prebiotics, immunostimulants, vitamins and nucleotides, is reported to increase growth and feed conversion efficiency, as well as having positive effects on the immune system associated with the intestinal tract and protection against bacterial infections (Tacchi et al., 2011). Moreover, macronutrients like proteins and lipids are reported to play key roles in the regulation of pathways of the immune system and have been widely studied due to the necessity of the aquafeed industry to replace dietary fishmeal (FM) and fish oil (FO) (Tacon and Metian, 2008). As global production of both FM and FO has been, at best, static over the last few years (FAO, 2010), the expansion of the aquaculture industry has increasingly relied on alternative sources of dietary protein and oil, such as plant proteins and vegetable oils (VO). There are several studies evaluating the consequences of FM and FO replacement in farmed fish and, although growth does not seem to be greatly affected by substantial levels of substitution, the detrimental consequences of dietary plant proteins and VO on the health status of fish has been an important concern in recent years (Bell et al., 1990, Montero et al., 2003; Bakke-McKellep et al., 2007). For instance, substitution of FM by some plant protein sources, particularly soybean meal, has been reported to affect the gastrointestinal tract and gut immune health causing enteritis and atrophy of mucosal folds (Kroghdal et al., 2003). Indeed, many of the plant protein sources potentially suitable for aquafeeds have significant levels of a variety of antinutritional factors (Francis et al., 2001). However, plant protein concentrates with reduced concentrations

of antinutrients (Gatlin et al., 2007), and the above-mentioned dietary supplements, have both been used successfully to reduce the negative impacts of FM substitution.

There are also many studies evaluating the replacement of FO by VO, recently reviewed by Turchini et al. (2009). A major concern of this replacement is the higher levels of n-6 polyunsaturated fatty acids (PUFA) contained in VO compared with FO, which are conversely rich in the n-3 long-chain PUFA (LC-PUFA) known to be essential for fish health. Dietary PUFA are involved in the regulation of the inflammatory response, and both innate and adaptive immune responses, through four potential mechanisms including gene expression, eicosanoid metabolism, cellular signalling and membrane organization (Raza Shaik and Eidin, 2006). Thus, specific transcription factors are activated by a number of PUFA, LC-PUFA and eicosanoid ligands (Moraes et al., 2006), and subsequently regulate the expression of genes related with inflammatory, B-cell and T-cell responses, which play important roles in viral infections. The roles of PUFA in cell membrane organization and signalling pathway mechanisms have been widely studied in humans (Shaik and Eidin, 2006). Inhibition of T-cell signalling pathways by PUFA is mainly linked to the suppression of the elevation of cytoplasmic calcium concentration through the phosphatidylinositol (PI) signalling system, which is a key event in T-cell activation (Stulnig and Zeyda, 2004). Among the aforementioned mechanisms, the eicosanoid pathway is the most extensively studied, not only *in vitro* but also *in vivo*, as eicosanoids are key mediators of inflammation and regulation of T and B lymphocytes. Arachidonic acid (20:4n-6; ARA) and eicosapentaenoic acid (20:5n-3; EPA) are released from membrane phospholipids through the action of phospholipases leading to the production of pro- or anti-inflammatory eicosanoids, respectively. The enzymes of eicosanoid metabolism act on both the above LC-PUFA and, although they have preference for ARA, increasing

levels of EPA in the membranes of immune cells inhibits the production of pro-inflammatory eicosanoids (Calder, 2008). Studies have demonstrated that LC-PUFA have similar roles in immune modulation in fish. The aforementioned substitution of dietary FO by VO, with the consequent reduction in the ratio of n-3/n-6 LC-PUFA, has been reported to promote the synthesis of pro-inflammatory eicosanoids (Bell et al., 1996), and alter humoral immunity and expression of pro-inflammatory cytokine genes (Montero et al., 2010b). Although most of these studies have been performed *in vitro* (Bell et al., 1993; 1995; Gjøen et al., 2007), little is known about the roles of LC-PUFA in viral infections.

In a previous study, the use of functional feeds led to a milder inflammatory response and consequently less severity of heart lesions in salmon experimentally infected by Atlantic salmon reovirus (ASRV), the causal agent of heart and skeletal muscle inflammation (HSMI) (Martinez-Rubio et al., 2012a- Chapter 2). In the present study we have employed a similar strategy to investigate the effects of similar functional feeds in controlling CMS. The functional feeds contained dietary lipid reduced from 31 % in a reference commercial feed to 18 % in the two functional feeds, along with dietary EPA levels increased from 3.6 % to 14 %. Supplementation of histidine was also assessed in one of the functional feeds as this amino acid and related compounds such as N-α-acetyl-histidine (NAH) have important roles in muscle pH buffering (Ogata, 2002; Suzuki et al., 1987) and tissue antioxidant systems (Kim et al., 1999; Wade et al., 1998). In particular, histidine has been associated with a cardio-protective role in human studies, being potentially beneficial in the alleviation of oxidative stress associated with viral diseases (Hasnain et al., 2003). Thus, we hypothesized that dietary supplementation of this amino acid could have a potentially beneficial effect in fish suffering CMS. Salmon were fed the functional feeds for

8-weeks prior to challenge with PMCV and throughout the 14-weeks post-infection period. The incorporation of LC-PUFA into membrane phospholipids of heart, the main organ affected by the disease, was assessed. Samples of both heart and liver tissue were subjected to histological evaluation. The inflammatory profile in the heart tissue was assessed after infection by determining gene expression by oligonucleotide microarray analysis.

5.2 Materials and methods

5.2.1 Experimental feeds and fish

Three fishmeal-based diets were formulated and manufactured by EWOS Innovation (Dirdal, Norway) (Table 5.1). The formulations were based on small-scale commercial screening trials (unpublished) and a previous study investigating HSMI (Martinez-Rubio et al., 2012- Chapter 2). The reference diet (REF) was essentially a standard, commercial formulation with 31 % lipid with the added oil being a blend of Northern hemisphere FO and rapeseed oil. The two functional feeds (CMS1 and CMS2) both contained a lower level of lipid (18 %) that was balanced by increased protein, provided by fishmeal and krill meal. Therefore, as a major factor differing between the REF and functional feeds was dietary lipid level, the feeds were not isolipidic or isoproteic. The added oil in the functional feeds was provided by a Southern hemisphere FO. As a result the CMS1 and CMS2 feeds had a similar fatty acid profile containing higher proportions of EPA (almost 14 %) and an n-3/n-6 PUFA ratio around 4, in comparison to the REF feed (< 4 % EPA and an n-3/n-6 ratio of 1.4) (Table 5.2). The only major difference between the functional feeds was that CMS1 was supplemented with additional histidine.

A total of 675 Atlantic salmon (*Salmo salar* L.), SalmonBreed IPN svak strain (average weight ca.150 g), were distributed into nine tanks (1 m^3) at the VESO facility,

Vikan, Norway and fed one of the three feeds (3 tanks of 75 fish each per dietary treatment; 225 fish in total) for a period of 8 weeks prior to being transferred to the challenge tanks. Feeding ration was a maximum of 2 %. Seawater/ brackish water (ca. 25 ‰-35 ‰) delivery was flow-through, being sufficient to maintain oxygen-satiation in effluent water > 70 %. Water temperature was maintained at 12 ± 1 °C, and a photoperiod 24:0 h light/dark regime was followed. Stocking density of the tanks was a maximum of 60 Kg/m^3.

Table 5.1 Formulation (g/Kg) and proximate composition (percentage) of the reference (REF) and functional (CMS1 and CMS2) feeds

Component[1]	REF	CMS1	CMS2
Fish meal and hydrolysates	421.0	530.0	530.0
Plant protein concentrates[2]	215.0	180.0	180.0
Northern fish oil	130.0	0.0	0.0
Southern fish oil	0.0	100.0	100.0
Rapeseed oil	107.0	0.0	0.0
Carbohydrate-based binders[3]	119.0	119.0	121.0
Micro premixes[4]	8.0	17.0	19.0
Krill meal[5]	0.0	50.0	50.0
Histidine (synthetic)	0.0	4.0	0.0
Proximate composition			
Moisture	6.5	6.5	6.5
Fat	31.0	18.0	18.0
Protein	42.2	53.4	53.4

1) All ingredients sourced from EWOS stocks unless otherwise stated.
2) Includes soy protein concentrate, pea protein concentrate, wheat gluten and sunflower meal
3) Includes wheat grain
4) Includes vitamins, minerals, crystalline amino acids, ammonium phosphate
5) Aker Biomarine AS

Table 5.2 Fatty acid compositions (percentage of total fatty acids) of the reference (REF) and functional (CMS1 and CMS2) feeds.

Fatty acid	REF	CMS1	CMS2
Saturated	16.5	26.6	25.8
Mounsaturated	59.1	36.4	36.1
18:2n-6	8.8	4.9	5.6
20:3n-6	0.2	0.3	0.3
20:4n-6	0.2	0.6	0.7
n-6 PUFA	9.8	6.9	7.2
18:3n-3	3.3	1.3	1.3
20:5n-3	3.7	13.7	13.4
22:6n-3	5.0	8.4	8.4
n-3 PUFA	13.9	27.5	27.8
PUFA	24.3	37.0	38.1
n-3/n-6	1.4	4.0	3.9
EPA/ARA	19.4	21.4	20.2

5.2.2 PMCV Challenge

After the pre-feeding period, the fish (non-vaccinated and HSMI-free) were transferred to challenge tanks (1 m^3) with the same rearing conditions described above. Of the available salmon, a total of 576 fish, pre-challenge average weight 339 ± 13g for the fish fed with the REF diet; 335 ± 13g for the fish fed with the CMS1 diet and 362±14g for the fish fed with the CMS2 diet; were distributed into 12 tanks (4 tanks per dietary treatment with 48 fish/tank). The fish were acclimated for 2 weeks prior to challenge and were fed with the same diets during the acclimation period and throughout the period of the challenge (14 weeks) that they were fed prior to transfer. No previous diseases were described.

For challenge, the fish were sedated using Aqui-S at final concentration of 5 mg/L of isoeugenol, followed by anaesthesia in benzocaine (20 %, Benzoak®) using a final concentration of 30 ml/L. Two fish from each pre-challenge tank, 18 in total (n = 6 per

dietary treatment) were collected prior to challenge (0 time-point) and then 576 fish (as described above) were challenged by intramuscular injection of 0.1 ml PMCV inoculum on each side of the fish in the lateral muscle tissue beneath the dorsal fin (total, 0.2 ml per fish).

Production of the PMCV inoculum has been described previously (Haugland et al., 2011). Briefly, the virus was originally isolated from heart tissue homogenate collected from a clinical outbreak of CMS and filtered through a 0.22-μm filter. Challenge of Atlantic salmon was performed using supernatant of GF-1 cell culture in which the virus was inoculated.

5.2.3 Sampling

Ten fish from each tank (total of 40 per dietary treatment) were sampled at 6-, 8-, 10-, 12- and 14-weeks post-challenge. Fish were anaesthetized as above and killed by a blow to the head and heart tissue collected for analyses. Liver tissue and a portion of each heart were transferred to 10% buffered formalin for histological analyses. Of the remaining portions of heart tissue, half were immediately transferred to RNAlater® (following manufacturers protocol) and stored at -20 ^{o}C prior to molecular analyses (i.e. 5 samples of heart tissue per tank, 20 per dietary treatment). The remaining samples of heart tissue were frozen in liquid N_2 and stored at -80 ^{o}C prior to lipid analyses (five per tank, 20 per treatment).

5.2.4 Growth performance

Growth performance of fish fed the three feeds was assessed using repeated measurements of tank mean weights. Tank mean weights were determined at the start of the trial (day 0), challenge-day (day 92) and then at 6-, 8-, 10-, 12- and 14-weeks post-

challenge. A linear mixed-effects model (multilevel model) was fitted between the response (weight) and predictor (diet) by allowing the intercepts and slopes of the time variable (day) to vary from tank to tank to account for the tank level correlations and variability in growth trajectories. The model was estimated with the lmer function in the lme4 package of the R language (R Development Core Team, 2008). All treatment effects were based on posterior simulation (n = 2,500) with 95 % credible intervals. Ninety-five percent credible intervals were interpreted as statistical significant at p = 0.05 % level when the interval did not overlap the reference value in question.

5.2.5 Histological examinations

The histological changes in heart were assessed as described in detail previously (Haugland et al., 2010). In brief, the inflammatory changes were evaluated separately for atrium and ventricle, and to the extent present, inflammatory scores of the epicardium were also recorded. Histological samples were collected to include atrium and ventricle in the same sample. Bulbus arteriosus was also included but was not assessed for histopathological changes. Samples were fixed in 10 % buffered formalin, embedded in paraffin wax and sectioned at 3 - 4 micron and stained with hematoxylin and eosin according to standard methods. All sections were evaluated randomly and without knowledge to which feeding group they belonged (double-blind).

Micrographs showing examples of the correspondence between different levels of heart lesions and histoscores assigned are shown in Figure 5.1

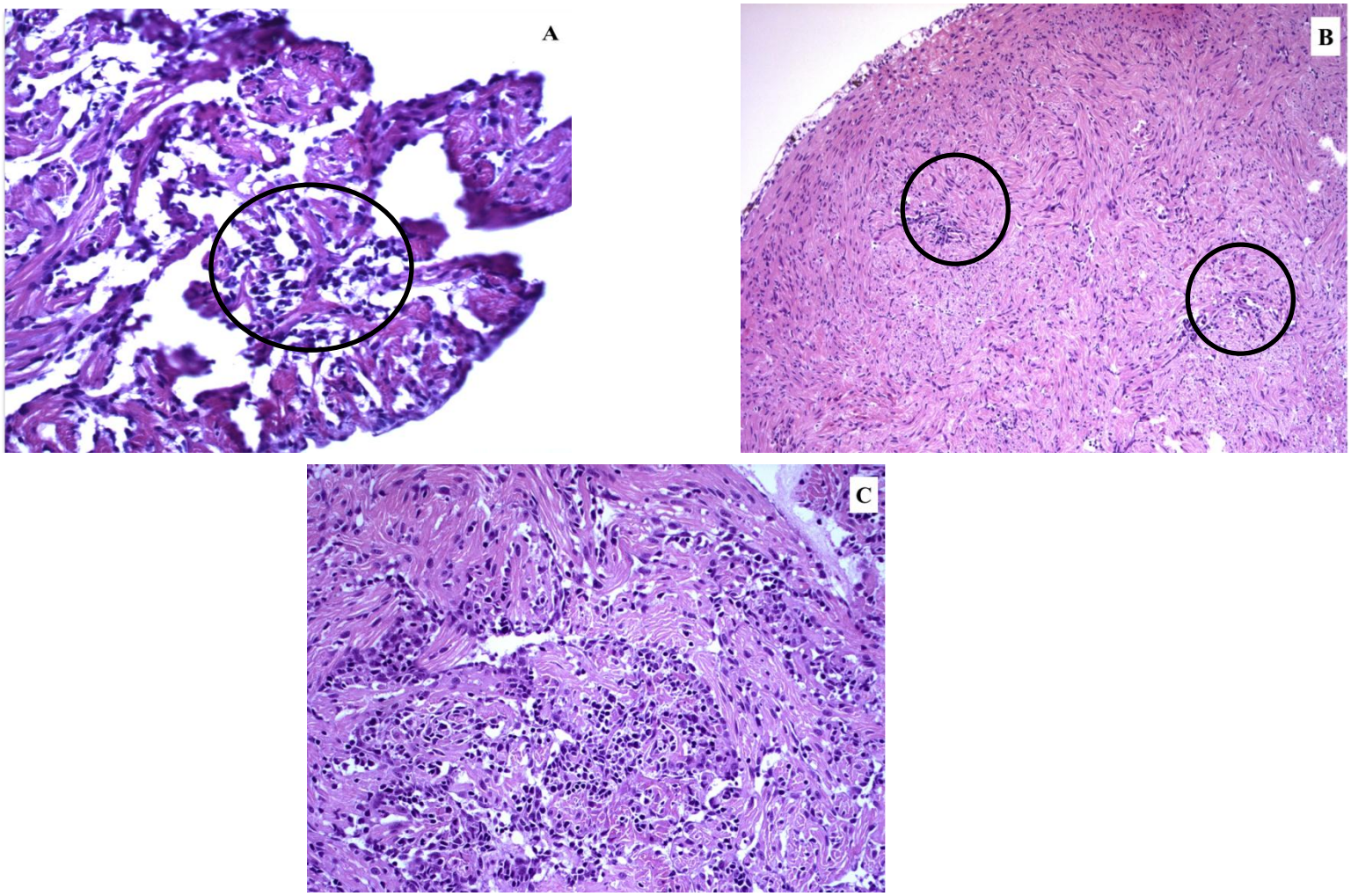

Figure 5.1 Micrographs showing different levels of pathology on the heart tissue. A, Atrium, infiltration of inflammatory cells (indicated), focal reaction (score 1); B, Ventricle, focal to multifocal infiltration of inflammatory cells (indicated) (score 1); C, diffuse infiltration of inflammatory cells and degeneration/necrosis (score 3)

Liver steatosis scores were ranked according to a non-continuous score grade from 0 to 5 (Table 5.3). Briefly, a score of 0 indicated the formation of vacuoles in the cytoplasm, involving less than 10 % of the hepatocytes and including less than 25 % of the area of the individual hepatocytes. A score of 5 indicated formation of vacuoles in the cytoplasm, involving more than 90 % of the hepatocytes and including more than 80 % of the area of the individual hepatocytes. The initial "continuous" scores were converted to a fewer number of discrete scores (3 - 6 classes depending on the response). Scores were an ordinal response and a multilevel ordinal regression was used to analyse the effects of feeds on the scores. This was achieved with the ordinal package Sweave le processed by LATEX of the R language (http://www.R-project.org) Each of the scores was used as the response variable in separate analyses that had feed type (REF, CMS1, CMS2) as the fixed effect term. Since multiple fish were examined from each replicate tank, a random effect of tank was added to the models.

Table 5.3 Scoring system for liver steatosis in individual sections.

0	formation of vacuoles in the cytoplasm, involving less than 10% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
1	formation of vacuoles in the cytoplasm, involving less than 25% of the hepatocytes and including less than 25% of the area of the individual hepatocytes
2	formation of vacuoles in the cytoplasm, involving less than 50% of the hepatocytes and including less than 50% of the area of the individual hepatocytes
3	formation of vacuoles in the cytoplasm, involving less than 75% of the hepatocytes and including less than 75% of the area of the individual hepatocytes
4	formation of vacuoles in the cytoplasm, involving less than 90% of the hepatocytes and including less than 80% of the area of the individual hepatocytes
5	formation of vacuoles in the cytoplasm, involving more than 90% of the hepatocytes and including more than 80% of the area of the individual hepatocytes

5.2.6 Lipid analyses

Lipid and fatty acid analyses were performed on the feeds and heart samples from all of the sampling points. Three pre-challenge heart samples per dietary treatment were randomly chosen for lipid analysis. Heart tissue samples from the post-challenge were

pooled per tank so that heart tissue of five fish became a pool resulting in 4 pools/treatment/sampling point. Total lipid from each pool was extracted by homogenisation in chloroform/methanol (2:1, by volume) according to Folch et al. (1957), and lipid content determined gravimetrically. Total lipid extracts were re-suspended in chloroform/methanol (2:1, v/v) + 0.1 % butylated hydroxytoluent (BHT), at a concentration of 10 mg lipid/ml and stored at -70 ^{o}C until analysed.

Phospholipid (PL) fractions were prepared from 0.5 mg of total lipid by thin-layer chromatography. Samples were applied to a 20 x 20 cm silica gel 60 TLC plate (VWR, Lutterworth, Leics, UK), and developed in isohexane/diethyl ether/glacial acetic acid (80:20:1, by vol.). The plate was sprayed lightly with 2, 7, dichlorofluorescein (0.1 %, w/v) in 97 % methanol (v/v) and the PL band on the origin scraped from the plate. Fatty acid methyl esters (FAME) were prepared by acid-catalysed transesterification directly on the silica by incubating in 2 ml of 1 % H_2SO_4 in methanol at 50 ^{o}C overnight (Christie, 2003). The samples were neutralised with 2.5 ml of 2 % $KHCO_3$ and extracted twice with 5 ml isohexane/diethyl ether (1:1, v/v) + BHT and the combined extracts dried under a stream of oxygen-free nitrogen and resuspended in 0.3 ml isohexane prior to fatty acid analysis.

FAME were separated and quantified by GLC (Fisons 8160; Carlo Erba, Milan, Italy) using a 60 m x 0.32 mm x 0.25 µm film thickness capillary column (ZB-WAX; Phenomenex, Macclesfield, Cheshire, UK). Hydrogen was used as the carrier gas at a flow rate of 4.0 ml/min and the temperature programme was from 50 to 150 ^{o}C at 40^{o}C/min then to 195 ^{o}C at 2 ^{o}C /min and finally to 215 ^{o}C at 0.5 ^{o}C /min. Individual FAME were identified by comparison with well-characterised in-house standards as well as commercial FAME mixtures (Supelco ™ FAME mix; Sigma-Aldrich Ltd, Gillingham, Dorset, UK).

Tissue and diet lipid class compositions were determined by single-dimension double-development high-performance thin-layer chromatography (HPTLC) and densitometry as described previously (Bell et al., 1993). Significance of differences due to diet and time were determined by two-way ANOVA ($p<0.05$) using the SPSS 19.0 statistical package (SPSS Inc., Chicago IL, USA).

5.2.7 Microarray experimental design

Heart tissue, as the main organ affected during a CMS outbreak, was selected for transcriptomic analysis. The choice of appropriate time-points for analysis was based on the description of expression of genes of the immune response after a PMCV infection described by Timmerhaus et al. (2011). Thus samples from fish at pre-challenge, 6-, 8-, and 14- weeks post-challenge/infection (wpc) were analysed. The pre-challenge sampling point was selected to evaluate the changes due to the composition of the diets, and 6- and 8-wpc sampling points were selected to specifically evaluate dietary effects on the peak of expression of the genes from various pathways of the immune response. As a plateau phase in the expression of the immune related genes at 10- and 12-wpc had been described previously (Timmerhaus et al., 2011), the 14-wpc sampling point was selected to evaluate gene expression changes in the possible recovery phase of the disease. Within each of these sampling points, the available samples were arranged by individual histoscore and 6 samples per dietary group were randomly selected from the interquartile range (25 – 75 %).

5.2.8 RNA extraction and purification

Heart tissue of the selected samples was homogenized in TRI Reagent (Ambion, Applied Biosystems, Warrington, U.K.) using an Ultra-Turrax homogeniser (Fisher Scientific, Loughborough, U.K.). Total RNA was isolated following manufacturer's

instructions, and RNA quality and quantity assessed by gel electrophoresis and spectrophotometry (NanoDrop ND-1000, Thermo Scientific, Wilmington, U.S.A.), respectively.

5.2.9 Microarray hybridisations and analysis

The transcriptomic experiment used an Atlantic salmon custom-made oligoarray with 44k features per array on a four-array-per-slide format, with each feature printed singly (Agilent Technologies UK Ltd., Wokingham, UK). The probes were co-designed by researchers at the Institute of Aquaculture, University of Stirling, U.K. and The Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima, Tromsø, Norway), and array design is available on request. A dual-labelled experimental design was employed for the microarray hybridisations. Each experimental sample was competitively hybridised against a common pooled-reference sample, which comprised equal amounts of each of the replicates used in the study. This design permits valid statistical comparisons across all treatments to be made. The entire experiment comprised 72 hybridisations; 4 time-points (pre-challenge, 6-, 8- and 14-wpc) × 3 diets (REF, CMS1 and CMS2) × 6 biological replicates.

Indirect labelling methodology was employed in preparing the microarray targets. Amplified antisense RNA (aRNA) was produced from each RNA sample using the TargetAmp™ 1-RoundAminoallyl-aRNA Amplification Kit (Ambion, Applied Biosystems, Warrington, UK), following the manufacturer's methodology, followed by Cy3 or Cy5 fluor incorporation through a dye-coupling reaction. Briefly, 250 ng of total RNA per sample were amplified and column-purified according to manufacturer's instructions. Resultant aRNA was quantified and quality assessed as above. Subsequently, Cy dye suspensions (Cy3 & Cy5) in sufficient quantity for all labelling reactions were prepared by adding 38 µL high purity dimethyl sulphoxide (Stratagene,

Agilent Technologies UK Ltd.) to each tube of Cy dye (PA23001 or PA25001, GE Healthcare, Chalfont St. Giles, UK). To attach the Cy dyes, 3 µg each aRNA sample was suspended in 10 µL nuclease-free H_2O and heated to 70 ºC for 2 min. When cooled to room temperature, 3 µL of coupling buffer (0.5 M $NaHCO_3$; pH 9.2) and 2 µL of Cy3 dye suspension stock was added and then incubated for 1 h at 25 ºC in the dark. To label the common pooled reference sample with Cy5, a scaled-up batch reaction was similarly performed. Unincorporated dye was removed by column purification (Illustra AutoSeq G-50 spin columns; GE Healthcare). Dye incorporation and aRNA yield were quantified by spectrophotometry (NanoDrop) and further quality controlled by separating 0.4 µL of the sample on a thin mini-agarose gel and visualising products on a fluorescence scanner (Typhoon Trio, GE Healthcare).

Hybridisation of a total of 6 slides (24 arrays) was performed in a single day, with sample order semi-randomised, using SureHyb hybridisation chambers in a DNA Microarray Hybridisation Oven (Agilent Technologies). For each hybridisation, 825 ng of Cy3-labelled experimental biological replicate and Cy5-labelled reference pool of all the samples were combined and total volume made up to 35 µl with nuclease-free water. A fragmentation master mix was prepared containing, per reaction, 11 µl 10× blocking agent, 2.2 µl 25× fragmentation buffer and 6.8 µl nuclease-free water, and 20 µl was dispensed into the Cy-dyes mix. After incubating in the dark at 60 ºC for 30 min, 57 µl 2×GE Hybridisation buffer (pre-heated to 37 ºC) was added, contents gently mixed, spun at 16,000 g for 1 min and kept on ice until loaded onto the microarray slides as per the manufacturers protocol. Hybridisation was carried out in the oven rotator (Agilent Technologies) at 65 ºC and 10 rpm for 17 h. Post-hybridisation washes were carried out in EasyDip^{TM} Slide staining containers (Canemco Inc., Quebec, Canada). After disassembling the array-gasket sandwiches were submerged in wash

buffer 1 at room temperature, the microarray slides were transferred to an EasyDip™ container and incubated in wash buffer 1 for 1 min at 31 ºC in an orbital incubator rotating at 150 rpm, and then a further 1 min at 31 ºC at 150 rpm in wash buffer 2. A final dip in wash buffer 2 at room temperature was performed, after which the slides were dried by centrifugation (500 x g for 6 min) and kept in a desiccator in the dark until scanned the same day. Unless otherwise stated, all reagents were from Agilent Technologies.

Scanning was performed at 5 μm resolution using an Axon GenePix 4200AL Scanner (MDS Analytical Technologies, Wokingham, Berkshire, U.K.). Laser power was kept constant (80 %) and the "auto PMT" function within the acquisition software (v.4) was enabled to adjust PMT for each channel such that less than 0.1 % of features were saturated and that the mean intensity ratio of the Cy3 and Cy5 signals was close to one. Agilent Feature Extraction Software (v 9.5) was used to identify features and extract fluorescence intensity values from the resultant TIF images. The remaining analysis was then performed in the GeneSpring GX version 12 analysis platform (Agilent Technologies). After removing control features and filtration of low quality spots (saturated, non-uniform, population out layers and spots non-significantly different from background), lowess normalisation of log2-expression ratios (ER) was performed.

As the primary objective of the present study was to evaluate the effects of functional feeding (i.e. comparison between diets), differentially expressed genes were selected based on expression changes between the functional feed groups (CMS1 and CMS2) and the fish fed the REF diet, or between the two functional feed groups at each sampling point (PreCh-, 6-, 8- and 14-wpc) (cutoff log2-ER = 0.8 (1.75-fold change), $p < 0.05$, one-way ANOVA. Annotation of selected genes was performed using STARS

software developed at the Norwegian Institute of Food, Fisheries and Aquaculture Research (Nofima, Tromsø, Norway) (Krasnov et al., 2011b), KEGG analysis, and information from literature and public databases. Selected genes from each of the one-way ANOVA lists were submitted to Gene Expression Omnibus (Liu et al., 2007) for enrichment analysis. The numbers of features corresponding to GO functional classes and KEGG pathways were compared in the lists of differentially expressed genes and whole microarray. Enrichment was assessed with Yates' corrected chi-square test ($p<0.05$).

5.3 Results

5.3.1 Growth

There were no significant differences overall between diets at the end of the trial (i.e. after 192 days) (Figure 5.2). However, separate statistical analysis at the beginning and the end of the trial showed a statistical different between the dietary groups before challenge. After 92 days of feeding (pre-challenge) fish fed diet CMS2 grew significantly better than those fed REF diet and CMS1.

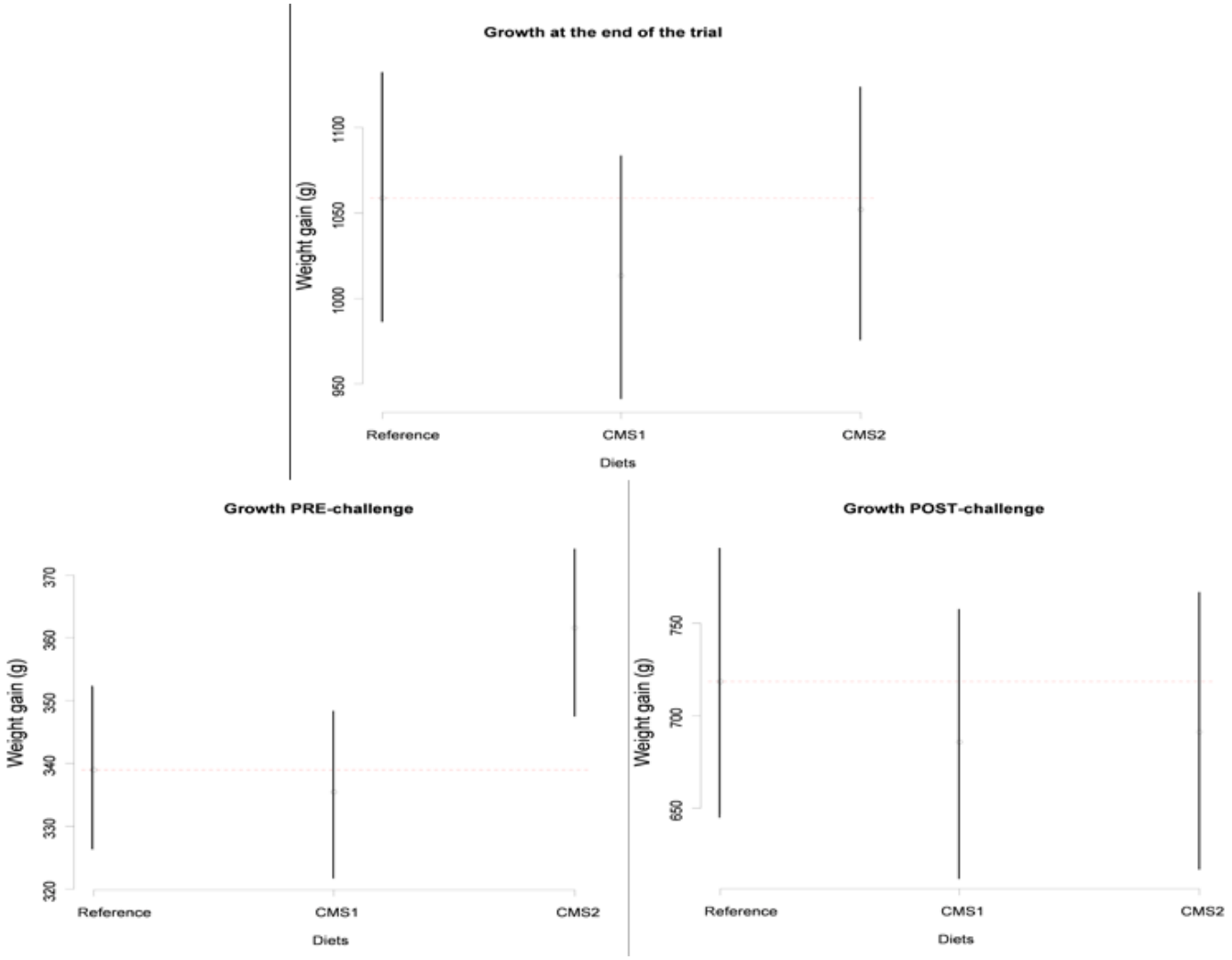

Figure 5.2 Growth performances over the course of the entire trial, before and after the viral challenge. Error bars denote approximate 95 % confidence limits.

5.3.2 Lipid and fatty acid composition of heart tissue

Total lipid content of heart tissue was similar irrespective of dietary treatment and did not show any significant changes over the course of infection (Table 5.4).

Table 5.4. Lipid contents (percentage of wet weight) of heart tissue of salmon fed the reference (REF) and functional (CMS1 and CMS2) feeds immediately prior to challenge with PMCV (PreCh) and at different weeks post-challenge (wpc).

	REF	CMS1	CMS 2
PreCh	2.8 ± 0.2	3.5 ± 0.1	3.6 ± 0.2
6-wpc	3.3 ± 0.2	3.3 ± 0.2	3.5 ± 0.2
8-wpc	3.8 ± 0.4	3.3 ± 0.2	3.6 ± 0.6
10-wpc	3.8 ± 0.3	3.6 ± 0.2	3.6 ± 0.3
12-wpc	3.8 ± 0.2	3.6 ± 0.1	3.5 ± 0.1
14-wpc	3.8 ± 0.3	3.4 ± 0.5	3.5 ± 0.4

There were no significant differences between time-points.

Levels of total phospholipids (PL) and the major PL classes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), were significantly higher in heart tissue in fish fed the functional feeds (Figure 5.3) compared with heart tissue of fish fed the REF diet (2-way ANOVA p-value diet, < 0.05). This was probably a consequence of the levels of triacylglycerols (TAG) being significantly higher in the REF group, with these differences being more obvious at the end of the initial feeding phase prior to infection with PMCV. After PMCV infection, PL levels generally decreased over the course of the infection with this effect being significant for phosphatidylinositol (PI) and phosphatidylserine (PS).

The fatty acid compositions of total PL of heart tissue of fish fed the functional feeds were characterised by lower proportions of monoenes and n-6 PUFA, and higher proportions of saturated fatty acids, n-3 PUFA, LC-PUFA (ARA, EPA and DHA), and EPA/ARA ratio (Figure 5.4). The two functional feeds presented similar levels in the relative proportions of the important LC-PUFA, ARA, EPA and DHA. Thus, heart

tissue phospholipids showed proportions of the LC-PUFA relevant to eicosanoid pathways, and that could therefore potentially influence the immune response (Calder 2009b), that reflected the composition of the diets. The proportions of EPA, ARA and the EPA/ARA ratio were significantly higher in fish fed the functional feeds compared to fish fed the REF diet (P value diet, <0.05). However, levels of DHA were similar between the different dietary groups, which is consistent with our previous study using similar feeds in which levels of DHA were more conserved despite differences in the composition of the diets (Martinez-Rubio et al., 2012b (Chapter 3).

Changes in the proportions of the immune and inflammation-related LC-PUFA in heart total PL during the time course of the infection were similar between the three different groups (Figure 5.4). The changes in ARA post-infection were not significant, but the proportions of EPA and DHA were significantly higher at 6-wpc compared with pre-challenge. After 6-wpc, levels of DHA progressively decreased during the time-course of the infection but proportions of EPA were significantly higher at 10-wpc. As a consequence of the latter, and the changes in ARA levels, the EPA/ARA ratio in heart total PL was also higher in all dietary treatments at 10-wpc. There were no differences in the EPA/ARA ratio in heart PL between fish fed the different diets in the later stages of the infection.

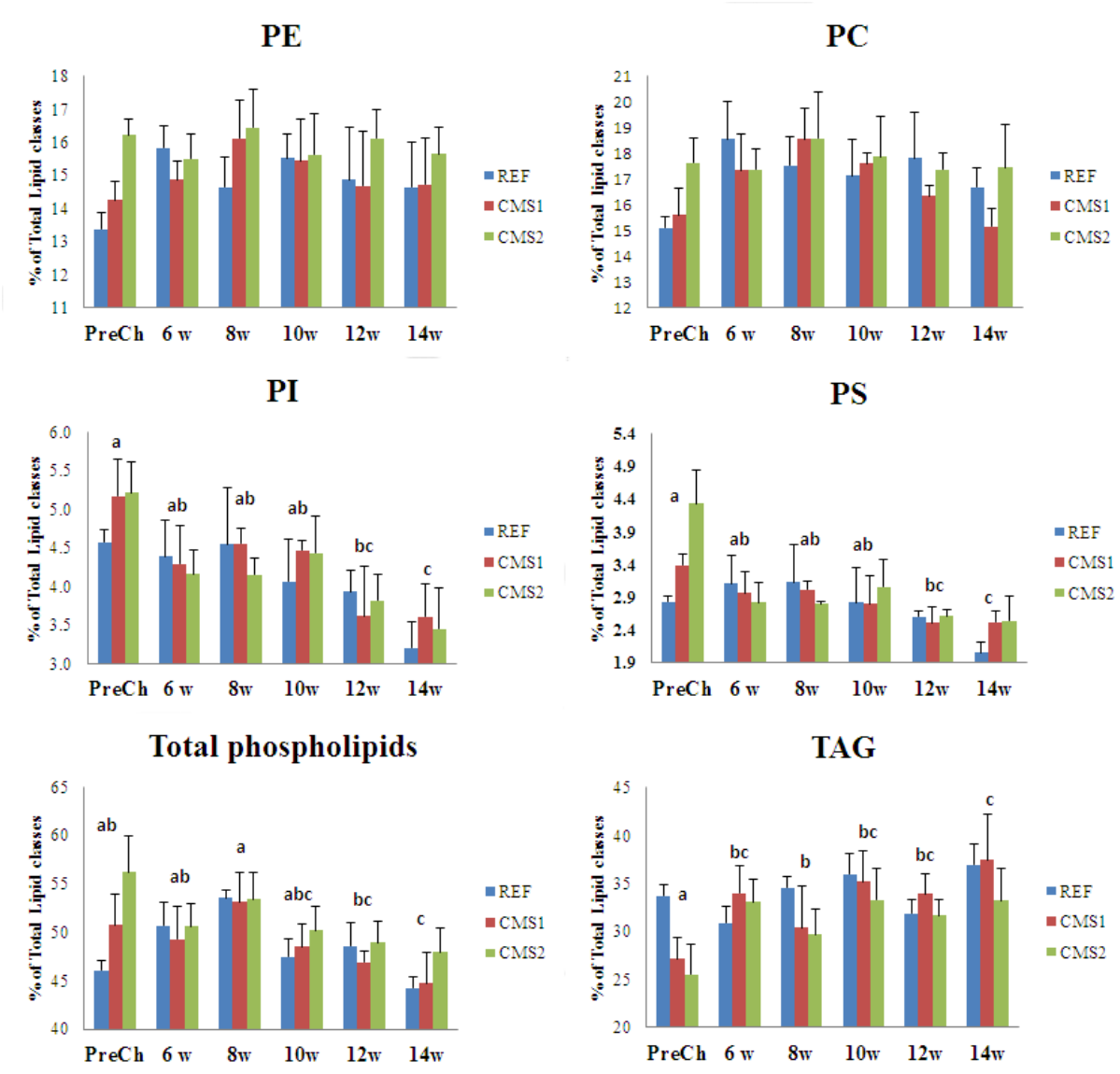

Figure 5.3 Proportions of lipid classes in total lipid of heart tissue from fish fed the reference (REF) and functional (CMS1 and CMS2) feeds at different times before (PreCh) and after (6, 8, 10, 12 and 14 weeks) infection with PMCV. PC, phosphatidycholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TAG, triacylglycerol. Letters are showing significant differences during the time-course of the infection, using the p-value time of the 2-way ANOVA. P-value diet was showing significant differences in PC, PE, TPL and TAG.

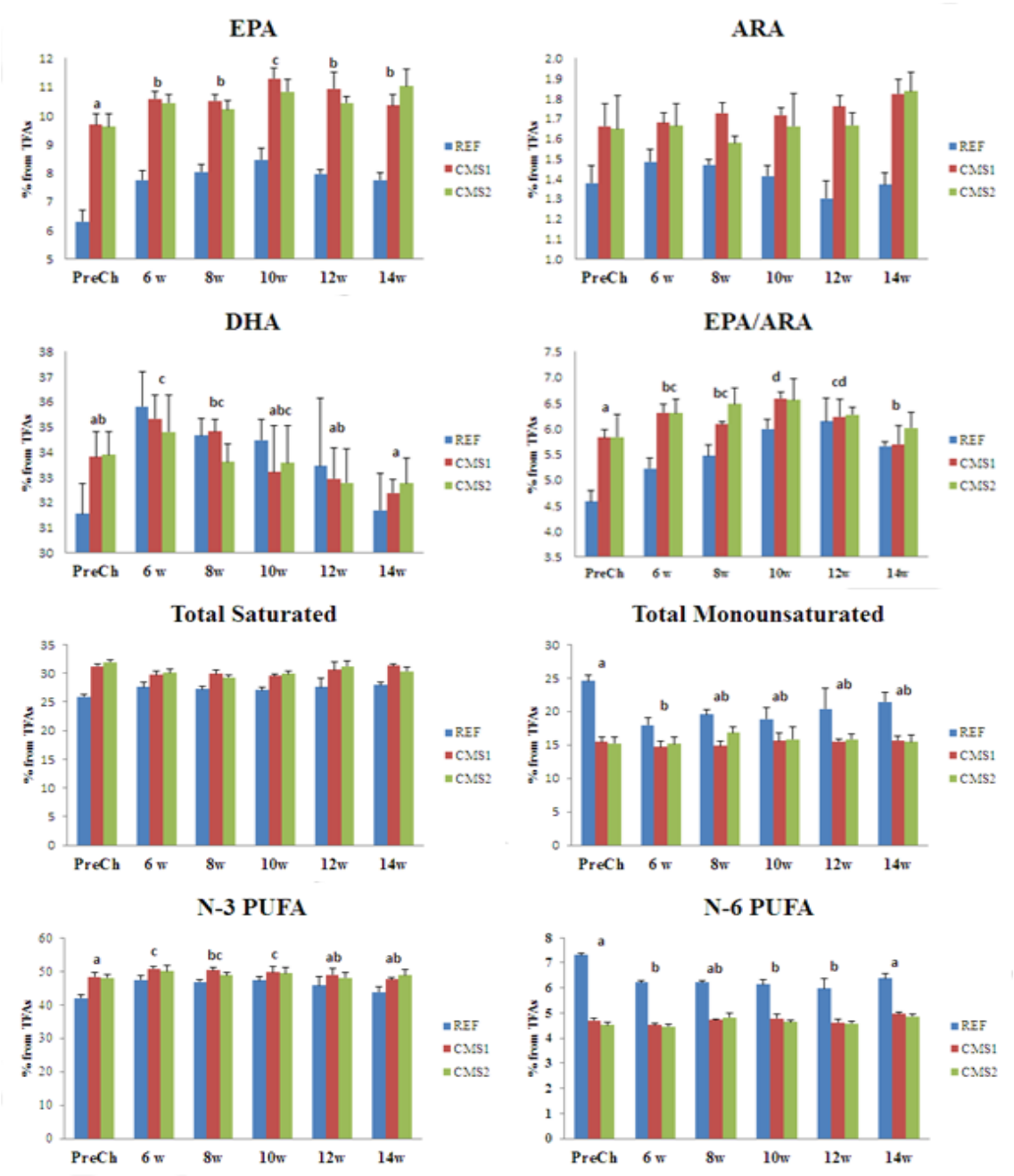

Figure 5.4 Fatty acid compositions (percentage of total fatty acids) of total phospholipids of heart tissue from fish fed the reference (REF) and functional (CMS1 and CMS2) feeds at different times before (PreCh) and after (6, 8, 10, 12 and 14 weeks) infection with PMCV. ARA, arachidonic acid; DHA, docosahexaenoic acid (DHA); EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid. Letters are showing significant differences during the time-course of the infection, using the p-value time of the 2-way ANOVA.P-value diet is showing significant differences between the three treatments in EPA, ARA and EPA/ARA.

5.3.3 Heart and liver tissue histopathology

Histological changes were first observed in the atrium at 6-wpc (Figure 5.5), characterised by focal infiltration of inflammatory cells dominated by lymphocytes. A degenerative process associated with the inflammatory changes was also observed in cardiomyocytes of the atrium. By 8-wpc the sum of the average scores for atrium and ventricle had increased to 4.1 in fish fed the REF diet, and 3.5 for fish fed both functional feeds. The most marked increase in histopathological changes (from 6- to 8-wpc) was found in the ventricle with a 5.6-fold increase in the REF group, and 5.1- and 9.1-fold for the CMS1 and CMS2 dietary groups, respectively. These changes were typified by multifocal infiltration of inflammatory cells, dominated by lymphocytes and macrophages, and concomitant degeneration and necrosis of myocytes. Another typical finding was hyperplasia of endothelial cells in inflamed areas. Inflammatory changes were greatest in the atrium from 6- to 8-wpc and significantly ($p < 0.01$) higher for all groups at both time points. Both groups of fish fed with the functional diets showed significantly lower histoscores in the atrium (Figure 5.6) at both 6- and 8-wpc. Lesions in the ventricle were also significantly lower for the group of fish fed with the functional feeds. By 10-wpc, the inflammatory changes and myocyte necrosis had levelled off in all groups, and was not different from 8-wpc. At 12- and 14-wpc there was a moderate decline in inflammatory scores (Figure 5.5). There were no differences between dietary groups at 10- and 12-wpc in both parts of the heart. At the end of the trial, 14-wpc, lesions in the atrium were not significantly different between groups, although fish fed with the CMS2 have lower histoscores compare with the other groups. In ventricle, fish fed both functional feeds showed lower histoscores although they were not significant (Figure 5.6).

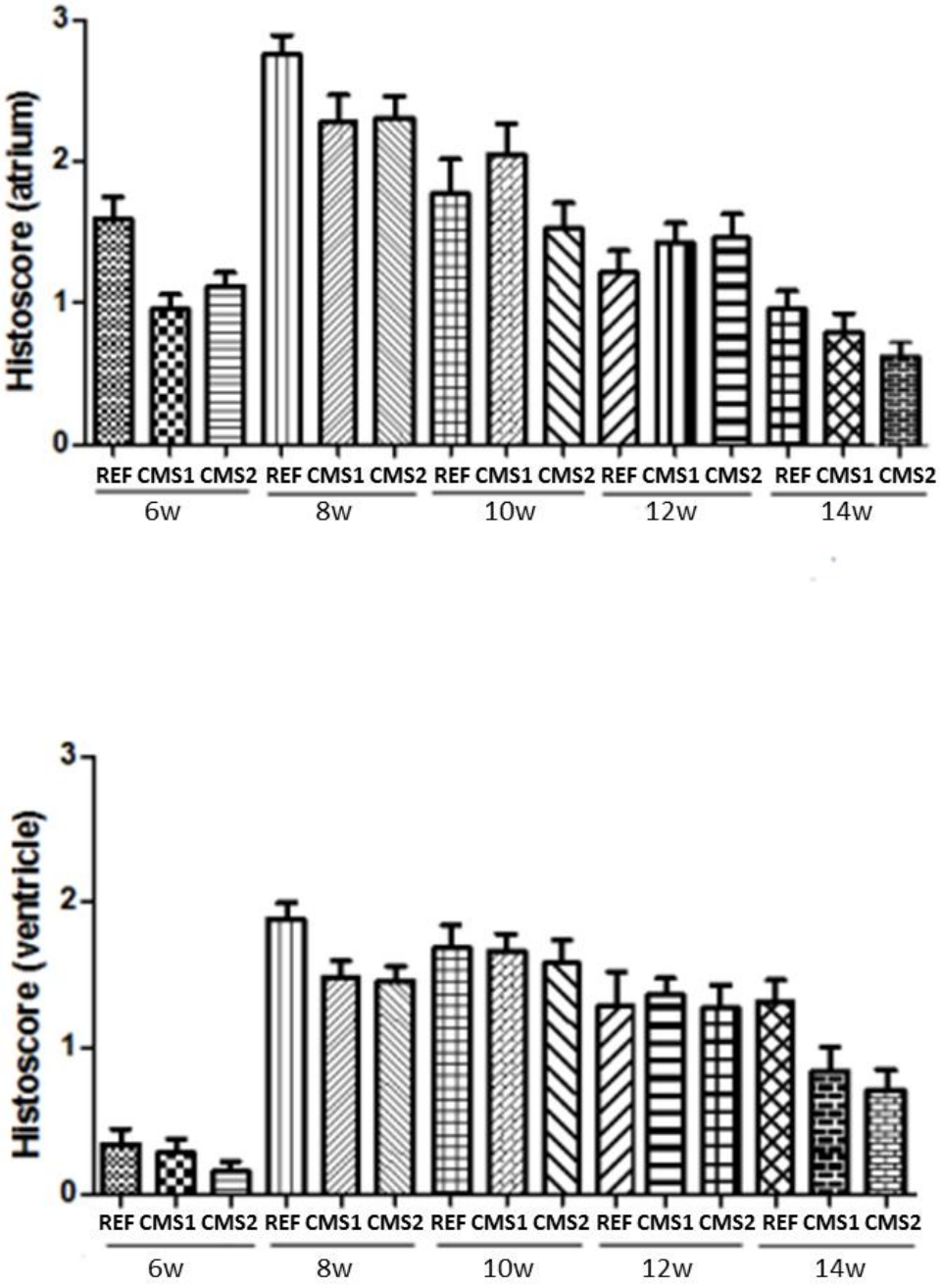

Figure 5.5 Average histoscores (± SEM) of atrium and ventricle in the different dietary treatment groups at 6-, 8-, 10-, 12- and 14-weeks post-challenge

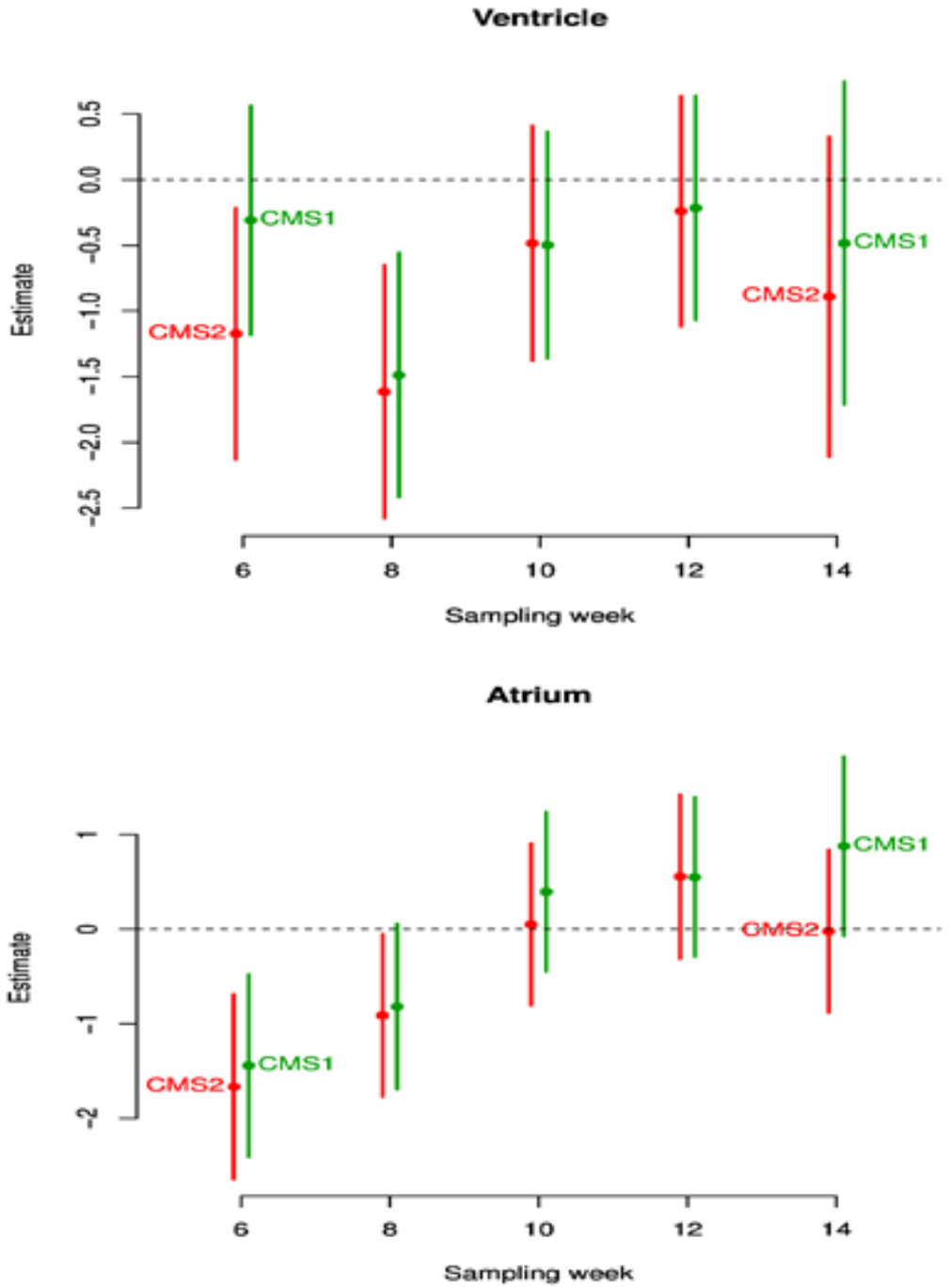

Figure 5.6 Statistical analysis of the atrium and ventriculum histoscores. Estimated effects of CMS1 and CMS2 diets in comparison to the REF diet by sampling weeks. Negative estimates mean there are lower scores and positive that there are higher scores than for the REF dietary group. Error bars denote approximate 95 % confidence limits.

The liver histology scores, based on the degree of steatosis were read as vacuole-formation in hepatocyte cytoplasm characterised by both micro- and macro-vesicular

lesions. There were clear differences between the fish fed the functional feeds and fish fed the REF diet over all the samplings points post-challenge, with the latter group presenting higher micro- and macro-vesicular steatosis. Severity of the liver histopathology (based on the scoring system in Table 5.3) was significantly higher at the beginning of the infection and greater at the end of the challenge (12- and 14-wpc) in salmon fed the REF diet compared to fish fed the two functional feeds (Figure 5.7).

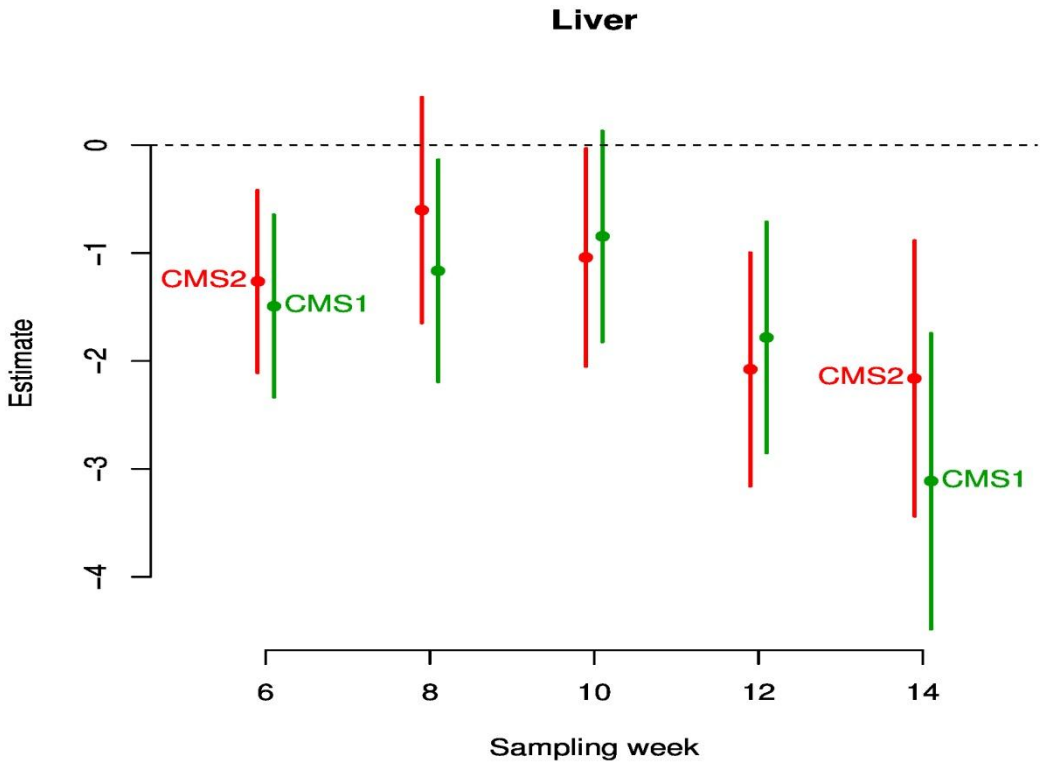

Figure 5.7 Statistical analysis of liver histoscores. Estimated effect of CMS1 and CMS2 diets in comparison to the REF diet by sampling weeks. Negative estimates means there are lower scores and positive that there are higher scores than for the REF dietary group. Error bars denote approximate 95 % confidence limits.

5.3.4 Gene expression (microarray analysis)

5.3.4.1 Pre-challenge.

Immediately prior to challenge, fish were sampled to specifically evaluate the differences in gene expression in heart tissue between fish fed the three dietary treatments. The genes showing significant and relevant differential expression between

the three dietary groups (criteria described above) was the largest list in this study highlighting the great influence of diet on metabolic pathways including the immune/inflammatory response (Supplementary Table 5.1). The enrichment analysis list at this point showed the relevance of the innate immune response and especially the MHC class I pathway with genes related to both pathways being significantly represented (p-value < 0.001) (Table 5.5). The list, with annotations from Kegg analysis, STARS software, and literature references, shows that there was a general lower expression of most of the genes related with the innate immune response, MHC class I response, B-cell and T-cell immune response and apoptosis in fish consuming the functional feeds compared with fish fed the REF diet (Table 5.6). This list also shows there was higher expression of genes involved in transcription, translation and nucleotide metabolism in fish fed the functional feeds compared with fish fed the REF diet, possibly indicating increased protein turnover and metabolism in those groups. Of the genes related to lipid metabolism (Table 5.7), some genes involved in membrane transport showed increased expression in fish fed the functional feeds, especially CMS2, compared with the REF fish, but genes associated with most other lipid metabolic processes generally showed lower expression in fish fed the functional feeds. Genes related with fatty acid biosynthesis such as *delta-6 fatty acyl desaturase* and *fatty acyl-CoA hydrolase precursor* were differently expressed between all three dietary groups, with higher expression in fish fed the CMS1 diet and lower expression in fish fed the CMS2 diet, compared to fish fed the REF diet.

Table 5.5 Enrichment analysis.

Prechallenge			
GO Biological process Category	**% Count in Selection**	**% Count in Total**	**Yates corrected Chi-square**
ErbB signaling pathway	1.73	0.72	0.0287
focal adhesion	1.51	0.58	0.0241
innate immune response	1.94	0.51	0.0002
Leukocyte transendothelial migration	2.38	1.06	0.0147
MHC class I protein complex	0.86	0.15	0.0017
steroid hormone receptor activity	1.30	0.43	0.0159
6-weeks post challenge			
GO Biological process Category	**% Count in Selection**	**% Count in Total**	**Yates corrected Chi-square**
basement membrane	1.45	0.32	0.0005
cell-cell adhesion	1.69	0.57	0.0094
ECM-receptor interaction	1.93	0.87	0.0477
extracellular space	3.86	2.19	0.0403
Focal adhesion	4.10	2.14	0.0142
immune response	6.02	1.57	0
protein serine/threonine kinase activity	4.82	2.90	0.0387
regulation of Rab GTPase activity	2.41	0.68	0.0002
8-weeks post challenge			
GO Biological process Category	**% Count in Selection**	**% Count in Total**	**Yates corrected Chi-square**
Cytokine-cytokine receptor interaction	1.80	0.56	0.0102
Hematopoietic cell lineage	2.40	0.45	0
protein serine/threonine kinase activity	5.69	2.90	0.0067
Rab GTPase activator activity	3.29	0.70	0
regulation of Rab GTPase activity	3.29	0.68	0
structural constituent of cytoskeleton	2.10	0.61	0.0028
T cell receptor signaling pathway	2.10	0.91	0.0547
14-weeks post challenge			
GO Biological process Category	**% Count in Selection**	**% Count in Total**	**Yates corrected Chi-square**
B cell receptor signaling pathway	2.18	0.69	0.0244
ErbB signaling pathway	2.62	0.72	0.0039
Insulin signaling pathway	3.49	1.16	0.0042
Jak-STAT signaling pathway	2.18	0.57	0.0066
lipid metabolic process	2.18	0.66	0.0191
Natural killer cell mediated cytotoxicity	2.18	0.58	0.0079
Phosphatidylinositol signaling system	2.18	0.61	0.0114
Purine metabolism	3.06	1.24	0.0361
Regulation of actin cytoskeleton	3.93	1.79	0.0349
regulation of Rab GTPase activity	2.18	0.68	0.0225
regulation of Rho protein signal transduction	3.49	0.99	0.0008
T cell receptor signaling pathway	3.06	0.91	0.0032
ubiquitin-dependent protein catabolic proc.	2.62	0.96	0.03

Table 5.6 Gene expression pre-challenge. Immune-related genes selected by significance (one-way ANOVA) and expression differences between the functional feeds (CMS1 and CMS2) and reference diet (REF) or between CMS dietary groups. At cut-off log2-ER = 0.8 (1.75-fold). Red/orange colour intensity was used to indicate higher expression and green/blue colour intensity was used to indicate lower expression.

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Antiviral and IFN response				
sacsin	Ssa#KSS1716	-1.90	-0.86	-1.04
interferon induced with helicase C domain 1	Ssa#S48413574	-1.67	-1.14	-0.52
Signal transducer and activator of transcription 3	Ssa#S32012561	-1.43	-2.07	0.64
similar to mKIAA2005 protein	Ssa#S30296047	-1.42	-1.25	-0.17
fish virus induced TRIM protein	Ssa#KSS4382	-1.38	-0.95	-0.43
VHSV-inducible protein-4	Ssa#KSS4396	-1.24	-1.37	0.12
VHSV-induced protein	Ssa#KSS1846	-1.22	-1.28	0.07
interferon, gamma	Ssa#S22497299	-1.08	-1.46	0.39
Ubiquitin-like protein-1	Ssa#S35549782	-0.94	-0.92	-0.03
retinoic acid-inducible gene-I	Ssa#DY714827	-0.90	-0.81	-0.09
Gig1-2	Ssa#S30282627	-0.88	-0.99	0.11
Interferon-induced protein 44	Ssa#S31978702	-0.87	-0.70	-0.18
Gig1-1	Ssa#KSS1939	-0.83	-1.24	0.41
Poly polymerase 12	Ssa#KSS366	-0.82	0.56	-1.38
Interferon-induced protein 44-1	Ssa#S30269828	-0.80	-0.78	-0.02
fish virus induced TRIM protein	Ssa#CL81Contig1	-0.80	-0.65	-0.15
interferon regulatory factor 7	Ssa#KSS4495	1.74	2.15	-0.41
Interferon regulatory factor 4	Omy#S18532156	1.49	1.40	0.09
Interferon-induced protein with tetratricopeptide repeats 5-1	Ssa#S31971912	-0.66	-1.93	1.27
Receptor-transporting protein 3	Ssa#KSSb2765	-0.55	-1.35	0.80
interferon-gamma receptor 1	Ssa#KSS450	0.07	-1.08	1.15
sacsin	Ssa#S35671378	-0.39	-0.96	0.58
fish virus induced TRIM protein	Omy#S22915797	-0.64	-0.80	0.17
VHSV-induced protein-10	Ssa#S35559165	-0.55	-0.64	0.09
Interferon-induced protein 44	Ssa#EG868694	-0.74	-0.74	-0.01
interferon regulatory factor 1	Ssa#S41489472	-0.64	-0.54	-0.10
Tripartite motif containing TRIM 25	Ssa#DY701534	-0.77	-0.39	-0.38
GTPase IMAP family member 7	Ssa#S35509469	0.91	0.41	0.50
Tripartite motif-containing protein 25	Ssa#S32009123	-1.17	-0.38	-0.80
52 kDa Ro protein	Omy#S15242638	0.82	1.63	-0.81
Toll-interacting protein	Ssa#S35591296	-0.69	-1.24	0.55
Apoptopsis				
Rho guanine exchange factor 16	Omy#S15320242	0.89	0.19	0.70
programmed cell death 2-like	Ssa#CK879149	-2.10	-1.90	-0.20
death associated transcription factor 1-like	Omy#CA384493	-1.13	-1.04	-0.09
caspase 7	Ssa#STIR00029	0.84	1.61	-0.77
membrane metallo-endopeptidase	Ssa#S30275401	-2.87	-1.15	-1.72
meprin A, beta	Ssa#CK884807	-1.63	-2.60	0.97
endopeptidase Clp precursor	Ssa#S30295980	-1.39	-1.31	-0.08
matrix metalloproteinase	Ssa#S25807320	-1.13	-0.10	-1.02
placental protein 11 precursor	Ssa#EG935968	-0.72	-1.01	0.30
SUMO-activating enzyme subunit 1	Ssa#TC81961	-1.63	-0.66	-0.97
E3 ubiquitin-protein ligase Hakai	Omy#S15282203	0.54	1.59	-1.05
ubiquitin specific protease 32	Ssa#S18861997	-1.45	-0.45	-1.01
E3 ubiquitin-protein ligase NRDP1	Ssa#S30262289	-0.83	-0.48	-0.35

Table 5.6 (continued 1)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
B-cell				
Lymphocyte antigen 75 precursor	Ssa#S48406409	1.48	1.46	0.02
AF361437_1 interleukin 13 receptor alpha-2	Omy#EXOB3_B11	0.62	0.02	0.60
Hematopoietic lineage cell-specific protein	Ssa#KSS4990	0.22	1.80	-1.58
Immunoglobulin light chain variable region	Ssa#S35665005	-0.94	-1.33	0.39
IG LC	Ssa#S18892313	-0.47	-1.39	0.92
Immunoglobulin mu heavy chain	Ssa#S18836426	-0.57	-1.02	0.44
Ig kappa chain V-IV region B17 precursor	Ssa#DW471462	-0.38	-0.97	0.59
AF273017_1 immunoglobulin light chain precursor	Ssa#S18892310	-0.29	-0.83	0.54
IG HC variable region	Ssa#S18892407	-0.63	-0.77	0.14
Complement				
complement factor H1 protein	Omy#CT568091	1.33	0.04	1.29
Eicosanoids				
cytochrome P450, family 2, subfamily J, polypeptide 25	Ssa#S31983866	1.11	-0.17	1.28
cytosolic phospholipase A2, group IVA	Omy#S34423720	0.90	0.37	0.53
Innate immune response				
CD83 antigen precursor	Ssa#KSS3110	-0.55	-0.25	-0.30
Signaling intermediate in Toll pathway	Ssa#S31970854	-0.81	-0.97	0.17
macrophage colony-stimulating factor receptor	Ssa#S31986130	0.66	0.07	0.59
TNF- receptor superfamily, member 19 isoform 2 prec	Ssa#S30280758	-0.81	-1.34	0.53
Ras-related and estrogen-regulated growth inhibitor	Ssa#DW542009	-0.82	-1.05	0.24
A-kinase anchor protein 10 precursor	Ssa#S48405355	-0.63	-1.70	1.07
kelch-like 26	Ssa#TC89603	-1.59	-1.11	-0.49
Suppressor of G2 allele of SKP1 homolog	Ssa#S35582811	-1.01	-1.10	0.09
Integrin, beta-like 1 (with EGF-like repeat domains)	Ssa#S30292184	-0.89	-1.40	0.51
integrin alpha 4 precursor	Ssa#CA049694	-0.74	-1.54	0.80
neutrophil cytosolic factor 2	Ssa#S35600971	0.90	0.08	0.81
neutrophil cytosolic factor 2	Ssa#KSS461	-0.65	-1.60	0.95
G protein-coupled receptor 137C	Ssa#DY708003	-1.14	-0.73	-0.41
similar to TGF-beta type II receptor	Ssa#S22709188	-1.94	-1.53	-0.41
mitogen-activated protein kinase 7 interacting prot2	Ssa#TC110578	-1.30	-0.99	-0.31
Mitogen-activated protein kinase 12	Ssa#S30240975	-0.88	-1.35	0.47
akirin 1(2a)	Ssa#KSS177	-0.81	-0.76	-0.05
MHC antigen presentation				
Cathepsin Z precursor	Ssa#AM402782	-0.66	-0.90	0.24
MHC class I antigen	Ssa#TC108524	-2.33	-1.57	-0.76
MHC class I antigen	Ssa#S35668662	-0.82	-0.60	-0.22
MHC class II beta chain	Omy#S15341284	1.58	0.33	1.25
MHC class I antigen	Ssa#S29966061	-0.73	-0.57	-0.16
AF508864_1 MHC class I	Ssa#S18892284	-0.71	-0.48	-0.24

Table 5.6 (continued 2)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Natural cell killer				
Granzyme A precursor	Ssa#DW578033	2.14	-1.33	3.47
CD2 antigen cytoplasmic tail-binding protein 2	Omy#S15249212	-0.85	-0.33	-0.52
protein serine/threonine kinase activity				
PREX1 protein	Omy#S15319930	2.24	2.05	0.19
Cyclic adenosine 3, 5-monophosphate dependent prot kinase	Omy#BX913234	0.94	0.72	0.22
protein kinase C, alpha	Omy#TC170368	0.68	1.69	-1.00
PREX1 protein	Ssa#CX357733	-0.43	-1.46	1.04
PTK2 protein tyrosine kinase 2 isoform a	Ssa#STIR25175	-0.60	-0.81	0.21
Serine/threonine-protein kinase Sgk1	Ssa#DY693244	1.28	1.62	-0.34
G protein-coupled receptor family C	Ssa#S30281123	0.69	3.49	-2.80
T-cell response				
T-lymphocite maturation assoc protein	Ssa#S35581943	-1.01	-0.23	-0.78
H2.0-like homeobox protein	Ssa#S35562668	0.64	0.59	0.05
T cell receptor alpha	Ssa#S37959053	-1.92	-2.10	0.18
T cell receptor alpha	Ssa#TC108495	-1.09	-1.38	0.29
T cell receptor alpha	Ssa#NP9934055	-1.05	-1.68	0.63
T cell receptor alpha	Ssa#S37959570	-0.92	-1.46	0.54
T-cell receptor beta chain 3 variable region	Ssa#DW473052	1.91	2.35	-0.44
T cell receptor alpha	Ssa#S37959185	-0.18	-2.02	1.83
T cell receptor alpha	Ssa#NP9934310	-0.12	-1.52	1.40
T cell receptor alpha	Ssa#S37959051	0.25	-1.46	1.71
T-cell activation Rho GTPase-activating protein	Ssa#S32008293	0.77	0.58	0.19
protein tyrosine phosphatase, receptor, C isoform 2 precursor	Omy#CA382452	-1.64	-0.93	-0.71
bridging integrator 3	Ssa#S31984691	-1.59	-1.75	0.16
proto-oncogene tyrosine-protein kinase fyn	Ssa#S35552559	-1.32	-1.30	-0.02
crystallin, gamma B	Ssa#S19189155	1.01	1.09	-0.08
Beta-crystallin A1-2	Ssa#S18849203	1.39	2.04	-0.66
SH2/SH3 adaptor crk	Omy#S15315949	1.77	1.22	0.55

Table 5.7 Gene expression pre-challenge. Metabolic genes selected by significance (one-way ANOVA) and expression differences between the functional feeds (CMS1 and CMS2) and reference diet (REF) or between CMS dietary groups. At cut-off log2-ER = 0.8 (1.75-fold). Red/orange colour intensity was used to indicate higher expression and green/blue colour intensity was used to indicate lower expression.

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Lipid Metabolism transport				
diacylglycerol kinase, zeta 104kDa isoform 4	Ssa#S31987853	-1.10	-0.93	-0.17
Ethanolamine kinase 1	Ssa#STIR44126	0.95	2.51	-1.57
O-acyltransferase domain containing 2	Omy#BX085588	0.34	1.52	-1.18
Choline-phosphate cytidylyltransferase A	Omy#S34309497	0.13	1.27	-1.14
ATP-binding cassette, sub-family A, member 2	Omy#TC156037	-1.32	0.03	-1.35
ATP-binding cassette, sub-family A member 1	Omy#DV200496	-0.11	-1.12	1.01
ATP-binding cassette, sub-family B, member 10	Omy#S15281990	-0.21	-0.97	0.77
Peroxisomal 2,4-dienoyl-CoA reductase	Ssa#S48403141	-1.22	-0.70	-0.52
carnitine O-octanoyltransferase	Ssa#DW564434	-1.83	-0.75	-1.07
Peroxisomal 2,4-dienoyl-CoA reductase	Ssa#S48403141	-1.22	-0.70	-0.52
delta-6 fatty acyl desaturase	Ssa#S18892244	1.00	-1.90	2.90
protein kinase, AMP-activated, alpha 1 subunit 2	Ssa#BM414052	-0.36	0.61	-0.98
Fatty acyl-CoA hydrolase precursor, medium chain	Ssa#CA041487	0.57	-2.03	2.60
Bile acid receptor	Ssa#S32008595	-1.10	-0.96	-0.14
Nucleotide Metabolism				
Cullin-4B	Ssa#TC111475	-1.21	-2.28	1.06
Uridine-cytidine kinase 2	Ssa#S32008884	-1.19	-0.94	-0.25
hypothetical protein LOC553671	Omy#S15300468	-1.11	-0.96	-0.15
Cytosolic 5-nucleotidase 1A	Omy#S34310160	2.63	2.55	0.07
phosphodiesterase 7A isoform a	Ssa#DY729436	-0.47	-1.47	0.99
Carbohydrate Metabolism				
Serine hydroxymethyltransferase, mitochondrial precursor	Ssa#S30288614	-1.96	-1.79	-0.17
Alpha-galactosidase A precursor	Ssa#S30239539	1.79	0.69	1.10
galactosidase, beta 1 isoform a	Omy#S18164426	1.76	1.40	0.35
hexose-6-phosphate dehydrogenase precursor	Ssa#DV106122	1.09	0.63	0.46
Glyoxylate reductase/hydroxypyruvate reductase	Ssa#CK896827	0.98	1.34	-0.36
6-phosphofructokinase type C	Ssa#DW182233	-1.46	0.70	-2.16
alpha amylase	Omy#TC139939	-0.93	-2.18	1.25
Solute carrier family 2	Ssa#DW563694	2.01	1.24	0.77
Triosephosphate isomerase	Ssa#EL697699	1.16	0.57	0.59
solute carrier family 2	Ssa#TC112180	0.68	1.83	-1.16
Amino Acid Metabolism				
cytokine-like nuclear factor n-pac	Omy#TC155552	-1.64	-0.09	-1.54
DNA cytosine methyltransferase 3 alpha isoform a	Ssa#DW561493	-1.55	-0.97	-0.58
medium-chain acyl-CoA dehydrogenase	Ssa#S35548423	-1.33	-0.68	-0.65
Myc protein	Ssa#TC82506	-1.23	-0.66	-0.57
similar to hCG2041215	Ssa#DW578787	0.86	1.24	-0.38
suppressor of variegation 3-9 homolog 1	Ssa#DY732310	-0.12	-1.53	1.42

5.3.4.2 6 weeks post-challenge (infection)

Six weeks after the viral infection the enrichment analysis showed that, at this point, differences between the three dietary groups were focussed on the immune response (Table 5.5). Moreover, the list of genes related with the immune response that were significantly differentially expressed between the three dietary groups was the largest over the course of the infection, highlighting the clear dietary influence on these responses at this point (Table 5.8). In general, genes related to the innate immune response, complement pathway, eicosanoids, B-cell and T-cell responses, showed lower expression in fish consuming the functional feeds compared with fish fed the REF diet. Most of the genes related to antiviral and interferon immune responses were, however, similarly or higher expressed in fish fed the CMS1 diet compared with the REF group, with fish fed the CMS2 diet showing lower expression of these genes. The expression of genes related with apoptosis was, in general, higher in fish fed the REF diet, although the expression of some important markers of the apoptotic process, such as *caspase recruitment domain family member 11*, was higher in fish consuming the functional feeds, which could indicate the beginning of apoptosis in these fish.

Enrichment analysis also showed the importance of cell signalling pathways at this point, with genes related with *basement membrane, cell-cell adhesion* and the *regulation of RAb GTPase activity* generally higher expressed in the REF dietary group compare with the functional feed groups (Table 5.5). This result correlated with the broad expression of genes related with different pathways of the immune response observed in the REF group as cell trafficking and signal transduction are crucial for the development of host responses after a viral infection.

Differences between the groups of fish fed the two different functional feeds were mainly found in genes related with the *Antiviral and IFN response* and *Apoptosis*, which were higher expressed in fish fed the CMS1 diet compared to those fed the CMS2 diet.

Table 5.8 Gene expression 6-weeks post-challenge. Immune related genes from different pathways selected by significance (one-way ANOVA) and expression differences between the functional feeds (CMS1 and CMS2) and reference diet (REF) or between CMS dietary groups. At cut-off log2-ER = 0.8 (1.75-fold). Red/orange colour intensity was used to indicate higher expression and green/blue colour intensity was used to indicate lower expression.

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Antiviral and IF response				
up-regulated gene 4 isoform 3	Omy#CA379360	-0.67	-0.98	0.31
interferon induced with helicase C domain 1	Ssa#S48413574	-0.49	-2.39	1.90
Very large inducible GTPase 1-3	Ssa#BQ035726	-0.38	-1.46	1.09
Interferon regulatory factor 2	Ssa#S41489471	-0.09	-0.72	0.62
Interferon-induced protein with tetratricopeptide repeats 5-1	Ssa#S31971912	1.00	-0.09	1.09
GTPase IMAP family member 7	Ssa#EG764760	-0.38	-1.24	0.87
retinoic acid induced 16	Ssa#S30239710	-0.15	-1.18	1.03
Retinol dehydrogenase 12	Omy#CX142563	-0.14	-1.89	1.75
ret finger protein	Ssa#S32007273	-1.58	-0.41	-1.17
v-akt murine thymoma viral oncogene 2-like protein	Ssa#DY694205	-1.70	-0.84	-0.86
coiled-coil domain containing 55	Ssa#BM414279	-0.39	-1.09	0.69
fish virus induced TRIM protein	Omy#S34422596	1.18	0.34	0.83
kelch-like 3	Ssa#DY738366	1.34	0.98	0.36
interferon-inducible double stranded RNA dependent inhibitor	Ssa#KSS4526	0.66	1.33	-0.67
dachshund homolog 1 isoform b	Ssa#S30240633	1.17	-0.01	1.18
very large inducible GTPase 1	Ssa#CA040423	1.24	-0.51	1.75
Apoptosis				
Regulator of G-protein signaling 1	Ssa#CL47Contig1	0.27	-1.13	1.40
ubiquitin protein ligase E3A isoform 2	Omy#S15322642	-0.51	1.41	-1.92
TNF receptor-associated factor 4	Ssa#S19109495	-0.98	-0.79	-0.18
ubiquitin specific protease 47	Omy#S34315100	1.88	1.54	0.34
apoptotic chromatin condensation inducer 1a	Ssa#S48412898	-1.41	-2.38	0.96
death effector domain-containing protein	Omy#TC135059	-0.53	-0.94	0.41
caspase recruitment domain family, member 11	Ssa#S48433478	2.44	1.81	0.63
calpain 5	Ssa#S31991645	-2.73	-2.46	-0.27
placental protein 11 precursor	Ssa#EG935968	-1.17	-1.12	-0.06
Cathepsin L1	Omy#TC159807	-0.76	-0.76	0.00
Serine protease HTRA1	Ssa#DW550176	0.01	-0.86	0.86

Table 5.8 (continued 1)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
B-cell response				
SH3 protein expressed in lymphocytes	Ssa#KSS3229	-0.79	-0.08	-0.72
immunoglobulin mu heavy chain	Ssa#S35560318	-1.86	-1.80	-0.05
immunoglobulin mu heavy chain	Omy#S15341231	-1.42	-1.09	-0.33
IG HC variable region	Ssa#S18892407	-1.34	-1.34	0.00
IG HC variable region	Ssa#S18892374	-1.34	-0.75	-0.59
immunoglobulin mu heavy chain	Omy#S15341233	-1.29	-1.52	0.22
immunoglobulin mu heavy chain	Omy#S15341239	-1.26	-1.61	0.35
IG HC variable region	Ssa#S30263508	-1.24	-0.95	-0.29
immunoglobulin mu heavy chain	Omy#S15341238	-1.22	-0.86	-0.36
immunoglobulin mu heavy chain	Ssa#S18836426	-1.20	-1.05	-0.15
AF273426_1 immunoglobulin heavy chain variable region	Ssa#S18892363	-1.15	-0.98	-0.17
IgM heavy chain membrane bound form	Ssa#S18892409	-1.10	-0.53	-0.57
AF273436_1 immunoglobulin heavy chain variable region	Ssa#S18892353	-0.93	-1.49	0.56
CD97 antigen precursor	Ssa#S31983538	-1.63	-1.83	0.21
Tyrosine-protein kinase Lyn	Omy#CX255862	-0.96	-1.10	0.14
Complement pathway				
Complement factor D precursor	Ssa#S35687715	-1.90	-1.06	-0.84
complement C4	Ssa#TC80864	-1.24	-0.95	-0.30
Complement C1q subcomponent subunit C precursor	Ssa#S30293441	-0.18	-1.33	1.15
Complement C1q subcomponent subunit C precursor	Ssa#S30254199	-0.07	-0.79	0.73
Complement C1q-like protein 2 precursor	Omy#S15323007	-1.09	1.88	-2.96
Eicosanoids				
Cytochrome P450 2U1	Ssa#DW576911	-2.43	-1.45	-0.98
leukotriene b4 12-hydroxydehydrogenase	Ssa#S35662467	-0.94	-0.59	-0.35
Cytochrome P450	Ssa#S31996544	0.60	0.71	-0.11
Phospholipase A1 member A precursor	Ssa#S35669014	-1.70	-2.31	0.61
Innate immune response				
macrophage expressed gene 1	Ssa#S48415409	-1.47	-1.01	-0.46
amyloid beta (A4) precursor-like protein 2	Ssa#CX727503	-1.09	0.43	-1.52
integrin alpha 2b preproprotein	Ssa#DW546887	0.13	-1.51	1.64
interleukin 17 isoform D-1	Ssa#S35583279	-0.62	-0.28	-0.34
cytokine receptor common gamma chain	Ssa#KSSb2566	-0.65	-0.30	-0.35
CD209 antigen-like protein A	Ssa#TC95863	-0.67	-1.18	0.50
megakaryocyte-associated tyrosine kinase isoform a	Ssa#KSS3516	-1.03	-1.87	0.84
tumor necrosis factor receptor associated factor 2	Omy#S15340564	0.04	-0.94	0.98
Tumor necrosis factor, alpha-induced protein 2	Ssa#KSS2955	-1.81	-0.43	-1.38
Proline-serine-threonine phosphatase-interacting protein 2	Ssa#KSS834	-1.73	-0.17	-1.55
Integrin alpha-10	Ssa#CK891865	-0.84	-0.89	0.05
catenin, alpha 1	Omy#S34424745	-0.18	-1.35	1.18

Table 5.8 (continued 2)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
T-cell response				
CD82 antigen	Ssa#S35505059	-1.10	-1.12	0.03
L-plastin	Ssa#EG803998	-0.78	-0.31	-0.47
T cell receptor alpha	Ssa#NP9933973	-2.14	-0.63	-1.51
T-cell receptor alpha chain V region HPB-MLT precursor	Ssa#S35603081	-1.96	-1.14	-0.81
T cell receptor alpha	Ssa#S37959529	-1.41	-1.86	0.45
T cell receptor alpha	Ssa#S37959531	-1.27	-1.16	-0.11
T cell receptor alpha	Ssa#S37959049	-1.25	-0.66	-0.59
T cell receptor alpha	Ssa#S37959473	-1.25	-1.05	-0.20
T cell receptor alpha	Ssa#S37959118	-1.14	-0.56	-0.58
T cell receptor alpha	Ssa#S37959090	-1.13	-0.74	-0.39
T cell receptor alpha	Ssa#S37959477	-1.12	-0.84	-0.28
T cell receptor alpha	Ssa#NP9934200	-1.11	-0.60	-0.52
T cell receptor alpha	Ssa#NP9934238	-1.08	-0.37	-0.72
CD8 beta	Ssa#S22938352	-1.05	-0.81	-0.24
T cell receptor alpha	Ssa#S37959570	-0.97	-0.58	-0.38
T cell receptor alpha	Ssa#S37959329	-0.96	-0.67	-0.29
T cell receptor alpha	Ssa#S37959572	-0.96	-0.55	-0.40
T cell receptor alpha	Ssa#S37959277	-0.91	-1.13	0.22
T cell receptor alpha	Ssa#NP9934393	-0.89	-0.90	0.02
T cell receptor alpha	Ssa#S37959654	-0.86	-0.44	-0.43
T cell receptor alpha	Ssa#TC107916	-0.81	-0.60	-0.22
T cell receptor alpha	Ssa#S37959481	-0.81	-0.30	-0.51
T-cell receptor beta chain	Omy#NP565024	-0.74	-0.54	-0.20
T-cell surface glycoprotein CD3 zeta chain precursor	Ssa#S37438815	-0.63	-0.47	-0.16
T cell receptor alpha chain	Ssa#S19630248	-0.61	-0.58	-0.03
T-cell antigen receptor	Ssa#S18892491	-0.60	-0.65	0.05
Plastin-2	Omy#S15275191	-0.53	-0.84	0.31
modified T cell receptor alpha	Ssa#NP9934223	-0.90	-2.24	1.34
Tyrosine-protein kinase SYK [Salmo salar]	Ssa#S31979655	-0.97	-0.07	-0.90
T cell receptor alpha [Salmo salar]	Ssa#TC109038	-0.91	0.04	-0.94
similar to granzyme [Danio rerio]	Ssa#S18889540	-0.84	-0.26	-0.59
T cell receptor alpha [Salmo salar]	Ssa#S37959673	-0.84	0.20	-1.04
discs, large homolog 1 isoform 1 [Homo sapiens]	Ssa#TC88397	-1.39	-1.01	-0.38
CTLA4-like protein [Oncorhynchus mykiss]	Ssa#S35533507	-0.69	-1.12	0.43
phosphoinositide-3-kinase, regulatory subunit 1 isoform 1	Ssa#S35572038	-0.05	-1.21	1.15
BNIP2 motif-containing molecule at the C-terminal region 1	Ssa#S31965741	-0.38	-1.56	1.18
RAS guanyl-releasing protein 2	Ssa#DY736725	0.62	1.70	-1.08
NOD3 protein	Ssa#S30240289	0.20	-1.06	1.25
SH2/SH3 adaptor crk	Omy#S15315949	1.96	0.66	1.30
Tyrosine-protein kinase SYK	Ssa#DY705451	-1.39	-0.95	-0.44
Nattectin precursor	Ssa#S31963042	1.68	1.14	0.54

Table 5.8 (continued 3)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Innate to adaptive immune response				
AF418561_1 CC chemokine with stalk CK2	Ssa#KSS1002	0.66	0.32	0.34
Chemokine receptor-like 1	Ssa#KSSb2250	-0.92	-0.28	-0.64
C-X-C motif chemokine 14 precursor	Ssa#S35552426	-0.86	-0.25	-0.61
C3a anaphylatoxin chemotactic receptor	Ssa#S30270134	-1.28	-1.12	-0.15
lymphocyte cytosolic protein 1 precursor	Omy#S34314234	0.14	1.13	-0.98
Leukocyte common antigen precursor	Ssa#KSS1719	-1.30	0.30	-1.60
MHC antigen presentation				
MHC class II beta chain	Omy#S15341284	0.24	-0.87	1.11
Transmembrane and TPR repeat-containing protein 4	Ssa#S31973974	-0.34	-0.86	0.52
Natural killer cells				
granzyme K precursor	Ssa#EG847002	-0.81	-0.06	-0.76
Protein serine/threonine kinase activity				
Serine/threonine-protein kinase Nek7	Ssa#S35580453	-1.47	-0.28	-1.19
Serine/threonine-protein phosphatase 2A 65 kDa A beta	Ssa#KSS3533	0.67	-0.94	1.61
AKT2 kinase	Ssa#CK874283	-0.71	-1.34	0.63
Proline-serine-threonine phosphatase-interacting protein 2	Ssa#KSS834	-1.73	-0.17	-1.55
Serine/threonine-protein kinase tousled-like 2	Ssa#S30279923	0.50	1.14	-0.64
inositol 1,4,5-triphosphate receptor, type 2	Omy#AB023964	-0.24	-0.84	0.60

5.3.4.3 8 weeks post-challenge

Enrichment analysis indicated that, in the list of genes showing significant differences between the dietary treatments, signalling pathways such as *cytokine-cytokine receptor interaction, Rab GTPase activity and protein serine/threonine kinase activity* were prominent at this time point (Table 5.5). However, expression of the annotated genes involved in those pathways was, in general, higher in fish fed the functional feeds compared with the fish fed the REF diet (Table 5.9).

Overall, the expression of genes related to the immune response was generally still lower in the groups of fish consuming the functional feeds compared with the REF group. However, the magnitude of the differences was lower at this time point compared with the magnitude of the differences observed at 6-wpc, especially in genes

related with the T-cell response that were, in some cases, even higher expressed in fish fed the functional feeds. Genes involved in this pathway are reported to be key to the host immune response after a PMCV infection (Haugland et al., 2011; Timmerhaus et al., 2011). Thus, the presence of several genes related with the T-cell response in the lists of genes significantly differentially expressed between the dietary groups at all sampling points assessed after viral infection was consistent with previous data. Furthermore, it showed that the expression of genes in this pathway could be used to track the stage of the disease in fish from the different dietary groups at the different time points, as well as possible modulation of the host immune response by diet.

Also interesting was the presence in the lists of genes involved in negative regulation of the immune response, like *fetuin B, lymphocyte antigen 75 precursor* or *zinc finger, NFX1-type containing 1*, which generally showed higher expression in fish consuming the functional feeds compared with fish fed the REF diet. Differences between the groups of fish fed the two different functional feeds still showed genes related to the *antiviral and IFN response* and *apoptosis* were higher expressed in fish fed the CMS1 diet compared to fish fed the CMS2 diet as at 6-wpi.

Table 5.9 Gene expression 8-weeks post-challenge. Metabolic genes selected by significance (one-way ANOVA) and expression differences between the functional feeds (CMS1 and CMS2) and reference diet (REF) or between CMS dietary groups. At cut-off log2-ER = 0.8 (1.75-fold). Red/orange colour intensity indicates higher expression and green/blue colour intensity indicates lower expression.

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Antiviral and IFN response				
GTPase IMAP family member 7	Omy#S23940134	-0.33	-1.63	1.30
interferon induced with helicase C domain 1	Ssa#S48413574	-0.72	-1.83	1.11
up-regulated gene 4 isoform 3	Omy#CA379360	-0.57	-1.12	0.55
fish virus induced TRIM protein	Omy#TC148385	-0.06	-1.72	1.65
fish virus induced TRIM protein	Omy#BX874038	-0.04	-0.71	0.67
Very large inducible GTPase 1-3	Ssa#BQ035726	-1.82	-2.26	0.44
52 kDa Ro protein-2	Ssa#S18855772	-1.26	-1.81	0.55
mucin 5, subtype B, tracheobronchial	Ssa#S35597062	-0.77	-0.74	-0.03
janus kinase 1	Ssa#DW006200	-0.70	-0.70	-0.01
similar to LDLR dan isoform 5	Ssa#STIR19388	-0.78	-0.47	-0.31
DEAH (Asp-Glu-Ala-His) box polypeptide 15	Ssa#S30293256	-0.82	0.79	-1.61
Apoptosis				
TNF decoy receptor	Ssa#S35595553	-1.56	-1.67	0.10
AF327708_1 retinoic acid receptor-gamma	Ssa#S18892416	2.12	2.95	-0.83
TNF decoy receptor	Ssa#S35595553	-1.56	-1.67	0.10
Pleckstrin homology domain-containing family A member 1	Ssa#S31982099	-0.27	-1.84	1.58
pleckstrin homology domain containing, family A member 7	Ssa#TC107691	0.87	0.26	0.61
Ras-related protein Rab-31	Ssa#DW559421	-0.24	-1.90	1.66
Programmed cell death protein 10	Ssa#S35563611	-0.03	-0.85	0.83
death associated transcription factor 1-like	Omy#CA384493	0.11	-1.14	1.25
programmed cell death 11	Ssa#DW565559	-1.27	-2.01	0.74
programmed cell death 2-like	Ssa#CK879149	-0.48	-0.83	0.35
Damage-regulated autophagy modulator	Ssa#DY714977	-0.48	-0.61	0.14
death inducer-obliterator 1 isoform b	Ssa#DY702496	1.50	0.85	0.65
ubiquitin specific protease 47	Omy#S34315100	1.35	2.41	-1.06
Damage-regulated autophagy modulator	Ssa#DY714977	-0.48	-0.61	0.14
endopeptidase Clp precursor	Ssa#S30295980	0.19	-0.63	0.82
cathepsin L	Omy#CX262263	-1.06	-1.16	0.09
Cathepsin L1	Omy#TC159807	-1.02	-1.13	0.10
Cathepsin L1	Omy#TC144585	-1.01	-1.09	0.08
membrane alanine aminopeptidase precursor	Ssa#S31973583	-0.80	-0.78	-0.02
matrix metalloproteinase	Ssa#S18857994	-2.31	-0.80	-1.51
B-cell response				
B-cell CLL/lymphoma 11B isoform 1	Omy#S23946132	1.01	0.05	0.96
B-cell receptor-associated protein 29	Ssa#S25806768	-0.27	-0.64	0.37
Ig lambda chain V-I region BL2 precursor	Ssa#S35587819	-1.46	-1.62	0.17
immunoglobulin tau heavy chain constant region secreted	Ssa#S35700501	-1.45	-1.50	0.05
immunoglobulin mu heavy chain	Omy#CF752815	-1.47	0.86	-2.33
Tyrosine-protein kinase Lyn	Ssa#TC65653	-0.47	-0.81	0.34
interleukin 4 induced 1 isoform 2	Ssa#S31970040	-0.89	-1.18	0.29
interleukin 4 induced 1 isoform 2	Omy#CA377250	-1.12	-2.12	1.00
Complement				
complement receptor-like	Ssa#S31993393	-0.17	-1.12	0.95
C3a anaphylatoxin chemotactic receptor	Ssa#S35585217	-1.34	-0.88	-0.46
complement factor Bf-1	Ssa#S18849877	-2.13	-0.90	-1.23

Table 5.9 (continued 1)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Eicosanoids				
cytochrome P450	Ssa#S35596838	0.51	1.67	-1.16
Innate immune system				
CD63 antigen	Ssa#S48427799	-0.80	1.00	-1.79
integrin alpha 4 precursor	Ssa#CA049694	-2.07	-1.95	-0.13
integrin alpha 4 precursor	Ssa#DY702132	-1.52	-0.77	-0.75
C-C chemokine receptor type 3	Ssa#S30243811	-0.79	-1.32	0.53
macrophage colony-stimulating factor receptor	Ssa#DW557277	-0.22	-1.69	1.47
interleukin-1 receptor-like protein	Ssa#S18892319	-1.35	-0.53	-0.82
Damage-regulated autophagy modulator	Ssa#DY714977	-0.48	-0.61	0.14
SLAM family member 8 precursor	Ssa#S35700201	-1.06	-0.57	-0.49
matrix metalloproteinase 13	Ssa#S35545523	-1.90	-1.97	0.07
similar to leukocyte immune-type receptor 3	Ssa#S35683164	-0.54	-0.61	0.08
vav 1 guanine nucleotide exchange factor	Ssa#S30295362	0.24	-1.14	1.37
Innate to adaptive				
High affinity immunoglobulin epsilon receptor gamma precursor	Ssa#KSSb2476	-1.99	-0.52	-1.47
minor histocompatibility antigen ha-1	Ssa#KSS1884	-1.45	-1.58	0.13
Natural cell killer				
Granzyme A precursor	Ssa#S31981622	-1.60	-1.55	-0.05
CD226 antigen	Ssa#S30266224	-2.61	-1.33	-1.29
Regulator of the immune response				
Lymphocyte antigen 75 precursor	Ssa#S48406409	1.05	0.64	0.41
zinc finger, NFX1-type containing 1	Ssa#S48395929	1.19	2.07	-0.88
Cysteine-rich secretory protein LCCL domain-containing 2 precursor	Ssa#TC107297	-1.80	-0.60	-1.20
fetuin B	Ssa#S19100169	1.42	0.58	0.83
NG,NG-dimethylarginine dimethylaminohydrolase 2	Ssa#CA048628	-0.64	-2.06	1.42
Protein serine/threonine kinase activity				
Serine/threonine-protein kinase 38	Ssa#DW578681	2.32	2.06	0.25
v-akt murine thymoma viral oncogene 2-like protein	Omy#CA354845	2.03	2.91	-0.88
CHK1 checkpoint homolog	Omy#S15332619	-0.63	2.24	-2.87
Serine/threonine-protein kinase 38	Ssa#DY695675	0.98	-0.44	1.42

Table 5.9 (continued 2)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
T-cell response				
IL-6 subfamily member M17	Ssa#S48442658	-1.76	-1.70	-0.06
Regulator of G-protein signaling 1	Ssa#S35664360	-0.51	-0.61	0.10
Interleukin-2 receptor subunit beta	Ssa#S48408611	-0.59	-0.64	0.05
T cell receptor alpha	Ssa#TC107842	-0.44	-0.98	0.54
CD8 beta	Ssa#S35494290	-0.35	-0.61	0.26
Leukocyte common antigen precursor	Ssa#TC103193	-0.24	-0.93	0.69
T-cell immunoglobulin and mucin domain-containing protein 4	Omy#CA368982	-2.09	-1.39	-0.70
T cell receptor alpha	Ssa#TC109235	-1.66	-1.85	0.19
T-cell receptor alpha chain V region HPB-MLT precursor	Ssa#S35603081	-1.24	-1.42	0.18
T cell receptor alpha	Ssa#S37959058	-1.07	-0.92	-0.14
T cell receptor alpha	Ssa#S37959267	-0.75	-1.08	0.33
CD3 epsilon	Ssa#S37438812	-0.70	-0.75	0.05
CD3 gammadelta-A	Ssa#S37438814	-0.65	-0.47	-0.18
T-cell leukemia homeobox 3	Omy#TC163062	1.44	-0.15	1.59
Nattectin precursor	Ssa#S31963042	2.41	0.33	2.08
Differentially expressed in FDCP 6 homolog	Ssa#S35552642	-0.40	-1.33	0.93
Fermitin family homolog 3	Ssa#KSS3812	0.62	1.10	-0.47
NCK interacting protein with SH3 domain isoform 2	Ssa#S31979794	-0.24	0.81	-1.05
solute carrier family 23 member 1 isoform b	Ssa#S30259049	0.74	0.86	-0.12
hypothetical protein LOC541529	Ssa#DW178537	1.43	2.05	-0.61
human caldesmon 1 transcript variant	Ssa#KSS4667	-0.47	-1.00	0.53
CD40 ligand	Ssa#S35485977	-0.82	-0.57	-0.24
T-cell receptor alpha chain V region 2B4 precursor	Ssa#S35536498	0.56	-1.14	1.70
interferon, gamma	Ssa#S22497299	-1.03	-1.03	0.00

5.3.4.4 14-weeks post-challenge

At this time point, the list of immune-related genes significantly differentially expressed between the dietary groups is the smallest compared with the lists reported above. However, the enrichment analysis showed the relevance of different immune related pathways in this list (Table 5.5). At 14-wpc, the main differences between the dietary groups were mainly in pathways related with cell signalling, but also in important players in the host immune response such as B-cell and T-cell immune response and Natural Killer cell-mediated cytotoxicity. The annotated list showed that, with the exception of the genes related to the innate immune response, the expression of genes related to the other pathways of the host immune response mentioned above was

similar or higher in the fish fed the functional feeds, especially the CMS1 diet (Table 5.10).

To better understand whether the shorter list of genes related with the immune response could be interpreted as remission of the immune response corresponding to a recovery stage of the disease, the expression of genes of the most relevant immune pathways that were higher expressed in fish fed the REF diet at 6-wpc, but were not differentially expressed at 14-wpc, was plotted (Figure 5.8). The, 6-wpc list was selected for this comparison as it was most representative of immune genes after PMCV infection (Timmerhaus et al., 2011). This showed that genes related with the immune response were actively transcribed 14-wpc with their expression generally being similar in all three dietary groups. Moreover, expression of those genes was higher in fish fed the functional feeds compared with their expression at 6-wpc (Figure 5.8). Therefore 14-wpc should be considered as the beginning of a recovery phase after infection with PMCV in this trial.

Table 5.10 Gene expression 14-weeks post-challenge. Immune genes selected by significance (one-way ANOVA) and expression between functional feeds (CMS1 and CMS2) and reference diet (REF) or between CMS groups. At cut-off log2-ER = 0.8 (1.75-fold). Red/orange colour intensity indicates higher expression and green/blue colour intensity indicates lower expression.

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Antiviral and IFN response				
RAP1 GTPase activating protein	Ssa#S48420291	1.65	1.35	0.30
fish virus induced TRIM protein	Omy#BX867355	-0.25	-0.69	0.44
dicer	Ssa#STIR40177	0.66	1.80	-1.14
Very large inducible GTPase 1-3	Ssa#BQ035726	-0.60	-1.89	1.30
fish virus induced TRIM protein	Omy#BX867355	-0.25	-0.69	0.44
Apoptosis				
Pleckstrin homology domain-containing family A	Ssa#S31982099	0.56	1.28	-0.72
cell death-inducing DFFA-like effector a isoform 1	Omy#CX043075	0.43	-1.25	1.67
son of sevenless homolog 1	Ssa#DW548235	-1.80	-0.67	-1.13
carboxypeptidase M precursor	Ssa#S30296241	0.75	1.32	-0.57
membrane metallo-endopeptidase	Ssa#S30275401	0.14	-0.84	0.98
meprin A, beta	Ssa#CK884807	-1.74	-1.40	-0.34
pleckstrin homology domain interacting protein	Omy#CA364148	0.15	-0.94	1.09
triple functional domain (PTPRF interacting)	Ssa#S30276444	1.45	0.34	1.11
Amyloid beta A4 precursor protein-binding family B	Omy#CA354955	0.46	1.86	-1.40
Inositol hexakisphosphate kinase 2	Ssa#S30279656	-0.88	0.01	-0.89
B-cell response				
CD97 antigen precursor	Ssa#DY730078	2.63	1.84	0.79
Eicosanoid				
Cytochrome P450 2U1	Ssa#DW576911	-1.23	-0.62	-0.61
Arachidonate-5-lipoxygenase	Ssa#STIR00023_2	-0.35	0.74	-1.10
Innate immune response				
stress protein HSP70	Ssa#KSS3301	0.12	2.31	-2.19
ccaat enhancer-binding protein zeta	Omy#BX297669	-0.91	-1.01	0.10
tubulin tyrosine ligase-like family member 6a	Ssa#TC82460	-0.46	-0.84	0.38
integrin, alpha 9 precursor	Ssa#TC91550	0.08	-1.03	1.11
CC chemokine	Ssa#KSSb2632	-0.61	-0.86	0.24
CD83 antigen precursor	Ssa#KSS3110	-0.45	-0.48	0.04
megakaryoblastic leukemia 2 protein	Omy#BX913242	-1.53	-2.07	0.54
AF345874_1 novel immune-type receptor 7	Omy#S15340598	-1.85	-1.80	-0.05
Metalloreductase STEAP4	Ssa#TC108295	-0.04	-0.92	0.88
TNF superfamily member 14	Ssa#S31975856	-0.96	0.14	-1.10
Coagulation factor XIII A chain precursor	Ssa#CL28Contig1	1.05	1.91	-0.86
talin 1	Ssa#TC81470	-1.02	0.53	-1.55
transforming growth factor beta	Omy#S23942215	1.12	0.49	0.63
similar to TGF-beta type II receptor	Ssa#S22709188	-0.71	-1.16	0.45
MHC antigen presentation				
beta-2 microglobulin	Ssa#CL57Contig1	-0.07	-0.78	0.71
beta-2 microglobulin type 2	Omy#S34310091	-0.04	-0.76	0.72

Table 5.10 (continued)

BlastxHit	ProbeName	CMS1-REF	CMS2-REF	CMS1-CMS2
Immune regulator				
suppressor of cytokine signaling 5	Omy#TC163366	0.57	1.07	-0.50
inhibitor of kappa light polypeptide enhancer in B-cells	Ssa#EG816410	1.37	-0.12	1.49
fetuin B	Ssa#S19100169	1.51	-0.56	2.07
BRCA1 associated protein-1	Ssa#CK886907	1.60	-0.31	1.91
Phosphatidylinositol signalling system				
Phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1	Ssa#DY718621	1.16	1.45	-0.29
inositol polyphosphate-5-phosphatase A	Omy#TC156380	0.74	0.82	-0.08
phosphoinositide-3-kinase, regulatory subunit 1 (alpha)	Ssa#DY716055	0.85	-0.30	1.15
phosphoinositide-3-kinase, catalytic, delta polypeptide	Ssa#AM042317	0.85	-0.50	1.35
phosphatidylinositol-4,5-bisphosphate phosphodiesterase delta-4	Omy#S23931398	0.36	-1.45	1.81
Phosphatidylinositol glycan anchor biosynthesis	Ssa#S30272457	0.61	0.49	0.12
protein serine/threonine kinase activity				
ankyrin repeat domain 3	Omy#S15268006	-1.98	-1.38	-0.60
T-cell response				
CD82 antigen	Ssa#S35505059	-0.70	-1.40	0.70
modified T cell receptor alpha	Ssa#NP9934285	-0.73	-2.13	1.41
CD8 alpha	Ssa#S43839059	-0.33	-0.77	0.44
T-cell leukemia homeobox 3	Ssa#DW588601	0.15	-0.74	0.89
IL2-inducible T-cell kinase	Ssa#S30292785	0.57	-0.78	1.35
T cell receptor alpha	Ssa#S37959057	-0.94	-1.23	0.29
CD8 beta	Ssa#S22938352	-0.68	-0.80	0.12
T cell receptor alpha	Ssa#S37959281	-0.65	-0.90	0.25
T cell receptor alpha	Ssa#S37959544	-0.44	-0.72	0.27
Rho-related GTP-binding protein RhoE precursor	Ssa#S30264152	1.00	0.78	0.22
Cas-Br-M (murine) ecotropic retroviral transforming sequence b	Omy#CA378941	1.08	1.82	-0.74
discs, large homolog 1 isoform 1	Omy#S34310120	-1.17	-1.58	0.41
phosphoinositide-3-kinase, catalytic, delta polypeptide	Ssa#AM042317	0.85	-0.50	1.35
Tyrosine-protein kinase SYK	Omy#CA346982	0.35	1.48	-1.12
Perforin-1 precursor	Ssa#S30280863	-1.24	-0.41	-0.84
Plastin-2	Ssa#TC105599	0.04	1.95	-1.92
human caldesmon 1 transcript variant	Ssa#KSS4667	-0.21	-0.85	0.64

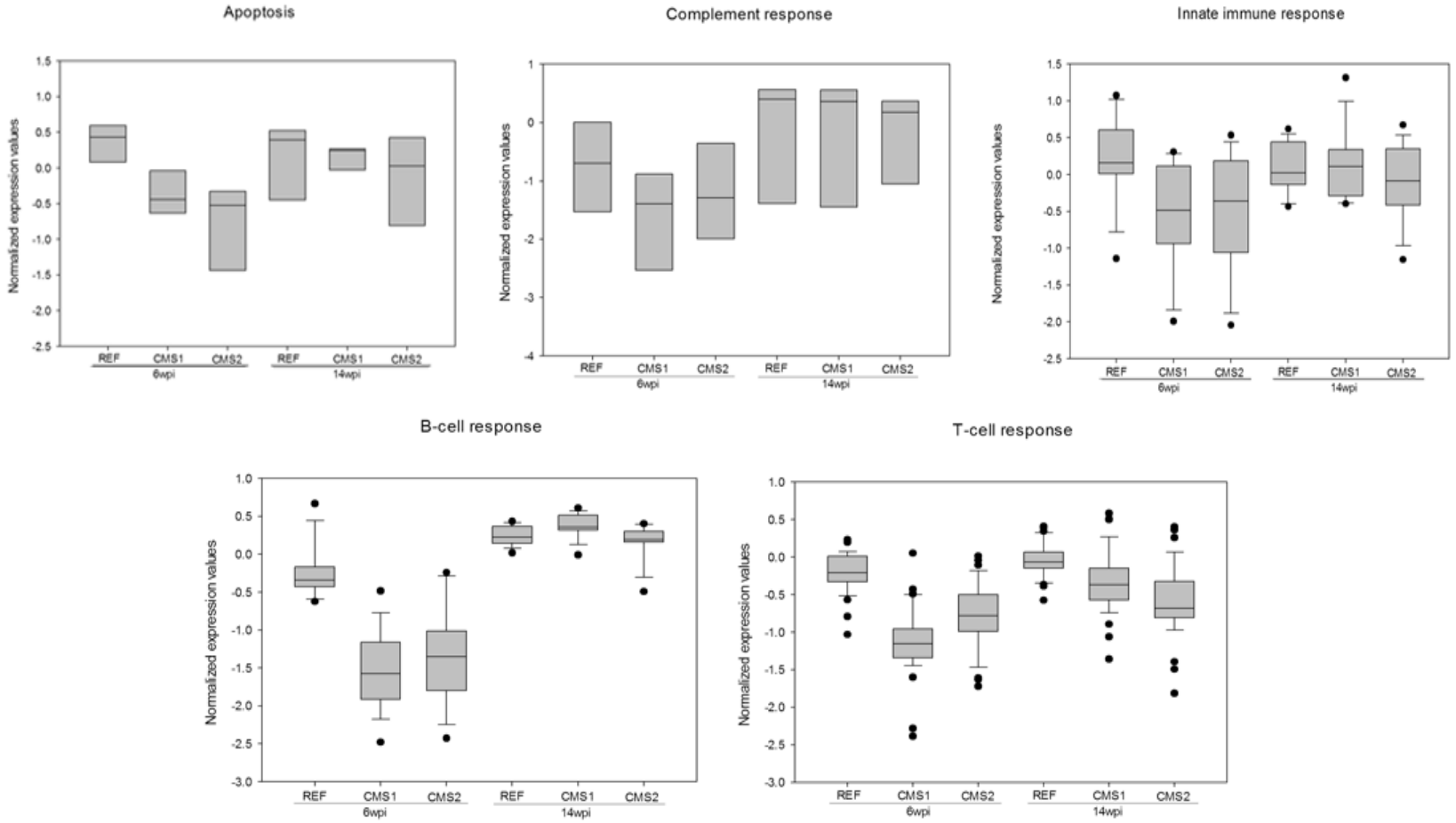

Figure 5.8 Box plots showing log2-ER (expression ratios) for all genes included in five gene sets comparing the expression of genes significantly different expressed at 6-wpc with those at14-wpc. Boxes represent 50 % of the values, while black bars mark the median log2-ER. Whiskers indicate the maximum length of 1.5 times the box length. Values beyond whiskers are plotted as a black circle.

5.4. Discussion

The present study has shown the important role that dietary immunomodulation can have in controlling salmonid heart viral diseases. Specifically, microarray analysis of heart transcriptome showed how reduced dietary lipid content and increased levels of EPA can lead to altered expression of genes involved in the immune response after challenge with PMCV.

The specific role and/or mechanism that reduced dietary lipid content has in mediating the effects of the functional feeds has been difficult to establish (Martinez-Rubio et al., 2012a,b,c). Previous studies on HSMI showed that there was reduced lipid deposition (steatosis) in livers of fish fed the functional feeds with lower lipid content at initial stages of the disease and the steatosis was usually more frequent when the severity of the heart lesions was high (Martinez-Rubio et al., 2012b). Thus, there was potential correlation between liver metabolism and ongoing viral infection, although earlier studies had found no correlation between the severity of heart and liver lesions during different stages of HSMI disease, associating the lesions to the circulatory disturbances as a consequence of the heart lesions (Kongtorp et al. 2006). Therefore, even though the precise mechanism of the reduced liver steatosis index at initial stages in fish fed the functional feeds was not identified in detail, reduced dietary lipid was undoubtedly a major contributing factor (Martinez-Rubio et al., 2012b,c). Functional feeds used in the present study were clearly beneficial for the liver pathology associated with CMS. Fish fed both functional feeds showed lower histoscores over the whole course of the infection, with the scores being significantly lower at 12- and 14-wpc in fish fed both functional feeds compared to fish fed the REF diet. Hence, despite increased pathology in heart tissue observed in fish fed CMS1 and CMS2 at these later

time points of the challenge, the functional feeds clearly moderated the hepatic steatosis after infection with PMCV, probably due to the reduced dietary lipid content.

As previously described in humans (Calder, 1997) and fish (Bell et al., 1991; Lin and Shiau, 2007; Montero et al., 2010b), the fatty acid compositions of membrane PL play a critical role in the regulation of the innate and adaptive immune response. Fatty acids such as ARA, EPA and DHA, released from membrane PL through the action of phospholipases, modulate the immune response not only through the production of eicosanoids, but also by modifying important signalling pathways and stimulating immune-related nuclear transcription factors (PPARs and NFkβ) and the release of certain cytokines (Shaik and Eidin, 2006). The n-6 LC-PUFA, ARA is associated with pro-inflammatory responses as the precursor of pro-inflammatory eicosanoids, and EPA, as well as being the precursor of anti-inflammatory eicosanoids, can also decrease the production of ARA-derived eicosanoids through substrate competition of the eicosanoid-synthesising enzymes. DHA has also an anti-inflammatory role in humans, particularly relevant at the recovery phase of an inflammatory process, as it is a precursor of immune-resolving resolvins and protectins (Calder, 2009b).

Although macrophages, primarily produced in the head kidney of fish, are a major source of eicosanoids, production of eicosanoids at the site of the infection is also highly relevant due to the short life of these LC-PUFA derivatives (Rowley et al., 1995) In the present study, fatty acid composition of the PL of heart tissue, as the main site of infection after a challenge with PMCV, was determined before the viral challenge and at five time points over the course of the infection. As previously reported in unchallenged (Bell et al., 1991) and challenged fish (Martinez-Rubio et al., 2012a Chapter 1), levels of DHA in heart tissue PL did not reflect the fatty acid composition of the diets, as levels of this fatty acid were generally similar between the three dietary

groups in the present trial. This perhaps reflected the fundamental role of DHA in the maintenance of the structure and fluidity of cellular membranes (Stillwell and Wassall, 2003). In our previous study investigating the effects of similar diets on HSMI in salmon, levels of DHA were not significantly affected during the time-course of the infection after challenge with ASRV (Martinez-Rubio et al. 2012a; Chapter 2). Thus, the precise immunomodulatory role of DHA described in humans did not appear to be as relevant in fish during the inflammatory response associated with HSMI (Calder, 2007). In contrast, in the present study, the levels of DHA in heart tissue PL significantly changed over the time-course of the infection after PMCV challenge. Levels of DHA were higher when there was enhanced immune response and the levels of heart lesions were more prominent. Whether DHA levels were indeed directly related with the activation of the different immune pathways involved in the control of the inflammatory process is something that requires further investigation in fish. However, the present results suggest that changes in the levels of DHA may be correlated with PMCV infection and therefore this fatty acid could have a role in the immune response in fish as has been described previously in humans (Calder, 2009a).

Levels of EPA and ARA, and the EPA/ARA ratio, in the PL of heart tissue were significantly altered by the functional feeds, and hence one of the main strategies of the experimental design of the present trial was achieved. Specifically, the potential bioavailability of EPA and its proportion relative to ARA was always higher over the time-course of the infection in fish fed the functional feeds compared with fish fed the REF diet. Interestingly, the use of Southern hemisphere FO in the functional feeds in the present trial also increased the level of ARA in heart tissue PL and thus the EPA/ARA ratio was actually lower than in heart tissue of fish in a previous trial on HSMI (Martinez-Rubio et al., 2012; Chapter 2). The relative proportions of ARA and

EPA remained generally constant during the time-course of the PMCV infection although, as described above for DHA, there were increased levels of both ARA and EPA when, according to gene expression analysis, there was activation of the immune response. However, this was only significant for EPA, possibly reflecting the higher absolute levels of EPA generated by the functional feeds in the present trial.

During a pathogen infection, there is considerable mobilisation to the main sites of infection of immune cells that are producers of eicosanoids, whose production is supported by release of the immune-related LC-PUFA from membrane PL (Balfry and Higgs, 2001). Although infiltration of inflammatory cells and their specific fatty acid compositions were not analysed in the present study, the correlations between levels of ARA, EPA and DHA in the membrane PL, the extent of heart histological lesions, and gene expression in the heart tissue analysed, clearly indicated that dietary effects on the incorporation of these fatty acids in tissues (heart, immune cells, etc.) were highly relevant to their immunomodulatory roles after a viral infection.

In addition to effects on fatty acid composition, the PL class composition was also affected by the viral infection, with the main change being the significant decrease in the proportions of PI and PS as the infection progresses. Phosphorylated derivatives of PI such as phosphatidylinositol 4,5-bisphosphate (PIP_2) are involved in the production of the intracellular second messengers diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3) through the action of the phospholipase C (Stulnig and Zeyda, 2004). DAG and IP_3 activate calcium channels, increasing Ca^{+2} concentration in the cytosol, with this being an essential step for the survival of some human virus (Chami et al., 2006). PS has also been related with this signalling pathway as it is an important activator of protein kinase C (Bell and Sargent, 1987; Tocher et al., 2008). As the gene expression data also highlight the relevance of this signalling pathway during the course

of the infection, the decreased proportions of PI and PS could reflect utilisation of these PL classes associated with an immunomodulatory role. This relationship between composition/content of specific PL classes is an interesting and important area for future studies on clinical nutrition in fish.

Immediately prior to challenge, genes related with the different pathways of the innate and adaptive immune response were generally expressed at lower levels in fish consuming the functional feeds. As this was pre-challenge, this effect must be entirely due to the dietary modulation. Broadly similar effects on gene expression were reported previously in a trial investigating substitution of dietary FO with VO in salmon (Morais et al., 2011). In that trial, expression levels of immune-related genes in liver of fish fed a FO diet, with a fatty acid composition similar to the functional feeds, were generally lower than in fish fed a VO diet, with a similar composition to the present REF diet. However, in the earlier study, genes related with the T-cell response were expressed at a lower level in fish fed the VO diet (Morais et al., 2011). This contradictory result may be explained by the higher levels of EPA present in the functional feeds used in the present study compared with the FO diet used in the earlier study, as the considerable influence of EPA in controlling T-cell signalling pathways has been documented previously (Shaik and Edidin, 2006; Calder, 2008). Expression of genes related with non-specific immune responses were also higher in fish fed a diet with a blend of VO and FO compared with fish fed FO in a study in grouper (*Epinephelus malabaricus*) (Lin & Shiau, 2007). So it appears that, even without any apparent infection, inclusion of FO could dampen both innate and adaptive immune responses in humans and fish.

Although no lesions in the heart were recorded before the PMCV challenge, the increased immune status of the fish fed with the REF diet could thus be a factor in the earlier development of the immune response and heart lesions associated with CMS in

these fish compared with the fish fed the functional feeds. Put another way, the functional feeds lowered the immune status prior to viral challenge and, in doing so, delayed and dampened the inflammatory/immune response to the infection. Whether this could generally improve resistance and/or outcome to this kind of viral infection in salmon requires further investigation.

Before challenge there were also dietary-induced differences in the expression of several genes related with other metabolic pathways. Thus, genes related with fatty acid oxidation such as *carnitine O-octanoyltransferase*, and *peroxisomal 2, 4-dienoyl-CoA reductase* showed higher expression in fish fed the REF diet. This was consistent with the higher lipid content of the REF diet compared to the functional feeds, and was in agreement with a previous study where increased expression of genes related with peroxisomal fatty acid oxidation was found in salmon fed diets including VO (Leaver et al., 2008b). Perhaps in contrast, some genes associated with lipid transport showed higher expression in fish fed the functional feeds, especially the CMS2 diet. However, this result was entirely consistent with data from a previous study in rainbow trout (*Oncorhynchus mykiss*) fed a low energy diet (Kolditz et al., 2008).

Genes related with fatty acid biosynthesis including *delta-6 fatty acyl desaturase* and *fatty acyl-CoA hydrolase* showed increased expression in fish fed the CMS1 diet compared to the REF diet, although the expression of both was unexpectedly lower in the fish fed the CMS2 diet. In a previous study with functional feeding and ASRV infection in salmon (Chapter 3) and the above study in trout (Kolditz et al. 2008a), these genes were up-regulated in fish fed lower energy diets. This is in agreement with the data for the CMS1 diet, but the contradictory result in fish fed CMS2 could be related with the higher LC-PUFA content, especially EPA, of the CMS2 diet compared with the diets used in the aforementioned earlier studies. However, as EPA levels were

similar in both functional feeds, interaction between supplementation with histidine and the LC-PUFA biosynthesis pathway requires further investigation.

The heart histology data obtained in the present trial were in agreement with previous studies describing the development of heart pathology in CMS (Timmerhaus et al., 2011; Haughland et al., 2011). Thus, ventricle and atrium were severely affected by the viral infection with the lesions characterised by inflammation, degeneration and necrosis. Similarly, there was correlation between the expression of genes related with the immune response and the lesions in the heart tissue in agreement with Timmerhaus et al. (2011). Importantly, however, the temporary differences in the expression of the immune genes observed between the different dietary treatments in the present study considerably strengthened this correlation. Thus, overall, the changes associated with the progress of CMS heart pathology, previously described by Timmerhaus et al. (2011), were temporally similar with the development of the infection in fish fed the REF diet. In addition, the present data show a clear delay in the appearance of these histological changes in fish fed both functional feeds, and slightly improved performance of fish fed the CMS2 diet. This could be associated with a lower up-regulation of expression of genes associated with the innate immune response in fish fed CMS2 compared with the other dietary groups.

Enrichment analysis of transcriptomic data is commonly used to identify genes significantly relevant in selected gene lists (Liu et al., 2007). In the present study, enrichment analysis was particularly effective in elucidating how different signalling pathways, especially protein serine-threonine kinase at the peak of the inflammatory process, PI at later stages, and genes related with the T-cell receptor signalling pathways, were significantly modulated by diet over the course of the infection. At 6-wpc, gene expression and heart tissue histopathology of fish fed the REF diet were in

agreement with those reported by Timmerhaus et al. (2011). Thus, atrium was clearly affected by the infection at 6-wpc with histoscores higher in the fish fed the REF diet compared to fish fed the functional feeds, whereas ventricle was only slightly affected. According to the aforementioned study of Timmerhaus et al. (2011), a peak in the expression of genes related with the complement response, B and T-cell responses and apoptosis, was expected at this time post-infection. Furthermore, most of the genes related with these pathways showed lower expression in fish fed the functional feeds compared to fish fed the REF diet. In addition, genes related with antiviral and interferon responses showed similar or higher expression in fish fed the functional feeds, especially CMS1, compared with fish fed the REF diet. The peak in the expression of the latter genes was reported to be at 2-4 wpc in the earlier study (Timmerhaus et al., 2011), so this also supported the conclusion that there was a delayed response in fish fed the functional feeds. A delayed response to viral infection was also observed after ASRV infection in a previous study on HSMI in salmon (Martinez-Rubio et al., 2012a; Chapter 2). In the HSMI study, the peak in expression of genes related with activation of the immune response was delayed by around two weeks in fish fed similar functional feeds compared to fish fed a standard commercial diet.

As the CMS disease progressed the extent of lesions in the heart increased in both atrium and ventricle with the differences between the fish fed the functional feeds and fish fed the REF diet being most pronounced at 8-wpc. At this time-point, the fish fed the functional feeds, especially CMS2, presented significantly lower histoscores in both atrium and ventricle compared with fish fed the REF diet. These differences could be a consequence of the striking up-regulation of genes related with the host immune response observed at 6-wpc in fish fed the REF diet. Again, this result was in agreement with the previous study by Timmerhaus et al. (2011). At 8-wpc, following

the hypothesis of delayed development of CMS in fish fed the functional feeds, higher expression of genes involved in antiviral and innate immune pathways could have been expected in these fish compared with fish fed the REF diet but, in contrast, these groups showed a lower activation of these pathways. Dietary modulation of the inflammatory response has, of course, been described previously in fish. Higher incorporation of n-3 LC-PUFA in biological membranes of immune cells led to a lower expression of pro-inflammatory cytokines in gilthead sea bream (*Sparus aurata*) (Montero et al., 2010b), decreased antibody production and macrophage killing activity in rainbow trout (Kiron et al., 1995), and lower production of pro-inflammatory eicosanoids in Atlantic salmon (Bell et al., 1996). Furthermore, dietary EPA suppressed production and release of tumor necrosis factor (TNF), interleukins and IFN in humans (Das, 2003). Dietary EPA also suppressed key components of the antiviral response in gilthead sea bream, and dietary VO inclusion modulated the expression of Mx proteins, interferon-induced mediators of innate resistance to RNA virus (Montero et al., 2008b). The increased proportions of dietary n-3 LC-PUFA, especially EPA, in the functional feeds used in the present study could, according to these previous studies, be a major factor in preventing an uncontrolled immune response that could be more harmful to the fish.

As expected, according to the description of gene expression related with the immune response 8-wpc reported by Timmerhaus et al. (2011), most of the genes related with the T-cell response showed lower expression in fish fed the functional feeds compared with fish fed the REF diet. However, genes involved in protein serine/threonine kinase activity, a group of enzymes crucially controlling transcriptional and metabolic pathways determining T-cell function (Finlay and Cantrell, 2011), showed higher expression in fish fed the functional feeds, suggesting possible initiation of the T-cell response in these fish at 8-wpc.

Although gene expression was not assessed at 10- and 12-wpc, increased transcription of genes involved in virus clearance may be predicted according to the histopathological analysis of lesions in the atrium and ventricle at these time-points. By 14-wpc, there was a clear remission in the lesions in both parts of the heart in all three dietary groups. Although the histopathology of the lesions was not significantly different between dietary groups, there was slightly better recovery in fish consuming the functional feeds, especially CMS2. As the expression of many of the genes related with the immune response was not significantly different between the three dietary groups, it is difficult to determine the precise mechanism whereby functional feeds could regulate the immune response towards a faster resolution of the inflammatory process associated with CMS. Nonetheless, some of the pathways represented in the list of genes significantly differentially expressed at 14-wpc in fish fed the different diets could be key to that regulation. Thus, important markers of the T-cell response such as *T cell receptor alpha, CD8 beta and alpha* and *CD82 antigen* showed lower expression in fish fed the functional feeds, but genes related with the PI signalling system were higher expressed in those fish compared with fish fed the REF diet. As previously mentioned, the signalling pathway mediated by PI is highly involved in the regulation of the T-cell response (Stulnig and Zeyda, 2004), and dependent on the levels of membrane PL classes and their fatty acid compositions. As the lesions in heart tissue in CMS were mainly associated with the action of CD8+ cytotoxic T-cells (Timmerhaus et al., 2011), any factor controlling this response, such as the anti-inflammatory n-3 LC-PUFA present in the functional feeds used in the present study, could be key to the better performance observed in the fish fed these feeds. Furthermore, results from the previous study evaluating the use of functional feeds in salmon suffering HSMI were in agreement with this hypothesis. Thus, milder expression of genes related with

inflammatory response and virus clearance, including those related with T-cell response, were reported in salmon consuming the functional feeds leading to a better performance of those fish over the course of the HSMI infection (Martinez-Rubio et al., 2012a; Chapter 1).

Histidine supplementation may be a promising alternative therapy in chronic heart failure patients (Kalantar-Zadeh et al., 2008), and a protector of the myocardial function after ischemia in humans (Koch et al., 2009). In addition, oxidative stress has been associated with viral infections (Hasnain et al., 2003). These studies led us to hypothesise that dietary histidine supplementation could be beneficial in modulating the level and extent of heart lesions associated with CMS. Although this amino acid has been reported to be crucial preventing cataract formation in adult salmonids in periods of increased risk (Waagbø et al., 2010), the present study showed that, in general, the addition of histidine did not improve the performance of the fish as the level of heart lesions and the expression of genes related with the immune response were not further reduced in fish fed the histidine supplemented diet. Further investigation is therefore required using different doses of histidine and evaluating possible interactions with other components of the diet.

Conclusion

The present study is the first to describe the effects of functional feeds on the expression of genes related with the immune response after infection with PMCV in Atlantic salmon. Significant differences in immune and inflammatory responses and pathology in heart tissue were found in fish fed the different dietary treatments over the course of the infection, highlighting the immune modulatory role of dietary lipid content and fatty acid composition in viral infections in Atlantic salmon. A previous study (Martinez-Rubio et al. 2012a), clearly associated reduced dietary lipid content

and increased EPA with a milder and delayed inflammatory response and, consequently, less severity of heart lesions after infection with ASRV. In the present study with PMCV infection, the same strategy was also effective albeit slightly less than in the previous HSMI study, with this difference possibly associated with the lower EPA/ARA ratio in the functional feeds used in the present trial.

Chapter 6

General Discussion

The primary overriding objective of the present thesis was to evaluate the effects of functional feeds as an alternative therapy to mitigate chronic inflammatory processes associated with two emerging viral diseases in Atlantic salmon aquaculture, namely heart and skeletal muscle inflammation (HSMI) and cardiomyopathy syndrome (CMS). Experimental challenges with the casual agents of both diseases, along with the use of oligoarray technology to determine global gene expression, identified the immunomodulatory roles of the functional feeds, confirmed their beneficial advantages in viral infection, and opened a new line of research for the implementation of clinical nutrition in aquaculture.

As the work in this thesis was in collaboration with, and partly sponsored by, an industrial partner, the major international aquafeed company, EWOS, through their research arm EWOS Innovation AS, experimental feeds were based on practical formulations and the trials were carefully designed to specifically deliver answers to important existing commercial problems. Thus, overall, the study closely linked modern, cutting-edge transcriptomic techniques that delivered detailed mechanistic data at a molecular level with traditional diagnostic histological data to deliver results that had the potential to have immediate commercial application. This short pathway to impact is one of the great benefits of a strong academic/industrial partnership. Therefore, all of the feed formulations investigated in this study were

designed to be suitable for future commercialization, enabling results from this thesis to be highly applicable and rapidly applied in the salmon industry.

Furthermore, the compositions of the reference diets used in the trials were closely based on commercial formulations currently in use within the industry. At the outset of this study, it was speculated that the increasing prevalence and severity of both diseases, HSMI and CMS, may be associated with changes in feed formulations that had been taking place over the same timescale. Thus, one hypothesis was that the trends for increasing dietary energy/lipid content along with increasing presence of plant raw materials as replacements for FM and FO including n-6 PUFA-rich VO in the feeds were possible important factors in the increasing importance of these diseases probably through disturbances in lipid and fatty acid metabolism affecting severity and regulation of the inflammatory responses. Consistent with this contention, the timings and incidence of lesions in the heart tissue of fish fed the standard, reference feeds were very similar to that previously described in the literature for natural outbreaks in fish fed commercial feeds (Ferguson et al., 1990; Kongtorp et al., 2004, 2006; Fritsvold et al., 2009). Therefore, changes in lipid mobilization and incorporation of dietary fatty acids related with the immune response supported by gene expression in fish fed the reference diets likely reflected general metabolic changes that farmed salmon are experiencing in response to changing dietary formulations and as observed in natural disease outbreaks. This fact strongly supported the rationale on which the formulations of the functional feeds were based and lent weight to the beneficial effects that were observed in fish fed the functional feeds in the different experiments conducted in this study.

The long duration of the two main experimental trials, continuing for up to 16 weeks post-challenge, and the evaluation of the changes at several points during the

clinical phases of both viral diseases enabled a detailed description and appreciation of the dietary modulation of the immune and inflammatory responses during the time course of the viral infections. Studies of this duration have no precedents in the literature as previous trials evaluating transcriptomic responses after different viral infections in Atlantic salmon lasted few days (Jørgensen et al., 2007; Miller et al., 2007; LeBlanc et al., 2010), 7 weeks (Jørgensen et al., 2008) and a maximum of 12 weeks (Timmerhaus et al., 2011). Moreover, the use of functional feeding as an alternative therapy for viral diseases, has also not been previously reported, being just only one study evaluating mortality and gene expression in Atlantic salmon suffering HSMI which included RT-qPCR expression of a few genes related with cardiac transcription and inflammation after supplementation of tetradecylthioacetic acid (Alne et al., 2009; Grammes et al., 2012).

The use of oligoarray technology to identify effects on gene expression in the present study helped to simultaneously identify the interactions between the altered lipid and fatty acid compositions of the diets and the main players in the immune and inflammatory responses that developed following infection with ASRV and PCMV. This was also a new approach for the evaluation of the influence of diet on the immune response in aquaculture as previous studies, in general, focused on the production of certain specific metabolites such as cytokines and eicosanoids, or the expression of a limited set of genes mostly related with the innate immune response (Bell et al., 1993, 1996; Tocher et al., 1997; Lin and Shiau, 2007; Montero et al., 2010b). Transcriptomic results from the oligoarray analyses conducted during both viral challenge studies in the present thesis pointed to a dietary influence beyond the traditional inflammatory pathways mentioned above. Thus, dietary influences on the adaptive immune response through several pathways such as T-cell signaling

pathways and antigen presentation, as well as the innate immune response, showed that expression of genes related with antiviral responses was particularly affected between the dietary treatments. The interactions between dietary LC-PUFA and their metabolism and important pathways related with viral clearance were previously described in humans (Stulnig and Zeyda, 2004; Shaik and Eidin, 2006), but, to the best of our knowledge, little was known about these relationships in fish.

There was a strong and clear effect that was consistent in both challenges with the salmon fed the functional feeds showing delayed immune/inflammatory responses in comparison to fish fed the reference feeds. These delays in the development of inflammatory responses were also clearly correlated with the histopathology results in both challenges, and also the viral load in the case of the ASRV infection. The precise mechanism underpinning the overall delayed response is not entirely clear but is probably related in some way with an initial repression of the replication of the virus. Whether this could ultimately increase disease resistance is also not clear and it is difficult to accurately predict the consequences of this delayed immune and inflammatory response in a natural outbreak. Therefore, this is very clearly something that would be particularly interesting to study in future investigations.

In general, the expression of the different pathways of the immune response, especially those related with the innate response, including antiviral and apoptotic pathways, were lower in salmon fed the functional feeds at time points at which the viral load was similar across all treatments. This was the case in both challenges, but was most evident in fish fed the FF1 diet in the experimental challenge with ASRV, the causal agent of HSMI. Therefore, selection of supplements and raw materials for the formulation of functional feeds designed for these types of viral disease should focus on the inclusion of potential inflammatory response modulators, which could

attenuate the lesions associated with the more uncontrolled response that occurs in fish fed with standard commercial feeds. The main strategy used in the design of the functional feeds tested in the present thesis was focused on lipid and fatty acid content and composition, with a combination of EPA supplementation along with a reduced lipid content, achieved through the use of different raw materials. In the HSMI trial, improved performance was observed in salmon fed the diet which contained both krill oil and krill meal (FF2), compared with the fish fed the reference feed. This was probably because krill products are not only particularly rich in the anti-inflammatory fatty acid EPA, but also it is largely in the form of phospholipid, which can facilitate the enrichment of EPA in the phospholipid membranes of cells. However, the salmon fed the FF1 diet, which contained krill meal but a blend of fish oil and rapeseed oil rather than krill oil, actually showed the best performance and the largest impact on both the level of heart lesions and expression of genes of the immune response. This possibly reflected the fact that the FF1 feed, although not the highest dietary EPA level, had the highest EPA/ARA ratio. In light of the data from the ASRV challenge, the formulations of the functional feeds in the subsequent PCMV trial were modified to include an even higher supplementation of EPA (more than 3-fold greater than in the REF feed, compared to a 2-fold uplift in the ASRV trial), which was achieved by using a Southern hemisphere fish oil. The EPA/ARA ratios were also higher in the functional feeds in the PCMV trial compared to the EPA/ARA ratios in the FF1 and FF2 diets in the previous ASRV trial. However, salmon fed both functional feeds following PCMV infection did not show as clear a performance benefit as was observed with fish fed the FF1 feed in the earlier ASRV trial. Although the viral infections were of course different, we can possibly also hypothesize that the difference between the performance of fish in the two trials may,

in fact, be due to the different levels of ARA in the feeds in the two trials. Although the dietary levels of ARA are relatively low, the high biological activity on this fatty acid in inflammatory pathways makes its level crucial. Therefore, an important consequence of using Southern hemisphere FO, in addition to increased EPA, is the higher level of ARA that they contain. In addition, the dietary ARA level in the REF feed in the PCMV trial was lower than in the STD feed in the ASRV trial. Consequently, the difference in the EPA/ARA ratio between the STD diet and the FF1 diet in the ASRV trial was greater than the difference between the REF diet and both functional feeds in the PCMV trial. As EPA/ARA ratio appeared to be particularly important in discriminating between the FF1 and FF2 feeds in the ASRV trial it is possible that the higher EPA/ARA ratio (due to lower ARA) in the REF feed meant that the difference between the diets in EPA/ARA ratio were not as pronounced in the PCMV trial. Thus, the increased level of ARA in the functional feeds (CMS1 and CMS2) along with the lower ARA level of the REF diet impacted the EPA/ARA ratios and lessened the impact of EPA supplementation in immunomodulation, confirming the importance of the EPA/ARA ratio in influencing the production of lipid mediators of the inflammatory response. Nevertheless, the results may also suggest interactions between different components or raw materials used, not necessarily related to lipid composition. Therefore, further analysis of the different macro- and micronutrients of the diets would be a relevant area for future research in this field.

Interactions between dietary fatty acid composition and lipid and fatty acid metabolism after infection with the causal agent of HSMI, ASRV, were evaluated in depth in Chapters 3 and 4. Mobilization of LC-PUFA precursors of eicosanoids was clearly observed in membrane phospholipids of both heart tissue and head kidney at

the time of peak expression of the genes specifically related to the inflammatory response. As far as the author is aware this is the first study analyzing the expression of genes involved in the major steps in the production of eicosanoids, from the release of the LC-PUFA precursors via the expression of different phospholipases, to the actual biosynthetic steps in the formation of leukotrienes and prostaglandins from LC-PUFA via the analysis of the expression of COX2, ALOX5 and FLAP genes. Although the expression of most of the genes was individually not significantly different between fish fed the dietary treatments, there was a clear trend across all the genes of lower expression in the fish showing improved performance. In combination therefore, the overall gene expression pattern added weight to the impression that there were biologically significant effects of diet on the eicosanoid pathways. The lack of more conclusive results in terms of gene expression in this pathway is very likely associated with the fact that both pro- and anti-inflammatory eicosanoids are produced through the action of the same enzymes. This makes it difficult to directly correlate the expression of the genes involved in this pathway with the actual production of either pro- or anti-inflammatory eicosanoids. However, the differences in expression of, say, phospholipase observed between the initial and latter stages of the infection were significant, pointing to an actively regulated release of LC-PUFA from the membrane phospholipids at the beginning of the infection. Nevertheless, higher production of the final products of this pathway was observed at the end of the trial when the inflammatory response was more active. Thus, the eicosanoid pathway is one of the key links between dietary fatty acid composition and cellular fatty acid metabolism, through membrane fatty acid compositions, and the inflammatory response. Consequently, the role of eicosanoids in these viral diseases, associated with a chronic inflammatory process, could be crucial in the

evaluation of the physiological and clinical effects of functional feeds. Closer monitoring of the changes in the composition of tissue membrane phospholipids, and the identification and quantification of the eicosanoids, particularly the balance between ARA- and EPA-derived derivatives, produced at different stages of the viral disease, may lead to a better understanding of this important link between nutrition and the inflammatory response.

Dietary-induced effects on the phospholipid class compositions and the fatty acid compositions of the individual phospholipids in tissue cell membranes changed both the expression of genes involved in the eicosanoid pathway and also expression of key transcription factors related with the immune response, such as PPARs. These effects were more pronounced in the head kidney, a major immune tissue in fish, compared with the heart tissue, the main organ affected by the disease, over the time course of the infection highlighting the important role of this immune tissue in mediating the effects of diet on immunomodulation. This reflects the fact that head kidney is the primary source of macrophages in fish and so these immune cells, which are associated with the production of eicosanoids (Balfry and Higgs, 2001), are produced in the head kidney and travel to the sites of infection, such as heart in the case of the viral infections in the present study.

Dietary modulation of eicosanoid production by tissue at the main organ site of the infection can also be hypothesized based on the different levels of the LC-PUFA related to inflammatory response in the phospholipids of the heart tissue and the different expression of the genes involved in the biosynthesis of the eicosanoids over the course of the infection. As these inflammatory mediators have a short half-life and so can only be effective in the local vicinity of their production, *in situ* tissue production of eicosanoids could be important in mounting a more effective response.

Thus, increased bioavailability of the anti-inflammatory fatty acids such as EPA in the heart tissue itself, as well as in macrophages migrating there, could also be an important factor to consider in future functional feeding studies in viral diseases affecting heart tissue.

Evaluation of the LC-PUFA biosynthesis pathway was also performed to evaluate possible interactions with viral infection and/or dietary fatty acid composition that could lead to increased bioavailability of anti-inflammatory fatty acids in fish fed the functional feeds. The initial hypothesis, based on the existing literature, was that there would be higher expression of the genes related with the LC-PUFA biosynthesis pathway in fish fed the reference diet (STD), as the fatty acid composition of this diet had lower levels of the LC-PUFA products of this pathway and higher levels of C_{18} PUFA precursors (Leaver et al., 2008; Morais et al., 2009, 2011). However, the expression of both fatty acyl desaturases, key regulatory enzymes in the LC-PUFA biosynthesis pathway were higher in fish fed the functional feeds. As this was an unexpected result, a further trial was conducted (Chapter 4) in order to determine if the other major difference in lipid composition between the reference feed and the functional feeds, dietary digestible energy (lipid content), was a factor influencing the LC-PUFA biosynthetic pathway. Dietary lipid content was identified as a possible factor influencing the expression of these genes as similar effects were previously described in rainbow trout fed diets with differing energy levels (Kolditz et al., 2008). Another possible option was that previous studies in humans had suggested some depression of the activity of the LC-PUFA biosynthesis enzymes after viral infections (Horrobin, 1990), and so the study conducted in Chapter 4 was performed in fish without viral challenge. This trial was therefore designed to clarify whether dietary lipid content and/or viral infection,

which resulted in liver lesions associated with the infection, were the primary cause of the unexpected lower expression of the LC-PUFA biosynthesis genes. The results obtained in this chapter clearly confirmed that the increased expression of the genes of LC-PUFA biosynthesis were not only dependent on the fatty acid composition of the diet, but also on the lipid content of the diet. The use of diets with similar proportions of both precursors and products of the LC-PUFA biosynthesis pathway in all the diets strengthens the result that dietary lipid content was the main factor responsible for the increased expression of genes in this pathway. This was the first direct demonstration of the effect of dietary lipid content on the expression and likely activity of the LC-PUFA pathway in salmon (Zheng et al., 2004; 2005). There was no evidence of an association or relationship between the viral infection and the expression of desaturase genes and thus LC-PUFA biosynthesis pathway. Whether the effect of reduced dietary lipid content in increasing the expression of desaturase genes and thus increasing the active synthesis of LC-PUFA could be beneficial in maintaining the proportions of the LC-PUFA involved in the immune response during the infection requires further investigation. Therefore, this should be included in future studies in order to elucidate the important links between the metabolic and physiological effects of reduced dietary lipid content in functional feeds and their association with inflammatory and immune responses and improved performance of fish undergoing viral challenge.

The overall conclusion of this thesis is that there is a strong influence of lipid content and fatty acid composition of the diet on the pathways of immune and inflammatory responses in Atlantic salmon after a viral infection. Furthermore, the results also demonstrate that great insight into the possible metabolic and physiological mechanisms involved can be effectively revealed by the use of oligo

microarrays that provide near genome-wide appreciation of the effects on gene expression. In summary, the thesis has shown that the concept of clinical nutrition could be a powerful alternative therapy against viral diseases in salmon and, likely, all fish species. This is particularly the case where a major consequence of the viral infection is a chronic inflammatory response as functional feeds could not only potentially mitigate the overall immune and inflammatory processes, but also offer an opportunity to reduce the use of traditional pharmacological treatments that are causing increasing concern in relation to their environmental impact.

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