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Effect of functional feeds on fatty acid 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 lipid (hence energy) content reduced the inflammatory response to ASRV infection and the 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 eicosanoid and fatty acid metabolism in liver and head kidney. Here we show 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 mobilization of lipids and changes in the LC-PUFA composition of membrane phospholipids, along with significant 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 results from the present study suggest that clinical nutrition through functional feeding could be an effective complementary therapy for emerging salmon viral diseases associated with long-term inflammation.

Introduction

Heart and Skeletal Muscle Inflammation (HSMI) is an emerging viral disease caused by a novel Atlantic salmon reovirus (ASRV) [1] particularly affecting farmed salmon in Norway [2]. Mortality varies between outbreaks from 0 to 20%, and the morbidity of the lesions can reach 100%, affecting almost all fish in a farm, leading to significant growth reduction and financial impact [3]. The main symptoms associated with HSMI are epi-, endo- and myocarditis, and myocardial and red skeletal muscle necrosis, indicating that these tissues experience a severe inflammatory process. Early stages of the disease have been reported around five months after transfer to sea water and the cardiac lesions persist for several months until the fish are able to control the infection and reduce tissue damage [4]. 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), have essential roles in the development and control of inflammatory responses. They are important components of plasma membranes that are integral in controlling membrane-signalling pathways. A key link between fatty acid composition of immune cell membranes and the inflammatory process are eicosanoids, lipid mediators including prostaglandins (PG), leukotrienes (LTB) and thromboxanes (TX) that modulate the intensity and duration of inflammatory responses in humans [5] and fish [6]. 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 in relation to immune-related pathways [7,8]. 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, thus being highly relevant in viral infections [9]. PGE_2 is generally related with pro-inflammatory and immunosuppressive functions, although it has also been associated with anti-inflammatory actions during resolution of the inflammatory process [10]. In contrast, the n-3 LC-PUFA, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are considered anti-inflammatory fatty acids, with EPA being particularly relevant as an antagonist of ARA, acting as a competitive substrate for the main enzymes of the eicosanoid synthesis pathways, cyclooxygenase (COX) and lipoxygenase (LOX), giving rise to anti-inflammatory eicosanoids and inflammation-resolving E-series resolvins [10,11]. 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 eicosanoid pathways in fish, as in mammals [12]. 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 [13]. Vegetable oils (VO) are generally rich in n-6 PUFA and do not contain n-3 LC-PUFA, however, the replacement of dietary fish oil (FO) by VO does not appear to negatively affect growth performance in salmonids [14]. Therefore VO have been used in the past few years as a sustainable alternative to dietary FO in fish farming. However, the fatty profiles of the alternative feeds, especially the reduction in n-3 LC-PUFA levels and the n-3/n-6 PUFA ratio, are directly reflected in fish tissue fatty acid compositions. This is becoming of increasing interest in relation to potential modulation of immune and inflammatory response pathways [15-17].

In this context, we recently applied the concept of clinical nutrition to moderate the long-term inflammatory process associated with HSMI in salmon [18]. 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 while the functional feeds had reduced DE levels (18 MJ/Kg) achieved through lower lipid contents (18%), 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 of progression of the inflammatory and immune responses to ASRV infection in salmon. However, the study did not enable the identification of the precise physiological and molecular mechanisms by which the functional feeds provided enhanced immuno-inflammatory protection.

The primary objective of the present study was to elucidate possible mechanisms underpinning the observed effects of the functional feeds. Given that the main differences between the standard and functional feeds were in their lipid fractions, this study focussed on lipid metabolism, membrane fatty acid composition, and fatty acid metabolic pathways involved in LC-PUFA biochemistry and inflammatory processes, which 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 were determined. In addition, liver inflammation was measured, given that it is one of the tissues affected in HSMI, and the expression of genes, including transcription factors controlling aspects of lipid metabolism, and those involved in LC-PUFA biosynthesis and eicosanoid metabolism, was determined in liver and head kidney.

Material and methods

Fish and feeds

Three fishmeal-based diets were formulated and manufactured by EWOS Innovation (Dirdal, Norway). Formulation and proximate composition of the feeds are indicated in Tables 1 and 2, details as previously reported [18]. A total of 450 Atlantic salmon (*Salmo salar* L.), AquaGen strain, were distributed into nine tanks (50 fish/tank) at the EWOS facility, Lønningdal, Norway and fed one of the experimental feeds in triplicate tanks for a period of 8 weeks prior to being transferred to the challenge facility. All fish were weighed at the start and end of the feeding phase and doubled their weight in the 8-week period. Initial weights (g) were 109 ± 18, 109 ± 19 and 111 ±17, respectively, and final weights were 199 ± 40, 220 ± 42 and 219 ± 37, respectively, for fish fed the ST, FF1 and FF2 diets (means ± SD, n = 150). There were no significant differences in initial weights or in final weights between fish fed any of the treatments.

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 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 as described in detail previously [18].

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 anaesthetized as above, killed by a

blow to the head, and heart, liver and head kidney were collected. A 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 also immediately frozen in liquid N_2 and stored at -80 ^{o}C.

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. Liver steatosis scores were ranked according to a non-continuous score grade from 0 to 5 (Table 3). All sections were scored double-blinded. A selected proportion of liver samples (n = 15) 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 [19].

All data preparation and simulation output analysis was conducted using the R language [20]. 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 language [21] and fitted with JAGS [22]. 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.

Lipid analyses

Total lipid from approximately 1g of liver, 0.5 g of head kidney and 0.2g of heart was extracted by homogenization in chloroform/methanol (2:1, by volume) according to Folch et al. [23], 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 [24]. Fatty acid methyl esters (FAME) of heart and head kidney phospholipid classes and liver total lipid were prepared by acid-catalyzed transmethylation [25]. FAME were separated and quantified by gas chromatography [26]. Tissue and diet lipid class compositions were determined by single-dimension double-development high-performance thin-layer chromatography (HPTLC) and densitometry [27]. 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.

Real-time quantitative PCR

Expression of 13 selected genes was 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 LC-PUFA biosynthesis, $\Delta 5$ and $\Delta 6$ fatty acyl desaturases (D5FAD, D6FAD) and elongases 5 and 2 (ELOVL5, ELOVL2). The remaining genes are transcription factors involved in the 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 primer sequences were obtained either by literature searches or designed from EST sequences corresponding to candidate genes of interest using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 4). In addition, amplification of two reference genes, *cofilin-2* and elongation factor-1α (*elf-1α*) was performed as 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 [18,28], and their expression was equally stable under the present study's conditions.

Details of the qPCR protocol have been reported previously [18]. Briefly, 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, using a mixture of random primers (1.5µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/ µl, Eurofins MWG Operon, Ebersberg, Germany). qPCR amplifications were carried out in duplicate (Quantica, Techne,

196 Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL (most genes) or 2 µL (reference
197 genes and other highly expressed genes) diluted (1/20) cDNA, 0.5 µM of each primer and 10 µL
198 AbsoluteTM QPCR SYBR® Green mix (ABgene). Results were analysed using the relative expression
199 software tool (REST 2009, http://www.gene-quantification.info/), which employs a pair-wise fixed
200 reallocation randomisation test (10,000 randomisations) with efficiency correction to determine the
201 statistical significance of expression ratios (or gene expression fold-changes) between two treatments
202 [29]. Additionally, sequencing of amplicons corresponding to new primer designs enabled the
203 confirmation of identities and presence of single sequences for all genes.
204
205 **Results**
206
207 *Liver histology*

208 The liver histology scores, based on the degree of steatosis was read as vacuole-formation in
209 hepatocyte cytoplasm characterised by both micro- and macro-vesicular lesions (Fig.1). There were
210 clear differences between fish fed the functional feeds and fish fed the ST diet at 8-weeks post-
211 challenge, with the latter group presenting higher micro- and macro-vesicular steatosis. Quantification
212 of the liver histopathology thus showed that both the incidence (percentage of fish sampled) and
213 severity (based on the scoring system in Table 3) of steatosis at 8-weeks post challenge was clearly
214 greater in salmon fed the ST diet compared to fish fed the two functional feeds (Fig. 2). At 16 weeks
215 post-challenge, there were no longer differences in liver histology between the three dietary treatments.

216 *Lipid content and composition of tissues*

217 The total lipid and triacylglycerol (TAG) contents of liver were lower in fish fed the functional feeds
218 compared to fish fed the ST diet with higher lipid (Table 5). In contrast, diet had no effect on the total
219 lipid or TAG contents of heart and head kidney. Total lipid levels in all three tissues decreased during
220 the time course of the infection irrespective of diet, but a significant interaction between diet and time
221 was observed for lipid class compositions, given that these showed a change over time that was
222 variable and dependent on diet. In liver, this decrease was greatest in fish fed diet ST, which also
223 showed a large decline in TAG. The decline in tissue lipid content over the course of the infection was
224 greatest in head kidney. This was associated with decreased percentages of TAG in fish fed the
225 functional feeds and, conversely, with reduced proportions of polar lipids in fish fed diet ST (Table 5).

In heart, the results were less clear and 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.

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 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 diets 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 therefore showed the same differences between the ST diet and functional feeds, and between the two functional feeds (Table 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, fish fed the ST diet showed increased proportions of DHA and n-3 PUFA, and decreased proportions of monoenes at 16 weeks.

The focus in head kidney was on eicosanoid metabolism, and thus fatty acid compositions were determined in the sources of precursor LC-PUFA, the membrane phospholipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). The fatty acid profiles of the major phospholipids (PC and PE) in head kidney generally showed some significant effects of diet, including increased proportions of saturated fatty acids and decreased proportions of n-6 PUFA in fish fed the functional feeds, but the differences were not as pronounced as in total lipids of liver (Table 7). Irrespective of diet, the proportions of the eicosanoid precursors, ARA and EPA and the EPA/ARA ratio, declined in all head kidney phospholipids, except PI, over the course of the infection, being significantly reduced in fish at 16-weeks compared to 8-weeks post-challenge (Tables 7 and 8). The percentage of DHA in PE, PC and PS was similar in all dietary groups and decreased in PC, but generally not PE or PS, over the time-course of the infection. In contrast, the percentage of DHA in PI was higher in fish fed the ST diet at 8 weeks compared to the level in fish fed the functional feeds, and the level in fish fed all of the diets at 16 weeks post-infection (Table 8).

As with head kidney, the fatty acid composition of the main phospholipid classes of heart also showed effects of diet over the course of the infection, but they were not very pronounced and occurred mainly in PC (Table 9). The changes in the proportions of the eicosanoid precursors 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. However, within an individual feed, these changes were minimal. In contrast, changes in EPA were more pronounced, declining in both PC and PE of heart over the course of the infection. Additionally, it was noteworthy that the decline was much lower in fish fed the functional feeds (Table 9). As a result, although the EPA/ARA ratio in heart phospholipids decreased over the course of the infection in all treatments, 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. Unfortunately, the small amount of tissue available and the low level of PI and PS precluded the determination of fatty acid compositions of these phospholipid classes in heart.

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 (Fig. 3). 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 (Fig. 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.

Expression of PPARs

There were no significant effects of diet on the expression of PPARs in liver (Fig. 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 (Fig. 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 fed the functional feeds (Fig. 5). The only 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.

290 *Expression of genes related to eicosanoid biosynthesis*

291

292 In head kidney, the expression of three phospholipases reported to be involved in the release of
293 bioactive fatty acids from cell membranes [8] was higher at 8-weeks compared to 16-weeks post-
294 challenge in fish fed all diets, significantly so in fish fed the functional feeds (Fig. 6). At 8-weeks, the
295 expression of sPLA2 (PLA2G1) was higher in fish fed the functional feeds compared to fish fed the ST
296 diet, albeit only significant in fish fed diet FF2. The expression of COX2 (prostaglandin synthesis),
297 ALOX5 and FLAP, both involved in the biosynthesis of LTs, showed generally few differences
298 between dietary treatments or over the course of the infection. However, the expression of ALOX5
299 was lower in fish fed diet FF1 compared to fish fed diet FF2 at 8 weeks, and expression of COX2 was
300 significantly higher at 16-weeks compared to 8-weeks post-challenge in fish fed diet FF1 (Fig. 6).
301 In heart, phospholipases showed a different pattern of expression compared to head kidney (Fig. 7).
302 Expression of sPLA2 (PLA2G1) was negligible in heart and therefore no qPCR data are shown for this
303 tissue. There were no major differences in expression of phospholipases over the course of the
304 infection, other than higher expression of cPLA2 (PLA2G4) at 8- compared to 16-weeks post-challenge
305 in fish fed diet FF2. Expression of cPLA2 at 16-weeks and iPLA2 (PLA2G6) at 8-weeks was
306 significantly lower in fish fed diet FF2 compared to fish fed the other diets. Expression of ALOX5 and
307 FLAP did not differ between the three dietary groups, but there was a trend for FLAP expression to be
308 higher at 16- compared to 8-weeks post-challenge, although this was only significant in fish fed diet
309 FF2 (Fig. 7). In general, the expression pattern of COX2 in heart was similar to that observed in head
310 kidney, with the expression being significantly lower at 8-weeks post-challenge in fish fed the FF1 diet
311 compared to fish fed the other diets, and with a general up-regulation of this gene in all dietary groups,
312 significant in diet FF1, at 16- compared to 8-weeks post-challenge (Fig. 7).

313 **Discussion**

314

315 As previously described, reduced dietary lipid along with increased dietary EPA and EPA/ARA ratio
316 can modulate the inflammatory response and consequently reduce the severity of heart lesions caused
317 by ASRV infection [18]. The present study investigated the metabolic pathways that link fatty acids,
318 especially LC-PUFA, with the inflammatory response, correlating the previous findings on dietary
319 immuno-modulation during ASRV infection, with availability of LC-PUFA and potential effects on
320 eicosanoid metabolism in the main organs involved in both immune and metabolic processes.

Fatty acids can impact immune responses in several 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 membranes, affecting signalling pathways and regulating the activation of transcription factors and, thus, gene expression [5,30]. 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 [7], 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 fish, as in mammals, are determined by both dietary input and hepatic fatty acyl desaturase and elongase activities, and delivery via plasma lipoproteins [31,32]. In addition to liver being a major organ involved in LC-PUFA biosynthesis, liver lesions were reported in fish affected by HSMI, occurring as a collateral effect of the heart lesions which likely impair blood flow and hence lipoprotein circulation [3]. Therefore, liver was also investigated in the present study to determine how the different dietary levels of lipid and EPA (in addition to C18 PUFA precursors) and disease progression affected the expression of genes of the LC-PUFA biosynthesis pathway. Eight weeks post-challenge there was higher micro- and macro-vesicular steatosis in fish fed the ST diet compared with fish fed the functional feeds, which was 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 the liver of ST-fed fish. Although the primary cause of the increased steatosis index in ST-fed fish was not known, the observed differences could simply reflect the differences in dietary fatty acid composition, leading to higher fatty acid synthesis and deposition. There is however a potential interaction with the on-going viral infection although previous studies have found no correlation between the severity of heart and liver lesions during HSMI [4]. 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 all dietary groups at the end of the trial [18].

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 the functional feeds compared with fish fed the ST diet. Although this correlates with viral load, it is likely to be due to a 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 the 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 [33]. 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 [34]. 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 endogenous production and, possibly, availability of EPA. The lack of major effects, whether related to nutrition and feed composition or to viral infection, on the expression of elongases was not unexpected as they do not appear to be under the same level of transcriptional control as desaturases [35-37]. 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 present data indicate that, irrespective of the precise molecular mechanism, 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 the crucial role of diet in maintaining 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 are associated with regulation of lipid metabolism in mammals and fish [36,38]. Therefore, as differences in lipid and energy contents of the feeds were expected to affect lipid metabolism pathways, the expression of PPARs was 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 at 16 weeks post infection. In contrast, there were substantial effects related to the progression of the viral infection or HSMI in the expression of all PPARs, particularly PPARα and γ, which decreased from 8 to 16 weeks, particularly in head kidney. This is noteworthy as, recently, there has been interest in the involvement of PPARs in inflammatory processes as they are expressed in immune cells and activated by eicosanoids [39]. Although all three isoforms of PPAR appear to be involved in the immune response, most studies in humans focus on the immunomodulatory roles of PPARα [40] and PPARγ [41]. The expression of PPAR genes decreased during acute inflammation in lung [40], adipose tissue [42] and activated lymphocytes [43]. In mammals, *in vitro* studies generally suggest that PPARs play roles in the resolution of inflammation, regulating 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 [39]. The results of the present study on ASRV/HSMI generally agree 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

been previously 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 mobilization 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 inflammatory mediator eicosanoids [6]. The fatty acid composition of the predominant phospholipid fractions at 8-weeks post-challenge, when no tissue damage in hearts was found irrespective of diet [18] and therefore before the major inflammatory response to ASRV, showed that the proportions of the main LC-PUFA involved in inflammatory responses 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 [44-45]. The dietary fatty acid composition affected the phospholipid compositions although this varied between the different LC-PUFA. In this respect, the proportion of ARA in PE of head kidney was not correlated with levels of dietary ARA, but reflected the levels of precursor 18:2n-6 in the feeds suggesting *in vivo* biosynthesis of ARA as previously reported [44]. In contrast, the levels of EPA in PC, but not in 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 led to increased proportions of this fatty acid in immune cells and heart [31,44]. However, the levels of LC-PUFA in PC and PE were significantly impacted during 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 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 [11].

The initial step in eicosanoid biosynthesis is the release of LC-PUFA precursors from phospholipids, mediated by phospholipase A_2 (PLA_2) that hydrolyzes 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 of phospholipases, particularly in head kidney, 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

419 classification system based on cellular distribution and calcium dependence, including calcium-
420 dependent ($cPLA_2$) and calcium-independent ($iPLA_2$), both cytosolic, and secretory PLA_2 ($sPLA_2$)
421 [46]. To date, most studies in fish have investigated $cPLA_2$ [44,47], but all three classes of PLA_2 could
422 be potentially involved in the inflammatory response. However, research in this field has usually
423 focussed on the immunomodulatory role of $cPLA_2$ and $sPLA_2$, as $iPLA_2$ is considered more important
424 for the remodelling of cell membrane phospholipids [8].
425 The present study is the first to report the effects of viral infection on the expression of PLA_2 genes in
426 fish. All three main classes of PLA_2 were expressed in head kidney, although expression of $sPLA_2$ was
427 negligible in heart. The higher expression of the three PLA_2 at the early (8-week) stage of the disease in
428 head kidney could indicate an activation of the eicosanoid pathway in macrophages and other immune
429 related cells present in this tissue, highlighting the importance of the fatty acid composition of the cell
430 membranes for the synthesis of either pro- or anti-inflammatory eicosanoids. In heart, this pattern of
431 expression was only observed for $cPLA_2$ in fish fed the FF2 diet. Diet also affected PLA_2 expression
432 with $sPLA_2$ being up-regulated in head kidney of fish fed the functional feeds, and $iPLA_2$ down-
433 regulated in fish fed diet FF2, but the mechanism or significance of these effects are not clear.
434 Obviously, the present results represent snapshots of gene expression and so can only provide an
435 indication of the possible inflammatory processes at specific time-points particularly in a largely non-
436 fatal disease such as HSMI that manifests as a long on-going infection. The choice of time points in the
437 present study was informed by histological data on the progression of inflammatory disease in heart
438 and liver [18], and may not represent ideal times for assessing all aspects of the inflammatory response.
439 In order to broaden scope of the study, the expression of enzymes further downstream in the
440 production of eicosanoids was assessed. FLAP and ALOX5 are both enzymes involved in the
441 biosynthesis of leukotrienes, with ARA-derived leukotriene B_4 (LTB_4) being one of the most critical in
442 the inflammatory response. LTB_4 has an important role during the inflammatory process stimulating
443 aggregation of neutrophils and promoting leukocyte adherence to the endothelium [48]. Regarding viral
444 immunity, LTB_4 enhances the activity of NK cells and IFNγ [31], which are two elements of the
445 immune system that have been shown to play important roles in the immune response following ASRV
446 infection [18]. Expression of ALOX5 and FLAP was previously analysed after infection with infectious
447 salmon anaemia virus (ISAV) and infectious pancreatic necrosis virus (IPNV) and, in both cases, fold
448 changes were not high even when the inflammatory process was more evident, suggesting that either
449 the transcriptional regulation of these genes occurs in short periods (pulses) or small changes in
450 expression are functionally effective [49,50]. In the present study, there was a clear trend for lower
451 expression, significant at 8-weeks, of ALOX5 and FLAP in head kidney of fish fed the FF1 diet, which

452 may indicate lower production of LTB_4 that would correlate with the milder inflammatory response in
453 this dietary group [18].
454 In general, viral infections stimulate the production of COX2 in humans and there is evidence that
455 PGE_2, the product of the reaction catalysed by COX2, plays important roles in controlling viral
456 replication and modulating the inflammatory responses to virus [51]. This prostanoid has also been
457 related to anti-inflammatory responses, inhibiting the production of LTB_4 and other inflammatory
458 cytokines during resolution of the inflammatory process [11]. However, PGE_2 has generally been
459 regarded as pro-inflammatory and, in the present study, there was a clear trend of higher expression of
460 COX2 at 16-weeks post-challenge, compared to 8-weeks, in both head kidney and heart in all diets,
461 particularly fish fed FF1. This was in agreement with previous studies in both humans [51] and fish
462 [52] where enhanced expression of COX2 along with cytokines involved in the inflammatory response
463 was reported. Furthermore, the present study also showed a trend of lower expression of COX2 in fish
464 fed the functional feeds and that could indicate lower production of PGE_2 in these fish, consistent with
465 results from *in vitro* studies in which increased levels of n-3 PUFA decreased production of PGE_2 [31].
466 In conclusion, this study on HSMI (ASRV infection) in salmon is the first to evaluate the
467 expression of a suite of important genes involved in several pathways relating diet, membrane
468 phospholipid LC-PUFA compositions, and inflammatory responses after a viral infection. Previously,
469 we showed the potential benefits of using diets with reduced dietary lipid and increased EPA on disease
470 outcomes [18], and the present study has supported the earlier results with data on the LC-PUFA status
471 of membrane phospholipids during the course of the infection. In addition, the present study has
472 demonstrated that liver was also a site for histopathological lesions in HSMI. The observed steatosis in
473 liver likely reflects a general impairment of lipid metabolism. However, the higher expression of
474 hepatic Δ6 and Δ5 desaturases in fish fed the FF diets would increase their capacity for the endogenous
475 production and availability of anti-inflammatory EPA. Furthermore, mobilization of lipids, changes in
476 the LC-PUFA composition of membrane PC and PE, along with changes in the expression of genes
477 related to eicosanoid metabolism pathways, showed the important role played by the head kidney in
478 inflammatory diseases caused by viral infections. Further studies will be required to elucidate the
479 crucial role of the production of eicosanoids at different stages of the inflammatory process, especially
480 at the resolution of inflammation, and how this might be influenced by diet. However, the effects on
481 gene expression reported in the present study further suggest that, as in humans, clinical nutrition
482 through functional feeding could be an effective complementary therapy for dealing with emerging
483 salmon viral diseases associated with long-term inflammation.

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628
629

630 Legends to Figures.

631

632 Fig.1. Liver histology. Micrographs showing different degrees of steatosis. A, Microvesicular steatosis,
633 0.5 score; B, Moderate steatosis with a mix of microvesicular and some macrovesicular, 2.0 score;
634 C, Pronounced steatosis dominated by macrovesicular, 3.5 score. The scale bar corresponds to 40
635 micron.

636

637 Fig. 2. Incidence (percentage of fish sampled) and severity of histopathology (based on the steatosis
638 scoring system in Table 3) in liver at 8-weeks and 16-weeks post-challenge with Atlantic salmon
639 reovirus in fish fed the Standard (ST) reference feed and the two functional feeds (FF1 and FF2).

640

641 Fig. 3. Expression of genes of LC-PUFA biosynthesis in liver. Expression of genes involved in long-
642 chain polyunsaturated fatty acid (LC-PUFA) biosynthesis in liver was determined by real-time
643 quantitative PCR. Values were normalized by dividing the number of copies of the target genes by
644 the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-
645 1α). Different letters represent significant differences between diets within time points and symbols
646 represents differences between time-points within diets (two-way ANOVA, $p<0.05$).

647

648 Fig. 4. Expression of PPAR genes in liver. Expression of peroxisome proliferator-activated receptor
649 (PPAR) genes in liver was determined by real-time quantitative PCR. Values were normalized by
650 dividing the number of copies of the target genes by the number of copies of reference genes (an
651 average value for the expression of cofilin-2 and elf-1α). Different letters represent significant
652 differences between diets within time points and symbols represents differences between time-points
653 within diets (two-way ANOVA, $p<0.05$).

654

655 Fig. 5. Expression of PPAR genes in head kidney. Expression of peroxisome proliferator-activated
656 receptor (PPAR) genes in head kidney was determined by real-time quantitative PCR. Values were
657 normalized by dividing the number of copies of the target genes by the number of copies of
658 reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters
659 represent significant differences between diets within time points and symbols represents differences
660 between time-points within diets (two-way ANOVA, $p<0.05$).

661

662 Fig. 6. Expression of genes related to eicosanoid pathways in head kidney. Expression of genes
663 involved in eicosanoid metabolism pathways was determined in head kidney by real-time
664 quantitative PCR. Values were normalized by dividing the number of copies of the target genes by
665 the number of copies of reference genes (an average value for the expression of cofilin-2 and elf-
666 1α). Different letters represent significant differences between diets within time points and symbols
667 represents differences between time-points within diets (two-way ANOVA, $p<0.05$).
668
669 Fig. 7. Expression of genes related to eicosanoid pathways in heart. Expression of genes involved in
670 eicosanoid metabolism pathways was determined in heart by real-time quantitative PCR. Values
671 were normalized by dividing the number of copies of the target genes by the number of copies of
672 reference genes (an average value for the expression of cofilin-2 and elf-1α). Different letters
673 represent significant differences between diets within time points and symbols represents differences
674 between time-points within diets (two-way ANOVA, $p<0.05$).
675
676

Figure(s)
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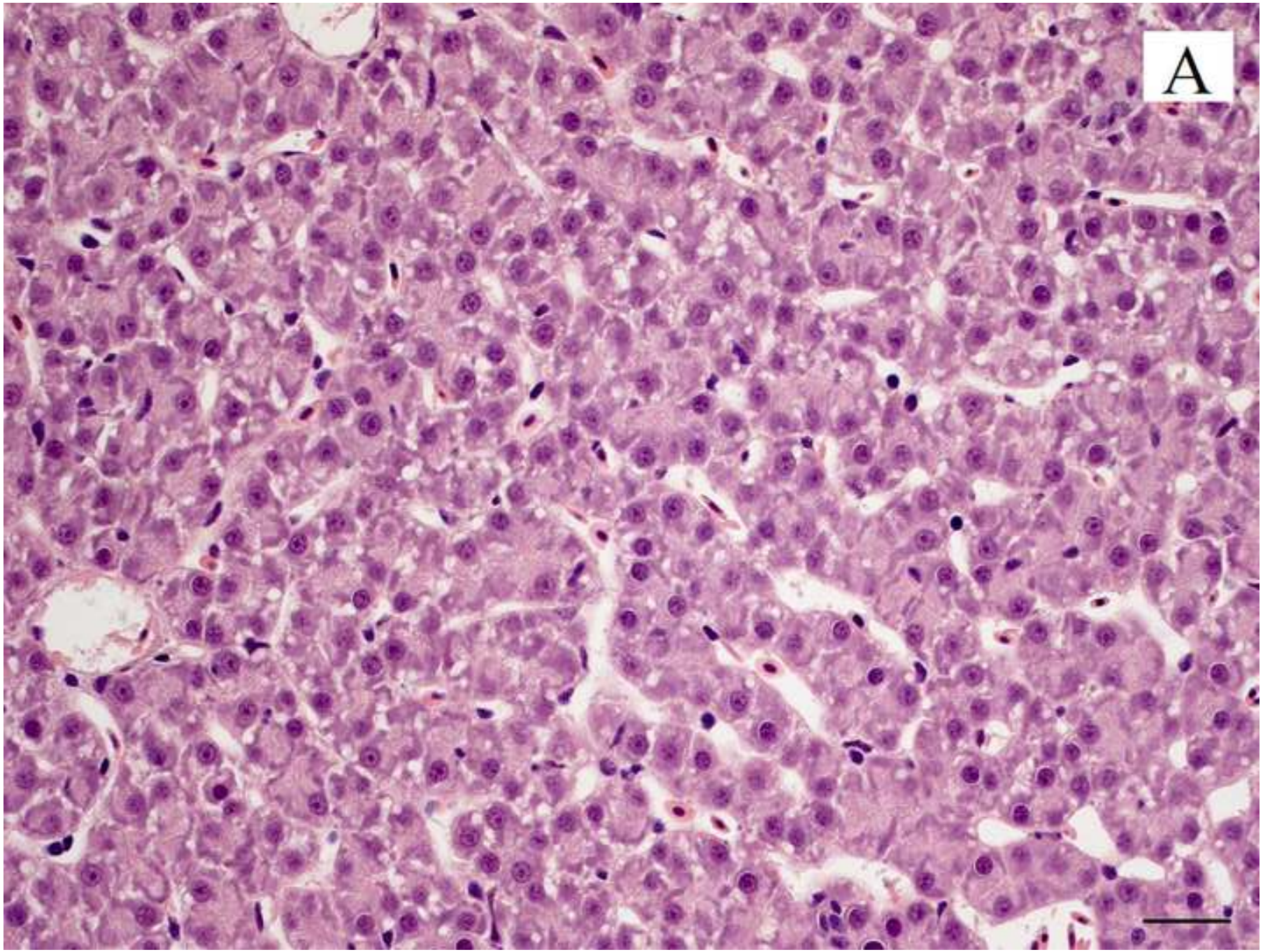

Figure 1B

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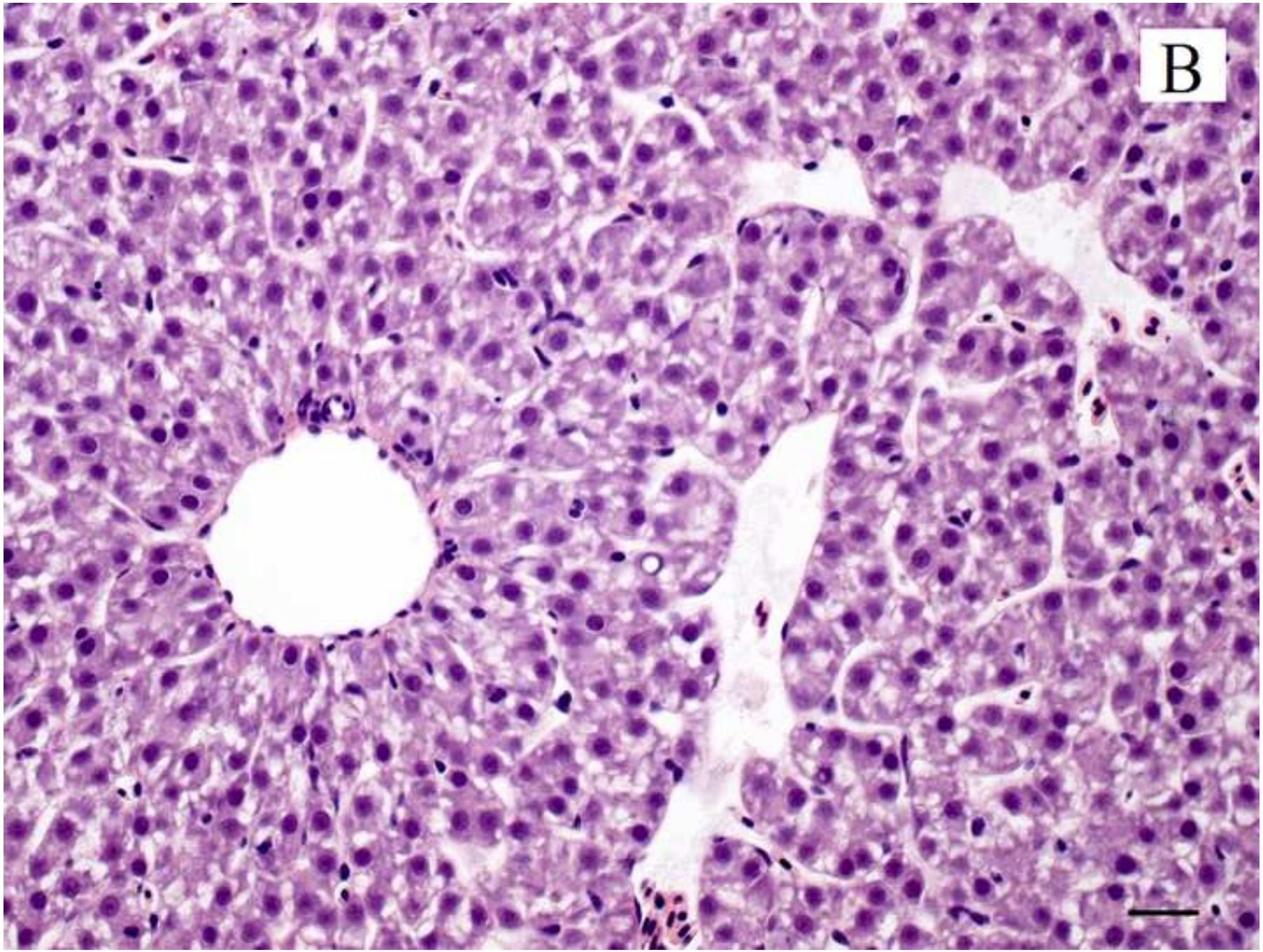

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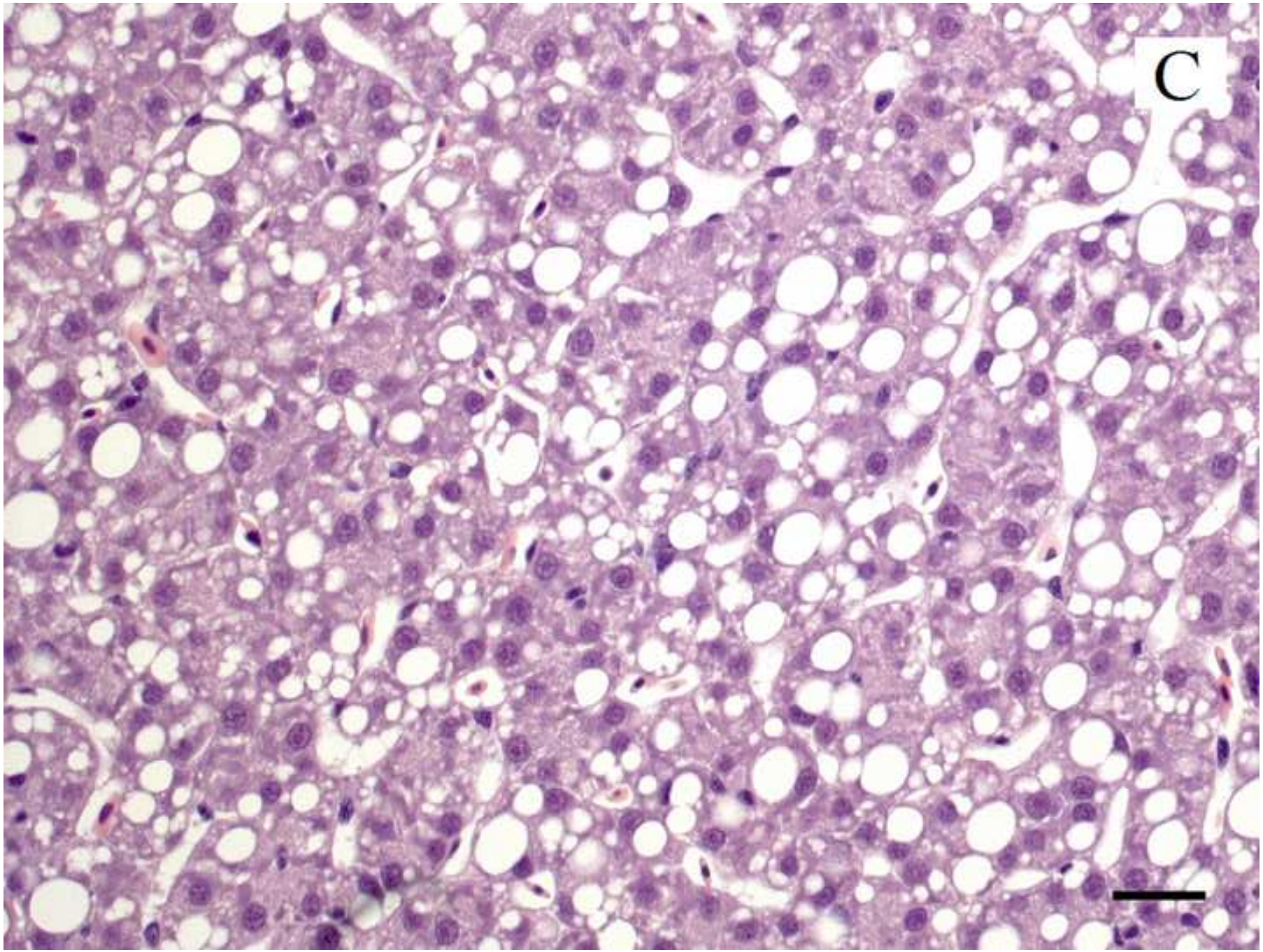

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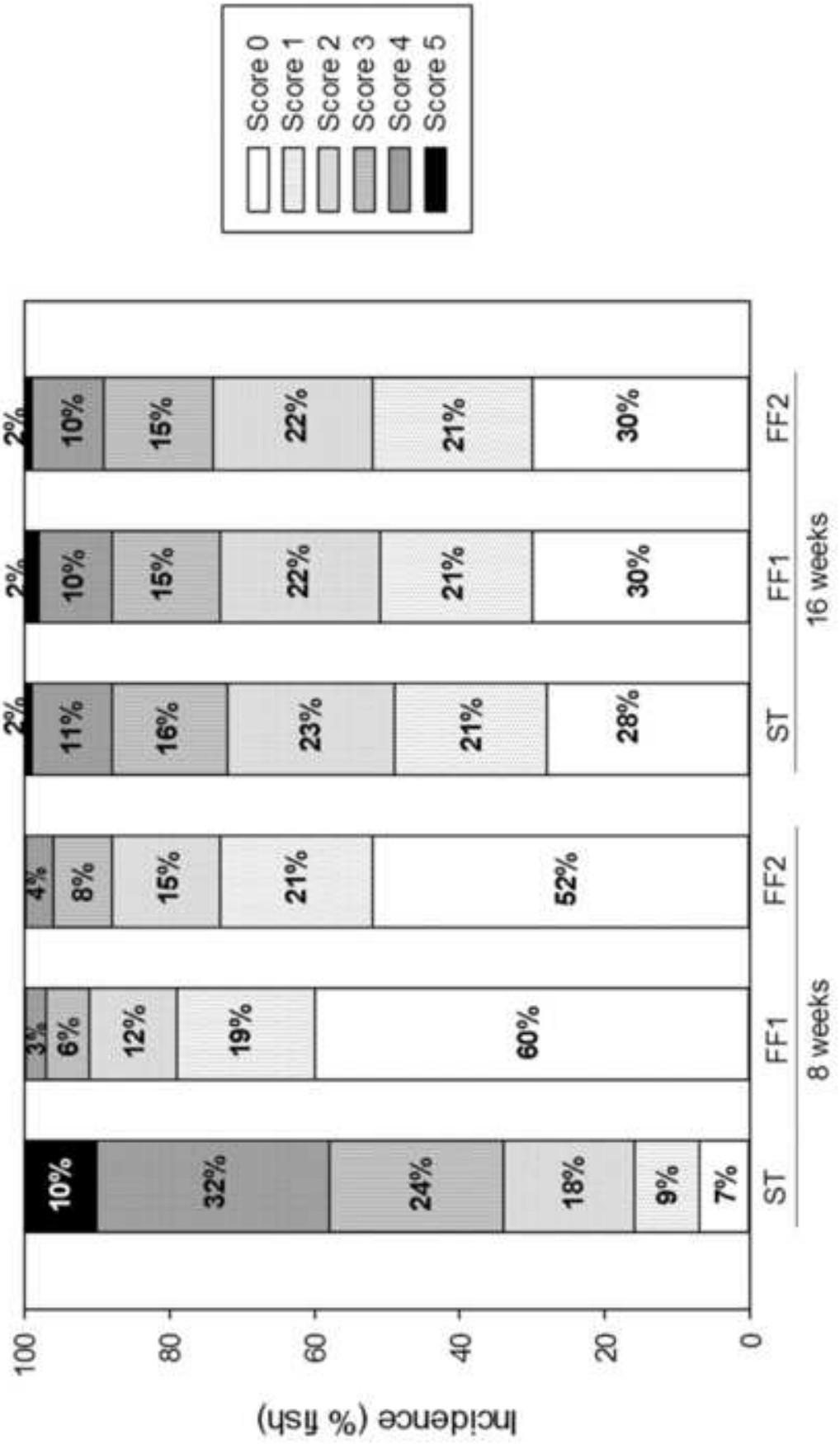

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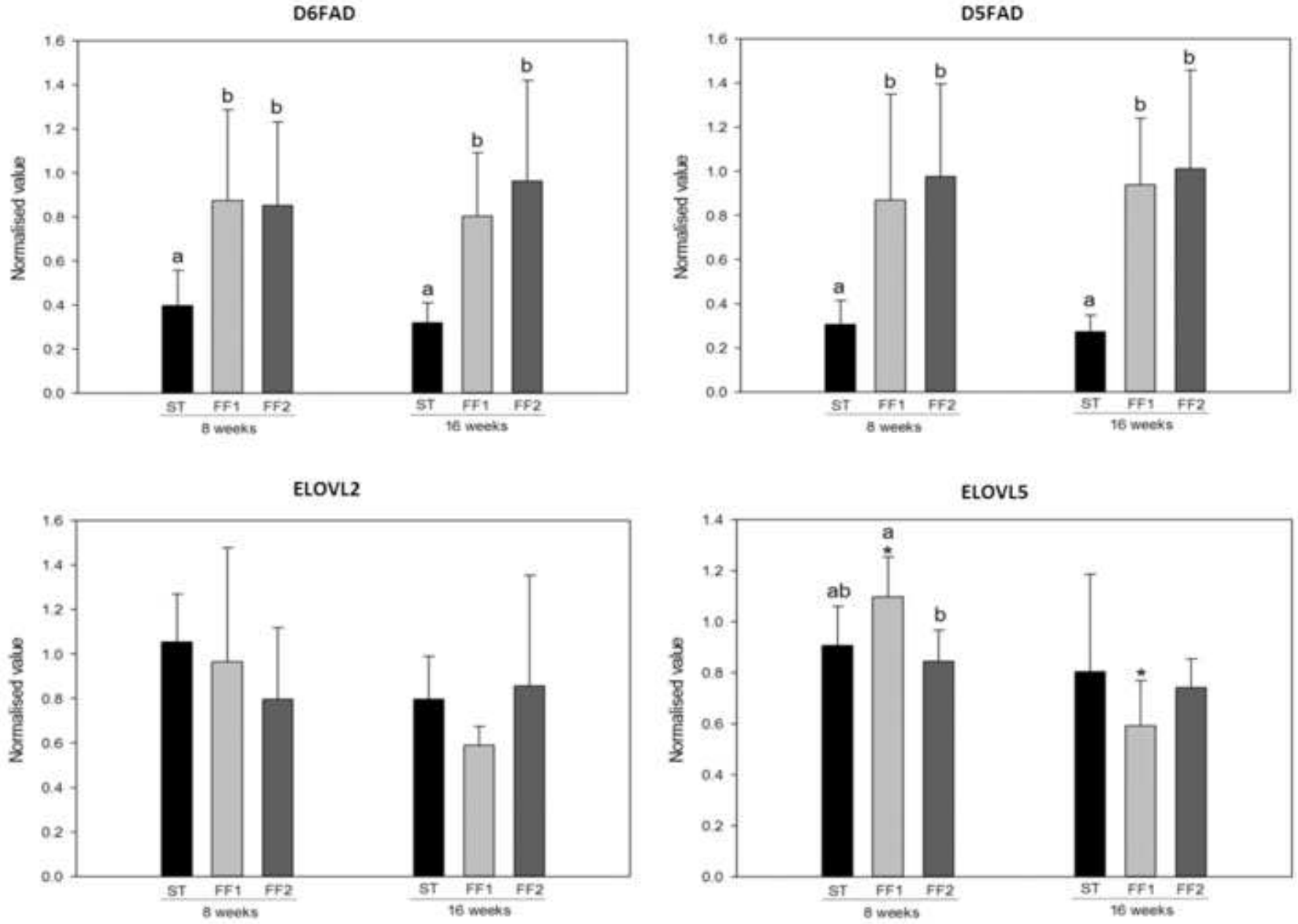

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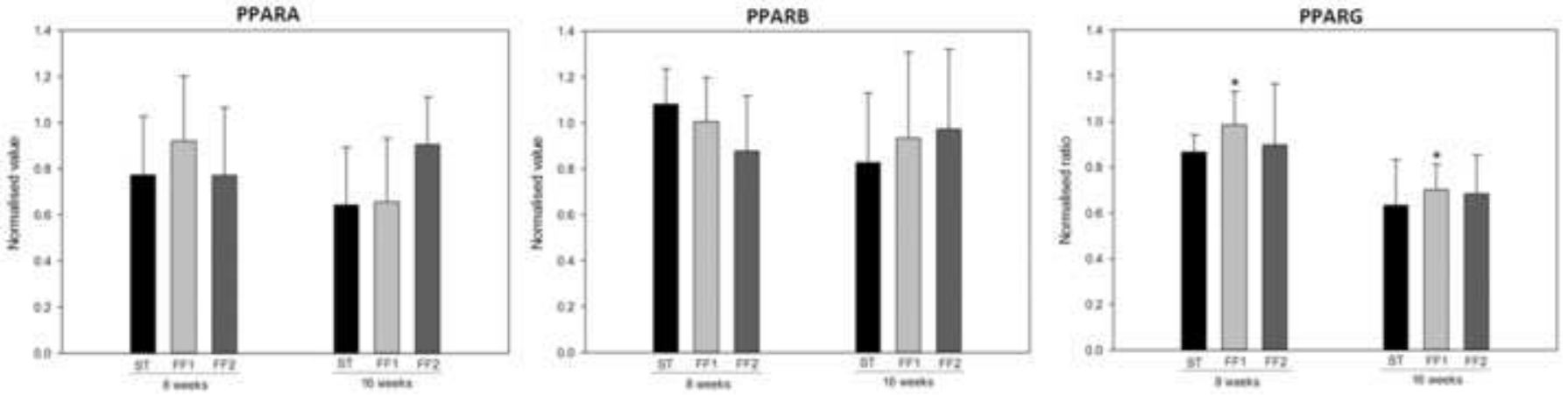

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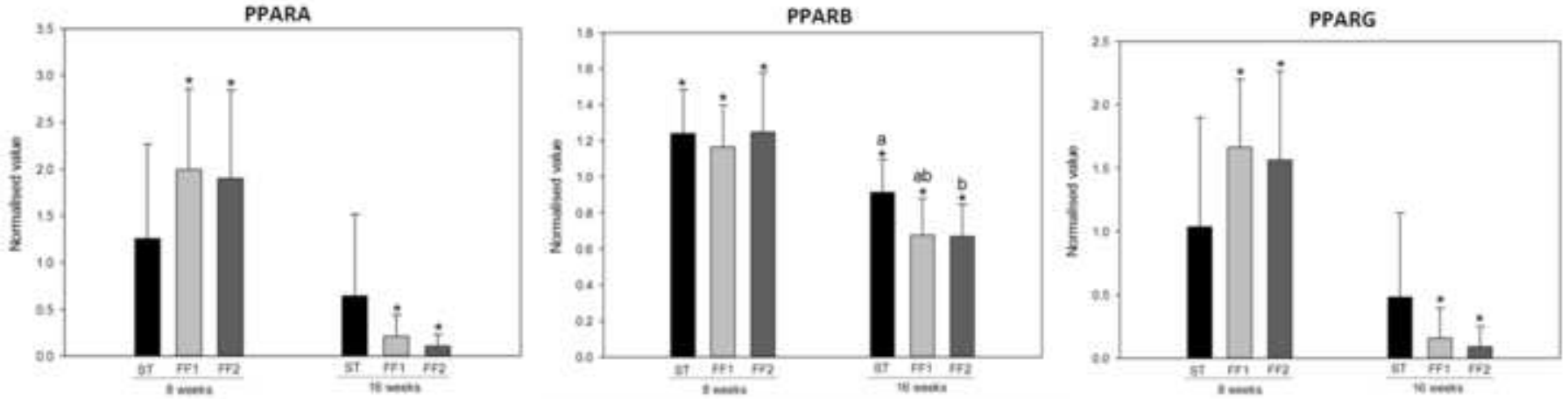

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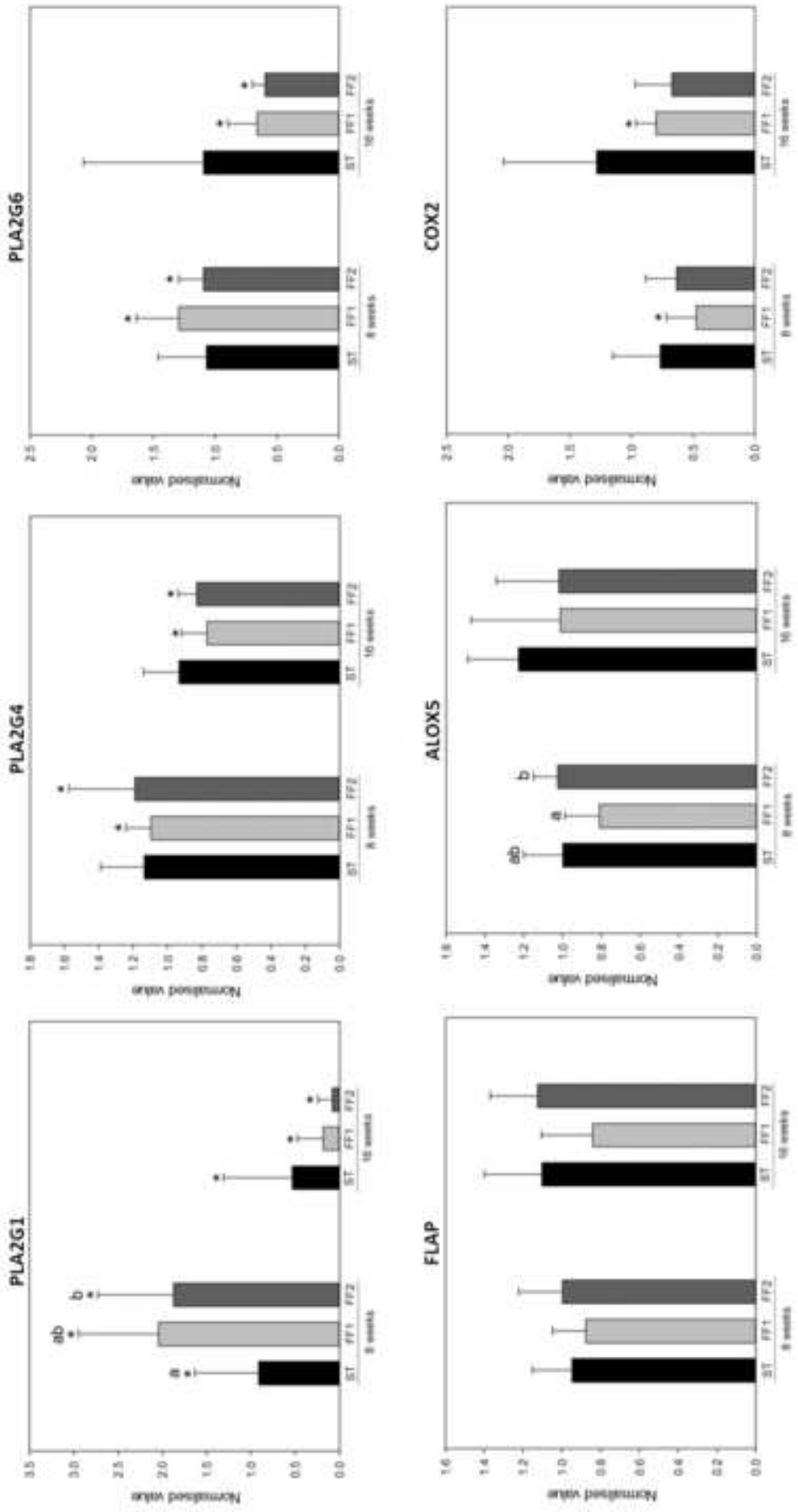

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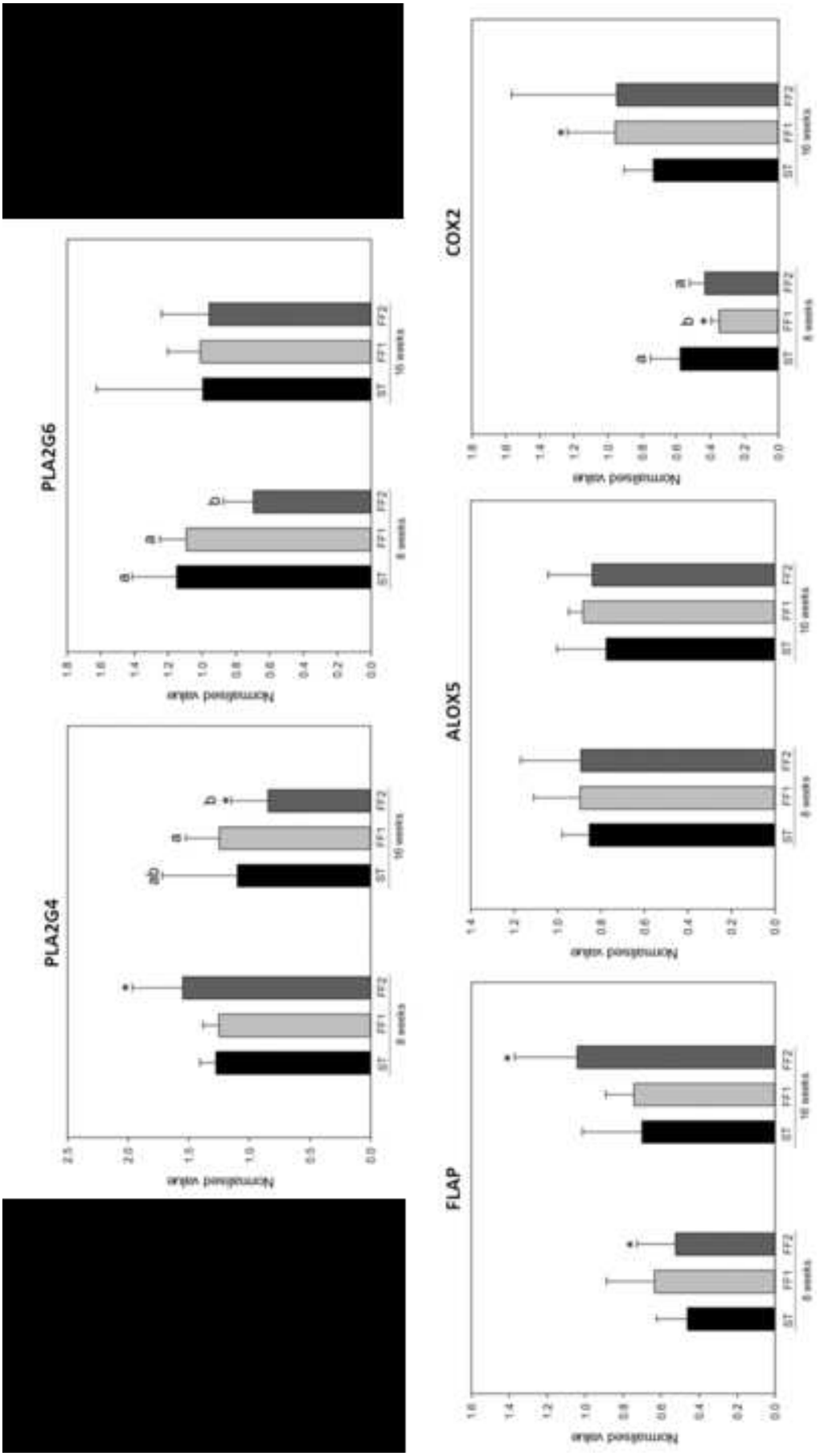

Table 1. Formulation and proximate compositions of the feeds

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 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
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

Table 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

Table 4

Table 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 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/tg)

Table 5

Table 5. 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 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

Table 6

Table 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

Table 7. Fatty acid compositions (percentage of total fatty acids) of phosphatidylethanolamine and phosphatidylcholine 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

	Phosphatidylethanolamine								
	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

	Phosphatidylcholine								
	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 8.docx

Table 8. Fatty acid compositions (percentage of total fatty acids) of phosphatidylinositol and phosphatidylserine 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.

	Phosphatidylinositol								
	ST		FF1		FF2		TWO WAY ANOVA *P-value*		
	8w	16w	8w	16w	8w	16w	Diet	Week	Diet x Week
Saturated	26.6 ± 2.2	30.5 ± 0.6	34.1 ± 1.4	35.2 ± 2.1	33.5 ± 2.1	34.4 ± 2.5	0.000	0.013	ns
Monounsaturated	15.7 ± 5.0	12.5 ± 1.6	12.9 ± 2.1	11.4 ± 2.2	12.4 ± 5.7	12.4 ± 2.8	ns	ns	ns
18:2n-6	2.5 ± 2.3	1.9 ± 0.4	1.2 ± 0.3	1.0 ± 0.3	1.3 ± 1.0	0.8 ± 0.2	0.042	ns	ns
20:3n-6	0.6 ± 0.2	0.9 ± 0.1	0.6 ± 0.1	0.6 ± 0.3	0.6 ± 0.1	0.5 ± 0.5	ns	ns	ns
20:4n-6	17.3 ± 6.4	23.8 ± 3.2	21.4 ± 1.6	23.1 ± 2.0	20.3 ± 4.2	21.0 ± 2.9	ns	0.048	ns
N-6 PUFA	21.6 ± 4.6	28.5 ± 2.8	23.8 ± 1.7	25.7 ± 1.7	22.8 ± 3.2	23.2 ± 2.5	ns	0.012	ns
18:3n-3	0.9 ± 0.6	0.5 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.4 ± 0.3	0.2 ± 0.0	0.000	0.008	ns
20:5n-3	6.8 ± 1.5	3.9 ± 0.9	6.1 ± 1.2	4.7 ± 0.8	8.5 ± 0.9	5.0 ± 1.0	0.022	0.000	ns
22:6n-3	21.1 ± 1.4	14.7 ± 1.3	14.5 ± 2.0	15.2 ± 2.0	16.1 ± 1.2	16.1 ± 2.6	0.010	0.017	0.002
N-3 PUFA	30.8 ± 3.4	20.4 ± 1.7	22.4 ± 2.4	21.5 ± 2.3	26.3 ± 1.0	22.5 ± 3.7	0.025	0.000	0.003
Total PUFA	52.4 ± 2.5	48.9 ± 3.3	46.2 ± 3.0	47.2 ± 3.0	49.2 ± 3.5	45.7 ± 3.8	0.032	ns	ns
EPA/ARA	0.4 ± 0.2	0.3 ±0.4	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.2 ± 0.1	ns	0.000	ns
n-3/n-6	1.5 ± 0.5	1.0 ± 0.6	0.9 ± 0.1	0.84 ± 0.1	1.2 ± 0.17	1.0 ± 0.22	ns	0.000	0.007

	Phosphatidylserine								
	ST		FF1		FF2		TWO WAY ANOVA *P-value*		
	8w	16w	8w	16w	8w	16w	Diet	Week	Diet x Week
Saturated	28.9 ± 2.8	29.8 ± 0.7	33.0 ± 0.6	36.1 ± 2.0	31.8 ± 1.9	33.5 ± 0.8	0.000	0.006	ns
Monounsaturated	11.8 ± 2.4	11.1 ± 1.4	10.4 ± 0.8	13.0 ± 4.1	12.2 ± 4.3	11.7 ± 3.4	ns	ns	ns
18:2n-6	1.4 ± 0.6	1.4 ± 0.2	0.8 ± 0.2	1.1 ± 0.1	1.1 ± 0.6	1.1 ± 0.6	0.018	ns	ns
20:3n-6	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	ns	ns	ns
20:4n-6	1.5 ± 0.4	0.9 ± 0.2	1.4 ± 0.3	0.9 ± 0.2	1.5 ± 0.2	0.8 ± 0.2	ns	0.000	ns
N-6 PUFA	4.5 ± 0.5	4.3 ± 0.5	3.6 ± 0.4	3.3 ± 0.2	4.0 ± 0.9	3.1 ± 0.5	0.000	0.004	0.062
18:3n-3	0.5 ± 0.2	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.2	0.1 ± 0.0	0.018	0.005	ns
20:5n-3	3.9 ± 1.4	1.8 ± 0.4	3.2 ± 0.6	1.6 ± 0.2	3.4 ± 0.3	1.8 ± 0.1	ns	0.000	ns
22:6n-3	40.9 ± 3.9	41.4 ± 2.3	39.1 ± 2.1	35.2 ± 3.0	39.0 ± 4.5	38.3 ± 5.8	0.034	ns	ns
N-3 PUFA	47.7 ± 3.2	45.9 ± 2.4	45.5 ± 2.6	40.1 ± 3.5	44.9 ± 4.1	42.8 ± 6.2	0.036	0.084	ns
Total PUFA	52.2 ± 3.1	50.2 ± 2.2	49.1 ± 2.4	43.4 ± 3.5	48.8 ± 3.3	45.8 ± 5.8	0.005	0.018	ns
EPA/ARA	3.0 ± 1.1	2.0 ± 0.50	2.3 ± 0.5	1.8 ±0.4	2.3 ± 0.5	2.5 ± 0.5	ns	ns	ns
n-3/n-6	9.5 ± 3.0	10.9 ± 1.7	12.6 ± 2.0	12.2 ± 1.5	11.9 ± 3.4	14.5 ± 3.9	0.049	ns	ns

Table 9. Fatty acid compositions (percentage of total fatty acids) of phosphatidylethanolamine and phosphatidylcholine 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.

	Phosphatidylethanolamine								
	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

	Phosphatidylcholine								
	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

Effect of functional feeds on fatty acid 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 lipid (hence energy) content reduced the inflammatory response to ASRV infection and the 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 eicosanoid and fatty acid metabolism in liver and head kidney. Here we show 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 mobilization of lipids and changes in the LC-PUFA composition of membrane phospholipids, along with significant 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 results from the present study suggest that clinical nutrition through functional feeding could be an effective complementary therapy for emerging salmon viral diseases associated with long-term inflammation.

Introduction

Heart and Skeletal Muscle Inflammation (HSMI) is an emerging viral disease caused by a novel Atlantic salmon reovirus (ASRV) [1] particularly affecting farmed salmon in Norway [2]. Mortality varies between outbreaks from 0 to 20%, and the morbidity of the lesions can reach 100%, affecting almost all fish in a farm, leading to significant growth reduction and financial impact [3]. The main symptoms associated with HSMI are epi-, endo- and myocarditis, and myocardial and red skeletal muscle necrosis, indicating that these tissues experience a severe inflammatory process. Early stages of the disease have been reported around five months after transfer to sea water and the cardiac lesions persist for several months until the fish are able to control the infection and reduce tissue damage [4]. 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), have essential roles in the development and control of inflammatory responses. They are important components of plasma membranes that are integral in controlling membrane-signalling pathways. A key link between fatty acid composition of immune cell membranes and the inflammatory process are eicosanoids, lipid mediators including prostaglandins (PG), leukotrienes (LTB) and thromboxanes (TX) that modulate the intensity and duration of inflammatory responses in humans [5] and fish [6]. 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 in relation to immune-related pathways [7,8]. 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, thus being highly relevant in viral infections [9]. PGE_2 is generally related with pro-inflammatory and immunosuppressive functions, although it has also been associated with anti-inflammatory actions during resolution of the inflammatory process [10]. In contrast, the n-3 LC-PUFA, eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), are considered anti-inflammatory fatty acids, with EPA being particularly relevant as an antagonist of ARA, acting as a competitive substrate for the main enzymes of the eicosanoid synthesis pathways, cyclooxygenase (COX) and lipoxygenase (LOX), giving rise to anti-inflammatory eicosanoids and inflammation-resolving E-series resolvins [10,11]. 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 eicosanoid pathways in fish, as in mammals [12]. 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 [13]. Vegetable oils (VO) are generally rich in n-6 PUFA and do not contain n-3 LC-PUFA, however, the replacement of dietary fish oil (FO) by VO does not appear to negatively affect growth performance in salmonids [14]. Therefore VO have been used in the past few years as a sustainable alternative to dietary FO in fish farming. However, the fatty profiles of the alternative feeds, especially the reduction in n-3 LC-PUFA levels and the n-3/n-6 PUFA ratio, are directly reflected in fish tissue fatty acid compositions. This is becoming of increasing interest in relation to potential modulation of immune and inflammatory response pathways [15-17].

In this context, we recently applied the concept of clinical nutrition to moderate the long-term inflammatory process associated with HSMI in salmon [18]. 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 while the functional feeds had reduced DE levels (18 MJ/Kg) achieved through lower lipid contents (18%), 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 of progression of the inflammatory and immune responses to ASRV infection in salmon. However, the study did not enable the identification of the precise physiological and molecular mechanisms by which the functional feeds provided enhanced immuno-inflammatory protection.

The primary objective of the present study was to elucidate possible mechanisms underpinning the observed effects of the functional feeds. Given that the main differences between the standard and functional feeds were in their lipid fractions, this study focussed on lipid metabolism, membrane fatty acid composition, and fatty acid metabolic pathways involved in LC-PUFA biochemistry and inflammatory processes, which 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 were determined. In addition, liver inflammation was measured, given that it is one of the tissues affected in HSMI, and the expression of genes, including transcription factors controlling aspects of lipid metabolism, and those involved in LC-PUFA biosynthesis and eicosanoid metabolism, was determined in liver and head kidney.

Material and methods

Fish and feeds

Three fishmeal-based diets were formulated and manufactured by EWOS Innovation (Dirdal, Norway). Formulation and proximate composition of the feeds are indicated in Tables 1 and 2, details as previously reported [18]. A total of 450 Atlantic salmon (*Salmo salar* L.), AquaGen strain, were distributed into nine tanks (50 fish/tank) at the EWOS facility, Lønningdal, Norway and fed one of the experimental feeds in triplicate tanks for a period of 8 weeks prior to being transferred to the challenge facility. All fish were weighed at the start and end of the feeding phase and doubled their weight in the 8-week period. Initial weights (g) were 109 ± 18, 109 ± 19 and 111 ±17, respectively, and final weights were 199 ± 40, 220 ± 42 and 219 ± 37, respectively, for fish fed the ST, FF1 and FF2 diets (means ± SD, n = 150). There were no significant differences in initial weights or in final weights between fish fed any of the treatments.

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 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 as described in detail previously [18].

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 anaesthetized as above, killed by a

blow to the head, and heart, liver and head kidney were collected. A 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 also immediately frozen in liquid N_2 and stored at -80 ^{o}C.

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. Liver steatosis scores were ranked according to a non-continuous score grade from 0 to 5 (Table 3). All sections were scored double-blinded. A selected proportion of liver samples (n = 15) 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 [19].

All data preparation and simulation output analysis was conducted using the R language [20]. 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 language [21] and fitted with JAGS [22]. 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.

Lipid analyses

Total lipid from approximately 1g of liver, 0.5 g of head kidney and 0.2g of heart was extracted by homogenization in chloroform/methanol (2:1, by volume) according to Folch et al. [23], 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 [24]. Fatty acid methyl esters (FAME) of heart and head kidney phospholipid classes and liver total lipid were prepared by acid-catalyzed transmethylation [25]. FAME were separated and quantified by gas chromatography [26]. Tissue and diet lipid class compositions were determined by single-dimension double-development high-performance thin-layer chromatography (HPTLC) and densitometry [27]. 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.

Real-time quantitative PCR

Expression of 13 selected genes was 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 LC-PUFA biosynthesis, $\Delta 5$ and $\Delta 6$ fatty acyl desaturases (D5FAD, D6FAD) and elongases 5 and 2 (ELOVL5, ELOVL2). The remaining genes are transcription factors involved in the 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 primer sequences were obtained either by literature searches or designed from EST sequences corresponding to candidate genes of interest using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) (Table 4). In addition, amplification of two reference genes, *cofilin-2* and elongation factor-1α (*elf-1α*) was performed as 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 [18,28], and their expression was equally stable under the present study's conditions.

Details of the qPCR protocol have been reported previously [18]. Briefly, 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, using a mixture of random primers (1.5µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng/ µl, Eurofins MWG Operon, Ebersberg, Germany). qPCR amplifications were carried out in duplicate (Quantica, Techne,

196 Cambridge, U.K.) in a final volume of 20 µL containing either 5 µL (most genes) or 2 µL (reference
197 genes and other highly expressed genes) diluted (1/20) cDNA, 0.5 µM of each primer and 10 µL
198 AbsoluteTM QPCR SYBR® Green mix (ABgene). Results were analysed using the relative expression
199 software tool (REST 2009, http://www.gene-quantification.info/), which employs a pair-wise fixed
200 reallocation randomisation test (10,000 randomisations) with efficiency correction to determine the
201 statistical significance of expression ratios (or gene expression fold-changes) between two treatments
202 [29]. Additionally, sequencing of amplicons corresponding to new primer designs enabled the
203 confirmation of identities and presence of single sequences for all genes.
204
205 **Results**
206
207 *Liver histology*

208 The liver histology scores, based on the degree of steatosis was read as vacuole-formation in
209 hepatocyte cytoplasm characterised by both micro- and macro-vesicular lesions (Fig.1). There were
210 clear differences between fish fed the functional feeds and fish fed the ST diet at 8-weeks post-
211 challenge, with the latter group presenting higher micro- and macro-vesicular steatosis. Quantification
212 of the liver histopathology thus showed that both the incidence (percentage of fish sampled) and
213 severity (based on the scoring system in Table 3) of steatosis at 8-weeks post challenge was clearly
214 greater in salmon fed the ST diet compared to fish fed the two functional feeds (Fig. 2). At 16 weeks
215 post-challenge, there were no longer differences in liver histology between the three dietary treatments.

216 *Lipid content and composition of tissues*

217 The total lipid and triacylglycerol (TAG) contents of liver were lower in fish fed the functional feeds
218 compared to fish fed the ST diet with higher lipid (Table 5). In contrast, diet had no effect on the total
219 lipid or TAG contents of heart and head kidney. Total lipid levels in all three tissues decreased during
220 the time course of the infection irrespective of diet, but a significant interaction between diet and time
221 was observed for lipid class compositions, given that these showed a change over time that was
222 variable and dependent on diet. In liver, this decrease was greatest in fish fed diet ST, which also
223 showed a large decline in TAG. The decline in tissue lipid content over the course of the infection was
224 greatest in head kidney. This was associated with decreased percentages of TAG in fish fed the
225 functional feeds and, conversely, with reduced proportions of polar lipids in fish fed diet ST (Table 5).

In heart, the results were less clear and 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.

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 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 diets 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 therefore showed the same differences between the ST diet and functional feeds, and between the two functional feeds (Table 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, fish fed the ST diet showed increased proportions of DHA and n-3 PUFA, and decreased proportions of monoenes at 16 weeks.

The focus in head kidney ~~and heart~~ was on eicosanoid metabolism, and thus fatty acid compositions were determined in the ~~main~~ sources of precursor LC-PUFA, the membrane phospholipid classes phosphatidylcholine (PC), ~~and~~ phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS). The ~~phospholipid~~ fatty acid profiles of the major phospholipids (PC and PE) in head kidney generally showed ~~in general,~~ some significant effects of diet, including increased proportions of saturated fatty acids and decreased proportions of n-6 PUFA in fish fed the functional feeds, but the differences were not as pronounced as in total lipids of liver (Table 7). Irrespective of diet, the proportions of the ~~major~~ eicosanoid precursors, ARA and EPA and the EPA/ARA ratio, declined in all head kidney phospholipids, exceptspecially PI~~E~~, over the course of the infection, being significantly reduced in fish at 16-weeks compared to 8-weeks post-challenge (Tables 7 and 8). The percentage of DHA in PE, PC and PS~~both phospholipid classes~~ was similar in all dietary groups and decreased in PC, but generally not PE or PS, over the time-course of the infection. In contrast, the percentage of DHA in PI was higher in fish fed the ST diet at 8 weeks compared to the level in fish fed the functional feeds, and the level in fish fed all of the diets at 16 weeks post-infection (Table 8).

As with head kidney, the ~~phospholipid~~ fatty acid composition of the main phospholipid classes of heart also showed effects of diet over the course of the infection, but they were not very pronounced and occurred mainly in PC (Table 9~~8~~). The changes in the proportions of the eicosanoid precursors 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. However, within an individual feed, these changes were minimal. In contrast, changes in EPA were more pronounced, declining in both PC and PE of heart over the course of the infection. Additionally, it was noteworthy that the decline was much lower in fish fed the functional feeds (Table 9~~8~~). As a result, although the EPA/ARA ratio in heart phospholipids decreased over the course of the infection in all treatments, 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. Unfortunately, the small amount of tissue available and the low level of PI and PS precluded the determination of fatty acid compositions of these phospholipid classes in heart.

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 (Fig. 3). 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 (Fig. 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.

Expression of PPARs

There were no significant effects of diet on the expression of PPARs in liver (Fig. 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 (Fig. 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 fed the functional feeds (Fig. 5). The only 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.

290

291 *Expression of genes related to eicosanoid biosynthesis*

292

293 In head kidney, the expression of three phospholipases reported to be involved in the release of
294 bioactive fatty acids from cell membranes [8] was higher at 8-weeks compared to 16-weeks post-
295 challenge in fish fed all diets, significantly so in fish fed the functional feeds (Fig. 6). At 8-weeks, the
296 expression of sPLA2 (PLA2G1) was higher in fish fed the functional feeds compared to fish fed the ST
297 diet, albeit only significant in fish fed diet FF2. The expression of COX2 (prostaglandin synthesis),
298 ALOX5 and FLAP, both involved in the biosynthesis of LTs, showed generally few differences
299 between dietary treatments or over the course of the infection. However, the expression of ALOX5
300 was lower in fish fed diet FF1 compared to fish fed diet FF2 at 8 weeks, and expression of COX2 was
301 significantly higher at 16-weeks compared to 8-weeks post-challenge in fish fed diet FF1 (Fig. 6).
302 In heart, phospholipases showed a different pattern of expression compared to head kidney (Fig. 7).
303 Expression of sPLA2 (PLA2G1) was negligible in heart and therefore no qPCR data are shown for this
304 tissue. There were no major differences in expression of phospholipases over the course of the
305 infection, other than higher expression of cPLA2 (PLA2G4) at 8- compared to 16-weeks post-challenge
306 in fish fed diet FF2. Expression of cPLA2 at 16-weeks and iPLA2 (PLA2G6) at 8-weeks was
307 significantly lower in fish fed diet FF2 compared to fish fed the other diets. Expression of ALOX5 and
308 FLAP did not differ between the three dietary groups, but there was a trend for FLAP expression to be
309 higher at 16- compared to 8-weeks post-challenge, although this was only significant in fish fed diet
310 FF2 (Fig. 7). In general, the expression pattern of COX2 in heart was similar to that observed in head
311 kidney, with the expression being significantly lower at 8-weeks post-challenge in fish fed the FF1 diet
312 compared to fish fed the other diets, and with a general up-regulation of this gene in all dietary groups,
313 significant in diet FF1, at 16- compared to 8-weeks post-challenge (Fig. 7).

314 **Discussion**

315

316 As previously described, reduced dietary lipid along with increased dietary EPA and EPA/ARA ratio
317 can modulate the inflammatory response and consequently reduce the severity of heart lesions caused
318 by ASRV infection [18]. The present study investigated the metabolic pathways that link fatty acids,
319 especially LC-PUFA, with the inflammatory response, correlating the previous findings on dietary

immuno-modulation during ASRV infection, with availability of LC-PUFA and potential effects on eicosanoid metabolism in the main organs involved in both immune and metabolic processes.

Fatty acids can impact immune responses in several 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 membranes, affecting signalling pathways and regulating the activation of transcription factors and, thus, gene expression [5,30]. 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 [7], 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 fish, as in mammals, are determined by both dietary input and hepatic fatty acyl desaturase and elongase activities, and delivery via plasma lipoproteins [31,32]. In addition to liver being a major organ involved in LC-PUFA biosynthesis, liver lesions were reported in fish affected by HSMI, occurring as a collateral effect of the heart lesions which likely impair blood flow and hence lipoprotein circulation [3]. Therefore, liver was also investigated in the present study to determine how the different dietary levels of lipid and EPA (in addition to C18 PUFA precursors) and disease progression affected the expression of genes of the LC-PUFA biosynthesis pathway. Eight weeks post-challenge there was higher micro- and macro-vesicular steatosis in fish fed the ST diet compared with fish fed the functional feeds, which was 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 the liver of ST-fed fish. Although the primary cause of the increased steatosis index in ST-fed fish was not known, the observed differences could simply reflect the differences in dietary fatty acid composition, leading to higher fatty acid synthesis and deposition. There is however a potential interaction with the on-going viral infection although previous studies have found no correlation between the severity of heart and liver lesions during HSMI [4]. 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 all dietary groups at the end of the trial [18].

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 the functional feeds compared with fish fed the ST diet. Although this correlates with viral load, it is likely to be due to a 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 the 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 [33]. 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 [34]. 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 endogenous production and, possibly, availability of EPA. The lack of major effects, whether related to nutrition and feed composition or to viral infection, on the expression of elongases was not unexpected as they do not appear to be under the same level of transcriptional control as desaturases [35-37]. 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 present data indicate that, irrespective of the precise molecular mechanism, 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 the crucial role of diet in maintaining 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 are associated with regulation of lipid metabolism in mammals and fish [36,38]. Therefore, as differences in lipid and energy contents of the feeds were expected to affect lipid metabolism pathways, the expression of PPARs was 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 at 16 weeks post infection. In contrast, there were substantial effects related to the progression of the viral infection or HSMI in the expression of all PPARs, particularly PPARα and γ, which decreased from 8 to 16 weeks, particularly in head kidney. This is noteworthy as, recently, there has been interest in the involvement of PPARs in inflammatory processes as they are expressed in immune cells and activated by eicosanoids [39]. Although all three isoforms of PPAR appear to be involved in the immune response, most studies in humans focus on the immunomodulatory roles of PPARα [40] and PPARγ [41]. The expression of PPAR genes decreased during acute inflammation in lung [40], adipose tissue [42] and activated lymphocytes [43]. In mammals, *in vitro* studies generally suggest that PPARs play roles in the resolution of inflammation, regulating 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 [39]. The results of the present study on ASRV/HSMI generally agree 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 been previously 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 mobilization 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 inflammatory mediator eicosanoids [6]. The fatty acid composition of the predominant phospholipid fractions at 8-weeks post-challenge, when no tissue damage in hearts was found irrespective of diet [18] and therefore before the major inflammatory response to ASRV, showed that the proportions of the main LC-PUFA involved in inflammatory responses 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 [44-45]. The dietary fatty acid composition affected the phospholipid compositions although this varied between the different LC-PUFA. In this respect, the proportion of ARA in PE of head kidney was not correlated with levels of dietary ARA, but reflected the levels of precursor 18:2n-6 in the feeds suggesting *in vivo* biosynthesis of ARA as previously reported [44]. In contrast, the levels of EPA in PC, but not in 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 led to increased proportions of this fatty acid in immune cells and heart [31,44]. However, the levels of LC-PUFA in PC and PE were significantly impacted during 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 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 [11].

The initial step in eicosanoid biosynthesis is the release of LC-PUFA precursors from phospholipids, mediated by phospholipase A_2 (PLA_2) that hydrolyzes 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 of phospholipases, particularly in head kidney, 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 distribution and calcium dependence, including calcium-dependent ($cPLA_2$) and calcium-independent ($iPLA_2$), both cytosolic, and secretory PLA_2 ($sPLA_2$) [46]. To date, most studies in fish have investigated $cPLA_2$ [44,47], 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 [8].

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 membranes for the synthesis of either pro- or anti-inflammatory eicosanoids. In heart, this pattern of expression was only observed for $cPLA_2$ in fish fed 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 represent snapshots of gene expression and so can only provide an indication of the possible inflammatory processes at specific time-points particularly in a largely non-fatal disease such as HSMI that manifests 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 [18], and may not represent ideal times for assessing all aspects of the inflammatory response.

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 [48]. Regarding viral immunity, LTB_4 enhances the activity of NK cells and IFNγ [31], which are two elements of the immune system that have been shown to play important roles in the immune response following ASRV infection [18]. Expression of ALOX5 and FLAP was previously analysed after infection with infectious salmon anaemia virus (ISAV) and infectious pancreatic necrosis virus (IPNV) and, in both cases, fold changes were not high even when the inflammatory process was more evident, suggesting that either the transcriptional regulation of these genes occurs in short periods (pulses) or small changes in

451 expression are functionally effective [49,50]. In the present study, there was a clear trend for lower
452 expression, significant at 8-weeks, of ALOX5 and FLAP in head kidney of fish fed the FF1 diet, which
453 may indicate lower production of LTB_4 that would correlate with the milder inflammatory response in
454 this dietary group [18].
455 In general, viral infections stimulate the production of COX2 in humans and there is evidence that
456 PGE_2, the product of the reaction catalysed by COX2, plays important roles in controlling viral
457 replication and modulating the inflammatory responses to virus [51]. This prostanoid has also been
458 related to anti-inflammatory responses, inhibiting the production of LTB_4 and other inflammatory
459 cytokines during resolution of the inflammatory process [11]. However, PGE_2 has generally been
460 regarded as pro-inflammatory and, in the present study, there was a clear trend of higher expression of
461 COX2 at 16-weeks post-challenge, compared to 8-weeks, in both head kidney and heart in all diets,
462 particularly fish fed FF1. This was in agreement with previous studies in both humans [51] and fish
463 [52] where enhanced expression of COX2 along with cytokines involved in the inflammatory response
464 was reported. Furthermore, the present study also showed a trend of lower expression of COX2 in fish
465 fed the functional feeds and that could indicate lower production of PGE_2 in these fish, consistent with
466 results from *in vitro* studies in which increased levels of n-3 PUFA decreased production of PGE_2 [31].
467 In conclusion, this study on HSMI (ASRV infection) in salmon is the first to evaluate the
468 expression of a suite of important genes involved in several pathways relating diet, membrane
469 phospholipid LC-PUFA compositions, and inflammatory responses after a viral infection. Previously,
470 we showed the potential benefits of using diets with reduced dietary lipid and increased EPA on disease
471 outcomes [18], and the present study has supported the earlier results with data on the LC-PUFA status
472 of membrane phospholipids during the course of the infection. In addition, the present study has
473 demonstrated that liver was also a site for histopathological lesions in HSMI. The observed steatosis in
474 liver likely reflects a general impairment of lipid metabolism. However, the higher expression of
475 hepatic Δ6 and Δ5 desaturases in fish fed the FF diets would increase their capacity for the endogenous
476 production and availability of anti-inflammatory EPA. Furthermore, mobilization of lipids, changes in
477 the LC-PUFA composition of membrane PC and PE, along with changes in the expression of genes
478 related to eicosanoid metabolism pathways, showed the important role played by the head kidney in
479 inflammatory diseases caused by viral infections. Further studies will be required to elucidate the
480 crucial role of the production of eicosanoids at different stages of the inflammatory process, especially
481 at the resolution of inflammation, and how this might be influenced by diet. However, the effects on
482 gene expression reported in the present study further suggest that, as in humans, clinical nutrition

483 through functional feeding could be an effective complementary therapy for dealing with emerging
484 salmon viral diseases associated with long-term inflammation.

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490 **References**

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