IMPACT OF PLANT-BASED OILS AND OTHER FEED INGREDIENTS ON GROWTH, TISSUE COMPOSITION, GENE EXPRESSION, AND HEALTH OF ATLANTIC SALMON (*SALMO SALAR*)

by

©Tomer Katan

thesis submitted to the School of Graduate Studies in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Marine Biology

Department of Ocean Sciences

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Abstract

The ω6 to ω3 (ω6:ω3) fatty acid (FA) ratio is known to affect many biological processes (e.g. inflammation, FA metabolism) and human diseases. However, its impacts on salmon physiology, immune response, and the underlying molecular mechanisms are less well understood. The current thesis applied a nutrigenomics and lipidomics approach to study the impacts of plant-based feeds with varying dietary ω6:ω3 ratios and ω3 long chain polyunsaturated fatty acid (LC-PUFA) levels on farmed salmon growth, tissue composition and lipid metabolism, gene expression, and antibacterial immune response.

In Chapter 2 Atlantic salmon were fed with diets containing the same sources and equal levels of marine and plant proteins, and differed in plant oil mixes to generate a range of ω6:ω3 (i.e. 0.3-2.7). A targeted qPCR study was used to measure the mRNA expression of lipid metabolism and eicosanoid synthesis-related genes in the liver. This study revealed that while growth performance and organ indices were not affected by dietary ω6:ω3, liver and muscle FA composition was highly reflective of the diet and suggested elongation and desaturation of 18:3ω3 and 18:2ω6. Compound-specific stable isotope analysis further demonstrated that liver 20:5ω3 and 20:4ω6 synthesis was largely driven by dietary 18:3ω3 and 18:2ω6, respectively. Moreover, significant correlations between LC-PUFA synthesis-related transcripts and liver LC-PUFA further supported FA biosynthesis. In Chapter 3, I used the same fish from the previous feeding trial in order to investigate how the two extreme ω6:ω3 diets (i.e. high ω6 and high ω3) affected the hepatic transcriptome (using 44K microarrays), and to identify novel biomarker genes that respond to variation in ω6:ω3. The microarray study identified transcripts with important roles in lipid metabolism

(*helz2a*), cell proliferation (*htra1b*), immune and inflammatory response (*lect2a, itgb5, helz2a, p43*), control of muscle and neuronal cell development (*mef2d*), and translation (*eif2a, eif4b1, p43*). Further, the PPARα activation-related transcript *helz2a* was down-regulated by high ω6 diet compared with high ω3 diet, and two of its paralogues showed significant correlations with ω6 and ω3 FA in two tissues (liver, muscle). These data indicated their potential as biomarkers of tissue response to dietary ω6:ω3 variation.

The focus of Chapter 4 was to apply a two-factorial design to evaluate if diets with different combinations of ω6:ω3 (high ω6, balanced, high ω3) and EPA+DHA levels (0.3, 1.0, or 1.4%, as formulated) impact head kidney lipid composition, and the transcript expression of genes involved in FA and eicosanoid metabolism (using qPCR). Head kidney FA composition was reflective of the diet and responded to ω6:ω3 variation. Proportions of 20:5ω3 were similar among fish fed 0.3% EPA+DHA with high ω3, 1% EPA+DHA (both high ω3 and high ω6 treatments) and 1.4% EPA+DHA/balanced fed fish, although dietary 20:5ω3 varied by 2.5- to 3-fold. Further, positive correlations were identified between head kidney ω3 LC-PUFA and *elovl5a* transcript levels. This suggested that high dietary 18:3ω3 promoted the synthesis of ω3 LC-PUFA in salmon fed lower dietary EPA+DHA levels (0.3%). This Chapter also showed significant correlations between head kidney FA composition and the expression of eicosanoid synthesis-related transcripts (i.e. *5loxa, 5loxb, cox1, cox2, ptges2, ptges3,* and *pgds*), which illustrated the constitutive relationships among FA and eicosanoid metabolism in salmon. In Chapter 5 salmon were fed high ω6 and high ω3 diets combined with two EPA+DHA levels (0.3 and 1.0% of diet) to investigate dietary impacts on antibacterial and eicosanoid responses. Eicosanoid-metabolism-related transcripts (e.g. *cox1, 5loxa, 5loxb, pgds, lkha4*) in the head kidney

were down-regulated in formalin-killed *Aeromonas salmonicida* (ASAL)-injected compared with the phosphate-buffered saline (PBS)-injected fish (within diet), suggesting an attempt to mitigate the proinflammatory response in bacterin-challenged fish. Fish fed 0.3% EPA+DHA with high ω6 diet showed the strongest fold-change induction (ASAL *vs.* PBS) of antibacterial genes (*ccl19b, il8, il10, il8, saa5, hamp*). Further, this Chapter showed positive correlations between head kidney ω6 PUFA and the transcript expression of immune-related genes (e.g. *il8*, *il10*, *hamp*, *camp*, *stlr5*, *lect2*). These findings suggested that 0.3%EPA+DHA with high ω6 diet may have enhanced the innate antibacterial immune response of Atlantic salmon. Finally, diet and ASAL and/or PBS injection significantly impacted the plasma prostaglandin (PGE_2, PGF3α) levels [measured with electrospray ionization mass spectrometry (ESI-MS/MS)] of salmon. The 0.3%EPA+DHA with high ω6 diet also resulted in lower levels of PGE_2 in ASAL- when compared with PBS-injected fish, and this was concurrent with lower head kidney expression of PGE_2 receptor-encoding transcript (i.e. *ptger4a*).

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List of abbreviations

AA:	amino acid
ACOX:	acyl-coenzyme A oxidase
AFI:	apparent feed intake
ALA:	α-linolenic acid
AMPL:	acetone mobile polar lipid
ARA:	arachidonic acid
aRNA:	anti-sense amplified RNA
ASAL:	formalin-killed atypical *A. salmonicida*
ASAL:	*Aeromonas salmonicida*
BAR:	bile acid receptor
BP:	biological process
CAMP:	cathelicidin
CC:	cellular component
CCL:	cc chemokine-like
CF:	condition factor
cGRASP:	consortium for genomic research on all salmonids project
CID:	collision-induced dissociation
COX:	cyclooxygenase
CPT:	carnitine palmitoyltransferase
CPL:	cytosolic phospholipase
CREAIT:	core research equipment and instrument training
CSIA:	compound-specific stable isotope analysis
C_T:	Cycle threshold
CYP7A1*:*	cholesterol 7 alpha-hydroxylase
DGLA:	dihomo-γ-linolenic acid
DHA:	docosahexaenoic acid
DHCR7:	7-dehydrocholesterol reductase
EEF1α:	elongation factor 1 alpha
EIF:	eukaryotic translation initiation factor
EFA:	essential fatty acids
ELOVL:	elongation of very long chain fatty acids
EPA:	eicosapentaenoic acid
ESI:	electrospray ionization
EST:	expressed sequence tags
FA:	fatty acids
FABP:	fatty acid binding protein
FAD:	fatty acyl desaturases

FAME:	fatty acid methyl ester
FAS:	fatty acid synthase
FDR:	false discovery rate
FM:	fish meal
FO:	fish oil
FXR:	farnesoid X receptor
g:	gram
GC-FID:	gas chromatography-flame ionization detector
GO:	gene ontology
GOI:	genes of interest
HAAF:	hemagglutinin / amebocyte aggregation factor
HAMP:	Hepcidin antimicrobial peptide
HELZ2:	helicase with zinc finger domain 2
HSC:	hematopoietic stem cells
HSI:	hepato-somatic index
HTRA1:	serine protease HTRA1
IDI:	isopentenyl-diphosphate delta isomerase
IFN:	interferons
Ig:	immunoglobulins
IL:	interleukins
IP:	intraperitoneal
IRF:	interferon regulatory factor
ISG:	interferon stimulated gene
ITGB5:	integrin beta-5-like
l:	litre
LC-MS/MS:	liquid chromatography tandem mass spectrometry
LC-PUFA:	long-chain polyunsaturated fatty acids
LECT2:	leukocyte cell-derived chemotaxin 2 precursor
LHPL:	lipoma HMGIC fusion partner-like protein
LKHA4:	leukotriene A4 hydrolase
LNA:	linoleic acid
LOX:	lipoxygenase
5LOX:	arachidonate 5-lipoxygenase
LPS:	lipopolysaccharides
LXR:	liver X receptor
MEF2D:	myocyte-specific enhancer factor 2D
MF:	molecular function
mg:	milligram
MHC:	major histocompatibility complex
min:	Minute

ml:	millilitre
MMC:	melanomacrophage centers
M-MLV:	moloney murine leukemia virus
mRNA:	messenger RNA
MTCO:	cytochrome c oxidase
MUFA:	monounsaturated fatty acids
NCBI:	National Center for Biotechnology Information
ND:	not detectable
NFKB:	NF-kappa-B
NLR:	nucleotide-binding domain, leucine-rich repeat containing receptors
NR:	non-redundant
NRC:	National Research Council
nt:	nucleotide
P43:	aminoacyl tRNA synthase complex-interacting multifunctional protein 1
PABPC:	polyadenylate-binding protein cytoplasmic
PAMP:	pathogen-associated molecular patterns
PBS:	phosphate-buffered saline
PCoA:	principal coordinates analysis
PERMANOVA	permutational multivariate analysis of variance
PFP:	percentage of false-positives
PGDS:	prostaglandin-D synthase
pIC:	polyriboinosinic polyribocytidylic acid
PIT:	passive integrated transponder
PL:	phospholipid
PMT:	photomultiplier tube
PO:	plant oils
PPARβ:	peroxisome proliferator-activated receptor beta
PPARa:	peroxisome proliferator-activated receptor alpha
PRR:	pattern recognition receptors
PTGER:	prostaglandin E receptor
PTGES:	prostaglandin E synthase
PTGIS:	prostacyclin synthase-like
PUFA:	polyunsaturated fatty acids
QC:	quality check
qPCR:	quantitative polymerase chain reaction
r:	delta
RC:	relative contribution
RIG:	retinoic acid-inducible gene
RP:	rank products
RPL:	60S ribosomal protein

RQ:	relative quantity
RT:	reverse transcriptase
SAA:	serum amyloid A
SAM:	significance analysis of microarrays
SFA:	saturated fatty acids
SGR:	specific growth rate
SIMPER:	similarity of percentages analysis
SREBP:	sterol regulatory element binding protein
ST:	sterol
STLR:	soluble toll-like receptor
SD:	standard deviation
TAG:	triacylglycerol
TCR:	T cell receptors
TGF:	transforming growth factor
TLC-FID:	thin-layer chromatography-flame ionization detector
TLR:	toll-like receptors
TNF:	tumor-necrosis factor
TL:	total lipids
VO:	vegetable oils
VPDB:	vienna pee dee belemnite
VSI:	viscero-somatic index
WG:	weight gain
ΔΔCT:	Comparative CT
µl:	microlitre
µM:	micromolar
ω:	omega
ω6:ω3:	omega-6 to omega-3 ratio

Co-authorship statement

I am the first author of all Chapters and manuscripts generated from this thesis. I was primarily responsible for experimental design and research proposal, sampling, data and statistical analyses, and manuscript preparation, editing and submission.

Work described in this thesis was conducted with supervision and guidance from Dr. Christopher Parrish and Dr. Matthew Rise. They contributed to the conception and design of all studies, and are co-authors on all manuscripts. Further, they reviewed and edited all manuscripts and sections of this thesis.

Dr. Albert Caballero-Solares was a co-author on the manuscripts resulting from Chapters 2, 3 and 4. He contributed to the experimental design, data analyses, sampling management, training, and manuscript reviewing. Xi Xue was a co-author on the manuscripts resulting from Chapters 3 and 4. He assisted with training, data analyses, and reviewing of these manuscripts. Further, Albert Caballero-Solares and Xi Xue helped with qPCR assays in Chapter 5.

Dr. Richard Taylor was a co-author on the manuscripts resulting from Chapters 2, 3 and 4. He participated in the formulation of the experimental diets and in the design of the feeding trials, and reviewed the manuscripts. Dr. JuDong Yeo is a co-author on the manuscript resulting from Chapters 5. He provided guidance with the operation and data analysis of ESI-MS/MS and prostaglandin identification.

Chapter 1. General Introduction

1.1. Background

Global fish production in 2018 was estimated to be 179 million tonnes, of which 82 million tonnes represented aquaculture production. Thus, aquaculture accounted for approximately one-half of global fish production, and this is predicted to increase in the future with human population growth and the resultant increase in fish consumption (Naylor et al., 2009; Turchini et al., 2009; FAO, 2020). Global aquaculture production for human consumption is predicted to increase by 30% from 2018 to 2030 (from ~85 million tonnes to ~110 million tonnes; FAO, 2020). However, total global capture fisheries have been relatively stable in the last 3.5 decades (i.e. fluctuated between ~87 million tonnes and 96 million tonnes year^{-1}), and in 2017 ~34% of marine fish stocks were overfished (an increase from 10% in 1974; FA0, 2020). Further, previous predictions showed a global collapse of all taxa currently fished by the year 2048 (Worm et al., 2006; Ayer et al., 2009). Global declines in wild Atlantic salmon (*Salmo salar*) populations have also been observed (Chaput, 2012; ICES 2016; Forseth et al., 2017). As seafood supplies from wild sources are mostly fully exploited, this provides a major opportunity for aquaculture to fill this shortage and become the main supplier of global seafood. However, a major challenge facing the aquaculture industry is to provide fish with nutritious, palatable, and high-quality feed ingredients while decreasing the dependence on threatened marine resources such as fish meal (FM) and fish oil (FO) (Tocher et al., 2006; Naylor et al., 2009; Pratoomyot et al., 2010; Froehlich et al., 2018). Annual global production of FM and FO has generally remained stagnant in the last 3 decades, and the prices of these commodities have

significantly increased (Tacon and Metian, 2015; FAO, 2016, 2020). Environmental parameters (climate change-driven effects, e.g. El Niño) and fishery regulations (Hermansen and Heen, 2012; Obilava, 2014; Murray and Munro, 2018) have a large impact on these prices. It was shown that about a third of the landings from wild fisheries are used for the production of FM and FO, which is unsustainable and unprofitable (Huntington and Hasan, 2009). Therefore, this has led to the development of diets that replace FM and FO with alternative sources of proteins and lipids which are nutritious, economical, and readily available (Turchini et al., 2009; Hodar et al., 2020). Alternative sources of FM and FO include animal sources (e.g. poultry meal, krill meal, insect protein, and animal-based oils such as poultry oil, beef tallow, and pork lard) and plant sources (e.g. soya bean meal, algae, and plant-based oils such as soy bean, camelina, canola, palm, flaxseed, and others: Bandara, 2018; Alhazzaa et al., 2019; Hodar et al., 2020). Plant oils have been heavily utilized as FO alternatives in aquafeeds due to their high availability, increasing production, and economic value (Tacon and Metian, 2008; Turchini et al., 2009; Olsen and Hasan, 2012; Hodar et al., 2020).

1.1.1. Atlantic salmon aquaculture

Atlantic salmon is one of the most popular and economically valuable fish species. Its global production increased by ~70% (i.e. from ~1437 thousand tonnes to 2436 thousand tonnes) from 2010 to 2018, and as such, it is also among the most successful aquaculture species (Asche et al., 2013; FAO, 2020). This growth was a result of improved production practices and technological advances, more efficient logistics and supply chains, and increased scale (Asche, 2008; Bergheim et al., 2009; Kumar and Engle, 2016). The five

leading salmon producing countries are Norway, Chile, Scotland, Canada, and the Faroe Islands (Iversen et al., 2020). Atlantic salmon is also considered the largest fish commodity by value, mainly due to strong demand in many developed and developing countries (FAO, 2020). Feed has a cost share of over 50% in salmon aquaculture and it is considered the most critical input-factor (Asche et al., 2016; Iversen et al., 2020). Although FM and FO were the predominant ingredients in salmonid feeds, previous estimations reported a drop in their inclusion rates in the past decade from 22% to 12% and from 12% to 8%, respectively (Tacon et al., 2011; Tacon and Metian 2015). One example is the aquafeed industry in Norway where in 2013 approximately 0.7 tonnes of wild fish were used to produce 1 tonne of salmon (Ytrestøyl et al., 2015). In comparison, three decades ago 2.8 tonnes wild fish were utilized to produce 1 tonne of salmon (Naylor et al., 1998). Further, infectious diseases (i.e. viral, bacterial, fungal, and parasitic) and co-infections have also severely influenced the salmon industry (Toranzo et al., 2005; Pettersen et al., 2015; Kotob et al., 2017; Semple and Dixon, 2020) and had major cost implications (Iversen et al., 2020).

Several culture conditions such as feeding, light, temperature, density, and oxygen saturation are manipulated in order to achieve optimized growth (Purser and Forteath, 2003; Bergheim et al., 2009; Thorarensen and Farrell, 2011). Salmon eggs are stripped from broodstock fish, fertilized with milt, hardened in water, and then placed in trays or silos for incubation in the hatchery. After they hatch, late alevins are transferred into tanks with recirculation or flow-through systems and fed inert feeds. When the smoltification process is completed, smolts (generally in the size range of 40-120 g) are transferred from

freshwater hatcheries to marine net cages. They are grown in cages for 1-2 years and then harvested at 2-6 kg (Stead and Laird 2002; Verspoor et al., 2007).

1.2. Lipid nutrition in aquafeeds

Lipids play an important role as a source of metabolic energy in fish for growth, reproduction, movement and migration (Sargent et al., 2002; Tocher, 2003). High-energy lipid feeds are commonly formulated to improve salmonids' growth performance for a given amount of feed. This allows more dietary protein to be converted into muscle protein rather than energy production (i.e. protein-sparing effect), as well as a decrease in nitrogenous losses from feeds into the environment (Izquierdo et al., 2002; Yigit et al., 2002). Lipids also play vital roles in membrane structure, and as precursors of vitamins, steroid hormones and prostaglandins (Tocher, 2003; Turner et al., 2006; Calder, 2007; Gomez-Abellan and Sepulcre, 2016). In addition, dietary lipids provide animals with essential fatty acids (EFA), and they carry fat-soluble vitamins.

1.2.1. Essential fatty acids

Vertebrate species cannot synthesize ω3 or ω6 polyunsaturated fatty acids (PUFA) *de novo* and therefore they must be obtained from their diet. These are known as EFA (Sargent et al., 1995; Sargent et al., 2002; Glencross, 2009). It was shown that EFA requirements vary among fish species, trophic levels (Trushenski et al., 2020), stage of development, and dietary lipid level (Izquierdo, 1996). The FA composition of fishes' natural diet, as well as their life history (e.g. herbivorous *vs* carnivorous), play a major role in their specific EFA requirements (Sargent et al, 1989). EFA deficiency negatively impacts

growth, reproduction, and survival in fish (Castel et al., 1972). Deficiency signs such as swollen, pale liver with fatty infiltration have been reported in fish (Sargent et al., 2002). Earlier studies revealed that salmonids require 1% of linoleic acid (LNA, 18:2ω6; Castell et al., 1972; Takeuchi and Watanabe, 1982), 0.5-1.0% of α-linolenic acid (ALA, 18:3ω3; Takeuchi and Watanabe, 1982; Turchini et al., 2009), and 0.5-1.0% of ω3 long-chain polyunsaturated fatty acids (LC-PUFAs; Sargent et al., 2002) in their diet. However, given the competitive interaction among PUFA, fishes' dietary EFA requirements cannot be considered in isolation, but rather relative to each other [e.g. ratios of docosahexaenoic (DHA, 22:6ω3), eicosapentaenoic (EPA, 20:5ω3) and arachidonic acids (ARA, 20:4ω6) such as 22:6ω3:20:5ω3:20:4ω6]. Further, the dietary ratio of ω6:ω3 requires careful consideration, given its impact on tissue 20:4ω6:20:5ω3 and on eicosanoid metabolism (Sargent et al., 1999, 2002; Turchini et al., 2009).

1.2.2. Dietary requirement of EPA and DHA

EPA and DHA play important roles in growth and metabolism, flesh quality, nervous system development, health, and reproduction of fishes (Sargent et al., 1999; Sargent et al., 2002; Tocher, 2010, 2015). They were also shown to have a positive impact on numerous human pathologies such as inflammatory and cardiovascular diseases (Gil et al., 2012; Calder, 2013). Therefore, it is crucial to establish optimal levels, and ensure sufficient dietary content of these ω3 LC-PUFAs. Ruyter et al. (2000) showed that the combination of EPA and DHA at 12.5% (of total FA) and 1.0% (as proportion of the diet) resulted in maximized growth of Atlantic salmon. In Rosenlund et al. (2016) it was reported that Atlantic salmon require EPA+DHA levels of 2.7 to 4.4% (of total FA) in the diet.

However, Bou et al. (2017) found that low EPA+DHA diets (i.e. 0.5 to 2.0% of diet) did not negatively influence Atlantic salmon growth. Further studies showed that Atlantic salmon have good growth potential even with low levels of ω3 LC-PUFA, as long as adequate levels of 18:3ω3 are provided in the diet (Ruyter et al., 2000; Menoyo et al., 2007; Glencross, 2009; Jalali et al., 2021).

1.3. Fish lipid classes

Lipids are a large group of compounds that are insoluble in water and soluble in organic solvents (e.g. chloroform, alcohols). Lipids containing FA are called complex lipids, while lipids without FA are termed simple lipids (Gurr et al., 2002; Parrish, 2013). Vertebrate lipids can be neutral or polar and this is based on their polarity and lipid class composition. Triacylglycerol (TAG) and sterols are neutral, while phospholipids are regarded as polar (Gurr et al., 2002). TAG is an important energy store in fish. Salmonids store lipid in the adipose tissue of their muscle, mostly as TAG (Polvi and Ackman, 1992; Bell, 1998). TAG is composed of three FA molecules which are esterified to glycerol. PUFA are preferentially located in the *sn*-2 [stereospecific numbering (*sn*)] position (i.e. middle glycerol carbon), while saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) are typically preferentially located in the *sn*-1 and *sn*-3 positions of the glycerol molecule (Tocher, 2003). Sterols have important roles in cell membrane structure (e.g. membrane fluidity and order) and as precursors of steroid hormones. Cholesterol, the main sterol in fish, is a tetracyclic alcohol compound and can be non-esterified (i.e. free cholesterol) or esterified to a FA (i.e. steryl esters; Sargent et al., 2002). Phospholipid is a general term that includes all lipids containing phosphorus. Phospholipids are amphipathic

molecules which play important roles in cell membrane structure and fluidity, and in the synthesis of numerous biologically active compounds (e.g. eicosanoids, diacylglycerol, platelet activating factors, and inositol phosphates; Tocher, 2003; Tocher et al., 2008). Phosphoglycerides are the most common of the phospholipids. They are characterized by a common backbone of phosphatidic acid, which is composed of glycerol and two FA. Generally, SFA and MUFA are preferentially esterified on position *sn*-1, while PUFA are preferentially esterified on position *sn*-2 of the phosphoglycerides (Sargent et al., 2002; Tocher et al., 2008).

1.4. FA structure and nomenclature

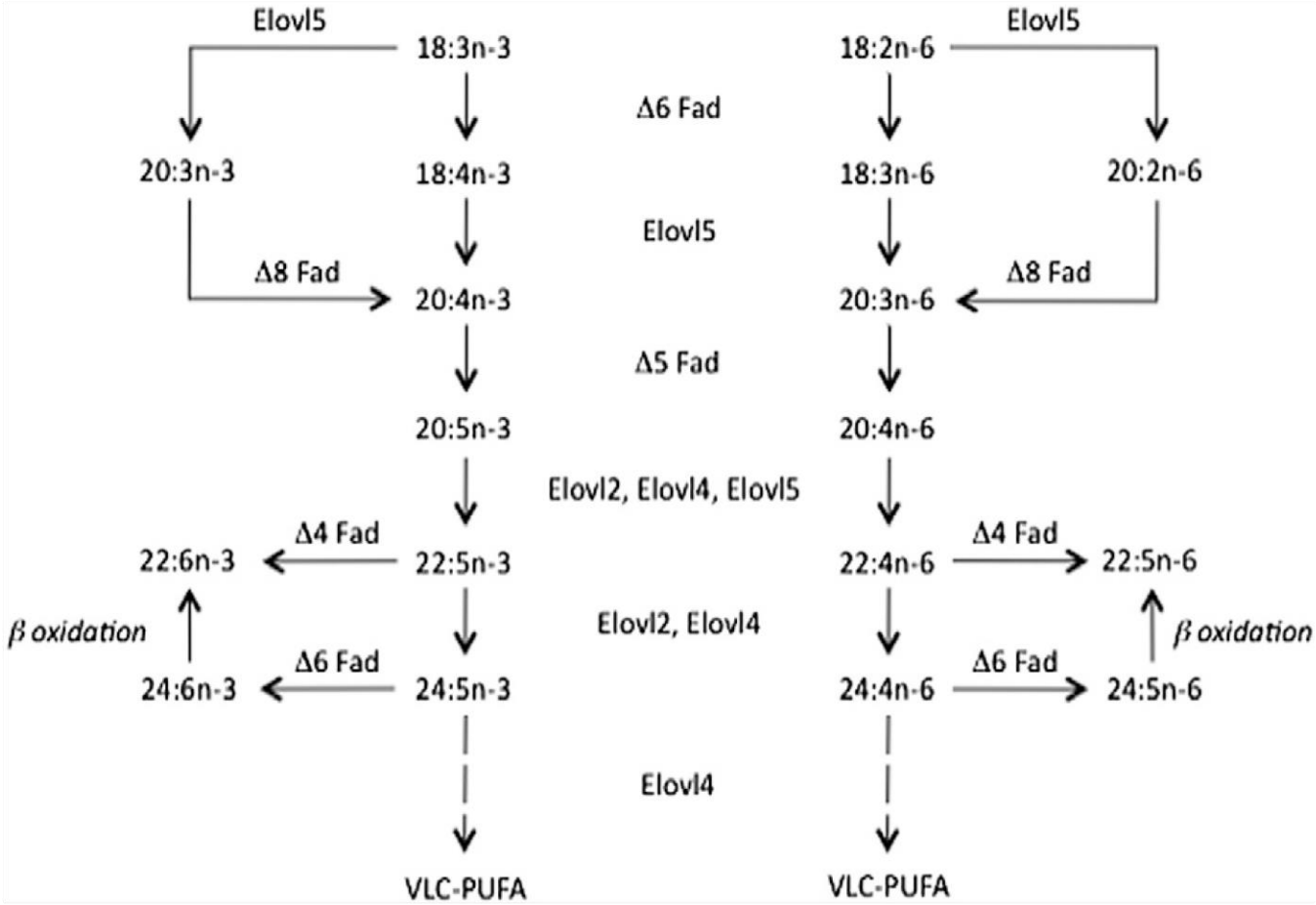
FA are classified based on their chain lengths, number of double bonds, and the position of double bonds. In vertebrates, FA contain from 14 to 24 carbons with a methyl and a carboxylic acid group at the molecule endings (Gurr and Harwood, 1991). In the shorthand systems (using omega/n/ω -designation), the position of the first double bond is relative to the methyl end, while in the delta configuration double bonds are in relation to the carboxyl terminus (Gurr et al., 2002; Sargent et al., 2003). For example, 16:0 represents a FA with 16 carbons and no double bonds, while 18:3ω3 designates an 18-carbon FA with three double bonds situated 3 carbons from the methyl end (using the shorthand system). The latter FA can also be described as $18{:}3^{\triangle 9,12,15}$ if following the delta nomenclature. SFA have the maximum number of hydrogens, and no carbon-carbon double bonds, while MUFA describe a hydrocarbon chain with one double bond. PUFA contain two or more carbon-carbon double bonds, while LC-PUFA describe a chain with 20 or more carbons, with 3 or more double bonds (Tocher, 2003). Finally, FA have common names (e.g. 16:0

is palmitic acid) and omega nomenclature (e.g. 20:5ω3 and 22:6ω3 represent eicosapentaenoic acid and docosahexaenoic acid, respectively).

1.5. Roles and synthesis of ω6 and ω3 PUFAs

All vertebrates lack the Δ12 (or ω6) and Δ15 (or ω3) desaturases, which are the enzymes responsible for the production of PUFA from 18:1ω9. Therefore, PUFA such as 18:2ω6 and 18:3ω3 must be supplied in the diet to satisfy EFA requirements in fish (Sargent et al., 2002; Glencross et al., 2009). However, these C_{18} PUFAs can be converted to the biologically active LC-PUFA (e.g. 20:4ω6, 20:5ω3 and 22:6ω3), and this ability varies with species and depends on the presence and expression of genes responsible for FA elongation and desaturation (Tocher, 2003; Tocher, 2015; Figure 1.1). Marine carnivorous fish species (e.g. *Sparaus aurata, Pagrus major, Paralichthys olivaceus*) have lost the ability to synthesize LC-PUFA, likely as a result of adaptation to environments that are rich in these FA, and therefore, they require them in their diets (Sargent et al., 1999; Sargent et al., 2002; Tocher et al., 2006). However, freshwater fish species (e.g. *Clarius gariepinus, Cyprinus carpio, Oreochromis niloticus*) have LC-PUFA synthesis abilities since their natural environments are more abundant with C_{18} PUFA (particularly 18:2ω6 and 18:3ω3), and are poor sources of EPA and DHA. Atlantic salmon is an anadromous fish and as such it has a limited capacity to synthesize LC-PUFA Sargent et al., 2002). However, this ability is also dependent on life stage, and salmon parr were shown to perform well on diets containing LC-PUFA precursors and low ω3 LC-PUFA (Bell et al., 1997; Miller et al., 2007; Qian et al., 2020).

Figure 1.1. Pathways of long-chain polyunsaturated fatty acid biosynthesis in fish. Adapted from Tocher (2015). Δ4 Fad, Δ5 Fad and Δ6 Fad, fatty acyl desaturases. Δ8 desaturation activity is an inherent characteristic of the Δ6 Fad of most teleosts. Elovl2, Elovl4 and Elovl5, fatty acid elongases.



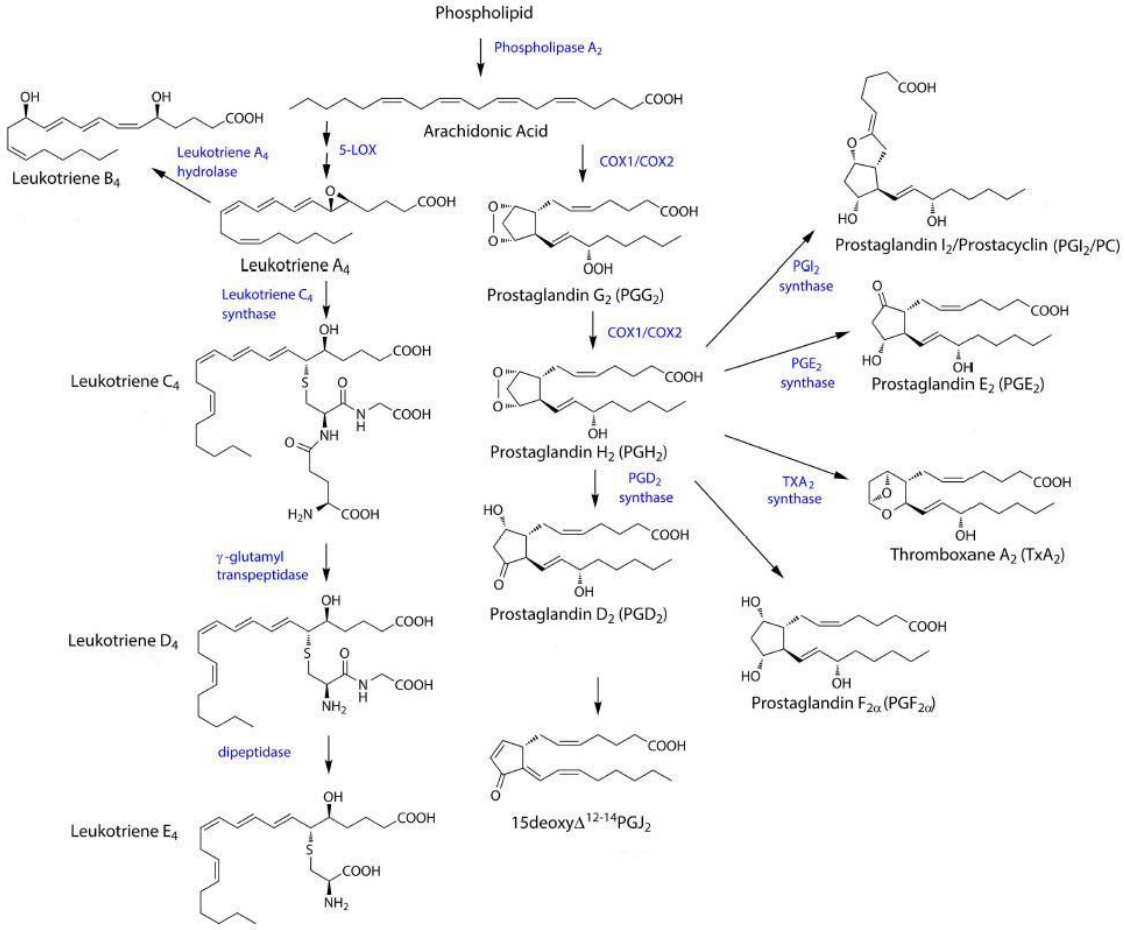
Indeed, salmon parr are exposed to rich dietary sources of C_{18} in their freshwater environment (e.g. invertebrates), and this adaptation may have influenced their ability to produce LC-PUFA *in vivo* (Bell et al., 1994).

The enzymes responsible for the bioconversion of the PUFA pathway are the fatty acyl desaturases (FADS2D5, FADS2D6a, FADS2D6b and FADS2D6c; Zheng et al., 2005; Monroig et al., 2010) and fatty acyl elongases (ELOVL5a, ELOVL5b, ELOVL2, ELOVL4a and ELOVL4b; Hastings et al., 2004; Morais et al., 2009; Carmona-Antoñanzas et al., 2011; Zhao et al., 2019). This pathway also involves transcription factors such as liver X receptor (LXR), sterol regulatory element binding protein (SREBP) 1 and 2, and peroxisome proliferator-activated receptor (PPAR) α, β and γ (Leaver et al., 2008; Carmona-Antoñanzas et al., 2014; Katan et al., 2019; Hixson et al., 2017).

As mentioned in section 1.2.2., LC-PUFA have important roles in growth, flesh quality, neuronal development, health, and reproduction in fish. They also have important functional and structural roles in cellular membranes (e.g. fluidity and membrane protein trafficking), TAG, and other storage lipids such as wax esters. Further, they act as ligands for transcription factors that control gene expression and metabolism (Tocher, 2010). In addition, ω6 [i.e. ARA and dihomo-γ-linolenic acid (DGLA, 20:3ω6)] and ω3 (i.e. EPA, DHA) LC-PUFAs are precursors to potent lipid mediator signaling molecules, termed eicosanoids, which have important roles in the regulation of inflammation and immune response [e.g. through their direct effects on immune cells or indirect effects via cytokines (Lall, 2000)]. In general, eicosanoids derived from ω6 PUFAs are pro-inflammatory, while eicosanoids derived from ω3 PUFAs are anti-inflammatory (Calder, 2007, 2013). EPA and ARA are competitively metabolized via cyclooxygenase (COX) and lipoxygenase (LOX)

to eicosanoids like prostaglandins, thromboxane, leukotrienes (Figure 1.2), lipoxins and epoxy compounds like 15(S)-HpETE or 15(S)-HETE (Calder, 2007; Holen et al., 2015). Furthermore, recent studies have identified non-classical eicosanoid derivatives such as resolvins, maresins and protectins from EPA and DHA which are anti-inflammatory mediators (Serhan and Petasis, 2011: Weylandt et al., 2012). These diverse regulatory roles underpin the importance of LC-PUFA as belonging to the ω6 or the ω3 pathway, and the concept of balance in dietary intake of the two PUFA series. This balance has a major impact on health and disease resistance in fishes (Yaqoob and Calder, 2007; Lands, 2014).

Figure 1.2. General overview of synthesis pathway for eicosanoids. Enzymes are shown in blue font. Adapted from Lone and Taskén (2013).



1.6. Challenges with plant-based oils in aquaculture

The FA composition of plant oils (PO) is one of the major bottlenecks to the sole use of these alternative lipid sources in aquafeeds. PO are generally poor sources of ω3 FA in comparison to marine FO. Rather, they are rich sources of ω6 and ω9 FA, such as 18:2ω6 and 18:1ω9, with the exception of linseed oil (rich in 18:3ω3). But most importantly, ω3 LC-PUFA are not present in conventional terrestrial PO. Furthermore, they generally (with the exception of linseed and camelina oils) provide inadequate ratios of ω6:ω3 FA. This limitation has resulted in lower levels of EPA and DHA in tissues of fish fed PO in comparison to FO, and consequently compromised flesh quality for consumers (Bransden et al., 2003; Alhazzaa et al., 2011; Sprague et al., 2016) and impacted fish health (e.g. immune status, intestinal morphology, liver steatosis (Montero et al., 2003; Ruyter et al., 2006). To overcome this limitation, significant efforts have been made to develop transgenic oilseed crops with *de novo* ω3 LC-PUFA synthesis capacity (Betancor et al., 2015, 2018; Napier et al., 2015). Further, in comparison to FO which contain low levels of ARA, PO lack ARA (Hertrampf and Piedad-Pascual, 2000). A complete replacement of FO with plant-based oils may also impair fishes' EFA profiles (Oliva Teles, 2015; Bandara, 2018). Previous studies showed that PO with adequate essential FA levels, ω3 PUFA content, and energy availability were best suited as a FO replacement (Peng et al., 2008; Turchini et al., 2009). Further, diets with a blend of PO (rather than a single PO) may provide a more balanced FA composition and maximize tissue LC-PUFA in fish (Turchini et al., 2009; Teoh et al., 2011; Thanuthong et al., 2011). Finally, another limitation with plant-based oils is that they contain phytosterols (e.g. β-sitosterol and campesterol), which

are the plant equivalents of animal cholesterol. Dietary phytosterols were shown to lower the uptake and synthesis of cholesterol in Atlantic salmon (Miller et al., 2008; Liland et al., 2013), and in some cases caused cholesterol deficiencies (Carmona-Antoñanzas et al., 2014; Liland, 2014).

1.7. The ratio of ω6:ω3 PUFAs

It was shown that optimal ratios of ω6:ω3 PUFAs are important for maintaining the metabolic homeostasis of many biological processes such as cell apoptosis, lymphocyte proliferation, phagocytosis, FA and cholesterol metabolism and others (de Pablo and Alvarez de Cienfuegos, 2000; Wymann and Schneiter, 2008). However, the underlying biological mechanisms are still poorly understood. One potential mechanism that has been suggested is that via interaction with nuclear receptors, PUFAs influence gene expression and regulate the activity of numerous transcription factors [e.g. SREBP, PPAR, LXR, and farnesoid X receptor (FXR)] (Leaver et al., 2008; Getek et al., 2013). Further, previous reports showed that 18:2ω6 and 18:3ω3 compete as substrates for the LC-PUFA synthesis enzymes (i.e. elongases and desaturases; Betancor et al., 2014; Gregory et al., 2016).

Earlier studies have suggested that humans evolved on a diet with ω6:ω3 of ~ 1:1, whereas in Western diets, the ratio is as high as 20-25:1 (Wijendran and Hayes, 2004; Simopoulos, 2011). Such high ratios promote the pathogenesis of many diseases that involve inflammatory processes, including cardiovascular diseases, inflammatory diseases (e.g. inflammatory bowel disease, rheumatoid arthritis), obesity, cancer, autoimmune and cognitive diseases, while increased levels of ω3 PUFA (a low ω6:ω3 ratio) exert protective effects. Therefore, increasing dietary intake of ω3 PUFA is one of the most efficient

strategies to correct this imbalance (Simopoulos et al., 2008; Wall et al., 2010). In fish, there have already been concerns that an extensive replacement of FO with PO causes a high incidence of cardiovascular and inflammatory disorders, and that farmed seafood does not supply an adequate ratio (Young, 2009; Oliva-Teles, 2012). Although many studies were conducted on the nature of this ratio in humans, very little is known about how variation in ω6:ω3 affects farmed fish. Furthermore, the upper limit of ω6:ω3 before fish growth and health are affected is still unknown. This information will be valuable, particularly when assessing the suitability of candidate plant-based oils in aquafeeds. Finally, as the aquafeed industry moves to higher levels of terrestrial-based oils, it will be essential to provide the minimum ω3 LC-PUFA requirements to meet market demands and ensure the health benefits of consuming farmed seafood.

1.8. Using gene expression to study LC-PUFA biosynthesis

One strategy to enhance or optimize the endogenous LC-PUFA production and storage in fish is by identification of the optimal levels of FA in the diets (Gregory et al., 2016; Colombo et al., 2018). Earlier studies have investigated how substitution of FO with vegetable oils influenced gene expression or enzyme activities. Collectively, it has been shown that the transcript levels of genes that regulate LC-PUFA and cholesterol biosynthesis are under dietary influence (e.g. Jordal et al., 2005; Leaver et al., 2008; Torstensen and Tocher, 2011; Gregory et al., 2016; Hixson et al., 2017). Multiple studies showed up-regulation of LC-PUFA synthesis-related genes in fish fed plant-based diets as compared with a marine diet (e.g. Jordal et al., 2005; Leaver et al., 2008; Morais et al., 2011; Xue et al., 2015, 2020). It was established that the LC-PUFA pathway is under a

feedback loop where dietary levels can promote or inhibit synthesis, and this has been predominantly based on manipulations of EPA and DHA (e.g. synthesis is reduced with increasing concentrations or enhanced by decreasing concentrations: Jordal et al., 2005; Glencross et al., 2015). However, the influence of LC-PUFA precursors on this pathway is less understood (Gregory et al., 2016).

1.9. Fish immune system – general overview

The immune system is an extremely complex network of cells, biological structures, chemical processes, and multilevel defense mechanisms (Danilova, 2006). Immune mechanisms could be constitutive (already present) or responsive (inductive), and the operation of the system is both at the local and systemic levels (Ellis, 2001). Vertebrate immune systems are differentiated into innate and adaptive immune systems. Both systems contain multiple cellular and humoral components. During an immune response, both the innate and the adaptive systems work together to eliminate pathogens such as viruses or bacteria (Tort et al., 2003; Secombes and Wang, 2012). The innate system is a non-specific immune defense that is the first to respond to infection and disease (Magnadottir, 2006). This system is triggered when pattern recognition receptors (PRR) on immune cells [e.g. Toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I), nucleotide-binding domain, leucine-rich repeat containing receptors (NLRs); (Reviewed by Li et al., 2017; Liao and Su, 2021)] bind to a conserved pathogen-associated molecular pattern (PAMP) on bacterial or viral pathogens [e.g. lipopolysaccharides (LPS), peptidoglycans, bacterial DNA, or viral DNA/RNA] (Boltaña, 2011; Reviewed by Smith et al., 2020). The innate system can be classified into three defence mechanisms: physical and chemical barriers,

cellular, and humoral components. The first line of defense in fish consists of epithelial barriers such as skin, scales, gills, and intestinal mucosa. In addition to these physical barriers, chemical mediators secreted from mucous cells (e.g. lysozyme, lectins, defensins, complement proteins, and antibacterial peptides) have a role in trapping the pathogens and preventing their entry. Mucosal tissues contain leucocyte populations responsible for local immune responses (Whyte, 2007; Rauta et al., 2012). If a pathogen is successful in passing these physical barriers, it will be faced with cellular and humoral components. Cellular defences are comprised of multiple cell types such as macrophages, neutrophils, monocytes, dendritic cells, non-specific cytotoxic cells, and natural killer cells (Secombes and Wang, 2012). Depending on the cell type, cells can participate in numerous defence responses such as phagocytosis, activation of humoral components, and activation of the adaptive immune system through antigen presentation. Further, immune cells can initiate an inflammatory response through interferons (IFN), interleukins (IL), chemokines, and factors like tumor-necrosis factor (TNF) (Whyte, 2007; Uribe et al., 2011). Humoral components may be cellular receptors or molecules that are soluble in plasma and other body fluids (Magnadóttir, 2006).

The adaptive system is a specific immune defence mechanism that develops immunological memory, which allows for an enhanced response when a second infection with the same pathogen occurs. Similarly to the innate system, the adaptive system includes both cellular and humoral components. Fish are equipped with immunoglobulins (Ig), major histocompatibility complex (MHC) proteins, and T- and B-lymphocytes, which can elicit specific immune responses against a wide diversity of antigens (Flajnik and Kasahara, 2010). B cells are key elements of the humoral response. Their main role is to produce

immunoglobulins against antigens, and they also can present processed antigens to T cells (Secombes and Wang, 2012; Lewis et al., 2014). T cells are the key element of the cellular adaptive response. They contain T cell receptors (TCR), which recognize a specific antigen when presented by MHC (class I or class II). T cells are generally classified into cytotoxic T cells and helper T cells (Castro et al., 2011; Secombes and Wang, 2012).

The major lymphoid tissues in teleost fish are the thymus, head kidney, spleen, as well as the mucosa-associated lymphoid tissues including the skin, gills, and gut (reviewed by Press and Evensen, 1999; Uribe et al., 2011). The thymus is responsible for the production of T and B cells, while myeloid cells and eosinophilic granular cells were also identified in this organ (Chilmonczyk, 1992; Zapata and Amemiya, 2000). The head kidney in fish is the functional homolog of the bone marrow in vertebrates and is the main site of haematopoiesis (Zapata et al., 2006). Hematopoietic stem cells (HSCs) have the ability to self-renew and generate mature blood cell types over the lifetime of an organism. They differentiate into erythroid, myeloid, and lymphoid lineages (Willett et al., 1999; Orkin and Son, 2008; Kobayashi et al., 2016). The anterior kidney contains macrophages, which aggregate into structures called melanomacrophage centres (MMC), lymphoid cells (mostly B cells), reticular (Press et al., 1994), and endothelial cells (Dannevig et al., 1994). The spleen is composed of a system of splenic ellipsoids (which are involved in the capture of antigens), MMC, and lymphoid tissue. Given the large number, diversity, and evolutionary divergence of teleost species, they may exhibit some species-dependent innate and adaptive immune responses (Grimholt, 2016; Yu et al., 2020; Liao and Su, 2021).

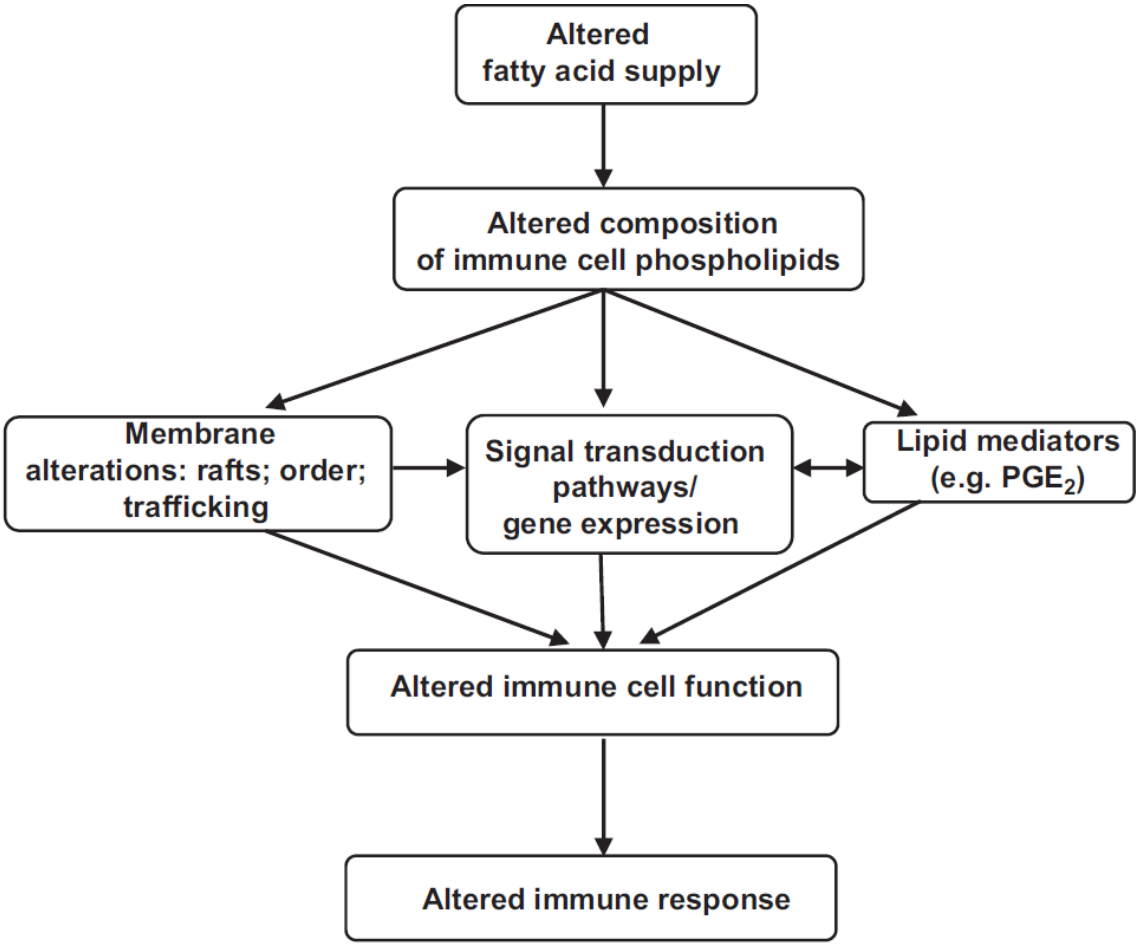
1.10. Dietary lipid immunomodulation

Dietary lipid composition can alter the FA composition of immune cells. This may influence immune responses in a variety of ways, such as alterations in the membrane physical (e.g. membrane order and fluidity) and functional properties (e.g. location and functions of proteins, carriers, or enzymes), as well as regulation of gene expression through effects on receptors, intracellular signal transduction, or transcription factors (Calder, 2007; Puertollano et al., 2008; Calder, 2013; Figure 1.3). Further, as mentioned in Section 1.5, EPA, DHA, DGLA, and ARA are precursors of potent lipid mediator signalling molecules, termed eicosanoids, which have important roles in the regulation of inflammation and immune response e.g. through their direct effects on immune cells such as macrophages and other leukocytes, or indirectly via cytokines (Rowley et al., 1995; Lall, 2000; Calder, 2013; Martinez-Rubio et al., 2013; Gomez-Abellan and Sepulcre, 2016).

As plant-based diets in aquafeeds have distinct FA compositions in relation to marine-based diets (e.g. higher ω6 PUFAs and higher ω6:ω3 FA ratios), this may affect immune and eicosanoid responses of fish consuming them (Montero et al., 2010; Alhazzaa et al., 2013; Montero et al., 2015; Holen et al., 2018). Research on the relationship between dietary FA composition, eicosanoid production, and immune response in teleost fish has been inconclusive and often contradictory [reviewed by Lall (2000), Kiron (2012), Oliva-Teles (2012), and Martin and Król (2017)]. Some studies showed that fish fed with diets containing high ω3 FA resulted in decreased survival and immunosuppression following an immune challenge, when compared with those fed with a high ω6 FA diets (Fracalossi and Lovell, 1994; Li et al., 1994; Montero et al., 2003), while other studies indicated a

positive effect of high dietary ω3 on the immune response (Ashton et al., 1994; Alhazzaa et al., 2013). For example, increased activity of head kidney macrophages following a challenge with *Edwardsiella ictaluri* has been associated with higher dietary levels of ω3 FA in channel catfish (*Ictalurus punctatus*) (Sheldon and Blazer, 1991). Previous research pointed out that the impacts of dietary FA on the immune response are complex and depend on factors such as competition between ω3 and ω6 FA during LC-PUFA metabolism, the cell type involved, and the sources of dietary lipids (Lall et al., 2001; Reviewed by Kiron, 2012; Martin and Król, 2017). These factors could modulate the production of eicosanoids, and the ability of organisms to fight diseases.

Figure 1.3. Mechanisms by which an altered supply of FA affect immune responses. Adapted from Calder (2007). Abbreviations used: PG, prostaglandin. FA, fatty acids.



1.11. Goals and objectives

The focus of this thesis was to examine the impacts of plant-based feeds with varying dietary ω6:ω3 ratios and ω3 LC-PUFA levels on farmed salmon growth, tissue composition and lipid metabolism, gene expression, and antibacterial immune response. Four experiments were performed, with each experiment representing a data Chapter (i.e. Chapters 2, 3, 4 and 5). Individual objectives were:

1- **To examine the effects of plant-based diets with varying ratios of ω6:ω3 on growth performance, tissue lipid composition, FA biosynthesis, and lipid-related gene expression.**

   The goal of this study was to investigate the impact of varying dietary ω6:ω3 (with low incorporation of FO; 12-week exposure) in Atlantic salmon feeds on growth performance and physiology. Diets contained the same sources and equal levels of marine and plant proteins and differed in PO mixes [i.e. soy (rich in ω6 FA), linseed (flax; rich in ω3 FA), and palm oils]. Dietary impacts on growth performance, tissue (liver, muscle) lipid composition, and liver LC-PUFA synthesis were examined. Further, a targeted qPCR study was used to measure the mRNA expression of lipid metabolism and eicosanoid synthesis-related genes in the liver. Correlation analysis was utilized to elucidate the relationships between FA composition and transcript expression in salmon liver. The guiding hypothesis of this study is that tissue composition will reflect dietary variation in ω6:ω3 and consequently influence FA biosynthesis. These metabolic changes will likely be associated with changes in the transcript levels of genes related to FA and eicosanoid metabolism.

2- **To examine the effects of plant-based diets with varying ratios of ω6:ω3 on the hepatic transcriptome, and association with phenotypic traits (growth, somatic indices, and tissue lipid composition).**

The ω6:ω3 ratio is known to affect many biological processes (e.g. inflammation, FA metabolism) and human diseases. However, its impacts on fish physiology and the underlying molecular mechanisms are less well understood. Further, it is not known which genes are involved in variation in dietary and tissue ω6:ω3 FA ratios in Atlantic salmon fed high levels of terrestrial-based oils. The aim of this Chapter was to utilize a 44K salmonid oligonucleotide microarray for the examination of the impact of the two extreme ω6:ω3 diets (i.e. high ω6 and high ω3; 12-week exposure) on the hepatic transcriptome, and to identify novel biomarker genes and molecular pathways that are altered by variation in ω6:ω3. To illustrate links between liver transcripts and phenotypic traits, correlation analyses were performed. I hypothesized that Atlantic salmon fed the two diets with the most extreme lipid compositions (i.e. High ω3 and High ω6) would show the most extensive transcriptomic differences, and that diets would modulate the expression of genes related to immune and inflammatory responses, eicosanoid synthesis and FA metabolism.

3- **To investigate the influence of plant-based diets with varying EPA+DHA levels and ω6:ω3 on head kidney lipid composition, and transcript expression of genes involved in FA and eicosanoid metabolism.**

The interaction of dietary ω6:ω3 with EPA+DHA levels, and their nutritional impacts on eicosanoid and FA metabolism in fish, have not been fully elucidated. Further, the constitutive regulation of LC-PUFA pathway in Atlantic salmon head kidney requires further investigation given the central role of this organ in haematopoietic and immune processes. Therefore, the focus of this two factorial design study was to assess how diets with different combinations of ω6:ω3 (high ω6, balanced, high ω3) and EPA+DHA levels (0.3, 1.0, or 1.4%, as formulated) impact head kidney lipid composition, and the transcript expression of genes involved in FA and eicosanoid metabolism. Furthermore, correlation analyses were used to relate head kidney lipid composition with transcript expression. I hypothesized that head kidney lipid composition would be reflective of the diet at week 12, and that dietary ω6:ω3 and EPA+DHA levels would impact the transcript expression of eicosanoid metabolism and LC-PUFA synthesis-related genes. For example, previous studies showed up-regulation of transcripts involved in LC-PUFA synthesis in Atlantic salmon fed plant-based diets (containing lower EPA and DHA) as compared with marine diets (Morais et al., 2011; Xue et al., 2015).

4- To investigate the influence of plant-based diets with varying EPA+DHA levels and ω6:ω3 on antibacterial and eicosanoid responses in Atlantic salmon challenged with formalin-killed *Aeromonas salmonicida* (ASAL) bacterin.

The goal of the fourth Chapter was to examine the impact of low EPA+DHA [i.e. below requirement (0.3%, as formulated) and at the minimum requirement level (1.0%, as formulated)] and two extreme ω6:ω3 diets (i.e. high ω6 and high ω3) on

the head kidney transcript expression of genes involved in antibacterial immune response and eicosanoid metabolism. Additionally, dietary and ASAL challenge impacts on plasma prostaglandin levels (i.e. PGE_2, PGF3α) were elucidated. Finally, to aid in linking head kidney lipid composition with antibacterial and eicosanoid-related transcripts correlation analysis was used. Dietary lipid composition (e.g. EPA, DHA, and ARA) was shown to influence immune responses by altering the FA composition of immune and inflammatory cell membranes (Calder, 2007; Wall et al., 2010; Calder, 2013). Thus, I hypothesized that changes in head kidney lipid composition would result in modifications in the expression of immune and eicosanoid metabolism-related transcripts.

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Chapter 2. Effect of plant-based diets with varying ratios of ω6 to ω3 fatty acids on growth performance, tissue composition, fatty acid biosynthesis and lipid-related gene expression in Atlantic salmon (*Salmo salar)*

Preface

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2.1. Abstract

Little is known about how variation in omega-6 to omega-3 (ω6:ω3) fatty acid (FA) ratios affects lipid metabolism and eicosanoid synthesis in salmon, and the potential underlying molecular mechanisms. The current study examined the impact of five plant-based diets (12-week exposure) with varying ω6:ω3 (0.3-2.7) on the growth, tissue lipid composition (muscle and liver), and hepatic transcript expression of lipid metabolism and eicosanoid synthesis-related genes in Atlantic salmon. Growth performance and organ indices were not affected by dietary ω6:ω3. The liver and muscle FA composition was highly reflective of the diet (ω6:ω3 of 0.2-0.8 and 0.3-1.9, respectively) and suggested elongation and desaturation of the ω3 and ω6 precursors 18:3ω3 and 18:2ω6. Furthermore, proportions of ω6 and ω3 PUFA in both tissues showed significant positive correlations with dietary inclusion (% of diet) of soy and linseed oils, respectively. Compound-specific stable isotope analysis (CSIA) further demonstrated that liver long-chain polyunsaturated fatty acid (LC-PUFA) synthesis (specifically 20:5ω3 and 20:4ω6) was largely driven by dietary 18:3ω3 and 18:2ω6, even when 20:5ω3 and 22:6ω3 were supplied at levels above minimum requirements. In addition, significant positive and negative correlations were identified between the transcript expression of LC-PUFA synthesis-related genes and liver ω6 and ω3 LC-PUFA, respectively, further supporting FA biosynthesis. Liver ω3 LC-PUFA also correlated negatively with the eicosanoid synthesis-related transcripts *pgds* and *cox1*. This is the first study to use CSIA, hepatic transcriptome, and tissue lipid composition analyses concurrently to demonstrate the impact of plant-based diets with varying ω6:ω3 on farmed Atlantic salmon.

2.2. Introduction

Aquaculture currently accounts for approximately one-half of the fish consumed globally, and this is predicted to increase in the future with human population growth and the resultant increase in fish consumption (Turchini et al., 2009; Merino et al., 2012; Liu et al., 2018). Furthermore, as seafood supplies from wild sources are mostly fully exploited (FAO, 2016) this provides a key opportunity for aquaculture to become the main supplier of global seafood. However, a major challenge the aquaculture industry now faces is to provide fish with nutritious and high-quality feed ingredients while decreasing the dependence on limited marine resources such as fish meal (FM) and fish oil (FO) (Naylor et al., 2009; Tacon and Metian, 2015; Froehlich et al., 2018). This has led to the development of diets that replace FM and FO with terrestrial sources of proteins, and lipids such as vegetable oils (VO), that are more readily available and cost-effective (Tacon and Metian, 2008; Turchini et al., 2009; Olsen and Hasan, 2012; Nasopoulou and Zabetakis, 2012; Gasco et al., 2018).

Earlier studies have shown that partial replacement of FO by VO does not affect growth, feed conversion and survival in Atlantic salmon (Rosenlund et al., 2001; Bransden et al., 2003; Tocher, 2010; Liland et al., 2013). However, the main limitation of VO is that they lack ω3 long-chain polyunsaturated fatty acids (LC-PUFA; $\geq$ 20 carbons in length) which are crucial for growth, health, reproduction, and other biological processes of fishes (Sargent et al., 1999; Sargent et al., 2002; Tocher, 2015; Gasco et al., 2018). This limitation has resulted in lower levels of eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) in tissues of fish fed VO in comparison to FO, and has consequently

compromised fish health [e.g. reduced serum alternative complement pathway and head kidney phagocytic activity (Montero et al., 2003), and increased lipid accumulation in the liver (Jordal et al., 2007; Liland, 2014)], and flesh nutritional quality for consumers (Bransden et al., 2003; Bell et al., 2010; Alhazzaa et al., 2011; Sprague et al., 2016).

Salmonids can synthesize LC-PUFA from their precursors, α-linolenic acid (ALA,18:3ω3) and linoleic acid (LNA, 18:2ω6) to a limited extent (Bell et al., 1993; Tocher et al., 2000; Hixson et al., 2014), and this is performed by the fatty acyl desaturase enzymes, FADS2D5 and FADS2D6 (Zheng et al., 2005; Monroig et al., 2010), and fatty acyl elongase (elongation of very long chain fatty acids - ELOVL) enzymes, ELOVL5 and ELOVL2 (Hastings et al., 2004; Morais et al., 2009). This pathway has been shown to be regulated at the mRNA level and involves transcription factors such as liver X receptor (LXR), sterol regulatory element binding protein (SREBP) 1 and 2, and peroxisome proliferator-activated receptor (PPAR) α, β and γ (Leaver et al., 2008; Carmona-Antoñanzas et al., 2014; Glencross et al., 2015; Hixson et al., 2017).

There have been concerns that an extensive replacement of FO with VO causes a high incidence of cardiovascular and inflammatory disorders in fish, and that farmed seafood fed with VO diets may not supply an adequate ratio of omega-6 to omega-3 (ω6:ω3) fatty acids (FA) for the consumer (Young, 2009; Oliva-Teles, 2012). Although some studies have shown that this ratio plays an important role in fish immune response (e.g. via pro- and anti-inflammatory eicosanoids) (Furne at al., 2013; Lopez-Jimena et al., 2015; Holen et al., 2018) and FA metabolism (Torstensen and Tocher, 2010; Vagner and Santigosa, 2011), very little is known about how variation in ω6:ω3 affects lipid metabolism and eicosanoid synthesis in salmon, and the potential molecular mechanisms

involved. In addition, it has been shown that LC-PUFA synthesis is under a feedback loop where dietary levels can inhibit or promote synthesis, and this has been predominantly based on manipulations of DHA and EPA. However, the impact of LC-PUFA precursors (ALA and LNA) is less understood (Gregory et al., 2016). Establishing this relationship is important, as it will reveal the full potential of the LC-PUFA precursors to act as a source for endogenous LC-PUFA production. One possible method to determine the extent of LC-PUFA synthesis is that of compound-specific stable isotope analysis (CSIA), in which the isotopic signatures of FA are determined by measuring their $^{13}C/^{12}C$ ratio. This ratio (expressed as $\delta^{13}C$) in animal tissues was shown to reflect their dietary composition (Voigt et al., 2008), and by using a mathematical mixing model it is possible to estimate the proportional contribution of a terrestrial FA to the isotopic composition of the tissue (e.g. EPA / DHA) (Phillips, 2012). Consequently, the goal of this study was to examine the effects of plant-based diets (with low incorporation of FO) with varying ratios of ω6:ω3 fatty acids on: 1) growth performance and tissue lipid composition (i.e. muscle and liver); and 2) expression of genes related to FA metabolism and eicosanoid synthesis in salmon liver. The levels of arachidonic acid (ARA, 20:4ω6), EPA and DHA were similar among the diets. Correlation analyses were used to relate FA composition in the liver with hepatic transcript expression. Furthermore, CSIA of liver tissues was used to determine the extent of LC-PUFA synthesis (i.e. the proportion of EPA and DHA synthesized from the dietary precursor ALA) in fish fed the two extreme ω6:ω3 diets.

2.3. Materials and Methods

2.3.1. Experimental diets

Five experimental diets with varying ratios of ω6 to ω3 were formulated and manufactured by Cargill Innovation Center (Dirdal, Norway). The experimental diets were as follows: ω6:ω3 of 1:3 (high ω3), 1:2 (medium ω3), 1:1 (balanced), 2:1 (medium ω6) and 3:1 (high ω6). Diets were formulated to be isonitrogenous and isoenergetic (Table 2.1) and to meet the nutritional requirements of salmonids [National Research Council (NRC), 2011]. Diets were stored at -20°C until needed.

2.3.2. Experimental fish and rearing conditions

Salmon pre-smolts were transported from Northern Harvest Sea Farms (Stephenville, NL, Canada) in October 2015, and reared in the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, Memorial University of Newfoundland, Canada). Ethical treatment of fish in this experiment was carried out in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (protocol # 16-74-MR). After arrival, salmon were graded to select the most uniform population and held in 3800 l tanks. Then they were PIT (Passive Integrated Transponder; Easy AV, Avid Identification Systems, Norco, CA, USA) - tagged and fed a standard commercial feed (Winter EP200, 4 mm, Skretting Canada, St. Andrews, NB, Canada) until they reached the appropriate size to begin the experiment.

Table 2.1 Formulation and nutrient composition (%) of experimental diets[a] fed to Atlantic salmon.

Ingredient (%)[b]	**High ω3**	**Med ω3**	**Balanced**	**Med ω6**	**High ω6**
Marine protein[c]	26.7	26.7	26.7	26.7	26.7
Plant protein[d]	44.6	44.6	44.6	44.6	44.6
Additives[e]	2.6	2.6	2.6	2.6	2.6
Fish oil	4.8	4.8	4.8	4.8	4.8
Linseed oil	16.7	12.5	8.3	4.2	0
Soy oil	0	4.9	9.8	14.7	19.6
Palm oil	4.6	3.9	3.2	2.4	1.7
Digestible energy (MJ Kg^{-1})	20.4	20.4	20.4	20.4	20.4
Digestible protein (g Kg^{-1})	421	421	421	421	421
Dry matter (%)[f]	94.8	94.9	94.8	95.0	94.8
Ash (%)[f]	6.2	6.2	6.0	6.0	6.2

[a] Means were calculated using 3 pellets per diet (n=3).

[b] All ingredients were sourced from Cargill Innovation stocks.

[c] Marine protein is comprised of fish meal.

[d] Plant protein concentrate is a proprietary blend of soy, corn and wheat.

[e] Additives are proprietary mineral, vitamin mix and amino acids. Mineral and vitamin composition is proprietary information to EWOS.

[f] Analysed as % of wet weight (n = 3).

Post-smolts (203 ± 24 g mean initial weight ± standard error (SE); 27 ± 0.12 cm mean initial fork length ± SE) were randomly distributed into twenty 620 l tanks, holding 40 fish each and subjected to an 18-day acclimation period (during which they were exposed to the commercial diet) followed by a 12-week feeding trial (4 tanks per diet). Throughout the trial, fish were fed overnight using automatic feeders and apparent feed intake was recorded. The uneaten pellets were collected in the morning and the daily amount of feed was adjusted based on the amount of uneaten pellets. All tanks were supplied with 11 °C flow-through filtered seawater at 12 l min^{-1}; the dissolved oxygen level was 10 mg l^{-1}, and the photoperiod was maintained at 24 h light. This photoperiod was followed in order to replicate the light regime currently used in feeding trials of the industry partner (Cargill). Mortalities were weighed and recorded throughout the trial (< 1% at week 12).

2.3.3. Fish sampling

Salmon tissue sampling occurred at week 0 (the day before fish were exposed to the experimental diets) and week 12. Fish were starved for 24 h prior to sampling. Morphometric parameters (length, weight), organ indices [hepato-somatic index (HSI), viscero-somatic index (VSI)] and tissue samples were collected from 10 randomly selected fish at week 0, and 5 fish per tank at week 12. Sampling occurred after the fish were euthanized by an overdose of MS-222 (400 mg l^{-1}, Syndel Laboratories, Vancouver, BC, Canada). For lipid analyses, liver and muscle (i.e. the skin was removed on the left side of the fish to obtain white skeletal dorsal muscle tissue near the dorsal fin) were removed, weighed and subsampled for dry matter and lipid analysis. Samples were collected in 15 ml glass test tubes that had been rinsed three times with methanol, followed by three rinses

with chloroform. Tissue samples for lipid analysis were stored on ice during sampling, their wet weights recorded, and they were covered with 2 ml of chloroform (HPLC-grade). Finally, glass test tubes were filled with nitrogen, the Teflon-lined caps were sealed with Teflon tape, and the samples were stored at −20°C. For gene expression analyses, liver (50–100 mg sample $^{-1}$) tissues were collected in 1.5 ml nuclease-free tubes, flash-frozen in liquid nitrogen, and stored at −80°C until RNA extractions were performed.

2.3.4. Lipid extracts

Lipid samples were extracted according to Parrish (1999). Samples were homogenized in a 2:1 mixture of ice-cold chloroform:methanol using a metal rod with Teflon coating. Chloroform-extracted water was added to bring the ratio of chloroform: methanol: water to 8:4:3. The sample was sonicated for 4 min in an ice bath and centrifuged at 3000 rpm for 2 min at room temperature. The bottom, organic layer was removed using a double pipetting technique, placing a 2 ml lipid cleaned glass Pasteur pipette inside a 1 ml glass pipette, in order to remove the organic layer without disturbing the top, aqueous layer. Chloroform was then added back to the extraction test tube and the entire procedure was repeated three times. All organic layers were pooled into a separate lipid-cleaned vial. Finally, samples were concentrated under a flow of nitrogen gas.

2.3.5. Lipid class separation

Lipid class composition was determined using an Iatroscan Mark 6 TLC-FID (thin-layer chromatography-flame ionization detector) (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), silica coated Chromarods, and a three-step development method (Parrish, 1987).

The lipid extracts were applied to the Chromarods and focused to a narrow band using 100% acetone. The first development system was hexane/diethyl ether/formic acid (98.95:1.0:0.05). The rods were developed for 25 min, removed from the system for 5 min to dry and replaced for 20 min. The second development was for 40 min in hexane/diethyl ether/formic acid (79:20:1, v/v/v). The final development system had two steps, the first was in 100% acetone for two 15 min time periods, followed by two 10 min periods in chloroform/methanol/chloroform extracted water (5:4:1, v/v/v). Before using each solvent system, the rods were dried in a constant humidity chamber. After each development system, the rods were partially scanned in the Iatroscan and the data were collected using Peak Simple software (SRI Instruments, version 3.67, Torrance, CA, USA,). The Chromarods were calibrated using standards from Sigma Chemicals (Sigma Chemicals, St. Louis, MO, USA).

2.3.6. Fatty acid methyl ester (FAME) derivatization

Fifty microliters of lipid extract were transferred into a lipid-cleaned 15 ml glass vial and concentrated under a flow of nitrogen to complete dryness. Then, 1.5 ml of methylene chloride and 3 ml of Hilditch reagent (1.5 sulfuric acid: 98.5 anhydrous methanol) were added to each sample vial, followed by vortexing and 4 min sonication (Fisher Scientific FS30, Pittsburgh, PA, USA). Vials were then filled with nitrogen, capped and heated at 100°C for 1 h. Subsequently, 0.5 ml of saturated sodium bicarbonate solution and 1.5 ml of hexane were added to each vial, followed by vortexing, and then the upper organic layer was removed into a separate lipid-cleaned glass vial. Each sample was then dried and refilled with ~0.5 ml of hexane. Finally, vials were filled with nitrogen, capped,

sealed with Teflon tape, and sonicated for 4 min to resuspend the fatty acids. All FAMEs were analyzed on a HP 6890 GC-FID (gas chromatography-flame ionization detector system) equipped with a 7683 autosampler (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Fatty acid peaks were identified against known standards (PUFA 1, PUFA 3, BAME and a Supelco 37 component FAME mixture) (Sigma-Aldrich Canada Ltd, Oakville, ON, Canada). Finally, chromatograms were integrated using the Varian Galaxie Chromatography Data System, version 1.9.3.2 (Walnut Creek, CA, USA). FA data were expressed as area percentage of FAME.

2.3.7. Compound-specific stable carbon isotope analysis

The $\delta^{13}C$ ($^{13}C/^{12}C$) values of identified FAME were measured and analyzed using an Agilent 6890N gas chromatograph coupled via a GC Combustion III interface to a Delta V Plus isotope ratio mass spectrometer (Thermo Fisher Scientific, Mississauga, ON, Canada) at the Core Research Equipment and Instrument Training Network (CREAIT Network) of Memorial University. FAME from the two extreme diets (i.e. high ω3 and high ω6), and from salmon liver tissues at the beginning (i.e. week 0) and at the end of the study (i.e. week 12) from fish that were exposed to these diets, were analysed. All $\delta^{13}C$ values were calculated relative to the Vienna Pee Dee Belemnite (VPDB) standard using the following equation:

$$\delta^{13}C = [\frac{R\,(sample)}{R\,(standard)} - 1] * 1000$$

Where R is the ratio of $^{13}C/^{12}C$. An isotopically characterized standard purchased from Indiana University (Fatty Acid mixture F8) was analyzed every 4 samples and was used to

correct the measured $\delta^{13}C$ values of the samples. In addition, an aliquot of the methanol used during the FAME derivatization of fatty acids was collected and analysed for $\delta^{13}C$ composition with an Aurora Total Organic Carbon analyzer in order to correct for the additional methyl group added to fatty acids during transesterification. This correction was applied to all fatty acids using the following equation:

$$\delta^{13}C = (n + 1) [\delta^{13}C_{FAME}] - n[\delta^{13}C_{FFA}]$$

where n is the number of C atoms in the fatty acid.

Finally, a two-end-member mixing model (Budge at al., 2008; Phillips, 2012) was used to determine the relative contribution of dietary ALA to liver EPA and DHA in the two dietary treatments:

$$\delta^{13}C \text{ Tissue}, _k = X_k \delta^{13}C_{pre} + (1- X_k) \delta^{13}C_{LC\text{-}PUFA}$$

where X_k is the proportion of precursor (ALA) C contribution to k, the fatty acid of interest (i.e. EPA or DHA). $\delta^{13}C_{pre}$ is the isotopic signature of ALA, while $\delta^{13}C_{LC\text{-}PUFA}$ is the isotopic signature of EPA or DHA in each of the two diets. Overall standard deviations were obtained through error propagation analysis, and calculated from coefficients of variation according to Caulcutt and Boddy (1983). The diet-tissue discrimination factor was calculated by subtracting the mean $\delta^{13}C$ signature of the diet from that of the tissue for each individual fatty acid [0.4 $\pm$ 0.5 parts per thousand (‰) overall mean ± standard error (SE) of the high ω3 and high ω6 treatments).

2.3.8. RNA extraction, DNase treatment, column purification and cDNA synthesis

RNA extraction, DNase treatment and column purification of collected samples were performed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), RNase-free DNase Set (QIAGEN, Mississauga, ON, Canada) and RNeasy Mini Clean-up Kit (QIAGEN), respectively. All procedures were conducted according to the manufacturers' instructions with modifications as described in Xue et al. (2015). RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity and quantity were assessed using NanoDrop spectrophotometry (Thermo Fisher Scientific) using the A260/280 and A260/230 ratios of all RNA samples. Column-purified RNA samples had A260/280 ratios of 2.0 - 2.2 and A260/230 ratios of 1.8 - 2.3 (data not shown). All cDNAs were prepared by reverse transcription of 1 µg of column-purified total RNA in each sample using 1 µl of random primers (250 ng; Invitrogen), 1 µl of dNTPs (10 mM each), 4 µl of 5x first-strand buffer, 2 µl of DTT (0.1 M) and 1 µl of Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (200 U; Invitrogen) at 37°C for 50 min in a 20 µl reaction volume following the manufacturer's instructions and as described in Xue et al. (2015). Finally, all cDNAs were diluted 40 times with nuclease-free water (Invitrogen) prior to qPCR.

2.3.9. Real-time quantitative polymerase chain reaction (qPCR) analysis

Expression of 23 genes of interest (GOI) (Table 2.2) related to FA, cholesterol and eicosanoid metabolism was quantified by qPCR using liver RNA templates from the following 3 dietary treatments at week 12: high ω3; balanced; and high ω6. The sequences of all primer pairs used in qPCR analyses and the GenBank accession number of sequences

used for primer design are presented in Table 2.2. It is worth noting that primers for the transcript *cox2* (GenBank accession number AY848944) failed quality testing due to low transcript levels. qPCR primer quality testing procedures, including standard curves and dissociation curves, were conducted to verify that a single product was amplified (melt curve analysis) with no detectable primer dimers as described elsewhere (Rise et al. 2010; Booman et al. 2011). In brief, the amplification efficiency (Pfaffl, 2001) of each primer pair was determined using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of input total RNA. Two pools were generated (i.e. high ω3 pool and high ω6 pool), and each pool consisted of 8 fish (1.25 ng of cDNA per fish). The reported primer efficiencies are an average of the two pools. A 5-point 1:2 dilution series was used for the primers: pparβ2a, 5loxa, 5loxb and cox1 as these transcripts had lower expression levels [fluorescence threshold cycle (C_T) values of ~28-31). Additionally, amplicons were checked by 1.5% agarose gel electrophoresis and compared with the 1 kb plus DNA Ladder (Invitrogen) to ensure that the correct size fragment was amplified.

Table 2.2. qPCR primers.

Transcript name (symbol)	Nucleotide sequence (5' - 3')[a]	Amp. effic. (%)[b]	Amplicon size (bp)	GenBank Acc.[c]
Elongation of very long chain fatty acids 2 (*elovl2*)[d]	**F:** GATGCCTGCTCTTCCAGTTC	99.0	113	FJ237532
	R: GCGACTGGACTTGATGGATT			
Elongation of very long chain fatty acids 5a (*elovl5a*)[e]	**F:** CAGTGTGGTGGGGACAAAG	99.7	115	AY170327
	R: TTCCCTCATGGACAAGCA			
Delta-5 fatty acyl desaturase (*fadsd5*)[d]	**F:** GTCTGGTTGTCCGTTCGTTT	96.5	135	AF478472
	R: GAGGCGATCAGCTTGAGAAA			
Delta-6 fatty acyl desaturase a (*fadsd6a*)[d]	**F:** CCCCAGACGTTTGTGTCAG	97.3	181	AY458652
	R: CCTGGATTGTTGCTTTGGAT			
Delta-6 fatty acyl desaturase b (*fadsd6b*)	**F:** TGACCATGTGGAGAGTGAGG	88.4	95	NM001172281
	R: CCAAAGCCAAGGCCTCTAGT			
Sterol regulatory element-binding protein 1 (*srebp1*)[d]	**F:** TCAACAAGTCGGCAATTCTG	93.6	100	HM561860
	R: GACATCTTCAGGGCCATGTT			
Sterol regulatory element-binding protein 2 (*srebp2*)[d]	**F:** GAGTGCTGAGGAAAGCCATC	91.7	129	HM561861
	R: TCTCCACATCGTCAGACAGC			
Peroxisome proliferator-activated receptor beta 1 (*pparβ1a*)[d]	**F:** AAGGAGGTCAACAACGCCTA	93.8	97	AJ416953
	R: ACTCTACTGGGCTGGAGCTG			
Peroxisome proliferator-activated receptor beta 2 (*pparβ2a*)	**F:** CCGTTTGTGATCCATGATGT	89.6	128	NM001123559
	R: GTGCACTGACAGCGGTAAAA			
Liver X receptor a *(lxra)*[d]	**F:** GGGCAAGATGGACAGATCAT	89.6	126	FJ470290
	R: CCTCACCAGGACCAACATCT			
Fatty acid binding protein 3 b (*fabp3b*)[g]	**F:** AACCCACCACCATCATTGAG	90.5	102	BT050105
	R: CGAACTCCTCTCCCAGTTTG			
Fatty acid binding protein 10 a (*fabp10a*)	**F:** ACATGGCAGGTGTATGCTCA	87.8	103	BG935057
	R: CAGTCACGGGCTTGATGTCT			
Fatty acid binding protein 10 b (*fabp10b*)	**F:** GATGGCAAAAAGATCAAGTGC	91.3	82	CA037797
	R: CCTGGACGCTGGAGAATTTA			
7-dehydrocholesterol reductase (*dhcr7*)[d]	**F:** CTTCTGGAATGAGGCATGGT	85.0	192	CK891712
	R: TAACCCACCAGACCCAAGAG			
isopentenyl-diphosphate delta isomerase 1 (*idi1*)[g]	**F:** GTCGGGATGCCTGAAATAAA	86.0	129	DY710563
	R: GAGGTGGCAGTTCTTTTTGC			
Bile acid receptor, alias: farnesoid X receptor (*bar*)[d]	**F:** GCCAAGAGGTAAGCATCTCG	88.0	120	GO063627
	R: TCAGGAGGTTCTGTGCAATG			
Fatty acid synthase a (*fasa*)	**F:** GGAGGGCACAATGGAGTAAA	91.6	136	DW563978
	R: TGAGACAGTGAATCGGATGG			
Fatty acid synthase b (*fasb*)	**F:** TGCCATACAAGTGATGTCCTG	104.8	105	EG872804
	R: AGTGGGCACCAAACATGAAC			
Cholesterol 7 alpha-hydroxylase b (*cyp7a1b*)[d]	**F:** CTGGCCGAGAACTTAAGCAA	94.4	94	CA042205
	R: TCAGGTCATTGAAGGTGGAC			

Arachidonate 5-lipoxygenase a (*5loxa*)[f]	**F:**CTGCTCACCATGCTGCTGTC **R:**GTGTGGGAGGAGGCTTCC	101.2	93	NM001139832
Arachidonate 5-lipoxygenase b (*5loxb*)[f]	**F:**ACTGCTGTGGGTTTCCCAAG **R:**GACAGCAGCGTGATGTGCAG	81.0	98	DW555519
Prostaglandin-D synthase (*pgds*, alias lipocalin-type *pgds*)[f]	**F:**GGTGCTCAACAAGCTCTACA **R:**GCAGGAAAGCGATGTTGTCA	86.8	114	BT048787
Cyclooxygenase-1 (*cox1*)[f]	**F:**CTCATGAGGGTGGTCCTCAC **R:**AGGCACAGGGGGTAGGATAC	91.0	135	BT045745
Elongation factor 1 alpha-2 (eef1α-2)[e]	**F:**GCACAGTAACACCGAAACGA **R:**ATGCCTCCGCACTTGTAGAT	86.4	132	BG933853
60S ribosomal protein 32 (*rpl32*)[d]	**F:**AGGCGGTTTAAGGGTCAGAT **R:**TCGAGCTCCTTGATGTTGTG	96.1	119	BT043656

[a] F is forward and R is reverse primer.

[b] Amplification efficiency (%).

[c] GenBank Accession number.

[d] Primers that were previously published in Hixson et al. (2017).

[e] Primers that were previously published in Xue et al. (2015).

[f] Primers that were previously published in Caballero-Solares et al. (2017).

[g] Primers that were designed in Caballero-Solares et al. (2018). Normalizer genes are underlined.

To select the most suitable normalizer genes for this study, six candidate normalizers were selected based on previous qPCR studies from the Rise lab (*rpl32*, *actb*, *eef1α-1*, *eef1α-2*, *abcf2*, *pabpc1*) (Xue et al. 2015; Caballero-Solares et al., 2017), and quality tested as mentioned above. Six fish per treatment (18 total) were randomly selected, and their C_T values were measured using cDNA corresponding to 5 ng of input total RNA. Then their expression stability was analysed using the geNorm algorithm (Vandesompele et al. 2002). *Rpl32* and eef1α-2 were shown to be the most stable (i.e. geNorm M-values of 0.30 and 0.25, respectively) across the 6 candidate genes (data not shown), and therefore were selected as normalizers. qPCR reactions, including no-template controls, were performed in technical triplicates using Power SYBR Green I dye chemistry in 384-well format with the ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). When a C_T value within a triplicate was greater than 0.5 cycles from the other two values, it was considered to be an outlier, discarded and the average C_T of the remaining two values was calculated.

All PCR amplifications were performed in a total volume of 13 µl and consisted of 4 µl of cDNA (5 ng input total RNA), 50 nM each of forward and reverse primer and 1× Power SYBR Green PCR Master Mix (Applied Biosystems). The Real-Time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 40 cycles (of 95°C for 15 s and 60°C for 1 min), with the fluorescence signal data collection after each 60°C step. The qPCR reaction setup, amplification and cycling were performed as in Xue et al. (2015). Finally, the relative quantity (RQ) of each transcript was calculated using ViiA 7 Software v1.2 (Applied Biosystems) for Comparative C_T (ΔΔCT) analysis (Livak and Schmittgen, 2001), with primer amplification efficiencies incorporated (Table

2.2). The expression levels of each GOI were normalized to both normalizers, and the sample with the lowest GOI expression was used as the calibrator sample (i.e. RQ = 1) for each GOI, as in Rise et al. (2015). Transcript expression data are presented as RQ values (mean ± SE) relative to the calibrator.

2.3.10. Statistical analysis

2.3.10.1. Growth performance

To examine the effect of diet on growth performance, a one-way ANOVA, with tank as a random factor nested within diet (Minitab 16 Statistical Software, State College, PA, USA) was used to determine significant differences between groups. Growth data were presented as mean ± standard deviation (SD).

2.3.10.2. Dietary and tissue composition

For analysis of lipid classes and FA composition in the diets, a one-way ANOVA followed by a Tukey pairwise comparison ($p \leq 0.05$) was used. In the tissue composition analyses (i.e. muscle and liver), a nested general linear model was used with tank as random factor, followed by a Tukey pairwise comparison ($p \leq 0.05$) to identify significant differences between tanks (i.e. to identify tank effect) and dietary treatments at week 12. Then, a one-way ANOVA followed by Tukey post-hoc tests were used to identify significant differences between week 0 and week 12. Finally, for CSIA data *t* tests were performed to detect differences in $\delta^{13}C$ values of each FA (comparing high ω3 to the high ω6 diet), and a one-way ANOVA followed by Tukey post-hoc tests were used to detect

differences between week 0 and week 12 in the high ω3 and high ω6 fed fish (Minitab 16 Statistical Software).

2.3.10.3. qPCR data

One-way ANOVA followed by Tukey pairwise comparison ($p \leq 0.05$) post-hoc tests were used to examine the effect of diet on the expression of each GOI (Minitab 16 Statistical Software). RQ data were presented as mean ± SE. Finally, each dietary treatment group was tested for outliers using Grubb's test ($p < 0.05$). Six samples in total were excluded from the study as they were outliers, and each GOI had a minimum of 6 samples per treatment (n = 6-8 per treatment).

In all analyses, the residuals were tested to verify normality, independence and homogeneity of variance. Normality was examined using the Anderson-Darling test. If the test failed ($p < 0.05$), a one-way ANOVA on ranks was performed and followed by Kruskal-Wallis / Dunn's multiple comparison tests (SigmaPlot,, Systat Software, Inc., Version 13, San Jose, CA, USA). In all cases, differences were considered statistically significant when $p \leq 0.05$.

2.3.10.4. Multivariate and correlation analyses

To relate hepatic qPCR transcript expression (RQ values), and dietary oil incorporation to tissue FA composition, Pearson correlation analyses were performed (Minitab 16 Statistical Software). Principal coordinates analysis (PCoA) was used to describe lipid and FA composition in the muscle and liver tissue (PRIMER, Plymouth Routines in Multivariate Ecological Research; PRIMER-E Ltd, version 6.1.15, Ivybridge,

UK). PCoA is a multidimensional statistical scaling method used to explore similarities in data sets. A correlation matrix was used, and the first two PCO axes (i.e. PCO1, PCO2) were plotted. Furthermore, SIMPER (Similarity of Percentages analysis) was used to quantify differences among treatments in lipid and FA data. This analysis calculates the contribution of variables to within group similarity and between group dissimilarity. PERMANOVA (Permutational Multivariate Analysis of Variance) was used to perform pairwise tests between treatments (9999 permutations). In all cases, the non-parametric Bray-Curtis similarity was used.

2.3.11. Fatty acid retention and turnover calculations

Fatty acid retention rates in the muscle and liver tissues were calculated for each fish using the following equations:

$$\%\ of\ original\ (no\ turnover) = \left[\frac{mg\ per\ liver\ at\ week\ 0}{mg\ per\ liver\ at\ week\ 12}\right] * 100$$

$$\%\ of\ original\ (24\%\ per\ month\ ) = \left[\frac{(mg\ per\ liver\ at\ week\ 0) * 0.76}{mg\ per\ liver\ at\ week\ 12}\right] * 100$$

Retention was set as 100% when the FA concentration (i.e. mg per liver) at week 12 was equal to or lower than the concentration at week 0. To calculate retention with turnover, a rate of 24% per month was applied for a period of 3 months.

2.4. Results

2.4.1. Diet composition

All experimental diets had the same levels of marine protein (26.7%), plant protein (44.6%), fish oil (4.8%) and other additives (2.6%) (Table 2.1) in order to investigate the impact of varying ω6:ω3 ratios on salmon metabolism. Furthermore, digestible energy (20.4 MJ Kg^{-1}), digestible protein (421 g Kg^{-1}), dry matter (94.8-95.0%) and ash (6.0-6.2%) were kept very similar among the five diets. The most abundant lipid classes were triacylglycerol (TAG) (76.9-85.4%), sterol (1.9-3.4%), phospholipid (2.6-3.3%) and acetone mobile polar lipid (AMPL) (0.4-6.4%) (Table 2.3). The high ω6 diet had the lowest levels, while the medium ω3 diet showed the highest levels of TAG. The high ω6 diet also had the lowest levels of sterols. The medium ω3 diet showed the lowest, while the high ω6 diet showed the highest levels of AMPL. No differences were observed among diets for phospholipids and total lipids ($p = 0.51$-0.94; Table 2.3). The fatty acid composition of the diets reflected the objectives of the formulations. Although some significant differences were observed among the 5 experimental diets, overall, they had similar levels of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) (except 18:1ω7).

Table 2.3. Lipid and FA composition (%) of experimental diets[a] fed to Atlantic salmon.

	High ω3	Med ω3	Balanced	Med ω6	High ω6
Lipid class composition (% of total lipid)					
TAG[b]	80.0	85.4	82.9	80.3	76.9
Sterol	3.1	3.4	3.4	3.0	1.9
AMPL[c]	4.5	0.4	1.6	4.4	6.4
Phospholipid	2.6	2.7	3.3	2.8	2.7
Total lipids (mg g^{-1} wet weight)	189	211	215	208	206
FA composition (% of total FAs)					
14:0	1.7	1.7	1.5	1.6	1.6
16:0	13.7	13.4	13.0	13.2	13.2
16:1ω7	1.7	1.7	1.7	1.7	1.7
18:0	2.9	2.9	3.0	3.1	3.0
18:1ω7	1.0	1.1	1.2	1.4	1.4
18:1ω9	20.1	19.4	19.6	20.9	19.4
18:2ω6 (LNA)	12.7	16.9	24.6	29.9	36.2
18:3ω3 (ALA)	29.8	26.2	18.9	11.5	6.4
20:1ω9	2.5	2.6	2.5	2.5	2.6
20:4ω6 (ARA)	0.1	0.1	0.1	0.1	0.1
20:5ω3 (EPA)	2.6	2.7	2.6	2.7	2.6
22:6ω3 (DHA)	3.2	3.3	3.2	3.2	3.2
Σ SFA[d]	19.1	18.8	18.5	18.9	19.0
Σ MUFA[e]	29.8	29.3	29.5	30.9	29.8
Σ PUFA[f]	50.9	51.7	51.9	49.9	51.0
Σ ω3	37.1	33.7	26.2	18.9	13.7
Σ ω6	13.0	17.2	24.9	30.2	36.5
ω6/ω3	0.4	0.5	0.9	1.6	2.7
DHA/EPA	1.3	1.2	1.2	1.2	1.3
EPA/ARA	20.7	20.4	22.9	18.3	20.5
EPA+DHA (% of wet weight)[g]	1.0	1.2	1.1	1.1	1.3

[a] Mean (n=3). [b] Triacylglycerol. [c] Acetone mobile polar lipid. [d] Total saturated fatty acids. [e] Total monounsaturated fatty acids. [f] Total polyunsaturated fatty acids. [g] EPA+DHA analysed as % of dietary wet weight. Fatty acids that showed the largest range among diets are bolded.

Similarly, diets had similar levels of 20:4ω6, 20:5ω3, 22:6ω3 and the sums of SFA and polyunsaturated fatty acids (PUFA). A large range was observed across diets for 18:2ω6 (from 12.7 to 36.2%), 18:3ω3 (from 6.4 to 29.8%) and the sums of ω3 and ω6 fatty acids (13.7-37.1% and 13-36.5%, respectively). Finally, the ω6:ω3 ratio ranged from 0.4 to 2.7.

2.4.2. Growth performance

Experimental diets did not affect any of the growth parameters [e.g. final weight, final length, condition factor (CF), weight gain and specific growth rate (SGR),] or organ indices (i.e. HSI and VSI) after 12 weeks of feeding (p = 0.30-0.95). Mean weight gain ranged from 344 to 368 g $fish^{-1}$. Furthermore, apparent feed intake was not significantly different among the dietary treatments (Table 2.4).

2.4.3. Tissue composition

2.4.3.1. Muscle

Lipid classes were mainly TAG (68.4-80.9%), phospholipid (11.1-22%) and sterol (4.1-6.1%). The high ω6 fed salmon had lower levels of TAG compared to fish fed the balanced diet, while phospholipid content was higher in the high ω6 compared to the balanced and medium ω3 fish (p = 0.04 and 0.003, respectively; Table 2.5). Finally, no differences were observed among dietary treatments in sterols, AMPL and total lipid contents (p = 0.07-0.35).

Muscle had similar levels of C_{14} – C_{16} fatty acids, 18:0 and 18:1ω9 among the five dietary treatments (p = 0.08-0.7), and reflected the dietary composition. Furthermore, the sums of SFA, MUFA and PUFA as well as DHA/EPA ratio were also not different among

Table 2.4. Growth performance of Atlantic salmon fed plant-based diets with varying ratios of ω6 to ω3 fatty acids for 12 weeks[a].

Growth parameter	High ω3	Med ω3	Balanced	Med ω6	High ω6
Initial weight (g)	211 ± 26	210 ± 25	210 ± 26	211 ± 26	210 ± 25
Final weight (g)	565 ± 101	564 ± 104	565 ± 102	563 ± 103	565 ± 103
Weight gain (g)[b]	344 ± 99	368 ± 94	350 ± 84	359 ± 90	354 ± 87
Initial length (cm)	27.0 ± 1.1	26.8 ± 1.2	26.8 ± 1.2	27.1 ± 1.1	26.9 ± 1.2
Final length (cm)	35.8 ± 1.8	35.4 ± 1.8	35.5 ± 1.7	35.6 ± 2.0	35.3 ± 2.0
SGR (% day^{-1})[c]	1.16 ± 0.2	1.16 ± 0.2	1.17 ± 0.2	1.16 ± 0.2	1.17 ± 0.2
CF[d]	1.22 ± 0.1	1.21 ± 0.1	1.21 ± 0.1	1.20 ± 0.1	1.18 ± 0.1
VSI (%)[e]	9.81 ± 2.4	9.80 ± 1.8	9.82 ± 2.5	9.90 ± 2.5	9.80 ± 1.8
HSI (%)[f]	1.20 ± 1.0	1.31 ± 1.2	1.22 ± 1.0	1.11 ± 0.3	1.24 ± 1.1
AFI (g $fish^{-1}$)[g]	273 ± 11	276 ± 11	275 ± 17	282 ± 12	280 ± 11

[a] Means ± standard deviation (n = 136). No significant dietary effects (p = 0.30-0.95).

[b] Weight gain (g $fish^{-1}$) = Final weight − initial weight.

[c] Specific growth rate (% day^{-1}) = 100 × [ln (final body weight) − ln (initial body weight)] / days

[d] Condition factor = weight / $length^3$.

[e] Viscero-somatic index (%) = 100 ∗ (viscera weight / body weight).

[f] Hepato-somatic index (%) = 100 ∗ (liver weight / body weight).

[g] Apparent feed intake (g $fish^{-1}$) = (Feed consumed, g) / (number of fish per tank).

Table 2.5. Lipid and FA composition (%) of Atlantic salmon muscle[a] before (initial) and after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids.

	Initial	High ω3	Med ω3	Balanced	Med ω6	High ω6
Lipid class composition (% of total lipid)						
TAG[b]	58.3 ± 23.0	73.6 ± 13.1[ab]	74.5 ± 14.3[ab]	80.9 ± 7.9[a]	70.5 ± 12.8[ab]	68.4 ± 14.3[b]
Sterol	1.6 ± 1.0	5.5 ± 2.7	5.9 ± 4.3	4.1 ± 3.0	6.1 ± 4.5	5.4 ± 2.9
AMPL[c]	1.6 ± 2.3	0.6 ± 1.0	1.5 ± 5.5	0.3 ± 0.7	0.7 ± 1.0	0.9 ± 1.2
Phospholipid	33.6 ± 23.0	14.6 ± 9.6[ab]	13.2 ± 6.5[b]	11.1 ± 5.8[b]	19.3 ± 8.3[ab]	22.0 ± 13.9[a]
Total lipids (mg g^{-1} wet weight)	20.9 ± 11.6	34.5 ± 15.2	28.9 ± 13.0	24.8 ± 15.7	29.5 ± 19.3	42.6 ± 30.3
FA composition (% of total FAs)						
14:0	2.5 ± 0.7	1.3 ± 0.2	1.3 ± 0.3	1.3 ± 0.2	1.1 ± 0.3	1.3 ± 0.3
16:0	15.4 ± 1.3	13.6 ± 1.0	13.5 ± 0.8	13.7 ± 0.9	13.9 ± 1.1	13.9 ± 1.0
16:1ω7	4.7 ± 1.1	1.8 ± 0.2	1.8 ± 0.3	1.8 ± 0.2	1.7 ± 0.3	1.9 ± 0.4
18:0	4.1 ± 0.5	3.4 ± 0.3	3.4 ± 0.3	3.5 ± 0.5	3.5 ± 0.2	3.6 ± 0.2
18:1ω9	23.1 ± 3.6	20.0 ± 2.0	19.5 ± 2.2	19.6 ± 1.5	19.4 ± 1.5	19.2 ± 1.5
18:1ω7	2.7 ± 0.2	1.5 ± 0.1[a]	1.5 ± 0.1[a]	1.7 ± 0.1[b]	1.8 ± 0.1[c]	1.9 ± 0.1[d]
18:2ω6 (LNA)	8.9 ± 1.2	10.8 ± 0.8[a]	13.9 ± 1.3[b]	19.6 ± 1.3[c]	23.3 ± 2.1[d]	27.9 ± 2.1[e]
18:3ω3 (ALA)	1.0 ± 0.1	20.0 ± 1.7[a]	17.6 ± 1.9[b]	12.4 ± 1.2[c]	7.5 ± 0.8[d]	3.3 ± 0.3[e]
18:4ω3	0.8 ± 0.2	2.5 ± 0.7[a]	2.3 ± 0.5[ab]	1.9 ± 0.3[b]	1.5 ± 0.3[c]	1.0 ± 0.1[d]
20:1ω9	3.3 ± 0.8	2.4 ± 0.4	2.4 ± 0.3	2.4 ± 0.3	2.3 ± 0.3	2.5 ± 0.3
20:2ω6	0.5 ± 0.1	0.6 ± 0.1[a]	0.7 ± 0.1[a]	1.0 ± 0.1[b]	1.1 ± 0.2[b]	1.5 ± 0.6[c]
20:3ω6	0.7 ± 0.1	0.3 ± 0.1[a]	0.4 ± 0.1[a]	0.6 ± 0.1[b]	0.8 ± 0.2[c]	1.0 ± 0.1[d]
20:4ω6 (ARA)	1.4 ± 0.4	0.4 ± 0.1[a]	0.4 ± 0.2[ab]	0.4 ± 0.1[ab]	0.6 ± 0.2[bc]	0.6 ± 0.3[c]
20:4ω3	0.5 ± 0.03	1.5 ± 0.2[a]	1.3 ± 0.2[a]	1.1 ± 0.1[b]	0.8 ± 0.1[c]	0.5 ± 0.1[d]
20:5ω3 (EPA)	4.0 ± 1.1	2.7 ± 0.8	2.7 ± 0.8	2.6 ± 0.5	2.7 ± 0.5	2.3 ± 0.5

22:6ω3 (DHA)	15.7 ± 6.4	9.8 ± 3.5	9.7 ± 4.2	9.2 ± 1.7	10.6 ± 3.6	9.5 ± 3.1
Σ SFA[d]	22.9 ± 1.2	18.8 ± 1.0	18.8 ± 1.0	19.0 ± 1.4	19.2 ± 1.3	19.6 ± 1.1
Σ MUFA[e]	39.8 ± 6.9	29.4 ± 3.2	29.1 ± 3.2	29.4 ± 2.7	29.0 ± 2.3	29.8 ± 2.7
Σ PUFA[f]	37.0 ± 6.3	51.6 ± 2.6	52.0 ± 3.0	51.4 ± 3.8	51.6 ± 2.0	50.4 ± 2.4
Σ ω3	23.5 ± 7.3	38.5 ± 3.2[a]	35.4 ± 3.7[a]	28.6 ± 2.9[b]	24.3 ± 3.5[c]	17.5 ± 3.3[d]
Σ ω6	12.4 ± 0.9	12.4 ± 0.7[a]	15.9 ± 1.1[b]	22.1 ± 1.4[c]	26.5 ± 1.9[d]	32.2 ± 2.4[e]
ω6/ω3	0.6 ± 0.2	0.3 ± 0.04[a]	0.5 ± 0.1[a]	0.8 ± 0.1[b]	1.1 ± 0.2[c]	1.9 ± 0.5[d]
DHA/EPA	3.9 ± 0.7	3.5 ± 0.5	3.5 ± 0.6	3.6 ± 0.5	3.9 ± 0.6	4.1 ± 0.8
EPA/ARA	3.0 ± 0.4	7.3 ± 1.3[a]	6.7 ± 1.1[a]	6.3 ± 1.4[a]	4.8 ± 0.7[b]	4.1 ± 1.4[b]

[a] Mean (n=10-20) ± standard deviation. Different superscripts in the same row indicate significant differences among treatments at week 12. Underlines represent values that are significantly different to week 0 (initial) ($p \leq 0.05$). [b] Triacylglycerol. [c] Acetone mobile polar lipid. [d] Total saturated fatty acids. [e] Total monounsaturated fatty acids. [f] Total polyunsaturated fatty acids.

treatments (p = 0.23-0.93). However, all treatments were different from each other in the levels of both precursors 18:2ω6 (LNA) and 18:3ω3 (ALA) ($p < 0.001$). LNA was ~ 2.5-fold higher in the high ω6 compared to the high ω3 fish, while for ALA the opposite was observed, with high ω3 being ~ 6-fold higher than the high ω6 fish. The intermediate ω3 and ω6 PUFA (i.e. 18:4ω3, 20:2ω6, 20:3ω6 and 20:4ω3) were also different among dietary treatments ($p < 0.001$), following dietary LNA and ALA composition. There were no differences in the contents of 20:5ω3 (EPA) and 22:6ω3 (DHA) among treatments (p = 0.27 and 0.82, respectively). However, it was evident that DHA levels in the muscle were ~ 3-fold higher than the dietary levels. Furthermore, ARA, EPA and DHA decreased after 12 weeks of feeding (as compared with week 0), while the opposite was true for the sum of PUFA ($p < 0.001$; Table 2.5). Finally, the ω6:ω3 ratio was different among treatments and ranged from 0.3 to 1.9 ($p < 0.001$).

Principal coordinates analysis (PCoA) showed that the ω6:ω3 treatments formed distinct, separated clusters (i.e. from left to right: high ω6, medium ω6, balanced, medium ω3, and high ω3) and indicated that their muscle lipid and FA profiles were different (Figure 2.1). PERMANOVA pairwise tests demonstrated that all treatments were significantly different from each other [p(perm) = 0.0001-0.0262)]. The FA vectors showed that the high ω3 fed fish largely differed in respect to proportions of 18:3ω3, 18:4ω3, 20:4ω3 and the sum of ω3, while the high ω6 fish differed in 18:1ω7, 18:2ω6, 20:3ω6 and the sum of ω6 (Figure 2.1). Furthermore, PCO1 and PCO2 accounted for 50.1% and 43.5% of the variability, respectively. Interestingly, the sum of PUFA, EPA, DHA and the lipid class sterol were grouped together and showed a positive association with phospholipid and ARA, while these were negatively associated with MUFA and TAG.

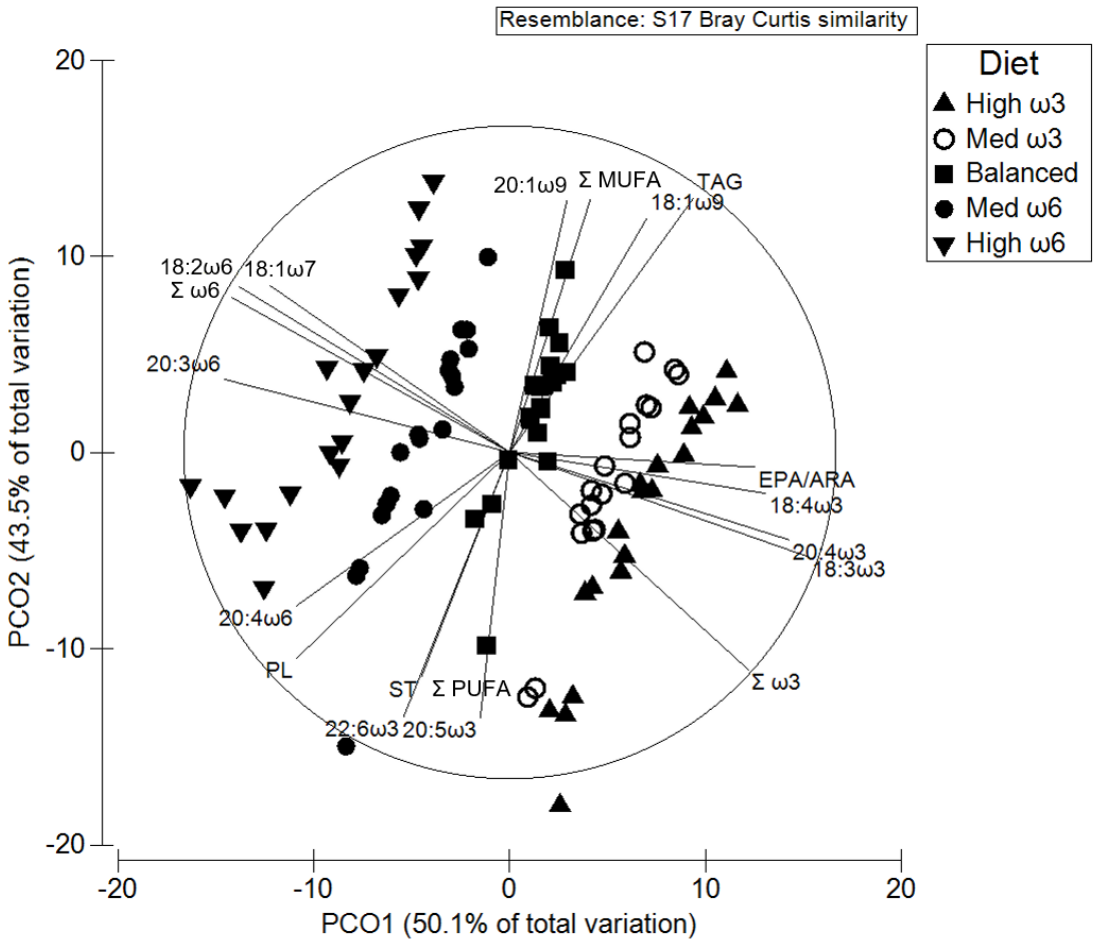

Figure 2.1. Principal coordinates analysis (PCoA) of lipid and FA composition (%) ($r^2 >$ 0.70) in the muscle of Atlantic salmon after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids. PL and ST represent the lipid classes phospholipid and sterol, respectively.

SIMPER analysis revealed that lipid classes and sum ω3 and sum ω6 were the main drivers of the FA difference among treatments. Sum ω3 and sum ω6 contributed 7.5-15.7% and 6.3-14.8% to the dissimilarity, respectively, while TAG and phospholipid were also major contributors (13.3-24.0% and 10.8-17.5% dissimilarity, respectively; Table S.2.1).

2.4.3.2. Liver

The predominant lipid class in the liver was phospholipid (47.7-59.9%), followed by sterol (19.8-25.4%), TAG (10.9-15.7%) and AMPL (1.9-6.4%). The high ω3 fed fish showed higher AMPL levels compared to the medium ω3 fish ($p = 0.003$), while no differences were shown for the other lipid classes or total lipids ($p = 0.17$-0.66). All treatments showed a decrease in TAG from week 0 to week 12 ($p = 0.001$; Table 2.6).

Liver FA composition was also very reflective of the diet and responded to changes in ω3 and ω6 PUFA. ALA and LNA were different among treatments ($p < 0.001$). While LNA showed a ~ 2.3-fold increase in the high ω6 compared to the high ω3 fish, the levels of ALA were 8.4-fold higher in the high ω3 compared to the high ω6 fed fish. The intermediate ω3 (20:3ω3 and 20:4ω3) and ω6 (20:2ω6 and 20:3ω6) PUFA were also different among dietary treatments ($p < 0.001$). ARA proportions were similar between the high ω3 and medium ω3, and between medium ω3 and the balanced treatment. These three treatments had lower ARA compared to the medium and high ω6 fish ($p < 0.001$). Furthermore, fish had 4.1% ARA at week 0 which was a result of the commercial diet; after 12 weeks of feeding the experimental diets, the levels of ARA were ~ 2.5-fold higher in the high ω6 compared to the high ω3 fish (Table 2.6). EPA proportions were similar among the high ω3, medium ω3 and the balanced treatments, and these groups had higher level.

Table 2.6. Lipid and FA composition (%) of Atlantic salmon liver[a] before (initial) and after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids.

	Initial	High ω3	Med ω3	Balanced	Med ω6	High ω6
Lipid class composition (% of total lipid)						
TAG[b]	28.9 ± 8.5	13.9 ± 8.1	10.9 ± 6.4	13.7 ± 7.5	15.7 ± 10.6	14.7 ± 8.2
Sterol	22.9 ± 7.7	23.5 ± 10.1	25.1 ± 13.5	19.8 ± 9.9	21.4 ± 11.5	25.4 ± 11.8
AMPL[c]	0.1 ± 0.1	6.4 ± 8.1^{a}	1.9 ± 2.4^{b}	2.9 ± 3.5^{ab}	2.1 ± 2.9^{ab}	6.0 ± 4.0^{ab}
Phospholipid	48.0 ± 15.5	49.4 ± 15.2	56.9 ± 18.7	59.9 ± 13.8	56.1 ± 19.1	47.7 ± 15.1
Total lipids (mg g^{-1} wet weight)	32.9 ± 11.3	37.8 ± 14.3	29.8 ± 8.5	31.9 ± 10.2	37.9 ± 12.9	33.0 ± 9.3
FA composition (% of total FAs)						
14:0	1.2 ± 0.3	0.8 ± 0.1^{a}	0.6 ± 0.1^{b}	0.7 ± 0.1^{b}	0.7 ± 0.1^{b}	0.7 ± 0.1^{ab}
16:0	15.4 ± 2.5	15.5 ± 2.2	14.7 ± 1.5	15.1 ± 1.3	15.0 ± 1.7	15.9 ± 0.9
16:1ω7	2.7 ± 0.5	1.1 ± 0.2^{a}	0.9 ± 0.2^{b}	0.8 ± 0.2^{bc}	0.9 ± 0.1^{bc}	0.8 ± 0.1^{c}
18:0	5.8 ± 0.5	5.5 ± 0.4^{a}	5.4 ± 0.5^{a}	5.4 ± 0.5^{a}	5.5 ± 0.5^{a}	6.3 ± 0.8^{b}
18:1ω9	20.1 ± 5.9	15.3 ± 3.3^{a}	13.3 ± 2.8^{ab}	11.8 ± 1.8^{b}	12.4 ± 2.5^{b}	11.6 ± 1.4^{b}
18:1ω7	2.4 ± 0.4	1.2 ± 0.3^{ab}	1.1 ± 0.2^{b}	1.1 ± 0.1^{b}	1.2 ± 0.2^{ab}	1.3 ± 0.1^{a}
18:2ω6 (LNA)	7.0 ± 1.1	6.8 ± 0.6^{a}	7.9 ± 0.7^{b}	11 ± 1.3^{c}	13.3 ± 1.5^{d}	15.5 ± 1.3^{e}
18:3ω3 (ALA)	0.5 ± 0.1	8.4 ± 1.2^{a}	6.6 ± 0.9^{b}	4.7 ± 0.8^{c}	2.7 ± 0.5^{d}	1.0 ± 0.2^{e}
20:1ω9	1.7 ± 0.6	1.9 ± 0.6^{a}	1.6 ± 0.6^{ab}	1.5 ± 0.4^{ab}	1.6 ± 0.5^{ab}	1.4 ± 0.4^{b}
20:2ω6	0.5 ± 0.2	1.2 ± 0.3^{a}	1.5 ± 0.4^{ab}	2.0 ± 0.5^{b}	2.6 ± 0.6^{c}	3.0 ± 0.9^{c}
20:3ω6	1.5 ± 0.7	1.0 ± 0.2^{a}	1.4 ± 0.2^{b}	1.9 ± 0.3^{c}	2.5 ± 0.7^{d}	3.5 ± 0.4^{e}
20:4ω6 (ARA)	4.1 ± 0.8	1.7 ± 0.2^{a}	2.1 ± 0.3^{ab}	2.4 ± 0.4^{b}	3.0 ± 0.5^{c}	4.2 ± 1.0^{d}
20:3ω3	0.03 ± 0.02	1.6 ± 0.6^{a}	1.2 ± 0.3^{b}	0.8 ± 0.2^{c}	0.5 ± 0.2^{c}	0.2 ± 0.1^{d}
20:4ω3	0.4 ± 0.1	2.4 ± 0.4^{a}	2.1 ± 0.3^{b}	1.7 ± 0.2^{c}	1.1 ± 0.2^{d}	0.5 ± 0.1^{e}

20:5ω3 (EPA)	4.7 ± 1.3	5.9 ± 1.0[a]	6.4 ± 1.1[a]	5.9 ± 0.9[a]	4.9 ± 0.8[b]	3.9 ± 0.5[c]
22:5ω3	1.7 ± 0.5	1.4 ± 0.3[ab]	1.5 ± 0.2[a]	1.4 ± 0.3[ab]	1.2 ± 0.2[bc]	1.0 ± 0.2[c]
22:6ω3 (DHA)	24.0 ± 5.9	24.7 ± 3.3[a]	27.5 ± 3.2[b]	27.5 ± 2.1[b]	26.8 ± 3.3[ab]	25.4 ± 1.9[ab]
Σ SFA[d]	23.0 ± 2.2	22.4 ± 2.3[ab]	21.2 ± 1.5[b]	21.7 ± 1.4[b]	21.6 ± 1.9[b]	23.6 ± 1.4[a]
Σ MUFA[e]	30.0 ± 7.6	21.3 ± 4.5[a]	19.0 ± 3.9[ab]	17.3 ± 2.4[b]	18.2 ± 3.4[b]	16.8 ± 1.9[b]
Σ PUFA[f]	46.8 ± 6.5	56.6 ± 3.3[a]	59.7 ± 2.6[b]	60.9 ± 1.3[b]	60.1 ± 2.2[b]	59.5 ± 1.6[b]
Σ ω3	31.9 ± 7.4	45.1 ± 2.9[a]	45.9 ± 3.0[a]	42.6 ± 2.0[b]	37.6 ± 3.4[c]	32.3 ± 2.0[d]
Σ ω6	14.1 ± 1.9	10.9 ± 0.6[a]	13.3 ± 0.6[b]	17.7 ± 0.9[c]	22.0 ± 1.6[d]	26.9 ± 1.1[e]
ω6/ω3	0.5 ± 0.2	0.2 ± 0.03[a]	0.3 ± 0.03[a]	0.4 ± 0.04[b]	0.6 ± 0.1[c]	0.8 ± 0.1[d]
DHA/EPA	5.2 ± 1.1	4.3 ± 0.7[a]	4.4 ± 0.8[a]	4.7 ± 0.7[a]	5.6 ± 0.8[b]	6.6 ± 0.9[c]
EPA/ARA	1.2 ± 0.2	3.6 ± 0.6[a]	3.1 ± 0.5[b]	2.5 ± 0.2[c]	1.6 ± 0.2[d]	1.0 ± 0.3[e]

[a] Mean (n=8-20) ± standard deviation. Different superscripts in the same row indicate significant differences between treatments at week 12. Underlines represent values that are significantly different to week 0 (initial; $p \leq 0.05$); [b] Triacylglycerol; [c] Acetone mobile polar lipid; [d] Total saturated fatty acids; [e] Total monounsaturated fatty acids; [f] Total polyunsaturated fatty acids.

compared to medium and high ω6 fish ($p < 0.001$). The latter treatment had the lowest EPA levels (3.9%). DHA proportions were lower in the high ω3 compared to the medium ω3 and balanced fed fish ($p = 0.006$). 18:1ω9 and the sum of MUFA were higher in the high ω3 compared to the balanced, medium and high ω6 treatments ($p < 0.001$), and 20:1ω9 was greater in the high ω3 compared to the high ω6 fed fish ($p = 0.048$). The sum of ω3 showed an increase between the initial time point to week 12 in all treatments (from 31.9 to 37.6-45.9%; $p < 0.001$) apart from the high ω6 fish, while sum ω6 was higher in the balanced, medium, and high ω6 fish at week 12 compared to week 0 (17.7-26.9% *vs* 14.1%; $p < 0.001$). Additionally, all treatments were different from one another in sum ω6, while for sum ω3, the high and medium ω3 were different from the other three treatments ($p < 0.001$). Finally, the ratios of ω6:ω3 and EPA/ARA were different among treatments ($p < 0.001$) and ranged from 0.2 to 0.8 and 1.0 to 3.6, respectively.

PCoA of lipid and FA composition in the liver (Figure 2.2) separated the dietary treatments. The high ω3 fed fish largely differed in respect to proportions of 18:3ω3, 20:4ω3, EPA/ARA and the sum of ω3, while the high ω6 fish differed in 18:2ω6, 20:3ω6, ARA and the sum of ω6 (Figure 2.2). Furthermore, PCO1 and PCO2 accounted for 36.8% and 33.5% of the variability, respectively. PERMANOVA pairwise tests demonstrated that all treatments were significantly different from each other [p(perm) = 0.0001-0.013)]. Similar to the muscle, the sum of PUFA and DHA grouped together and showed negative associations with MUFA and TAG. However, the latter was grouped with sterol.

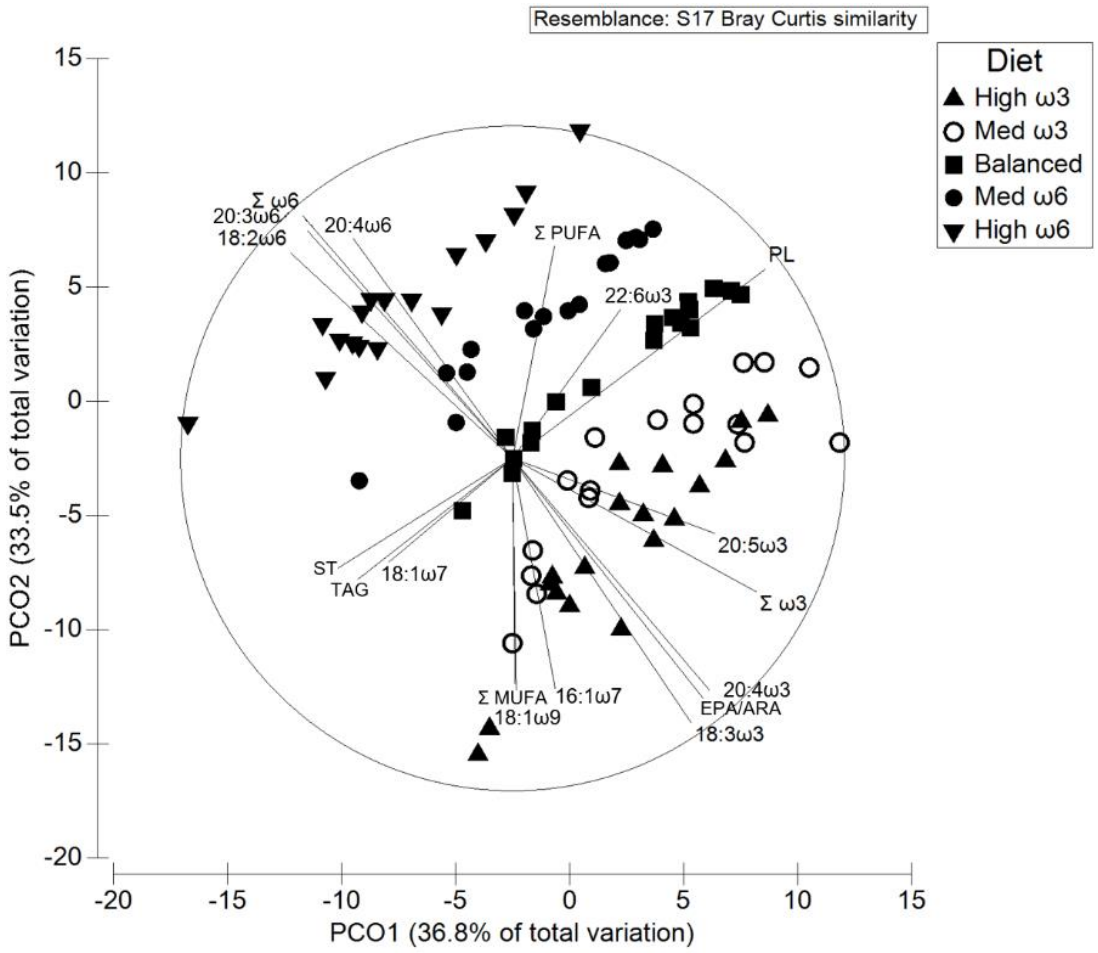

Figure 2.2. Principal coordinates analysis (PCoA) of lipid and FA composition (%) ($r^2 > 0.55$) in the liver of Atlantic salmon after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids. PL and ST represent the lipid classes phospholipid and sterol, respectively.

Finally, as shown in the muscle, SIMPER analysis identified lipid classes and sum ω3 and sum ω6 as the main drivers of the FA difference among treatments. Sum ω3 and sum ω6 contributed 7.3-11.4% and 7.7-13.1% to the dissimilarity, respectively, and phospholipid and sterol were also major contributors (13.0-25.4% and 9.7-18.0% dissimilarity, respectively; Table S.2.2).

2.4.4. Hepatic qPCR analysis

2.4.4.1. Genes related to LC-PUFA synthesis and FA metabolism

The transcript expression of *srebp1* was lower in the high ω6 compared to the balanced fed fish (0.53-fold; p = 0.047), while *lxra* was lower in the high ω6 compared to the balanced and high ω3 fed fish (0.83 and 0.85-fold, respectively; p = 0.01) (Table 2.7). *Elovl2* and *elovl5a* appeared to increase (although not significant) in the high ω6 compared to the other two treatments (~1.34-1.40 and 1.36-1.40-fold; p = 0.08 and 0.27, respectively), while *pparβ1a* transcript expression was 1.17-fold higher in the high ω3 compared to the high ω6 fish (p = 0.08). Furthermore, the transcript *fas* paralog b (*fasb*) showed 1.48-fold higher expression in the high ω3 compared with the other 2 treatments (p = 0.08). Interestingly, the fatty acid binding protein-encoding transcripts examined (i.e. *fabp3b*, *fabp10a* and *fabp10b*) showed increasing trends (although not significant) in the high ω3 compared to the other 2 treatments (2.78, 1.28 and ~1.3-fold, respectively; p = 0.06-0.14). Notably, no significant differences or trends were observed in the expression levels of *fadsd5* and *fadsd6* (both paralogs; p = 0.31-0.97).

Table 2.7. Relative transcript expression of genes related to lipid metabolism and eicosanoid synthesis in the liver of Atlantic salmon after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids.

Gene[a]	High ω3	Balanced	High ω6	p-value[b]
elovl2	2.02 ± 0.10	1.94 ± 0.26	2.70 ± 0.34	0.08
elovl5a	2.09 ± 0.25	2.14 ± 0.31	2.92 ± 0.57	0.27
fadsd5	2.33 ± 0.12	2.27 ± 0.35	2.36 ± 0.32	0.97
fadsd6a	1.85 ± 0.14	2.06 ± 0.28	1.57 ± 0.15	0.31
fadsd6b	1.81 ± 0.13	1.86 ± 0.27	1.91 ± 0.26	0.96
fabp10a	1.89 ± 0.14	1.48 ± 0.11	1.47 ± 0.15	0.06
fabp10b	2.90 ± 0.33	2.07 ± 0.30	2.35 ± 0.22	0.14
fabp3b	4.31 ± 1.55	1.55 ± 0.13	1.54 ± 0.25	0.11
fasa	4.28 ± 0.58	3.06 ± 0.65	4.61 ± 0.87	0.28
fasb	2.91 ± 0.41	1.96 ± 0.30	1.97 ± 0.19	0.08
lxra	1.38 ± 0.06[a]	1.43 ± 0.04[a]	1.18 ± 0.05[b]	**0.01**
pparβ1a	1.55 ± 0.09	1.37 ± 0.06	1.32 ± 0.06	0.08
pparβ2a	1.83 ± 0.21	2.38 ± 0.46	1.64 ± 0.08	0.27
srebp1	2.53 ± 0.25[ab]	2.72 ± 0.47[a]	1.44 ± 0.13[b]	**0.05**
srebp2	1.81 ± 0.32	1.74 ± 0.15	1.73 ± 0.16	0.97
bar	1.88 ± 0.09[a]	1.38 ± 0.09[b]	1.80 ± 0.13[a]	**0.01**
cyp7a1b	7.45 ± 1.70	7.08 ± 0.76	4.50 ± 1.11	0.21
dhcr7	2.10 ± 0.24	2.30 ± 0.34	2.98 ± 0.48	0.23
idi1	4.10 ± 1.14	4.16 ± 0.79	3.48 ± 0.15	0.85
cox1	3.29 ± 0.63	2.94 ± 0.47	2.39 ± 0.59	0.55
5loxa	3.79 ± 0.69[a]	7.35 ± 0.94[b]	3.88 ± 0.97[a]	**0.01**
5loxb	2.57 ± 0.41	2.86 ± 0.46	2.34 ± 0.34	0.68
pgds	2.10 ± 0.50	2.62 ± 0.34	3.39 ± 0.47	0.16

[a] Gene expression data presented as mean relative quantity (RQ) ± standard error (n = 6-8). RQ values were normalized to elongation factor 1 alpha-2 (*eef1α-2)* and 60S ribosomal protein 32 (*rpl32*), and calibrated to the lowest expressing individual for each gene of interest.
[b] Different letters indicate significant differences between treatments (One-way ANOVA followed by Tukey pairwise comparison; $p \leq 0.05$).

2.4.4.2. Genes related to cholesterol and bile acid metabolism

The transcript expression of *bar* was lower in the balanced as compared with the high ω3 and high ω6 fish (0.73 and 0.77-fold, respectively; p = 0.01) (Table 2.7). No significant differences were shown in the mRNA levels of *cyp7a1b*, when comparing the high ω6 to the balanced and high ω3 fish (0.64 and 0.60-fold, respectively; p = 0.21). Furthermore, no dietary effect was observed in the expression level of *dhcr7, srebp2* and *idi1* (p = 0.23-0.97).

2.4.4.3. Genes related to eicosanoid synthesis

5loxa was higher in the balanced as compared with the high ω3 and high ω6 fish (1.94 and 1.89-fold, respectively; p = 0.01), while no dietary effect was shown in the expression level of *5loxb* (p = 0.68; Table 2.7). mRNA levels of the other two eicosanoid synthesis relevant transcripts (*pgds* and *cox1*) were also not significantly altered by diet (p = 0.16-0.55); however, *pgds* showed a gradual increase from high ω3 to high ω6 (1.61-fold), while the latter transcript showed the opposite trend (1.38-fold).

2.4.5. Correlations between liver transcript expression and fatty acid composition

Although most of the transcripts examined in this study were not significantly altered by diet (Table 2.7), Pearson correlation identified multiple significant correlations between FA composition and relative transcript expression (RQ values) in the liver (Table 2.8).

Table 2.8. Significant Pearson correlations[a] ($p \leq 0.05$) between hepatic transcript expression (RQ values) of genes involved in lipid metabolism and eicosanoid synthesis with liver fatty acid composition (% of total FAs).

Transcript	FA	Pearson R	p-Value
elovl2	20:2ω6	0.484	0.026
	20:5ω3 (EPA)	-0.565	0.008
	Σ ω3	-0.511	0.018
	22:5ω3	-0.438	0.047
elovl5a	20:2ω6	0.671	0.001
	20:5ω3 (EPA)	-0.590	0.005
	Σ ω3	-0.513	0.017
	22:5ω3	-0.502	0.020
lxra	18:1ω9	0.517	0.016
	20:1ω9	0.512	0.018
	Σ MUFA	0.537	0.012
	18:3ω3 (ALA)	0.528	0.014
	20:3ω3	0.570	0.007
	Σ SFA	-0.534	0.013
	20:3ω6	-0.458	0.037
	20:4ω6 (ARA)	-0.512	0.018
	22:6ω3 (DHA)	-0.446	0.043
fasb	20:3ω3	0.459	0.037
	20:3ω6	0.458	0.037
bar	20:1ω9	0.458	0.037
	Σ MUFA	0.627	0.002
	20:4ω6 (ARA)	0.628	0.002
	22:5ω3	0.596	0.004
	Σ SFA	-0.638	0.002
	Σ PUFA	-0.636	0.002
	20:5ω3 (EPA)	-0.603	0.004
cyp7a1b	20:2ω6	-0.479	0.033
pgds	Σ MUFA	0.437	0.047
	20:1ω9	0.505	0.019
	22:6ω3 (DHA)	-0.668	0.001
	EPA+ARA+DHA	-0.594	0.005
cox1	20:1ω9	0.467	0.033
	Σ PUFA	-0.432	0.050

[a] Pearson correlations were calculated using individual fish from all dietary treatments (n = 6-8).

Elovl2 and *elovl5a* were negatively correlated with EPA, the sum of ω3 FA and 22:5ω3 (p = 0.005-0.047), and positively correlated with 20:2ω6 (p = 0.026 and 0.001, respectively). These transcripts also showed positive association with ω6/ω3 (p = 0.064 and 0.051, respectively; Table S.2.3). *Lxra* was negatively correlated with the sum of SFA, 20:3ω6, ARA, and DHA (p = 0.013-0.043), and showed positive correlations with the sum of MUFA, 18:1ω9, 20:1ω9, ALA and 20:3ω3 (p = 0.007-0.018). Furthermore, *fasb* was positively correlated with the intermediate PUFA 20:3ω3 and 20:3ω6 (p = 0.037). *Bar* was negatively correlated with the sum of SFA, the sum of PUFA, and EPA (p = 0.002-0.004), and positively correlated with the sum of MUFA, 20:1ω9, ARA, and 22:5ω3 (p = 0.002-0.037), while *cyp7a1b* showed negative correlation with 20:2ω6 (p = 0.033). Finally, *pgds* was negatively correlated with DHA and EPA+ARA+DHA (p = 0.001-0.005), and showed positive correlation with the sum of MUFA and 20:1ω9 (p = 0.019-0.047), while *cox1* was correlated negatively with the sum of PUFA (p = 0.050) and positively with 20:1ω9 (p = 0.033).

2.4.6. CSIA of fish fed the two extreme ω6:ω3 diets

The $\delta^{13}C$ values of the diets (high ω6 *vs* high ω3) were only different from each other in respect to the precursors 18:2ω6 and 18:3ω3 (p = 0.028 and 0.050, respectively; Table 2.9). The proportions of 20:4ω6 in both diets were extremely low (Table 2.3) and therefore their $\delta^{13}C$ values could not be measured. Liver $\delta^{13}C$ values of fish fed the two extreme diets were only different for 18:2ω6, 20:4ω6 and 20:5ω3 (p = 0.001-0.007). However, the concentrations of 18:3ω3 in the week 0 livers and in the high ω6 fed fish were too low to measure and the values of $\delta^{13}C$ were not detectable.

Table 2.9. $\delta^{13}C$ values[a] (‰) of fatty acids in the two extreme ω6:ω3 diets and in the liver of Atlantic salmon at week 0 and after 12 weeks of feeding.

				Week 0	Week 12			RC (%) [b]	
Fatty acid	**High ω3 diet**	**High ω6 diet**	**p-value**	**Initial liver**	**High ω3 liver**	**High ω6 liver**	**p-value**	**High ω3 liver**	**High ω6 liver**
16:0	-29.5 ± 0.2	-29.5 ± 0.2	0.95	-20.7 ± 0.7[a]	-28.1 ± 0.2[b]	-28.1 ± 0.3[b]	< 0.001		
18:0	-29.7 ± 0.03	-30.2 ± 0.3	0.11	-19.9 ± 0.5[a]	-27.6 ± 0.4[b]	-27.9 ± 0.5[b]	< 0.001		
18:1ω9	-29.6 ± 0.1	-29.7 ± 0.3	0.45	-21.9 ± 0.4[a]	-30.3 ± 0.4[b]	-29.9 ± 0.6[b]	< 0.001		
18:2ω6 (LNA)	-30.7 ± 0.1	-30.4 ± 0.01	0.05	-24.9 ± 0.4[a]	-30.3 ± 0.6[b]	-31.2 ± 0.5[c]	< 0.001		
18:3ω3 (ALA)	-30.4 ± 0.1	-32.2 ± 0.5	0.03	ND[c]	-30.8 ± 0.6	ND[c]	-		
20:4ω6 (ARA)	ND[c]	ND[c]	-	-26.5 ± 0.9[a]	-29.0 ± 0.5[b]	-30.4 ± 0.5[c]	< 0.001		
20:5ω3 (EPA)	-24.9 ± 0.3	-25.0 ± 0.3	0.81	-30.1 ± 0.2[a]	-29.5 ± 0.9[a]	-28.0 ± 0.4[b]	< 0.001	90.8 ± 17.8	44.1 ± 3.9
22:6ω3 (DHA)	-25.1 ± 0.5	-25.5 ± 0.9	0.59	-29.9 ± 0.6[a]	-26.9 ± 0.2[b]	-26.6 ± 0.3[b]	< 0.001	35.8 ± 4.2	17.9 ± 2.9

[a] $\delta^{13}C$ data presented as mean ± standard deviation. Different superscripts in the same row indicate significant differences between treatments at week 12, and between week 0 (initial) and week 12 ($p \leq 0.05$) (n = 3 and n = 8 in the dietary and liver data, respectively). [b] Relative percent contribution (RC) of dietary 18:3ω3 to EPA and DHA in the liver, using a discrimination factor of 0.4‰. RC data presented as mean ± standard deviation. [c] Not detectable.

Notably, all FA showed a significant difference in liver $\delta^{13}C$ between week 0 and week 12 ($p < 0.001$). Finally, the mixing model estimated the relative contribution (RC) of dietary 18:3ω3 to EPA in the liver was 90.8 $\pm$ 17.8 and 44.1 $\pm$ 3.9% in the high ω3 and high ω6 fish, respectively, while DHA showed much lower precursor incorporation (35.8 $\pm$ 4.2% and 17.9 $\pm$ 2.9%, respectively).

2.5. Discussion

2.5.1. Growth performance

Experimental diets in the current study did not have a significant effect on growth performance (e.g. weight gain, SGR, and CF) or organ indices (HSI and VSI) of Atlantic salmon. This result is in agreement with earlier studies that have used VO such as linseed, sunflower or soy to replace fish oil with varying ratios of 18:2ω6 and 18:3ω3 (Bell et al., 2004; Menoyo et al., 2007; Thanuthong et al., 2011), and demonstrates that high dietary levels of 18:3ω3 or 18:2ω6 do not have a negative effect on salmon growth performance. The morphometric values obtained in this study are similar to those reported in earlier studies of Atlantic salmon smolts (~ 130-300 g mean initial weight) feeding on high fish oil (~ 25%) and low to high fish meal (~ 10-60%)-based diets in seawater (Leaver et al., 2008; Torstensen et al., 2008; Hixson et al., 2017). It is also important to mention that multivariate statistical analysis in the current study showed that overall growth performance was not significantly affected by diet [(p(perm) = 0.62]. EPA+DHA levels were not significantly different among diets and ranged from 1.0 to 1.3% (of dietary wet weight; Table 2.3), and as these levels are sufficient to satisfy the essential FA requirement for

Atlantic salmon (NRC, 2011), it is not surprising that growth was not significantly different among the five dietary treatments.

2.5.2. Tissue lipid composition

2.5.2.1. Muscle

Salmon muscle of fish fed with the experimental diets in this study was mainly composed of TAG (68.4-80.9%; Table 2.5) which is typical for a storage tissue in salmonids (Bell et al., 1998; Hixson et al., 2017). As further indicated by the PCoA analysis (Fig. 2.1), TAG levels in the muscle were associated with the monounsaturates 18:1ω9, 20:1ω9 and the sum of MUFA. It was shown that MUFA are preferentially stored as TAG in fish muscle by earlier studies, particularly 18:1ω9 (Henderson and Tocher, 1987; Ruiz-Lopez et al., 2015; Kacar et al., 2016). Collectively, these results could suggest that MUFA were preferentially used as storage lipids to fulfill energetic requirements. Furthermore, fish fed the high ω6 diet showed significantly higher phospholipid proportions as compared with the balanced and medium ω3 fed fish (Table 2.5). Interestingly, ARA was associated with phospholipid proportions in the muscle (Fig. 2.1) and correlated positively with dietary soy levels ($r^2 = 0.447$; $p < 0.0001$; Table 2.10). This close association between PL proportions and ARA proportions was likely based on membrane incorporation, especially in the high ω6 fed fish. Higher incorporation of ARA in membrane phospholipids could modulate the synthesis of pro-inflammatory eicosanoids (Schmitz and Ecker, 2008; Calder, 2013). However, as no direct measurements of eicosanoid secretion or transcript expression of eicosanoid genes were made in the muscle, this can only be postulated.

Table 2.10. Significant Pearson correlations[a] ($p \leq 0.05$) between ω6 and ω3 PUFA (% of total FAs) in the tissues (muscle and liver) and dietary soy and linseed oil incorporation (% of diet).

Tissue	Tissue FA	Dietary oil	Pearson R	p-Value
Muscle	18:2ω6 (LNA)	Soy	0.969	< 0.0001
	20:2ω6		0.774	< 0.0001
	20:3ω6		0.899	< 0.0001
	20:4ω6 (ARA)		0.447	< 0.0001
	Σ ω6		0.975	< 0.0001
	18:3ω3 (ALA)	Linseed	0.977	< 0.0001
	18:4ω3		0.785	< 0.0001
	20:4ω3		0.902	< 0.0001
	Σ ω3		0.916	< 0.0001
Liver	18:2ω6 (LNA)	Soy	0.940	< 0.0001
	20:2ω6		0.738	< 0.0001
	20:3ω6		0.926	< 0.0001
	20:4ω6 (ARA)		0.789	< 0.0001
	Σ ω6		0.980	< 0.0001
	18:3ω3 (ALA)	Linseed	0.959	< 0.0001
	20:3ω3		0.860	< 0.0001
	20:4ω3		0.927	< 0.0001
	20:5ω3 (EPA)		0.594	< 0.0001
	Σ ω3		0.829	< 0.0001

[a] Pearson correlations were calculated using individual fish from all dietary treatments (n = 16-20).

Total sterols in the muscle was not significantly affected by diet when expressed as a proportion; however, quantitatively (in mg g^{-1} wet weight) the balanced fed fish had lower levels of this lipid class compared to the high ω6 and high ω3 fish (Table 2.11), and PCoA revealed that sterol proportions were associated with EPA and DHA in the muscle. Notably, high within-treatment variation was observed in total lipids, particularly in the Balanced, medium ω6 and high ω6 fed fish. Fat deposition is influenced by region and sample size (Bell et al., 1998; Katikou et al., 2001), and as such sampling differences could be a potential driver of these large SD.

The FA composition of the muscle was highly reflective of the diet and responded to changes in ω3 and ω6 FA. Significant differences were observed among treatments in 18:2ω6, 18:3ω3 and ω6:ω3 proportions, while DHA, EPA, total SFA, MUFA and PUFA did not differ among treatments and reflected dietary levels (except DHA). The PCoA plot and SIMPER analysis revealed that the differences in FA composition were mainly driven by 18:3ω3, the sum of ω3 (highest in the high ω3 fish), 18:2ω6 and the sum of ω6 (highest in the high ω6 fish). However, intermediate PUFA such as 18:4ω3, 20:4ω3 and 20:3ω6 were also significantly different among treatments and their prevalence was higher in week 12 than in week 0, which suggests that LC-PUFA synthesis has occurred (Table 2.5). In addition, although proportions of EPA and DHA significantly decreased at week 12 as compared with the initial time point (week 0), their concentrations (measured as g $muscle^{-1}$) in all treatments (apart from the balanced fed fish) significantly increased from week 0 to week 12 (Table S.2.4). This suggests that EPA and particularly DHA were selectively incorporated and retained in the muscle throughout the duration of the trial as has been demonstrated in other studies (Bell et al., 2004; Leaver et al., 2011; Hixson et al., 2014).

Table 2.11. Lipid composition (mg g^{-1} wet weight) of Atlantic salmon muscle and liver tissues[a] before (initial) and after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids.

	Initial	High ω3	Med ω3	Balanced	Med ω6	High ω6
Lipid class (mg g^{-1} wet weight)						
Muscle						
TAG[b]	15.7 ± 10.0	26.7 ± 14.7	22.8 ± 12.9	20.5 ± 13.6	22.2 ± 17.8	31.8 ± 27.8
Sterol	0.3 ± 0.1	1.6 ± 0.6[a]	1.3 ± 0.5[ab]	0.8 ± 0.6[b]	1.4 ± 0.6[ab]	1.9 ± 0.9[a]
Phospholipid	4.9 ± 0.7	4.2 ± 2.6[a]	3.3 ± 1.7[a]	2.7 ± 2.1[a]	5.0 ± 2.5[ab]	7.1 ± 4.3[b]
Liver						
TAG[b]	9.5 ± 4.3	5.2 ± 3.7	3.2 ± 2.0	4.2 ± 2.2	5.5 ± 3.2	4.7 ± 3.1
Sterol	7.3 ± 3.0	7.9 ± 1.8[a]	6.9 ± 2.9[ab]	5.6 ± 1.9[b]	6.9 ± 1.7[ab]	7.6 ± 2.3[ab]
Phospholipid	16.0 ± 8.8	19.7 ± 11.2	17.6 ± 9.5	19.7 ± 9.1	22.7 ± 10.7	16.7 ± 9.0

[a] Mean (n=9-20) ± standard deviation. Different superscripts in the same row indicate significant differences among treatments at week 12. Underlined values are significantly different to week 0 (initial) ($p \leq 0.05$). [b] Triacylglycerol.

The concentration of ω3 LC-PUFA in farmed fish fillets is extremely important for the health of the consumer (Turchini et al., 2010; Nichols et al., 2014). The sum of EPA and DHA in the white muscle of fish fed the five ω6:ω3 diets ranged from 2.3 to 3.6 mg g^{-1} wet weight (Table 2.12) and showed significantly lower concentration in the balanced compared to the high ω6 fed fish. Based on Canada's Food Guide, one serving of fish is 75 g (Health Canada, 2011). Therefore, these EPA+DHA levels would translate to 170 to 273 mg for 75 g serving, and the high ω3 and high ω6 fed fish would be sufficient to satisfy daily EPA and DHA requirements for the consumer (250 mg), set by the World Health Organization (Burlingame et al., 2009). Previous studies showed that increasing dietary supply of ALA resulted in higher concentrations of EPA and DHA in fish fillets (Turchini et al., 2009; Thanuthong et al., 2011). However, the present study did not observe a clear trend between dietary ALA and muscle EPA and/or DHA concentration. The discrepancy between studies could be related to the differences in the dietary content of EPA and DHA, and the species examined. Finally, ω6:ω3 ratios in the muscle ranged from 0.3 to 1.9 which is lower than the maximum ω6:ω3 reported by several studies as a healthy ratio for the human consumer (i.e. 4:1) (Simopoulos, 2002; Young, 2009).

Table 2.12. EPA+DHA concentrations (mg g^{-1} wet weight) in Atlantic salmon muscle[a] before (initial) and after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids.

	Initial	**High ω3**	**Med ω3**	**Balanced**	**Med ω6**	**High ω6**
EPA+DHA (mg g^{-1} wet weight)	3.3 ± 1.3^{ab}	3.3 ± 1.0^{ab}	2.7 ± 0.8^{ab}	2.3 ± 0.9^{a}	3.0 ± 1.5^{ab}	3.6 ± 1.8^{b}
EPA+DHA (mg 75 g^{-1} fillet)	247	248	202	170	224	273

[a] Mean (n=10-20) ± standard deviation. Different superscripts in the first row indicate significant differences among treatments at week 12, and between week 12 and week 0 (initial) ($p \leq 0.05$).

2.5.2.2. Liver

In contrast to the muscle, no differences were observed among treatments in the lipid classes TAG (~11-16%) or phospholipid (~ 48-60%), and total lipids concentrations were also similar in the liver (~30-38 mg g^{-1} wet weight; Table 2.6). The fact that total lipid content or HSI were not significantly influenced by diet could suggest that the VO and precursor levels used in the current study did not have an adverse effect on liver lipid deposition. This is in agreement with Blanchard et al. (2008) who fed juvenile Eurasian perch VO with varying ratios of ω6:ω3 FA (0.17-1.23) and Bell et al. (1994) who used safflower or linseed oil diets (ω6:ω3 of 12.5 and 0.35, respectively) for turbot.

The FA composition of the liver was very reflective of the diet and responded to changes in ω3 and ω6 FA. Similar to the pattern observed in the muscle, the difference among treatments was mainly driven by the sums of ω3 and ω6 FA which was a result of variation in dietary and tissue levels of the precursors 18:3ω3 and 18:2ω6, respectively. It is also important to mention that proportions of ω3 PUFA (i.e. 18:3ω3, 18:4ω3 and 20:4ω3) in the liver were positively correlated with dietary levels of linseed oil ($p < 0.0001$), while ω6 PUFA (i.e. 18:2ω6, 20:3ω6 and 20:4ω6) showed positive correlations with soy oil levels ($p < 0.0001$; Table 2.10). However, unlike muscle, liver showed a significant increase in EPA in the high ω3, medium ω3 and balanced treatments in comparison with the medium and high ω6 fish (Table 2.6). This may suggest a higher requirement for EPA in the liver tissue and consequently increased synthesis. Surprisingly, DHA was significantly lower in the high ω3 compared with the medium ω3 and balanced fish, and this was coupled with the lowest PUFA and highest MUFA proportions in the former treatment. The reason for

this is not fully understood. However, when DHA was measured as concentration (mg g^{-1} wet weight) there was no significant difference among treatments ($p = 0.6$). Furthermore, ARA proportions showed a ~ 2.5-fold increase in the high ω6 compared with the high ω3 fish. The levels of the former treatment (4.2%) were similar to that detected in the initial time point (4.1%). However, quantitatively (mg $liver^{-1}$), the high ω6 fish showed a significantly higher amount of ARA compared to the high ω3 fish, and when compared to the initial time point (week 0) (Table S.2.5).

Interestingly, when applying a conservative turnover rate of 24% $month^{-1}$, only ~25% of ARA at week 12 was incorporated from the initial time point (i.e. week 0) in the high ω6 fish, while the high ω3 showed a retention of ~57% (Table S.2.5). Thus, it is likely that the high ARA levels observed in the high ω6 fish were a result of biosynthesis from dietary precursors to a large extent. Indeed, Trueman et al. (2005) observed a turnover rate of 20 to 40% per month in Atlantic salmon smolts, while Jardine et al. (2004) showed muscle turnover of 24 to 66% per month. Mean growth rates in the latter study were 1% day^{-1}, which is relatively close to the growth observed in the present study.

Diets in this study also clearly altered the EPA/ARA ratio in the liver, which was positive in all treatments except the high ω6 fish. Since EPA and ARA compete as substrates for eicosanoid synthesis in vertebrates (Calder, 2013; Holen et al., 2015), I speculate that the high dietary proportions of ALA coupled with low ARA may have increased the synthesis of anti-inflammatory eicosanoids.

2.5.3. Hepatic qPCR analysis

2.5.3.1. LC-PUFA synthesis and FA metabolism

The transcript expression of key genes encoding desaturases and elongases (*fadsd5, fadsd6, elovl2* and *elovl5a*), which regulate the LC-PUFA pathway, were not significantly altered by diet (Table 2.7). This may be due to the fact that dietary levels of EPA+DHA were kept similar among all treatments and above the minimum requirement for Atlantic salmon. Indeed, hepatic LC-PUFA synthesis in salmonids is stimulated by increased precursor (18:2ω6 and/or 18:3ω3) availability and negatively affected by the concentration of LC-PUFA (i.e. EPA, ARA, DHA) in a feedback loop manner (Jordal et al., 2005; Jump et al., 2005; Glencross et al., 2015). However, significant negative correlations were observed between both elongases (*elovl2* and *elovl5a*) and ω3 FA (EPA, the sum of ω3 and 22:5ω3), and positive relationship with 20:2ω6 in the liver (Table 2.8). Furthermore, although not significant ($p = 0.064$ and 0.051, respectively), *elovl2* and *elovl5a* showed positive associations with ω6:ω3 ratio (Table S.2.3). Interestingly, similar trends of negative correlations with EPA and positive with ω6 PUFA were identified between these hepatic transcripts and salmon muscle FA in Hixson et al. (2017). These results further support the notion that the ω3 LC-PUFA synthesis pathway is suppressed when dietary requirement of EPA and DHA are met. Furthermore, the positive correlation identified between the *elovl* transcripts and 20:2ω6 show that the alternative LC-PUFA pathway (i.e. elongation of 18:2ω6 to 20:2ω6 followed by Δ8 desaturation to 20:3ω6) (Monroig et al., 2011; Kabeya et al., 2017) was likely activated in these fish.

Dietary PUFA can also regulate lipid metabolism genes (e.g. related to FA and cholesterol metabolism) by affecting the expression of transcription factors such as *srebp1*, *lxr* and *pparβ* (Betancor et al., 2014; Glencross et al., 2015; Hixson et al., 2017). mRNA levels of *lxra* and *srebp1* in the current study showed down-regulation in the high ω6 compared to the balanced and high ω3 fed fish. These differences were statistically significant except when comparing high ω6 with high ω3 fish in the latter transcript. ω3 and ω6 PUFA can influence the expression of *lxr* via the DR1 element (Tobin et al., 2002) and result in inhibition or activation of *srebp1* (Joseph et al., 2002). It was shown that diets rich in EPA and DHA or 18:2ω6 reduce hepatic pre-mature and nuclear SREBP-1 (Schmitz and Ecker, 2008). Thus, it is possible that the down-regulation observed in the high ω6 fish is influenced by dietary 18:2ω6. Furthermore, the positive correlations identified between *lxra* and ω3 PUFA (i.e. 18:3ω3 and 20:3ω3) and negative with ARA may suggest that *lxra* and *srebp1* were induced by ω3 rather than ω6 PUFA.

Finally, *fasb* and *srebp1* agreed in the direction of expression between the 2 extreme treatments. This result is in agreement with Morais et al. (2011) who showed that these transcripts had similar directions of expression in salmon with high lipid content that were fed with VO blend diet. Although it was suggested that *fas* is a target of SREBP1 in salmon cell lines (Minghetti et al., 2011), the interaction between *fas* and *srebp1* is complex and requires further research in fish (Zheng et al., 2013; Carmona-Antoñanzas et al., 2014).

2.5.3.2. Cholesterol and bile acid metabolism

Earlier studies have shown that in salmon that were fed plant-based diets, genes related to cholesterol biosynthesis were up-regulated in the liver as compared with fish fed

FO diet (Leaver et al., 2008; Morais et al., 2011). Indeed, cholesterol biosynthesis is responsive to the availability of dietary cholesterol (Norambuena et al., 2013). In this study, all diets had identical proportions of marine products (fish oil and fish meal) and plant proteins. However, VO proportions (i.e. linseed/soy/palm) varied, and total sterol was significantly lower in the high ω6 compared to the balanced and medium ω3 diets. Furthermore, as it was shown that soy, linseed, and palm oils have different phytosterol compositions (Fedeli et al., 1966; Itoh et al., 1973), this could have affected the absorption of cholesterol in these fish. Despite these differences, the examined transcripts related to cholesterol synthesis (*idi1*, *srebp2* and *dhcr7*) were not differentially expressed among treatments. The transcript encoding bile acid receptor, *bar*, showed a significant down-regulation in the balanced as compared with the two extreme ω6:ω3 treatments. BAR is a nuclear receptor that is activated by cholesterol derived bile acids, predominantly in the liver. Sterol concentrations in the liver (Table 2.11) showed a similar pattern of lowest levels in the balanced treatment, which may explain the observed down-regulation. Lowest concentration of sterol in the balanced as compared with the high ω3 and high ω6 treatments was also observed in the muscle (Table 2.11). Furthermore, *bar* was negatively correlated with EPA and the sum of PUFA, and positively with ARA. It was shown by Hixson et al. (2017) that hepatic *bar* mRNA levels were also negatively correlated with muscle EPA in salmon. Indeed, earlier *in vitro* studies showed that PUFA (e.g. ARA, DHA and 18:3ω3) can also act as ligands targeting this receptor (Zhao et al., 2004; Claudel at al., 2005). Finally, the correlation observed between 20:2ω6 and *cyp7a1b* further demonstrates that cholesterol catabolism was influenced by PUFA, as the latter transcript is involved in

the rate-limiting step in bile acid synthesis (Kortner et al., 2014). However, the direction of change and interaction between ω3 and ω6 PUFA are still unclear.

2.5.3.3. Eicosanoid synthesis

Fish fed the balanced diet had significantly higher levels of *5loxa* compared to the high ω3 and high ω6 fish. This was positively associated with levels of ARA+EPA in the liver ($p = 0.059$, $r^2 = 0.419$; Table S.2.3). Furthermore, negative correlations were identified between the transcript expression of hepatic *pgds* with DHA and the sum of EPA+ARA+DHA, while *cox1* was negatively correlated with the sum of PUFA. 5-Lipoxygenase (5LOX) catalyzes two steps in the biosynthesis of leukotrienes derived from ARA, EPA, and DHA (Rowley at al., 1995). COX1 and PGDS convert ARA and EPA into prostaglandins H and D, respectively (Gómez-Abellán and Sepulcre, 2016). These eicosanoids have important roles in the regulation of inflammation and immune response (Lall, 2000; Calder, 2007; Holen et al., 2015). Holen et al. (2018) examined the effect of VO with varying ω6:ω3 ratios (0.7-4.1) on eicosanoid production in salmon head kidney tissue and head kidney leukocytes. It was shown that tissue *pgds* was significantly up-regulated in the fish fed with soy oil as compared with the other diets, and this coincided with higher PGE2 levels in LPS induced leukocytes. A similar trend of higher *pgds* expression was observed in the current study in the high ω6 fed fish. Interestingly, Holen et al. (2015) showed that the combined effect of ARA+EPA in salmon head-kidney cells resulted in up-regulation of the eicosanoid genes *cox2*, *5lox*, *pges*, and *pgds*, when compared with LC-PUFA when they were introduced separately (i.e. 50 µM of ARA/EPA/DHA). This is in agreement with this study, where eicosanoid-related

transcripts examined (not including *5loxb*) showed strong associations with the sums of ARA+EPA or EPA+ARA+DHA in the liver (Table S.2.3). To the best of my knowledge, there are no other studies that examined the combined effects of LC-PUFA on eicosanoid-related genes in salmon liver.

2.5.4. CSIA of fish fed the two extreme ω6:ω3 diets

The high ω3 and high ω6 diets were isotopically distinct with respect to both PUFA precursors, however, 18:3ω3 was more isotopically depleted in the heavier isotope. ALA was enriched in the high ω3, while LNA was enriched in the high ω6 diet. These isotopic signatures are in the range of those reported for terrestrial plants including soy, palm and linseed (Woodbury et al., 1998; Kelly and Rhodes, 2002). It was also evident that ALA was isotopically distinct from EPA and DHA, in both diets. These dietary components constitute the two end members in the mixing model.

Liver ARA in the high ω6 was isotopically significantly lighter (-30.4‰) than the high ω3 fish (-29.0‰) and showed the same value as dietary LNA (-30.4‰). Furthermore, a significant shift in $\delta^{13}C$ from a marine to a terrestrial signature was demonstrated in both treatments between week 0 and week 12. This demonstrated a substantial ARA fractionation and precursor incorporation. Metherel et al. (2017) showed that in rats fed with a high LNA (~ 30% of FA) and low ALA (~ 2% of FA) and ARA (~ 0.07% of FA) diet for 12 weeks, 81% of liver ARA was derived from the dietary LNA (using CSIA and the mixing model). Precursor incorporation could not be quantified in this study as ARA values in the diets were undetected. Notwithstanding, the current findings further illustrated the large impact of LNA on ARA synthesis in vertebrates.

The high ω3 fish showed the opposite in respect to ω3 FA, with significantly lighter / depleted EPA (-29.5 *vs* -28.0‰) that closely resembled dietary ALA (-30.4‰). The fact that both EPA and DHA showed depleted isotopic signatures at week 0 compared to week 12 is surprising and could suggest a high precursor incorporation in fish fed with a low fish oil commercial diet. The mixing model indicated that ~ 91 $\pm$ 18 and 44 $\pm$ 4% of liver EPA was synthesized from the dietary terrestrial ALA in the high ω3 and high ω6 fish, respectively. The discrepancy between relative contribution (Table 2.9) and retention rate estimates (Table S.2.5) may suggest that EPA had a greater turnover than the 24% month^{-1}. DHA showed much lower precursor incorporation as compared with EPA (~36 $\pm$ 4 and 18 $\pm$ 3%). These data, along with the FA proportion data, suggest that DHA in the liver (similar to the muscle) was selectively incorporated and retained throughout the trial and, thus, much less was synthesized. Notably, the relative contribution values obtained in the current study are in the range of the conversion rate estimated from the SIAR Bayesian isotopic mixing model (Parnell et al., 2010). The percent contributions differed by ~ 5 to 6% for DHA and by as little as 1.3% for EPA (Table S.2.6).

It was demonstrated in previous studies that dietary PUFA precursors play a major role in LC-PUFA synthesis (Buzzi et al 1996; Cleveland et al., 2012; Masiha et al., 2013). However, EPA and DHA varied with precursor levels, making it difficult to truly determine the precursor role in the synthesis. Hixson (2014) used CSIA to compare isotopic signatures of rainbow trout fed with camelina oil (100% replacement) with that of fish oil in the muscle. That analysis demonstrated that ~ 27% of DHA and ~ 14% of EPA were synthesized from the terrestrial ALA in the camelina diet. Differences in the magnitude of ALA incorporation into EPA could be attributed to dietary ALA/LNA which were ~1:1 as

compared with 2.3:1 in the present study, as well as differences between species, and in bioconversions between muscle and liver.

One of the mixing model assumptions is that the stable isotope ratio of the tissue reflects the diet ± a correction factor, which is termed "diet-tissue discrimination" (Arneson and MacAvoy, 2005). This correction, also known as fractionation, occurs because physiological processes of lighter isotopes often occur at higher rates than heavier isotopes (Fry, 2006). Discrimination factors vary among species, tissues within a species and diets (Phillips, 2012). In the current study the mean discrimination was ~ 0.4‰. This correction factor is identical to the mean discrimination reported by Townsend (2015) (0.4 ± 0.1‰ mean ± SE), and in the lower range of Budge et al. (2016) (1.0 ± 1.3‰ mean ± SE) who used CSIA to analyse the $\delta^{13}C$ of PUFA in fish livers.

2.6. Conclusions

The present study demonstrated that plant-based diets with varying ratios of ω6 to ω3 FA (0.3 to 2.7) did not affect growth performance or organ indices (i.e. HSI and VSI) of cultured Atlantic salmon smolts. This indicated that the high dietary levels of 18:3ω3 (~ 30% of total FA) or 18:2ω6 (~ 35% of total FA) were similarly deposited in the liver, and did not have a negative effect on salmon growth performance. FA composition of liver and muscle tissues was highly reflective of the diet and suggested elongation and desaturation of the ω3 and ω6 precursors. CSIA analysis further demonstrated that liver LC-PUFA synthesis was largely driven by dietary PUFA precursors even when EPA and DHA were supplied at levels above minimum requirements.

Furthermore, quantities of EPA+DHA in white muscle of fish fed with the 2 extreme diets (i.e. high ω3 and high ω6) were sufficient to satisfy daily EPA and DHA requirements for human consumers. Finally, correlation analyses between liver FA composition and hepatic gene expression: 1) further supported the relationship between liver ω6:ω3 and LC-PUFA biosynthesis; and 2) demonstrated that the transcript expression levels of key eicosanoid synthesis genes were associated with the proportions (% of total fatty acids) of EPA+ARA, EPA+ARA+DHA, and the sum of PUFA.

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2.8. Supplemental material

Table S.2.1. SIMPER dissimilarities of muscle FA and lipid composition compared across dietary treatments.

Dietary treatments	Average dissimilarity (%)	Major lipid class / FA contributor	Con. (%)[a]
High ω3 & medium ω3	8.2	Triacylglycerol	24.0
		Phospholipid	15.9
		Σ ω3	7.5
		Σ ω6	6.3
High ω3 & balanced	11.6	Triacylglycerol	16.1
		Σ ω3	12.2
		Σ ω6	11.9
		Phospholipid	10.9
Medium ω3 & balanced	8.9	Triacylglycerol	17.7
		Phospholipid	10.8
		Σ ω3	10.6
		Σ ω6	9.7
		18:3ω3	8.8
High ω3 & medium ω6	14.9	Triacylglycerol	13.9
		Σ ω3	13.7
		Σ ω6	13.6
		18:3ω3	12.0
Medium ω3 & medium ω6	12.5	Triacylglycerol	15.9
		Σ ω3	12.2
		Σ ω6	12.0
		18:3ω3	11.8
Balanced & medium ω6	9.4	Triacylglycerol	21.2
		Phospholipid	15.6
		Σ ω3	8.6
		18:3ω3	7.3
High ω3 & high ω6	19.1	Σ ω3	15.7
		Σ ω6	14.8
		18:2ω6	12.8
		18:3ω3	12.5
Medium ω3 & high ω6	16.8	Σ ω3	14.7
		Σ ω6	13.7
		Triacylglycerol	13.3
		18:3ω3	12.4
Balanced & high ω6	13.6	Triacylglycerol	17.1
		Phospholipid	14.6
		Σ ω3	11.8
		Σ ω6	10.6
Medium ω6 & high ω6	10.4	Triacylglycerol	20.9
		Phospholipid	17.5
		Σ ω3	9.5
		Σ ω6	7.9

[a] Precent contribution.

Table S.2.2. SIMPER dissimilarities of liver FA and lipid composition compared across dietary treatments.

Dietary treatments	Average diss. (%)[a]	Major lipid class / FA contributor	Con. (%)[b]
High ω3 & medium ω3	11.2	Phospholipid	25.4
		Sterol	16.4
		Triacylglycerol	10.5
High ω3 & balanced	12.4	Phospholipid	21.2
		Sterol	12.9
		Triacylglycerol	9.5
		Σ ω6	7.7
Medium ω3 & balanced	10.6	Phospholipid	25
		Sterol	18
		Triacylglycerol	9.8
High ω3 & medium ω6	14.8	Phospholipid	17.3
		Σ ω6	10.7
		Sterol	10
		Triacylglycerol	8.3
		Σ ω3	7.3
Medium ω3 & medium ω6	13.1	Phospholipid	19.5
		Sterol	13.7
		Σ ω6	9.3
		Σ ω3	8.6
Balanced & medium ω6	9.6	Phospholipid	21.4
		Sterol	14.2
		Triacylglycerol	11.7
		Σ ω3	7.5
High ω3 & high ω6	17.4	Σ ω6	13.1
		Phospholipid	13
		Σ ω3	10.5
		Sterol	9.7
		Triacylglycerol	7.4
Medium ω3 & high ω6	16.6	Phospholipid	17.2
		Sterol	11.5
		Σ ω6	11.5
		Σ ω3	11.4
Balanced & high ω6	13.5	Phospholipid	20
		Sterol	13.3
		Σ ω3	10.9
		Σ ω6	9.5
Medium ω6 & high ω6	11.1	Phospholipid	23.3
		Sterol	15
		Triacylglycerol	11.2
		Σ ω3	7.6

[a] Average dissimilarity. [b] Precent contribution.

Table S.2.3. Pearson correlations[a] ($p < 0.1$) between hepatic transcript expression (RQ values) of genes involved in lipid metabolism and eicosanoid synthesis with liver fatty acid composition (% of total FAs).

Transcript	FA	Pearson R	p-Value
elovl2	20:2ω6	0.484	**0.026**
	18:2ω6 (LNA)	0.375	0.094
	ω6/ω3	0.411	0.064
	20:5ω3 (EPA)	-0.565	**0.008**
	Σ ω3	-0.511	**0.018**
	22:5ω3	-0.438	**0.047**
elovl5a	20:2ω6	0.671	**0.001**
	18:2ω6 (LNA)	0.376	0.093
	Σ ω6	0.379	0.090
	ω6/ω3	0.432	0.051
	20:5ω3 (EPA)	-0.590	**0.005**
	Σ ω3	-0.513	**0.017**
	22:5ω3	-0.502	**0.020**
lxra	18:1ω9	0.517	**0.016**
	20:1ω9	0.512	**0.018**
	Σ MUFA	0.537	**0.012**
	18:3ω3 (ALA)	0.528	**0.014**
	20:3ω3	0.570	**0.007**
	Σ SFA	-0.534	**0.013**
	20:3ω6	-0.458	**0.037**
	20:4ω6 (ARA)	-0.512	**0.018**
	22:6ω3 (DHA)	-0.446	**0.043**
	Σ PUFA	-0.402	0.071
	Σ ω6	-0.411	0.064
	ω6/ω3	-0.421	0.057
pparβ1a	Σ ω3	0.427	0.054
	18:2ω6 (LNA)	-0.381	0.088
	20:2ω6	-0.392	0.078
	20:3ω6	-0.395	0.076
	Σ ω6	-0.407	0.067
	ω6/ω3	-0.375	0.094
fasb	20:3ω3	0.459	**0.037**
	20:3ω6	0.458	**0.037**
	18:1ω9	0.419	0.058
	18:3ω3 (ALA)	0.377	0.092
	22:6ω3 (DHA)	0.413	0.063

	Σ ω3	0.377	0.092
	18:2ω6 (LNA)	-0.390	0.081
	Σ ω6	-0.390	0.081
fabp10a	18:2ω6 (LNA)	-0.379	0.090
	20:3ω6	-0.418	0.060
	Σ ω6	-0.386	0.084
fabp3b	Σ PUFA	-0.382	0.087
bar	20:1ω9	0.458	**0.037**
	Σ MUFA	0.627	**0.002**
	20:4ω6 (ARA)	0.628	**0.002**
	22:5ω3	0.596	**0.004**
	20:3ω6	0.406	0.067
	20:3ω3	0.418	0.060
	Σ SFA	-0.638	**0.002**
	Σ PUFA	-0.636	**0.002**
	20:5ω3 (EPA)	-0.603	**0.004**
cyp7a1b	20:4ω3	0.401	0.080
	20:2ω6	-0.479	**0.033**
	ω6/ω3	-0.400	0.081
dhcr7	20:3ω6	0.382	0.087
	Σ ω3	-0.421	0.057
pgds	Σ MUFA	0.437	**0.047**
	20:1ω9	0.505	**0.019**
	18:1ω9	0.415	0.061
	22:6ω3 (DHA)	-0.668	**0.001**
	EPA+ARA+DHA	-0.594	**0.005**
	Σ PUFA	-0.408	0.066
cox1	20:1ω9	0.467	**0.033**
	18:1ω9	0.378	0.091
	Σ MUFA	0.393	0.078
	Σ PUFA	-0.432	**0.050**
	ARA+EPA	-0.395	0.076
5loxa	ARA+EPA	0.419	0.059

Pearson correlations were calculated using individual fish (n = 6-8).

Table S.2.4. Polyunsaturated fatty acid concentrations and retention rates in Atlantic salmon muscle[a] at week 0 (initial) and after 12 weeks of feeding diets with varying ratios of ω6 to ω3 fatty acids.

Fatty acid	Dietary treatment / time point	mg g^{-1} wet weight[b]	g $muscle^{-1}$	% of original (no turnover)[c]	% of original (24% $month^{-1}$)[c]
18:2ω6 (LNA)	Initial	1.7 ± 1.1^{a}	0.1 ± 0.1^{a}		
	High ω3	3.3 ± 1.7^{a}	1.0 ± 0.6^{ab}	26.7 ± 25.6	11.7 ± 11.3
	Med ω3	3.6 ± 1.9^{a}	1.0 ± 0.6^{ab}	23.5 ± 21.6	11.6 ± 14.5
	Balanced	4.3 ± 2.6^{a}	1.3 ± 0.9^{ab}	21.0 ± 24.9	9.5 ± 11.9
	Med ω6	6.2 ± 4.7^{a}	1.8 ± 1.4^{bc}	15.0 ± 14.7	6.6 ± 6.5
	High ω6	10.4 ± 7.9^{b}	3.0 ± 2.4^{c}	11.9 ± 17.6	5.2 ± 7.7
18:3ω3 (ALA)	Initial	0.2 ± 0.1^{a}	0.02 ± 0.01^{a}		
	High ω3	6.0 ± 2.9^{c}	1.8 ± 1.1^{d}	1.5 ± 1.4^{a}	0.6 ± 0.6^{a}
	Med ω3	4.5 ± 2.3^{c}	1.3 ± 0.8^{cd}	2.1 ± 2.6^{a}	0.9 ± 1.2^{a}
	Balanced	2.7 ± 1.5^{b}	0.8 ± 0.5^{bc}	3.4 ± 3.9^{a}	1.5 ± 1.7^{a}
	Med ω6	2.0 ± 1.5^{ab}	0.6 ± 0.5^{ab}	4.9 ± 4.8^{ab}	2.1 ± 2.1^{ab}
	High ω6	1.2 ± 0.9^{ab}	0.3 ± 0.3^{ab}	10.6 ± 15.8^{b}	4.6 ± 6.9^{b}
20:4ω6 (ARA)	Initial	0.2 ± 0.1^{a}	0.02 ± 0.01^{a}		
	High ω3	0.1 ± 0.04^{b}	0.03 ± 0.01^{a}	65.4 ± 22.5^{ab}	34.2 ± 20.3^{ab}
	Med ω3	0.1 ± 0.03^{b}	0.02 ± 0.01^{a}	76.7 ± 21.9^{a}	37.9 ± 16.0^{ab}
	Balanced	0.1 ± 0.03^{b}	0.02 ± 0.01^{a}	79.1 ± 20.3^{a}	45.0 ± 26.3^{a}
	Med ω6	0.1 ± 0.1^{b}	0.04 ± 0.02^{ab}	59.2 ± 24.8^{ab}	28.1 ± 15.8^{ab}
	High ω6	0.2 ± 0.1^{a}	0.05 ± 0.04^{b}	47.1 ± 27.7^{b}	23.5 ± 18.6^{b}
20:5ω3 (EPA)	Initial	0.6 ± 0.3	0.1 ± 0.02^{a}		
	High ω3	0.7 ± 0.3	0.2 ± 0.1^{b}	33.3 ± 21.0	14.7 ± 9.2
	Med ω3	0.6 ± 0.2	0.2 ± 0.1^{b}	40.2 ± 20.8	17.7 ± 9.2
	Balanced	0.5 ± 0.2	0.1 ± 0.1^{ab}	45.2 ± 24.2	22.5 ± 17.8
	Med ω6	0.6 ± 0.3	0.2 ± 0.1^{b}	41.4 ± 25.1	18.2 ± 11.0
	High ω6	0.8± 0.4	0.2 ± 0.1^{b}	37.1 ± 25.5	19.3 ± 20.5
22:6ω3 (DHA)	Initial	2.3 ± 0.7^{ab}	0.2 ± 0.1^{a}		
	High ω3	2.6 ± 0.8^{ab}	0.7 ± 0.3^{b}	33.2 ± 19.5	14.6 ± 8.6
	Med ω3	2.1 ± 0.6^{ab}	0.6 ± 0.2^{b}	40.5 ± 17.8	17.8 ± 7.8
	Balanced	1.8 ± 0.7^{b}	0.5 ± 0.2^{ab}	45.3 ± 23.3	22.9 ± 19.0
	Med ω6	2.4 ± 1.1^{ab}	0.7 ± 0.3^{b}	36.9 ± 17.3	16.2 ± 7.6
	High ω6	2.9 ± 1.4^{a}	0.8 ± 0.4^{b}	34.6 ± 25.1	16.1 ± 14.0

[a] Mean (n=10-20) ± standard deviation. Different superscripts in the same column indicate significant differences among treatments at week 12, and between week 12 and week 0 (initial) ($p \leq 0.05$). [b] Fatty acid concentrations in mg per g of muscle wet weight. [c] Percent of original week 0 fatty acid at week 12 when no turnover had occurred, and when turnover rate is 24% $month^{-1}$ for 3 months.

Table S.2.5. Fatty acids concentrations and retention rates in Atlantic salmon liver[a] at week 0 (initial) and after 12 weeks of feeding the two extreme ω6:ω3 diets.

Fatty acid	Dietary treatment/time point	mg g^{-1} wet weight[b]	mg $liver^{-1}$	% of original (no turnover)[c]	% of original (24% $month^{-1}$)[c]
16:0	Initial	3.1 ± 1.4	7.8 ± 3.4[a]		
	High ω3	3.0 ± 1.4	18.3 ± 10.1[b]	55.5 ± 28.1	25.2 ± 14.1
	High ω6	2.8 ± 1.2	18.0 ± 6.6[b]	51.4 ± 24.5	22.5 ± 10.7
18:0	Initial	1.1 ± 0.5	2.9 ± 1.2[a]		
	High ω3	1.1 ± 0.5	6.6 ± 3.8[b]	56.5 ± 28.7	27.2 ± 17.3
	High ω6	1.1 ± 0.5	7.3 ± 3.0[b]	49.5 ± 27.2	21.7 ± 12.0
18:1ω9	Initial	4.1 ± 2.2[a]	10.2 ± 5.6		
	High ω3	3.0 ± 1.8[ab]	18.5 ± 12.4	69.2 ± 31.0	36.9 ± 24.6
	High ω6	2.1 ± 0.9[b]	13.3 ± 5.0	76.1 ± 19.1	41.1 ± 22.2
18:2ω6 (LNA)	Initial	1.4 ± 0.6[a]	3.5 ± 1.5[a]		
	High ω3	1.3 ± 0.7[a]	8.4 ± 5.1[a]	55.7 ± 29.0[a]	26.9 ± 17.7[a]
	High ω6	2.8 ± 1.2[b]	17.6 ± 6.5[b]	23.4 ± 11.3[b]	10.3 ± 4.9[b]
18:3ω3 (ALA)	Initial	0.1 ± 0.04[a]	0.3 ± 0.1[a]		
	High ω3	1.6 ± 0.9[b]	10.1 ± 6.5[b]	3.9 ± 2.6[a]	1.7 ± 1.1[a]
	High ω6	0.2 ± 0.1[a]	1.2 ± 0.4[a]	26.8 ± 12.3[b]	11.7 ± 5.4[b]
20:4ω6 (ARA)	Initial	0.8 ± 0.3[a]	2.0 ± 0.8[a]		
	High ω3	0.3 ± 0.2[b]	2.0 ± 1.2[a]	86.2 ± 19.3[a]	57.3 ± 29.8[a]
	High ω6	0.8 ± 0.4[a]	4.9 ± 2.8[b]	52.8 ± 29.5[b]	25.3 ± 17.1[b]
20:5ω3 (EPA)	Initial	0.9 ± 0.3[ab]	2.2 ± 0.9[a]		
	High ω3	1.2 ± 0.6[a]	7.2 ± 4.1[b]	42.8 ± 25.5	19.2 ± 12.5
	High ω6	0.7 ± 0.3[b]	4.4 ± 1.7[a]	57.7 ± 24.2	26.3 ± 12.5
22:6ω3 (DHA)	Initial	4.6 ± 1.6	11.6 ± 3.9[a]		
	High ω3	4.8 ± 2.2	29.6 ± 16.8[b]	51.5 ± 27.5	23.3 ± 13.5
	High ω6	4.5 ± 1.9	28.7 ± 11.0[b]	48.0 ± 22.9	21.1 ± 10.1

[a] Mean (n=9-20) ± standard deviation. Different superscripts in the same column indicate significant differences among treatments at week 12, and between week 12 and week 0 (initial) ($p \leq 0.05$). [b] Fatty acid concentrations in mg per g of liver wet weight. [c] Percent of original week 0 fatty acid at week 12 if no turnover had occurred, and if turnover had occurred at 24% $month^{-1}$ for 3 months.

Table S.2.6. Mean relative contribution (%) and 95% confidence intervals of dietary 18:3ω3 to EPA and DHA in the liver[a] of Atlantic salmon after 12 weeks of feeding the two extreme ω6:ω3 diets. Calculated using the SIAR Bayesian isotopic mixing model.

Fatty acid	**High ω3**		**High ω6**	
20:5ω3 (EPA)	89.5	$78.6 < \bar{X} < 99.9$	47.1	$40.3 < \bar{X} < 54.0$
22:6ω3 (DHA)	41.0	$33.8 < \bar{X} < 48.0$	23.9	$14.2 < \bar{X} < 33.1$

[a] Mean relative contribution and 95% confidence intervals (n = 8). Discrimination factor of 0.4‰ (n = 8).

Chapter 3. Influence of varying dietary ω6 to ω3 fatty acid ratios on the hepatic transcriptome, and association with phenotypic traits (growth, somatic indices, and tissue lipid composition), in Atlantic salmon (*Salmo salar*)

Preface

A version of the study described in Chapter 3 was published in the journal *Biology*. Katan, T., Xue, X., Caballero-Solares, A., Taylor, R. G., Parrish, C. C., and Rise, M. L. (2021). Influence of varying dietary ω6 to ω3 fatty acid ratios on the hepatic transcriptome, and association with phenotypic traits (growth, somatic indices, and tissue lipid composition), in Atlantic salmon (*Salmo salar*). *Biology* 10, 578-599

3.1. Abstract

The importance of dietary omega-6 to omega-3 (ω6:ω3) fatty acid (FA) ratios for human health has been extensively examined. However, its impact on fish physiology, and the underlying molecular mechanisms, are less well understood. This study investigated the influence of plant-based diets (12-week exposure) with varying ω6:ω3 (0.4–2.7) on the hepatic transcriptome of Atlantic salmon. 44K microarray analysis identified genes involved in immune and inflammatory response (*lect2a*, *itgb5*, *helz2a*, *p43*), lipid metabolism (*helz2a*), cell proliferation (*htra1b*), control of muscle and neuronal development (*mef2d*) and translation (*eif2a*, *eif4b1*, *p43*), that were differentially expressed between the two extreme ω6:ω3 dietary treatments (high ω6 *vs*. high ω3) at week 12. Eight out of 10 microarray-identified transcripts showed an agreement in the direction of expression fold-change between the microarray and qPCR studies. The PPARα activation-related transcript *helz2a* was confirmed by qPCR to be down-regulated by high ω6 diet compared with high ω3 diet. The transcript expression of two *helz2* paralogues was positively correlated with ω3, and negatively with ω6 FA in both liver and muscle, thus indicating their potential as biomarkers of tissue ω6:ω3 variation. *Mef2d* expression in liver was suppressed in the high ω6 compared to the balanced diet (ω6:ω3 of 2.7 and 0.9, respectively) fed fish, and showed negative correlations with ω6:ω3 in both tissues. The hepatic expression of two *lect2* paralogues was negatively correlated with viscerosomatic index, while *htra1b* correlated negatively with salmon weight gain and condition factor. Finally, *p43* and *eif2a* were positively correlated with liver Σω3, while these transcripts and *eif4b2* showed negative correlations with 18:2ω6 in the liver. This suggested that some

aspects of protein synthesis were influenced by dietary ω6:ω3. In summary, this nutrigenomic study identified hepatic transcripts responsive to dietary variation in ω6:ω3, and relationships of transcript expression with tissue (liver, muscle) lipid composition and other phenotypic traits.

3.2. Introduction

Plant-based oils are commonly used in aquafeeds to replace fish oil (FO), due to decreasing global availability, rising market price, and concerns regarding the ecological sustainability of the finite fishery resources upon which FO production depends (Wilberg and Miller, 2007; Naylor et al., 2009). Indeed, plant oils (PO) were shown to be more economical and environmentally sustainable (Turchini et al., 2009), and their inclusion as alternatives to FO in many experimental diets did not affect the growth and survival of farmed Atlantic salmon (*Salmo salar*) (Bransden et al., 2003; Tocher, 2010; Liland et al., 2013). However, terrestrial oils are devoid of long-chain polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA, 20:5ω3), docosahexaenoic acid (DHA, 22:6ω3), and arachidonic acid (ARA, 20:4ω6), which are abundant in FO. These LC-PUFA have important functions in vertebrate health, reproduction, neural development, and growth, among other biological processes (Tocher, 2010, 2015). This has resulted in decreased fillet EPA and DHA levels in farmed fish that were fed with PO as a partial or full replacement for FO, compromising their nutritional quality for human consumers (Alhazzaa et al., 2011; Calder, 2015; Sprague et al., 2016). Further, previous studies reported impacts on fish health and physiology with the dietary replacement of FO by PO (e.g. liver steatosis, altered complement pathway and phagocytic activity, and modulated expression of genes involved in immune response; Montero et al., 2003; Ruyter et al., 2006; Jordal et al., 2007; Liland, 2014; Caballero-Solares et al., 2017). Another concern is that most terrestrial oils used in aquafeeds, and the farmed seafood consuming them, may not provide adequate ratios of ω6 to ω3 (ω6:ω3) fatty acids (FA) due to high ω6 FA content

(Pickova and Mørkøre, 2007; Weaver et al., 2008; Young, 2009; Sprague et al., 2016). Previous human nutrition studies reported that high dietary ω6:ω3 promotes the pathogenesis of many diseases, including cardiovascular, inflammatory, autoimmune, and cognitive, as well as obesity and cancer (Simopoulos, 2003, 2008; Wijendran and Hayes, 2004; Gómez Candela et al., 2011). An optimal ratio of ω6:ω3 is important for maintaining the homeostasis of many biological processes such as cell apoptosis, inflammation, fatty acid and cholesterol metabolism, and others (de Pablo and De Cienfuegos, 2000; Wymann and Schneiter, 2008; Duan et al., 2014). However, the underlying molecular mechanisms are still poorly understood in fish, and it is not known which genes are involved in variation in dietary and tissue ω6:ω3 in salmon fed high levels of terrestrial-based oils.

In the previous Chapter, a feeding trial was performed to examine the impact of five plant-based diets with varying ω6:ω3 on salmon growth, tissue (i.e. muscle, liver) lipid composition, liver LC-PUFA synthesis, and transcript expression (targeted qPCR) of lipid metabolism and eicosanoid synthesis-related genes (Katan et al., 2019). The objective of the current study was to utilize a 44K salmonid oligonucleotide microarray (Jantzen et al., 2011; Sahlmann et al., 2013; Xue et al., 2015) for the examination of the impact of the two extreme ω6:ω3 diets (i.e. high ω6 and high ω3) on the hepatic transcriptome at week 12. I hypothesized that salmon fed the two diets with the most extreme lipid compositions (i.e. High ω3 and High ω6) would show the most extensive transcriptomic differences. The current study used the same fish as in the previous Chapter. The aim was to identify novel biomarker genes and molecular pathways that are altered by variation in ω6:ω3. To aid in the elucidation of the relationships between liver transcripts and phenotypic traits (i.e.

growth parameters, somatic indices, tissue FA and lipid class composition), correlation analyses were also performed.

3.3. Materials and Methods

3.3.1. Fish and experimental diets

Five experimental diets with varying ratios of ω6:ω3 were formulated and manufactured by Cargill Innovation Center (Dirdal, Norway). The diets had ω6:ω3 of 1:3 (high ω3), 1:2 (medium ω3), 1:1 (balanced), 2:1 (medium ω6) and 3:1 (high ω6). Dietary formulations and their lipid composition were shown in Chapter 2. However, as they pertain to the current study, the formulation and lipid profiles of the relevant diets (i.e. high ω3, balanced, and high ω6) are also included in this Chapter (Tables 3.1 and 3.2). All diets contained the same sources and equal levels of marine and plant proteins, but had different mixes of plant-based oils [i.e. linseed (flax), soy, and palm]. All diets were formulated to be isonitrogenous and isoenergetic (Table 3.1), and to meet the nutritional requirements of salmonids [National Research Council (NRC), 2011].

Atlantic salmon pre-smolts were transported from Northern Harvest Sea Farms (Stephenville, NL, Canada) in October 2015, and held in the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, Memorial University of Newfoundland, Canada) in 3800 l tanks. After their arrival, fish were graded in order to select the most uniform population, and this was followed by PIT (Passive Integrated Transponder; Easy AV, Avid Identification Systems, Norco, CA, USA)-tagging for individual identification.

Table 3.1. Formulation and nutrient composition (%) of experimental diets[a] fed to Atlantic salmon. This diet information was shown in Chapter 2. However, it is included here as this information is pertinent to the current study as well.

Ingredient (%)[b]	High ω3	Balanced	High ω6
Marine protein[c]	26.7	26.7	26.7
Plant protein[d]	44.6	44.6	44.6
Additives[e]	2.6	2.6	2.6
Fish oil	4.8	4.8	4.8
Linseed oil	16.7	8.3	0
Soy oil	0	9.8	19.6
Palm oil	4.6	3.2	1.7
Digestible energy (MJ Kg^{-1})	20.4	20.4	20.4
Digestible protein (g Kg^{-1})	421	421	421
Dry matter (%)[f]	94.8	94.8	94.8
Ash (%)[f]	6.2	6.0	6.2

[a] Means were calculated using 3 pellets per diet (n=3).

[b] All ingredients were sourced from Cargill Innovation stocks.

[c] Marine protein is comprised of fish meal.

[d] Plant protein concentrate is a proprietary blend of soy, corn and wheat.

[e]Additives are proprietary mineral, vitamin mix and amino acids. Mineral and vitamin composition is proprietary information to Cargill Innovation.

[f] Analysed as % of wet weight (n = 3).

Table 3.2. Lipid and FA composition (%) of experimental diets[a] fed to Atlantic salmon. This diet information was shown in Chapter 2. However, it is included here as this information is pertinent to the current study as well.

	High ω3	Balanced	High ω6
Lipid class composition (% of total lipid)			
TAG[b]	80.0	82.9	76.9
Sterol	3.1	3.4	1.9
AMPL[c]	4.5	1.6	6.4
Phospholipid	2.6	3.3	2.7
Total lipids (mg g^{-1} wet weight)	189	215	206
FA composition (% of total FAs)			
14:0	1.7	1.5	1.6
16:0	13.7	13.0	13.2
16:1ω7	1.7	1.7	1.7
18:0	2.9	3.0	3.0
18:1ω7	1.0	1.2	1.4
18:1ω9	20.1	19.6	19.4
18:2ω6 (LNA)	12.7	24.6	36.2
18:3ω3 (ALA)	29.8	18.9	6.4
20:1ω9	2.5	2.5	2.6
20:4ω6 (ARA)	0.1	0.1	0.1
20:5ω3 (EPA)	2.6	2.6	2.6
22:6ω3 (DHA)	3.2	3.2	3.2
Σ SFA[d]	19.1	18.5	19.0
Σ MUFA[e]	29.8	29.5	29.8
Σ PUFA[f]	50.9	51.9	51.0
Σ ω3	37.1	26.2	13.7
Σ ω6	13.0	24.9	36.5
ω6:ω3	0.4	0.9	2.7
DHA/EPA	1.3	1.2	1.3
EPA/ARA	20.7	22.9	20.5
EPA+DHA (% of wet weight)[g]	1.0	1.1	1.3

[a] Mean (n=3). [b] Triacylglycerol. [c] Acetone mobile polar lipid. [d] Total saturated fatty acids. [e] Total monounsaturated fatty acids. [f] Total polyunsaturated fatty acids. [g] EPA+DHA analysed as % of dietary wet weight. Fatty acids that showed the largest range among diets are bolded.

Then, post-smolts (203 ± 24 g mean initial weight ± SE; 27 ± 0.12 cm mean initial fork length ± SE) were randomly distributed into twenty 620 l tanks (40 fish $tank^{-1}$), and subjected to a 2.5-week acclimation period. After the completion of the acclimation period, fish were switched from the commercial diet (Nutra Transfer NP, 3 mm, Skretting Canada, St. Andrews, NB, Canada), and fed with the experimental diets (4 tanks $diet^{-1}$) for 12 weeks. The photoperiod was maintained at 24 h light. Fish were fed overnight using automatic feeders, and apparent feed intake was recorded throughout the trial. Mortalities were also recorded during the trial (< 1% at week 12). For additional details regarding the rearing conditions and recordings, refer to Chapter 2.

3.3.2. Sample collection

Growth performance parameters (e.g. fork-length, weight, organ indices) were measured at the beginning and the end of the 12-week feeding trial (Chapter 2). At the end of the trial, salmon were starved for 24 h, and then 5 fish per tank were euthanized with an overdose of MS-222 (400 mg l^{-1}; Syndel Laboratories, Vancouver, BC, Canada) and dissected for tissue collection. For gene expression analyses, liver samples (50-100 mg) were collected in 1.5 ml nuclease-free tubes, flash-frozen in liquid nitrogen, and stored at −80°C until RNA extractions were performed. Liver and muscle samples for lipid analyses, were collected, processed, and stored as described in Katan et al. (2019). Only liver samples from fish that showed weight gains within one standard deviation below and above the mean value of each tank were utilized for this study, in order to reduce biological variability in the gene expression data among fish. Tank means rather than dietary treatment means,

were chosen for sample selection, so that variability between tanks could be included in the statistical analysis.

3.3.3. RNA extraction, DNase treatment, column purification and cDNA synthesis

The TissueLyser system (at 25 Hz for 2.5 min) with 5 mm stainless steel beads, (QIAGEN, Mississauga, ON, Canada) was used to homogenize liver samples in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Samples were subjected to RNA extraction according to manufacturer's instructions. Due to low 260/230 ratios (i.e. 1.0-1.6) following TRIzol extraction, all RNA samples were then re-extracted (phenol-chloroform) and precipitated following standard methods (Xu et al., 2013). This was followed by DNaseI treatment and column purification using RNase-free DNase Set and RNeasy Mini Kit (QIAGEN). All procedures were conducted according to manufacturer instructions, and as described in Xue et al. (2015). RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity and quantity were assessed by NanoDrop UV spectrophotometry (NanoDrop, Thermo Scientific, Mississauga, ON, Canada). DNased and column-purified RNA samples had A260/280 and A260/230 ratios of 1.8 – 2.2. All cDNAs were synthesized by reverse transcription of 1 µg of DNaseI-treated, column-purified total RNA from each sample, with 1 µl of random primers (250 ng; Invitrogen), 1 µl of dNTPs (0.5 mM final concentration; Invitrogen), 4 µl of 5× first-strand buffer (1× final concentration; Invitrogen), 2 µl of DTT (10 mM final concentration; Invitrogen) and 1 µl of Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (200 U; Invitrogen) at 37°C for 50 min, following manufacturer instructions, and as described in

Xue et al. (2015). The total reaction volume was 20 µl. Finally, all cDNAs were diluted 40× with nuclease-free water (Invitrogen) prior to the qPCR.

3.3.4. Microarray hybridization and data acquisition

Eight fish (2 from each of the 4 dietary tanks) from each of the 2 extreme ω6:ω3 treatments (high ω6 or high ω3) were used in the microarray analysis (i.e. 16 fish total), using a common reference design. Four arrays were used in the current study, and each array contained 2 fish per treatment which were randomly selected. The common reference was made by an equal quantity of each DNase I-treated, column-purified total RNA liver sample. The microarray experiment was performed as described in Xue et al. (2015). Briefly, anti-sense amplified RNA (aRNA) was in vitro transcribed from 1 µg of each column-purified RNA or reference pooled RNA using Ambion's Amino Allyl MessageAmp II aRNA Amplification kit (Life Technologies), following manufacturer instructions. The quantity and quality of aRNA were assessed using NanoDrop spectrophotometry and 1% agarose gel electrophoresis, respectively. Then, 20 µg of each aRNA were precipitated overnight, following standard molecular biology procedures, and re-suspended in coupling buffer. Each individual aRNA sample was labeled with Cy5 (i.e. experimental samples), whereas the reference pool was labeled with Cy3 (i.e. common reference) fluor (GE HealthCare, Mississauga, ON, Canada), following manufacturer instructions. The “microarray” function of the NanoDrop spectrophotometer was used to measure the labeling efficiency of the aRNA. The labeled aRNA (825 ng) from each experimental sample (i.e. Cy5) was mixed with an equal quantity of labeled aRNA from the common reference (i.e. Cy3), for each array, and the resulting pool was fragmented

following manufacturer instructions (Agilent, Mississauga, ON, Canada). Each pool was co-hybridized to a consortium for Genomic Research on All Salmonids Project (cGRASP)-designed 4 x 44K salmonid oligonucleotide microarray (GEO accession # GPL11299) (Jantzen et al., 2011) (Agilent). Finally, the arrays were hybridized at 65°C for 17 h with rotation (10 rpm), using an Agilent hybridization oven. The microarray slides were washed immediately after hybridization as per the manufacturer's instructions.

Each microarray slide was scanned at 5 μm resolution with 90% laser power using a ScanArray Gx Plus scanner and ScanExpress v4.0 software (Perkin Elmer, Waltham, MA, USA), and the Cy3 and Cy5 channel photomultiplier tube (PMT) settings were adjusted to balance the fluorescence signal between channels. The resulting raw data were saved as TIFF images, and the signal intensity data were extracted using Imagene 9.0 (BioDiscovery, El Segundo, CA, USA). Removal of low-quality or flagged spots on the microarray, as well as $\log_2$-transformation and Loess-normalization of the data, were performed using R and the Bioconductor package mArray (Booman et al., 2011). Features absent in more than 25% (i.e. 4 out of 16 arrays) of the arrays were omitted, and the missing values were imputed using the EM_array method and the LSimpute package (Bø et al., 2004; Celton et al., 2010). The final dataset used for statistical analyses consisted of 10,264 probes for all arrays (GEO accession number: GSE139418; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139418).

3.3.5. Microarray data analysis

The Significance Analysis of Microarrays (SAM) algorithm (Tusher et al., 2001) was performed to identify genes that were significantly differentially expressed between

the two extreme ω6:ω3 treatments. A false discovery rate (FDR) threshold of 10% was used with the Bioconductor package siggenes (Schwender et al., 2006) in R. For the identification of additional transcripts that were differentially expressed between the two dietary treatments, the Rank Products (RP) method was also used, as this method is less sensitive to high biological variability (Breitling et al., 2004; Jeffery et al., 2006). The latter analysis was performed at percentage of false-positives (PFP) threshold of 10%, using the Bioconductor package RankProd (Hong et al., 2006). Due to high background signal in the first slide (i.e. slide # 11502), no genes were initially identified as significantly differentially expressed; therefore, this slide was removed from the analyses. In order to maximize our capacity to identify differentially expressed genes, gene lists were obtained with 2 and 3 of the remaining slides (consisting of 4 and 6 fish per treatment, respectively). Each slide is composed of 4 arrays, i.e., 4 biological replicates analyzed per slide.

The resulting gene lists were annotated using the contiguous sequences (contigs) that were used for the design of the 60mer oligonucleotide probes of the array (Jantzen et al., 2011). Annotations were performed manually with a BLASTx alignment against the NCBI non-redundant (nr) amino acid database using an E-value threshold of 10^{-5}. The best BLASTx hits corresponding to putative *Homo sapiens* orthologues were used to obtain gene ontology (GO) terms manually from the UniProt Knowledgebase (http://www.uniprot.org/, accessed on 4 November 2020).

3.3.6. qPCR study and data analysis

Transcript expression levels of 10 genes of interest (GOI) (Table 3.3), identified as differentially expressed in the microarray analyses, were assayed by qPCR.

Table 3.3. qPCR primers

Gene name (symbol)[a]	Nucleotide sequence (5' - 3')[b]	Amp. effic. (%)[c]	Amplicon size (bp)	GenBank accession number
Serine protease HTRA1 a (*htra1a*)[d]	F:GCTGATGTGGTGGAGGAGAT	113.3	127	NM001141717
	R:TCAAGCCGTCCTCTGACAC			
Serine protease HTRA1 b (***htra1b***)[d]	F:ATGATGACTCTCACACCAATGC	95.4	104	EG831192
	R:GTTTTTGGGATGACCTCGATT			
Aminoacyl tRNA synthase complex-interacting multifunctional protein 1 (***p43***)	F:GGAAGACGAATGCAGAGGAC	97.2	82	BT044000
	R:GGAGCGGTCATTCACACTTT			
Eukaryotic translation initiation factor 2A (***eif2a***)	F:TAAACCCAGATGCCCTTGAG	94.9	143	NM001140088
	R:GGCTTTCAGCTCGTCGATAG			
Eukaryotic translation initiation factor 4B 1 (***eif4b1***)	F:CGCAGGGACCGGGATGAT	85.3	123	BT072661
	R:TCGGTCCTC5CTGTCCGC			
Eukaryotic translation initiation factor 4B 2 (*eif4b2*)	F:CACATCCAGGAAGTACCTCT	87.4	94	DY739566
	R:TCGTCCTCCTTACCGCTGA			
Cytochrome c oxidase subunit 2 (***mtco2***)[e]	F:CACCGATTACGAAGACTTAGGC	107.9	136	DW554935
	R:TGAAACTAGGACCCGGATTG			
Leukocyte cell-derived chemotaxin 2 precursor a (***lect2a***)[d]	F:CAGATGGGGACAAGGACACT	94.6	150	BT059281
	R:GCCTTCTTCGGGTCTGTGTA			
Leukocyte cell-derived chemotaxin 2 precursor b (*lect2b*)[d]	F:ACAACTGGGGACAAGGACAG	84.8	125	DV106130
	R:CACTTTGCCGTTGAGTTTCA			
60S ribosomal protein L18 (***rpl18***)	F:AGTTCCACGACTCGAAGATC	93.8	143	DW535031
	R:TTTTATTGTGCCGCACAAGGT			
Myocyte-specific enhancer factor 2D (***mef2d***)	F:GCAGCAACATCAACAACAGC	89.5	160	XM014177143
	R:CTCATCTCTACCCAAGAGGA			
Helicase with zinc finger domain 2 a (***helz2a***, alias *pric285a*)[f]	F:GCAAGGTTGGGTATGAGGAA	91.3	149	BT072427
	R:TTCGGAGTTGCTCCAGTCTT			
Helicase with zinc finger domain 2 b (*helz2b*, alias *pric285b*)[f]	F:AGACGTAGTGGTTCGGATCG	82.0	145	EG928625
	R:GACCGTGATTTCGTCCAGTT			
Integrin beta-5-like (***itgb5***)[g]	F:CCTGCCAGCGGCTATGCAA	94.1	147	DW540995;
	R:AGGACTGACATGCCGTTGG			XM014165323
Elongation factor 1 alpha-2 (*eef1α-2*)[h]	F:GCACAGTAACACCGAAACGA	86.4	132	BG933853
	R:ATGCCTCCGCACTTGTAGAT			
60S ribosomal protein 32 (*rpl32*)[h]	F:AGGCGGTTTAAGGGTCAGAT	96.1	119	BT043656
	R:TCGAGCTCCTTGATGTTGTG			

[a] Bolded gene symbols refer to microarray-identified transcripts. [b] F is forward and R is reverse primer. [c] Amplification efficiency (%) [d] Primers that were previously published in Caballero-Solares et al. (2018). [e] The *Salmo salar* sequence of *mtco2* used in the qPCR assay showed 87% identity with the 60mer microarray probe (C060R108) affiliated with rainbow trout (*Oncorhynchus mykiss*). [f] Primers that were previously published in Caballero-Solares et al. (2017) (annotated as VHSV-induced protein

in that study). Alias *pric285* stands for peroxisomal proliferator-activated receptor A interacting complex 285. [g] The Atlantic salmon sequences of *itgb5* used in the qPCR assay showed 86% identity with the 60mer microarray probe (C002R106) affiliated with rainbow trout. Primers were designed based on common regions between DW540995 and XM014165323. [h] Primers that were previously published in Katan et al. (2019) and shown in Chapter 2. Normalizer genes are underlined.

In addition to the high ω6 and high ω3 treatments, the qPCR analysis also included liver samples from fish fed the balanced diet. In addition to the microarray-identified GOI, BLASTn searches using publicly available Atlantic salmon cDNA sequences [i.e. in NCBI's non-redundant nucleotide (nt) and expressed sequence tags (EST) databases] were used to identify paralogues for each GOI, as described in Caballero-Solares et al. (2018).

Paralogue-specific primers were used for *eif4b*, *htra1*, *lect2* and *helz2* (Table S.3.1 and Figures S.3.1 to S.3.4). The sequences of the primer pairs used in qPCR, GenBank accession number of sequences used for primer design, and other details are presented in Table 3.3. Notably, primers for the transcript *lhpl4* (GenBank accession number NM_001146670) failed quality testing, and thus this transcript was not included in the qPCR study. In addition, the 60mer microarray probe for *mtco1* (C188R069) is affiliated with a rainbow trout sequence, and had relatively low identity (i.e. <85%) with available *Salmo salar* sequences (using NCBI's EST and nt databases), and therefore was excluded from the qPCR study. The program Primer 3 (http://frodo.wi.mit.edu) was used for primer design. Each primer pair was quality-tested, including standard curve and dissociation curve to ensure that a single product was amplified with no primer dimers (Rise et al., 2010; Booman et al., 2011). Primer pairs were quality-tested using the 7500 Fast Real Time PCR system (Applied Biosystems/Life Technologies, Foster City, CA, USA). The amplification efficiency (Pfaffl, 2001) of each primer pair was determined using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of input total RNA. Two pools were generated (i.e. high ω3 pool and high ω6 pool), with each pool consisting of 8 fish (and each fish contributing an equal quantity to the pool). The reported primer pair amplification efficiencies are an average of the two pools, except if one pool showed poor efficiency or

spacing (i.e. *p43*, *eif2a*, *htra1a*, *helz2a* and *helz2b*, where one pool was used due to low expression levels). A 5-point 1:2 dilution series was used for the primers *mtco2* and *helz2b* as these transcripts had lower expression levels [fluorescence threshold cycle (C_T) values of ~ 30 and 31, respectively). Furthermore, amplicons were checked by 1.5% agarose gel electrophoresis and compared with the 1 kb plus DNA Ladder (Invitrogen) to ensure that the correct size fragment was amplified.

To select the most suitable normalizer genes, six candidate normalizers were tested based on our previous qPCR studies (*rpl32*, *actb*, *eef1α-1*, *eef1α-2*, *abcf2*, *pabpc1*) (Xue et al., 2015; Caballero-Solares et al., 2017)], and salmon literature on reference genes (*actb*, *eef1α-1*, *eef1α-2*) (Olsvik et al., 2005). Their qPCR primers were quality-tested as described above. Then, their C_T values were measured using cDNA (corresponding to 5 ng of input total RNA) of 6 randomly selected fish per treatment (18 total). The geNorm algorithm (Vandesompele et al., 2002) was used to analyze their expression stability. *Rpl32* and *eef1α-2* were shown to be the most stable (i.e. geNorm M-values of 0.30 and 0.25, respectively) among the 6 candidate reference genes, and therefore were selected as normalizers.

All PCR amplifications were performed in a total reaction volume of 13 µl and consisted of 4 µl of cDNA (5 ng input total RNA), 50 nM each of forward and reverse primer and 1× Power SYBR Green PCR Master Mix (Applied Biosystems), and nuclease-free water (Invitrogen). The qPCR reactions, including no-template controls, were performed in technical triplicates using the ViiA 7 Real-Time PCR System (384-well format) (Applied Biosystems) and the Power SYBR Green I dye chemistry. The Real-Time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min,

followed by 40 cycles (of 95°C for 15 s and 60°C for 1 min), with the fluorescence signal data collection after each 60°C step. When a C_T value within a triplicate was greater than 0.5 cycles from the other two values, it was considered to be an outlier, discarded and the average C_T of the remaining two values was calculated. The relative quantity (RQ) of each transcript was calculated using ViiA 7 Software v1.2 (Applied Biosystems) for Comparative C_T ($\Delta\Delta C_T$) analysis (Livak and Schmittgen, 2001), with primer amplification efficiencies incorporated (Table 3.3). The expression levels of each GOI were normalized to both normalizer genes, and the sample with the lowest normalized expression was used as the calibrator sample (i.e. RQ = 1.0) for each GOI, as in (Rise et al., 2015). Transcript expression data are presented as RQ values (mean ± SE) relative to the calibrator.

3.3.7. Statistical analyses

3.3.7.1. qPCR data

A general linear model with tank nested in diet, followed by a Tukey pairwise comparison ($p < 0.05$) was used to identify significant differences among dietary treatments at week 12. In cases where significant tank effect was observed ($p < 0.05$), a one-way ANOVA followed by a Tukey pairwise comparison post-hoc tests was performed (Minitab 17 Statistical Software, State College, PA, USA). The RQ data are presented as mean ± SE. Each dietary treatment group was tested for outliers using Grubb's test ($p < 0.05$). In total, 9 RQ values were identified as statistical outliers in the entire dataset (i.e. out of 322 values), and excluded from the study. Each GOI had a minimum of 6 samples per dietary treatment, while most GOI had a sample size of 7-8 per dietary treatment. The qPCR fold-changes were calculated by dividing the mean RQ value of the high ω6 fish by that of the

high ω3 fish. Finally, residuals were tested to verify normality, independence, and homogeneity of variance. Normality was examined using the Anderson-Darling test. If the test failed ($p < 0.05$), a one-way ANOVA on ranks was performed, and was followed by Kruskal-Wallis test (SigmaPlot, Systat Software, Inc., Version 13, San Jose, CA, USA). In all cases, differences were considered statistically significant when $p < 0.05$.

3.3.7.2. Correlation analyses of qPCR and lipid composition data

Tissue lipid composition (muscle and liver) and growth performance of salmon fed varying ω6:ω3 diets were shown in Chapter 2. Pearson correlation analyses were performed in the current study to identify the relationships between hepatic transcript expression (i.e. qPCR data), tissue composition (i.e. % FA and lipid classes), and growth parameters [i.e. weight gain (WG), condition factor (CF)], using individual fish. All GOI in the qPCR study were used in the correlation analysis in order to identify differences between the liver and muscle tissue. Only ω3 and ω6 FA that accounted for > 0.5% of the total FA in the tissue (average of each treatment) were included in the analyses. Furthermore, hierarchical clustering was used to group transcripts and lipid composition [using group average in PRIMER (PRIMER-E Ltd., version 6.1.15, Ivybridge, UK)]. IBM SPSS Statistics was used for the correlation analyses.

3.4. Results

3.4.1. Liver microarray analysis

RP analysis detected 9 differentially expressed features (PFP < 10%; Table 3.4). Eight of these features (i.e. *lhpl4*, *htra1b*, *mtco2*, *lect2a*, *rpl18*, *helz2a*, *itgb5*, and *mtco1*)

were identified analyzing data from 2 slides (slides # 11504-11505; comprising 4 fish per treatment), and one (i.e. *mef2d*) was identified analyzing data from 3 slides (slides # 11503-11505; comprising 6 fish per treatment). Two features (i.e. *lhpl4* and *htra1b*) showed higher expression in the high ω6 fish (4.78- and 3.57-fold change, respectively), while the other 7 RP-identified features (i.e. *mtco2*, *lect2a*, *rpl18*, *mef2d*, *helz2a*, *itgb5*, and *mtco1*) showed down-regulation in the high ω6 fish (fold-change ranged from -3.27 to -7.11).

SAM analysis identified *p43*, *eif2a*, *eif4b1*, and *itgb5* as differentially expressed genes (FDR < 10%) between the high ω6 and high ω3 fed salmon, using 3 slides (slides # 11503-11505) (Table 3.4). These genes were down-regulated in the high ω6 compared with the high ω3 fed fish (fold-change values ranged from -2.79 to -5.12). One feature (*itgb5*) was represented in both SAM and RP analysis, and was down-regulated in the high ω6 compared to the high ω3 fed fish, in both analyses (-5.12 and -5.25- fold-change, respectively).

Putative identities were determined for the 12 microarray-identified features, and functional annotations (i.e. GO terms) were collected for them (Table 3.4). The microarray-identified gene *lhpl4* (4.78-fold up-regulated) is involved in the nervous system, with GO annotations "regulation of inhibitory synapse assembly" and "GABA receptor binding". The feature *htra1b* (3.57-fold up-regulated) was classified as a gene involved in cell proliferation, and showed the functional annotations "positive regulation of epithelial cell proliferation", "proteolysis" and "extracellular space" (Table 3.4).

Table 3.4. Microarray-identified transcripts that were significantly differentially expressed in the liver of salmon fed high ω6 compared to high ω3 diet.

Probe ID[a]	BLASTx identification[b]				Gene ontology (GO) of putative human orthologues[d]	Fold-change[e]
	Best named BLASTx hit [species][c]	Accession no.	E-value	% ID (AA)		
C187R103	Lipoma HMGIC fusion partner-like 4 protein (*lhpl4*) [*Salmo salar*]*	NP_001140142	0	272/272 (100%)	BP: regulation of inhibitory synapse assembly, gamma-aminobutyric acid receptor clustering. MF: protein binding, GABA receptor binding. CC: inhibitory synapse, postsynaptic membrane, cell projection, plasma membrane, cell junction.	4.78
C231R170	Serine protease HTRA1 (*htra1b*) [*Salvelinus alpinus*]	XP_023864611	4e-171	248/256 (97%)	BP: proteolysis, extracellular matrix disassembly, negative regulation of transforming growth factor beta receptor signaling pathway, negative regulation of defense response to virus, positive regulation of epithelial cell proliferation. MF: serine-type endopeptidase and peptidase activity, insulin-like growth factor binding, hydrolase activity. CC: collagen-containing extracellular matrix, extracellular space, plasma membrane, cytoplasm.	3.57
C103R052	Aminoacyl tRNA synthase complex-interacting multifunctional protein 1 (*p43*) [*Salmo trutta*]	XP_029622221	0	321/326 (98%)	BP: inflammatory response, apoptotic process, response to wounding, tRNA aminoacylation for protein translation, defense response to virus, leukocyte migration, angiogenesis, chemotaxis, positive regulation of glucagon secretion. MF: RNA binding, tRNA binding, protein binding, cytokine activity, protein homodimerization activity. CC: aminoacyl-tRNA synthetase multienzyme complex, nucleus, cytosol, endoplasmic reticulum, extracellular region.	-2.79
C067R040	Eukaryotic translation initiation factor 2A (*eif2a*) [*Salmo salar*]	NP_001133560	0	576/576 (100%)	BP: translational initiation, ribosome assembly, protein phosphorylation, SREBP signaling pathway, response to amino acid starvation. MF: translation initiation factor activity, cadherin binding, ribosome binding, tRNA binding, protein binding. CC: blood microparticle, extracellular space, cytosolic small ribosomal subunit.	-3.13
C253R093	Eukaryotic translation initiation factor 4B (*eif4b1*) [*Salvelinus alpinus*]	XP_023852969	6e-11	37/40 (93%)	BP: translational initiation, eukaryotic translation initiation factor 4F complex assembly. MF: RNA binding, protein binding, translation initiation factor activity, RNA strand annealing activity. CC: polysome, cytosol, eukaryotic translation initiation factor 4F complex.	-3.23
C060R108	Cytochrome c oxidase subunit 2 (*mtco2*) [*Oncorhynchus masou masou*]	ASB29545	7e-74	115/182 (63%)	BP: electron transport chain, oxidation-reduction process. MF: cytochrome-c oxidase activity, copper ion binding, metal ion binding, oxidoreductase activity. CC: membrane, respirasome, mitochondrion.	-3.27
C159R112	Leukocyte cell-derived chemotaxin 2 precursor (*lect2a*)[*Salmo salar*]	ACI67916	6e-102	155/156 (99%)	BP: chemotaxis, skeletal system development. MF: protein binding, metal ion binding. CC: cytoplasm, extracellular space.	-3.48
C152R057	60S ribosomal protein L18 (*rpl18*) [*Salmo trutta*]	XP_029599741	3e-122	172/173 (99%)	BP: translation, viral transcription, SRP-dependent cotranslational protein targeting to membrane. MF: structural constituent of ribosome, protein binding, RNA binding. CC: ribosome, cytosolic large ribosomal subunit, cytosol.	-4.37

C133R018	Myocyte-specific enhancer factor 2D (*mef2d*) [*Oncorhynchus mykiss*]	XP_021427816	3e-70	193/193 (100%)	BP: positive regulation of vascular smooth muscle cell proliferation, muscle organ development, skeletal muscle and neuronal cell differentiation, apoptotic process, positive regulation of transcription by RNA polymerase II, adult heart development, nervous system development. MF: DNA-binding transcription factor activity, RNA polymerase II-specific, protein binding, histone deacetylase binding, protein heterodimerization activity. CC: nucleus, nuclear chromatin, intracellular membrane-bounded organelle, nucleoplasm.	-4.54
C065R088	Helicase with zinc finger domain 2 (*helz2a* alias, *pric285a*) [*Salmo trutta*]	XP_029548942	0	694/714 (97%)	BP: regulation of lipid metabolic process, positive regulation of transcription by RNA polymerase II, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay. MF: nuclear receptor transcription activity, helicase activity, ribonuclease activity, hydrolase activity, RNA binding, ATP binding, protein binding, metal ion binding. CC: nucleus, membrane, nucleoplasm.	-4.71
C002R106 *	Integrin beta-5-like (*itgb5*) [*Oncorhynchus mykiss*]	XP_021453113	0	283/315 (90%)	BP: cell adhesion mediated by integrin, integrin-mediated signaling pathway, muscle contraction, antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent, viral process, transforming growth factor beta receptor signaling pathway. MF: protein binding, signaling receptor activity, virus receptor activity. CC: cell surface, extracellular exosome, phagocytic vesicle, plasma membrane, integrin complex.	-5.12
C188R069	Cytochrome c oxidase subunit 1 (*mtco1*) [*Oncorhynchus tshawytscha*]*	NP_148940	0	410/437 (94%)	BP: oxidation-reduction process, oxidative phosphorylation, electron transport chain, aerobic respiration. MF: oxidoreductase activity, cytochrome-c oxidase activity, heme binding, metal ion binding. CC: mitochondrial inner membrane, respiratory chain complex IV, respirasome.	-7.11

[a] Refers to the identity of the probe on the 44K array. Probes that are shown in bold font are features that were identified by SAM (FDR < 10%), and the remaining features were identified by RP analysis (PFP < 10%). Probe with an asterisk represents a feature that was identified in both SAM and RP analysis. Two 4x 44K array slides (slides # 11504-11505; representing 4 fish per treatment) were used in the RP analysis. However, the RP-identified *mef2d* was obtained using 3 slides (slides # 11503-11505; representing 6 fish per treatment). SAM-identified features were obtained using three slides (slides # 11503-11505). [b] Genes were identified by BLASTx, using the contig from which the microarray probe was designed against the NCBI non-redundant database. The best BLASTx hit with E-value < 10^{-5} and an informative protein name was used, and presented with species name, GenBank accession number, E-value and % amino acid (AA) identity. [c] All microarray-identified genes, with the exception of *lhpl4* and *mtco1*, were quantified by qPCR (see Materials and Methods). [d] Gene Ontology (GO) terms were selected from putative *Homo sapiens* orthologues (i.e. best BLASTx hit). Representative GO terms were identified (i.e. redundancies were not included), and divided into the categories: biological process (BP), molecular function (MF) and cellular component (CC). [e] Fold-change values between the 2 dietary treatments (high ω6/high ω3) for each of the significant microarray features. Down-regulated transcripts are shown with negative values [- (1/fold-change)]. The SAM- and RP-identified *itgb5* showed fold-changes of -5.12 and -5.25, respectively.

Several informative microarray features represented genes involved in translation, such as *p43*, *eif2a*, *eif4b1*, and *rpl18* (-2.79 to -4.37-fold down-regulated), with associated GO terms "tRNA binding", "aminoacyl-tRNA synthetase multienzyme complex", "translational initiation", "ribosome assembly", and "structural constituent of ribosome". Furthermore, the GO terms "defense response to virus", "inflammatory response" and "response to wounding" were also identified with *p43*. The features *mtco2* and *mtco1* (-3.27- and -7.11-fold down-regulated, respectively) were classified as mitochondrion respiratory chain components, and showed the GO terms "electron transport chain", "oxidation-reduction process" and "cytochrome-c oxidase activity". Other microarray-identified features corresponded to immune- and inflammation-related genes such as *lect2a* (with the GO terms "chemotaxis" and "metal ion binding") and *itgb5* ("antigen processing and presentation" and "phagocytic vesicle"), showed down-regulation in the high ω6 fed fish (-3.48 to -5.25-fold-change, respectively). The gene *mef2d* (-4.54-fold down-regulated) is involved in muscle cell proliferation, and in neuronal cell differentiation and survival, with associated GO terms "muscle organ development", "skeletal muscle cell differentiation" "nervous system development", "apoptotic process" and "DNA-binding transcription factor activity" (Table 3.4). Further, the microarray-identified feature *itgb5* was associated with the GO term "muscle contraction". Finally, the gene *helz2a* (-4.71-fold down-regulated) was classified as a gene involved in lipid metabolism regulation by peroxisome proliferator-activated receptor alpha (PPARα), and showed the functional annotations "regulation of lipid metabolic process", "nuclear receptor transcription activity", "ATP binding", "metal ion binding", "hydrolase activity", and "ribonuclease activity".

3.4.2. qPCR study

Ten microarray-identified genes were used in the qPCR study. All genes with the exception of *mtco2* and *rpl18* showed an agreement in the direction of expression fold-change (i.e. up- or down-regulation) between the microarray and qPCR studies (Table 3.5). The microarray-identified *helz2a* showed significantly lower transcript expression in the high ω6 compared to the high ω3 fed fish (-1.49-fold; p = 0.04). The paralogue *helz2b* showed significantly lower expression in both the high ω6 and balanced groups compared to the high ω3 fed fish (-1.61-fold; p = 0.002). The transcript *mef2d* showed significantly lower expression in the high ω6 compared to the balanced fed fish (-1.27-fold; p = 0.03), and lower expression trend in the high ω6 compared to the high ω3 fish (-1.22-fold; p = 0.06). Both paralogues of *htra1* were numerically higher (although not statistically significant) in the high ω6 compared to the balanced and high ω3 fish (1.34-1.57-fold and 3.75-2.09-fold; p = 0.25 and 0.07, respectively) (Table 3.5).

3.4.3. Correlations between hepatic qPCR transcript expression and liver lipid composition

Hierarchical clustering of the qPCR transcripts showed 4 separate clusters (Figures 3.1 and 3.2). The first cluster consisted of both paralogues of *htra1*. The second cluster comprised *rpl18* only. The third cluster included some of the immune- and inflammation-related transcripts such as *lect2*, *p43*, and *helz2a*, as well as *mef2d*, *eif2a*, *eif4b*, and *mtco2*. The transcripts *helz2b* and *itgb5* composed the fourth cluster. Cluster analysis of liver lipid composition and somatic indices showed 4 clusters (Figure 3.1).

Table 3.5. Hepatic qPCR analysis of microarray-identified transcripts, and comparison between the microarray and qPCR results.

Microarray probe[a]	Transcript name	qPCR RQ values[b]			p-value (qPCR)[c]	Fold-change[d]	
		High ω3	Balanced	High ω6		44K	qPCR
N/A	*htra1a*	2.2 ± 0.41	1.9 ± 0.29	3.0 ± 0.65	0.25	N/A	1.34
C231R170	*htra1b*	6.0 ± 2.14	10.7 ± 3.56	22.4 ± 7.43	0.07	3.57	3.75
C103R052	*p43*	3.4 ± 0.66	2.9 ± 0.41	2.2 ± 0.47	0.24	-2.79	-1.59
C067R040	*eif2a*	5.2 ± 0.40	5.3 ± 0.74	3.5 ± 0.92	0.19	-3.13	-1.47
C253R093	*eif4b1*	8.8 ± 1.78	6.7 ± 1.43	5.5 ± 1.53	0.29	-3.23	-1.59
N/A	*eif4b2*	2.7 ± 0.30	2.7 ± 0.40	2.2 ± 0.30	0.55	N/A	-1.22
C060R108	*mtco2*	1.4 ± 0.10	1.2 ± 0.06	1.4 ± 0.14	0.43	-3.27	1.07
C159R112	*lect2a*	7.6 ± 2.58	4.0 ± 1.20	4.2 ± 0.96	0.38	-3.48	-1.79
N/A	*lect2b*	3.4 ± 0.74	3.7 ± 0.89	3.8 ± 0.61	0.96	N/A	1.12
C152R057	*rpl18*	2.0 ± 0.17	2.1 ± 0.25	2.2 ± 0.16	0.88	-4.37	1.08
C133R018	*mef2d*	1.9 ± 0.10[ab]	2.0 ± 0.11[a]	1.5 ± 0.13[b]	0.03	-4.54	-1.22
C065R088	*helz2a*	2.3 ± 0.32[a]	1.6 ± 0.15[ab]	1.5 ± 0.14[b]	0.04	-4.71	**-1.49**
N/A	*helz2b*	2.3 ± 0.21[a]	1.4 ± 0.09[b]	1.4 ± 0.11[b]	0.002	N/A	**-1.61**
C002R106	*itgb5*	2.1 ± 0.23	1.9 ± 0.08	1.6 ± 0.08	0.13	-5.25	-1.34

[a] Refers to the identity of the probe on the 44K array. Transcripts with no probe ID are paralogues of microarray-identified transcripts. [b] Mean relative quantity (RQ) ± standard error (n=6–8). RQ values were normalized to *elongation factor 1 alpha-2* (*eef1α-2*) and *60S ribosomal protein 32* (*rpl32*), and calibrated to the lowest expressing individual for each gene of interest. Different letters indicate significant differences among treatments (General linear model followed by Tukey pairwise comparison). [c] p-values obtained in the qPCR study. Differences were considered statistically significant when $p < 0.05$. [d] Microarray and qPCR comparison of fold-changes (i.e. high ω6/high ω3). Down-regulated transcripts are negative values [- (1/fold-change]. qPCR fold-changes corresponding to GOI with significant differences between the high ω6 and high ω3 treatments are bolded.

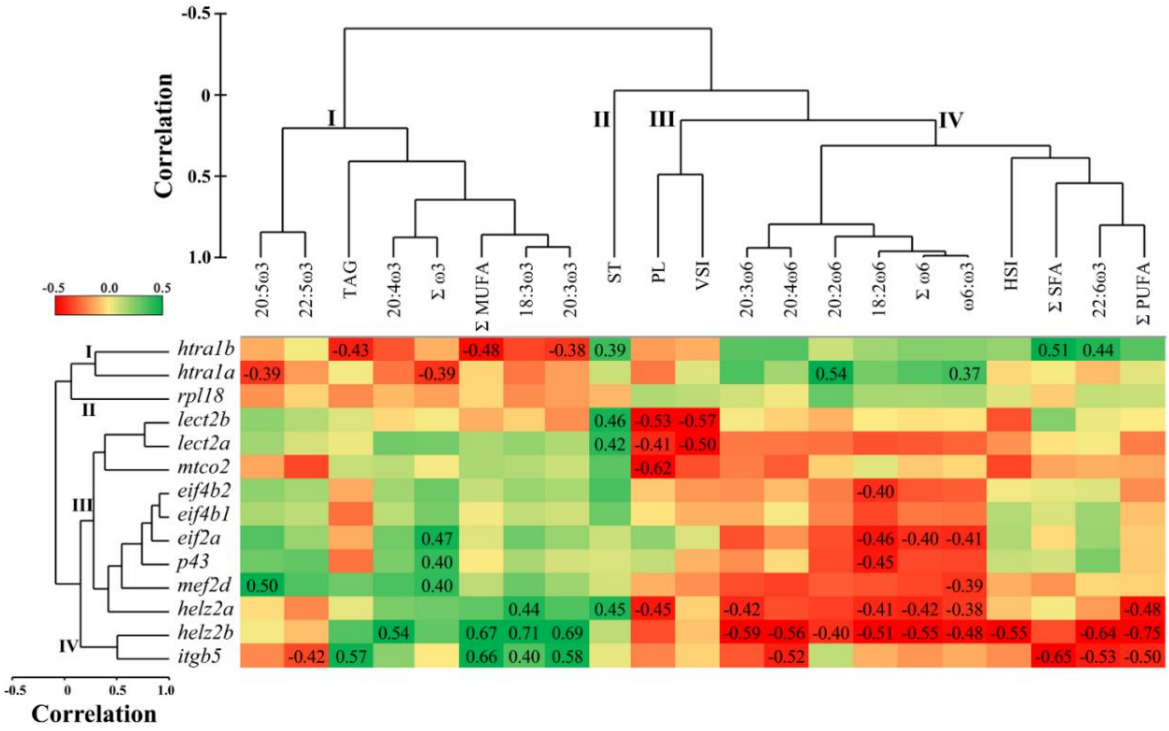

Figure 3.1. Pearson correlation matrix and hierarchical clustering of liver transcript expression [qPCR relative quantity values (RQ)], liver lipid composition, and somatic indices in Atlantic salmon fed diets with varying ω6 to ω3 fatty acid ratios. Correlation coefficients were described when correlations were statistically significant ($p < 0.05$). Red signifies negative and green signifies positive relationships. ΣSFA, ΣMUFA, and ΣPUFA represents total saturated, monounsaturated and polyunsaturated fatty acids, respectively. 20:5ω3, 22:6ω3, and 20:4ω6 represent EPA, DHA, and ARA, respectively. TAG, ST and PL represent triacylglycerols, sterols, and phospholipids, respectively. HSI and VSI represent hepatosomatic and viscerosomatic indices, respectively.

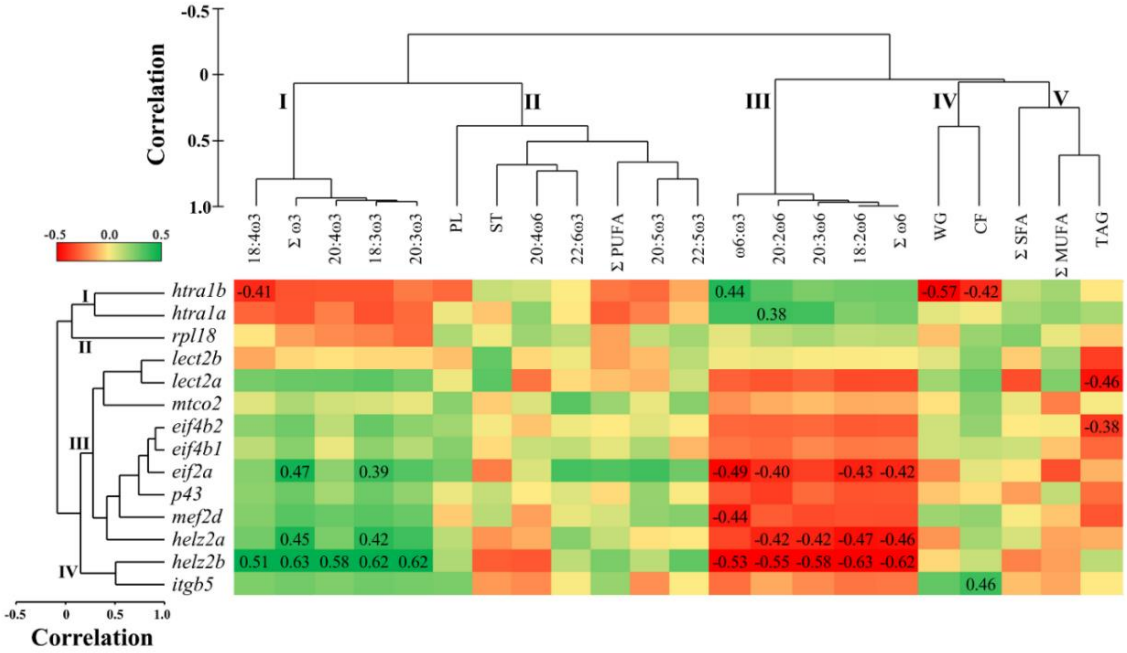

Figure 3.2. Pearson correlation matrix and hierarchical clustering of liver transcript expression [qPCR relative quantity values (RQ)], muscle lipid composition, and growth in Atlantic salmon fed diets with varying ω6 to ω3 fatty acid ratios. Correlation coefficients were described when correlations were statistically significant ($p < 0.05$). Red signifies negative and green signifies positive relationships. ΣSFA, ΣMUFA, and ΣPUFA represent total saturated, monounsaturated and polyunsaturated fatty acids, respectively. 20:5ω3, 22:6ω3, and 20:4ω6 represent EPA, DHA, and ARA, respectively. TAG, ST and PL represent triacylglycerols, sterols, and phospholipids, respectively. WG and CF represent, weight gain and condition factor, respectively.

Cluster one consisted of the ω3 FA: 18:3ω3, 20:3ω3, 20:4ω3, 20:5ω3, 22:5ω3, as well as the sums of ω3 (Σω3) and monounsaturated FA (ΣMUFA), and the lipid class triacylglycerols (TAG). The lipid class sterols (ST) represented cluster two, while total phospholipids (PL) segregated with viscerosomatic index (VSI) in cluster three. Cluster four consisted of the ω6 FA: 18:2ω6, 20:2ω6, 20:3ω6, 20:4ω6, Σω6, and the ratio ω6:ω3; in addition, 22:6ω3, the sums of PUFA (ΣPUFA) and saturated fatty acids (ΣSFA), and the hepatosomatic index (HSI) were associated with this cluster.

The hepatic transcript expression of *htra1b* was negatively correlated with TAG, ΣMUFA, and 20:3ω3, and positively with ST, ΣSFA, and 22:6ω3 (p = 0.009–0.043; Figure 3.1). *Htra1a* showed negative correlation with 20:5ω3 and Σω3, and positive with 20:2ω6, and ω6:ω3 (p = 0.006– 0.047). Both paralogues of *lect2* were correlated negatively with PL and VSI, and positively with ST (p = 0.004–0.032). Transcript expression of *mtco2* was negatively correlated with PL (p = 0.001), while that of *eif4b2* was negatively correlated with 18:2ω6 (p = 0.036; Figure 3.1). Both *eif2a* and *p43* transcript expression correlated negatively with 18:2ω6 (p = 0.019 and 0.021, respectively) and positively with Σω3, whereas *eif2a* alone correlated negatively with Σω6 and ω6:ω3 (p=0.037 and 0.033, respectively). *Mef2d* was correlated negatively with ω6:ω3 and positively with 20:5ω3 (p =0.042, and 0.011, respectively), while the three transcripts *eif2a, p43*, and *mef2d* showed positive correlations with Σω3 (p = 0.016–0.037). Furthermore, both paralogues of *helz2* correlated negatively with ω6 PUFA (i.e. 18:2ω6, 20:3ω6, Σω6), ω6:ω3, and ΣPUFA, and positively with 18:3ω3 (p = 0.0001–0.047). However, *helz2b* had negative correlations with additional ω6 (i.e. 20:2ω6, 20:4ω6; p = 0.038 and 0.004, respectively), and positive correlations with ω3 PUFA (i.e. 20:3ω3, 20:4ω3; p = 0.0001 and 0.005, respectively). Also,

helz2b correlated negatively with 22:6ω3 and HSI, and positively with ΣMUFA (p = 0.0001–0.005). In contrast, *helz2a* correlated negatively with PL, and positively with ST (p = 0.021). Finally, *itgb5* had negative correlations with 22:5ω3, 20:4ω6, ΣSFA, 22:6ω3, and ΣPUFA, and positive correlations with TAG, ΣMUFA, 18:3ω3, and 20:3ω3 (p =0.001–0.034; Figure 3.1).

3.4.4. Correlations between hepatic qPCR transcript expression and muscle lipid composition

Muscle tissue lipid composition and growth showed 5 separate clusters (Figure 3.2). Cluster one consisted of the ω3 FA: 18:3ω3, 18:4ω3, 20:3ω3, 20:4ω3, and Σω3. Cluster two included ΣPUFA, and the LC-PUFA: 20:5ω3, 22:5ω3. 22:6ω3, and 20:4ω6; Furthermore, the lipid classes ST and PL were grouped in cluster two. Cluster three grouped the ω6 FA: 18:2ω6, 20:2ω6, 20:3ω6, Σω6 and the ratio ω6:ω3. Cluster four showed the growth parameters (i.e. WT and CF), while cluster five grouped TAG, ΣSFA and ΣMUFA.

The hepatic transcript expression of *htra1b* was correlated negatively with muscle 18:4ω3 and growth parameters WG and CF, and positively with ω6:ω3 (p = 0.004–0.037), while that of *htra1a* showed positive correlation with 20:2ω6 (p = 0.049; Figure 3.2). The transcript expression of *lect2a* and *eif4b2* was negatively correlated with TAG (p = 0.021 and 0.049, respectively). *Eif2a* was correlated negatively with muscle ω6 FA (i.e. 18:2ω6, 20:2ω6, Σω6) and ω6:ω3, and positively with ω3 FA (18:3ω3 and Σω3) (p = 0.014–0.045; Figure 3.2). *Mef2d* was negatively correlated with ω6:ω3 (p = 0.026). Further, both paralogues of *helz2* were negatively correlated with ω6 FA (18:2ω6, 20:2ω6, 20:3ω6, Σω6), and positively correlated with ω3 (i.e. 18:3ω3 and Σω3) (p = 0.001–0.032). *Helz2b* alone

correlated negatively with ω6:ω3, and positively with 18:4ω3, 20:3ω3, and 20:4ω3 (p = 0.002-0.01). Finally, *itgb5* showed positive correlation with CF (p = 0.024).

3.4.5. Overlapping lipid-gene correlations between the liver and muscle analyses

Some significant correlations showed an overlap between the liver and muscle analyses (Figure 3.3). The hepatic transcript expression of *htra1a* was correlated positively with 20:2ω6 in both tissues. The expression of *eif2a* was correlated positively with Σω3, and negatively with 18:2ω6, Σω6 and ω6:ω3, while that of *mef2d* showed negative correlations with ω6:ω3 in both liver and muscle. The transcript expression of *helz2a* was correlated positively with 18:3ω3, and negatively with 18:2ω6, 20:3ω6 and Σω6, while that of *helz2b* correlated positively with 18:3ω3, 20:3ω3 and 20:4ω3, and negatively with 18:2ω6, 20:2ω6, 20:3ω6, Σω6 and ω6:ω3 (Figure 3.3). The hepatic transcript expression of most genes (all except *helz2b* with 20:4ω3) showed stronger positive correlations with liver compared to muscle FA. However, negative correlations were mostly (all except *eif2a* with 18:2ω6, and both *helz2* paralogues with 20:3ω6) more significant with muscle compared with the liver FA (Figure 3.3).

		18:3ω3	20:3ω3	20:4ω3	Σ ω3	20:2ω6	18:2ω6	20:3ω6	ω6	ω6:ω3
A	*htra1a*					0.54, 0.38				
	eif2a				0.47, 0.47		-0.46, -0.43		-0.40, -0.42	-0.41, -0.49
	mef2d									-0.39, -0.44
	helz2a	0.44, 0.42					-0.41, -0.47	-0.42, -0.42	-0.42, -0.46	
	helz2b	0.71, 0.62	0.69, 0.62	0.54, 0.58		-0.40, -0.55	-0.51, -0.63	-0.59, -0.58	-0.55, -0.62	-0.48, -0.53
B	*htra1a*					0.006, 0.049				
	eif2a				0.016, 0.019		0.019, 0.031		0.037, 0.033	0.033, 0.014
	mef2d									0.042, 0.026
	helz2a	0.023, 0.032					0.033, 0.019	0.031, 0.032	0.029, 0.021	
	helz2b	0.0001, 0.002	0.0001, 0.002	0.005, 0.004		0.038, 0.006	0.010, 0.002	0.003, 0.004	0.005, 0.002	0.015, 0.008

Figure 3.3. Overlapping Pearson correlations between the liver and muscle analyses. Liver transcript expression [qPCR relative quantity values (RQ)] of GOIs was correlated with liver and muscle lipid composition in Atlantic salmon fed diets with varying ω6 to ω3 fatty acid ratios. Statistically significant ($p < 0.05$) correlations are shown. Green and red cells signify positive and negative relationships, respectively. Upper panel shows correlation coefficients (A), and lower panel depicts p-values (B). Commas-separated values from the liver and muscle analyses, respectively (A, B).

3.5. Discussion

The microarray study indicated that dietary variation in ω6:ω3 resulted only in small changes in the liver transcriptome of salmon fed plant-based diets. This can partly be explained by the fact that growth performance and somatic indices were not significantly affected by diet (Chapter 2, published as Katan et al. 2019). It was previously shown that different replacements of FO with camelina oil had no impact on Atlantic cod (*Gadus morhua*) growth, and resulted in only one microarray-identified gene that showed a significant difference in spleen basal expression between treatments (Booman et al., 2014). Furthermore, the current results are in line with previous microarray studies, which demonstrated that dietary replacement of fish meal (FM) and FO with terrestrial ingredients, resulted in subtle gene expression changes in Atlantic salmon distal intestine (Brown et al., 2016), head-kidney (Eslamloo et al., 2017), and liver (Morais et al., 2011). However, Atlantic salmon fed soy and linseed oils showed large alterations in hepatic gene expression compared to those fed FO (Leaver et al., 2008). Differences in the numbers of responsive transcripts between Leaver et al. (2008) and the current study could be related to dietary lipid sources and studied time points.

Several transcripts that play important roles in immune and inflammatory response (*lect2a*, *itgb5*, *helz2a*, *p43*), lipid metabolism (*helz2a*), cell proliferation (*htra1b*), muscle and neuronal cell development (*mef2d*), and translation (*eif2a*, *eif4b1*, *p43*) were identified by the current microarray study as diet-responsive. All transcripts with the exception of *mtco2* and *rpl18* showed an agreement in the direction of expression fold-change between the microarray and the qPCR analyses (Table 3.5). The 60mer microarray probe

representing the transcript *mtco2*, which was designed using a rainbow trout cDNA sequence, showed only 87% similarity (see Materials and Methods) with the *Salmo salar* cDNA sequence that was used in the qPCR study (and other *S. salar* sequences in NCBI databases). This fact, as well as other limitations (e.g. mRNA regions targeted by the qPCR primers and microarray probe may not be the same; possibility of contig misassembly) could have contributed to the disagreement between microarray and qPCR results (Booman et al., 2011).

Hepatic *helz2a* showed a significant differential expression between the high ω6 and high ω3 fed fish in the microarray experiment, and both paralogues of this transcript (i.e. *helz2a* and *helz2b*) were significantly down-regulated in the high ω6 compared to the high ω3 fed fish in the qPCR analysis. Interestingly, the transcript expression of *helz2b* was positively correlated with ω3 (i.e. 18:3ω3, 20:3ω3, 20:4ω3), and negatively with ω6 PUFA (i.e. 18:2ω6, 20:2ω6, 20:3ω6, 20:4ω6), Σω6 and ω6:ω3, in the liver tissue (Figure 3.1). In the muscle tissue, these PUFA (with the exception of 20:4ω6) were also correlated with hepatic *helz2b* expression (Figure 3.2). These data suggest that *helz2* is a potential novel molecular biomarker of tissue variation in ω6:ω3. The protein encoded by this gene is a nuclear transcriptional co-activator for PPARα (Surapureddi et al., 2002; Katano-Toki et al., 2013; Yoshino et al., 2014), which is a master regulator of numerous genes involved in lipid metabolism processes (e.g. FA oxidation, and metabolism of bile acids, triacylglycerols, and retinoids) (Kersten, 2014). Additionally, HELZ2 was shown to have an antiviral function in mammals (Fu and Blackshear, 2017; Fusco et al., 2017), and its gene was identified as an ancestral (between mammals and fish) interferon stimulated gene (ISG) with conserved components of antiviral immunity (Levraud et al., 2019). *Helz2*

transcripts [referred to as VHSV-induced protein (*vig1*)] showed up-regulation in the head kidney of Atlantic salmon exposed to the viral mimic polyriboinosinic polyribocytidylic acid (pIC) (Caballero-Solares et al., 2017). The negative correlation between liver *helz2b* and HSI is not surprising, given the involvement of PPARα in hepatic FA β-oxidation, and in liver steatosis (Kersten et al., 2000; Van Raalte et al., 2004). In addition, the observed positive correlations with ω3 PUFA are in line with the anti-inflammatory properties of PPARα (Van Raalte et al., 2004). In the previous Chapter, it was observed that the fatty acid binding protein-encoding transcript *fabp10* showed an upregulation trend ($p = 0.06$) in the high ω3 compared to the balanced and high ω6 fed fish. Thus, this suggests that the high ω3 diet may have influenced the transport of ω3 FA in liver cells, and played a role in the activation of PPARα. The interaction between liver fatty acid transport and PPARα activation has been shown in previous mammalian studies (Huang et al., 2002; Hostetler et al., 2009). Another potential mechanism that could explain the positive correlation between *helz2b* expression with ω3 PUFA, is that ω3 PUFA bind to PPARα with higher affinity than ω6 PUFA (Desvergne and Wahli, 1999). However, there is still a lack of knowledge on the interaction between dietary ω3 and ω6 PUFA, and the mechanisms by which they regulate PPARα activators (Schroeder et al., 2008).

Mef2d was identified in the microarray as down-regulated by the high ω6 diet, with qPCR showing significantly lower expression in the high ω6 compared to the balanced diet fed fish. Furthermore, hepatic *mef2d* expression was correlated positively with liver 20:5ω3 and Σω3, and negatively with the ratio of ω6:ω3 in both liver and muscle tissues. The gene *mef2*, characterized in zebrafish (*Danio rerio*) (Ticho et al., 1996) and common carp (*Cyprinus carpio*) (He et al., 2019), is involved in skeletal and cardiac muscle development

and differentiation, as well as in neuronal cell development (Black and Olson, 1998; Flavell et al., 2006, 2008; Haberland et al., 2007). Wei et al. (2016) reported a significant increase in skeletal muscle *mef2c* transcript expression in pigs fed with linseed-enriched (10%) as compared with a control diet (0%). Additionally, a study with Atlantic salmon revealed that feeding with a synthetic FA [i.e. tetradecylthioacetic acid (0.25%] compared to a control diet (0%) increased the cardiosomatic index, and the cardiac expression of *mef2c* (Grammes et al., 2012). In relation to the liver, previous studies showed that members of the MEF2 family regulate the activation of hepatic stellate cells –a type of cell involved in liver fibrosis– in mice (Xiaomeng et al., 2016) and rats (Wang et al., 2004), and ω3 PUFA inhibited the proliferation and activation of these cells in mouse liver (Zhang et al., 2016). Further, Wang et al. (2004) reported that *mef2d* was induced during hepatic stellate cells activation. These data may support the idea that the transcript *mef2* is responding to dietary FA (particularly ω3 PUFA) in vertebrates. However, as most studies examined the role of *mef2* expression in skeletal (Fuentes et al., 2013; Wei et al., 2016) and cardiac (Lien et al., 2006; Grammes et al., 2012) muscle development, the interactions between *mef2d* and liver physiology are less understood in fish. Future studies should investigate the influence of dietary ω6:ω3 on liver *mef2d* expression, and their interaction with hepatic stellate cells in fish.

Serine protease HTRA1-encoding transcript (*htra1b*) was up-regulated in the high ω6 compared to the high ω3 fed fish, in the microarray study. *Htra1b* showed a similar trend of higher expression in the high ω6 fed fish ($p = 0.07$) in the qPCR analysis, and a similar fold-change (i.e. high ω6/high ω3) in the microarray and qPCR studies (Table 3.5). Further, hepatic *htra1b* was positively correlated with ΣSFA, 22:6ω3 and ST, and

negatively with ΣMUFA, 20:3ω3 and TAG in the liver, while in the muscle it showed positive and negative correlations with ω6:ω3 and 18:4ω3, respectively. The transcript *htra1a* was positively correlated with 20:2ω6 and ω6:ω3, and negatively with ω3 PUFA (i.e. 20:5ω3 and Σω3) in the liver, and showed positive correlation with 20:2ω6 in the muscle. Serine protease HTRA1 function was linked to cell growth and apoptosis, as well as immune and inflammatory responses (by inhibiting the TGF-beta pathway) in mammalian tissues (e.g. eye, bone and liver) (Clausen et al., 2002; Oka et al., 2004; Zhu et al., 2010; Graham et al., 2013). It was previously shown that dietary FM replacement with terrestrial plant alternatives induced higher hepatic *htra1* transcript expression in Atlantic salmon (Tacchi et al., 2012). Conversely, replacing both dietary FM and FO by terrestrial plant alternatives down-regulated the transcription of *htra1a* and *htra1b* in Atlantic salmon liver (Caballero-Solares et al., 2018). Discrepancies between Caballero-Solares et al. (2018) and the present study extend to the FA-transcript correlation analyses; while in the present study the transcript expression of *htra1* paralogues correlated positively and negatively with tissue ω6 and ω3 FA levels, respectively, the opposite tendency was observed in Caballero-Solares et al. (2018). However, unlike the previous studies (Tacchi et al., 2012; Caballero-Solares et al., 2018), the current study tested different mixes of vegetable oils while keeping equal FM/FO inclusion levels across diets. Therefore, although the studies cannot be directly compared, such discrepancies suggest that the regulation of HTRA1-mediated processes in the liver of Atlantic salmon depends on the combination of protein and lipid sources included in the diet. Finally the negative correlation observed between *htra1b* and growth parameters (i.e. WG and CF) in the current study (Figure 3.2) is interesting, as mammalian HTRA1 was negatively linked to skeletal

muscle development and bone formation (Bakay et al., 2002; Graham et al., 2013; Tiaden and Richards, 2013).

The immune related microarray features *lect2a* and *itgb5* were down-regulated in the high ω6 compared to the high ω3 fed fish, and an agreement was observed in the direction of expression fold-change between the microarray and qPCR studies. LECT2 is a multifunctional protein that plays a role in cell growth, neutrophil chemotactic activity, and innate immune response against pathogens in fish (Kokkinos et al., 2005; Wei et al., 2011; Chen et al., 2014; Fu et al., 2014). LECT2 also function as a hepatokine that modulate the inflammatory response in mammals (Lan et al., 2014; Jung et al., 2018). Earlier microarray studies reported down-regulation of hepatic *lect2* in Atlantic salmon fed terrestrial as compared with marine diets (Xue et al., 2015; Caballero-Solares et al., 2018). This may indicate that pro-inflammatory plant-based diets suppress the constitutive transcript expression of hepatic *lect2*. The observed negative correlation between both paralogues of *lect2* and VSI suggests that *lect2* suppression is related to higher lipid deposition. However, the interaction between fat deposition and immune response is very complex, and requires further investigation in fish (Todorčević et al., 2010; Jung et al., 2018). Additionally, this study showed a positive correlation between the transcript expression of both paralogues of *lect2* and liver sterol content. Interestingly, a previous study reported down-regulation of hepatic *lect2* in Atlantic salmon fed cholesterol supplemented as compared with a non-supplemented plant-based diet, and this coincided with reduced plasma phytosterols (i.e. sitosterol and campesterol; Kortner et al., 2014). Although dietary sterol levels were not significantly different in the present study, liver sterol concentration did vary among treatments (Chapter 2). Clearly, more studies are required in order to elucidate the impact

of dietary and tissue cholesterol and phytosterols on the constitutive transcript expression of *lect2* in fish. Finally, the correlations observed in this Chapter between *itgb5* and liver FA are in line with the notion that FA can regulate the mRNA and protein levels of integrins and other adhesion proteins, in leukocytes and endothelial cells (Calder, 1998; Pompéia et al., 2000). Interestingly, European seabass (*Dicentrarchus labrax*) fed a plant-based diet showed a down-regulation in hepatic *Integrin beta-2* compared to those fed a marine diet (Geay et al., 2011), while a reduction in the ω6:ω3 ratio of human lung cancer cells resulted in a delayed adhesion, and down-regulation of *integrin-α2* (Xia et al., 2005). Taken together, these data suggest that the transcript expression of integrins may be impacted by dietary or tissue ω6:ω3.

Similar to *lect2a* and *itgb5*, the transcripts *p43*, *eif2a*, and *eif4b1* were down-regulated in the high ω6 fed fish in the microarray experiment, and they showed an agreement in the direction of dietary modulation with the qPCR study. The transcript expression of *p43*, *eif2a*, and *eif4b2* was negatively correlated with 18:2ω6, and both *p43* and *eif2a* were positively correlated with liver Σω3. Further, *eif2a* expression was positively correlated with 18:3ω3 and Σω3, and negatively correlated with 18:2ω6, 20:2ω6, Σω6 and ω6:ω3 in the muscle. The protein p43 is associated with a multi-tRNA synthetase complex, and regulates tRNA channeling in mammals (Ivakhno et al., 2004). In addition, p43 also encodes an apoptosis-induced cytokine, which regulates inflammation, wound healing, and angiogenesis (Ko et al., 2001; Park et al., 2002; Sang et al., 2005). Phosphorylation of the protein eIF4B was shown to stimulate translation in zebrafish (Le et al., 2012; Faught et al., 2019) and yeast (Sen et al., 2016). However, phosphorylation of eIF2A repressed translation in response to accumulation of misfolded proteins in the ER of

several fish species (Harding et al., 2000; Howarth et al., 2014; Kavaliauskis et al., 2016). Thus, changes in the expression patterns of *p43*, *eif2a* and *eif4b*, and the correlations observed with tissue lipid composition, suggest that some aspects of protein synthesis were influenced by dietary and tissue ω6:ω3. Previous mammalian studies demonstrated that translation is inhibited in apoptotic cells, and this was correlated with enhanced cleavage of the eukaryotic translation initiation factors eIF4B, eIF2, and others (Saelens et al., 2001; Morley et al., 2005). Thus, the fact that the high ω6 fed fish showed up-regulation of *htra1b* (Table 3.4) may suggest that apoptosis was associated with the observed modulation of translation-related transcripts (Table 3.4), and their correlations with tissue FA (Figures 3.1 and 3.2). However, as this microarray study did not identify other well-known apoptosis biomarkers (e.g. genes encoding caspases and Bcl-2 family members), this can only be postulated. Further, the stimulatory effects of ω3 PUFA on protein synthesis (Smith et al., 2011) could be another potential mechanism explaining the positive correlations observed between *p43*, *eif2a*, and liver ω3 FA. Research examining the impact of replacing FO/FM with plant-based diets on protein synthesis in salmonids has been contradictory. Some authors showed an induction (Tacchi et al., 2012), while others a suppression (Panserat et al., 2009) of these and other translation-related transcripts. Indeed, protein synthesis regulation in fish is a dynamic process, and is influenced by dietary formulations, genetic (Morais et al., 2011) and abiotic factors, protein requirement, growth, and the tissues examined (Houlihan and Carter, 2001; Kaushik and Seiliez, 2010).

3.6. Conclusions

This 44K microarray study demonstrated that high ω6 and high ω3 plant-based diets with varying ratios of ω6:ω3 (i.e. 2.7 and 0.4, respectively) resulted in a relatively low number of differentially expressed transcripts in salmon liver. However, the identified transcripts and/or their functional annotations suggested important roles in lipid metabolism (*helz2a*), cell proliferation (*htra1b*), immune and inflammatory response (*lect2a*, *itgb5*, *helz2a*, *p43*), control of muscle and neuronal cell development (*mef2d*), and translation (*eif2a*, *eif4b1*, *p43*). Two paralogues of *helz2* were down-regulated in the high ω6 compared to the high ω3 fed fish in the qPCR study. Significant positive correlations were observed between the hepatic transcript expression of *helz2b* and ω3 PUFA, while negative correlations were identified with ω6 PUFA and ω6:ω3, in both the liver and muscle tissues. This indicated that the PPARα activation-related transcript *helz2* is a potential novel molecular biomarker of tissue variation in ω6:ω3. Given these data and the importance of *helz2* as an ancestral vertebrate interferon stimulated gene, future studies should investigate the dietary ω6:ω3 impact on Atlantic salmon anti-viral response. The transcript *mef2d* was suppressed in the high ω6 compared to the balanced fed fish, and was negatively correlated with ω6:ω3 in both tissues. The present microarray study revealed that the upregulation of hepatic *htra1b* concurred with the suppression of immune- and inflammatory-related transcripts (i.e. *lect2a*, *p43*, *helz2a*, *helz2b*, and *itgb5*). This supported the idea proposed by other researchers (Tacchi et al., 2012; Caballero-Solares et al., 2018) of a link between the dietary modulation of *htra1* and that of immune-related transcripts. Finally, the transcripts *p43*, *eif2a*, and *eif4b1* were significantly down-regulated in the high

ω6 compared to the high ω3 fed fish in the microarray, and showed an agreement in the direction of expression fold-change between the microarray and qPCR studies. These data, along with the significant correlations observed between *p43*, *eif2a* and *eif4b2* expression and tissue PUFA, suggested that the molecular regulation of protein synthesis in the liver may have been impacted by dietary ω6:ω3.

3.7. References

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3.8. Supplemental material

Table S.3.1. Paralogues of genes involved in the qPCR study, and their identity (%)[a] over the aligned nucleotide regions.

Transcript name (symbol)	GenBank Accession no.	Aligned region (bp)	ID (%)[a]	E-value
Serine protease HTRA1 precursor a (*htra1a*)	NM001141717	591	91	0
Serine protease HTRA1 precursor b (*htra1b*)	EG831192			
Eukaryotic translation initiation factor 4B 1 (*eif4b1*)	BT072661	756	82	0
Eukaryotic translation initiation factor 4B 2 (*eif4b2*)	DY739566			
Leukocyte cell-derived chemotaxin 2 precursor a (*lect2a*)	BT059281	531	88	0
Leukocyte cell-derived chemotaxin 2 precursor b (*lect2b*)	DV106130			
Helicase with zinc finger domain 2 a (*helz2a*, alias *pric285a*)	BT072427	828	67	1e-60
Helicase with zinc finger domain 2 b (*helz2b*, alias *pric285b*)	EG928625			

[a] Percent identity over the aligned nucleotide regions.

```
                      1                                                50
                        1                                                50
htra1a_NM001141717      (1) CAACAAAGCTCATTTTAGTGAAAAGCGTCTGACATTTATTTGCAAATGTA
   htra1b_EG831192      (1) --------------------------------------------------
                           51                                               100
htra1a_NM001141717     (51) TGTAACGTTTATTTATGCAGATCAAACATATTTTTGTAGCAAGTCGTAAA
   htra1b_EG831192      (1) --------------------------------------------------
                          101                                               150
htra1a_NM001141717    (101) GTTTTAAGAGTCGTTTTTTTTGGCAACTTTGTACCTTTTTAGATACTTTA
   htra1b_EG831192      (1) --------------------------------------------------
                          151                                               200
htra1a_NM001141717    (151) ATATTTAACAATGTTTTGGTGCGTCTTCTGCGCAACTTTTATTCTTGCTC
   htra1b_EG831192      (1) --------------------------------------------------
                          201                                               250
htra1a_NM001141717    (201) CTTTAGTTTGCGAGTCAAGAGCCAAGCGATATGTCATCGGCTGTCCAGAT
   htra1b_EG831192      (1) --------------------------------------------------
                          251                                               300
htra1a_NM001141717    (251) AAATGTGACAAATTTCTATGTCCCCCGATCCCTGCGGACTGTTTGGCCGG
   htra1b_EG831192      (1) --------------------------------------------------
                          301                                               350
htra1a_NM001141717    (301) CGACATCCTTGACCAATGCGACTGCTGTCCGGTCTGTGCGCACGGAGAAG
   htra1b_EG831192      (1) --------------------------------------------------
                          351                                               400
htra1a_NM001141717    (351) GTGAGGTGTGCGGCGGCACGGGGAGACTAGGGGACCCGGAGTGCGGAGAG
   htra1b_EG831192      (1) --------------------------------------------------
                          401                                               450
htra1a_NM001141717    (401) GGCATGGACTGCTCGATATCGGACGGAATTGGGGTGTCCGCCACAGTAAG
   htra1b_EG831192      (1) --------------------------------------------------
                          451                                               500
htra1a_NM001141717    (451) GCGTCGGGGCAAAAACGGTGTGTGCGTCTGCAAAGTTGCGGACCCGGTGT
   htra1b_EG831192      (1) --------------------------------------------------
                          501                                               550
htra1a_NM001141717    (501) GCGGCAGTGACGGGGTGTCCTACCGAAACATCTGCGAACTGAAGAGATTG
   htra1b_EG831192      (1) --------------------------------------------------
                          551                                               600
htra1a_NM001141717    (551) AGTAACCGGGCTCTGAAGCTTCAGCAGCCACCGGTCATCTTCATACAGAG
   htra1b_EG831192      (1) --------------------------------------------------
                          601                                               650
htra1a_NM001141717    (601) AGGAACCTGTAGCAAAGGCCAGGAGAATCCAGACAGTCTGCGCCACAGAT
   htra1b_EG831192      (1) --------------------------------------------------
                          651                                               700
htra1a_NM001141717    (651) ATAACTTCATCGCTGATGTGGTGGAGGAGATCGCTCCCGCTGTGGTTCAT
   htra1b_EG831192      (1) --------------------------------------------------
                          701                                               750
htra1a_NM001141717    (701) ATTGAACTTTACCGCAAGATGGTGTTCTCTAAGCGTGAGGTGGCGGTGGC
   htra1b_EG831192      (1) --------------------------------------------------
                          751                                               800
htra1a_NM001141717    (751) CAGCGGGTCTGGCTTCGTGGTGTCAGAGGACGGCTTGATTGTGACCAACG
   htra1b_EG831192      (1) --------------------------------------------------
                          801                                               850
htra1a_NM001141717    (801) CCCACGTGGTGGCCAATAAGCACCGGGTGAAGGTGGAGCTGAAGAGTGGC
   htra1b_EG831192      (1) --------------------------------------------------
                          851                                               900
htra1a_NM001141717    (851) GCCACCTTCGACGCCAAGATCACAGACGTGGACGAGAAGGCAGACATTGC
   htra1b_EG831192      (1) --------------------------------------------------
                          901                                               950
htra1a_NM001141717    (901) CCTCATCAAGATCGACACCCCGATGAAACTGCCGGTGCTGCTGCTGGGTC
   htra1b_EG831192      (1) --------------------------------------------------
                          951                                              1000
htra1a_NM001141717    (951) GTTCAGCTGACCTGAGGCCTGGTGAGTTTGTTGTGGCCATCGGCAGCCCC
   htra1b_EG831192      (1) ------------------GGGGGAGTTTGTGGTGGCCATCGGCAGCCCC
                         1001                                              1050
```


htra1a_NM001141717 (1001) TTCTCCCTGCAGAACACGGTCACCACAGGTATCGTCAGCACCACCCAAAG
htra1b_EG831192 (32) TTCTCCCTGCAGAACACGGTCACCACAGGTATCGTCAGCACCACCCAGAG
1051 1100
htra1a_NM001141717 (1051) AGGAGGCAAGGAGCTTGGCCTGAGGAACTCTGATATGGAATACATCCAGA
htra1b_EG831192 (82) AGGAGGCAAGGAGCTGGGCCTGAGGAACTCTGATATGGACTACATCCAGA
1101 1150
htra1a_NM001141717 (1101) CGGACGCTATCATCAACGAAGGAAGTGTAGATCTACCATCTGATCATCCC
htra1b_EG831192 (132) CGGACGCTATCATCAAC----------T------
1151 1200
htra1a_NM001141717 (1151) TTTAACCTGAATGCTGTTCCTTTTCAGTATGGGAACTCTGGCGGACCCCT
htra1b_EG831192 (150) ----------ATGGGAACTCTGGCGGACCCCT
1201 1250
htra1a_NM001141717 (1201) GGTCAATCTGGACGGAGAGGTGATTGGGATCAACACACTGAAGGTGACAG
htra1b_EG831192 (172) GGTCAATCTGGATGGAGAGGTGATTGGGATCAACACACTGAAGGTGACAG
1251 1300
htra1a_NM001141717 (1251) CAGGAATCTCCTTCGCCATCCCCTCAGACAAGATCCGTCAGTTCTTGGCA
htra1b_EG831192 (222) CAGGAATCTCCTTCGCCATCCCCTCAGACAAGATCCGTCAGTTCCTGGCA
1301 1350
htra1a_NM001141717 (1301) GAGTCCCACGGCAGACAATCTAAAGGTAGA----------
htra1b_EG831192 (272) GAGTCCCATGACAGGCAATCTAAAGGTACAGATTACCAATCTAAAGGGAA
1351 1400
htra1a_NM001141717 (1331) -TTATTACCAAAGAAGAAGTATATCGGTGTGAGGATGATGACTCTCACTA
htra1b_EG831192 (322) ATTATCAACAAGTAAGAAATATATCGGTGTGAGGATGATGACTCTCACAC
1401 1450
htra1a_NM001141717 (1380) CAACGCTTGCAAAGGAGCTGAAGGAGAGAACATCAGACTTCCCTGATGTT
htra1b_EG831192 (372) CAATGCTTGCAAAGGAGCTGAAGGAGAGACAATCAGACTTCCCCGATGTT
1451 1500
htra1a_NM001141717 (1430) ACCTCAGGGGCATATGTCATCGAGGTCATCCCAAAAACACCAGCTGAGAC
htra1b_EG831192 (422) ACCTCAGGGGCATATGTAATCGAGGTCATCCCAAAAACACCAGCTGAGAC
1501 1550
htra1a_NM001141717 (1480) AGGTGGCCTGCAGGAGAGTGACGTCATAATCACCATCAACAGCCAGCGAA
htra1b_EG831192 (472) AGGTGGCCTGCAGGAGAGTGATGTCATAATCACCATCAACAGCCAGCGAA
1551 1600
htra1a_NM001141717 (1530) TCACCTCGGCCAGTGATGTCAGCAGCTCTATCAAGAGGGACGACACGCTG
htra1b_EG831192 (522) TCACCTCAGCCAGTGACGTCAGCAGCTCCATCAAGAGGGAAGACACGCTG
1601 1650
htra1a_NM001141717 (1580) CGAATGGTGGTCCGGCGGGGGAACGAGGACATCATGCTCACCGTCGTCCC
htra1b_EG831192 (572) CGCATGGTGGTCCGGCGGGGGAACGAGGACGTCATGCTCACCGTCGTCCC
1651 1700
htra1a_NM001141717 (1630) CGAGGACATTGACCCTTGACCTCTCAGCAACCACGAGCTGGTTCTCAGTG
htra1b_EG831192 (622) AGAGGAGATTGACCCTTGACCTCTCAGCAACCACGAGCTGGTTCTCAGTG
1701 1750
htra1a_NM001141717 (1680) TTTAAAACCACGGAC-TTAAACCGGTTGTGTGTCTGGATCCACACCCTAC
htra1b_EG831192 (672) TTGAAAATCACGGACCTTAAACCGG--GTGTGTCTGGAGCCACACCCTAC
1751 1800
htra1a_NM001141717 (1729) CATAGAACCTGTAACTACGGGTG----CTCTTCGCTCTCAGTGAA--ACT
htra1b_EG831192 (720) CTTAGAACCTGTAACTACTAGTGTCTCCCCCCACTCCCTCAAACTACA
1801 1850
htra1a_NM001141717 (1773) GATTATTGTTTTATACTCTACTAACGTCCTTGCACAGAAAACCCACCTCA
htra1b_EG831192 (770) GAGGTCTGTGAGGCCCACAACTAACCCTCTGGGAGCTCAAATGTGT----
1851 1900
htra1a_NM001141717 (1823) ATCAGATAGAAAAGTGGGTGTCACAGATTACCGTAGTTTTGTAGTTGTTT
htra1b_EG831192 (816) ----------
1901 1950
htra1a_NM001141717 (1873) TTGTTGCTGTTGATATTATGTTTTTTTTTTATTGTATTTTAATTGCATTT
htra1b_EG831192 (816) ----------
1951 2000
htra1a_NM001141717 (1923) CTCTTGATACGTTTTGACGACAATTTCACAGAGGGAATAAAAAGATTTTG
htra1b_EG831192 (816) ----------
2001 2027
htra1a_NM001141717 (1973) AAAAAAAAAAAAAAAAAAAAAAAAA
htra1b_EG831192 (816) ----------

Figure S.3.1. Alignment of the nucleotide sequences of *htra1a* (GenBank accession number NM_001141717) and *htra1b* (GenBank accession number EG831192). Conserved regions are highlighted in yellow. Alignments were performed using AlignX (Vector NTI Advance 11). Forward primers are bolded and underlined, whereas reverse primers are bolded without an underline.

1 50

```
eif4b1_BT072661     (1) ATAAGAAGGGGAAGACCCTGACCCTCACTGACTTCCTGGCAGAGGACAAT
eif4b2_DY739566     (1) --------------------------------------------------
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eif4b2_DY739566     (1) --------------------------------------------------
                       101                                              150
eif4b1_BT072661   (101) CAGCTGGGCCGACGAGACGGATGACCTGGAGGGAGATGTGTCCACCTCCT
eif4b2_DY739566     (1) --------------------------------------------------
                       151                                              200
eif4b1_BT072661   (151) GGCACTCAGGGGAGGACAGTTACCGGGCCCCGGCCATAGATCGCAACATC
eif4b2_DY739566     (1) --------------------------------------------------
                       201                                              250
eif4b1_BT072661   (201) CTGCCCACGGCGCCACGGTCGGCCCGCGAGCCCAATGTGGACCGGTCACG
eif4b2_DY739566     (1) --------------------------------------------------
                       251                                              300
eif4b1_BT072661   (251) CCTCCCCCGCAGCCCCCCCTACACCGCCTTCCTGGGCAACCTGCCCTACG
eif4b2_DY739566     (1) --------------------------------------------------
                       301                                              350
eif4b1_BT072661   (301) ACGTCTCCGAGGAATCTATCATGGACTTCTTCCGGGGCCTAGCGATCAGT
eif4b2_DY739566     (1) --------------------------------------------------
                       351                                              400
eif4b1_BT072661   (351) GCTGTGCGCTTGCCACGGGAGCCCAGTAACCCAGAGAGGCTGAAGGGCTT
eif4b2_DY739566     (1) --------------------------------------------------
                       401                                              450
eif4b1_BT072661   (401) TGGCTACGCAGAGTTTGATGATGTGGACTCCCTCCTGAGGGCGCTGACTC
eif4b2_DY739566     (1) --------------------------------------------------
                       451                                              500
eif4b1_BT072661   (451) TCAATGAGGAGAACCTGGGAAACCGCAGGATCCGGGTGGATATTGCAGAT
eif4b2_DY739566     (1) --------------------------------------------------
                       501                                              550
eif4b1_BT072661   (501) CAGTCCAACGACAAGGAGGGGAGAGATAATGGCCAGATGGGTGGACGGGA
eif4b2_DY739566     (1) --------------------------------------------------
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eif4b1_BT072661   (551) CAGGATGGGCCGTATGGGAGACATGGGGGGCCCCGACAAGACAGACAGTG
eif4b2_DY739566     (1) --------------------------------------------------
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eif4b1_BT072661   (601) ACGACTGGAGGGCCCGGCCCACTGCTGACGCTGATGACGGACCCCCAAAG
eif4b2_DY739566     (1) --------------------------------------------------
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eif4b1_BT072661   (651) AGAGAGGAATCCACTTTCGGGTCACGCGACCGCTATGGAGACCGTGACGG
eif4b2_DY739566     (1) --------------------------------------------------
                       701                                              750
eif4b1_BT072661   (701) GCTGAGACGGGACAACGACCGCGGATTTGGCGGCGACCGGGACC-CGCGGA
eif4b2_DY739566     (1) --------------------------------CCGCCTTCGGGTCACGCGAC
                       751                                              800
eif4b1_BT072661   (750) TTTGGCGGCGACCGGGACCGCGGATTTGGCGGCGACCGGGACCGCGGATT
eif4b2_DY739566    (21) CGCTACGGAGACCGTGA---CGGGCTGCGAAGGGACAACGACCGCGGCTT
                       801                                              850
eif4b1_BT072661   (800) TGGCGGCGACCGGGACGGCGGGAGAGACCGCGGGTTCGGTGGCAGAGACC
eif4b2_DY739566    (68) CGGCGGCGACCGGGAC------------CGCGGCTTCGGCGGCAGAGACC
                       851                                              900
eif4b1_BT072661   (850) GCTATGATGACCGGGGAGGTGAGAGCGGAGCCTTTGGCTCCCGCAGGGACC
eif4b2_DY739566   (106) GCTATGACGACCGGGGAGGTGAGAACGGAGCCTTTGGCTCCCGCAGGGATT
                       901                                              950
eif4b1_BT072661   (900) CGGGATGATGGCGGGCGACGTGCCTTTGGCAGTGGCTACCGCCGTGATGA
eif4b2_DY739566   (156) AGGGATGACGGCGGGCGACGTGCCTTCGGCAGTGGGTACCGCCGTGACGA
                       951                                             1000
eif4b1_BT072661   (950) TGAC------------GGGGGTGGTGGTGGCCGCTACGGGGAACGGGATC
eif4b2_DY739566   (206) TGACTATCAGAGTGGCGGAGGTGGCGGTAGCCGCTATGGGGCCCGGGATC
                      1001                                             1050
eif4b1_BT072661   (988) GCTACGGCGCGGACAGAGAGGACCGATATGAGAGGCGCGAGGA-GAGAG-
eif4b2_DY739566   (256) GCTATGGCGGAGACCGGGAGGGCCGGTATGAGAGGCAGGACGAAGGGCGT
                      1051                                             1100
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eif4b1_BT072661  (1036) GAGGTGAG-GGTGGTCCCACGCAGAGACCCAAGCTGGTCCTGAAGCCACG
eif4b2_DY739566   (306) GAGGAGAGAGGTGGTCCGATCGAAAGGCCCAAGTTGGTCCTGAAGCCGCG
                       1101                                          1150
eif4b1_BT072661  (1085) CAGCACGCCCAAGGAGGAGGAGCAGGCCCGCAGCGGTGGTGCGGGAGCTG
eif4b2_DY739566   (356) CAGCATGGCCAAGGAGGAGCAGCAGGCCGGCAGCGGTGGGGC------TA
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eif4b1_BT072661  (1135) CCCCAGCTGCTCCAGCGACTGCCCCCAGCTCCGGCCGTGCCTCCATCTTC
eif4b2_DY739566   (400) C---------TCCGGCAGCCGCCCGCAGCTCTGGTCGAGCCTCCATCTTC
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eif4b1_BT072661  (1185) GGAGCAGCCAAGCCCGTGGACACTGCAGCCAAGGAGCGGGAGGTGGAGGA
eif4b2_DY739566   (441) GGAGCGGCCAAGCCCGTGGACACAGCGGCTAAGGAGCGGGAGGTGGAGGA
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eif4b1_BT072661  (1235) GAAACTCCAGAGGCAGCTGGAAGAGGACAAGTCCAGGGGCTTTGATAGGA
eif4b2_DY739566   (491) GAAGCTCCAGAGGCAGCTGGAAGAGGACAAGTCCAAGGGCTTCGATAGGA
                       1301                                          1350
eif4b1_BT072661  (1285) AACCCCGCGATAGAGACAGGGACCCAAGTTGGAGGAGTGAGGAGCCACCT
eif4b2_DY739566   (541) AACCTCGCGACAGAGACAGGGACCCAAGCTGGAGGAATGAGGAGCCACCT
                       1351                                          1400
eif4b1_BT072661  (1335) TCTGAGCGACCTGCTACACGCTCCCGCACAGGAAGTGAGTCATCACAGAC
eif4b2_DY739566   (591) TCTGAGC----------GGTCCCGCACAGGAAGTGAGTCATCACATCC
                       1401                                          1450
eif4b1_BT072661  (1385) GGGAAGTACATCTGGAGGAAGAGTCTCGCGGCGCAGAGAGAGCGAGCGAT
eif4b2_DY739566   (629) AGGAAGTACCTCTGGAGGGAGAGCCTCACGGCGCAGAGAGAGCGAGCGCT
                       1451                                          1500
eif4b1_BT072661  (1435) CGGTTGAGAATGAAGTATTCAGTGGTAAGGAGGATGACCCTCCCTCTCCC
eif4b2_DY739566   (679) CAGTGGAGAACGAAGTATTCAGCGGTAAGGAGGACGACCCTCCCTCTCCC
                       1501                                          1550
eif4b1_BT072661  (1485) GGAGCTCGCCCTACTTCCGCCAACTCTTCCACATCTTCCTCCAAGGAGCC
eif4b2_DY739566   (729) GGGTCTCGCCCCTCTTCCGCCAACTCTTCCACCTTCTCCTCCAAG-----
                       1551                                          1600
eif4b1_BT072661  (1535) CCTCAAGGTGATGCCCGCACCACCACCCAAGGAGAATGCCTGGGCCAAGC
eif4b2_DY739566   (774) --------------------------------------------------
                       1601                                          1650
eif4b1_BT072661  (1585) GCCCTGCGGTGAGCGCAGGGTCCACCCCTGTCTCCCCCAGTGACGCGGCA
eif4b2_DY739566   (774) --------------------------------------------------
                       1651                                          1700
eif4b1_BT072661  (1635) TGTCCCAAACTGAGCTCAAGTTCTGCAGATGAAAGAGGATCTGGAAGGGA
eif4b2_DY739566   (774) --------------------------------------------------
                       1701                                          1750
eif4b1_BT072661  (1685) AATTCAGCTCAGCCAGTAAATACGCCGCTTTGCTAATGGACAGCGAGCAA
eif4b2_DY739566   (774) --------------------------------------------------
                       1751                                          1800
eif4b1_BT072661  (1735) GGAGACGACGCAGAGGACAGCGTAGAATAAAGCGAGAGCGGGGGAGTGAA
eif4b2_DY739566   (774) --------------------------------------------------
                       1801                                          1850
eif4b1_BT072661  (1785) AAGAAGATCCAGCCAATGAAAGAATCTCACATTAAATATGAAAGTCTCCT
eif4b2_DY739566   (774) --------------------------------------------------
                       1851                                          1900
eif4b1_BT072661  (1835) CACCTGGTGTAGAGGCACCTGGGAGGTCATCACTTCTACCTACTTTCCTT
eif4b2_DY739566   (774) --------------------------------------------------
                       1901                                          1950
eif4b1_BT072661  (1885) ACTCGTAACCCTCCTTCCCCTTTTCAGGACTTTTCAGTTTCTAAAAAGAA
eif4b2_DY739566   (774) --------------------------------------------------
                       1951                                          2000
eif4b1_BT072661  (1935) TAGTCATATACTGACTTGTTTTAAAATGGAAAAGGAAAGGAATAGTCGAC
eif4b2_DY739566   (774) --------------------------------------------------
                       2001                                          2050
eif4b1_BT072661  (1985) ACAAGAAAATATGGAAAATTGAATTAATTGTAATAAATAAAAAAAGTGGG
eif4b2_DY739566   (774) --------------------------------------------------
                       2051                                          2100
eif4b1_BT072661  (2035) GTAAAAACAAATTGTTTACATTTGTGATGGTATGAGTTGGGCTCCATCCT
eif4b2_DY739566   (774) --------------------------------------------------
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eif4b1_BT072661  (2085) TCTCCCCAGTGGAAAAGGATGAAGAGTTGGTTACTGGATGCTTGCTTGTC
eif4b2_DY739566   (774) --------------------------------------------------
                        2151                                          2200
eif4b1_BT072661  (2135) AGCCTGACCTATTAAATCTCATGTGCAGTTAAAAAAAAAAAAAAAAAAAA
eif4b2_DY739566   (774) --------------------------------------------------
                        2201
eif4b1_BT072661  (2185) AAAAAAAAGA
eif4b2_DY739566   (774) ----------
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Figure S.3.2. Alignment of the nucleotide sequences of *eif4b1* (GenBank accession number BT072661) and *eif4b2* (accession number DY739566). Conserved regions are highlighted in yellow. Alignments were performed using AlignX (Vector NTI Advance 11). Forward primers are bolded and underlined, whereas reverse primers are bolded without an underline.

```
lect2a_BT059281     (1) -CAAGACTTTCTTGTAGATTTTCTCCTTGA-GTGAAAGCATCATCTAAGG
lect2b_DV106130     (1) GCAAGACCTTCTTGCAGAGTTTTTCCTTGAAGCGAAAGCATCCTCTCAGG
                        51                                              100
lect2a_BT059281    (49) CTTTACCATGAGGACTGCTGTTCTTTTGTTTACTGTGGTGCTCATAGCTG
lect2b_DV106130    (51) CTTCAACATGAAGACAGCTATTCTTCTGCTTACTGTAGTCCTTATGGCTG
                       101                                             150
lect2a_BT059281    (99) TGTTGTCAGAGTGCGAGATGGTCAAGTTTGGTCAGCTGTGCAGCGGCAAC
lect2b_DV106130   (101) TGTTGCCAGAGTGTGAGATGGCCAAGTTTGGTCAGCTGTGTAGCGGAAAT
                       151                                             200
lect2a_BT059281   (149) TCCAGTAACAGGAGGAGGACAGGGGACAGATGGGGACAAGGACACTACGG
lect2b_DV106130   (151) TCCAGTAACAGGAGGAGGACAGGGGACAACTGGGGACAAGGACAGTACGG
                       201                                             250
lect2a_BT059281   (199) CGCACGCAGAGGAAACCGTGAGCATCAGGGCCTGGACATTGTGTGTAATG
lect2b_DV106130   (201) AGCAAGCAGAACAGACCATGTGCATAAGGGCATTGACATCGTGTGTAACG
                       251                                             300
lect2a_BT059281   (249) ATGGGGCCACAGTGTACGCTCCATTTGATGTGAAACTCAATGGGAAAGTG
lect2b_DV106130   (251) ACGGGGCCACAGTGTACGCTCCATTTGACGTGAAACTCAACGGCAAAGTG
                       301                                             350
lect2a_BT059281   (299) ATCGTGTACACAGACCCGAAGAAGGCAGCCATCAATGATGGGATCAACCT
lect2b_DV106130   (301) ACAGTGTACACAAACCCAAAGAAAGCAGCCATCAACGATGGGATCAACCT
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lect2a_BT059281   (349) CAGTGGAGAGGGTCTGTGCTTTAAGCTGTTCTACGTAAAGCCTGACAAGT
lect2b_DV106130   (351) CAGTGGGGAAGGTCTGTGCTTTAAGCTGTTCTACGTGAAGCCTGACAGTT
                       401                                             450
lect2a_BT059281   (399) ACTCTGGGGTGGTGAAGAAGGGCCAGAGGATTGGGACCCTGCTGACCATG
lect2b_DV106130   (401) ACTCTGGGGTGGTGAAGAAGGGCCAGAGGATTGGGACCCTGCTCCCCATG
                       451                                             500
lect2a_BT059281   (449) CAAAGTGTCTACCCAGGGATCACTTCTCACGTCCACGTCCAGATGTGTGA
lect2b_DV106130   (451) CAGAGTGTCTACCCAGGGATCACTTCTCACGTCCACGTCCAGATGTGTGA
                       501                                             550
lect2a_BT059281   (499) CAAGTCTGACCCCACCAAGTTTTTCTAATGGAGTCCCCTTTGGCCTCTCC
lect2b_DV106130   (501) CAAGTCCGACCCCACCAAGTACTTCTGATTGA-------TTGG---TTAA
                       551                                             600
lect2a_BT059281   (549) ATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCAAT
lect2b_DV106130   (541) ATAAATCATCTAAATTATCAC-CAATG----------------------
                       601                                             650
lect2a_BT059281   (599) CAATCAATCAATCAATCAATCAATCAATCAATCAATCAATCTACAAAATG
lect2b_DV106130   (567) --------------------------------------------------
                       651                                             700
lect2a_BT059281   (649) ATTGTAATCATTGGCCAATAGATGGGCTTACTGTGTTTAAAAATAATAAT
lect2b_DV106130   (567) --------------------------------------------------
                       701                                             750
lect2a_BT059281   (699) TTGCTTATTATAATAAACATTTTTTATTACAGTATAAAATACATAAAAGT
lect2b_DV106130   (567) --------------------------------------------------
                       751                                             800
lect2a_BT059281   (749) TGCACACTTCTGGAATAAAGTTTTAACCCTTTAAGCATCAACAGGGGTAA
lect2b_DV106130   (567) --------------------------------------------------
                       801                                             850
lect2a_BT059281   (799) TTCATAATTTCAAAAACCTTTTTTTTATCCTTTTATGCAACTAGTTACAG
lect2b_DV106130   (567) --------------------------------------------------
                       851                                             900
lect2a_BT059281   (849) ACATGAAAATAATTTACAATGATTTCTGTGTTAATATATAATTTCTGTGG
lect2b_DV106130   (567) --------------------------------------------------
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lect2a_BT059281   (899) TATATATATATATATATATATATATATATATATATATATATATATATA
lect2b_DV106130   (567) --------------------------------------------------
                       951                                            1000
lect2a_BT059281   (949) TAAAATCTCTTTTTTGTGTACTAGTTTTACGTGGCTAATCTGACAATGTG
lect2b_DV106130   (567) --------------------------------------------------
                      1001                                            1050
lect2a_BT059281   (999) GACTCAATAGTAAACTTTGAAAAAAACATAGATTTAAAACAAATACACGT
lect2b_DV106130   (567) --------------------------------------------------
                      1051                                            1100
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lect2a_BT059281  (1049) GTCAAGGCTGTGGGTAACTGGTGAAAGGAGTCAGGCGCAGGAGAGCTGAG
lect2b_DV106130   (567) --------------------------------------------------
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lect2a_BT059281  (1099) ATGCGTGGACAAGGTATTTAATACAAGAAAACATCAGTATGAACACAATA
lect2b_DV106130   (567) --------------------------------------------------
                       1151                                          1200
lect2a_BT059281  (1149) CTATGGTGCTGGAAAAAAAAACGGTACCACGAAATTAACAGGCGTAATAAA
lect2b_DV106130   (567) --------------------------------------------------
                       1201                                          1250
lect2a_BT059281  (1199) AAAACCCGGTAACAATATACCAGCCGTCAGATACAGCCTTACAATAAAAC
lect2b_DV106130   (567) --------------------------------------------------
                       1251                                          1300
lect2a_BT059281  (1249) AAAGACGCACACAAACATCGGGGAAACCAGAGGGTTAAATAATGAACATG
lect2b_DV106130   (567) --------------------------------------------------
                       1301                                          1350
lect2a_BT059281  (1299) TAATGGGGGAATTGAAACCAGGTGTGTAAAAAAACAACAACAAAACAAAT
lect2b_DV106130   (567) --------------------------------------------------
                       1351                                          1400
lect2a_BT059281  (1349) GGAAAATGAAAAGTGGATCGGTGATGGCTACCGCCGAATGCCGCTCGAAC
lect2b_DV106130   (567) --------------------------------------------------
                       1401                                          1450
lect2a_BT059281  (1399) AAGGAGCGGGACCGACTCCGGCGGAAGTCGTGACAACATGTGTGTCATTG
lect2b_DV106130   (567) --------------------------------------------------
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lect2a_BT059281  (1449) AACAACCCCATACCATGACTAGATGGTGGAAAAAAACACCCATCTCTTTA
lect2b_DV106130   (567) --------------------------------------------------
                       1501                                          1550
lect2a_BT059281  (1499) ATAAGTTCTTTAGACAATTAAAGCTAATTTACCAACATTTCTGAAAATTT
lect2b_DV106130   (567) --------------------------------------------------
                       1551                                          1600
lect2a_BT059281  (1549) GGAATGAAACTTCTATTGATAAAAATAACTGTTTAAAACATGAAAAACAT
lect2b_DV106130   (567) --------------------------------------------------
                       1601                                          1650
lect2a_BT059281  (1599) ACTAAAATGTATATTTTTAAACATGAAATTTGACTATAAATGTGTGAAAA
lect2b_DV106130   (567) --------------------------------------------------
                       1651                                          1700
lect2a_BT059281  (1649) TTTAAAATATATGCATATTTTGGGGAATTTAATAAAAGAAAAAGTGTTAC
lect2b_DV106130   (567) --------------------------------------------------
                       1701                                          1750
lect2a_BT059281  (1699) TGTTTGACAGAAAATGCCATTTTGCCATTATTTCCAATTTGGGGTTAAAA
lect2b_DV106130   (567) --------------------------------------------------
                       1751                                          1800
lect2a_BT059281  (1749) TGACCCATGTTGATGCATTTAGGGGTCAAAATATGTTGGTCCTTTTGGGG
lect2b_DV106130   (567) --------------------------------------------------
                       1801                                          1850
lect2a_BT059281  (1799) TATTAACAACAAAATAAATACATCATAAATCATTCCTAATAAATAATGTG
lect2b_DV106130   (567) --------------------------------------------------
                       1851                                          1900
lect2a_BT059281  (1849) TATTTTTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
lect2b_DV106130   (567) --------------------------------------------------
                       1901      1914
lect2a_BT059281  (1899) AAAAAAAAAAAAGA
lect2b_DV106130   (567) --------------
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Figure S.3.3. Alignment of the nucleotide sequences of *lect2a* (GenBank accession number BT059281) and *lect2b* (GenBank accession number DV106130). Conserved regions are highlighted in yellow. Alignments were performed using AlignX (Vector NTI Advance 11). Forward primers are bolded and underlined, whereas reverse primers are bolded without an underline.

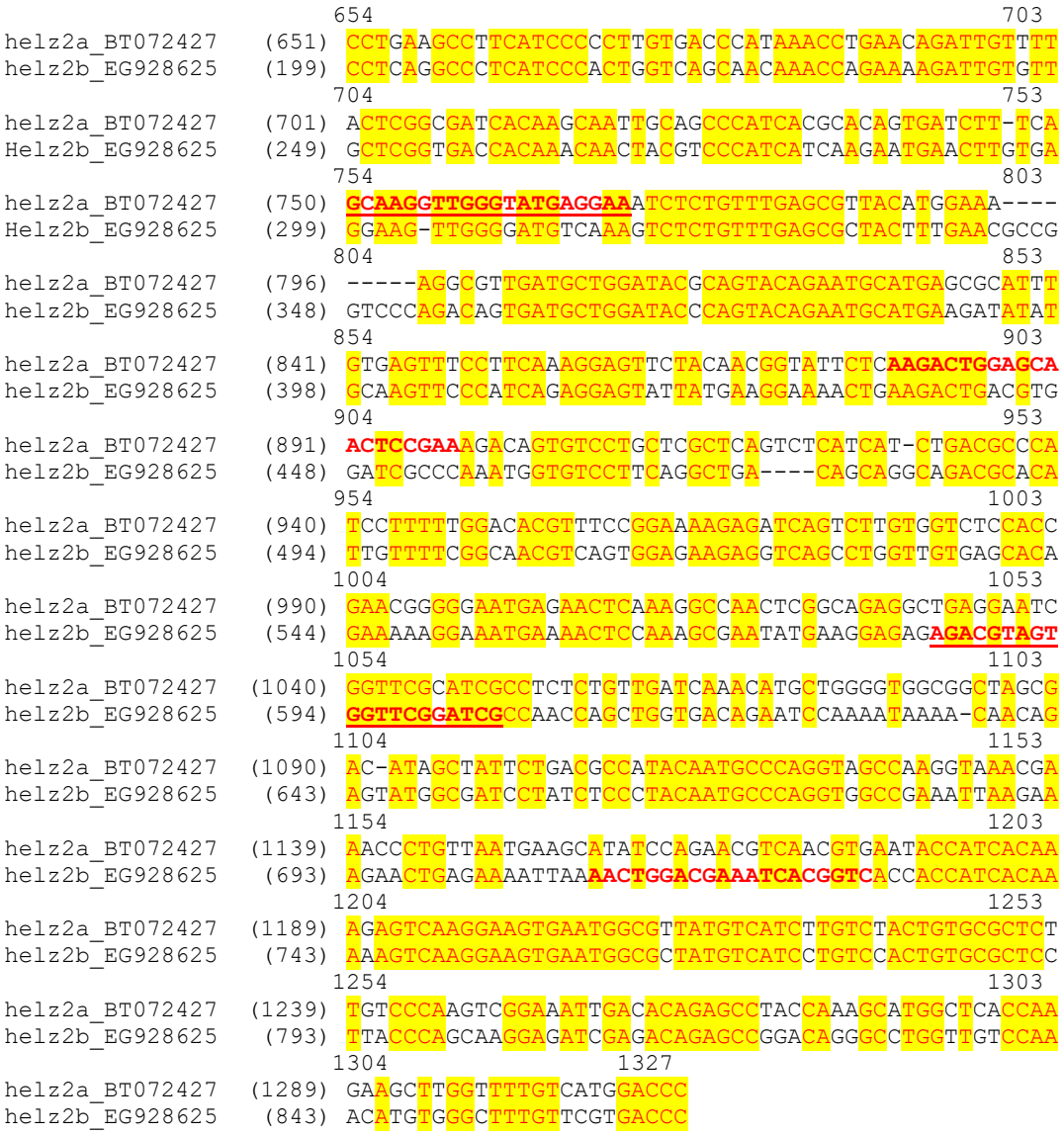


Figure S.3.4. Alignment of the nucleotide sequences of *helz2a* (GenBank accession number BT072427) and *helz2b* (GenBank accession number EG928625). Conserved regions are highlighted in yellow. Alignments were performed using AlignX (Vector NTI Advance 11). Forward primers are bolded and underlined, whereas reverse primers are bolded without an underline.

Chapter 4. Influence of dietary long-chain polyunsaturated fatty acids and ω6 to ω3 ratios on head kidney lipid composition and expression of fatty acid and eicosanoid metabolism genes in Atlantic salmon (*Salmo salar*)

Preface

A version of the study described in Chapter 4 was published in the journal *Frontiers in Molecular Biosciences*. Katan, T., Xue, X., Caballero-Solares, A., Taylor, R. G., Rise, M. L., and Parrish, C. C. (2020). Influence of dietary long-chain polyunsaturated fatty acids and ω6 to ω3 ratios on head kidney lipid composition and expression of fatty acid and eicosanoid metabolism genes in Atlantic salmon (*Salmo salar*). *Frontiers in Molecular Biosciences* 7, 602587.

4.1. Abstract

The interaction of dietary eicosapentaenoic acid and docosahexaenoic acid (EPA+DHA) levels with omega-6 to omega-3 ratios (ω6:ω3), and their impact on head kidney lipid metabolism in farmed fish, are not fully elucidated. The present study investigated the influence of five plant-based diets (12-week exposure) with varying EPA+DHA levels (0.3, 1.0, or 1.4%) and ω6:ω3 (high ω6, high ω3, or balanced) on tissue lipid composition, and transcript expression of genes involved in fatty acid and eicosanoid metabolism in Atlantic salmon head kidney. Tissue fatty acid composition was reflective of the diet with respect to C_{18} PUFA and MUFA levels (% of total FA), and ω6:ω3 (0.5-1.5). Fish fed 0.3% EPA+DHA with high ω6 (0.3%EPA+DHA↑ω6) had the highest increase in proportions (1.7-2.3-fold) and in concentrations (1.4-1.8-fold) of arachidonic acid (ARA). EPA showed the greatest decrease in proportion and in concentration (by ~ ½) in the 0.3%EPA+DHA↑ω6 fed fish compared to the other treatments. However, no differences were observed in EPA proportions among salmon fed the high ω3 (0.3 and 1.0% EPA+DHA) and balanced (1.4% EPA+DHA) diets, and DHA proportions were similar among all treatments. Further, the transcript expression of *elovl5a* was lowest in the 0.3%EPA+DHA↑ω6 fed fish, and correlated positively with 20:3ω3, 20:4ω3 and EPA:ARA in the head kidney. This indicates that high dietary 18:3ω3 promoted the synthesis of ω3 LC-PUFA. Dietary EPA+DHA levels had a positive impact on *elovl5a*, *fadsd5* and *srebp1* expression, and these transcripts positively correlated with tissue ΣMUFA. This supported the hypothesis that LC-PUFA synthesis is positively influenced by tissue MUFA levels in Atlantic salmon. The expression of *pparaa* was higher in the

0.3%EPA+DHA↑ω6 compared to the 0.3%EPA+DHA↑ω3 fed fish. Finally, significant correlations between head kidney fatty acid composition and the expression of eicosanoid synthesis-related transcripts (i.e. *5loxa*, *5loxb*, *cox1*, *cox2*, *ptges2*, *ptges3*, and *pgds*) illustrated the constitutive relationships among fatty acids and eicosanoid metabolism in salmon.

4.2. Introduction

Long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic (EPA, 20:5ω3), docosahexaenoic (DHA, 22:6ω3) and arachidonic (ARA, 20:4ω6) acids play important roles in fish growth, metabolism (Sargent et al., 2002), neural development, health and reproduction (Sargent et al., 1999; Tocher, 2010, 2015). They are also important structural components of cell membranes (Calder, 2013), and act as precursors to eicosanoid metabolites such as prostaglandins, leukotrienes, thromboxanes, docosanoids, maresins and resolvins which regulate inflammatory and immune response processes (Calder, 2007, 2013; Arts and Kohler, 2009; Martínez-Rubio et al., 2013; Holen et al., 2015; Montero et al., 2019). The levels of EPA and DHA in fish feeds showed a large reduction (from ~ 20 to 5% of dietary lipid) over the past ~ 3 decades (Einen and Thomassen, 1998; Ytrestøyl et al., 2015; Sprague et al., 2016), due to decreasing global availability and rising market price of their fish oil (FO) sources (Turchini et al., 2009; Tacon and Metian, 2015; Froehlich et al., 2018). The reduction in FO and fish meal (FM), and the concomitant rise in incorporation of plant-based ingredients have influenced fish health (e.g. liver steatosis, altered phagocytic activity, and modulation of immune-related transcript expression) (Montero et al., 2003; Ruyter et al., 2006; Caballero-Solares et al., 2017), and their nutritional quality for human consumers (Sprague et al., 2016; De Roos et al., 2017).

Salmonids have a capacity to desaturate and elongate the precursors 18:3ω3 and 18:2ω6 to LC-PUFA metabolites (Tocher et al., 2000; Hixson et al., 2014; Katan et al., 2019). The enzymes involved in the bioconversion of both ω3 and ω6 pathways are the

fatty acyl desaturases (FADS2D5, FADS2D6a, FADS2D6b and FADS2D6c: Zheng et al., 2005; Monroig et al., 2010) and fatty acyl elongases (ELOVL5a, ELOVL5b, ELOVL2, ELOVL4a and ELOVL4b: Hastings et al., 2004; Morais et al., 2009; Carmona-Antoñanzas et al., 2011; Zhao et al., 2019). Hepatic LC-PUFA synthesis in Atlantic salmon (*Salmo salar*) is influenced by precursor (18:2ω6 and/or 18:3ω3) availability, as well as the concentration of LC-PUFA (i.e. EPA, ARA, DHA) (Jordal et al., 2005; Glencross et al., 2015; Katan et al., 2019). This pathway is regulated at the transcriptional level and is mediated by transcription factors [e.g. liver X receptor (LXR), sterol regulatory element binding protein (SREBP) 1 and 2, and peroxisome proliferator-activated receptor (PPAR) α, β and γ (Carmona-Antoñanzas et al., 2014; Hixson et al., 2017; Katan et al., 2019; Emam et al., 2020)]. However, the constitutive regulation of this pathway in salmon head kidney is less well understood (Betancor et al., 2014), and requires further investigation given the central role of this organ in haematopoetic and immune processes (Tort et al., 2003; Zapata et al., 2006; Gjøen et al., 2007).

Plant oils used in aquafeeds generally contain high levels of ω6 FA, and as such, these oils and farmed fish consuming them may provide inadequate ratios of ω6 to ω3 (ω6:ω3) fatty acids for the consumer (Pickova and Mørkøre, 2007; Weaver et al., 2008; Young, 2009). Further, ω6:ω3 plays an important role in fish immune response [through their conversion into pro- and anti-inflammatory eicosanoids (Furne et al., 2013; Holen et al., 2018)] and FA metabolism [e.g. impacting LC-PUFA synthesis and transcript expression of genes involved in this pathway (Torstensen and Tocher, 2010; Vagner and Santigosa, 2011; Katan et al., 2019)]. Previous studies revealed that diets with varying ω6:ω3 ratio (i.e. ~0.3-4.0) influenced the transcription of inflammation- and FA

metabolism-related genes in Atlantic salmon head kidney (Martinez-Rubio et al., 2013; Holen et al., 2018). However, the interaction of dietary ω6:ω3 with EPA+DHA levels, and their nutritional impacts on eicosanoid and fatty acid metabolism in salmon, have not been fully elucidated.

Emam et al. (2020) recently showed that diets with different combinations of ω6:ω3 (high ω6, high ω3, or balanced) and EPA+DHA levels (0.3, 1.0, or 1.4%) affected muscle lipid composition, and the hepatic transcript expression of fatty acid synthesis-related genes in Atlantic salmon. The current study used the same diets and fish population as in Emam et al. (2020), with the aim to investigate how diets with low FO inclusion levels impact head kidney lipid composition, and the transcript expression of genes involved in fatty acid and eicosanoid metabolism. Furthermore, correlation analyses were used to relate head kidney lipid composition with transcript expression.

4.3. Materials and Methods

4.3.1. Experimental diets and animals

Five experimental diets were manufactured by Cargill Canada (formerly EWOS Canada; Surrey, BC, Canada), top-coated with different oil mixes at the Chute Animal Nutrition Centre (Dalhousie University, Truro, NS, Canada), and stored at −20°C until needed. Experimental diets contained different levels of FO, poultry fat, and vegetable oils (VO; i.e. soy oil, linseed oil, and rapeseed oil) to generate three levels of EPA+DHA (0.3, 1.0 and 1.4%, as formulated) and contrasting ω6:ω3 ratios (high ω6, high ω3, and balanced). The diets were as follows: 0.3% EPA+DHA with higher ω6 (0.3%EPA+DHA↑ω6), 0.3% EPA+DHA with higher ω3 (0.3%EPA+DHA↑ω3), 1.0% EPA+DHA with higher ω6 (1%EPA+DHA↑ω6), 1.0% EPA+DHA with higher ω3 (1%EPA+DHA↑ω3), and 1.4% EPA+DHA with a more balanced ω6:ω3 ratio (1.4%EPA+DHA/balanced). All diets were formulated to be isonitrogenous and isoenergetic and to meet the nutritional requirements of salmonids [National Research Council (NRC), 2011]. Dietary formulations and their lipid composition were published previously (Emam et al., 2020), and are shown in Tables 4.1 and 4.2 herein.

Atlantic salmon smolts were transported from a regional salmon farm to the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, Memorial University of Newfoundland, Canada; October 2016). After their arrival, fish were graded by size in order to select the most uniform population, PIT (Passive Integrated Transponder; Easy AV, Avid Identification Systems, Norco, CA, USA)-tagged for individual identification, and kept in 3800 l tanks until the beginning of the feeding trial.

Table 4.1. Formulation and nutrient composition (%) of experimental diets fed to Atlantic salmon for 12 weeks.

Ingredient (% of diet)[a]	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	1%EPA+DHA ↑ω6	1%EPA+DHA ↑ω3	1.4%EPA+DHA balanced
Fish oil	0.1	-	4.3	4.3	6.8
Soy oil	12.5	-	10.1	-	-
Linseed oil	-	7.9	-	6.5	-
Poultry fat[b]	2.4	7.1	0.6	4.3	3.4
Rapeseed oil	-	-	-	-	4.8
Proximate composition (% as fed basis)					
Nitrogen[c]	7.3	7.5	7.8	7.3	7.2
Crude lipid[d]	19.9	20.6	20.0	20.7	20.4
Dry matter[d]	97.4	97.6	98.0	97.5	97.8
Ash[d]	5.7	5.7	5.4	5.7	6.0

[a] All ingredients were sourced from Cargill Innovation stocks. Each experimental diet contained 24.7% marine protein, 57.4% plant protein, and 2.9% additives.

[b] Poultry fat contained EPA+DHA and 18:2ω6 levels of 0.4 and 2.9% of total FA, respectively. This contributed to their level in experimental diets.

[c] Analysed as % of dry weight (n=3).

[d] Analysed as % of wet weight (n=3).

Table 4.2. Lipid and fatty acid composition (%) of experimental diets[a] fed to Atlantic salmon for 12 weeks.

	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	1%EPA+DHA ↑ω6	1%EPA+DHA ↑ω3	1.4%EPA+DHA balanced
Lipid classes composition (% of total lipid)					
TAG (%)[b]	62.2	68.4	70.6	60.9	71.6
ST (%)[c]	3.8	5.5	3.8	3.4	3.4
AMPL (%)[d]	2.1	2.7	1.8	1.7	2.1
PL (%)[e]	11.7	6.0	4.4	10.2	6.3
TL (mg/g)[f]	174	189.1	168.9	210.1	201.8
Fatty acid composition (% of total FA)					
14:0	0.7	0.8	1.9	2.0	2.7
16:0	14.0	14.0	13.5	13.3	12.9
16:1ω7	1.2	2.3	1.8	2.7	3.2
18:0	5.2	5.8	4.7	5.0	4.0
18:1ω7	1.7	1.6	1.9	2.0	2.9
18:1ω9	23.2	28.1	18.6	22.5	29.5
18:2ω6 (LNA)	**41.4**	**18.3**	**33.4**	**14.8**	**13.3**
18:3ω3 (ALA)	**6.0**	**22.4**	**5.0**	**18.4**	**4.1**
20:1ω9	0.3	0.4	1.0	1.0	1.7
20:1ω11	0.1	0.1	2.5	2.6	4.1
20:4ω6 (ARA)	0.3	0.4	0.3	0.4	0.4
20:5ω3 (EPA)	**1.1**	**1.2**	**3.1**	**3.1**	**4.3**
22:1ω11	0.2	0.1	2.6	2.7	4.2
22:6ω3 (DHA)	**1.7**	**1.8**	**3.6**	**3.5**	**4.5**
ΣSFA[g]	21.1	21.4	21.4	21.3	20.9
ΣMUFA[h]	27.2	33.2	29.6	34.8	47.3
ΣPUFA[i]	51.6	45.2	48.8	43.6	31.4
Σω3	**9.4**	**25.9**	**14.1**	**27.4**	**16.4**
Σω6	**41.9**	**19.0**	**34.0**	**15.6**	**14.2**
ω6:ω3	4.5	0.7	2.4	0.6	0.9
EPA+DHA	2.8	3.0	6.7	6.6	8.8
DHA:EPA	1.5	1.5	1.2	1.1	1
EPA:ARA	3.7	3.0	10.3	7.8	10.8

[a] Mean (n=3). [b] Triacylglycerol. [c] Sterols. [d] Acetone mobile polar lipid. [e] Phospholipids. [f] Total lipids. [g] Total saturated fatty acids. [h] Total monounsaturated fatty acids. [i] Total polyunsaturated fatty acids. Fatty acids in bold font were key to the experimental design.

Then, salmon post-smolts [210 ± 44 g mean initial weight ± standard deviation (SD); 29.0 ± 0.19 cm mean initial fork length ± SD] were randomly distributed into twenty 620 l tanks (40-41 fish $tank^{-1}$), and subjected to an 8-week acclimation period during which they were given a commercial diet (EWOS Dynamic S, Cargill Inc., Elk River, MN, USA). After the completion of the acclimation period, fish were switched from the commercial feed and fed with the experimental diets (4 tanks $diet^{-1}$) for 12 weeks. At all stages, fish were fed to satiation overnight using automatic feeders (AVF6 Vibrating Feeders, Pentair Aquatic Eco-Systems, Inc., Apopka, FL, USA). Uneaten pellets were collected every morning, and the daily amount of feed was adjusted based on the number of uneaten pellets. All tanks were supplied with 12°C flow-through filtered seawater at 12 l min^{-1}; the dissolved oxygen level was ~10 mg l^{-1}, and the photoperiod was maintained at 24-h light. Mortalities were weighed and recorded (i.e. ≤ 1% at week 12) throughout the trial. Ethical treatment of fish in this experiment was carried out in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (protocol # 16-75-MR).

4.3.2. Sample collection

Salmon samples were collected from 10 opportunistically netted fish (each from a different tank) at week 0 (the day before fish were exposed to the experimental diets), and from 5 fish per tank at week 12. Sampling occurred after the fish were starved for 24 h and euthanized with an overdose of MS-222 (400 mg l^{-1}; Syndel Laboratories, Vancouver, BC, Canada). For lipid analyses, head kidney samples were collected in 15 ml glass test tubes that had been rinsed three times with methanol, followed by three rinses with chloroform.

Samples were stored on ice during sampling, their wet weights recorded, and they were covered with 2 ml of chloroform (HPLC-grade). Finally, the glass test tubes were filled with nitrogen, the Teflon-lined caps were sealed with Teflon tape, and samples were stored at −20°C. For gene expression analyses, head kidney (50–100 mg sample^{-1}) tissues were collected in 1.5 ml nuclease-free tubes, flash-frozen in liquid nitrogen, and stored at −80°C until RNA extractions were performed.

4.3.3. Lipid extractions

Lipid samples were extracted according to Parrish (1999). Samples were homogenized in a 2:1 mixture of ice-cold chloroform:methanol using a Polytron PCU-2-110 homogenizer (Brinkmann Instruments, Rexdale, ON, Canada). Chloroform-extracted water was added to bring the ratio of chloroform:methanol:water to 8:4:3. Each sample was sonicated for 4 min in an ice bath and centrifuged at 3000 rpm for 2 min at room temperature. The bottom, organic layer was removed using a double pipetting technique, placing a 2 ml lipid-clean glass Pasteur pipette inside a 1 ml glass pipette, in order to remove the organic layer without disturbing the top, aqueous layer. Chloroform was then added back to the extraction test tube, and the entire procedure was repeated three times. All organic layers were pooled into a separate lipid-clean vial. Finally, samples were concentrated under a flow of nitrogen gas.

4.3.4. Lipid class separation

Iatroscan Mark 6 TLC-FID (thin-layer chromatography-flame ionization detector) (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan), and a three-step development method were

used to determine the lipid class composition (Parrish, 1987). Lipid extracts and standards were applied to the Chromarods and focused to a narrow band using 100% acetone. The first development system was hexane:diethyl ether:formic acid (98.95:1.0:0.05). The rods were developed for 25 min, removed from the system for 5 min to dry, and replaced for 20 min. The second development was for 40 min in hexane:diethyl ether:formic acid (79:20:1). The final development system had two steps, the first was in 100% acetone for two 15 min time periods, followed by two 10 min periods in chloroform:methanol:chloroform extracted water (5:4:1). Before using each solvent system, the rods were dried in a constant humidity chamber. After each development system, the rods were partially scanned in the Iatroscan, and the data were collected using Peak Simple software (SRI Instruments, version 3.67, Torrance, CA, USA). The Chromarods were calibrated using standards from Sigma Chemicals (Sigma Chemicals, St. Louis, MO, USA).

4.3.5. Fatty acid methyl ester (FAME) derivatization

Lipid extracts (50 µl) were transferred into lipid-clean 15 ml glass vials and concentrated under a flow of nitrogen to complete dryness. Then, 1.5 ml of methylene chloride and 3 ml of Hilditch reagent (1.5 sulfuric acid: 98.5 anhydrous methanol) were added to each sample vial, and this was followed by a brief vortexing and 4 min sonication (Fisher Scientific FS30, Pittsburgh, PA, USA). Vials were then filled with nitrogen, capped and heated at 100°C for 1 h. Subsequently, 0.5 ml of saturated sodium bicarbonate solution and 1.5 ml of hexane were added to each vial, followed by a brief vortexing, and then the upper organic layer was removed into a separate lipid-clean glass vial. Each sample was then dried and refilled with ~0.5 ml of hexane. Finally, vials were filled with nitrogen,

capped, sealed with Teflon tape, and sonicated for 4 min to resuspend the fatty acids. All solvents were OmniSolv grade (VWR International, Mississauga, ON, Canada).

All FAMEs were analysed on a HP 6890 GC-FID (gas chromatograph-flame ionization detector) system equipped with a 7683 autosampler (Agilent Technologies Canada Inc., Mississauga, ON, Canada). Additional details regarding the GC conditions (e.g. column length, diameter, temperature, carrier gas) are described in Hixson et al. (2017). Fatty acid peaks were identified against known standards (PUFA 1, PUFA 3, BAME and a Supelco 37 component FAME mixture: Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). Finally, chromatograms were integrated using the Varian Galaxie Chromatography Data System, version 1.9.3.2 (Walnut Creek, CA, USA). FA data were expressed as area percentage of FAME.

4.3.6. RNA extraction, DNase treatment, column purification and cDNA synthesis

Head kidney samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), using the TissueLyser II system at 25 Hz for 2.5 min, using 5 mm stainless steel beads (QIAGEN, Mississauga, ON, Canada), and subjected to RNA extraction. This was followed by DNaseI treatment and column purification using the RNase-free DNase Set (QIAGEN) and the RNeasy Mini Clean-up Kit (QIAGEN), respectively. All procedures were conducted according to manufacturer instructions and as described in Xue et al. (2015). RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity and quantity were assessed by Nanodrop UV spectrophotometry (Thermo Fisher Scientific, Waltham, MA, USA). DNaseI-treated and column-purified RNA samples had A260/280

and A260/230 ratios of 1.8-2.3 (data not shown). All cDNAs were prepared by reverse transcription of 1 μg of DNaseI-treated, column-purified total RNA for each sample, using 1 μl of random primers (250 ng; Invitrogen), 1 μl of dNTPs (0.5 mM final concentration; Invitrogen), 4 μl of 5X first-strand buffer (1× final concentration; Invitrogen), 2 μl of DTT (10 mM final concentration; Invitrogen), and 1 μl of Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (200 U; Invitrogen) at 37°C for 50 min. The total reaction volume was 20 μl, following manufacturer instructions, and as described in Xue et al. (2015). Finally, all cDNAs were diluted 40 times with nuclease-free water (Invitrogen) prior to real-time quantitative polymerase chain reaction (qPCR).

4.3.7. qPCR analysis

Expression of 25 genes of interest (GOI) (Table 4.3) related to FA and eicosanoid metabolism was quantified by qPCR using head kidney cDNA templates from fish fed the 5 dietary treatments at week 12. For each dietary treatment, eight individuals (two from each tank) were used in the qPCR study. Only head kidney samples from fish that had specific growth rates within 1.5 standard deviations below and above the mean value of each tank were selected for the qPCR study, in order to reduce biological variability. The sequences of all primer pairs used in qPCR analyses, GenBank accession numbers of sequences used for primer design, and other details are presented in Table 4.3. Notably, primers for the transcripts *fadsd6a*, *elovl2* and *elovl5b* (GenBank accession numbers AY458652, FJ237532 and FJ237532, respectively) failed quality testing due to low transcript expression levels, and thus were not included in the qPCR study.

Table 4.3. qPCR primers.

Gene name (symbol)	Nucleotide sequence (5' - 3')[a]	Amp. Effic. (%)[b]	Amplicon size (bp)	GenBank Accession number
Elongation of very long chain fatty acids 4 b (*elovl4b*)[c]	F:TAGCAGAGTTGGGGATCAGC	99.4	101	XM_014144655
	R:CGAGATTTAGGTGCGTGTACTG			
Elongation of very long chain fatty acids 5 a (*elovl5a*)[d]	F:CAGTGTGGTGGGGACAAAG	90.2	115	AY170327
	R:TTCCCTCATGGACAAGCA			
Delta-5 fatty acyl desaturase (*fadsd5*)[d]	F:GTCTGGTTGTCCGTTCGTTT	89.0	135	AF478472
	R:GAGGCGATCAGCTTGAGAAA			
Delta-6 fatty acyl desaturase b (*fadsd6b*)[d]	F:TGACCATGTGGAGAGTGAGG	90.1	95	NM001172281
	R:CCAAAGCCAAGGCCTCTAGT			
Delta-6 fatty acyl desaturase c (*fadsd6c*)	F:CCAGTTGGAATCACGTACTGC	102.7	162	NM001171780
	R:GTGTGTCTCCCAGGACGAAG			
Sterol regulatory element-binding protein 1 (*srebp1*)[d]	F:TCAACAAGTCGGCAATTCTG	103.1	100	HM561860
	R:GACATCTTCAGGGCCATGTT			
Sterol regulatory element-binding protein 2 (*srebp2*)[d]	F:GAGTGCTGAGGAAAGCCATC	103.1	129	HM561861
	R:TCTCCACATCGTCAGACAGC			
Liver X receptor a (*lxra*)[d]	F:GGGCAAGATGGACAGATCAT	102.6	126	FJ470290
	R:CCTCACCAGGACCAACATCT			
Liver X receptor b (*lxrb*)[e]	F:CTCGCCTGTGTTCCTGTTTT	92.6	103	GE770391
	R:GAAACGCAAGACCTTCTGCT			
Peroxisome proliferator-activated receptor alpha a (*pparaa*)[c]	F:CCCTGGTGGCTAAGATGGT	103.1	132	XM_014124067
	R:AGACTTGGCGAACTCGGTTA			
Peroxisome proliferator-activated receptor beta 1 (*pparb1*)	F:CAGCTGATCAACGGTACGAC	93.4	112	NM001123635
	R:TGCTCTTGGCAAACTCAGTG			
Peroxisome proliferator-activated receptor beta 2 a (*pparb2a*)[d]	F:CCGTTTGTGATCCATGATGT	103.7	128	NM001123559
	R:GTGCACTGACAGCGGTAAAA			
Fatty acid synthase a (*fasa*)[d]	F:GGAGGGCACAATGGAGTAAA	97.7	136	DW563978
	R:TGAGACAGTGAATCGGATGG			
Fatty acid synthase b (*fasb*)[d]	F:TGCCATACAAGTGATGTCCTG	99.1	105	EG872804
	R:AGTGGGCACCAAACATGAAC			
Acyl-coenzyme A oxidase 1 (*acox1*)[c]	F:GTGCACCTACGAGGGAGAGA	99.9	111	DW555884
	R:TAGGACACGATGCCACTCAG			
Carnitine palmitoyltransferase 1 a (*cpt1a*)[c]	F:CGGTGGCAGATGATGGATATG	104.6	82	XM_014176428
	R:GAGTGCTTGCTGGAGATGTG			
Cytosolic phospholipase A2 (*cpla2*)	F:GACGTGGCAGATTCAGACAA	92.3	147	NM_001141333
	R:GAACCAGAGAGATGGCAGGT			
Cyclooxygenase-1 (*cox1*)[d]	F:CTCATGAGGGTGGTCCTCAC	104.1	135	BT045745
	R:AGGCACAGGGGGTAGGATAC			
Cyclooxygenase-2 (*cox2*)[f]	F:ACCTTTGTGCGAAACGCTAT	97.0	113	AY848944
	R:GAGTAGGCCTCCCAGCTCTT			
Arachidonate 5-lipoxygenase a *(5loxa*)[d]	F:CTGCTCACCATGCTGCTGTC	97.0	93	NM001139832
	R:GTGTGGGAGGAGGCTTCC			

Arachidonate 5-lipoxygenase b (*5loxb*)[d]	F:ACTGCTGTGGGTTTCCCAAG R:GACAGCAGCGTGATGTGCAG	102.6	98	DW555519
Prostaglandin-D synthase (*pgds*, alias *lipocalin-type pgds*)[d]	F:GGTGCTCAACAAGCTCTACA R:GCAGGAAAGCGATGTTGTCA	90.3	114	BT048787
Prostaglandin E synthase 2-like (*ptges2*)	F:TTCTGCGCTGTTACCCAGAG R:GTACATCGTCTGACCTTCAG	99.8	112	XM_014171682; XM_014160437
Prostaglandin E synthase 3 (*ptges3*)	F:TGGCCTAGGCTAACGAAAGA R:TTGCCTAGTTCCTCGTCTGAG	97.7	101	BT056895
Leukotriene A4 hydrolase (*lkha4*, alias *lta4h*)	F:AAGGTCTCCAAGGTAACAGC R:AATGGCAGTGTGATCTCCAA	94.8	97	NM_001140120
Eukaryotic translation initiation factor 3 subunit D (*eif3d*)[f]	F:CTCCTCCTCCTCGTCCTCTT R:GACCCCAACAAGCAAGTGAT	94.2	105	GE777139
Polyadenylate-binding protein cytoplasmic 1 (*pabpc1*)[f]	F:TGACCGTCTCGGGTTTTTAG R:CCAAGGTGGATGAAGCTGTT	93.6	108	EG908498

[a] F is forward and R is reverse primer.

[b] Amplification efficiency (%)

[c] Primers that were previously published in Emam et al. (2020).

[d] Primers that were previously published in Katan et al. (2019) and shown in Chapter 2.

[e] Primers that were previously published in Hixson et al. (2017).

[f] Primers that were previously published in Caballero-Solares et al. (2017). Normalizer genes are underlined.

Each primer pair was quality-tested to verify that a single product was amplified with no primer dimers, and included standard curves and dissociation curves, as described in Rise et al. (2010) and Booman et al. (2011). In brief, the amplification efficiency (Pfaffl, 2001) of each primer pair was determined using a 5-point 1:3 dilution series starting with cDNA representing 10 ng of input total RNA. Two pools were generated (i.e. 0.3%EPA+DHA↑ω6 and 0.3%EPA+DHA↑ω3), and each pool consisted of 8 fish (with each fish contributing an equal quantity to the pool). The reported primer pair amplification efficiencies were an average of the two pools, except if one pool showed poor efficiency or spacing due to low expression levels (i.e. the 0.3%EPA+DHA↑ω3 pool was used for *fadsd6b* and *fadsd6c,* while the 0.3%EPA+DHA↑ω6 pool was used for *lxrb*).

To select the most suitable normalizer genes, six candidate normalizers were tested based on our previous qPCR studies (*rpl32*, *eef1α-1*, *eef1α-2*, *actb*, *eif3d*, *pabpc1*) (Xue et al., 2015; Caballero-Solares et al., 2017), and salmon literature on reference genes (*actb*, *eef1α-1*, *eef1α-2*) (Olsvik et al., 2005). Their qPCR primers were quality-tested as mentioned above. Half of the fish population involved in the qPCR study was utilized for normalizer testing (i.e. 20 total which consisted of 4 fish per treatment). Cycle threshold (C_T) values were measured using cDNA corresponding to 5 ng of input total RNA. Expression stability was then analysed using the geNorm algorithm (Vandesompele et al., 2002). *Eif3d* and *pabpc1* were shown to be the most stable (i.e. geNorm M-values of 0.23 and 0.22, respectively) among the 6 candidate genes, and therefore were selected as normalizers.

All PCR amplifications were performed in a total reaction volume of 13 µl and consisted of 4 µl of cDNA (5 ng input total RNA), 50 nM each of forward and reverse

primer and 1× Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and nuclease-free water (Invitrogen). The qPCR reactions, including no-template controls, were performed in technical triplicates using the ViiA 7 Real-Time PCR System (384-well format) (Applied Biosystems) and the Power SYBR Green I dye chemistry. The Real-Time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 40 cycles (of 95°C for 15 s and 60°C for 1 min), with the fluorescence signal data collection after each 60°C step. When a C_T value within a triplicate was greater than 0.5 cycle from the other two values, it was considered to be an outlier, discarded and the average C_T of the remaining two values was calculated. The relative quantity (RQ) of each GOI was calculated using a qBase relative quantification framework (Hellemans et al., 2007; Booman et al., 2014) with primer amplification efficiencies incorporated (Table 4.3). The expression levels of each GOI were normalized to both normalizer genes, and the sample with the lowest normalized expression was used as the calibrator sample (i.e. RQ = 1.0) for each GOI (Rise et al. 2015). Transcript expression data are presented as RQ values relative to the calibrator. The qPCR fold-change values were calculated by dividing the mean RQ value of each dietary treatment by that of the 0.3%EPA+DHA↑ω6 fish.

4.3.8. Statistical analyses

4.3.8.1. Tissue lipid composition and qPCR data

Nested general linear models were used with tank nested in diet (Minitab 17 Statistical Software, State College, PA, USA). This was followed by Tukey post-hoc tests ($p < 0.05$) to identify significant differences among treatments at week 12 (Minitab 17

Statistical Software). However, when a significant tank effect was identified ($p < 0.05$), a one-way ANOVA followed by Tukey post-hoc tests was performed (Minitab 17 Statistical Software). To identify significant differences in head kidney lipid composition between week 0 (i.e. initial) and week 12, a one-way ANOVA followed by Tukey post-hoc tests was used. To show the effects of dietary EPA+DHA (i.e. 0.3 and 1%) and ω6:ω3 (i.e. high ω6 and high ω3) factors on the transcript expression of each GOI, a two-way ANOVA was performed (Minitab 17 Statistical Software). The 1.4% EPA+DHA/balanced treatment was not included in this analysis since it was not formulated following the 2x2 factorial design of the other four treatments (i.e., 0.3 or 1.0% EPA+DHA *vs*. high ω6 or high ω3).

For the qPCR analysis, each dietary treatment was tested for outliers using Grubb's test ($p < 0.05$). In total, 7 RQ values were identified as statistical outliers in the entire dataset (i.e. out of 1000 values comprising all samples and all GOIs), and excluded from the study. All GOIs had a sample size of 7-8 per dietary treatment. Finally, residuals were tested to verify normality, independence and homogeneity of variance. Normality was examined using the Anderson-Darling test. If the test failed ($p < 0.05$), a one-way ANOVA on ranks was performed, and followed by a Kruskal-Wallis test (SigmaPlot, Systat Software, Inc., Version 13, San Jose, CA, USA). In all cases, differences were considered statistically significant when $p < 0.05$.

4.3.8.2. Multivariate and Pearson correlation analyses

Principal coordinate analysis (PCoA) was used to describe head kidney lipid and FA composition in the five dietary treatments (PRIMER, Plymouth Routines in Multivariate Ecological Research; PRIMER-E Ltd., version 6.1.15, Ivybridge, UK). A

similarity matrix was used, and the first two PCO axes (i.e. PCO1, PCO2) were plotted. SIMPER (Similarity of Percentages Analysis) was performed to quantify differences among treatments in lipid and FA data, while PERMANOVA (Permutational Multivariate Analysis of Variance) was used to perform pairwise tests between treatments (9999 permutations). In all cases, the non-parametric Bray-Curtis similarity was used.

Pearson correlation analysis was performed to identify relationships between transcript expression (i.e. qPCR RQ data) and tissue lipid composition (i.e. % FA and lipid classes) in the head kidney, using individual fish from all dietary treatments. All GOIs and lipid classes [i.e. triacylglycerols (TAG), sterols (ST) and phospholipids (PL)] were used in the correlation analysis. However, transcripts and lipid classes with no significant correlations were removed. Individual saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) that accounted for $\geq$ 5% of the total FA, and all ω6 and ω3 FA were included in the analysis. IBM SPSS (IBM SPSS Statistics, Version 25, Armonk, NY, USA) was used for the correlation analyses. In order to separately group transcripts and lipid composition (% fatty acids and lipid classes), hierarchical clustering was used with group average in PRIMER.

4.4. Results

4.4.1. Head kidney lipid composition

The predominant lipid class in the head kidney was TAG (46.6-52.0%), followed by PL (22.8-28.6%), and ST (18.4-23.7%) (Table 4.4). No significant differences were observed among dietary treatments in any lipid class or in total lipids (mg g^{-1}) at week 12

Table 4.4. Lipid and fatty acid composition (%) of Atlantic salmon head kidney[a] before (Initial) and after 12 weeks of feeding diets with different ratios of ω6:ω3 and levels of EPA+DHA.

	Initial	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	1%EPA+DHA ↑ω6	1%EPA+DHA ↑ω3	1.4%EPA+DHA balanced
Lipid classes composition (% of total lipid)						
TAG (%)[b]	38.9 ± 18.7	46.6 ± 12.5	48.5 ± 12.8	47.8 ± 9.8	47.0 ± 15.9	52.0 ± 14.7
ST (%)[c]	36.7 ± 15.2	23.7 ± 6.9	20.5 ± 7.2	23.5 ± 6.0	21.8 ± 6.8	18.4 ± 6.8
PL (%)[d]	23.5 ± 11.4	27.2 ± 14.1	28.6 ± 11.2	22.8 ± 9.1	28.2 ± 12.3	25.4 ± 11.0
TL (mg g^{-1})[e]	32.1 ± 12.0	26.9 ± 7.2	33.8 ± 9.0	31.9 ± 8.3	35.1 ± 14.7	36.2 ± 13.4
Fatty acid composition (% of total FA)						
14:0	2.4 ± 0.5	0.9 ± 0.4^{a}	1.1 ± 0.3^{a}	1.4 ± 0.3^{ab}	1.7 ± 0.8^{bc}	2.1 ± 0.9^{c}
16:0	15.4 ± 2.0	14.9 ± 1.2	14.1 ± 0.8	15.6 ± 3.2	14.9 ± 3.7	14.8 ± 2.7
16:1ω7	5.6 ± 1.0	2.1 ± 0.8^{a}	2.9 ± 0.5^{bc}	2.4 ± 0.8^{ab}	3.1 ± 1.0^{bc}	3.4 ± 0.9^{c}
18:0	5.0 ± 0.7	5.6 ± 1.0^{a}	5.2 ± 0.4^{ab}	5.7 ± 1.9^{a}	4.9 ± 0.5^{ab}	4.4 ± 0.3^{b}
18:1ω9	31.5 ± 4.7	20.7 ± 3.1^{a}	24.8 ± 2.3^{bc}	20.0 ± 2.9^{a}	22.6 ± 2.5^{ab}	25.7 ± 2.5^{c}
18:1ω7	3.4 ± 0.4	2.2 ± 0.2^{a}	2.3 ± 0.1^{ab}	2.4 ± 0.3^{ab}	2.4 ± 0.2^{b}	3.0 ± 0.1^{c}
18:2ω6 (LNA)	10.1 ± 1.1	19.3 ± 3.1^{a}	11.6 ± 1.2^{c}	16.3 ± 3.6^{b}	10.5 ± 1.4^{c}	9.7 ± 1.0^{c}
18:3ω6	0.3 ± 0.1	0.9 ± 0.2^{a}	0.5 ± 0.1^{b}	0.5 ± 0.1^{b}	0.3 ± 0.1^{c}	0.3 ± 0.1^{c}
18:3ω3 (ALA)	1.1 ± 0.3	1.9 ± 0.4^{a}	6.5 ± 1.2^{b}	1.7 ± 0.5^{a}	6.3 ± 1.5^{b}	1.7 ± 0.3^{a}
18:4ω3	0.6 ± 0.2	0.7 ± 0.2^{a}	1.8 ± 0.4^{c}	0.7 ± 0.2^{a}	1.5 ± 0.3^{b}	0.9 ± 0.2^{a}
20:1ω11	0.5 ± 0.1	0.2 ± 0.1^{a}	0.2 ± 0.3^{a}	1.0 ± 0.3^{b}	0.9 ± 0.3^{b}	1.5 ± 0.4^{c}
20:1ω9	2.9 ± 0.5	1.2 ± 0.4^{a}	1.4 ± 0.2^{ab}	1.6 ± 0.3^{b}	1.6 ± 0.5^{b}	2.1 ± 0.4^{c}
20:2ω6	0.6 ± 0.1	1.4 ± 0.3^{a}	0.7 ± 0.1^{b}	1.2 ± 0.4^{a}	0.6 ± 0.2^{b}	0.8 ± 0.1^{b}
20:3ω6 (DGLA)	0.4 ± 0.1	2.6 ± 0.7^{a}	1.0 ± 0.2^{c}	1.5 ± 0.5^{b}	0.7 ± 0.2^{d}	0.7 ± 0.1^{cd}
20:4ω6 (ARA)	1.2 ± 0.8	4.3 ± 1.7^{a}	2.5 ± 0.6^{b}	2.4 ± 0.8^{b}	2.0 ± 0.7^{b}	1.9 ± 0.6^{b}
20:3ω3	0.1 ± 0.04	0.1 ± 0.03^{a}	0.4 ± 0.1^{b}	0.2 ± 0.03^{a}	0.4 ± 0.1^{b}	0.2 ± 0.02^{a}
20:4ω3	0.4 ± 0.2	0.4 ± 0.1^{a}	1.0 ± 0.2^{c}	0.6 ± 0.1^{ab}	0.9 ± 0.3^{c}	0.7 ± 0.2^{b}

20:5ω3 (EPA)	2.9 ± 1.7	2.6 ± 0.6[a]	3.7 ± 0.7[b]	3.8 ± 1.2[b]	4.4 ± 1.3[b]	4.3 ± 1.1[b]
22:1ω11	2.4 ± 0.4	0.7 ± 0.3[a]	0.8 ± 0.3[a]	1.4 ± 0.3[b]	1.4 ± 0.5[b]	1.8 ± 0.5[c]
22:4ω6	0.1 ± 0.05	0.2 ± 0.1[a]	0.1 ± 0.01[b]	0.2 ± 0.1[ab]	0.1 ± 0.1[b]	0.2 ± 0.2[ab]
22:5ω6 (ω6 DPA)	0.1 ± 0.1	0.4 ± 0.1[a]	0.2 ± 0.05[b]	0.2 ± 0.04[b]	0.2 ± 0.05[b]	0.2 ± 0.04[b]
22:5ω3 (ω3 DPA)	1.0 ± 0.5	0.8 ± 0.3[a]	1.0 ± 0.1[abc]	1.0 ± 0.4[ab]	1.1 ± 0.4[bc]	1.3 ± 0.4[c]
22:6ω3 (DHA)	6.8 ± 4.3	12.4 ± 2.9	12.8 ± 3.0	13.5 ± 3.4	13.6 ± 3.8	14.0 ± 3.8
ΣSFA[f]	23.8 ± 2.9	22.3 ± 2.2	21.2 ± 1.0	23.8 ± 5.6	22.4 ± 4.9	22.2 ± 3.7
ΣMUFA[g]	48.5 ± 6.9	28.3 ± 4.7[a]	33.8 ± 3.0[ab]	30.7 ± 4.5[ab]	33.7 ± 3.6[b]	39.3 ± 3.7[c]
ΣPUFA[h]	27.3 ± 8.8	49.0 ± 4.5[a]	44.6 ± 2.4[ab]	45.1 ± 8.3[ab]	43.6 ± 5.0[b]	38.1 ± 4.3[c]
Σω3	13.1 ± 7.2	19.1 ± 3.0[a]	27.4 ± 2.9[c]	21.7 ± 5.0[ab]	28.5 ± 4.8[c]	23.4 ± 4.4[b]
Σω6	12.9 ± 1.9	28.9 ± 3.4[a]	16.4 ± 0.8[c]	22.1 ± 4.6[b]	14.3 ± 1.2[cd]	13.6 ± 0.5[d]
ω6:ω3	1.2 ± 0.7	1.5 ± 0.3[a]	0.6 ± 0.1[c]	1.0 ± 0.2[b]	0.5 ± 0.1[c]	0.6 ± 0.1[c]
EPA+DHA	9.7 ± 6.0	15.0 ± 3.3	16.5 ± 3.4	17.3 ± 4.4	18.0 ± 4.8	18.3 ± 4.6
DHA:EPA	2.4 ± 0.2	4.9 ± 1.1[a]	3.4 ± 0.6[b]	3.7 ± 0.7[b]	3.2 ± 0.7[b]	3.3 ± 0.5[b]
EPA:ARA	2.4 ± 0.6	0.7 ± 0.3[a]	1.6 ± 0.3[b]	1.6 ± 0.3[b]	2.2 ± 0.4[c]	2.3 ± 0.5[c]

[a] Mean (n = 10-20) ± standard deviation (SD). Different superscripts in the same row indicate significant differences among treatments at week 12. Underlines represent values that are significantly different to week 0 (Initial) ($p < 0.05$).

[b] Triacylglycerol.

[c] Sterols.

[d] Phospholipids.

[e] Total lipids.

[f] Total saturated fatty acids.

[g] Total monounsaturated fatty acids.

[h] Total polyunsaturated fatty acids.

(p = 0.09-0.72). ST showed a decrease from week 0 (initial) to week 12 in all treatments (p < 0.0001, Table 4.4).

Individual SFA showed differences among treatments (i.e. 14:0 and 18:0; p < 0.0001 and 0.001, respectively) at week 12. The 1.4%EPA+DHA/balanced treatment had higher 14:0 than the 0.3%EPA+DHA↑ω6, 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω6 fed fish. Lower 18:0 levels were observed in the 1.4%EPA+DHA/balanced compared to the high ω6 (i.e. with 0.3% and 1.0% EPA+DHA) treatments. However 16:0 and total SFA (ΣSFA) were similar among dietary treatments (p = 0.60 and 0.45, respectively) at week 12 (Table 4.4).

Salmon fed the 1.4%EPA+DHA/balanced diet had the highest level of total MUFA (ΣMUFA), while the 1%EPA+DHA↑ω3 fed fish showed higher ΣMUFA than the 0.3%EPA+DHA↑ω6 fish at week 12 (p < 0.0001). These differences were also observed in some individual MUFA (i.e. 18:1ω7, 20:1ω11, 20:1ω9, 22:1ω11; p < 0.0001). The 0.3%EPA+DHA↑ω3 fish had higher 18:1ω9 compared to fish fed the high ω6 diets (i.e. 0.3 and 1% EPA+DHA), and this FA was higher in the 1.4%EPA+DHA/balanced than the other treatments (not including 0.3%EPA+DHA↑ω3; p < 0.0001). All treatments showed a significant decrease in ΣMUFA, 18:1ω9 and other MUFA (i.e. 16:1ω7, 18:1ω7, 20:1ω9, 22:1ω11) when comparing week 12 to week 0 (p < 0.0001).

The levels of LC-PUFA precursors 18:2ω6 and 18:3ω3 reflected experimental diet levels (i.e., highest 18:2ω6 in the high ω6 fed salmon; highest 18:3ω3 in the high ω3 fed salmon; p < 0.0001; Table 4.4). Further, compared with week 0, 18:2ω6 and 18:3ω3 increased after 12 weeks of feeding high ω6 (i.e. 0.3%EPA+DHA↑ω6 and 1%EPA+DHA↑ω6) and high ω3 (0.3%EPA+DHA↑ω3 and 1%EPA+DHA↑ω3) diets,

respectively ($p < 0.0001$; Table 4.4). The intermediate ω6 (18:3ω6, 20:2ω6 and 20:3ω6) and ω3 PUFA (18:4ω3, 20:3ω3 and 20:4ω3), and the ω6:ω3 ratios were different among treatments at week 12 ($p < 0.0001$) and responded to dietary changes in ω6 and ω3 PUFA. The proportion of 20:4ω6 was highest in fish fed 0.3%EPA+DHA↑ω6 diet ($p < 0.0001$), while no significant differences were observed among the remaining four treatments at week 12. In contrast, the 0.3%EPA+DHA↑ω6 fish showed the lowest 20:5ω3 ($p < 0.0001$), and similar levels of this ω3 LC-PUFA were observed among the remaining 4 treatments ($p = 0.18$) at week 12. Further, after 12 weeks of feeding, no differences were shown among dietary treatments in 22:6ω3 ($p = 0.53$). Compared with week 0, 20:4ω6 showed an increase after 12 weeks of feeding high ω6 (i.e. 0.3 and 1%EPA+DHA) and the 0.3%EPA+DHA↑ω3 diet, while 20:5ω3 increased in the 1%EPA+DHA↑ω3 and 1.4%EPA+DHA/balanced fed fish. All 5 treatments increased in 22:6ω3 at week 12 compared to week 0 ($p < 0.0001$). Fish fed the 0.3%EPA+DHA↑ω6 diet had the highest ω6:ω3, and the high ω6 fish (i.e. 0.3 and 1% EPA+DHA) had higher ω6:ω3 compared to high ω3 (i.e. 0.3 and 1% EPA+DHA) and 1.4%EPA+DHA/balanced fed fish at week 12 ($p < 0.0001$). The DHA:EPA ratio was higher in the 0.3%EPA+DHA↑ω6 fed fish compared with the other four treatments at week 12 ($p < 0.0001$; Table 4.4). Finally, EPA:ARA was highest in the 1%EPA+DHA↑ω3 and the 1.4%EPA+DHA/balanced treatments, and lowest in the 0.3%EPA+DHA↑ω6 fed fish ($p < 0.0001$).

PCoA showed a clear separation between the high ω6 and high ω3 treatments (i.e. 0.3%EPA+DHA↑ω6 and 1%EPA+DHA↑ω6 on top, and 0.3%EPA+DHA↑ω3 and 1%EPA+DHA↑ω3 on the bottom; Figure 4.1). The 1.4%EPA+DHA/balanced fed fish clustered with the high ω3 treatments. PCO1 and PCO2 accounted for 49.8 and 30.3% of

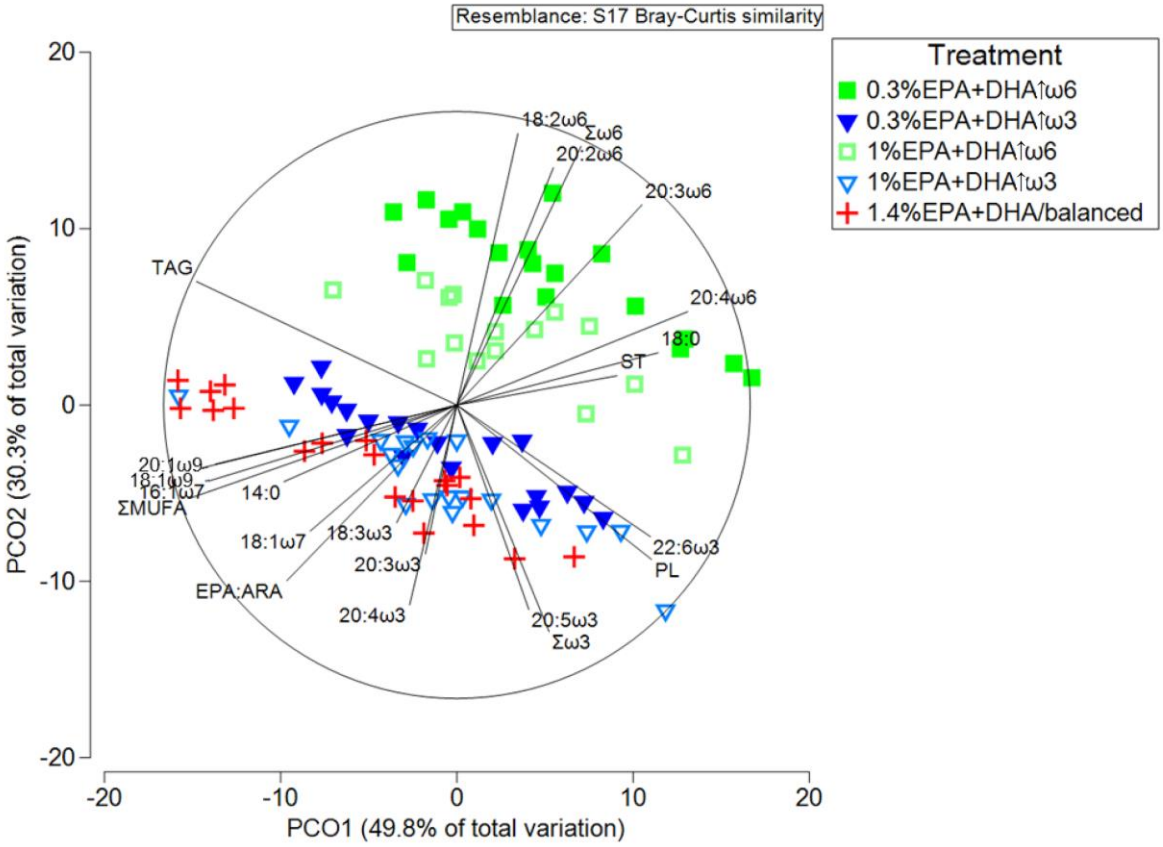


Figure 4.1. Principal coordinates analysis (PcoA) of lipid and FA composition (%) (r^2 > 0.45) in the head kidney of Atlantic salmon after 12 weeks of feeding diets with different ratios of ω6:ω3 and levels of EPA+DHA. PL, ST and TAG represent the lipid classes phospholipid, sterol and triacylglycerol, respectively. ΣMUFA and EPA:ARA represent total monounsaturated fatty acids and the ratio of eicosapentaenoic (20:5ω3) to arachidonic (20:4ω6) acid, respectively.

the variability, respectively. PERMANOVA pairwise tests showed that the 0.3%EPA+DHA↑ω3 were not different from 1%EPA+DHA↑ω3 fed fish [p(perm) = 0.12]. However, all other treatments were significantly different from each other [p(perm)=0.0001–0.003] (Table S.4.1). PcoA vectors showed that fish fed high ω6 diets (particularly 0.3%EPA+DHA↑ω6) were associated with 18:2ω6, 20:2ω6, 20:3ω6, 20:4ω6 and Σω6, while the high ω3 and 1.4%EPA+DHA/balanced diet fed fish were associated with 18:3ω3, 20:3ω3, 20:4ω3, 20:5ω3, Σω3, EPA:ARA, the MUFA 18:1ω7, 16:1ω7, 18:1ω9 and 20:1ω9, as well as ΣMUFA (Figure 4.1). The lipid classes ST and PL clustered with the high ω6 and high ω3 (including 1.4%EPA+DHA/balanced) treatments, respectively.

SIMPER analysis indicated that the dissimilarity between 0.3%EPA+DHA↑ω6 and 0.3%EPA+DHA↑ω3, and between 0.3%EPA+DHA↑ω6 and 1%EPA+DHA↑ω6 were mainly caused by Σω6, Σω3, and the lipid classes TAG, PL and ST (Table S.4.2). The main FA contributors to the dissimilarities between the remaining high ω6 and high ω3 comparisons were Σω6 and 18:2ω6. Finally, the FA dissimilarity between 1.4%EPA+DHA/balanced and the high ω6 treatments were mainly driven by Σω6, ΣPUFA and ΣMUFA, while ΣPUFA contributed to the FA dissimilarity between 1.4%EPA+DHA/balanced and the high ω3 treatments (Table S.4.2). This analysis also showed that the highest dissimilarity in lipid composition profile was between 0.3%EPA+DHA↑ω6 and 1.4%EPA+DHA/balanced fed fish (average dissimilarity = 18.7%), while the lowest dissimilarity was between the two high ω3 (i.e. 0.3%EPA+DHA↑ω3 and 1%EPA+DHA↑ω3; average dissimilarity = 10.0%) and the two

high ω6 (i.e. 0.3%EPA+DHA↑ω6 and 1%EPA+DHA↑ω6; average dissimilarity = 11.0%) treatments.

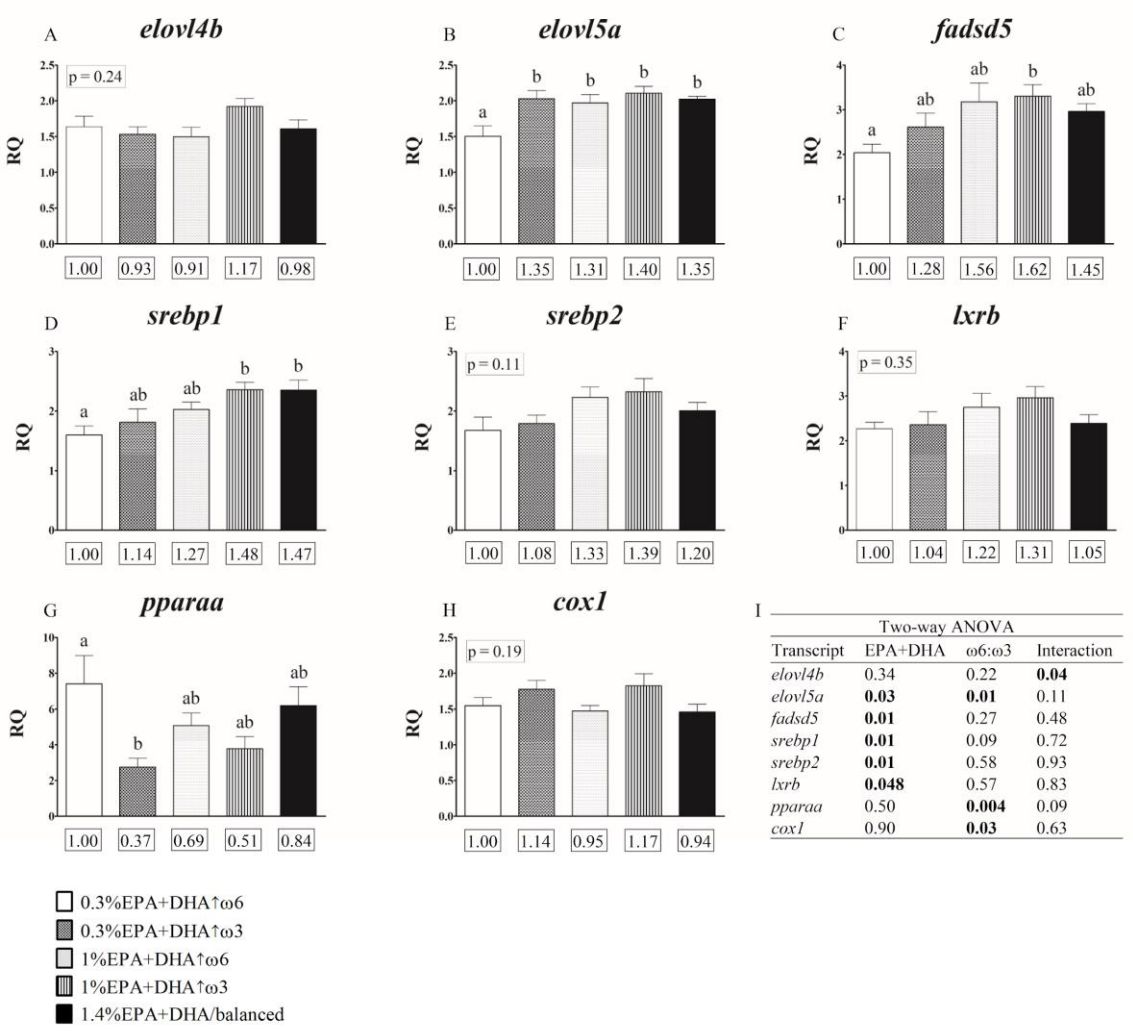
4.4.2. qPCR analysis of transcripts involved in fatty acid metabolism and eicosanoid synthesis

4.4.2.1. Dietary impacts on transcript expression

One-way ANOVA indicated that salmon fed 0.3%EPA+DHA↑ω3, 1.4%EPA+DHA/balanced, and the 1%EPA+DHA diets had higher expression levels (1.31-1.40-fold; p = 0.01) of the transcript *elovl5a* compared with the 0.3%EPA+DHA↑ω6 fed fish (Figure 4.2B and Table S.4.3). The head kidney transcript expression of *fadsd5* was higher in the 1%EPA+DHA↑ω3 compared with the 0.3%EPA+DHA↑ω6 fed fish (1.62-fold; p = 0.04; Figure 4.2C and Table S.4.3), while *srebp1* mRNA levels were higher in the 1%EPA+DHA↑ω3 and 1.4%EPA+DHA/balanced treatments compared with the 0.3%EPA+DHA↑ω6 fed fish (1.48- and 1.47-fold, respectively; p = 0.02; Figure 4.2D and Table S.4.3). The transcript *pparaa* showed lower expression in the 0.3%EPA+DHA↑ω3 compared with the 0.3%EPA+DHA↑ω6 fed fish (0.37-fold; p = 0.02; Figure 4.2G and Table S.4.3). The prostaglandin synthesis-related transcript *ptges3* showed an increasing trend (although not significant) in the 0.3%EPA+DHA↑ω3 compared with the 0.3%EPA+DHA↑ω6 fed fish (1.30-fold; p = 0.06; Table S.4.3).

4.4.2.2. Effects of EPA+DHA and ω6:ω3 factors on transcript expression

Two-way ANOVA analysis showed a significant interaction between the dietary factor EPA+DHA and ω6:ω3, impacting the transcript expression of *elovl4b* (Figure 4.2I and Table



I

Two-way ANOVA

Transcript	EPA+DHA	ω6:ω3	Interaction
elovl4b	0.34	0.22	**0.04**
elovl5a	**0.03**	**0.01**	0.11
fadsd5	**0.01**	0.27	0.48
srebp1	**0.01**	0.09	0.72
srebp2	**0.01**	0.58	0.93
lxrb	**0.048**	0.57	0.83
pparaa	0.50	**0.004**	0.09
cox1	0.90	**0.03**	0.63

Figure 4.2. Head kidney qPCR transcripts related to fatty acid metabolism (A-G) and eicosanoid synthesis (H) in salmon fed diets with different ratios of ω6:ω3 and levels of EPA+DHA for 12 weeks. Transcripts identified as statistically significant in the two-way ANOVA analysis are presented (A-I). Transcript expression data of these and other genes of interest (GOIs) are presented in Table S.4.3. Transcript expression values presented as mean relative quantity (RQ) ± SE (n=7–8). Different letters above error bars indicate significant differences among treatments. When differences were not statistically significant (one-way ANOVA and Tukey post-hoc tests; $p > 0.05$), p-values are shown above error bars. Values below each GOI represent fold-changes relative to the 0.3%EPA+DHA↑ω6 treatment (see Materials and Methods). (I) Two-way ANOVA results. Significant differences ($p < 0.05$) are shown in bold font. Two-way ANOVA analysis of the entire set of GOIs is presented in Table S.4.4.

S.4.4). The transcript expression of *elovl5a* was significantly affected by both EPA+DHA and ω6:ω3, while *fadsd5*, *srebp1*, *srebp2* and *lxrb* were significantly affected by dietary EPA+DHA levels. Finally, the transcript expression of *pparaa* and *cox1* was significantly affected by the factor ω6:ω3 (Figure 4.2I and Table S.4.4).

4.4.3. Hierarchical clustering and Pearson correlation analyses

4.4.3.1. Hierarchical clustering of head kidney transcript expression and lipid composition

Hierarchical clustering of the qPCR analyzed transcripts identified three separate clusters (Figure 4.3). Cluster I consisted of the transcript *pparb2a*, and the eicosanoid metabolism-related transcripts *ptges3*, *ptges2*, *5loxa*, *5loxb* and *cox1*. Cluster II included *pparaa* and *lxra*, while cluster III included the lipid metabolism-related transcripts *srebp2*, *elovl5a*, *srebp1*, *acox1*, *fadsd5*, and the eicosanoid synthesis-related *cox2* and *pgds*. Cluster analysis of head kidney lipid composition also showed three clusters. Cluster I was composed of the ω3 FA (i.e. 20:5ω3, 22:6ω3, 20:4ω3, 18:4ω3, 18:3ω3, 20:3ω3 and 22:5ω3), Σω3, 18:1ω9, ΣMUFA, EPA:ARA, and the lipid class TAG. Cluster II included 18:0, 16:0, ΣSFA and 22:4ω6. Cluster III showed the ω6 FA (i.e. 18:3ω6, 20:2ω6, 18:2ω6, 22:5ω6, 20:3ω6, 20:4ω6), Σω6, ω6:ω3, as well as ΣPUFA and the lipid class ST (Figure 4.3).

4.4.3.2. Correlations between lipid composition and fatty acid metabolism transcripts

The head kidney transcript expression of *pparb2a* was correlated positively with 20:4ω3 ($p = 0.031$; Figure 4.3), while *pparaa* showed negative correlations with several ω3 FA (i.e. 18:3ω3, 18:4ω3, 20:3ω3, 20:4ω3) and Σω3 ($p = 0.002$-0.044), and positive correlations with several ω6 FA (i.e. 18:2ω6, 20:2ω6, 20:3ω6, 20:4ω6, 22:5ω6), Σω6, and the

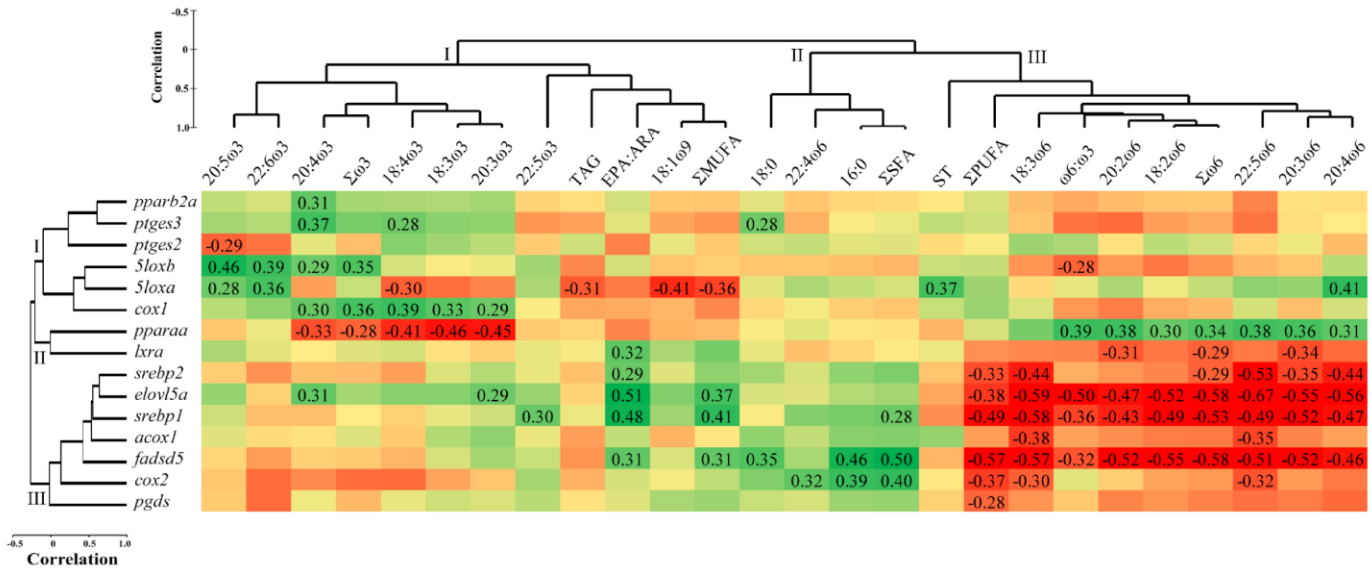


Figure 4.3. Hierarchical clustering and Pearson correlation matrix of head kidney transcript expression [qPCR relative quantity (RQ) values] and lipid composition (% fatty acids and lipid classes) in Atlantic salmon fed diets with different ratios of ω6:ω3 and levels of EPA+DHA for 12 weeks. Transcripts, fatty acids, and lipid classes that were not significantly correlated were excluded (see Materials and Methods). Statistically significant ($p < 0.05$) correlation coefficients are shown. Red cells signify negative relationships, and green cells signify positive relationships. ΣSFA, ΣMUFA, and ΣPUFA represent total saturated, monounsaturated, and polyunsaturated fatty acids, respectively. 20:5ω3, 22:5ω3, 22:6ω3, and 20:4ω6 represent EPA, DPA, DHA, and ARA, respectively. TAG and ST represent triacylglycerol and sterols, respectively.

ratio of ω6:ω3 (p = 0.009-0.038). The five transcripts *lxra*, *srebp2*, *elovl5a*, *srebp1* and *fadsd5* showed positive correlations with EPA:ARA (p = 0.001-0.032; Figure 4.3). The mRNA levels of *elovl5a* also showed positive correlations with 20:3ω3, 20:4ω3 and ΣMUFA (p = 0.013-0.044), while *srebp1* correlated positively with 22:5ω3 and ΣMUFA and ΣSFA (p = 0.006-0.047). The transcript *fadsd5* correlated positively with ΣMUFA, ΣSFA, and the FA 16:0 and 18:0 (p = 0.001-0.032). The same five transcripts (i.e. *lxra*, *srebp2*, *elovl5a*, *srebp1* and *fadsd5*) and *acox1* correlated negatively with ω6 FA; *lxra* with 20:2ω6, 20:3ω6 and Σω6 (p = 0.021-0.042), *srebp2* with 18:3ω6, 20:3ω6, 20:4ω6, 22:5ω6, as well as Σω6 and ΣPUFA (p = 0.0001-0.041). *Elovl5a*, *srebp1* and *fadsd5* correlated negatively with 18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, 20:4ω6, 22:5ω6, Σω6, ω6:ω3 and ΣPUFA (p = 0.0001-0.028). The transcript *acox1* showed negative correlations with 18:3ω6 and 22:5ω6 (p = 0.011 and 0.016, respectively; Figure 4.3).

4.4.3.3. Correlations between lipid composition and eicosanoid metabolism transcripts

The transcript expression of *ptges3* was correlated positively with 18:4ω3, 20:4ω3 and 18:0 (p = 0.012-0.049; Figure 4.3), while *ptges2* showed a negative correlation with 20:5ω3 (p = 0.041). The transcript expressions of *5loxa* and *5loxb* showed positive correlations with 20:5ω3 and 22:6ω3 (p = 0.002-0.045). *5loxb* also correlated positively with 20:4ω3 and Σω3 (p = 0.042 and 0.017, respectively), and negatively with ω6:ω3 (p = 0.044), while *5loxa* correlated positively with ST and 20:4ω6 (p = 0.012 and 0.006, respectively), and negatively with 18:4ω3, TAG, 18:1ω9 and ΣMUFA (p = 0.006-0.038; Figure 4.3). The mRNA levels of *cox1* showed positive correlations with the ω3 FA: 18:3ω3, 18:4ω3, 20:3ω3, 20:4ω3 and Σω3 (p = 0.009-0.043). The transcript *cox2* correlated positively with 16:0, ΣSFA and 22:4ω6 (p =

0.007-0.029), and negatively with 18:3ω6, 22:5ω6 and ΣPUFA (p = 0.013-0.037). Finally, *pgds* correlated negatively with ΣPUFA (p = 0.045; Figure 4.3).

4.5. Discussion

4.5.1 Head kidney lipid composition

The head kidney was mainly composed of TAG (46.6-52.0%), and this was followed by the lipid classes PL (22.8-28.6%), and ST (18.4-23.7%) (Table 4.4). The observed lipid class proportions (i.e. TAG and ST) are in the range of what was found in earlier studies on Atlantic salmon head kidney (Martinez-Rubio et al., 2013; Foroutani et al., 2020). Similar to these findings, Foroutani et al. (2020) reported that TAG was the predominant lipid class in Atlantic salmon head kidney. Head kidney is a principal component of the immune system and the major site of haematopoiesis in fish (Tort et al., 2003; Zapata et al., 2006). Therefore, high levels of TAG may allow fish to fulfill the energy required to fuel cell differentiation processes and other immune functions. Interestingly, Thompson et al. (1995) reported that TAG proportions in Atlantic salmon head kidney leucocytes, macrophages, T- and B-cells varied between ~14 and 20%, and indicated that TAG may serve as an energy store and/or a source of free fatty acids that are used in cellular functions in salmon. The current study also showed that lipid class composition [when expressed as % or as concentration (mg g^{-1} wet weight)] was not significantly affected by diet (Table 4.4, Table S.4.5). This result is in agreement with Betancor at al. (2014) who reported that dietary variation in LC-PUFA did not impact head kidney lipid classes in Atlantic salmon. However, the current study revealed that PL proportions were correlated positively with 20:5ω3 and negatively with 18:2ω6, 20:2ω6,

and ω6:ω3 in the head kidney (Table S.4.6). Further, PcoA indicated associations between PL and the ω3 PUFA 22:6ω3, 20:5ω3 and Σω3 (Figure 4.1). These data suggest a preferential incorporation into the membrane of ω3 over ω6 PUFA. This can potentially increase the availability of anti-inflammatory eicosanoids in the head kidney (Martinez-Rubio et al., 2013). However, this can only be postulated, as fatty acid composition of the PL portion of the head kidney was not determined in this Chapter.

The fatty acid composition of the head kidney reflected that of the experimental diets. For example, LC-PUFA precursor composition (i.e. 18:2ω6 and 18:3ω3) and ω6:ω3 ratio of the head kidney reflected the dietary composition (i.e., highest 18:2ω6 and ω6:ω3 in the high ω6 fed fish; highest 18:3ω3 in the high ω3 fed fish). Previous studies also indicated that head kidney lipid composition of Atlantic salmon (Gjoen et al., 2004; Foroutani et al., 2020), gilthead seabream (*Sparus aurata*) (Montero et al., 2003) and Nile tilapia (*Oreochromis niloticus*) (Chen et al., 2016) reflected dietary variation in 18:2ω6 and 18:3ω3. Gjoen et al. (2004) demonstrated that dietary replacement of FO with soy oil (i.e. 50%) modified head kidney ω6:ω3 (from 0.3 to 1.1), which reflected the dietary ω6:ω3. These ratios are comparable to the ω6:ω3 detected in the present Chapter (i.e. 0.5-1.5). At week 12, the 0.3%EPA+DHA↑ω6 fed fish had 1.7-2.3-fold more 20:4ω6 in terms of proportions and 1.4-1.8-fold in terms of concentrations, when compared with the other treatments. However, 20:5ω3 proportions (0.6-0.7-fold) and concentrations (0.4-0.6-fold) were lowest in this group, compared to the other treatments. Additionally, both proportions and concentrations of 20:5ω3 were similar between the 0.3%EPA+DHA↑ω3 and the 1%EPA+DHA↑ω3 fed fish. A similar effect on head kidney 20:4ω6 proportions was shown in a previous study where soy oil fed Atlantic salmon had 3-fold higher 20:4ω6 than FO

fed fish (Gjoen et al., 2004). Furthermore, the differences in 20:4ω6 and 20:5ω3 proportions are in line with a previous study from our laboratory with Atlantic salmon muscle (Emam et al., 2020), who used the same diets and fish population as this Chapter. Taken together, these data highlight the importance of dietary LC-PUFA precursors (i.e. 18:2ω6 and 18:3ω3) when salmon are fed with lower inclusion of EPA and DHA (0.3%), and their impact on head kidney LC-PUFA. Although LC-PUFA synthesis was not quantified in the current study, the fact that intermediate ω6 and ω3 PUFAs were also significantly different among dietary treatments, and their head kidney proportions were higher than those of the diet, suggest that the LC-PUFA synthesis pathway was activated. Interestingly, 22:6ω3 proportions were not affected by diet at week 12, and 22:6ω3 concentrations (within the high ω3 and high ω6 treatments) did not vary with increasing dietary EPA+DHA levels. This suggests that in addition to elongation and desaturation, selective retention had also occurred. Indeed, earlier studies indicated higher 22:6ω3 retention in Atlantic salmon with decreasing dietary EPA+DHA (Glencross et al., 2014; Bou et al., 2017). However, the fact that 1%EPA+DHA/high↑ω3 and 1.4%EPA+DHA/balanced treatments showed higher concentration of 22:6ω3 than the 0.3%EPA+DHA/high↑ω6 fed fish suggests that both dietary EPA+DHA and ω6:ω3 influenced head kidney DHA content.

Salmon fed the 1.4%EPA+DHA/balanced diet had the highest level of ΣMUFA, and the 1%EPA+DHA↑ω3 fed fish showed higher ΣMUFA than the 0.3%EPA+DHA↑ω6 fed fish. These differences in ΣMUFA, and the observed changes in individual MUFA (i.e. 16:1ω7, 18:1ω7, 18:1ω9 and 20:1ω9) were also a reflection of the diets. Notably, the 1.4%EPA+DHA/balanced diet contained rapeseed oil which is rich in 18:1ω9 (Orsavova

et al., 2015), and this may have influenced the observed changes in tissue MUFA. Emam et al. (2020) reported a similar response in salmon muscle, and showed that hepatic MUFA synthesis-related transcript *scdb* was positively correlated with muscle 20:1ω7 and 20:1ω9, among other MUFA. This suggests that head kidney MUFA differences between treatments in the present Chapter were also related to changes in MUFA synthesis. However, further analyses are required in order to test this hypothesis (e.g. transcript expression of MUFA synthesis-related genes in the head kidney).

4.5.2. Transcript expression of lipid metabolism-related genes

4.5.2.1. Transcripts involved in LC-PUFA synthesis

Transcript expression levels of key genes encoding enzymes involved in LC-PUFA synthesis were modulated by diet in this Chapter. Salmon fed with 0.3%EPA+DHA↑ω6 had the lowest expression of *elovl5a,* while the remaining four treatments had similar levels of this transcript (Figure 4.2B and Table S.4.3). The transcript expression of other genes encoding elongases (*elovl4b*) and desaturases (*fadsd5, fadsd6b* and *fadsd6c)* was also similar among 0.3%EPA+DHA↑ω3, 1%EPA+DHA (both high ω3 and high ω6) and 1.4%EPA+DHA/balanced fed fish (Table S.4.3). Further, *elovl5a* was correlated positively with ω3 (20:3ω3 and 20:4ω3), while both *elovl5a* and *fadsd5* were correlated negatively with ω6 PUFA (18:2ω6, 18:3ω6, 20:2ω6, 20:3ω6, 20:4ω6, 22:5ω6) in the head kidney. These transcript expression changes along with the fatty acid data suggest that 20:5ω3 synthesis was lower in fish fed lower dietary levels of 18:3ω3 and EPA+DHA (i.e. 0.3%), and further support the notion that higher dietary 18:3ω3 promoted the synthesis of ω3 LC-PUFA in the head kidney. Indeed, LC-PUFA synthesis in salmonids is influenced by the

availability of tissue LC-PUFA (i.e. EPA, DHA, ARA) and the precursors 18:3ω3 and 18:2ω6 (Jordal et al., 2005; Jump et al., 2005; Glencross et al., 2015; Katan et al., 2019). *Fadsd5* expression was higher with 1%EPA+DHA↑ω3 compared with 0.3%EPA+DHA↑ω6 fed fish, while *srebp1* showed higher expression levels in the 1%EPA+DHA↑ω3 and 1.4%EPA+DHA/balanced treatments compared with the 0.3%EPA+DHA↑ω6 fed fish. These transcripts, as well as *srebp2* and *lxrb* were significantly positively impacted by dietary EPA+DHA levels (Figure 4.2C-D and Figure 4.2I). These results are in contrast with previous studies reporting up-regulation of hepatic *fadsd5* in Atlantic salmon fed plant-based (containing lower EPA and DHA) compared to FO diets (Zheng et al., 2004; Morais et al., 2011; Xue at al., 2015). However data from the current Chapter are in agreement with Betancor et al. (2014) who reported up-regulation of head kidney *srebp1* and a ~1.5-fold increase in *fadsd5* in Atlantic salmon fed higher dietary levels of EPA+DHA (2% compared to 1% of diet). FO diets used in the former studies contained higher proportions of EPA+DHA (~11-24% of total FA) and higher inclusions of FO (~14-30% of diet) compared to the present study (EPA+DHA proportions and FO inclusions were 2.8-8.8% and 0.1-6.8%, respectively). These as well as organ (i.e. liver *vs* head kidney) differences may explain the discrepancies between studies. Further, dietary ratios of ALA:LNA were shown to influence LC-PUFA accumulation in salmonids and African catfish (*Clarias gariepinus*) muscle tissues (Colombo et al., 2018; Sourabié et al., 2019), and this may have played a role in the regulation of LC-PUFA synthesis in the present Chapter. The transcript expression of *elovl4b* was impacted by the interaction of EPA+DHA and ω6:ω3 in this Chapter. Previous studies in fish showed that *elovl4b* expression was influenced by dietary ω3 LC-PUFA (typically repressed by higher levels;

Li et al., 2017a; Li et al., 2017b), however, the interaction of dietary EPA+DHA with ω6:ω3 and their influence on this transcript is less explored. Finally, the positive impact of dietary EPA+DHA levels in our study could be related to MUFA levels, as the transcript expression of several lipid metabolism genes (*elovl5a*, *fadsd5* and *srebp1*) showed a positive correlation with head kidney ΣMUFA (Figure 4.3). Interestingly, Emam et al. (2020) showed that hepatic *elovl5a* and *srebp2* were positively correlated with the levels of individual MUFA in salmon muscle. Indeed, an earlier study with Atlantic salmon revealed that LC-PUFA synthesis is stimulated by dietary MUFA levels, and suggested that the sparing effect of SFA and MUFA on ω3 LC-PUFA could be the underlying mechanism (Emery et al., 2016).

The transcript expression of head kidney *pparaa* was higher in the 0.3%EPA+DHA↑ω6 compared with the 0.3%EPA+DHA↑ω3 fed fish (Figure 4.2G), and correlation analysis indicated that this transcript was positively correlated with ω6 PUFA (18:2ω6, 20:2ω6, 20:3ω6, 20:4ω6, 22:5ω6), Σω6 and ω6:ω3, and negatively with ω3 PUFA (18:3ω3, 18:4ω3, 20:3ω3, 20:4ω3) and Σω3 (Figure 4.3). Ppara is a transcription factor that regulates numerous genes involved in lipid-related processes such as FA oxidation, as well as bile acid and triacylglycerol metabolism (Kersten, 2014). Further, several studies in fish demonstrated involvement of Ppara in LC-PUFA synthesis (Dong et al., 2017; Zhu et al., 2019). Thus, data shown herein suggest that *pparaa* may have played a regulatory role in ω6 PUFA synthesis, particularly in the 0.3%EPA+DHA↑ω6 fed fish. Emam et al. (2020) showed that hepatic *pparaa* expression was higher in the 0.3%EPA+DHA↑ω3 than the 0.3%EPA+DHA↑ω6 and 1% EPA+DHA treatments (i.e. high ω3 and high ω6), and also a positive correlation between liver *pparaa* and muscle ω3 PUFA. These contrasting results

suggest that *pparaa* response in Atlantic salmon is tissue dependent. Finally, the fact that the transcript expression of *pparaa* was significantly impacted by dietary ω6:ω3 (Figure 4.2I), and the correlation with head kidney ω6:ω3 (Figure 4.3), suggest that this transcript may have been involved with inflammatory processes in this Chapter. This is in line with previous studies with mammals (Jones et al., 2002; Becker et al., 2008; Varga et al., 2011) and Atlantic salmon (Martinez-Rubio et al., 2013) which showed that Ppara and *ppara* play a role in the resolution of inflammation. More studies are required in order to investigate the dietary-induced impacts of *ppara* on inflammatory processes in Atlantic salmon head kidney.

4.5.2.2 Transcripts involved in eicosanoid metabolism

My qPCR results (Table S.4.3) are in line with previous studies which indicated no differences in head kidney constitutive transcript expression of several genes related to eicosanoid synthesis (i.e. *5lox, cox2*) in fish exposed to varying EPA levels [i.e. 0-20 µM (Furne et al., 2013), 1.1 and 19 mg g^{-1} lipid (Salini et al., 2016)] or ω6:ω3 ratios (0.7-4.1) (Holen et al., 2018). However, Holen et al. (2018) reported that Atlantic salmon fed with soybean oil as a FO replacement had higher expression of *pgds* and *pges* in head kidney leucocytes compared to fish fed palm and rapeseed oils. Differences in dietary and tissue EPA:ARA, EPA+DHA, the tissues/cells examined (head kidney *vs.* isolated leukocytes), and variation between *in vivo* and *in vitro* models could have contributed to these discrepancies between studies. Further, this Chapter revealed that head kidney transcript expression of *cox1* was negatively impacted by dietary ω6:ω3 levels (Figure 4.2H-I), and positively correlated with head kidney ω3 fatty acids (i.e. 18:3ω3, 18:4ω3, 20:3ω3, 20:4ω3

and Σω3; Figure 4.3). The enzyme COX1 is constitutively expressed in several tissues (e.g. liver, kidney, spleen, gill, muscle, gut) and has maintenance and homeostatic functions (Tapiero et al., 2002; Olsen et al., 2012). These data suggest that *cox1* expression could result in production of anti-inflammatory prostaglandins in Atlantic salmon head kidney at the constitutive level. Further, differences among treatments in head kidney EPA:ARA (Table 4.4) may have altered the production of eicosanoids via *cox1* transcription. Indeed, changes in cell membrane EPA:ARA could alter eicosanoid production and the inflammatory response in vertebrates (Calder, 2003; Arts and Kohler, 2009; Salini et al., 2016). However, since eicosanoid levels were not measured in this Chapter, these hypotheses could not be tested. Further, the implications of FA-transcript correlations on eicosanoid production could not be assessed.

In addition to *cox1*, transcript expression of *5loxa*, *5loxb* and *ptges3* was also positively correlated with head kidney ω3 LC-PUFA. However, *5loxa* showed positive correlations with both ω3 (i.e. 20:5ω3, 22:6ω3) and ω6 (20:4ω6) LC-PUFA. The latter result is in line with Chapter 2 and Katan et al. (2019) who reported that hepatic *5loxa* showed a positive relationship ($p = 0.059$) with liver EPA+ARA in Atlantic salmon fed different mixes of plant oils as a FO replacement. However, this is in contrast with Caballero-Solares et al. (2020) who showed that hepatic *5loxa* correlated negatively with liver EPA and DHA in salmon fed terrestrial plant ingredients as FO and FM alternatives. Discrepancies between studies suggest that the interaction of *5loxa* transcription with tissue LC-PUFA in salmon depends on tissue (i.e. head kidney *vs* liver) and/or dietary inputs (e.g. protein and lipid sources). This Chapter also showed positive correlations between ω3 PUFA (i.e. 18:4ω3, 20:4ω3) and *ptges3* expression, and negative correlations between EPA

and *ptges2*. In mammals, both PTGES2 and PTGES3 convert PGH_2 into PGE_2 (Neuman et al., 2017; Xu et al., 2019). Wei et al. (2016) showed that muscle *ptges3* expression was induced in pigs fed with linseed-enriched diets for 60 days. However, previous studies reported no impacts of dietary fatty acids on the transcript expression of *ptges2* and *ptges3* in bovine oocytes (Ponter et al., 2012) and Eurasian perch (*Perca fluviatilis*) liver, brain and intestine (Geay et al., 2015). Clearly, more research is required to elucidate the influence of dietary and tissue PUFA on the transcript expression of *ptges2* and *ptges3* in fish.

Cox2 showed a positive correlation with 16:0, ΣSFA and 22:4ω6, and negative correlations with ΣPUFA, 18:3ω6 and 22:5ω6 in the current study (Figure 4.3). This finding supports previous human macrophage studies which indicated that SFA induced *cox2* expression (Lee et al., 2001; Rocha et al., 2016). Astiz et al. (2012) showed that addition of 22:4ω6 and 22:5ω6 did not impact the production of COX2 in rat testis cells. However, a previous study on mouse brain indicated that reduction in the levels of ARA metabolites 22:4ω6 and 22:5ω6 reflected a shift of ARA from LC-PUFA biosynthesis to prostaglandins or leukotrienes synthesis (McNamara et al., 2009). Thus, the negative correlation between 22:5ω6 and *cox2* could be a result of higher availability of ARA for COX-2 mediated prostaglandin synthesis. Finally, similarly to *cox2*, *pgds* correlated negatively with ΣPUFA. This latter finding is in line with Chapter 2 who showed that hepatic *pgds* was negatively correlated with liver EPA+ARA+DHA in Atlantic salmon. However, Holen et al. (2015) reported that the combination of EPA+ARA+DHA did not modulate *cox2* transcript expression in Atlantic salmon head kidney cells. In summary, further examination is needed in order to elucidate the influence of ARA metabolites (i.e. 22:4ω6 and 22:5ω6) on *cox2*

expression, and the interactive impacts of PUFA on the transcript expression of *pgds* and *cox2* in Atlantic salmon head kidney.

4.6. Conclusion

This study investigated head kidney lipid composition and transcript expression of genes involved in fatty acid and eicosanoid metabolism in Atlantic salmon fed varying dietary levels of EPA+DHA (0.3, 1.0 and 1.4%) and ω6:ω3 ratios (high ω6, high ω3, and balanced) for 12 weeks. TAG was the predominant lipid class in all treatments, regardless of diet. This suggests that this lipid class could play an important role in immune and/differentiation processes in Atlantic salmon head kidney. Head kidney fatty acid composition was reflective of the diet with respect to C_{18} PUFA, MUFA levels (% of total fatty acids) and responded to ω6:ω3 variation. Proportions of 20:5ω3 were similar among 0.3%EPA+DHA↑ω3, 1%EPA+DHA (both high ω3 and high ω6 treatments) and 1.4%EPA+DHA/balanced fed fish, although dietary 20:5ω3 varied by 2.5- to 3-fold. Tissue fatty acid composition changes agreed with positive correlations between head kidney 20:3ω3 and 20:4ω3 and *elovl5a* transcription. This suggested that high dietary 18:3ω3 promoted the synthesis of ω3 LC-PUFA in salmon fed lower dietary EPA+DHA levels (0.3%). Further, head kidney transcript expression of several genes involved in FA metabolism (e.g. *elovl5a, fadsd5* and *srebp1)* was positively impacted by dietary EPA+DHA levels, and showed a positive correlation with head kidney ΣMUFA. This supported the hypothesis that LC-PUFA synthesis is stimulated by increasing MUFA levels in Atlantic salmon.

Several eicosanoid synthesis-related transcripts were significantly correlated with head kidney fatty acid composition. The head kidney transcript expression of *5loxb* correlated positively with ω3 LC-PUFA and Σω3, and correlated negatively with ω6:ω3. *Cox1* and *ptges3* transcript expression correlated positively with ω3 PUFA, while *cox1* was negatively impacted by dietary ω6:ω3 levels. I hypothesize that these transcript expression changes may have increased the production of anti-inflammatory eicosanoids in salmon head kidney. Finally, this study revealed significant positive and negative correlations of *cox2* expression with SFA (i.e. 16:0, ΣSFA) and 22:5ω6, respectively. Given the important role of head kidney in salmon immune response and the interaction with dietary LC-PUFA future studies should elucidate how ω6:ω3 and EPA+DHA levels affect LC-PUFA synthesis (e.g. stable isotope and FA mass balance methods) and eicosanoid production [e.g. Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)] in this organ.

4.7. References

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4.8. Supplemental material

Table S.4.1. PERMANOVA pairwise test[a] of head kidney FA and lipid composition compared across dietary treatments.

Dietary treatments	t	P(perm)[b]
0.3%EPA+DHA↑ω6, 0.3%EPA+DHA↑ω3	5.11	**0.0001**
0.3%EPA+DHA↑ω6, 1%EPA+DHA↑ω6	2.40	**0.003**
0.3%EPA+DHA↑ω6, 1%EPA+DHA↑ω3	5.64	**0.0001**
0.3%EPA+DHA↑ω6, 1.4%EPA+DHA/balanced	5.91	**0.0001**
0.3%EPA+DHA↑ω3, 1%EPA+DHA↑ω6	3.64	**0.0001**
0.3%EPA+DHA↑ω3, 1%EPA+DHA↑ω3	1.50	0.12
0.3%EPA+DHA↑ω3, 1.4%EPA+DHA/balanced	3.13	**0.0002**
1%EPA+DHA↑ω6, 1%EPA+DHA↑ω3	3.76	**0.0001**
1%EPA+DHA↑ω6, 1.4%EPA+DHA/balanced	4.19	**0.0001**
1%EPA+DHA↑ω3, 1.4%EPA+DHA/balanced	2.84	**0.002**

Significant P(perm)-values (i.e. $p < 0.05$) are in bold font.

[a] The non-parametric Bray-Curtis similarity was used with 9999 permutations (see Materials and Methods).

[b] PERMANOVA p-values for all dietary treatment comparisons.

Table S.4.2. SIMPER dissimilarities of head kidney FA and lipid composition compared across dietary treatments.

Dietary treatments	Ave. diss. (%)[a]	Major lipid class / FA contributor[b]	Con. (%)[c]
0.3%EPA+DHA↑ω6 & 0.3%EPA+DHA↑ω3	15.6	TAG	12.9
		PL	12.6
		Σω6	12.1
		Σω3	7.7
		ST	7.4
0.3%EPA+DHA↑ω6 & 1%EPA+DHA↑ω6	11.0	TAG	15.6
		PL	15.6
		ST	9.2
		Σω6	7.7
		Σω3	6.2
0.3%EPA+DHA↑ω3 & 1%EPA+DHA↑ω6	12.5	TAG	15
		PL	13.8
		ST	9.2
		Σω6	8.5
		18:2ω6	6.8
0.3%EPA+DHA↑ω6 & 1%EPA+DHA↑ω3	16.6	Σω6	13.1
		TAG	12.2
		PL	11.2
		Σω3	8.8
		18:2ω6	7.9
0.3%EPA+DHA↑ω3 & 1%EPA+DHA↑ω3	10.0	TAG	22.1
		PL	17.9
		ST	10.9
1%EPA+DHA↑ω6 & 1%EPA+DHA↑ω3	12.7	TAG	14.7
		PL	12.4
		Σω6	10.6
		ST	8
		18:2ω6	7.9
0.3%EPA+DHA↑ω6 & 1.4%EPA+DHA/balanced	18.7	Σω6	12.4
		TAG	12.1
		PL	10.4
		ΣMUFA	9.2
		ΣPUFA	9.2
0.3%EPA+DHA↑ω3 & 1.4%EPA+DHA/balanced	12.7	TAG	18.3
		PL	14.8
		ST	9.2

		ΣPUFA	7.8
1%EPA+DHA↑ω6 & 1.4%EPA+DHA/balanced	14.9	TAG	14.3
		PL	11
		Σω6	10
		ΣMUFA	9.7
		ΣPUFA	9.7
1%EPA+DHA↑ω3 & 1.4%EPA+DHA/balanced	12.3	TAG	19.6
		PL	14.5
		ST	9.2
		ΣPUFA	8

[a] Average dissimilarity (%).

[b] TAG, PL and ST represent triacylglycerol, phospholipids, and sterols, respectively. ΣMUFA and ΣPUFA represent total monounsaturated and polyunsaturated fatty acids, respectively.

[c] Percent contribution.

Table S.4.3. Relative transcript expression of genes related to FA and eicosanoid metabolism in the head kidney of Atlantic salmon after 12 weeks of feeding diets with different ratios of ω6:ω3 and levels of EPA+DHA.

Transcript[a]	0.3%EPA +DHA ↑ω6	0.3%EPA +DHA ↑ω3	1%EPA+ DHA ↑ω6	1%EPA+ DHA ↑ω3	1.4%EPA +DHA balanced	p-value[b]
elovl4b	1.6 ± 0.4	1.5 ± 0.3	1.5 ± 0.4	1.9 ± 0.3	1.6 ± 0.4	0.24
elovl5a	1.5 ± 0.4[a]	2.0 ± 0.3[b]	2.0 ± 0.3[b]	2.1 ± 0.3[b]	2.0 ± 0.1[b]	**0.01**
fadsd5	2.0 ± 0.5[a]	2.6 ± 0.9[ab]	3.2 ± 1.2[ab]	3.3 ± 0.7[b]	3.0 ± 0.5[ab]	**0.04**
fadsd6b	3.1 ± 1.3	3.2 ± 1.6	2.7 ± 0.8	2.8 ± 0.3	2.9 ± 0.7	0.70
fadsd6c	3.0 ± 1.2	2.9 ± 1.1	3.1 ± 1.3	3.9 ± 1.3	3.5 ± 1.1	0.50
srebp1	1.6 ± 0.4[a]	1.8 ± 0.6[ab]	2.0 ± 0.3[ab]	2.4 ± 0.3[b]	2.3 ± 0.5[b]	**0.02**
srebp2	1.7 ± 0.6	1.8 ± 0.4	2.2 ± 0.5	2.3 ± 0.6	2.0 ± 0.4	0.11
lxra	1.5 ± 0.3	1.5 ± 0.3	1.5 ± 0.2	1.7 ± 0.2	1.8 ± 0.3	0.23
lxrb	2.3 ± 0.4	2.4 ± 0.8	2.8 ± 0.9	3.0 ± 0.7	2.4 ± 0.6	0.35
pparaa	7.4 ± 4.5[a]	2.8 ± 1.4[b]	5.1 ± 2.0[ab]	3.8 ± 1.9[ab]	6.2 ± 3.0[ab]	**0.02**
pparb1	2.1 ± 0.5	2.0 ± 0.2	1.9 ± 0.6	2.0 ± 0.4	1.8 ± 0.3	0.68
pparb2a	1.4 ± 0.3	1.5 ± 0.3	1.5 ± 0.3	1.6 ± 0.3	1.4 ± 0.3	0.33
fasa	2.0 ± 0.9	2.0 ± 0.7	2.0 ± 0.6	2.3 ± 0.6	1.9 ± 0.6	0.85
fasb	4.0 ± 1.9	3.6 ± 1.4	3.5 ± 0.9	3.7 ± 1.1	3.0 ± 0.8	0.78
acox1	1.5 ± 0.4	1.7 ± 0.4	1.7 ± 0.3	1.8 ± 0.3	1.6 ± 0.2	0.62
cpt1a	1.5 ± 0.3	1.5 ± 0.1	1.5 ± 0.3	1.4 ± 0.3	1.5 ± 0.2	0.93
cpla2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.2	1.3 ± 0.1	1.4 ± 0.2	0.97
cox1	1.6 ± 0.3	1.8 ± 0.4	1.5 ± 0.2	1.8 ± 0.5	1.5 ± 0.3	0.19
cox2	3.3 ± 2.1	3.3 ± 0.8	4.2 ± 1.5	3.1 ± 1.5	3.9 ± 1.7	0.58
5loxa	1.9 ± 0.2	1.7 ± 0.4	1.9 ± 0.5	1.7 ± 0.5	1.8 ± 0.6	0.88
5loxb	1.5 ± 0.3	1.7 ± 0.3	1.6 ± 0.4	1.7 ± 0.4	1.7 ± 0.5	0.82
pgds	2.6 ± 1.1	3.4 ± 1.7	3.0 ± 1.1	3.1 ± 0.4	2.8 ± 0.9	0.74
ptges2	2.1 ± 0.6	2.1 ± 0.5	2.0 ± 0.8	2.0 ± 0.6	1.6 ± 0.4	0.57
ptges3	1.6 ± 0.5	2.1 ± 0.2	1.9 ± 0.5	1.9 ± 0.4	1.6 ± 0.4	0.06
ikha4	1.6 ± 0.4	1.5 ± 0.2	1.7 ± 0.7	1.6 ± 0.2	1.4 ± 0.3	0.71

Significant p-values (i.e. $p < 0.05$) are in bold font. [a] Transcript expression values presented as mean relative quantity (RQ) ± SD (n = 7–8). RQs were normalized to eukaryotic translation initiation factor 3 subunit D (*eif3d*) and polyadenylate-binding protein cytoplasmic 1 (*pabpc1*), and calibrated to the lowest expressing individual for each gene of interest (see Materials and Methods). [b] Different letters indicate significant differences among treatments (one-way ANOVA followed by Tukey post-hoc tests; see Materials and Methods).

Table S.4.4. Two-way ANOVA analysis[a] illustrating effects of dietary EPA+DHA (i.e. 0.3 and 1%) and ω6:ω3 (i.e. high ω6 and high ω3) on the transcript expression of genes related to FA and eicosanoid metabolism in Atlantic salmon head kidney.

Transcript	EPA+DHA	ω6:ω3	Interaction
elovl4b	0.34	0.22	**0.04**
elovl5a	**0.03**	**0.01**	0.11
fadsd5	**0.01**	0.27	0.48
fadsd6b	0.31	0.77	0.90
fadsd6c	0.24	0.43	0.27
srebp1	**0.01**	0.09	0.72
srebp2	**0.01**	0.58	0.93
lxra	0.28	0.31	0.39
lxrb	**0.048**	0.57	0.83
pparaa	0.50	**0.004**	0.09
pparb1	0.53	0.86	0.45
pparb2a	0.35	0.19	0.89
fasa	0.48	0.55	0.55
fasb	0.67	0.87	0.56
acox1	0.32	0.43	0.76
cpt1a	0.55	0.69	0.77
cpla2	0.88	0.68	0.99
cox1	0.90	**0.03**	0.63
cox2	0.53	0.35	0.35
5loxa	0.65	0.20	0.93
5loxb	0.70	0.30	0.92
pgds	0.85	0.31	0.40
ptges2	0.77	0.86	0.87
ptges3	0.65	0.14	0.09
ikha4	0.54	0.30	0.93

[a] p-values corresponding to the factors EPA+DHA, ω6:ω3, and their interaction (see Materials and Methods). Significant p-values (i.e. $p < 0.05$) are in bold font.

Table S.4.5. Lipid class and fatty acid composition (mg g^{-1} wet weight) of Atlantic salmon head kidney[a] before (Initial) and after 12 weeks of feeding diets with different ratios of ω6:ω3 and levels of EPA+DHA.

	Initial	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	1%EPA+DHA ↑ω6	1%EPA+DHA ↑ω3	1.4%EPA+DHA balanced
Lipid classes composition (mg g^{-1})						
TAG[b]	14.2 ± 10.2	12.9 ± 6.0	17.3 ± 8.1	15.7 ± 6.4	18.2 ± 14.9	20.3 ± 13.1
ST[c]	9.9 ± 2.1	6.1 ± 1.7	6.5 ± 1.8	7.1 ± 1.5	7.1 ± 2.1	6.0 ± 1.5
PL[d]	6.6 ± 2.9	7.2 ± 3.4	9.2 ± 3.7	7.0 ± 2.9	8.8 ± 2.8	8.5 ± 3.4
Fatty acid composition (mg g^{-1})						
14:0	0.4 ± 0.3	0.2 ± 0.1^{a}	0.3 ± 0.1^{ab}	0.3 ± 0.2^{ab}	0.4 ± 0.3^{bc}	0.5 ± 0.3^{c}
16:0	2.7 ± 1.7	2.6 ± 0.8	3.3 ± 1.1	3.3 ± 1.4	3.2 ± 1.6	3.6 ± 1.7
16:1ω7	0.9 ± 0.6	0.4 ± 0.2^{a}	0.7 ± 0.3^{ab}	0.5 ± 0.3^{ab}	0.8 ± 0.7^{ab}	0.9 ± 0.6^{b}
18:0	0.9 ± 0.7	1.0 ± 0.3	1.2 ± 0.4	1.2 ± 0.6	1.1 ± 0.5	1.1 ± 0.5
18:1ω9	5.1 ± 3.1	3.7 ± 1.6^{a}	5.9 ± 2.4^{ab}	4.3 ± 1.9^{ab}	5.7 ± 4.0^{ab}	7.0 ± 4.3^{b}
18:1ω7	0.6 ± 0.4	0.4 ± 0.1^{a}	0.5 ± 0.2^{a}	0.5 ± 0.2^{a}	0.6 ± 0.4^{ab}	0.8 ± 0.4^{b}
18:2ω6 (LNA)	1.6 ± 1.3	3.5 ± 1.7	2.8 ± 1.1	3.3 ± 1.3	2.7 ± 1.9	2.6 ± 1.6
18:3ω6	0.1 ± 0.05	0.2 ± 0.1^{a}	0.1 ± 0.1^{ab}	0.1 ± 0.04^{b}	0.1 ± 0.1^{b}	0.1 ± 0.1^{b}
18:3ω3 (ALA)	0.2 ± 0.2	0.3 ± 0.2^{a}	1.6 ± 0.7^{b}	0.4 ± 0.2^{a}	1.6 ± 1.0^{b}	0.5 ± 0.3^{a}
18:4ω3	0.1 ± 0.1	0.1 ± 0.1^{a}	0.4 ± 0.2^{c}	0.2 ± 0.1^{a}	0.4 ± 0.2^{bc}	0.3 ± 0.2^{ab}
20:1ω11	0.1 ± 0.1	0.02 ± 0.01^{a}	0.1 ± 0.1^{a}	0.2 ± 0.1^{b}	0.2 ± 0.2^{b}	0.4 ± 0.2^{c}
20:1ω9	0.4 ± 0.3	0.2 ± 0.1^{a}	0.3 ± 0.2^{a}	0.3 ± 0.2^{ab}	0.4 ± 0.4^{ab}	0.6 ± 0.4^{b}
20:2ω6	0.1 ± 0.1	0.3 ± 0.1^{a}	0.2 ± 0.1^{b}	0.2 ± 0.1^{a}	0.2 ± 0.1^{ab}	0.2 ± 0.1^{ab}
20:3ω6 (DGLA)	0.1 ± 0.1	0.5 ± 0.1^{a}	0.2 ± 0.1^{bc}	0.3 ± 0.1^{b}	0.2 ± 0.1^{c}	0.2 ± 0.1^{c}
20:4ω6 (ARA)	0.2 ± 0.2	0.7 ± 0.2^{a}	0.5 ± 0.1^{b}	0.5 ± 0.1^{b}	0.4 ± 0.1^{b}	0.4 ± 0.1^{b}
20:3ω3	0.01 ± 0.01	0.03 ± 0.01^{a}	0.1 ± 0.04^{b}	0.03 ± 0.01^{a}	0.1 ± 0.1^{b}	0.04 ± 0.02^{a}
20:4ω3	0.1 ± 0.1	0.1 ± 0.03^{a}	0.2 ± 0.1^{b}	0.1 ± 0.1^{a}	0.2 ± 0.1^{b}	0.2 ± 0.1^{b}
20:5ω3 (EPA)	0.5 ± 0.5	0.4 ± 0.1^{a}	0.8 ± 0.2^{b}	0.7 ± 0.2^{b}	1.0 ± 0.4^{bc}	1.1 ± 0.4^{c}

22:1ω11	0.4 ± 0.2	0.1 ± 0.1[a]	0.2 ± 0.1[ab]	0.3 ± 0.1[ab]	0.4 ± 0.3[bc]	0.5 ± 0.4[c]
22:4ω6	0.02 ± 0.03	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
22:5ω6 (ω6 DPA)	0.02 ± 0.02	0.1 ± 0.02[a]	0.1 ± 0.01[b]	0.04 ± 0.01[b]	0.04 ± 0.02[b]	0.05 ± 0.02[b]
22:5ω3 (ω3 DPA)	0.2 ± 0.2	0.1 ± 0.1[a]	0.2 ± 0.1[ab]	0.2 ± 0.1[ab]	0.3 ± 0.2[b]	0.3 ± 0.2[b]
22:6ω3 (DHA)	1.2 ± 1.1	2.1 ± 0.5[a]	2.8 ± 0.7[abc]	2.6 ± 0.7[ab]	3.1 ± 1.1[bc]	3.4 ± 0.9[c]
ΣSFA[e]	4.3 ± 2.7	3.9 ± 1.2	4.9 ± 1.6	5.0 ± 2.2	4.9 ± 2.5	5.5 ± 2.6
ΣMUFA[f]	8.5 ± 5.2	5.0 ± 2.2[a]	8.0 ± 3.3[ab]	6.6 ± 2.9[a]	8.5 ± 6.1[ab]	10.6 ± 6.5[b]
ΣPUFA[g]	4.5 ± 3.9	8.7 ± 2.8	10.3 ± 3.3	9.0 ± 2.6	10.4 ± 5.2	9.7 ± 4.2
Σω3	2.2 ± 2.1	3.3 ± 0.9[a]	6.2 ± 1.9[c]	4.3 ± 1.2[ab]	6.7 ± 2.8[c]	5.9 ± 2.1[bc]
Σω6	2.0 ± 1.6	5.3 ± 2.0	3.9 ± 1.4	4.5 ± 1.5	3.6 ± 2.2	3.6 ± 2.0
EPA+DHA	2.0 ± 1.6	2.5 ± 0.6[a]	3.6 ± 0.9[bc]	3.4 ± 0.9[ab]	4.1 ± 1.5[bc]	4.5 ± 1.3[c]

[a] Mean (n = 10-20) ± standard deviation (SD). Different superscripts in the same row indicate significant differences among treatments at week 12. Underlines represent values that are significantly different to week 0 (Initial) ($p < 0.05$). [b] Triacylglycerol. [c] Sterols. [d] Phospholipids. [e] Total saturated fatty acids. [f] Total monounsaturated fatty acids. [g] Total polyunsaturated fatty acids.

Table S.4.6. Significant Pearson correlations[a] ($p < 0.05$) between head kidney lipid and fatty acid composition (% of total) in Atlantic salmon fed diets with different ratios of ω6:ω3 and levels of EPA+DHA. TAG, PL, and ST represent triacylglycerol, phospholipids, and sterols, respectively. ΣSFA, ΣMUFA, and ΣPUFA represent total saturated, monounsaturated, and polyunsaturated fatty acids, respectively.

Lipid class	FA	Pearson r	p-value
TAG	16:0	-0.36	0.03
	18:0	-0.44	0.01
	18:1ω9	0.55	0.0001
	20:4ω6 (ARA)	-0.41	0.01
	22:6ω3 (DHA)	-0.45	0.01
	ΣSFA	-0.35	0.04
	ΣMUFA	0.49	0.002
PL	18:2ω6 (LNA)	-0.40	0.01
	20:2ω6	-0.34	0.04
	20:5ω3 (EPA)	0.41	0.01
	ω6:ω3	-0.34	0.04
ST	18:1ω9	-0.70	0.0001
	20:3ω6 (DGLA)	0.43	0.01
	20:4ω6 (ARA)	0.57	0.0001
	22:6ω3 (DHA)	0.44	0.01
	ΣMUFA	-0.71	0.0001
	ΣPUFA	0.49	0.002
	EPA:ARA	-0.49	0.002

[a] Pearson correlations were calculated using individual fish from all dietary treatments (n = 6–8).

Chapter 5. Antibacterial immune gene expression and eicosanoid responses in Atlantic salmon (*Salmo salar*) fed diets with varying EPA+DHA levels and ω6 to ω3 ratios

Preface

The study described in Chapter 5 is prepared for submission to the journal *Fish and Shellfish Immunology* as Katan, T., Caballero-Solares, A., Xue, X., Taylor, R., Yeo, J., Parrish, C. C., and Rise, M. L. (2021). Antibacterial immune gene expression and eicosanoid responses in Atlantic salmon (*Salmo salar*) fed diets with varying EPA+DHA levels and ω6 to ω3 ratios.

5.1. Abstract

The interaction of dietary ω6 to ω3 ratios (ω6:ω3) with low eicosapentaenoic acid and docosahexaenoic acid (EPA+DHA) levels, and their influence on antibacterial and eicosanoid responses in farmed fish, have not been fully elucidated. In the current study, Atlantic salmon (*Salmo salar*) were fed high ω6 and high ω3 fatty acid diets combined with two EPA+DHA levels (0.3 and 1.0% of diet) for 12 weeks. At the end of the trial, salmon were intraperitoneally injected with formalin-killed *Aeromonas salmonicida* (ASAL) or phosphate-buffered saline (PBS), and their head kidneys were sampled at 24 h post-injection to measure the transcript expression of antibacterial and inflammation-relevant genes using qPCR. Further, plasma prostaglandins (i.e. PGE2, PGF3α) were measured by ESI-MS/MS to investigate the impact of diet and injection. Most immune-related transcripts in the study (i.e. *stlr5*, *ccl19a*, *ccl19b*, *saa5*, *il8*, *il1b*, *il10*, *irf1*, *camp*, *haaf*, *hamp*) showed a significant ASAL induction in salmon head kidney in all dietary treatments. The magnitude of induction varied among transcripts encoding chemokines, interleukins, and antimicrobial peptides, and was influenced by diet. Head kidney transcript expression of several immune-related genes (i.e. *ccl19b*, *il8*, *il10*, *saa5*, *hamp*, *haaf*) showed the strongest ASAL fold-change induction in fish fed 0.3% EPA+DHA with high ω6 (0.3%EPA+DHA↑ω6). Two-way ANOVA revealed that the responsiveness of antibacterial transcripts to ASAL-injection was predominantly impacted by the interaction of dietary ω6:ω3 with EPA+DHA. Further, positive correlations were identified between head kidney ω6 PUFA and the transcript expression of genes involved in immune response and eicosanoid metabolism. These findings suggest that 0.3%EPA+DHA↑ω6 diet may

have enhanced the innate antibacterial immune response of Atlantic salmon. Most transcripts involved in eicosanoid metabolism were down-regulated in ASAL when compared with the PBS-injected fish (within diet), and this is suggested to be an attempt to mitigate the proinflammatory response in bacterin-challenged fish. Finally, fish injected with PBS and fed 0.3%EPA+DHA↑ω6 showed higher plasma PGE_2 and PGF3α as compared with 0.3%EPA+DHA↑ω3 fed fish. The former diet also resulted in lower levels of PGE2 in ASAL- when compared with PBS-injected fish, and this was concurrent with lower head kidney expression of PGE2 receptor-encoding transcript (i.e. *ptger4a*). In summary, this Chapter showed a dietary modulation of the innate immune response to bacterial antigen. In light of these data, more research is needed to investigate the impact of high ω6 plant-based diets with low EPA+DHA levels on antibacterial immune responses in Atlantic salmon.

5.2. Introduction

The aquaculture industry is growing faster (i.e. average annual growth rate of 5.8% since 2010) than any other animal food production sector, and is predicted to further expand with human population growth and the increase in fish consumption (FAO, 2018, 2020). However, infectious diseases (i.e. bacterial, viral, and parasitic) are causing major stock and economic losses, and represent a bottleneck to global aquaculture growth (World Bank Group, 2014; Lafferty et al., 2015; FAO, 2020). Atlantic salmon (*Salmo salar*), which is one of the most economically valuable fish species, is susceptible to several bacterial pathogens such as *Piscirickettsia salmonis* (causing Piscirickettsiosis), *Renibacterium salmoninarum* (causing Bacterial Kidney Disease), *Flavobacterium branchiophilum* (causing Bacterial Gill Disease), *Vibrio anguillarum* (causing Vibriosis), *Yersinia ruckeri* (causing Enteric Redmouth Disease), *Aeromonas salmonicida* (causing Furunculosis), *Moritella viscosa* (causing Winter Ulcer Disease), and others (Toranzo et al., 2005; Oyarzún et al., 2019; Semple and Dixon, 2020). The Gram-negative bacillus *A. salmonicida* (subspecies *salmonicida*) is an important bacterial pathogen due to high mortality and antibiotic use associated with furunculosis outbreaks in salmonid aquaculture (Toranzo et al., 2005; Janda and Abbott, 2010; Braden et al., 2019). This pathogen colonizes the head kidney, spleen, liver, brain and blood of fish (Bartkova et al., 2017; Valderrama et al., 2019; Vasquez et al., 2020).

The innate immune system is initiated when bacterial pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharides (LPS), peptidoglycans, flagellin, and bacterial DNA/RNA are recognized by animal immune cells, via germline-encoded

pattern recognition receptors (PRRs; Boltaña et al., 2011). This detection triggers intracellular signalling cascades that stimulate soluble mediators (e.g. chemokines, cytokines) and transcription factors, resulting in upregulation of genes involved in inflammatory responses (Secombes and Wang, 2012; Semple and Dixon, 2020). Formalin-killed typical *A. salmonicida* (ASAL) bacterin has been shown to elicit a robust anti-bacterial immune response in fish (e.g. including induction of inflammatory-related transcripts such as *hamp*, *camp*, *il8*, *il1b*, *fth*, and IRF-encoding transcripts; Hori et al., 2013; Inkpen et al., 2015; Zanuzzo et al., 2015).

Nutritionally adequate dietary formulations and feeding regimes have been considered as a disease prevention strategy, which could reduce the use of chemotherapeutics and antibiotics in aquaculture (Kiron, 2012; Oliva-Teles, 2012; Pohlenz and Gatlin, 2014). Dietary lipid composition can alter the fatty acid composition of immune cells and influence immune responses in a variety of ways [e.g. alterations in the membrane physical and/or functional properties, regulation of immune-related transcripts (Calder, 2007, Puertollano et al., 2008; Calder, 2013)]. Further, eicosapentaenoic (EPA, 20:5ω3), docosahexaenoic (DHA, 22:6ω3), dihomo-γ-linolenic (DGLA, 20:3ω6), and arachidonic (ARA, 20:4ω6) acids are precursors to potent lipid mediator signaling molecules, termed eicosanoids, which have important roles in the regulation of inflammation and immune response (e.g. through their direct effects on immune cells such as macrophages and other leukocytes, or indirectly via cytokines (Rowley et al., 1995; Lall, 2000; Calder 2013; Martinez-Rubio et al., 2013; Gómez-abellán and Sepulcre, 2016).

In the last two decades, significant research was undertaken on the development of diets that replace fish meal and fish oils with terrestrial sources of proteins and lipids such

as plants and vegetable oils, which are more available and cost-effective (Tacon and Metian, 2008; Turchini et al., 2009; Olsen and Hasan, 2012). Most plant oils (PO) in aquaculture feeds contain high levels of ω6 and high ω6 to ω3 (ω6:ω3) FA ratios (Pickova and Mørkøre, 2007), which have been shown to affect fish immune and eicosanoid responses (Montero et al., 2010; Alhazzaa et al., 2013; Montero et al., 2015; Holen et al., 2018). However, the nature of these effects are contradictory as reviewed by Kiron (2012), Oliva-Teles (2012), and Martin and Król (2017). Some studies suggested that Atlantic salmon fed PO with high ω6:ω3 (~3) were less resistant to bacterial infections (i.e. *A. salmonicida* and *V. anguillarum*) than those fed with low ω6:ω3 (~ 0.5) diets (Thompson et al., 1996; Carter et al., 2003). However, other studies indicated that PO with varying ω6:ω3 ratios (~ 0.1–4.0) had no impacts on immune responses (e.g. alternate complement, lysozyme and phagocytic activities, lymphocyte proliferation, expression of pro-inflammatory cytokine-related genes) and disease resistance in salmonids challenged with bacterial pathogens (Gjøen et al., 2004; Seierstad et al., 2009; Kiron et al., 2011). Further, Holen et al. (2018) previously reported that head kidney leukocytes from Atlantic salmon fed soy oil diet (ω6:ω3 of 2.4) and treated with LPS, had higher expression levels of inflammatory and eicosanoid-related genes (i.e. *il1b*, *tnfa*, *cox2*, *pgds*, *pges*) when compared to LPS-treated fish fed other PO with varying ω6:ω3 (i.e. 0.7, 0.9 and 4.1). These authors also reported that PGE_2 concentrations of head kidney leucocytes were higher in palm oil when compared with high soy oil (ω6:ω3 of 0.7 and 4.1, respectively) fed fish, while PGE_3 and LTB_5 levels were highest in the palm oil fed fish (Holen et al., 2018).

However, the interaction of dietary ω6:ω3 with low EPA+DHA levels, and their impact on antibacterial and eicosanoid responses in Atlantic salmon have not been fully

elucidated. The current study used the same diets and fish population as in Chapter 4, in order to examine how high ω6 and high ω3 plant-based diets containing low FO and EPA+DHA levels (i.e. 0.3% and 1.0%, as formulated) influence the head kidney transcript expression of genes involved in immune response and eicosanoid metabolism. Further, the impacts of diet and ASAL challenge on plasma prostaglandin (i.e. PGE_2, PGF3α) levels were assessed. Finally, correlation analysis was used to link head kidney lipid composition with transcript expression of immune- and eicosanoid-metabolism-related genes.

5.3. Materials and Methods

5.3.1. Experimental diets and fish

Experimental diets in this study contained different levels of fish oil, PO (i.e., soy oil, linseed oil, and rapeseed oil) and poultry fat, and had two levels of EPA+DHA (0.3% and 1.0%, as formulated) and contrasting ω6:ω3 ratios (high ω6 and high ω3). The four experimental diets were as follows: 0.3% EPA+DHA with higher ω6 (0.3% EPA+DHA↑ω6), 0.3% EPA+DHA with higher ω3 (0.3% EPA+DHA↑ω3), 1.0% EPA+DHA with higher ω6 (1% EPA+DHA↑ω6), and 1.0% EPA+DHA with higher ω3 (1% EPA+DHA↑ω3). All experimental diets were manufactured by Cargill Canada (formerly EWOS Canada; Surrey, BC, Canada), top-coated with different oil mixes at the Chute Animal Nutrition Centre (Dalhousie University, Truro, NS, Canada), and formulated to be isonitrogenous and isoenergetic, and to meet the nutritional requirements of salmonids [National Research Council (NRC), 2011]. Dietary formulations and their lipid composition are presented in Table 5.1 and Table 5.2, respectively.

Table 5.1. Formulation and nutrient composition (%) of experimental diets[a] fed to Atlantic salmon for 12 weeks.

Ingredient (% of diet)[b]	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	1%EPA+DHA ↑ω6	1%EPA+DHA ↑ω3
Fish oil	0.1	-	4.3	4.3
Soy oil	12.5	-	10.1	-
Linseed oil	-	7.9	-	6.4
Poultry fat[c]	2.4	7.1	0.6	4.3
Rapeseed oil	-	-	-	-
Proximate composition (% as fed basis)				
Nitrogen[d]	7.3	7.5	7.8	7.3
Crude lipid[e]	19.9	20.6	20.0	20.7
Dry matter[e]	97.4	97.6	98.0	97.5
Ash[d]	5.7	5.7	5.4	5.7

[a] Means were calculated using 3 pellets per diet (n=3).

[b] All ingredients were sourced from Cargill Innovation stocks. Each experimental diet contained 24.7% marine protein, 57.4% plant protein, and 2.9% additives.

[c] Poultry fat contained EPA+DHA and 18:2ω6 levels of 0.4 and 2.9% of total FA, respectively. This contributed to their level in experimental diets.

[d] Analysed as % of dry weight (n=3).

[e] Analysed as % of wet weight (n=3).

Table 5.2. Lipid and fatty acid composition (%) of experimental diets[a] fed to Atlantic salmon for 12 weeks.

	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	1%EPA+DHA ↑ω6	1%EPA+DHA ↑ω3	1.4%EPA+DHA balanced
Lipid classes composition (% of total lipid)					
TAG (%)[b]	62.2	68.4	70.6	60.9	71.6
ST (%)[c]	3.8	5.5	3.8	3.4	3.4
AMPL (%)[d]	2.1	2.7	1.8	1.7	2.1
PL (%)[e]	11.7	6.0	4.4	10.2	6.3
TL (mg/g)[f]	174.0	189.1	168.9	210.1	201.8
Fatty acid composition (% of total FA)					
14:0	0.7	0.8	1.9	2.0	2.7
16:0	14.0	14.0	13.5	13.3	12.9
16:1ω7	1.2	2.3	1.8	2.7	3.2
18:0	5.2	5.8	4.7	5.0	4.0
18:1ω7	1.7	1.6	1.9	2.0	2.9
18:1ω9	23.2	28.1	18.6	22.5	29.5
18:2ω6 (LNA)	**41.4**	**18.3**	**33.4**	**14.8**	**13.3**
18:3ω3 (ALA)	**6.0**	**22.4**	**5.0**	**18.4**	**4.1**
20:1ω9	0.3	0.4	1.0	1.0	1.7
20:1ω11	0.1	0.1	2.5	2.6	4.1
20:4ω6 (ARA)	0.3	0.4	0.3	0.4	0.4
20:5ω3 (EPA)	**1.1**	**1.2**	**3.1**	**3.1**	**4.3**
22:1ω11	0.2	0.1	2.6	2.7	4.2
22:6ω3 (DHA)	**1.7**	**1.8**	**3.6**	**3.5**	**4.5**
ΣSFA[g]	21.1	21.4	21.4	21.3	20.9
ΣMUFA[h]	27.2	33.2	29.6	34.8	47.3
ΣPUFA[i]	51.6	45.2	48.8	43.6	31.4
Σω3	**9.4**	**25.9**	**14.1**	**27.4**	**16.4**
Σω6	**41.9**	**19.0**	**34.0**	**15.6**	**14.2**
ω6:ω3	4.5	0.7	2.4	0.6	0.9
EPA+DHA	2.8	3.0	6.7	6.6	8.8
DHA:EPA	1.5	1.5	1.2	1.1	1
EPA:ARA	3.7	3.0	10.3	7.8	10.8

[a] Mean (n=3). [b] Triacylglycerol. [c] Sterols. [d] Acetone mobile polar lipid. [e] Phospholipids. [f] Total lipids. [g] Total saturated fatty acids. [h] Total monounsaturated fatty acids. [i] Total polyunsaturated fatty acids. Fatty acids in bold font were key to the experimental design.

Atlantic salmon smolts were transported from a regional salmon farm and reared in the Dr. Joe Brown Aquatic Research Building (Ocean Sciences Centre, Memorial University of Newfoundland, Canada; October 2016) in 3800 l tanks until re-distribution into the 620-l tanks used in the feeding trial. After their arrival, fish were graded by size to select the most uniform population, and PIT (Passive Integrated Transponder; Easy AV, Avid Identification Systems, Norco, CA, USA) - tagged for individual identification. Then, when they reached the appropriate size, salmon post-smolts [210 ± 44 g mean initial weight ± standard deviation (SD); 29.0 ± 0.19 cm mean initial fork length ± SD] were randomly distributed into twenty 620-l tanks (40-41 fish $tank^{-1}$), and were fed a standard commercial diet (EWOS Dynamic S, Cargill Inc., Elk River, MN, USA) for 8 weeks (i.e. acclimation period). Then, fish were switched from the commercial feed and fed with the experimental diets (4 tanks $diet^{-1}$) for 12 weeks. Throughout the trial, salmon were fed to satiation, overnight, using automatic feeders (AVF6 Vibrating Feeders, Pentair Aquatic Eco-Systems, Inc., Apopka, FL, USA). Uneaten pellets were collected daily and were used to adjust feed amounts to each tank. All tanks were supplied with 12°C flow-through filtered seawater at 12 l min^{-1}; the dissolved oxygen level was ~10 mg l^{-1}, and the photoperiod was maintained at 24-h light. Mortality rates were noted (i.e. $\leq$ 1% at week 12) and dead fish were weighed and recorded throughout the trial. Ethical treatment of fish in this experiment was carried out in accordance with the guidelines of the Canadian Council on Animal Care, and approved by the Institutional Animal Care Committee of Memorial University of Newfoundland (protocol # 16-75-MR).

5.3.2. Immune challenge and fish sampling

At week 12, head kidney samples were collected (i.e. 5 fish $tank^{-1}$ and a total of 20 $diet^{-1}$) for lipid composition analyses (Chapter 4 and Katan et al., 2020). Then, following a 24 h starvation period, fish (i.e. 3 $tank^{-1}$ and a total of 12 $diet^{-1}$) were lightly anesthetized (MS-222, 50 mg l^{-1}; Syndel Laboratories, Vancouver, BC, Canada), and subjected to an intraperitoneal (IP) injection (25-gauge needle) of sterile phosphate-buffered saline (PBS) or formalin-killed *A. salmonicida* (ASAL) bacterin (1 µl g^{-1} of wet mass). Formalin-killed typical ASAL was obtained in the form of a vaccine [Furogen Dip, Elanco (formerly Novartis), Charlottetown, PEI, Canada], which was centrifuged (2000 × *g* for 10 min at 4°C) and washed with ice-cold sterile PBS (Thermo Fisher Scientific, Waltham, MA, USA; 0.2 µm filtered) three times (Hori et al., 2013). After the third wash, the pelleted cell debris was resuspended in ice-cold PBS to an optical density of 1.0 at 600 nm wavelength (OD_{600}).

After injection, fish were allowed to recover from anesthesia in 4 620-l auxiliary tanks. Fish density in the auxiliary tanks was kept below 30 kg/m^3. Then, following euthanasia (MS-222, 400 mg l^{-1}; Syndel Laboratories) head kidney (50-100 mg $sample^{-1}$) and plasma (~1 ml $sample^{-1}$) were collected 24 h post-injection in 1.5 ml nuclease-free tubes, flash-frozen in liquid nitrogen, and stored at −80°C. Indomethacin (1 µl of a 10 mM solution; Sigma-Aldrich Canada Ltd., Oakville, ON, Canada) and BHT antioxidant (10 µl of a 0.005% solution; Sigma-Aldrich Canada Ltd.) were added to and mixed with all plasma tubes, before they were flash-frozen. This was done to inhibit *in vitro* synthesis and oxidation of eicosanoids, as per Ubhayasekera et al. (2018) and Tacconelli et al. (2010).

5.3.3. RNA extraction, DNase treatment, column purification and cDNA synthesis

Head kidney samples were homogenized in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) with the TissueLyser II system at 25 Hz for 2.5 min, using 5 mm stainless steel beads (QIAGEN, Mississauga, ON, Canada), and subjected to RNA extraction. This was followed by DNaseI treatment and column purification using the RNase-free DNase Set (QIAGEN) and the RNeasy Mini Clean-up Kit (QIAGEN), respectively. All procedures were conducted according to manufacturer instructions and as described in Xue et al. (2015). RNA integrity was verified by 1% agarose gel electrophoresis, and RNA purity and quantity were assessed by NanoDrop UV spectrophotometry (Thermo Fisher Scientific). DNaseI-treated and column-purified RNA samples had A260/280 and A260/230 ratios of 1.8-2.3. All cDNAs were prepared by reverse transcription of 1 µg of DNaseI-treated, column-purified total RNA for each sample, using 1 µl of random primers (250 ng; Invitrogen), 1 µl of dNTPs (0.5 mM final concentration; Invitrogen), 4 µl of 5X first-strand buffer (1× final concentration; Invitrogen), 2 µl of DTT (10 mM final concentration; Invitrogen), and 1 µl of Moloney murine leukemia virus (M-MLV) reverse transcriptase (RT) (200 U; Invitrogen) at 37°C for 50 min. The total reaction volume was 20 µl, following manufacturer instructions, and as described in Xue et al. (2015). Finally, all cDNAs were diluted 40 times with nuclease-free water (Invitrogen) prior to real-time quantitative polymerase chain reaction (qPCR).

5.3.4. qPCR analysis

Expression levels of 23 genes of interest (GOI; Table 5.3) known to play roles in antibacterial immune response (e.g. genes encoding chemokines, cytokines, transcription factors, and antimicrobial peptides) and in eicosanoid metabolism, were quantified by qPCR using head kidney cDNA templates from fish fed the 4 dietary treatments and injected with PBS or ASAL. Eight individuals per dietary treatment in each condition (i.e. PBS- and ASAL-injected) were used in the qPCR study. Only head kidney samples from fish that had specific growth rates within 1.5 standard deviations below and above the mean value of each tank were selected for the qPCR study, in order to reduce biological variability. The sequences of all primer pairs used in qPCR analyses, GenBank accession numbers of sequences used for primer design, and other details are presented in Table 5.3. Notably, primers for other paralogues of the transcripts *tlr5*, *il8*, *il10* and *hamp* (GenBank accession numbers DY712024, EG838475, XM_014134151 and DV105974, respectively) failed quality testing due to low transcript expression levels, and thus were not included in the qPCR study. Each primer pair was quality-tested to verify that a single product was amplified with no primer dimers, and included standard curves and dissociation curves, as described in Rise et al. (2010) and Booman et al. (2011).

In brief, the amplification efficiency (Pfaffl, 2001) of each primer pair was determined using a 5-point 1:3 dilution series starting with pooled cDNA representing 10 ng of input total RNA. Two cDNA pools were generated (i.e. PBS and ASAL), and each pool consisted of 16 fish from the 4 dietary treatments (i.e. 4 fish per diet, with each fish contributing an equal quantity to the pool).

Table 5.3. qPCR primers

Transcript name (symbol)	Nucleotide sequence (5'–3')[a]	Amp. effic. (%)[b]	Amplicon size (bp)	GenBank Accession number
Cathelicidin (*camp*)	F:AGACTGGCAACACCCTCAAC	101.7	112	AY360357
	R:TTGCCTCTTGTCCGAAT			
CC chemokine-like 19 a (*ccl19a*)[c]	F:CTCATCAAGAAGTGCCACGA	95.9	188	BT125321
	R:CACCCTGTTCTTCACCCACT			
CC chemokine-like 19 b (*ccl19b*)[c]	F:CTGCTTGACAACGACCGATA	94.1	151	BT058161
	R:GTTGTTCTTGGTGGCAGGAG			
Hemagglutinin / amebocyte aggregation factor (*haaf*)	F:AAGACACGTTTGTCTATGAGC	89.0	134	CB510444
	R:CTCATGTGGGTTGGAATGAT			
Hepcidin (*hamp*)[e]	F:ATGAATCTGCCGATGCATTTC	91.8	134	BT125319
	R:AATGGCTTTAGTGCTGGCAG			
Interferon regulatory factor 1 (*irf1*)[c]	F:GCAATGAAGTAGGCACAGCA	95.1	100	BT048538
	R:CGCAGCTCTATTTCCGTTTC			
Interleukin 1 beta (*il1b*)[e]	F:GTATCCCATCACCCCATCAC	94.5	119	AY617117
	R:TTGAGCAGGTCCTTGTCCTT			
Interleukin 8 (*il8*)	F:GAAAGCAGACGAATTGGTAGAC	93.4	99	BT046706
	R:GCTGTTGCTCAGAGTTGCAAT			
Interleukin 10 (*il10*)	F:TGGAGACTTCCCTGTTGGAC	98.8	131	EF165028
	R:CTGCGTTCTGTTGTTCATGG			
Leukocyte cell-derived chemotaxin 2 (*lect2*)[d]	F:CAGATGGGGACAAGGACACT	94.0	150	BT059281
	R:GCCTTCTTCGGGTCTGTGTA			
NF-kappa-B p105 subunit-like (*nfkb1*)	F:GGTATTTGTCTTCTCTCAGACG	96.7	142	GE792962
	R:GTCTATCTGCTGTCCGGAGT			
Serum amyloid A-5 (*saa5*)	F:AGGAGCTGGAAGTTTGTTGC	89.7	143	BT057477
	R:TATGCACGCCACATGTCTTT			
Soluble toll-like receptor 5 (*stlr5*)[e]	F:ATCGCCCTGCAGATTTTATG	92.6	103	AY628755
	R:GAGCCCTCAGCGAGTTAAAG			
Transforming growth factor beta-1 (*tgfb1*)	F:TCACTGGACTTGGAGCTGAT	97.7	134	BT059581
	R:ATCAGGGAGGTTGGGACTTT			
Arachidonate 5-lipoxygenase a *(5loxa*)[f]	F:CTGCTCACCATGCTGCTGTC	97.0	93	NM001139832
	R:GTGTGGGAGGAGGCTTCC			
Arachidonate 5-lipoxygenase b (*5loxb*)[f]	F:ACTGCTGTGGGTTTCCCAAG	102.6	98	DW555519
	R:GACAGCAGCGTGATGTGCAG			
Cyclooxygenase-1 (*cox1*)[f]	F:CTCATGAGGGTGGTCCTCAC	104.1	135	BT045745
	R:AGGCACAGGGGGTAGGATAC			
Cyclooxygenase-2 (*cox2*)[f]	F:ACCTTTGTGCGAAACGCTAT	97.0	113	AY848944
	R:GAGTAGGCCTCCCAGCTCTT			
Leukotriene A4 hydrolase (*lkha4*, alias *lta4h*)[f]	F:AAGGTCTCCAAGGTAACAGC	94.8	97	NM_001140120
	R:AATGGCAGTGTGATCTCCAA			
Prostacyclin synthase-like (*ptgis*)[g]	F:TGGTGACATTTTTACGGTGC	94.1	119	XM_014167834;

	R:ACCTGGGCGTAGCGACTGA			XM_014132335
Prostaglandin-D synthase (*pgds*, alias *lipocalin-type pgds*)[f]	F:GGTGCTCAACAAGCTCTACA	90.3	114	BT048787
	R:GCAGGAAAGCGATGTTGTCA			
Prostaglandin E synthase 2-like (*ptges2*)[f]	F:TTCTGCGCTGTTACCCAGAG	99.8	112	[h]XM_014171682;
	R:GTACATCGTCTGACCTTCAG			XM_014160437
Prostaglandin E2 receptor EP4a (*ptger4a*)	F:ATAACGGCACGATGACAAGT	87.6	114	NM_001173955
	R:TCCTTCCGAGACTTTCGAAG			
Eukaryotic translation initiation factor 3 subunit D (*eif3d*)[c]	F:CTCCTCCTCCTCGTCCTCTT	94.2	105	GE777139
	R:GACCCCAACAAGCAAGTGAT			
Polyadenylate-binding protein cytoplasmic 1 (*pabpc1*)[c]	F:TGACCGTCTCGGGTTTTTAG	93.6	108	EG908498
	R:CCAAGGTGGATGAAGCTGTT			

[a] F is forward and R is reverse primer.
[b] Amplification efficiency (%)
[c] Primers that were previously published in Caballero-Solares et al. (2017).
[d] Primers that were previously published in Caballero-Solares et al. (2018).
[e] Primers that were previously published in Eslamloo et al. (2020).
[f] Primers that were previously published in Katan et al. (2020).
[g] Primers were designed based on conserved regions between XM_014167834 and XM_014132335.
[h] Primers were designed based on conserved regions between XM_014171682 and XM_014160437.
Normalizer genes are underlined.

The reported primer pair amplification efficiencies were an average of the two pools, except if one pool showed poor efficiency or spacing due to low expression levels (i.e. the ASAL pool was used for *il10*, *saa*, and *cox2*).

To select the most suitable normalizer genes, six candidate normalizers were tested based on previous qPCR studies (*rpl32*, *eef1a-1*, *eef1a-2*, *actb*, *eif3d*, *pabpc1*) (Xue et al., 2015; Caballero-Solares et al., 2017), and salmon literature on reference genes (*actb*, *eef1α-1*, *eef1α-2*) (Olsvik et al., 2005). Their qPCR primers were quality-tested as described above. Half of the fish population involved in the qPCR study was utilized for normalizer testing. Cycle threshold (C_T) values were measured using cDNA corresponding to 5 ng of input total RNA. Expression stability was then analysed using the geNorm algorithm (Vandesompele et al., 2002). *Eif3d* and *pabpc1* were shown to be the most stable (i.e. geNorm M-values of 0.23 and 0.22, respectively) among the 6 candidate genes, and therefore were selected as normalizers. All PCR amplifications were performed in a total reaction volume of 13 µl and consisted of 4 µl of cDNA (5 ng input total RNA), 50 nM each of forward and reverse primer and 1× Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and nuclease-free water (Invitrogen). qPCR reactions, including no-template controls, were performed in technical triplicates using the ViiA 7 Real-Time PCR System (384-well format) (Applied Biosystems) and the Power SYBR Green I dye chemistry. The real-time analysis program consisted of 1 cycle of 50°C for 2 min, 1 cycle of 95°C for 10 min, followed by 40 cycles (of 95°C for 15 s and 60°C for 1 min), with the fluorescence signal data collection after each 60°C step. When a C_T value within a triplicate was greater than 0.5 cycle from the other two values, it was considered to be an outlier and discarded. The relative quantity (RQ) of each GOI was calculated using

a qBase relative quantification framework (Hellemans et al., 2007; Booman et al., 2014) with primer amplification efficiencies incorporated (Table 5.3). The expression levels of each GOI were normalized to both normalizer genes, and the sample with the lowest normalized expression was used as the calibrator sample (i.e. RQ = 1.0) for each GOI (Rise et al. 2015). Transcript expression data are presented as RQ values relative to the calibrator. qPCR fold-change values were calculated by dividing the RQ value of each ASAL-injected fish by the mean of the PBS-injected group in each dietary treatment.

5.3.5. Plasma eicosanoid analysis

Each plasma sample was thawed, briefly vortexed, and centrifuged at 3000 rpm for 1 min at room temperature. Then 10 µl of formic acid (98%; Thermo Fisher Scientific) were added into a separate tube containing 50 µl plasma. This was followed by vortexing for 1 min and centrifuging at 12,000 × *g* for 2 min (at 4°C), to remove any precipitate and to collect the supernatant. The prostaglandin extraction protocol followed modifications from Farndale et al. (1999) and Araujo et al. (2014). Each sample was diluted (1:20) by adding 50 µl of plasma into a separate glass tube containing a 4:1 mixture of methanol:chloroform, and then passed through a 0.45 µM filter (Thermo Fisher Scientific) into a new tube prior to electrospray ionization (ESI)-MS/MS injection.

Each sample (~300 µl) was injected with a high-pressure syringe pump (F100T2, Chemyx Inc., Stafford, TX, USA) at a flow of 10 µl min^{-1} into the mass spectrometer, using direct infusion. Identifications of PGE_2 (ω6-derived) and PGF3α (ω3-derived) were carried out with a triple quadrupole mass spectrometer (TSQ Quantis™ Triple Quadrupole Mass Spectrometer, Thermo Fisher Scientific) fitted with a mass selective detector ion trap

system for electrospray ionization (ESI). The temperature and drying gas used for the mass spectrometer were 350°C and N_2, respectively. The identification of the two prostaglandins was performed in the negative mode using 4490 and 4363 (v) ion spray voltage, 4.0 and 2.4 (arb) sheath gas, 6.1 and 20.7 (arb) aux gas, 7.1 and 0.4 (arb) sweep gas, 18.26 and 10.23 eV of collision energy, respectively. Vaporizer temperature was set to 30 °C, the ion transfer tube was kept at 325 °C, and the collision-induced dissociation (CID) gas was maintained at 1 mTorr, for both PGE_2 and PGF3α analyses. The product ions of m/z 351→333, 315, 271, 189 were used for the identification of PGE_2, while the ion intensity of 271 was used for quantification (Araujo et al., 2014; Canzi et al., 2019). For the identification of PGF3α the product ions of m/z 351→307, 247, 192 were used, while the ion intensity of 307 was used for quantification (Pier et al., 2018). Quantification of the two compounds was performed using standards [i.e. prostaglandin HPLC mixture for PGE_2 (11.36 µM) and EPA oxylipin Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) mixture for PGF3α (0.57 µM)] from Cayman Chemical (Cayman Chemical, Ann Arbor, MI, USA). The software Xcalibur (Thermo Fisher Scientific) was used for data processing.

5.3.6. Statistical analyses

5.3.6.1. qPCR data

To identify significant differences among dietary treatments in RQ values (within the PBS and ASAL groups), and in ASAL fold-change induction (ASAL/PBS) a one-way ANOVA followed by Tukey post-hoc tests was performed. A Student's *t*-test was used to assess the effect of ASAL-injection within each dietary treatment. To show the effects of

dietary EPA+DHA and ω6:ω3 on ASAL fold-change of each GOI, a two-way ANOVA was performed (Minitab 17 Statistical Software, State College, PA, USA). Each dietary treatment within the PBS and ASAL groups was tested for outliers using Grubb's test ($p < 0.05$). In total, 26 RQ values were identified as statistical outliers in the entire dataset (i.e. out of 1472 values comprising all samples and all GOIs), and excluded from the study. All GOIs had a sample size of 7-8 per dietary treatment in each of the PBS and ASAL groups. Finally, residuals were tested to verify normality, independence, and homogeneity of variance. Normality was examined using the Anderson-Darling test. If the test failed ($p < 0.05$), a one-way ANOVA on ranks was performed, and was followed by a Kruskal-Wallis test (SigmaPlot, Systat Software, Inc., Version 13, San Jose, CA, USA). In all cases, differences were considered statistically significant when $p < 0.05$.

5.3.6.2. Cluster and Pearson correlation analyses

Hierarchical clustering analyses were performed in order to separately group the transcript expression of GOIs (i.e. fold-change induction values) and lipid composition (% fatty acids and lipid classes) in the head kidney. The cluster mode was group average, using PRIMER (PRIMER-E Ltd., version 6.1.15, Ivybridge, UK). Pearson correlation analysis was performed to identify relationships between transcript expression (i.e. fold-change induction) and tissue lipid composition (i.e. % FA and lipid classes), using tank means. All lipid classes [i.e. triacylglycerols (TAG), sterols (ST) and phospholipids (PL)] and GOIs were included in the correlation analysis. However, FA, lipid classes, and transcripts with no significant correlations were removed. Individual saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) that accounted for > 5% of the total FA (average per

dietary treatment), and all ω6 and ω3 FA were included in the analysis. The correlation analysis was performed using IBM SPSS (IBM SPSS Statistics, Version 25, Armonk, NY, USA).

5.3.6.3. Plasma prostaglandin data

To identify significant differences between dietary treatments within the PBS and ASAL groups, and between PBS- and ASAL-injected fish, within a given diet, two-sample *t*-tests were performed. To show the effects of diet and injection on the levels of PGE_2 and PGF3α, two-way ANOVA was performed (Minitab 17 Statistical Software). Each dietary treatment within the PBS and ASAL groups was tested for outliers using Grubb's test ($p < 0.05$). In total, one value was identified as a statistical outlier in the entire dataset (i.e. out of 60 values comprising all samples in both, PGE_2 and PGF3α analyses), and excluded from the study. Analyses of both compounds had a sample size of 6-9 per dietary treatment in each of the PBS and ASAL groups. Residuals were tested to verify normality, independence, and homogeneity of variance. Normality was examined using the Anderson-Darling test. In all cases, differences were considered statistically significant when $p < 0.05$.

5.4. Results

5.4.1. Transcript expression of genes involved in immune response

5.4.1.1. Impact of ASAL injection on transcript expression

Most immune-related transcripts in the current study showed a significant ASAL induction compared with time-matched PBS injected controls (Figure 5.1 and Figure 5.2). However, the transcripts *lect2* and *nfkb1* were significantly induced by ASAL injection

only in certain dietary treatments [i.e. 0.3%EPA+DHA↑ω6 and 1.0%EPA+DHA↑ω6 ($p < 0.0001$), and in all treatment with the exception of 1.0%EPA+DHA↑ω3 ($p = 0.001$), respectively; Figures 5.1D and 5.2B]. Interestingly, the transcript *tgfb1* (Figure 5.1E) was down-regulated in ASAL- compared to PBS-injected fish in two of the specific dietary treatments (i.e. 0.3%EPA+DHA↑ω6 and 1.0%EPA+DHA↑ω3; $p = 0.03$ and 0.04, respectively).

The magnitude of induction varied between genes and dietary treatments. The pathogen recognition-related transcript *stlr5* had ASAL fold-change induction values (i.e. ASAL/PBS) of 5.2 to 15.9 (Figure 5.1A). Among the transcripts encoding chemokines (Figures 5.1B-F) *ccl19* paralogues showed the highest fold-change induction (fold-changes of 7.3 to 12.6 and 5.1 to 16.3 in *ccl19a* and *ccl19b*, respectively), followed by *il8* (fold-change of 2.6 to 8.2), *lect2* (fold-change of 0.9 to 2.1), and *tgfb1*. Fold-change values below 1.0 (i.e. down-regulation by ASAL) were observed in some dietary treatments. The transcript *tgfb1* showed a fold-change range of 0.4 to 1.5. Among the interleukins examined (Figure 5.1F-H) *il10* showed the strongest ASAL induction (fold-change of 15.7 to 85.9), followed by *il1b* (fold-change of 5.9 to 12.9) and *il8* (fold-change of 2.6 to 8.2). The acute phase protein-encoding transcript *saa5* had ASAL induction values of 25.2 to 87.9 (Figure 5.1I). Further, the transcription factor-encoding transcripts *irf1* and *nfkb1* had fold-change induction ranges of 2.4 to 4.3 and 1.2 to 2.3, respectively (Figure 5.2A-B). Among the genes encoding antimicrobial peptides (Figure 5.2C-D**)** *hamp* showed stronger ASAL induction (fold-change of 28.8 to 66.9) than *camp* (fold-change of 25.3 to 57.6). Finally, the transcript *haaf* had ASAL fold-change induction values of 6.2 to 25.6 (Figure 5.2E).

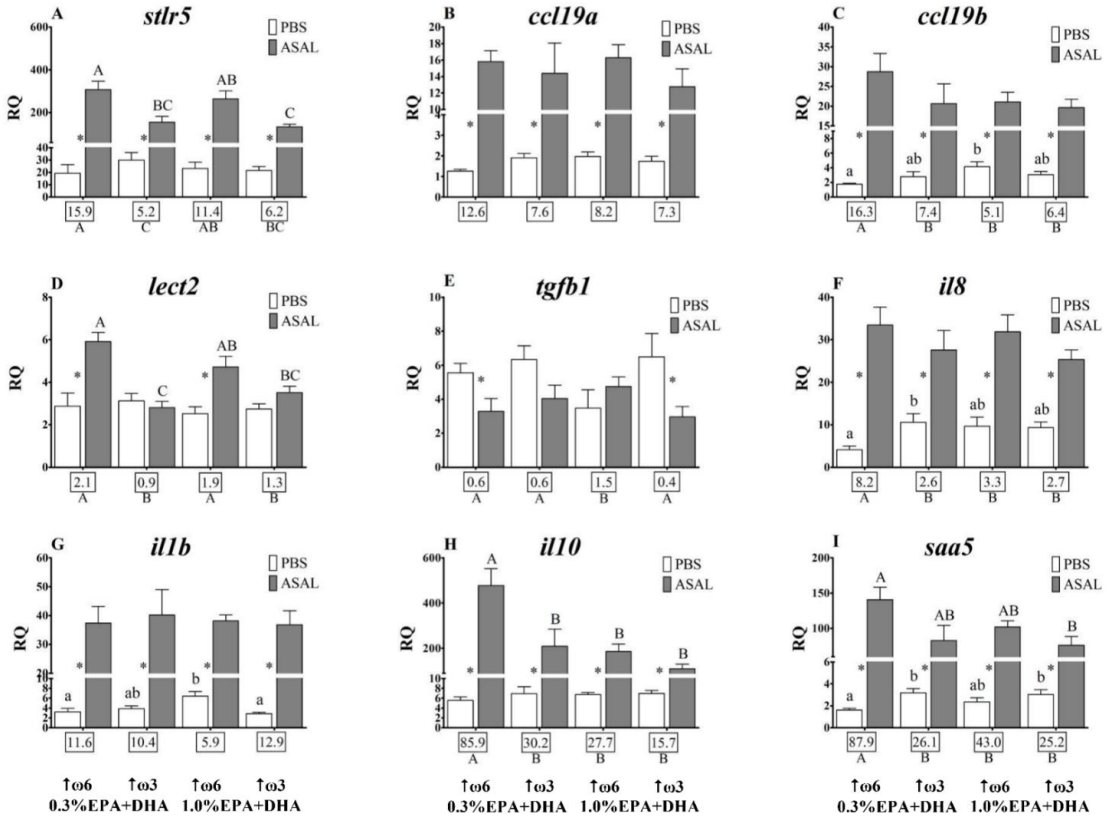

Figure 5.1. Head kidney qPCR transcript expression of genes related to pathogen recognition (A), chemokines (B-F), cytokines (F-H), and acute phase response (I) in salmon fed different ratios of ω6:ω3 and levels of EPA+DHA for 12 weeks. Head kidney tissues were sampled at 24 h post-injection. Transcript expression values presented as mean relative quantity (RQ) ± SE (n = 7-8). An asterisk represents significant difference between PBS- and ASAL-injected fish in each dietary treatment (Student's t-test; $p < 0.05$). Upper-case letters above error bars indicate significant differences among dietary treatments within ASAL-injected fish. Lower-case letters signify dietary differences within PBS-injected fish (one-way ANOVA and Tukey post-hoc tests; $p < 0.05$). Values below the figures represent fold-change (ASAL/PBS) for each dietary treatment, and significant differences among fold-changes are denoted with upper-case letters. Bottom legends describe the dietary treatments of all panels.

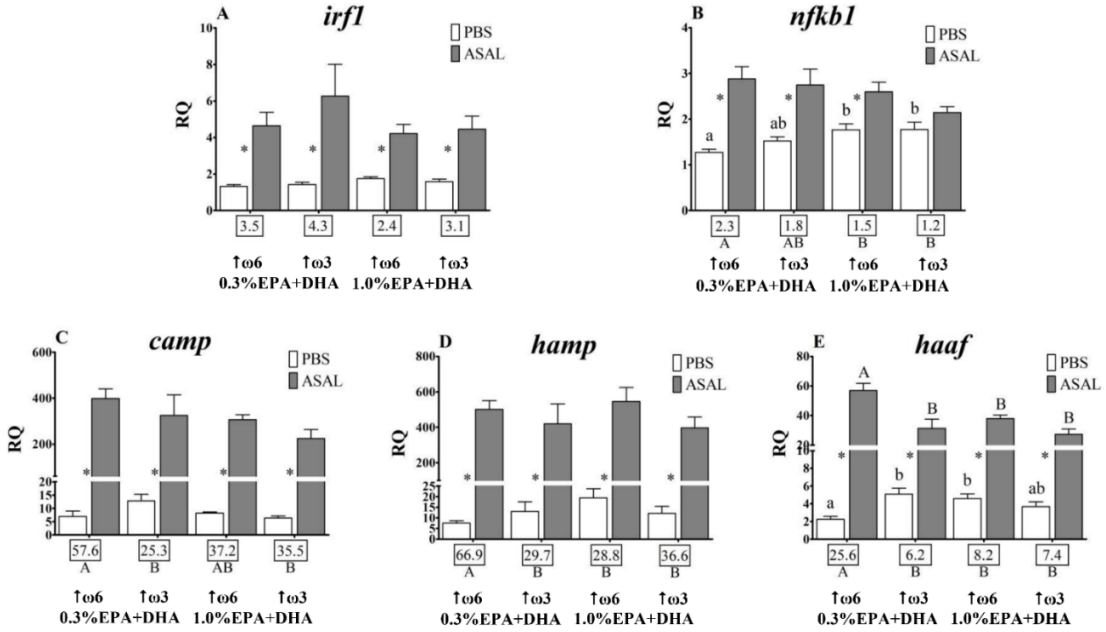

Figure 5.2. Head kidney qPCR transcript expression of genes encoding transcription factors (A-B), antimicrobial peptides (C-D), and agglutination-aggregation factor (E) in salmon fed different ratios of ω6:ω3 and levels of EPA+DHA for 12 weeks. Head kidney tissues were sampled at 24 h post-injection. Transcript expression values presented as mean relative quantity (RQ) ± SE (n = 7-8). An asterisk represents significant difference between PBS- and ASAL-injected fish in each dietary treatment (Student's t-test; $p < 0.05$). Upper-case letters above error bars indicate significant differences among dietary treatments within ASAL-injected fish. Lower-case letters signify dietary differences within PBS-injected fish (one-way ANOVA and Tukey post-hoc tests; $p < 0.05$). Values below the figures represent ASAL fold-change induction (ASAL/PBS) for each dietary treatment, and significant differences among fold-changes are denoted with upper-case letters.

5.4.1.2. Effect of diet on transcript expression

Transcript expression levels of *stlr5* were significantly higher in the ASAL-treated 0.3%EPA+DHA↑ω6 compared with the ASAL-treated 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω3 dietary treatments, and significantly higher in the ASAL-treated 1.0%EPA+DHA↑ω6 compared with the ASAL-treated 1.0%EPA+DHA↑ω3 fed fish ($p = 0.01$; Figure 5.1A). Further, *stlr5* ASAL fold-change induction was stronger in the 0.3%EPA+DHA↑ω6 compared with the two high ω3 treatments ($p < 0.0001$), and higher in the 1.0%EPA+DHA↑ω6 compared with 0.3%EPA+DHA↑ω3 fed fish ($p = 0.007$; Figure 5.1A). ASAL fold-change induction of *ccl19b* was significantly higher with 0.3%EPA+DHA↑ω6 than with the other dietary treatments ($p < 0.0001$), while the PBS-treated 1.0%EPA+DHA↑ω6 had higher mRNA levels of *ccl19b* compared with the 0.3%EPA+DHA↑ω6 fed fish ($p = 0.04$; Figure 5.1C). The transcript levels of *lect2* in the ASAL-injected salmon (i.e. RQs) and the ASAL fold-change induction were higher with 0.3%EPA+DHA↑ω6 than with high ω3 treatments (i.e. 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω3; $p < 0.0001$). Further, fish fed 1.0%EPA+DHA↑ω6 had stronger *lect2* fold-change induction than 1.0%EPA+DHA↑ω3 fed fish ($p = 0.022$; Figure 5.1D). Salmon fed 1.0%EPA+DHA↑ω6 had higher fold-change of *tgfb1* compared with the other dietary treatments ($p < 0.0001$; Figure 5.1F). Notably, 1.0%EPA+DHA↑ω6 was the only diet group in which this transcript was ASAL-induced (1.5-fold, although not significantly) compared with time- and diet-matched PBS controls ($p = 0.32$; Figure 5.1E). ASAL-treated fish fed 0.3%EPA+DHA↑ω6 had the highest expression levels of *il10* ($p = 0.001$; Figure 5.1H), and this diet also resulted in the strongest ASAL induction of *il10* and *il8* ($p < 0.0001$; Figures

5.1F and 5.1H) compared with the other three dietary treatments. The PBS-treated group had higher expression levels *il8* in 0.3%EPA+DHA↑ω3 compared with 0.3%EPA+DHA↑ω6 fed fish ($p = 0.03$; Figure 5.1F), while *il1b* showed higher expression in 1.0%EPA+DHA↑ω6 than in 1.0%EPA+DHA↑ω3 and 0.3%EPA+DHA↑ω6 fed fish ($p = 0.004$; Figure 5.1G). Transcript expression levels of *saa5* were significantly higher in the ASAL-treated 0.3%EPA+DHA↑ω6 compared with the ASAL-treated 1.0%EPA+DHA↑ω3 dietary treatment ($p = 0.03$; Figure 5.1I), while *saa5* ASAL fold-change induction was strongest in the 0.3%EPA+DHA↑ω6 fed fish when compared with the remaining dietary treatments ($p < 0.0001$; Figure 5.1I). However, PBS-treated fish, fed 0.3%EPA+DHA↑ω6 diet, had lower expression levels of *saa5* than the two high ω3 dietary treatments (i.e. 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω3; $p = 0.02$). Finally, it is worth noting that most GOIs in Figure 5.1 (i.e. *stlr5*, *ccl19a*, *ccl19b*, *lect2*, *il8*, *il10*, and *saa5*) numerically (i.e. differences were not statistically significant in all cases) showed the highest ASAL induction in 0.3%EPA+DHA↑ω6 compared with the other dietary treatments (Figure 5.1A-I).

ASAL induction of *nfkb1* was stronger in 0.3%EPA+DHA↑ω6 compared with 1.0%EPA+DHA↑ω6 and 1.0%EPA+DHA↑ω3 dietary treatments ($p < 0.0001$; Figure 5.2B). Further, *nfkb1* mRNA levels were higher in PBS-treated 1.0%EPA+DHA↑ω6 and 1.0%EPA+DHA↑ω3 fed fish compared with 0.3%EPA+DHA↑ω6 fed fish ($p = 0.03$; Figure 5.2B). ASAL induction of *camp* was higher in 0.3%EPA+DHA↑ω6 compared with high ω3 dietary treatments (i.e. 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω3; $p = 0.004$; Figure 5.2C). The transcript expression levels of *haaf* (i.e. RQ) in the ASAL-treated fish and ASAL induction were highest in 0.3%EPA+DHA↑ω6 compared with the remaining

dietary treatments ($p < 0.0001$; Figure 5.2E). However, *haaf* expression levels were lower in PBS-treated fish fed 0.3%EPA+DHA↑ω6 compared with 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω6 treatments ($p = 0.006$; Figure 5.2E). Similarly to *haaf*, ASAL induction of *hamp* was strongest in fish fed 0.3%EPA+DHA↑ω6 compared with the other three dietary treatments ($p = 0.001$; Figure 5.2D). Finally, most GOIs in Figure 5.2 (i.e. *nfkb1*, *camp*, *hamp* and *haaf*) numerically (i.e. differences were not statistically significant in all cases) showed the highest ASAL induction in 0.3%EPA+DHA↑ω6 compared with the other dietary treatments (Figure 5.2B-E).

5.4.2. Transcript expression of genes involved in eicosanoid metabolism

5.4.2.1. Impact of ASAL injection and diet on transcript expression

The transcript *cox2* was significantly induced by ASAL injection in all dietary treatments (fold-change of 18.7 to 64.0; $p < 0.0001$; Figure 5.3A). However, for all other eicosanoid metabolism-related transcripts, with the exception of *ptges2*, (i.e. *cox1*, *5loxa*, *5loxb*, *pgds*, *ptger4a*, *ptgis*, *lkha4*) the ASAL-injected fish had lower expression than the PBS group in some or in all dietary treatments ($p = 0.0001$-0.04; Figures 5.3B-I). The expression of *cox1*, *ptgis* and *lkha4* in 0.3%EPA+DHA↑ω6 fed fish, and that of *ptger4a* and *ptgis* in the 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω6 treatments also appeared to be ASAL-suppressed (although not significant; $p = 0.09$-0.25 and $p = 0.051$-0.4, respectively).

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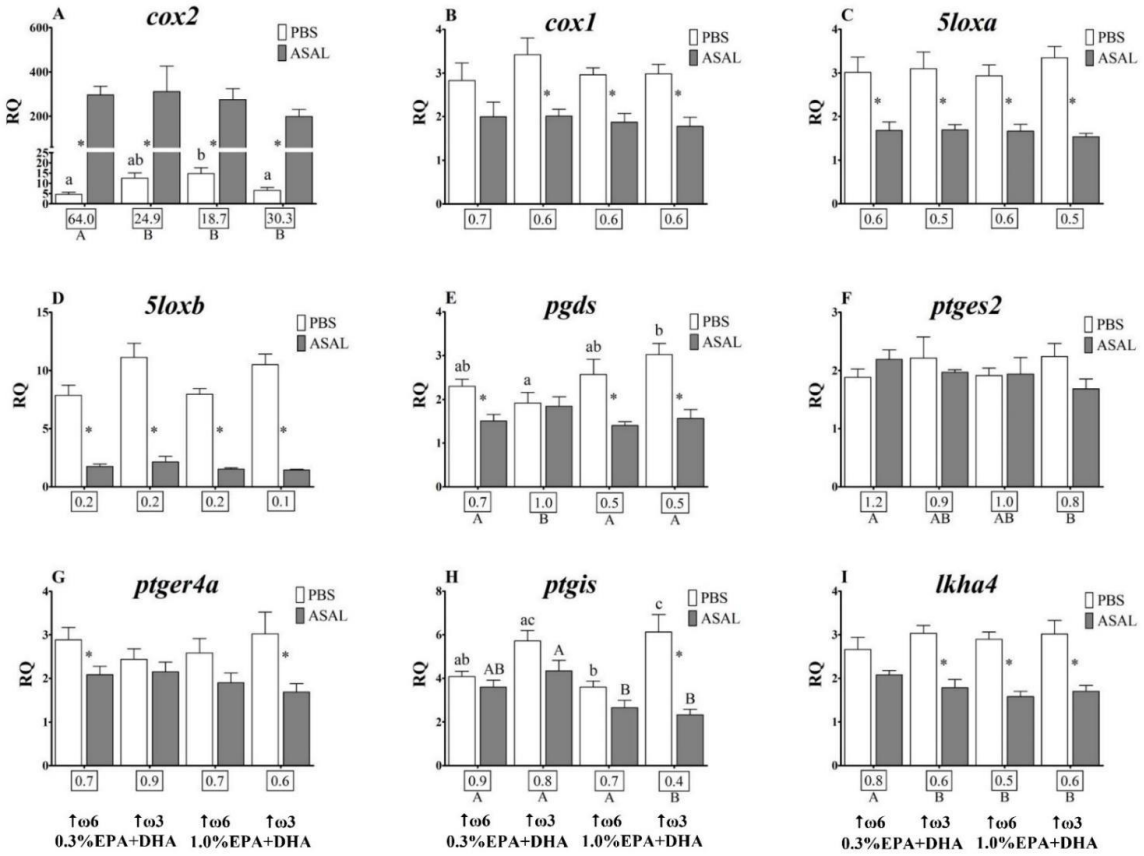

Figure 5.3. Head kidney qPCR transcript expression of genes involved in eicosanoid metabolism (A-I) in salmon fed different ratios of ω6:ω3 and levels of EPA+DHA for 12 weeks. Head kidney tissues were sampled at 24 h post-injection. Transcript expression values presented as mean relative quantity (RQ) ± SE (n = 7-8). An asterisk represents significant difference between PBS- and ASAL-injected fish in each dietary treatment (Student's t-test; $p < 0.05$). Upper-case letters above error bars indicate significant differences among dietary treatments within ASAL-injected fish. Lower-case letters signify dietary differences within PBS-injected fish (one-way ANOVA and Tukey post-hoc tests; $p < 0.05$). Values below the figures represent fold-change (ASAL/PBS) for each dietary treatment, and significant differences among fold-changes are denoted with upper-case letters. Bottom legends describe the dietary treatments of all panels.

The 0.3%EPA+DHA↑ω6 diet resulted in significantly higher ASAL fold-change induction of *cox2* when compared with the other dietary treatments ($p < 0.0001$; Figure 5.3A). Transcript expression levels of *cox2* were higher in PBS-treated 1.0%EPA+DHA↑ω6 compared with PBS-treated 0.3%EPA+DHA↑ω6 and PBS-treated 1.0%EPA+DHA↑ω3 fed fish ($p = 0.01$; Figure 5.3A). ASAL fold-change induction values of *pgds* were higher in 0.3%EPA+DHA↑ω3 compared with the other dietary treatments ($p = 0.001$; Figure 5.3E), and *pgds* expression levels were higher in PBS-treated 1.0%EPA+DHA↑ω3 than 0.3%EPA+DHA↑ω3 fed fish ($p = 0.04$; Figure 5.3E). ASAL induction of *ptges2* was higher in 0.3%EPA+DHA↑ω6 compared with 1.0%EPA+DHA↑ω3 fed fish ($p = 0.03$; Figure 5.3F). Transcript expression levels of *ptgis* were higher in ASAL-injected 0.3%EPA+DHA↑ω3 compared with the ASAL-injected 1.0%EPA+DHA↑ω6 and 1.0%EPA+DHA↑ω3 dietary treatments ($p = 0.002$; Figure 5.3H), while the 1.0%EPA+DHA↑ω3 diet resulted in stronger ASAL suppression (i.e. fold-change) of *ptgis* compared with the other three dietary treatments ($p = 0.001$; Figure 5.3H). The mRNA levels of *ptgis* were higher in PBS-injected 1.0%EPA+DHA↑ω3 than in PBS-injected 0.3%EPA+DHA↑ω6, and lower in PBS-treated 1.0%EPA+DHA↑ω6 compared with PBS-treated 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω3 treatments ($p = 0.004$; Figure 5.3H). Finally, the 0.3%EPA+DHA↑ω6 diet resulted in weaker ASAL suppression (i.e. fold-change) of *lkha4* compared to the other dietary treatments ($p = 0.01$; Figure 5.3I).

5.4.3. Influence of dietary EPA+DHA and ω6:ω3 on ASAL response

Two-way ANOVA analysis revealed a significant interaction between the dietary factors EPA+DHA and ω6:ω3 influencing ASAL fold-change values of most immune-

related transcripts (i.e. *ccl19b, tgfb1, il8, il1b, il10, saa5, camp, hamp, haaf*; Table 5.4). ASAL induction of *nfkb1* was significantly affected by both EPA+DHA and ω6:ω3, while *stlr5, ccl19a* and *lect2* ASAL fold-change was impacted by dietary ω6:ω3 alone. ASAL fold-change values (i.e. ASAL/PBS) of the eicosanoid metabolism-related transcripts *cox2, pgds, ptger4a*, and *lkha4* were also significantly influenced by the interaction of EPA+DHA with ω6:ω3. Finally, *ptgis* fold-change was impacted by EPA+DHA and ω6:ω3, while that of *ptges2* was impacted by dietary ω6:ω3 alone.

5.4.4. Hierarchical clustering and correlations analyses

5.4.4.1. Hierarchical clustering of head kidney lipid composition and fold-change transcript expression

Hierarchical clustering analysis of head kidney lipid composition identified five separate clusters (Figure 5.4). Cluster I consisted of the lipid class PL, while cluster II included 18:0, 16:0, ΣSFA, ω6 fatty acids (i.e. 20:2ω6, 18:2ω6, 20:4ω6, 22:5ω6, 22:4ω6, 18:3ω6, 20:3ω6), Σω6, ω6:ω3, and ΣPUFA. The third and fourth clusters were composed of ST and 22:5ω3, respectively. The fifth cluster consisted of ω3 fatty acids (i.e. 20:5ω3, 20:4ω3, 18:4ω3, 18:3ω3, 20:3ω3), Σω3, the ratio of EPA:ARA, 18:1ω9 and ΣMUFA. Cluster analysis of qPCR analyzed transcript expression (i.e. fold-change) also showed five clusters (Figure 5.4). Cluster I included the eicosanoid metabolism-related genes *ptgis, ptges2, lkha4, 5loxb,* and the transcript *nfkb1*, while cluster II consisted of *ptger4a* and *pgds*. Cluster III was the largest and included the immune-related transcripts *saa, haaf, hamp, ccl19a, ccl19b, camp, il8, il10, stlr5, lect2* and *cox2*. Cluster IV included only *irf1*, while cluster V included only *tgfb1*.

5.4.4.2. Correlations between immune-related transcripts and lipid composition

All transcripts involved in immune response in cluster III correlated positively with 18:0 (except *lect2*), while some correlated positively with 16:0 (i.e. *saa, haaf, ccl19a, il10* and *lect2*) and ΣSFA (i.e. *saa* and *lect2*; p = 0.0001-0.047; Figure 5.4). Interestingly, genes in this cluster correlated positively with ω6 fatty acids (i.e. 20:2ω6, 18:2ω6, 20:4ω6, 22:4ω6, 18:3ω6 and 20:3ω6), Σω6 and ω6:ω3 (p = 0.0001-0.025), while showing negative correlations with ω3 fatty acids [i.e. 20:4ω3, 18:4ω3, 18:3ω3 (excluding *hamp*), 20:3ω3 (excluding *hamp*)], Σω3, and EPA:ARA (p = 0.0001-0.046). Some transcripts from cluster III correlated negatively with 20:5ω3 (*saa, haaf, hamp, ccl19b, il8* and *stlr5*) and 22:5ω3 (*haaf* and *ccl19b*; p = 0.016-0.049). Finally, genes in cluster III correlated negatively with ΣMUFA and 18:1ω9 (excluding *hamp, cc19a* and *irf1*; p = 0.0001-0.047). Similar to the pattern of cluster III, *nfkb1* from cluster I correlated positively with 18:0, all of ω6 fatty acids, Σω6, ω6:ω3 (p = 0.0001-0.009), and showed negative correlations with ω3 fatty acids (i.e. 20:5ω3, 20:4ω3, 18:3ω3, 20:3ω3), Σω3, EPA:ARA, and ΣMUFA (p = 0.0001-0.026). Finally, cluster IV-associated *irf1* was correlated positively with 18:0 (p = 0.043), and *tgfb1* from cluster V showed negative correlations with ST, Σω3, 18:4ω3, 18:3ω3 and 20:3ω3 (p = 0.007-0.046).

Table 5.4. Two-way ANOVA analysis[a] showing the effects of dietary EPA+DHA and ω6:ω3 on ASAL fold-change response of genes involved in immune response and eicosanoid metabolism in salmon fed different ratios of ω6:ω3 and levels of EPA+DHA for 12 weeks.

Transcript	EPA+DHA	ω6:ω3	Interaction
stlr5	0.226	**< 0.0001**	0.064
ccl19a	0.100	**0.036**	0.140
ccl19b	**0.001**	**0.031**	**0.005**
lect2	0.499	**< 0.0001**	0.059
tgfb1	**0.033**	**0.003**	**0.001**
il8	**0.001**	**< 0.0001**	**< 0.0001**
il1b	0.377	0.114	**0.027**
il10	**0.001**	**0.002**	**0.033**
saa5	**0.003**	**< 0.0001**	**0.004**
irf1	0.089	0.413	0.771
nfkb1	**< 0.0001**	**0.043**	0.561
camp	0.375	**0.006**	**0.012**
hamp	**0.005**	**0.025**	**0.004**
haaf	**< 0.0001**	**< 0.0001**	**< 0.0001**
cox2	**0.007**	0.055	**0.001**
cox1	0.678	0.347	0.625
5loxa	0.408	0.227	0.323
5loxb	0.104	0.133	0.672
pgds	**0.001**	0.076	**0.037**
ptges2	0.151	**0.010**	0.949
ptger4a	0.058	0.908	**0.039**
ptgis	**0.003**	**0.006**	0.145
lkha4	**0.011**	0.079	**0.038**

[a] p-values of all GOIs are shown.

Significant p-values (i.e. $p < 0.05$) are shown in bold font.

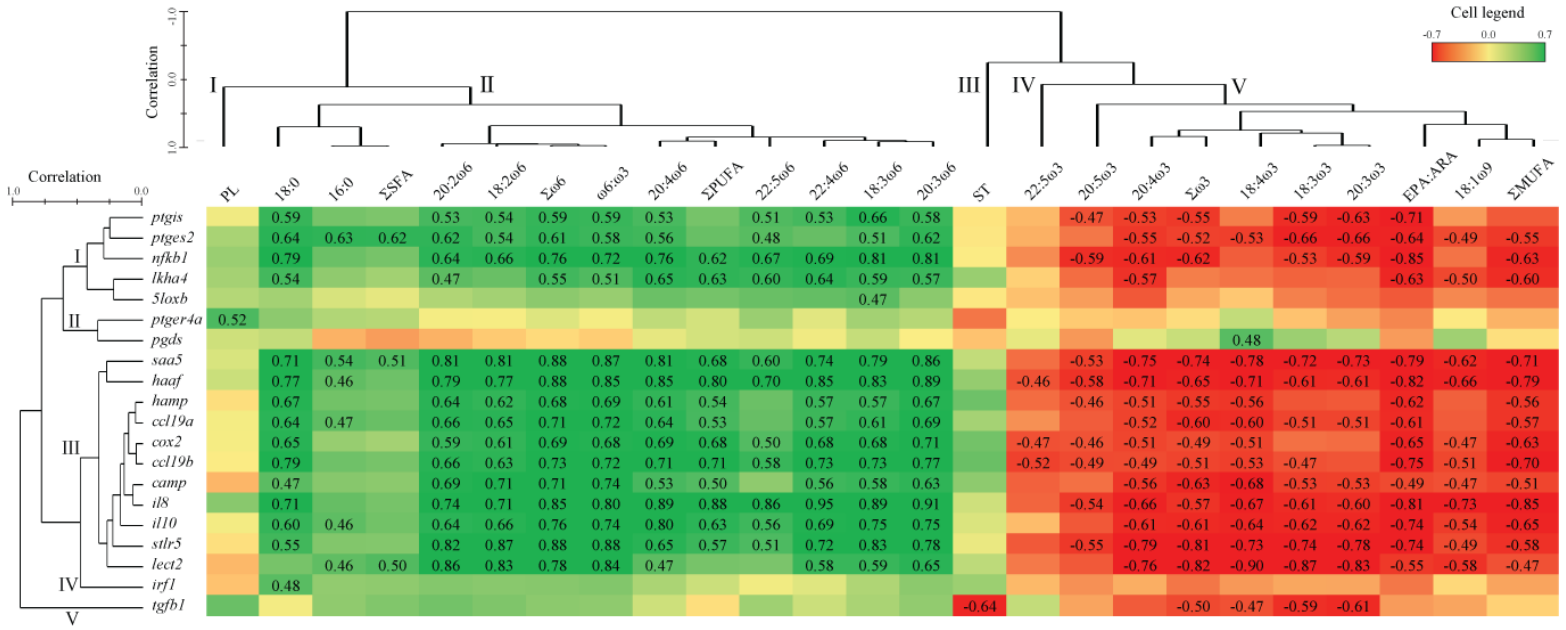

Figure 5.4. Hierarchical clustering and Pearson correlation matrix of transcript expression [ASAL fold-change values] of genes involved in immune response and eicosanoid metabolism, and head kidney lipid composition (% fatty acids and lipid classes) in Atlantic salmon fed diets with different ratios of ω6:ω3 and levels of EPA+DHA for 12 weeks. For ASAL fold-change calculations refer to Materials and Methods. Transcripts, fatty acids, and lipid classes that were not significantly correlated were excluded (see Materials and Methods). Statistically significant ($p < 0.05$) correlation coefficients are shown. Red cells signify negative relationships, and green cells signify positive relationships. ΣSFA, ΣMUFA, and ΣPUFA represent total saturated, monounsaturated, and polyunsaturated fatty acids, respectively. 20:5ω3, 22:5ω3, and 20:4ω6 represent EPA, ω3DPA, and ARA, respectively. PL and ST represent phospholipids and sterols, respectively.

5.4.4.3. Correlations between eicosanoid metabolism-related transcripts and lipid composition

The transcript expression of *ptgis*, *ptges2* and *lkha4* from cluster I correlated positively with 18:0, ω6 fatty acids [i.e. 20:2ω6, 18:2ω6 (excluding *lkha4)*, 20:4ω6, 22:5ω6, 22:4ω6 (excluding *ptges2*), 18:3ω6, 20:3ω6], Σω6 and ω6:ω3 (p = 0.0001-0.044), while showing negative correlations with ω3 fatty acids [20:4ω3, 18:3ω3 and 20:3ω3 (excluding *lkha4*)], Σω3 (excluding *lkha4*), EPA:ARA and ΣMUFA (excluding *ptgis*; p = 0.0001-0.028; Figure 5.4). The transcripts *ptgis* and *ptges2* were negatively correlated with 20:5ω3 and 18:4ω3, respectively (p = 0.012-0.046), while *ptges2* and *lkha4* correlated negatively with 18:1ω9 (p = 0.036 and 0.033, respectively). The transcript *5loxb* was positively correlated with 18:3ω6 (p = 0.043). Cluster II-associated *ptger4a* and *pgds* correlated positively with PL (p = 0.029) and 18:4ω3 (p = 0.040), respectively. Finally, correlations of *cox2* from cluster 3 were very similar to other immune-related transcripts, and showed positive correlations with 18:0 and all of ω6 fatty acids (including Σω6 and ω6:ω3; p = 0.002-0.035), and negative correlations with all of ω3 fatty acids (including Σω3, and excluding 18:3ω3 and 20:3ω3; p = 0.006-0.049). Further, *cox2* was negatively correlated with EPA:ARA, 18:1ω9 and ΣMUFA (p = 0.006-0.046).

5.4.5. Plasma levels of prostaglandins

Within the PBS group, salmon fed with 0.3%EPA+DHA↑ω6 diet had significantly higher concentrations (ng ml^{-1}) of PGE_2 and PGF3α (p = 0.03 and 0.02, respectively) than 0.3%EPA+DHA↑ω3 fed fish (Table 5.5). Further, 0.3%EPA+DHA↑ω6 fed fish in the ASAL group had a lower concentration of PGE_2 than the PBS-injected fish fed

0.3%EPA+DHA↑ω6 ($p = 0.02$). No dietary differences in prostaglandins were identified within the ASAL group ($p = 0.70$ and 0.49), and no differences were detected (Table 5.5) in PGF3α when comparing the ASAL with the PBS groups ($p = 0.2$ and 0.3). Finally, two-way ANOVA analysis showed a significant interaction between diet and injection in PGE_2 ($p = 0.036$), and PGF3α was significantly affected by diet ($p = 0.018$; Table 5.5).

Table 5.5. Plasma levels[a] of PGE_2 and PGF3α in salmon fed different ratios of ω6:ω3 and 0.3% EPA+DHA for 12 weeks, and 24 h post-injection with phosphate buffered saline (PBS) or formalin-killed *A. salmonicida* (ASAL) bacterin.

	PBS			**ASAL**		
	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	p-value	0.3%EPA+DHA ↑ω6	0.3%EPA+DHA ↑ω3	p-value
PGE_2	145.6 ± 23.3[a]	115.3 ± 21.3[b]	**0.032**	112.9 ± 19.1[*]	116.8 ± 17.1	0.697
PGF3α	300.6 ± 131.1[a]	157.3 ± 54.8[b]	**0.020**	227.3 ± 90.5	195.8 ± 85.2	0.488
[b]PG	Diet	Injection	Interaction			
PGE_2	0.100	0.054	**0.036**			
PGF3α	**0.018**	0.618	0.117			

[a]Average values (ng ml^{-1}) ± standard deviations (n = 6-9).
Lower-case letters indicate significant differences between dietary treatments within PBS-injected fish.
*Asterisks represent a significant injection effect within a dietary treatment (Student's *t*-test; $p < 0.05$).
[b]p-values from the Two-way ANOVA analysis of prostaglandins.

5.5. Discussion

5.5.1. Influence of ASAL injection on transcripts involved in immune response and eicosanoid metabolism

Most immune-related transcripts in the present study showed a significant ASAL induction in Atlantic salmon head kidney. This induction was generally independent of dietary treatment (not including *lect2*, *tgfb1* and *nfkb1*). The transcript *stlr5* responded to the ASAL injection. TLR5 is a PRR that detects bacterial flagellin and activates the Myeloid differentiation primary response 88 (MyD88)-dependent pathway in mammals and fish (Zhang et al., 2014; Muñoz-Flores et al., 2018). STLR5 is a soluble form that is unique to fish, and lacks the transmembrane region and Toll-IL-1 receptor (TIR) domain (Zhang et al., 2014). This PRR was identified in Atlantic salmon (Salazar et al., 2016), and *stlr5* expression was shown to be induced by *A. salmonicida* infection in the head kidney, spleen (Zhang et al., 2011), liver (Tsoi et al., 2006; Ewart et al., 2005), and gills (Meng et al., 2017) of this fish. Further, Meng et al. (2017) reported that gills *stlr5* expression reached a maximum at 24 h after *A. salmonicida* infection. Interestingly, TLR5 binding by flagellin induces NF-kB activation and generates pro-inflammatory cytokines in Atlantic salmon (Hynes et al., 2011; Arnemo et al., 2014; Salazar et al., 2016). Thus, these data suggest that *stlr5* was involved in the activation of the innate immune response to ASAL in the current study.

The chemokine-encoding genes *ccl19* (two paralogues), *lect2* (in the two high ω6 dietary treatments), and *il8* responded to the bacterial challenge in the current study. CCL19 is a cytokine and part of the CC chemokine subfamily (Leick et al., 2009). Previous turbot (*Scophthalmus maximus*) and striped murrel (*Channa striatus*) studies reported that

it plays a role in head kidney and peripheral blood leucocyte trafficking, cell proliferation, and antibacterial immune response (Chen et al., 2013; Arockiaraj et al., 2015). The paralogues *ccl19a* and *ccl19b* were induced in Atlantic salmon head kidney at 24 h post-injection with polyriboinosinic polyribocytidylic acid (pIC) when compared with the PBS group (Caballero-Solares et al., 2017). Further, the transcript *ccl19* and *ccl19*-like transcripts were up-regulated in several fish species [i.e. half-smooth tongue sole (*Cynoglossus semilaevis*), Dabry's sturgeon (*Acipenser dabryanus*), channel catfish (*Ictalurus punctatus*), ayu (*Plecoglossus altivelis*)] upon bacterial infection (Peatman et al., 2007; Li et al., 2011; Chen et al., 2018; Luo et al., 2018). More studies are required in order to elucidate the impact of ASAL infection on *ccl19* response in Atlantic salmon. We also showed that the head kidney transcript *lect2* responded to ASAL injection (in the high ω6 diet fed fish). The magnitude of induction was lower (i.e. ~2-fold) compared to other fish studies which reported dramatic *lect2* induction (~10 to 500-fold) by bacterial (*A. salmonicida*, *Vibrio alginolyticus*, LPS) challenges (Lin et al., 2007; Li et al., 2008; Holen et al., 2012). However, it was comparable to Rozas-Serri et al. (2018) who indicated head kidney *lect2* fold-change of ~ 2.5 in Atlantic salmon challenged with *Piscirickettsia salmonis*. This confirms that *lect2* response to bacterial infection depends upon species, tissue, time, and pathogen.

The interleukin-related transcripts in the current study (i.e. *il8*, *il1b*, *il10*) showed a significant ASAL induction compared with the time-matched PBS injected controls. IL8 is a member of the CXC chemokine family, and function as chemotactic factor and neutrophil activator in mammals (Zeilhofer and Schorr, 2000; Lin et al., 2004). It was previously shown that IL8 induced chemotaxis in head kidney leucocytes of mandarin fish (*Siniperca*

chuasti) (Wang et al., 2017). IL1B is regulating IL8 and they are considered as early pro-inflammatory cytokines in the inflammatory response (Dinarello, 1997; Petering et al., 1999; Laing and Secombes, 2004). In contrast, IL10 inhibits cytokine production (including IL1B and IL8) and is generally considered an anti-inflammatory cytokine in mammals (Sabat et al., 2010; Pestka et al., 2004; Mosser and Zhang, 2008) and fish (Piazzon et al., 2015; Huo et al., 2019). In line with results from this Chapter, previous studies with Atlantic cod (*Gadus morhua*) and Atlantic salmon indicated that these interleukin-encoding transcripts were strongly induced in the head kidney by bacterial challenges and stimulations (i.e. *V. anguillarum*, LPS, *A. salmonicida*; Seppola et al., 2008; Fast et al., 2009; Stenberg et al., 2019). Interestingly, this Chapter also showed that *il10* had the strongest ASAL fold-change induction among the three transcripts. This result is in line with Tran et al. (2019) who reported a strong up-regulation of *il10* in head kidney (~15-fold) and spleen (~25-fold) of cobia (*Rachycentron canadum*) at 24 h post-infection with the Gram-negative bacteria *Photobacterium damselae* subsp. *piscicida*. Thus, head kidney induction of *il10* was presumably a response to depressed inflammation as observed in previous studies with sea bass (*Dicentrarchus labrax*) and rainbow trout (*Oncorhynchus mykiss*) (Sepulcre et al., 2007; Harun et al., 2011). The transcript *saa5* also responded to ASAL injection in the current study. SAA is highly conserved and a major acute phase protein in mammals (Buck et al., 2016) and fish (Kania et al., 2014). Human SAA was shown to bind to Gram-negative bacteria and to have cytokine and chemokine inducing capacity (Hari-Dass et al., 2005; Eklund et al., 2012; Buck et al., 2016), but also to have important roles in lipid metabolism (Faty et al., 2012). Goldfish (*Carassius auratus* L.) SAA exhibited antibacterial properties and induced chemotaxis of neutrophils and

macrophages (Kovacevic and Belosevic, 2015). The SAA gene family consists of 5 members, and SAA5 was identified as a pseudogene in mice (Butler and Whitehead, 1996; Sack, 2018). The transcript *saa5* plays an important role in Atlantic salmon acute phase response (Lee et al., 2017; Chalmers et al., 2018). The expression of this transcript was massively induced (i.e. ~500 to 1000-fold) in the liver and internal organs of *A. salmonicida*-infected zebrafish (*Danio rerio*) and Atlantic salmon, respectively (Lin et al., 2007; Lee et al., 2017). Lee et al. (2017) also revealed that salmon primary head kidney leucocytes treated with recombinant IL1B had a strong induction of *saa5* at 4 and 8 h post-stimulation (i.e. fold-changes of ~ 60 and 40, respectively). The induction observed in head kidney leucocytes is in the range of that observed in the current study at 24 h post-injection. Taken together, these data confirm that *saa5* play important roles in Atlantic salmon antibacterial immune response.

TLR signalling results in the activation of several transcription factors including IRF1 and NFKB1. Both transcription factor-encoding transcripts *irf1* and *nfkb1* responded to ASAL injection in this Chapter. Atlantic salmon IRF1 induced type I interferons (Bergan et al., 2010). Although many fish studies examined the role of *irf1* in antiviral immune responses (Ordas et al., 2006; Jia and Guo, 2008; Rise et al., 2008), several reports showed it responsiveness to bacterial challenges (e.g. *Renibacterium salmoninarum*, ASAL, *Edwardsiella tarda*) in Atlantic salmon (Rozas-Serri et al., 2018; Eslamloo et al., 2020) and other species [i.e. Japanese flounder (*Paralichthys olivaceus*), Atlantic cod, half-smooth tongue sole] (Yabu et al., 1998; Feng et al., 2009; Zhang et al., 2015). Collectively, these results suggest that interferon-mediated immune response was activated by bacterial infections and injections (include ASAL) in fish. The nuclear factor kappa B (NFkB)

complex is a master regulator of the inflammatory response (Rius-Pérez et al., 2020) and considered an important transcription factor, regulating many cytokines (e.g. interleukins, chemokines, interferons) involved in the antibacterial immune response (reviewed by Blackwell and Christman, 1997; Ghosh and Karlin, 2002). The subunit p105 is the precursor of the p50 subunit and also functions as NFkB inhibitor by binding to RelA and preventing nuclear transport (Yurochko et al., 1995; Blackwell and Christman, 1997). Mammalian studies indicated an important role of p105 as a suppressor of inflammation (Ishikawa et al., 1998; Pereira and Oakley, 2008). Interestingly, microarray analyses by Škugor et al. (2009) revealed that hepatic transcript encoding NF-kappa B-p105 was up-regulated in Atlantic salmon with high furunculosis resistance as compared with the low resistance fish. Taken together, these data suggest that higher ASAL induction of *nfkb1* limited NFKB activation and may have limited the inflammatory response in the current study.

The transcripts *camp* and *hamp* also showed a significant ASAL induction in the current study. CAMP and HAMP are well characterized antimicrobial peptides in fish (Chang et al., 2005; Douglas et al., 2003; Bridle et al., 2011; Álvarez et al., 2014). They showed bactericidal activities against Gram-positive and Gram-negative bacteria in Atlantic salmon (Bridle et al., 2011), rainbow trout (Alvarez et al., 2014; Zhang et al., 2015), European seabass (Álvarez et al., 2016), among other fish (Cai et al., 2012; Katzenback, 2015). Data from this Chapter are in line with previous studies who showed that *camp* and *hamp* were induced in fish (i.e. Atlantic salmon, rainbow trout, Atlantic cod) head kidney at 24 h post-injection of live *A. salmonicida*. (Chang et al., 2006; Martin et al., 2006) and ASAL (Feng et al., 2009; Furlan et al., 2018). HAAF is an ortholog of the

horseshoe crab (*Limulus* sp.) amebocyte aggregation factor, known to promote aggregation of amebocytes and agglutination of erythrocytes (Fujii et al., 1992). Amebocytes are hemocytes that are functionally comparable to mammalian macrophages, T- and B-lymphocytes, and platelets (Gupta, 1991; MacPherson and Jacobs, 2000). Horseshoe crab amebocytes showed bactericidal activity against Gram-positive and Gram-negative bacteria (Pistole and Britko, 1978; Iwanaga et al., 1998). Interestingly, the expression of *haaf* was up-regulated in the liver and head kidney of salmonids (i.e. Atlantic salmon, rainbow trout) exposed to *A. salmonicida* (Ewart et al., 2005; Martin et al., 2006), *Piscirickettsia salmonis* (Rise et al., 2004) and *Vibrio anguillarum* (Gerwick et al., 2007), when compared to non-infected control fish. Rise et al. (2004) reported a head kidney fold-change induction of ~11 at 14 days post-infection, which is comparable to that observed in the current study at 24 h post-injection. These data suggest an important role of *haaf* in the molecular regulation and response to bacterial infections in Atlantic salmon.

The expression of head kidney *cox2* was strongly induced by ASAL injection in this Chapter. However, all other transcripts involved in eicosanoid metabolism (with the exception of *ptges2*) showed down-regulation in the ASAL when compared with the PBS group (within diet). This suppression occurred in some or in all dietary treatments. Eicosanoids are inflammatory mediators (Wall et al., 2010) generated by ω3 (i.e. EPA, DHA) and ω6 (primarily ARA) PUFA, and metabolized to prostaglandins, prostacyclins, thromboxanes, leukotrienes, docosanoids, maresins and resolvins (Calder, 2007, 2013; Arts and Kohler, 2009; Lone and Taskén, 2013). COX1 (constitutive) and COX2 (inducible) catalyze the synthesis of prostanoids (i.e. prostaglandin G and H), while PGDS and PTGES convert prostaglandin H into prostaglandin D and E, respectively. The enzyme

PTGIS is also involved in prostanoids synthesis and converts prostaglandin H into prostaglandin I. However, 5LOX and IKHA4 are involved in leukotrienes synthesis by the catalysis of leukotriene A and B, respectively (Rowley, 1995; Lone and Taskén, 2013; Gómez-Abellán and Sepulcre, 2016; Figure 1.2). The molecular regulation of genes involved in eicosanoid metabolism and their response to bacterial challenges is poorly understood in fish (Geay et al., 2015; Holen et al., 2018; Stenberg et al., 2019). Data from this Chapter are in line with Caballero-Solares et al. (2017) who showed that *cox2* was induced, while other eicosanoid-related transcripts (i.e. *cox1*, *5loxa*, *5loxb*, *pgds*) were supressed in the head kidney of Atlantic salmon treated with the viral mimic polyriboinosinic polyribocytidylic acid (pIC) when compared with the PBS group. Further, my results are in agreement with previous Atlantic salmon studies who indicated down-regulation of head kidney *5lox* (Stenberg et al., 2019) and *lkha4* (Martin et al., 2006) in response to bacterial infection (*A. salmonicida*) and bacterial PAMP (LPS). A reduction in the synthesis of prostaglandins and leukotrienes could be an attempt to mitigate the proinflammatory response in salmon subjected to viral and bacterial pathogens. Interestingly, previous insect studies reported that bacterial pathogens immunosuppressed host eicosanoid synthesis (Hwang et al., 2013; Kim et al., 2018). More studies are necessary to examine the relationship between transcripts involved in eicosanoid metabolism and fish immunosuppression by bacterial pathogens.

5.5.2. Dietary impacts on transcripts involved in immune response and eicosanoid metabolism

The transcript expression of multiple immune-related genes (i.e. *ccl19b*, *il8*, *il10*, *saa5*, *hamp*, *haaf*) showed the strongest ASAL fold-change induction in fish fed 0.3%EPA+DHA↑ω6 diet (Figures 5.1-5.2). Other antibacterial transcripts had higher fold-change induction in fish fed 0.3%EPA+DHA↑ω6 when compared to 0.3%EPA+DHA↑ω3 and 1.0%EPA+DHA↑ω3 diets (*stlr5*, *lect2*, *camp*), or compared to 1.0%EPA+DHA (both high ω6 and high ω3 diets; *nfkb1*). Further, two-way ANOVA analysis revealed that the responsiveness of antibacterial transcripts to ASAL-injection was predominantly impacted by the interaction of dietary ω6:ω3 with EPA+DHA (Table 5.4). Dietary lipid composition (particularly EPA, DHA, and ARA) can influence immune responses by altering the FA composition of immune and inflammatory cell membranes (Calder, 2006; Wall et al., 2010; Calder, 2013). Chapter 4 of this thesis (published as Katan et al., 2020) demonstrated that salmon fed 0.3%EPA+DHA↑ω6 diet had the highest head kidney DGLA and ARA, and lowest EPA (measured as proportions and concentrations) when compared to the other dietary treatments. Further, the current Chapter showed positive correlations between head kidney ω6 PUFA and the transcript expression (measured as fold-change induction) of genes involved in immune response and eicosanoid metabolism (Figure 5.4).

These data are in line with Montero et al. (2015) who reported that TLR-encoding transcripts *tlr1* and *tlr9* were up-regulated in Senegalese sole (*Solea senegalensis*) fed soy oil rich diets as a complete replacement of FO. In Chytilová et al. (2014), pre-feeding with flaxseed oil (rich in 18:3ω3) resulted in down-regulation of intestine *tlr5* in gnotobiotic pigs challenged with *Escherichia coli*. Several papers showed that the expression of

intestinal *tlr5* in fish was influenced by feeds containing probiotics (Sun et al., 2014; Pérez-Sánchez et al., 2015). However, the impact of dietary fatty acids on the molecular regulation of *stlr5* in fish is less understood. The PRR TLR5 activates NF-kB and generates pro-inflammatory cytokines in Atlantic salmon (Hynes et al., 2011; Arnemo et al., 2014; Salazar et al., 2016). The higher ASAL fold-change induction of *nfkb1* in fish fed 0.3%EPA+DHA↑ω6 suggests an attempt to reduce inflammation thorough NFKB inhibition (see section 4.1). The influence of fatty acids on *nfkb1* expression was previously shown in human endothelial cells (Baker et al., 2020) and mouse brain (Alashmali et al., 2019). The latter study reported higher expression of *nfkb1* in mouse fed deprived *vs* adequate ω6 PUFA diet (i.e. 2% and 23% of 18:2ω6), 1-day following LPS-injection. Discrepancies with the current study may be attributed to species, the organ examined, and immune challenge (i.e. LPS *vs*. ASAL bacterin).

ASAL fold-change induction of acute phase response-related (i.e. *saa5*) and chemokine-related transcripts (i.e. *ccl19b*, *il8*) was also positively influenced by the 0.3%EPA+DHA↑ω6 diet in this Chapter. In mammalian literature it was shown that SAA regulates the expression of several genes involved in lipid metabolism (Chen et al., 2008; Faty et al., 2012). Interestingly, varying dietary ω6:ω3 ratios impacted the constitutive hepatic expression levels of *saa* in zebrafish (Powell et al., 2015), while feeding of trans-cinnamic acid (plant metabolite) resulted in up-regulation of head kidney *saa* in rainbow trout challenged with *Yersinia ruckeri* (Yılmaz and Ergün, 2018). Further, data from this Chapter are in agreement with previous studies with mice which showed that dietary ω6 FA positively influenced the expression of *ccl19* at pre- and post-immune challenge (Lazic et al., 2014; Alashmali et al., 2019). This study also revealed that head kidney *lect2* was

ASAL-induced only in fish fed high ω6 diets (i.e. 0.3 and 1.0%). Morais et al. (2012) and Xue et al. (2020) indicated a positive influence of high ω3 LC-PUFA diets on the constitutive expression of hepatic *lect2* in Atlantic salmon. Taken together, these data suggest that *lect2* responsiveness to dietary FA varies among challenged, non-challenged and tissue-dependent in salmon. Given the important role of LECT2 in fish inflammatory response (Lin et al., 2007; Li et al., 2008), I hypothesize that *lect2* induction was related to the inflammatory status of fish fed high ω6 diets. Clearly, more studies that specifically examine the impacts of high ω6 plant-based diets on *saa5, ccl19 and lect2* expression in immune-challenged fish are required. Interestingly the expression of *tgfb1* was lower in the ASAL compared to the PBS-injected fish in salmon fed 0.3%EPA+DHA↑ω6 and 1.0%EPA+DHA↑ω3 diets. In gilthead seabream (*Sparus aurata*), head kidney *tgfb1* expression was modified by soybean protein as a fish meal replacement (Kokou, 2015), while Zeng et al. (2016) showed that distal intestine *tgfb1* expression levels were increased with increasing dietary 18:3ω3:18:2ω6. A previous study with rainbow trout revealed that PAMP (i.e. LPS, pIC), pro-inflammatory cytokines, and pathway activators did not induce the first paralogue of *tgfb1* in head kidney macrophages (Maehr et al., 2013). This explains the lack of induction in the current study which targeted the first paralogue of *tgfb1*.

The interleukin-encoding transcripts *il8* and *il10* showed the highest ASAL fold-changes in salmon fed 0.3%EPA+DHA↑ω6. These data agree with Montero et al. (2015) who reported up-regulation of intestine *il10* in Senegalese sole fed soy oil as compared with linseed and FO diets. In Zeng et al. (2016) the mRNA levels of intestine *il8* increased with decreasing dietary ratios of 18:3ω3:18:2ω6 in grass carp (*Ctenopharyngodon idella*). Further, increasing levels of ARA+EPA positively influenced the transcript expression of

il8 and *il10* in Atlantic cod head kidney leucocytes (Furne et al., 2013). The latter study supports the findings from this Chapter as the 0.3%EPA+DHA↑ω6 fed fish had the highest head kidney EPA+ARA (Chapter 4). The current study also showed dietary impacts on ASAL fold-change induction of genes encoding antimicrobial peptides (i.e. *camp, hamp*), and agglutination-aggregation factor (i.e. *haaf*). Previous work reported up-regulation of liver *haaf* in rainbow trout fed plant-based diet as a complete replacement to FO and fish meal (Panserat et al., 2009), while in Limtipsuntorn et al. (2014) Japanese flounder fed plant-based oils as a 100% replacement to FO showed up-regulation of liver *hamp*. However, Tacchi et al. (2012) showed that Atlantic salmon fed plant-proteins had lower liver *hamp* compared to the marine diet fed fish. In other fish studies the constitutive expression of *hamp* and *camp* was unaffected by plant-based diets or high ω6 feeds (Betancor et al., 2018; Nayak et al., 2020). Thus, although the studies cannot be directly compared with the current study (i.e. constitutive *vs* post-immune challenge expression), these data suggest that the regulation of *haaf* and *hamp* is influenced by plant-based diets (i.e. protein and lipid sources) in fish. Finally, mammalian studies reported that short chain FA induce the expression of *camp* (Sunkara et al., 2012; Jiang et al., 2013). However, the impacts of dietary PUFA and LC-PUFA on *camp* responses to immune stimuli in fish is less explored.

Salmon fed 0.3%EPA+DHA↑ω6 had the highest ASAL fold-change induction of head kidney *cox2*, and the expression (i.e. ASAL fold-change induction) of this transcript was positively correlated with ω6 PUFA in the head kidney. This suggests that higher levels of pro-inflammatory prostaglandins were generated in 0.3%EPA+DHA↑ω6 fed fish. Similar results were reported in previous studies which showed up-regulation of head

kidney (*in vitro* and *in vivo*) *cox2* in fish fed high ω6 or soy-based diets prior to bacterial challenge (i.e. LPS, *Streptococcus iniae*) (Holen et al., 2011; Holen et al., 2018; Nayak et al., 2020). In Holen et al. (2018) head kidney leucocyte *cox2* was up-regulated in Atlantic salmon fed soybean oil when compared with rapeseed and palm oils. *Ptgis* was the only eicosanoid-related transcript in the current study that showed dietary modulation in the ASAL-injected fish. This result confirms the data of Morais et al. (2012) who showed that liver *ptgis* was negatively influenced by ω3 LC-PUFA content in Atlantic salmon. Although not significant, fish fed 0.3%EPA+DHA↑ω3 had lower (quantitatively) proportion of EPA+DHA than 1.0%EPA+DHA↑ω6 and 1.0%EPA+DHA↑ω3 fed fish (Chapter 4). These data also agree with Soni et al. (2017) who indicated that EPA+DHA supplementation (i.e. ~ 0.7% of diet) resulted in down-regulation of spleen *ptgis* in mice fed corn oil-based diets. This suggests that the expression of *ptgis* is influenced by dietary and tissue EPA+DHA levels.

5.5.3. Plasma levels of prostaglandins

The current study measured two prostaglandins (i.e. PGE2, PGF3α) in Atlantic salmon plasma. The detected PGE_2 levels are comparable to the previous studies that used LC-ESI/MS/MS to measure PGE_2 in Atlantic cod head kidney leucocytes (Furne et al., 2013) and bluntnose minnows (*Pimephales notatus*) gills (Bhandari and Venables, 2011). Earlier studies measured PGF3α levels in Atlantic salmon organs (i.e. kidney, midgut; Bell et al., 1996; Olsen et al., 2012) and Senegalese sole plasma (Norambuena et al., 2013), and reported lower levels (i.e. 0.8-15 ng g^{-1}, and 1-7 ng ml^{-1}, respectively) compared to this Chapter. Both Bell et al. (1996) and Norambuena et al. (2013) used enzyme immunoassay

kits. Thus, differences among studies could be related to species, organ examined and/or method of measurement (i.e. enzyme immunoassay *vs*. ESI-MS/MS). To the best of my knowledge, there are no previous studies that utilized LC-MS/MS to quantify these prostaglandins in Atlantic salmon plasma. The current study showed that salmon fed 0.3%EPA+DHA↑ω6 had higher plasma PGE_2 than 0.3%EPA+DHA↑ω3 fed fish, in the PBS-injected fish. Further, the 0.3%EPA+DHA↑ω6 diet resulted in lower levels of PGE_2 in ASAL- when compared with PBS-injected fish (within dietary treatment). No significant differences were observed in any of the transcripts encoding eicosanoid enzymes between these dietary treatments in the PBS group (i.e. 0.3%EPA+DHA↑ω6 *vs* 0.3%EPA+DHA↑ω3). However, 0.3%EPA+DHA↑ω6 fed fish had lower expression of *ptger4a* in ASAL when compared to the PBS group (within dietary treatment), which agrees with the plasma data. PGE_2 binding to its receptors leads to activation of intracellular signal transduction and regulation of gene transcription (Sugimoto and Narumiya, 2007). Four PGE_2 subtype receptors (i.e. EP1-EP4) were identified in mammals (Narumiya et al., 1999; Sugimoto and Narumiya, 2007) and zebrafish (Iwasaki et al., 2013; Tsuge et al., 2013). However, only EP4 receptor was identified in Atlantic salmon (Leong et al., 2010; Guo et al., 2015). As an ARA derivative, PGE_2 is mostly regarded as pro-inflammatory. Nonetheless, it has been shown to play an important role in both the induction and suppression of fish inflammation (Fast et al., 2005; Belmonte et al., 2014; Guo et al., 2015). Interestingly, in Holen et al. (2012) *Pseudomonas aeruginosa* LPS treatment did not increase PGE_2 production in Atlantic cod head kidney leucocytes, while in Boltana et al. (2014) seabream injected with LPS from ASAL had similar plasma PGE_2 levels as the control (i.e. PBS-injected) group at 24 h post-injection. This Chapter confirms

the notion that the impact of bacterial stimulation on PGE_2 production varies between fish, origin of stimulation, doses, and time points (Gómez-abellán and Sepulcre, 2016). Our data regarding dietary modulated changes in PGE_2 are in line with Hundal et al. (2021) who showed increased liver PGE_2 in Atlantic salmon fed high ω6:ω3 (i.e. 6 *vs*. 1) plant-based oils. Other studies with Atlantic salmon also revealed a positive influence of high ω6 (Petropoulos et al., 2009) and ω6:ω3 (Berge et al., 2009) PO diets on plasma PGE_2 concentration. However, the latter two studies failed to show statistical significance due to large variation among fish.

Salmon fed 0.3%EPA+DHA↑ω6 also showed higher concentration of PGF3α than the 0.3%EPA+DHA↑ω3 fed fish (within PBS) in this Chapter. The oxidation of EPA was shown to result in PGF3α formation in plasma and urine of mammals (Nourooz-Zadeh et al., 1997; Fischer et al., 1988; Massaro et al., 2008). PGF3α is synthesized by the COX pathway and is regarded as anti-inflammatory (Tanaka et al., 2014) and less potent pro-inflammatory than 2-series prostaglandins (Kremmyda et al., 2011). However, relatively little is known about this prostaglandin and its dietary modulation in salmonids (Ghioni et al., 2002). Bell et al. (1997) reported higher PGF3α in the gills of Atlantic salmon fed PO as compared with FO, and this was concurrent with lower gill EPA:ARA in the PO fed fish. The current study did not profile the lipid composition of the plasma. Nevertheless, salmon fed 0.3%EPA+DHA↑ω6 diet had lower head kidney EPA:ARA (Chapter 4). Further, in Norambuena et al. (2013) plasma PGF3α was reduced with increasing dietary ARA and decreasing blood EPA:ARA in Senegalese sole. Thus, data from this Chapter support the notion that prostaglandin production of 2- and 3-series is influenced by tissue EPA:ARA in fish (Norambuena et al., 2012; Norambuena et al., 2013; Salini et al., 2016).

However, the interaction between dietary ω6:ω3 and tissue EPA:ARA, and the mechanisms by which they influence PGF3α production in immune-challenged fish is less researched.

5.6. Conclusions

The present study demonstrated the impacts of high ω6 and high ω3 plant-based diets containing low FO and EPA+DHA levels (i.e. 0.3 and 1.0%, as formulated) on the antibacterial immune and eicosanoid responses in Atlantic salmon. Most antibacterial transcripts were significantly induced by ASAL in salmon head kidney, in all dietary treatments (*stlr5, ccl19a, ccl19b, saa5, il8, il1b, il10, irf1, camp, haaf, hamp*). The magnitude of induction varied among transcripts encoding chemokines, interleukins, and antimicrobial peptides. All eicosanoid-metabolism-related transcripts (not including *cox2* and *ptges2*) were down-regulated in ASAL when compared with the PBS-injected fish (within diet), and this is suggested to be an attempt to mitigate the proinflammatory response in bacterin-challenged salmon. Further, higher expression of head kidney *nfkb1* and *il10* may have been another mechanism to suppress inflammation in the ASAL-injected fish. Fish fed 0.3%EPA+DHA↑ω6 diet showed the strongest ASAL fold-change induction of genes encoding chemokines (i.e. *ccl19b, il8*), interleukins (*il10, il8*), acute phase protein (i.e. *saa5*), antimicrobial peptide (*hamp*), and agglutination-aggregation factor (*haaf*). Two-way ANOVA analysis showed that ASAL fold-change levels of antibacterial and eicosanoid metabolism-related transcripts were largely impacted by the interaction of dietary ω6:ω3 with EPA+DHA. Further, this Chapter showed positive correlations between head kidney ω6 PUFA and the transcript expression of genes involved in immune response

and eicosanoid metabolism. These findings suggest that 0.3%EPA+DHA↑ω6 diet may have enhanced the innate antibacterial immune response of Atlantic salmon. Finally, fish fed 0.3%EPA+DHA↑ω6 had higher plasma PGE_2 and PGF3α when compared with 0.3%EPA+DHA↑ω3 fed fish, in the PBS group. The 0.3%EPA+DHA↑ω6 diet also resulted in lower levels of PGE_2 in ASAL- when compared with PBS-injected fish, and this was concurrent with lower head kidney expression of PGE_2 receptor-encoding transcript (i.e. *ptger4a*). Future studies should examine the impact of high ω6 plant-based diets with low EPA+DHA levels on antibacterial immune responses in Atlantic salmon.

5.7. References

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Chapter 6. Summary

6.1. General summary and future research

The current study investigated the effect of plant-based feeds with varying dietary ω6:ω3 ratios and ω3 long chain polyunsaturated fatty acid (LC-PUFA) levels on farmed Atlantic salmon physiology. By applying a multidisciplinary approach [e.g. lipid instrumental analyses, compound-specific stable isotope analysis (CSIA), gene expression and transcriptomic analyses], this thesis was able to link the dietary-induced tissue composition changes (i.e. liver, muscle, head kidney) of salmon with the transcript expression of genes with important functional roles [e.g. fatty acid (FA) metabolism, eicosanoid synthesis, immune response].

The aim of Chapter 2 was to elucidate the impact of plant-based diets with varying ratios of ω6:ω3 on growth performance, tissue lipid composition, FA biosynthesis, and transcript expression of genes involved in lipid metabolism. Levels of arachidonic (ARA, 20:4ω6), eicosapentaenoic (EPA, 20:5ω3) and docosahexaenoic (DHA, 22:6ω3) acids were similar among the 5 experimental diets, while a large range was observed in the long-chain polyunsaturated fatty acids (LC-PUFA) precursors α-linolenic (ALA, 18:3ω3) and linoleic (LNA, 18:2ω6) acids (from 12.7 to 36.2% and from 6.4 to 29.8%, respectively). This study hypothesized that dietary variation in ω6:ω3 ratio will influence tissue composition and FA biosynthesis; and that these metabolic changes will be associated with modifications in the transcript levels of FA and eicosanoid metabolism- related genes. Experimental diets had no impact on farmed Atlantic salmon growth performance (e.g. weight gain, specific growth rate, and condition factor) or organ indices [i.e. hepato-somatic index (HSI) and

viscero-somatic index]. This result is in line with previous studies which used linseed, sunflower or soy oil to replace fish oil with varying ratios of 18:2ω6 and 18:3ω3 (Bell et al., 2004; Menoyo et al., 2007; Thanuthong et al., 2011), and demonstrates that high dietary 18:3ω3 or 18:2ω6 may not have a negative effect on salmon growth performance. The fact that total lipid content or HSI were not significantly influenced by diet could suggest that the plant oils (PO) and precursor levels used in Chapter 2 did not have an adverse effect on liver lipid deposition. FA composition of liver and muscle tissues reflected dietary composition and responded to changes in ω3 and ω6 FA. CSIA indicated that liver EPA and ARA synthesis was largely driven by dietary PUFA precursors even when EPA and DHA were supplied at levels above minimum requirements. The current mixing model analysis revealed that ~91% and 44% of liver EPA was synthesized from dietary 18:3ω3 in the high ω3 and high ω6 fed fish, respectively. Although it was previously demonstrated that dietary PUFA precursors play a major role in LC-PUFA synthesis, EPA and DHA varied with precursor levels (Buzzi et al 1996; Cleveland et al., 2012; Masiha et al., 2013), making it difficult to truly determine the precursor role in the synthesis. Thus, the present thesis enhanced our understanding of the impact of dietary LC-PUFA precursors on salmon tissue composition and LC-PUFA synthesis. Further, diets in Chapter 2 of this thesis influenced the hepatic transcript expression of genes encoding transcription factors (i.e. *lxra*, *srebp1*). Dietary PUFA can regulate lipid metabolism genes by affecting the expression of transcription factors such as *srebp1*, *lxr* and *pparβ* (Betancor et al., 2014; Carmona-Antoñanzas et al., 2014; Glencross et al., 2015; Hixson et al., 2017). Future studies should investigate the interaction between dietary ω3 and ω6 FA and transcription factors that play regulatory roles in the LC-PUFA pathway of salmon. Finally, Chapter 2

showed that quantities of EPA+DHA in white muscle of fish fed with the 2 extreme diets (i.e. high ω3 and high ω6) were sufficient to satisfy daily EPA and DHA requirements for human consumers according to the World Health Organization (Burlingame et al., 2009). This is important because the quantitative amount of ω3 LC-PUFA in the fillet of farmed fish is crucial for the health of the consumer (Turchini et al., 2010; Nichols et al., 2014). Thus, data from this Chapter suggests that the high ω6 diet used herein may be suitable for enhancing ARA synthesis without compromising fillet quality in salmon.

In Chapter 3, the impacts of the two extreme ω6:ω3 diets (i.e. high ω6 and high ω3) on Atlantic salmon hepatic transcriptome (using 44K microarrays) were examined. The goal of this study was to identify novel biomarker genes and molecular pathways that are altered by variation in ω6:ω3. This study hypothesized that salmon fed the two diets with the most extreme lipid compositions would show the most extensive transcriptomic differences. My microarray analyses identified genes related to immune and inflammatory response (*lect2a, itgb5, helz2a, p43*), lipid metabolism (*helz2a*), cell proliferation (*htra1b*), control of muscle and neuronal development (*mef2d*), and translation (*eif2a, eif4b1, p43*) that were differentially expressed between the two extreme ω6:ω3 dietary treatments. Most transcripts (i.e. 8 out of 10) showed an agreement in the direction of expression fold-change between the microarray and qPCR studies. I also found that the PPARα activation-related transcript *helz2* is a potential novel molecular biomarker of tissue variation in ω6:ω3, based on dietary-induced transcript expression changes and correlation with tissue (muscle and liver) ω3 and ω6 PUFA. Given the importance of *helz2* as an ancestral vertebrate interferon stimulated gene (Levraud et al., 2019), future studies should investigate the dietary ω6:ω3 impact on Atlantic salmon anti-viral response. Further, correlation analyses illustrated the

relationships between liver transcript expression and tissue (liver, muscle) lipid composition, and other phenotypic traits in salmon fed varying dietary ω6:ω3. This study is also unique in that it identified several lipid-gene correlations that overlapped between the liver and the muscle (i.e. *htra1a, eif2a, mef2d, helz2a,* and *helz2b*, with ω3 and ω6 PUFA). Further research is needed to elucidate the influence of ω6:ω3 on these transcripts, and their regulatory pathways in salmon liver. For example, in this Chapter, *mef2d* was identified by microarray, and confirmed by qPCR to be diet-responsive. However, most previous studies examined the role of *mef2* expression in skeletal (Fuentes et al., 2013; Wei et al., 2016) and cardiac (Lien et al., 2006; Grammes et al., 2012) muscle development, and the interactions between *mef2d* and liver physiology are less understood in fish. Future studies should investigate the influence of dietary ω6:ω3 on liver *mef2d* expression, and their interaction with hepatic stellate cells in fish. My microarray study also identified transcripts that play a role in translation as diet responsive. The expression of *p43* and *eif2a* were positively correlated with liver Σω3, while these transcripts and *eif4b2* showed negative correlations with 18:2ω6 in the liver. This suggested that the molecular regulation of protein synthesis in salmon liver may have been impacted by dietary ω6:ω3. Given these data, future studies should investigate the impact of plant-based diets with varying ω6:ω3 on protein synthesis and their molecular mechanisms in salmonids. Finally, the upregulation of hepatic *htra1b* in this study concurred with the suppression of immune- and inflammatory-related transcripts (i.e. *lect2a, p43, helz2a, helz2b,* and *itgb5*). This supported the idea proposed by previous studies (Tacchi et al., 2012; Caballero-Solares et al., 2018) of a link between the dietary modulation of *htra1* and that of immune-related transcripts. In summary, this Chapter enhanced the current understanding of Atlantic salmon hepatic

transcriptome response to varying dietary ω6:ω3, and relationships with tissue lipid composition and phenotypic traits.

In the fourth Chapter of this thesis fish were fed with diets that varied in EPA+DHA levels (0.3, 1.0, or 1.4%, as formulated) and ω6:ω3 (high ω6, high ω3, or balanced). Diets were designed to be relatively low in EPA+DHA levels (i.e. 0.3% and 1% represented below and near the requirement level, respectively) and above the requirement level for Atlantic salmon (1.4%). After the completion of the feeding trial, head kidney lipid composition and transcript expression of genes involved in fatty acid and eicosanoid metabolism were examined. The hypotheses of this study were that head kidney lipid composition would be reflective of the diet, and that dietary ω6:ω3 and EPA+DHA levels would impact the constitutive transcript expression of genes involved in eicosanoid metabolism and LC-PUFA synthesis. Chapter 4 showed that triacylglycerol (TAG) was the predominant lipid class in all treatments, regardless of diet. This finding was in line with Foroutani et al. (2020) who investigated the lipid class composition in Atlantic salmon head kidney. This suggested that TAG may play an important role in immune and/differentiation processes in Atlantic salmon head kidney. However, as the present study focused solely on lipid metabolism, future research should investigate the link between tissue TAG levels and immune functions in salmon head kidney. Head kidney fatty acid composition was reflective of the diet with respect to C_{18} PUFA and monounsaturated fatty acid (MUFA) levels (% of total fatty acids), and responded to ω6:ω3 variation. This study also revealed that 20:5ω3 proportions at week 12 were similar among 0.3% EPA+DHA with high ω3 diet (0.3%EPA+DHA↑ω3), 1%EPA+DHA (both high ω3 and high ω6 treatments) and 1.4%EPA+DHA/balanced fed fish, although dietary 20:5ω3 varied by 2.5- to 3-fold.

Further, the 0.3% EPA+DHA↑ω6 fed fish had 1.7–2.3-fold more 20:4ω6 in terms of proportion and 1.4–1.8-fold in terms of concentration, when compared with the other treatments. DHA proportions were similar among all treatments. Tissue fatty acid composition changes agreed with positive correlations between head kidney 20:3ω3 and 20:4ω3 and *elovl5a* transcription. This suggested that high dietary 18:3ω3 promoted the synthesis of ω3 LC-PUFA in salmon fed lower dietary EPA+DHA levels (0.3%). Dietary EPA+DHA levels had a positive impact on *elovl5a, fadsd5* and *srebp1* expression, and these transcripts positively correlated with tissue ΣMUFA. This supported the hypothesis presented by previous studies that LC-PUFA synthesis is stimulated by increasing MUFA levels in Atlantic salmon (Emery et al., 2016; Emam et al., 2020). In Chapter 4 I also identified significant correlations between head kidney fatty acid composition and the expression of eicosanoid synthesis-related transcripts (i.e. *5loxa, 5loxb, cox1, cox2, ptges2, ptges3,* and *pgds*). These data shed light on the constitutive relationships among fatty acids and eicosanoid metabolism in salmon head kidney. The mRNA levels of several transcripts involved in eicosanoid synthesis showed positive correlations with tissue ω3 FA (e.g. *5loxb, cox1, ptges3*). Thus, I hypothesized that these transcript expression changes may have increased the production of anti-inflammatory eicosanoids in salmon head kidney. In summary, findings from this study highlighted the importance of dietary 18:2ω6 and 18:3ω3 when salmon are fed with lower inclusion of EPA and DHA (0.3%), and their impact on head kidney LC-PUFA. Given the important role of the head kidney in salmon immune responses and the interaction with dietary LC-PUFA, future studies should elucidate how ω6:ω3 and EPA+DHA levels affect LC-PUFA synthesis (e.g. using stable

isotope and FA mass balance methods) and eicosanoid production [e.g. Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS)] in this organ.

The aim of Chapter 5 was to examine how high ω6 and high ω3 diets with low fish oil (FO) and EPA+DHA levels (i.e. 0.3 and 1.0%, as formulated) impact the antibacterial immune and eicosanoid responses of Atlantic salmon. After the completion of the feeding trial fish were intraperitoneally injected with formalin-killed *Aeromonas salmonicida* (ASAL) bacterin or phosphate-buffered saline (PBS), and the transcript expression of head kidney (24 h post-injection) antibacterial and inflammation-relevant genes (using qPCR) was examined. Earlier studies quantified prostaglandin levels (i.e. PGE_2, PGF3α) in Atlantic salmon organs [e.g. kidney (Bell et al., 1996), midgut (Olsen et al., 2012), liver (Hundal et al., 2021, plasma (Berge et al., 2009), gills (Bell et al., 1996)]. However, this Chapter is unique in utilizing electrospray ionization (ESI)-MS/MS to quantify these prostaglandins in Atlantic salmon plasma. I hypothesized that dietary lipid composition (e.g. EPA, DHA, and ARA) will alter head kidney FA composition, and result in modifications in the expression of immune and eicosanoid metabolism-related transcripts. Most antibacterial-related transcripts showed a significant induction by ASAL in salmon head kidney (*stlr5*, *ccl19a*, *ccl19b*, *saa5*, *il8*, *il1b*, *il10*, *irf1*, *camp*, *haaf*, *hamp*), and the magnitude of induction varied among transcripts encoding chemokines, interleukins, and antimicrobial peptides. An important finding of this Chapter was that transcripts involved in eicosanoid metabolism (e.g. *cox1*, *5loxa*, *5loxb*, *pgds*, *lkha4*) were down-regulated in ASAL when compared with the PBS-injected fish (within diet). These results are in line with previous Atlantic salmon studies (Martin et al., 2006; Stenberg et al., 2019), and could be an attempt to mitigate the proinflammatory response in bacterin-challenged fish. Further,

fish fed 0.3%EPA+DHA↑ω6 diet had lower levels of PGE_2 in ASAL- when compared with PBS-injected fish, and this was concurrent with lower head kidney expression of PGE_2 receptor-encoding transcript (i.e. *ptger4a*). Interestingly, previous studies with insects reported that bacterial pathogens immunosuppressed host eicosanoid synthesis (Hwang et al., 2013; Kim et al., 2018). More studies are needed in order to elucidate the relationship between eicosanoid metabolism and antibacterial response in salmon. The 0.3%EPA+DHA↑ω6 diet resulted in the strongest ASAL fold-change induction of genes encoding chemokines (i.e. *ccl19b*, *il8*), interleukins (*il10*, *il8*), acute phase protein (i.e. *saa5*), antimicrobial peptide (*hamp*), and agglutination-aggregation factor (*haaf*). Chapter 4 indicated that salmon fed 0.3%EPA+DHA↑ω6 diet had the highest head kidney dihomo-γ-linolenic acid (DGLA, 20:3ω6), and ARA, and lowest EPA (measured as proportions and concentrations) when compared to the other dietary treatments. In Chapter 5 I used a two-way ANOVA analysis to show that the responsiveness of antibacterial transcripts to ASAL-injection was predominantly impacted by the interaction of dietary ω6:ω3 ratios with EPA+DHA proportions. Another important result of this Chapter was the positive correlations identified between head kidney ω6 PUFA and the transcript expression of genes involved in immune response and eicosanoid metabolism. These data are in line with previous studies which revealed that high ω6 diets resulted in up-regulation of immune- and inflammation-related transcripts in Senegalese sole (*Solea senegalensis*; Montero et al., 2015) and zebrafish (*Danio rerio*; Nayak et al., 2020). These findings suggest that 0.3%EPA+DHA↑ω6 diet may have enhanced the innate antibacterial immune response of Atlantic salmon. Notably, it is important to recognize that Chapter 5 used formalin-killed ASAL bacterin to evaluate the antibacterial immune response. However, salmon exposed

to pathogenic bacterial infection may show a different immune response to the one observed herein. More research is necessary in order to enhance our understanding of the impact of high ω6 plant-based diets with low EPA+DHA levels on antibacterial immune responses (including live infectious agents such as *A. salmonicida*, *Renibacterium salmoninarum*, *Vibrio anguillarum*, and others) in Atlantic salmon. Further, the influence of diet on antibacterial immune response in other immune-relevant organs such as spleen also warrants investigation. Chapter 5 represented a single time point study (i.e. 24 h post-injection) and provided a snapshot of the antibacterial immune response in Atlantic salmon. As such, future studies should investigate additional time points post-injection to determine expression profiles of key immune genes over time. Finally, as the focus of this study was on the interaction of dietary ω6:ω3 with two EPA+DHA levels (i.e. 0.3 and 1%), future work should evaluate a wider EPA+DHA range and their influence on antibacterial immune responses in fish.

6.2. Conclusions

This thesis revealed that Atlantic salmon tissues (liver, muscle, head kidney) reflected the dietary composition of plant-based feeds with varying ratios of ω6:ω3 (0.4 to 4.5). CSIA indicated that liver EPA and ARA synthesis was largely driven by dietary PUFA precursors even when EPA and DHA were supplied at levels above minimum requirements. High dietary 18:3ω3 also promoted the synthesis of ω3 LC-PUFA in the head kidney of salmon fed lower dietary EPA+DHA levels (0.3%). This resulted in similar EPA proportions among fish fed 0.3% EPA+DHA with high ω3 diet (0.3%EPA+DHA↑ω3), 1%EPA+DHA (both high ω3 and high ω6 treatments) and 1.4%EPA+DHA/balanced fed

fish, although dietary levels varied by 2.5- to 3-fold. Head kidney transcript expression of genes involved in LC-PUFA synthesis (*elovl5a*, *fadsd5* and *srebp1*) was positively impacted by dietary EPA+DHA levels, and these transcripts positively correlated with tissue ΣMUFA. The latter results are in line with previous studies (Emery et al., 2016; Emam et al., 2020) and suggest that future studies should investigate the link between LC-PUFA synthesis and MUFA levels in Atlantic salmon.

This thesis demonstrated that high ω6 and high ω3 plant-based diets with varying ratios of ω6:ω3 (0.4–2.7) resulted in a relatively low number of differentially expressed transcripts in salmon liver. However, the identified transcripts and/or their functional annotations suggested important roles in lipid metabolism, cell proliferation, immune and inflammatory response, control of muscle and neuronal cell development, and translation. The PPARα activation-related transcript *helz2* was identified as a potential novel molecular biomarker of tissue variation in ω6:ω3 fatty acid ratio. Given the importance of *helz2* as an ancestral vertebrate interferon stimulated gene, future studies should investigate the dietary ω6:ω3 impact on Atlantic salmon anti-viral response.

This thesis also enhanced our understanding of the link between head kidney FA composition and the constitutive expression of genes involved in eicosanoid synthesis. Several transcripts were positively correlated with tissue ω3 FA (e.g. *5loxb, cox1, ptges3*) and this may be an attempt to increase the production of anti-inflammatory eicosanoids in the head kidney of unchallenged salmon. In the ASAL bacterin-injected salmon most eicosanoid metabolism-related transcripts were down-regulated when compared with the PBS-treated fish (within diet; 24 h post-injection), and fish fed 0.3%EPA+DHA↑ω6 diet had lower plasma levels of PGE_2 in ASAL- when compared with PBS-injected fish. This

could be an attempt to mitigate the proinflammatory response in bacterin-challenged fish. Finally, this thesis revealed that the responsiveness of head kidney antibacterial transcripts to ASAL-injection was predominantly impacted by the interaction of dietary ω6:ω3 ratios with EPA+DHA proportions. The 0.3%EPA+DHA↑ω6 diet resulted in the strongest ASAL fold-change induction of genes encoding chemokines, interleukins, acute phase protein, antimicrobial peptide, and agglutination-aggregation factor. This diet likely enhanced the innate antibacterial immune response of Atlantic salmon.

6.3. References

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