The Utilisation and Effects of Early Nutritional Programming on Atlantic Salmon (*Salmo salar*. L)

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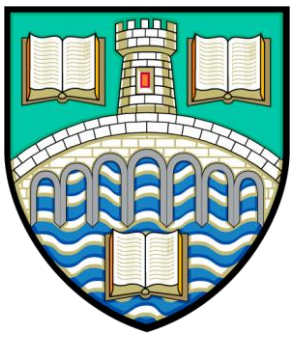

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‘Do not lead a life

overcome by

failure and hardship.’

Declaration

I hereby declare that the results and findings presented in this thesis have been composed entirely by the candidate. The work presented in the thesis, except where specifically acknowledged, has been conducted independently by the candidate. This thesis has not been submitted for any other degree or qualification.

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Abstract

Atlantic salmon (*Salmo salar*) farming has traditionally relied upon the inclusion of omega-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA), especially eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic (DHA; 22:6n-3) acids, supplied from the finite marine ingredients, fish oil and fish meal. However, sustainable aquafeeds, primarily comprised from terrestrial origin sources as alternatives, lack n-3 LC-PUFA, which can potentially result in some adverse effects such as growth or health impairment as well as a decline in the nutritional value. Strategies such as nutritional programming, where fish are subjected to early feeding events that may result in effects in adult fish, aim to improve utilisation of sustainable aquafeeds based on epigenetics as its underlying mechanism.

This thesis investigated the application of nutritional programming in fingerlings at the freshwater phase (Chapter 4, 5). The effects on tissue fatty acid composition as well as LC-PUFA biosynthesis were addressed by first studying the quantitative endogenous production of n-3 LC-PUFA from first feeding in salmon fed marine-free diets with varying levels of linolenic:α-linoleic acid (ALA:LA) ratios. All experimental groups exhibited a significantly lower weight gain than the commercial marine control group. Endogenous n-3 LC-PUFA production was 5.9, 4.4 and 2.8 mg/g of fish when dietary ALA:LA ratios were 3:1, 1:1 and 1:3, respectively. Similarly, the decreasing dietary ALA:LA ratios also resulted in a decline in EPA:arachidonic acid (ARA) and DHA:ARA ratios.

The second study (Chapter 4), further explored the optimal period of a plant-based stimulus and the response to a similar challenge at a later period, in order to achieve an efficient programming. Results provide evidence that a one-week stimulus may be best in improving growth performance and helping salmon adapt better to a plant-based diet (V feed), although different duration periods (1 or 2 week) had little or no effect on the fatty acid composition of both total and polar lipid in several tissues. Molecular analysis

revealed that the one-week stimulus upregulated several LC-PUFA biosynthetic enzymes following the challenge phase, which further supports the belief that a one-week stimulus elicits better performance and adaptation to V feed.

Further research was performed in order to investigate the long-term effect(s) of nutritional programming and challenge (Chapter 5). Applying a similar challenge to fish stimulated by a plant-based diet did not reduce salmon growth but did, however, result in a higher intracytoplasmic lipid vacuolization and lower n-3 LC-PUFA content in liver when compared to salmon fed a commercial diet. Nevertheless, the application of a stimulus elicited n-3 LC-PUFA biosynthesis in liver via the upregulation of the biosynthetic enzymes, indicating that a plant-based challenge could be considered as a booster for the early programming effects. However, further, more obvious, impacts might appear had the challenge phase been prolonged. Overall, results from the present work provide further insights into the refinement and long-term impacts of nutritional programming in order to improve the utilisation of sustainable aquafeeds for Atlantic salmon within the industry.

Key words: Atlantic salmon; endogenous biosynthesis; n-3 LC-PUFA; nutritional programming; stimulus; challenge

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Knowing all of you has been my miracle.

List of Abbreviations

aco	acyl-CoA oxidase
AI	atherogenic index
ALA	α-linolenic acid (18:3n-3)
ANFs	antinutritional factors
ANOVA	analysis of variance
AOAC	Association of Official Analytical Chemists
ARA	arachidonic acid (20:4n-6)
ATP	adenosine triphosphate
β-oxidation	beta-oxidation
BCP	1-bromo-3-chloropropane
BHT	butylated hydroxytoluene
cDNA	complementary DNA
CHA	challenge
C:M	chloroform:methanol
cpt	carnitine palmitoyl transferase
Ct	cycle threshold
DE	digestible energy
DHA	docosahexaenoic acid (22:6n-3)
DHGLA	di-homo-γ-linolenic (20:3n-6)
DPA	docosapentaenoic acid (22:5n-3)
DNA	deoxyribonucleic acid
DNMT	DNA methyltransferase
DP	digestible protein
EB	ethidium bromide
EFA	essential fatty acids
ef1α	elongation factor 1 alpha
elovl	elongases of very long chain fatty acids
EPA	eicosapentaenoic acid (20:5n-3)
fads	fatty acid desaturase
FAO	Food and Agriculture Organization
FAME	fatty acid methyl esters
fas	fatty acid synthase
FM	fishmeal
FO	fish oil

GM	genetic modification
GOI	gene of interest
H&E	haematoxylin and eosin
HK	housekeeping gene
hmgcr	3-hydroxy-3-methyl-glutaryl-CoA reductase
HNF	hepatic nuclear factor
hprt	hypoxanthine-guanine phosphoribosyltransferase
HPTLC	high-performance thin layer chromatography
IMR	Institute of Marine Research
INT	interaction
$KHCO_3$	potassium bicarbonate
KCL	potassium chloride
LA	linoleic acid (18:2n-6)
LC-PUFA	long chain polyunsaturated fatty acid
LIM	lipid inflammatory mediators
LT	leukotrienes
LX	lipoxins
lxr	liver X receptor
miRNA	micro-RNA
mRNA	messenger RNA
MS-222	tricaine methanesulfonate
MUFA	monounsaturated fatty acid
n.s.	not significant
NTC	non-template control
OFN	oxygen-free nitrogen
PAS-AB	periodic acid-Schiff with Alcian blue
PC	phosphatidylcholine
PCA	multivariate Principal Component Analysis
PE	phosphatidylethanolamine
PG	prostaglandins
PGI_2	prostacyclins
PI	peroxidation index
PI*	phosphatidylinositol
PL	phospholipids
ppar	peroxisome proliferators-activated receptor
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid

RNA	ribonucleic acid
rpl2	ribosomal protein L2
RRBS	reduced representation bisulfite sequencing
RT	reverse transcriptase
RT-qPCR	real-time polymerase chain reaction
SD	standard deviation
SFA	saturated fatty acid
SGR	specific growth rate
srebp	sterol regulatory element-binding proteins
STI	stimulus
TAE	tris-acetate-EDTA
TAG	triacylglycerols
TI	thrombogenic index
TL	total lipid
Tm	annealing temperatures
TX	thromboxanes
UoS	University of Stirling
UV	ultraviolet
VO	vegetable oils
v:v	volume per volume
w:v	weight per volume
5hmC	5-hydroxymethylcytosine

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Glossary Index of Common and Scientific Names

Common name (according to FishBase)	**Scientific name**
Antarctic krill	*Euphasia superba*
Arctic char	*Salvelinus alpinus*
Atlantic cod	*Gadus morhua*
Atlantic herring	*Clupea harengus*
Atlantic salmon	*Salmo salar*
Brown trout	*Salmo trutta*
Chinook salmon	*Oncorhynchus tshawytscha*
Cobia	*Rachycentron canadum*
European seabass	*Dicentrarchus labrax*
Gilthead sea bream	*Sparus aurata*
Golden pompano	*Trachinotus ovatus*
Japanese seabass	*Lateolabrax japonicus*
Murray cod	*Maccullochella peelii peelii*
Nile tilapia	*Oreochromis niloticus*
Pikeperch	*Sander lucioperca*
Rabbitfish	*Siganus canaliculatus*
Rainbow trout	*Oncorhynchus mykiss*
Red drum	*Sciaenops ocellatus*
Redfish	*Sebastes marinus*
Senegalese sole	*Solea senegalensis*
Turbot	*Scophthalmus maximus*
Yellowtail	*Seriola quinqueradiata*
Zebrafish	*Danio rerio*

1 CHAPTER

General Introduction & Objectives

1.1 Aquaculture industry

The rapid increase in the worldwide population will be accompanied by demands for greater volumes of seafood, being a valuable segment of the human food system in providing a rich supply of protein, essential amino acids, fatty acids, minerals and vitamins, which play a crucial role in human nutrition. Traditionally, wild fisheries were the main source of seafood. However, due to the high demand for seafood together with ecological issues, climate change and over fishing, a decrease in natural fish stocks is apparent. Therefore, the growing human demand for seafood cannot be satisfied by the wild fisheries alone. Currently, the seafood supply has been transformed into a farming-dependent mode. Indeed, as the wild fishery production stagnated since 1990s, aquaculture production continually increases and has progressively taken the major place of global seafood supply (Figure 1.1). The annual growth rate of aquaculture production from 2000 to 2020 was 5.3 % (FAO, 2022). Over 50 % of the seafood is currently supplied from aquaculture and it is predicted to reach up to 62 % and supply around 110 million tons by the year 2030 (Kobayashi *et al*., 2015). Asia is the major contributor, with almost 90 % of the global production including finfish, crustaceans, invertebrates etc. Among them, countries like China, India and Indonesia are the main producers of inland aquaculture, while Norway, Japan, Canada, Britain and Chile are the main mariculture producers according to their geographical environment (FAO, 2022).

Given the present situation, industrial expansion in a sustainable and practical way to have a continued supply of farmed seafood without compromising the ecosystems or economic detriment is required. To achieve this sustainable intensification of aquaculture, several approaches have been taken such as professional management, appropriate production systems and updated technologies. As a technology-based industry, the rapid growth of aquaculture over the past few decades has been due to breakthroughs in technological barriers including reproduction, larval rearing, disease and immunology, cultivation systems performance as well as nutrition and diet formulation (Browdy *et al*. 2010; Dawood *et al*., 2018; Joffre *et al*., 2017; Yue and Shen, 2022).

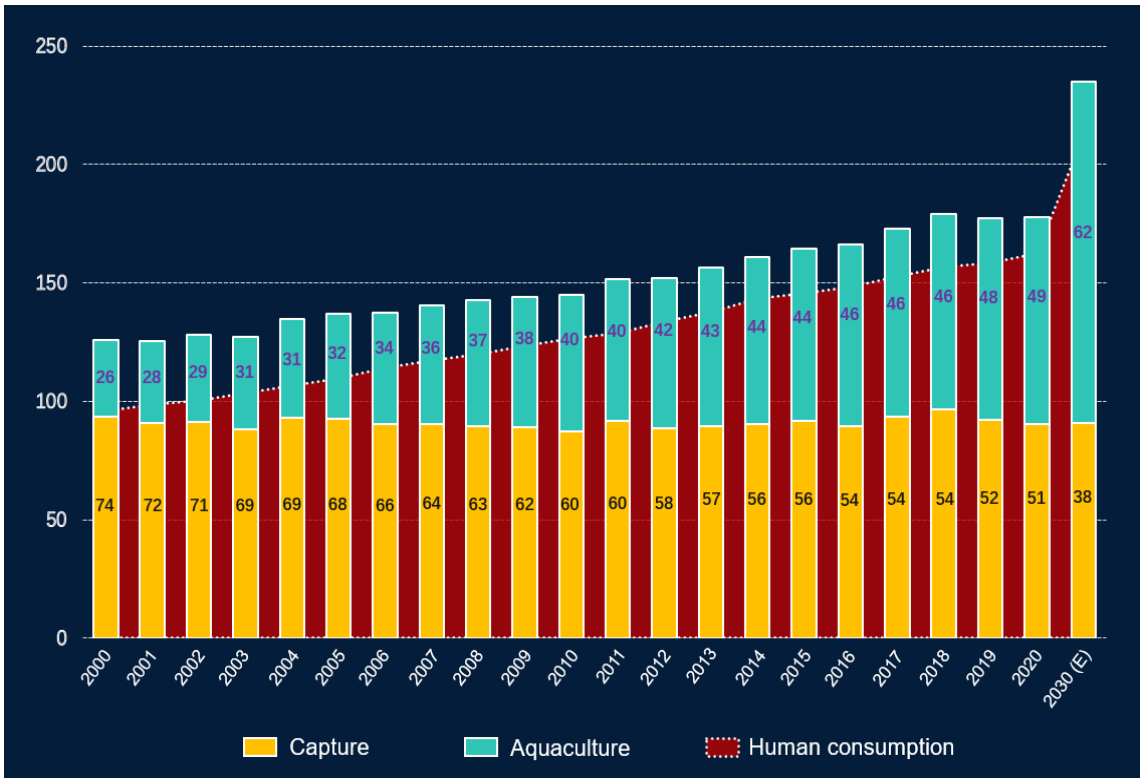


Figure 1.1 Annual global aquaculture production, wild captures and human consumption in million tonnes during 2000-2020. Data on the bar denote the percentage of the total production for each section (FAO, 2022).

Feed is the largest component of the aquaculture budget, accounting for approximately 70 % of the total farm expenses (Dossou *et al*., 2018). Traditionally, the main raw materials used in aquafeed production originated from processed marine ingredients, fish oil (FO) and fish meal (FM), in order to provide the balanced nutrition for carnivorous fish. However, this method of feeding has been argued to be non-sustainable as it results in an input of marine sources exceeding the output of culture production, thereby leading to a net loss, Whereas, this loss is neither environmentally sustainable nor the economically efficient and calls for improvements in various areas such as the use of heat treatment and microbial fermentation in feed manufacturing (Liang *et al*., 2008; Peres *et al*., 2003), including insects, algae, and bacteria as alternative ingredients (Hodar *et al*., 2020), optimally evaluating the use of ingredients for aquaculture feeds (Glencross, 2020)

as well as efficient feeding strategies (Cho and Bureau, 2001). Even though different ingredients are widely used in aquafeeds, the nutritional profile of fish is subsequently altered which can impact both the fish and consumers (Sprague *et al*., 2016).

1.2 Salmon

The Atlantic salmon (*Salmo salar*) is a migratory anadromous teleost belonging to the salmonidae family, which consists of the three main genera: Salmo, such as Atlantic salmon and brown trout (*Salmo trutta*); Oncorhynchus, such as Chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*); and Salvelinus such as Arctic char (*Salvelinus alpinus*). As a cold-water species, they can be found in both the east and west coasts of the North Atlantic Ocean as well as the northern Arctic zones (Klemetsen *et al*., 2003).

1.2.1 Overview

In general, the life cycle of salmon involves spawning in freshwater, a seaward migration where rapid growth and maturation due to the abundance of rich food resources (Klemetsen *et al*., 2003), and adults seasonally returning to streams and rivers to spawn as a cycle. Specifically, Atlantic salmon spawns in the autumn and the eggs after hatching become “alevins or larvae” which continue to utilize the nutrients embedded in yolk sacs. Once the yolk sac is fully absorbed, salmon become “fry” and are capable of feeding exogenously. The “parr” stage is the last freshwater juvenile stage and some salmon may remain at this stage for up to eight years. With the exception of some precocious males, smolt migration occurs in the early summer when they reach around 10-15 cm in body length and experience morphological, physiological and behavioural adaptions (smoltification) to prepare for the smolt stage, which include a slim body shape, dark back, white belly and body salt tolerance (Gibson 1993; Saltveit, 1990; Thorstad *et al*., 2011). Once at sea, the smolt progress through a period of intensive feeding, accumulating fat and gaining weight rapidly, spending on average one to four years at sea in order to

reserve enough energy for the return journey to their natal freshwater streams, around summer time, and reproductively develop for a spawning event in spring. Being iteroparous, Atlantic salmon are capable of surviving the spawning process to return to sea to repeat the process (Figure 1.2). Furthermore, some populations of salmon follow a diverse migration pattern of exclusive large lakes residence, known as "landlocked", spending their entire lives in freshwater. More general information of Atlantic salmon ecology has been reviewed by Aas (2010).

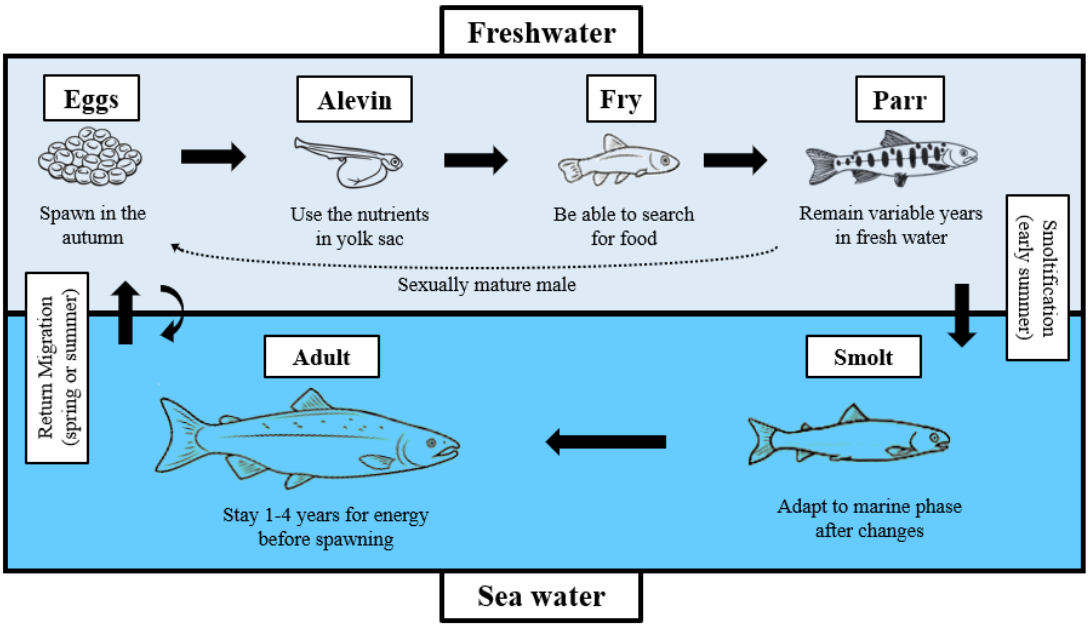


Figure 1.2 Schematic diagram of full life cycle of Atlantic salmon including both freshwater and seawater stage.

The micronutrient content (vitamins A, D, and E), highly digestible protein with essential amino acid and fatty acid content, especially the omega-3 long chain fatty acids (n-3 LC-PUFA), in salmon prove its value as a commercial species. Consequently, salmon aquaculture has made a significant contribution to seafood supply, economic and employment security in many nations, such as Norway, Chile and the United Kingdom (Houston *et al*., 2019). In contrast to Norway and Chile, the Scottish salmon industry brings its distinctive position into play to supply the differentiated products at higher unit

value rather than a standardised product in order to offset potentially higher production costs. From 1980s the salmon aquaculture industry has grown substantially in the past decades with a rapid industry expansion and explosive spread all over the word, accounting for 70% (2.8 million tons) of the market as the consumption is three times higher than 1980s. The successful process of salmon product is owing to its versatile attributes, apart from being sold as fresh, the high yield of salmon can be also sold in many forms such as frozen, readymade or other forms, which gives salmon a broad market appeal. Thus, considerable attention is paid to the different types of aquafeed with specialist formulations and the nutritional requirements to support customized salmon products (Shepherd *et al*., 2017).

1.2.2 Salmon nutrition

Salmon, as a carnivorous fish, is able to satisfy their demand by the diet mainly based on protein and lipid. Although carbohydrates are a low-cost energy substrate, the physiology and metabolism of salmon result in inefficient use of dietary carbohydrate (Villasante *et al*., 2019). Therefore, the input of nutritive carbohydrates is low and the presence of these is generally related to the physical characteristics of the pellets (sinking extruded pellets). Protein is the main component for Atlantic salmon feeds, with requirements for digestible protein estimated to be between 40-54%, depending on the fish size (NRC, 2011). Historically, salmon feeds contained very high levels of fish meal but due to advancements in fish requirements the level of protein ingredients represented by vegetable sources used within feeds stands at 97% (Aas *et al*., 2022). Nevertheless, the use of such ingredients has led to synthetic amino acids being used to supplement deficiencies. A similar trend of substitution, although not as dramatic as with protein, has been observed in fish oil, with vegetable sources representing approximately 66% of the oil composition in salmon aquafeeds (Aas *et al*., 2022). This has resulted in a variation in the flesh fatty acid profile of farmed salmon being reported with a reduced content of the health beneficial n-3 LC-PUFA, having potential implications for both the fish and the end consumer (Sprague *et al*., 2016).

1.3 Lipid nutrition

Lipids are a diverse group of compounds that can be classified by different criteria such as function, solubility and structure. Currently, they have been divided into eight categories: fatty acids, glycerolipids, phospholipids (PL), sphingolipids, saccharolipids, polyketides, sterol lipids and prenol lipids, among which the first four are commonly discussed in the present thesis. Through different ways, lipids are the source of metabolic energy, components of cell membranes, precursors of essential compounds and carriers of some nutrients, such as the lipid-soluble vitamins A, D, E and K. So far, lipid nutrition in fish, especially with the irreplaceable role has attracted great interest and has been extensively researched for deeper insight (Glencross *et al*., 2014; Tocher, 2003; 2010).

1.3.1 Phospholipid and triacylglycerol

Lipids can be further divided into polar and neutral classes based on their polarity (Figure 1.3). Polar lipids are mainly phosphoglycerides including phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI*). They are formed by choline, ethanolamine, serine, and inositol, respectively, being esterified to the common glycerol backbone with two esterified fatty acids and a phosphate group. Being abundant components in biological membranes, especially PC and PE, indicates their important contribution to the regulation of the membrane structure, fluidity and signal transduction in fish (Tocher, 2003).

Triacylglycerols (TAG) are the predominant neutral lipid which are characterized by three free fatty acids esterified to the glycerol backbone. Owing to the preferential binding of saturated fatty acids, which are primarily used as energy source due to their high caloric content (Henderson, 1996), TAG is used for energy production or stored within body tissues for use at a later date.

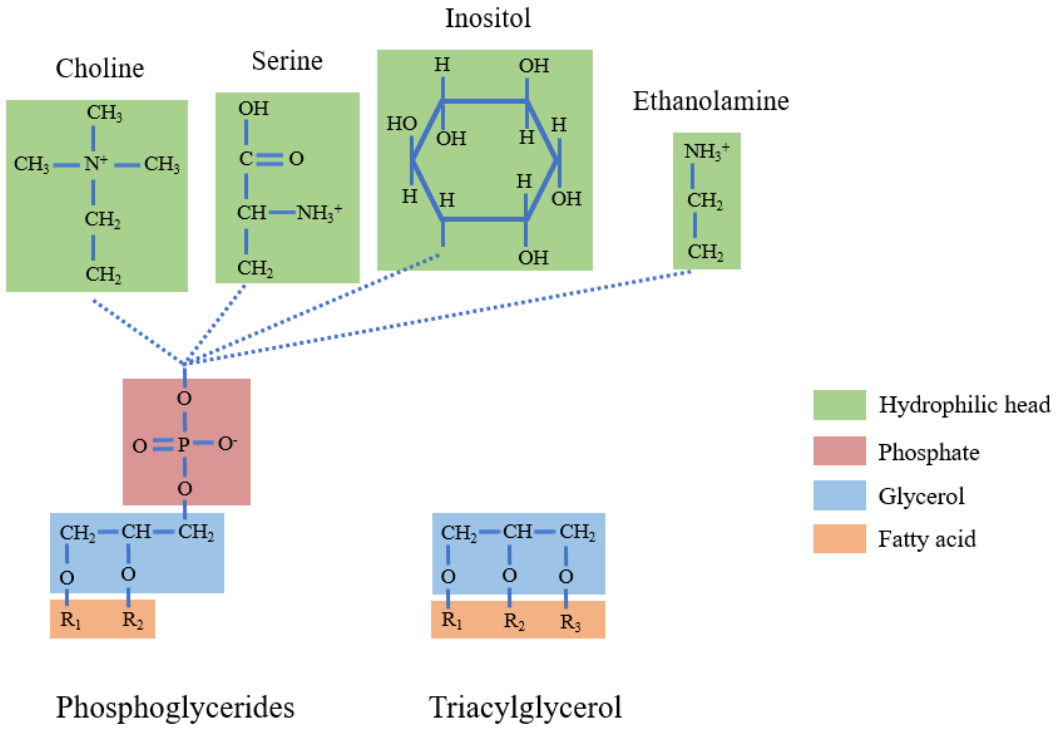


Figure 1.3 The structures of phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI*), phosphatidylethanolamine (PE), and triacylglycerol.

1.3.2 General fatty acids

Fatty acids are the basic building blocks of complex lipids. They consist of a carbon chain with an even number of carbon atoms, a carboxyl group and a methyl group at the end of both sides. Fatty acids have different saturation levels, which are defined by the number of carbon double bonds. They can be classified as saturated fatty acid (SFA, zero double bonds), monounsaturated fatty acid (MUFA, one double bonds) and polyunsaturated fatty acid (PUFA, more than one double bonds). Furthermore, the position of the closest double bonds to the methyl end indicates its series. So, the nomenclature of fatty acids including three features (chain length, saturation level, series) can be expressed as below (Figure 1.4). In addition, the series of PUFA with a carbon chain longer than twenty (twenty included) and more than three double bonds (three included) is also named as long chain PUFA (LC-PUFA) (Sargent *et al.*, 2002). Usually, fatty acids have a carbon chain containing 12-22 (more often 16-20) carbon atoms, they normally present as the even number due to the two-carbon unit synthesis. However, small amount of odd-chain fatty

acids is still detected in human tissues, plants or microbes.

1.3.2.1 Energy Production

Being that there is no dietary requirement for carbohydrate in fish (Wilson, 1994), and with a high calorific content (38.5 kJ/g) lipids, especially fatty acids, are the most favoured nutrient for energy intake and storage in fish. Metabolic energy is generated by mitochondrial beta-oxidation (β-oxidation) of fatty acids through the form of adenosine triphosphate (ATP) (Sargent *et al*., 2002). In fish, the SFA's (primarily 16:0) and MUFA's (mainly 18:1n-9, 20:1n-9, and 22:1n-11) are generally selectively catabolized. This is believed to be due to the high consumption of these fatty acids when growth occurs, with their high level in the feed reflecting the saying "increased dietary concentrations result in increased oxidation" (Stubhaug *et al*., 2007; Tocher, 2015). Although the n-3 LC-PUFA series is less catabolized than SFA and MUFA, this selective catabolism occurs more with eicosapentaenoic acid (EPA; 20:5n-3) than docosahexaenoic acid (DHA; 22:6n-3). The retention of n-3 LC-PUFA as a sparing effect implies that they are essential to the organism rather than as an energy source. Besides, the position of the first carboxyl-side double bond in DHA requires an unusual oxidation process which is well established in peroxisome (Balakrishnan, *et al*., 2021; Madsen *et al*., 1998), so DHA is not considered as the ideal substrate for mitochondrial β-oxidation to produce energy. This energy supply mode where DHA is relatively stored while certain FA such as SFA and MUFA are oxidized exists not only for body growth and heat production but also for reproduction, swimming, gonad development or egg production (Dos Santos *et al*., 2016; Gladyshev *et al*., 2018; Luo *et al*., 2015). For instance, MUFA and EPA will be selectively catabolized to produce energy for gonadogenesis so that the DHA can be transferred to the eggs and stored in a high proportion for early development. For the yolk sac and early larvae stage, DHA and EPA are utilized for energy in turn before being retained for the future larval development (Tocher, 2003).

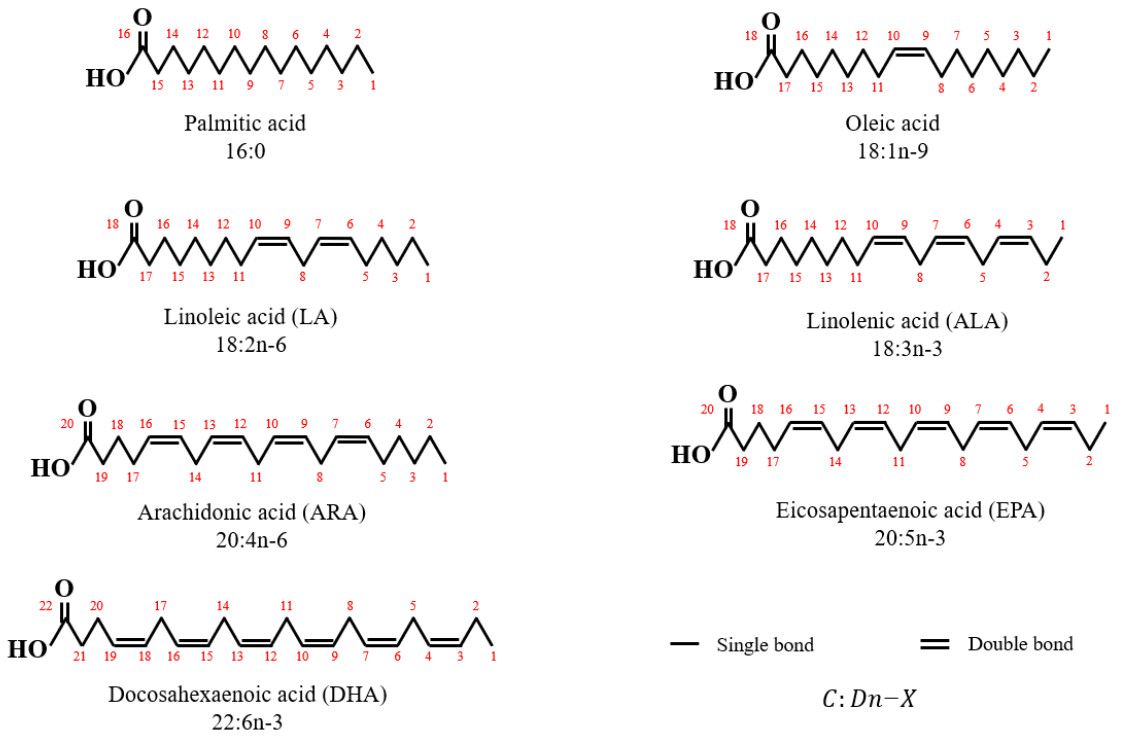


Figure 1.4 The nomenclature and structural formula of some fatty acids. Where *C* is the carbon chain length, *D* is the double bonds amount, *X* is the first double bonds position. Numbers in red are the carbon atom positions.

1.3.2.2 Cellular membrane structure

Apart from providing energy, fatty acids play a vital role in the cell membrane structure because of their being an integral part of phosphoglycerides, which is the fundamental component of the lipid bilayer in the cell membrane (Gylfason *et al*. 2010). Therefore, the variation in fatty acid composition affects membrane functions. Generally, EPA and DHA are the major fatty acids of the phosphoglycerides from fish neural tissues (Tocher and Harvie, 1988). The gross change of fatty acid composition or the redistribution both impact the homeostatic adjustments (Farkas, *et al*., 2001), such as a lower temperature induced restructuring of phosphoglycerides, which increase PUFA and MUFA proportion while reducing SFA content to maintain the fluidity of the membrane (Liu *et al*., 2020; Tocher, 2003). In particular, DHA is notable for its special importance in membrane function. Unlike other fatty acid conformations in conventional bilayer, the six methylene-interrupted double bonds in its structure provides flexibility and strength for

its unique hexagonal conformation (Gawrisch *et al*., 2003), enabling DHA to remain stable to external environmental changes including temperature and diet (Jobling and Bendiksen, 2003), which explains the preferential conservation of DHA in the body.

The significance of DHA is truly reflected by its high content in some neural and visual related tissues, especially at the larval stages during their normal development of functions (Tocher *et al*., 2019). In the brain and eye cell membranes, there is an abundance of PE with 40 % of it consisting of DHA. Previous research demonstrated that dietary deficiency of DHA resulted in inferior schooling skills and behavioural developments such as sound-stimulated escape response in turbot (*Scophthalmus maximus*), yellowtail (*Seriola quinqueradiata*) and sea bream (*Sparus aurata*) (Benítez-Santana *et al*., 2014; Castell *et al*., 1994; Ishizaki *et al*., 2001; Masuda *et al*., 1998). Furthermore, the absence of DHA in Atlantic herring (*Clupea harengus*) larvae retinal cells led to a reduced capture ability under low light (Bell *et al*., 1995), and a delayed appearance of response to visual stimulus which consequently impaired feed intake in larval gilthead sea bream (*Sparus aurata* L.) (Benítez-Santana *et al*., 2007). Undoubtedly, the normal function of brain and visual tissues cannot be maintained without the sufficient supply of DHA (Sissener *et al*., 2016).

1.3.2.3 Eicosanoids

The term “eicosanoid” is the collective name for a group of biologically active compounds derived from C20 PUFA that play a role in immunity as they are required to cope with inflammation, pathology and stressful situations (Arts and Kohler, 2009; Bell *et al*., 1996). Eicosanoids are generated from two types of enzymes: cyclooxygenase (produce prostaglandins, PG; prostacyclins, PGI2; thromboxanes, TX) and lipoxygenases (produce leukotrienes, LT; lipoxins, LX) which function widely in blood clotting, immune response, inflammatory response, cardiovascular tone, renal function, neural function, and reproduction in humans (Schmitz and Ecker 2008).

One of the important functions of n-3 and n-6 PUFA is to provide precursors for eicosanoid production. Eicosanoids are primarily derived from three fatty acids: Arachidonic acid (20:4n-6; ARA); di-homo-γ-linolenic (20:3n-6; DHGLA) and EPA. However, compared to EPA, ARA is the preferred precursor as eicosanoids formed from ARA have a higher activity than those formed from EPA (Tocher, 2003). Whereas, the competition between ARA and EPA occurs at several stages in the metabolism through the same enzymatic pathway, both fatty acids can be metabolized via the same enzymes in the eicosanoid production pathway (Norambuena *et al*., 2015). Moreover, in addition to ARA and EPA, DHA can also affect competition as well in humans (Tapieo *et al*., 2002). Therefore, the production of eicosanoids depends on not only the absolute amount but also the ratio between ARA and EPA. It has been reported that high levels of EPA impacted the inflammatory response by decreasing the secretion of high activity pro-inflammatory eicosanoids (Holen *et al*., 2015), and the genes related to eicosanoid production were highly expressed in pikeperch larvae (*Sander lucioperca*) fed a low DHA diet (El Kertaoui *et al*., 2021). Both reports further suggested that DHA, EPA and ARA participate in the inflammatory response by regulation of eicosanoids production. The balance between these key fatty acids is believed to be crucial within the human body as an excessive eicosanoid production can be pathological, potentially resulting in cardiovascular diseases, inflammatory responses and even cancer (Okuyama *et al*., 1996).

1.3.2.4 Essential fatty acid and n-3 LC-PUFA

All vertebrates require an adequate supply of n-3 and n-6 PUFA for growth, reproduction and physiological processes. Insufficient dietary amounts will interrupt body functions, result in various pathologies and can lead to death. As linoleic acid (18:2n-6, LA) and α-linolenic acid (18:3n-3, ALA) cannot be produced endogenously, they are termed as "essential fatty acids (EFA)" meaning that they are required in the diet (Glencross, 2009; Sargent *et al*., 1999). Which fatty acid is essential varies according to the biosynthetic ability of the species. For some vertebrates, capable of synthesizing C20 and C22 from C18 precursors *de novo*, LA and ALA are generally believed as EFA. For those with a

limited biosynthetic capacity, ARA, EPA and DHA are also considered as EFA.

Similar to other fatty acids, EFA also serve as an energy source and are constituents of phosphoglycerides. Their importance is underpinned by being regulators of metabolism and LC-PUFA biosynthesis. Among all the EFA, the n-3 LC-PUFA series has been the most widely researched, not only in terms of fish nutrition but also its nutritional and health promoting value for humans (Glencross *et al*., 2014; Sprague *et al*., 2016; Tocher, 2010). Indeed, it is reported that n-3 LC-PUFA shows a protective effect in reducing the risk of cardiovascular disease by a possible reduction in serum TAG and very low-density lipoprotein (Adkins and Kelley, 2010; Das, 2000). For humans, being deficient in n-3 LC-PUFA will more likely lead to suffering from cognitive and visual impairment when the demand is not fulfilled such as Alzheimer's disease (Conquer *et al*., 2000; Morris *et al*., 2003). In fact, n-3 LC-PUFA acts as cellular mediators and can improve neural health, neurogenesis, and neural function resulting in the reduction or elimination of inflammation (Calder and Yaqoob, 2009; Devassy *et al*., 2016). Those benefits are reported to be more vital for infant neurodevelopment (Lapillonne and Jensen, 2009). It has also been reported that n-3 LC-PUFA contribute to cancer chemoprevention, and are able to inhibit pro-inflammatory agents, tumour formation and progression via multiple mechanisms such as the inhibition of ARA and its derivative eicosanoids (Gu *et al*., 2015). In addition, n-3 LC-PUFA improve cancer treatment outcomes, chemotherapy tolerance and prevent recurrence (Hardman, 2004; Morland *et al*., 2016). Paradoxically, as eicosanoids also play a critical role on the immune response, the eicosanoid content should be balanced between deficiency and excessive production. Once more, this suggests that both the absolute content and ratio of n-3 and n-6 matters. Unfortunately, the modern diet, particularly in westernized nations, has an extreme disparity with an n-3:n-6 ratio up to 1:15 (Simopoulos, 1998), far away from the recommended ratio of 1:4 (Wijendran and Hayes, 2004). Although the previous large-scale research indicated that the global n-3 LC-PUFA consumption varies among the regions and nations, the general supply of n-3 LC-PUFA was far insufficient to meet the demand from human health (Naylor *et al*., 2009; Micha *et al*., 2014; Salem and Eggersdorfer, 2015). To rebalance the

ratio of dietary n-3:n-6, including higher n-3 LC-PUFA value seafood is seems to be an effective strategy, and salmon being a unique and rich source of n-3 LC-PUFA are the ideal choice (Sprague *et al*., 2017a; 2020).

1.3.3 Fatty acid biosynthesis and catabolism

1.3.3.1 Biosynthesis

There are two series of enzymes involved in fatty acid biosynthesis, fatty acid desaturase (*FADS*) and elongases of very long chain fatty acids (*ELOVL)*. Most investigations have drawn a conclusion that the fatty acid biosynthetic capacity is notably related to the *fads* and *elovl* genes. The biosynthetic pathways of polyunsaturated fatty acids are shown in Figure 1.5 and the genes involved are briefly introduced in Table 1.1. In the present thesis, it should be mentioned that uppercase abbreviations referred to the general protein or enzyme category, while lowercase abbreviations referred to genes.

Fatty acid desaturases

FADS enzymes desaturate single bonds to form double bonds on the certain position counting from the carboxyl-terminus of the fatty acid carbon chain. They are termed "front-end" desaturases, but can also be termed according to the position where the double bond is added. For instance, the desaturases introducing double bond on the 4^{th}, 5^{th} and 6^{th} carbon atom from carboxy side are named as Δ4, Δ5, Δ6 desaturase, respectively. Most teleosts only have a single type of *FADS2* (predominantly encoding Δ6 desaturases), in contrast to other mammals which possess two types *FADS1* (mainly encoding Δ5 desaturases) and *FADS2* (Guillou *et al*. 2010). However, in Atlantic salmon there are four diverse *fads2* genes including three Δ6-like (Δ6_a, Δ6_b and Δ6_c) and one Δ5 desaturase (Monroig *et al*., 2010a; 2011a), which enable salmon to conduct all the desaturation reactions. Importantly, Δ4 desaturase has been described in rabbitfish (*Siganus canaliculatus*) (Li *et al*., 2010), which suggests that Δ4 desaturation is widely spread and takes place during biosynthesis to provide an alternative pathway to produce DHA from EPA directly by Δ4 desaturase. Studies in Senegalese sole (*Solea senegalensis*) confirmed

Δ4 activity and its potential to effectively utilise more sustainable diets (Morais *et al*., 2012). Furthermore, Δ8 desaturation activity has been explored in teleost (Monroig *et al*., 2011b), so the "Δ8 pathway" seems to be an alternative route to the conventional "Δ6 pathway" (Castro *et al*., 2016), via 20:3n-3 as an intermediate.

Elongases of very long chain fatty acids

For the C18 to C24 PUFA, *elovl* enzymes are responsible of adding two carbon units to the carboxyl-terminus of the fatty acid chain, which catalyzes the rate-limiting step. Vertebrates normally have seven members in the *elovl* family (*elovl 1-7*), although only *elovl*2, *4* and *5* are involved in PUFA biosynthesis (Jakobsson *et al*., 2006). The most common in all the teleosts is *elovl5* which mainly conducts the elongation of C18 and C20 as well as C22, albeit at a very low efficiency. In contrast, *elovl2* exhibits more favourable activity towards C20 and C22 substrates rather than C18 (Castro *et al*., 2016; Morais *et al*., 2009). It is notable that *elovl5* can go through the Δ6 pathway or, alternatively, elongate both LA and ALA to 20:2n-6 and 20:3n-3 followed through the Δ8 pathway, which to some extent implies evidence of a Δ8 pathway. Apart from *elovl2* and *5*, *elovl4* is believed to elongate PUFA longer than C20, such as DHA via docosapentaenoic acid (DPA) to 24:5n-3 which shares the similar function with *elovl2*. Therefore, the hypothesis of *elovl4* playing a role in DHA biosynthesis has been investigated and confirmed in both cobia (*Rachycentron canadum*) and zebrafish (*Danio rerio*) (Monroig *et al*., 2010b; 2011c).

Synthesis of saturated fatty acid

All living organisms, including fish, are able to endogenously biosynthesis fatty acids *de novo*. In the conventional pathway, acetyl-CoA is the essential molecule from mitochondria obtained through the oxidative decarboxylation of carbohydrate-originated pyruvate or the oxidative degradation of protein-originated amino acids. Acting as the carbon source, two-carbon acetyl-CoA is carboxylated into malonyl-CoA. This activated acetyl-CoA is assembled in the cytosol by fatty acid synthase (FAS). By the action of FAS,

acetyl-CoA and other malonyl-CoA experience a series of condensation, reduction and dehydration. Next, the acetyl-CoA chain is gradually elongated, after seven or eight repetitions of these process the saturated palmitic acid (16:0) and stearic acid (18:0) are produced. Also, the further elongation can be achieved as well after more repetitions.

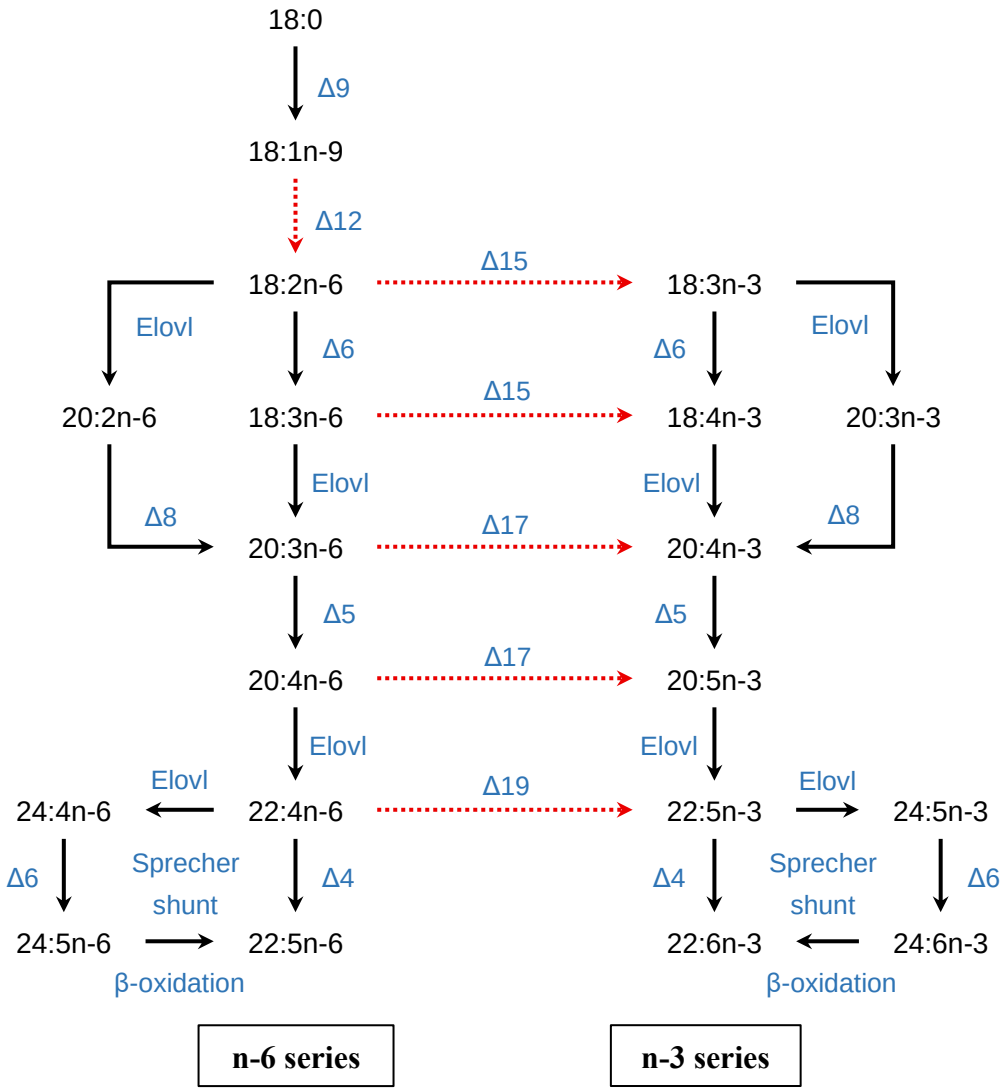


Figure 1.5 Biosynthetic pathways of polyunsaturated fatty acids. Elovl: elongase; Δ: desaturase; Red arrows denote the inaccessible paths in mammals.

Synthesis of monounsaturated fatty acid

Similar to all organisms, fish are also capable of desaturating endogenously synthesized 16:0 and 18:0 into 16:1n-7 (palmitoleic acid) and 18:1n-9 (oleic acid), respectively, by

microsomal Δ9 desaturase. Longer chain fatty acids, such as 18:1n-7, 20:1n-9 or 22:1n-9, can be produced by *elovl*. However, unlike plants and algae, in vertebrates, MUFA cannot be further desaturated into PUFA as they lack the necessary Δ12 and Δ15 desaturase to introduce double bonds beyond the position for Δ9 desaturase (Tocher *et al*., 1998). Therefore, it is impossible to endogenously synthesize n-3 and n-6 from 18:1n-9 and they have to be obtained from diet, which means that LA and ALA are EFA as mentioned before.

Synthesis of polyunsaturated fatty acid

Once LA and ALA are ingested from the diet, they can be further elongated and saturated for LC-PUFA. Both FA share the same pathway: for instance, at the first stage ALA is saturated to 18:4n-3 by Δ6 FADS then elongated to 20:4n-3, followed by Δ5 desaturation to become EPA. At the second stage, the EPA is elongated twice and desaturated by Δ6 FADS to 24:6n-3, then DHA is obtained after β-oxidation in peroxisomes via the "Sprecher shunt" (Sprecher, 2000). The pathway for 22:5n-6 synthesis from 18:2n-6 is exactly the same as DHA.

However, there are some aspects to consider according to the pathway. At the first stage, as aforementioned in some fish like Atlantic cod (*Gadus morhua*) and gilthead sea bream, ALA can be subjected to the Δ8 pathway and 20:3n-3 might not be the product, but can, alternatively, be desaturated to 20:4n-3 (Monroig, *et al*., 2011b). At the second stage, previous research indicated that DHA can be synthesized by the direct Δ4 desaturation from 22:5n-3 (Li *et al*., 2010; Morais *et al*., 2012). In addition, since both the n-3 and n-6 series ultilize the same set of enzymes, competition for these enzymes exists. It has been reported that the affinities of the desaturases and elongases to the fatty acids are different, being higher for n-3 than n-6 or n-9. Therefore, it can be concluded that the enzymes have a higher affinity towards ALA, in turn producing more DHA as the end product. On the contrary, as a lower priority for enzymes, LA generates less product, namely ARA. To some extent, ARA can be considered as the end product of LA pathway. In fact, further

desaturation or elongation steps will gradually decelerate with 22:5n-6 is hardly accumulated.

The EFA demands of freshwater fish have been proven to be met by LA and ALA, whereas marine fish require n-3 LC-PUFA since they are uncapable of converting LA and ALA to ARA, EPA and DHA (Tocher, 2003; Sargent *et al*., 2002). Some further *in vitro* trials have been conducted on turbot, salmon, sea bream and trout to explore why DHA is not produced as expected. It was revealed that, among the pathway, the activities of either *elovl* extending C18 to C20 or Δ5 *Fads* desaturating 20:3n-6 and 20:4n-3 limits the synthesis process (Ghioni *et al*., 1999; Tocher and Ghioni, 1999). Furthermore, the low enzymatic activity is in agreement with the poor gene expression of both desaturases and elongases. It has been speculated that the reason for a poor LC-PUFA biosynthesis is related to the fact that marine species remain in an EPA and DHA-abundant environment, where n-3 LC-PUFA demand can be easily satisfied from the natural food web. Therefore, their biosynthetic capacity turns out to be unnecessary, which leads to inactivity (Tocher, 2003). Taking all this into account, switching on or boosting the required genes to produce LC-PUFA at sufficient levels might be the solution.

1.3.3.2 Catabolism

As described above, fatty acids are an important energy provider through fatty acid catabolism. In mammals, the catabolism can occur in two different organelles, the mitochondrial matrix and peroxisomes, in which β-oxidation is performed to produce energy (Figure 1.6). Although both organelles share several enzymes involved in the whole pathway, there are still significant differences that need to be mentioned. Genes involved are briefly introduced in Table 1.1.

Mitochondrion

Fatty acids are transformed into fatty acyl-CoA in the cytosol before the reaction. Translocation of fatty acyl-CoA into mitochondria is initiated by being converted to acyl-

carnitine using carnitine palmitoyl transferase-1 (CPT1) at the outside membrane and then delivered into the mitochondria followed by its resetting back to the acyl-CoA via carnitine palmitoyl transferase-2 (CPT2). Afterwards, the acyl-CoA is β-oxidized. In brief, acyl-CoA is subjected to two rounds of dehydrogenation and one round of hydration, then two carbon atoms are cleaved accompanied by the production of ATP as energy. The shortened acyl-CoA will repeat β-oxidation for several rounds to split two carbon atoms sequentially and produce ATP via the tricarboxylic cycle every time. The cleavage of the bond in each cycle always occurs on the second carbon (also called the β carbon), hence why it is termed β-oxidation.

It is worthy to note that CPT1 is found in skeletal muscle and liver and is able to use a diverse range of fatty acids including some LC-PUFA as well as fatty acids shorter than 20 carbons. Due to its key role for the acyl-CoA transportation in the reaction, CPT1 is regarded as the rate-limiting enzyme for mitochondrial β-oxidation (Harpaz, 2005). The oxidation ratio is different according to the fatty acid substrate, but typically following the order as SFA/MUFA > PUFA > LC-PUFA, shorter chain > longer chain and n-6 > n-3 (Tocher and Glencross, 2015). The order partially reflects the fact that PUFA is not generally an ideal energy substrate and that LC-PUFA is preferred to be retained, especially those belonging to the n-3 series. Another factor that can impact the ratio is malonyl-CoA, which regulates the first step of fatty acid synthesis. Malonyl-CoA is a potent inhibitor of the CPT1 and acyl-CoA reaction, so the entry of fatty acids into mitochondria and further β-oxidation ratio is controlled by malonyl-CoA leading to the balance between synthesis and oxidation (Bremer, 1997; Frøyland *et al*., 1998).

Peroxisome

Like mitochondria, peroxisome is another site of β-oxidation. Since not all fatty acids can be thoroughly oxidized in mitochondria, some very long chain fatty acids, normally more than 20 carbons, are specifically stepwise shortened to certain lengths before they can be transported into the mitochondria for full oxidation to occur (Wanders *et al*., 2016).

The enzymes involved between peroxisome and mitochondria β-oxidation are different even though they have the same machinery. Briefly, from the fatty acid perspective these differences can be summarized as: 1) Translocation is not CPT1-dependant but in the form of acyl-CoA or free fatty acid; 2) Acyl-CoA oxidase (ACO) catalyzes the first reaction in peroxisomal β-oxidation and produces hydrogen peroxide (H_2O_2), so it is regarded as the rate-limiting enzyme (Poirier *et al*., 2006); 3) Peroxisomes are only responsible for the shortening of fatty acids but cannot completely oxidize fatty acids like mitochondria; 4) Some fatty acids cannot be oxidised in mitochondria (Hiltunen *et al*., 1986). For instance, DHA requires local specific enzymes after the first step of dehydrogenation to complete the β carbon cleavage on its Δ4 double bond, which occurs in peroxisomes. Thus, DHA is a poor substrate for mitochondrial oxidation and is better stored in tissues.

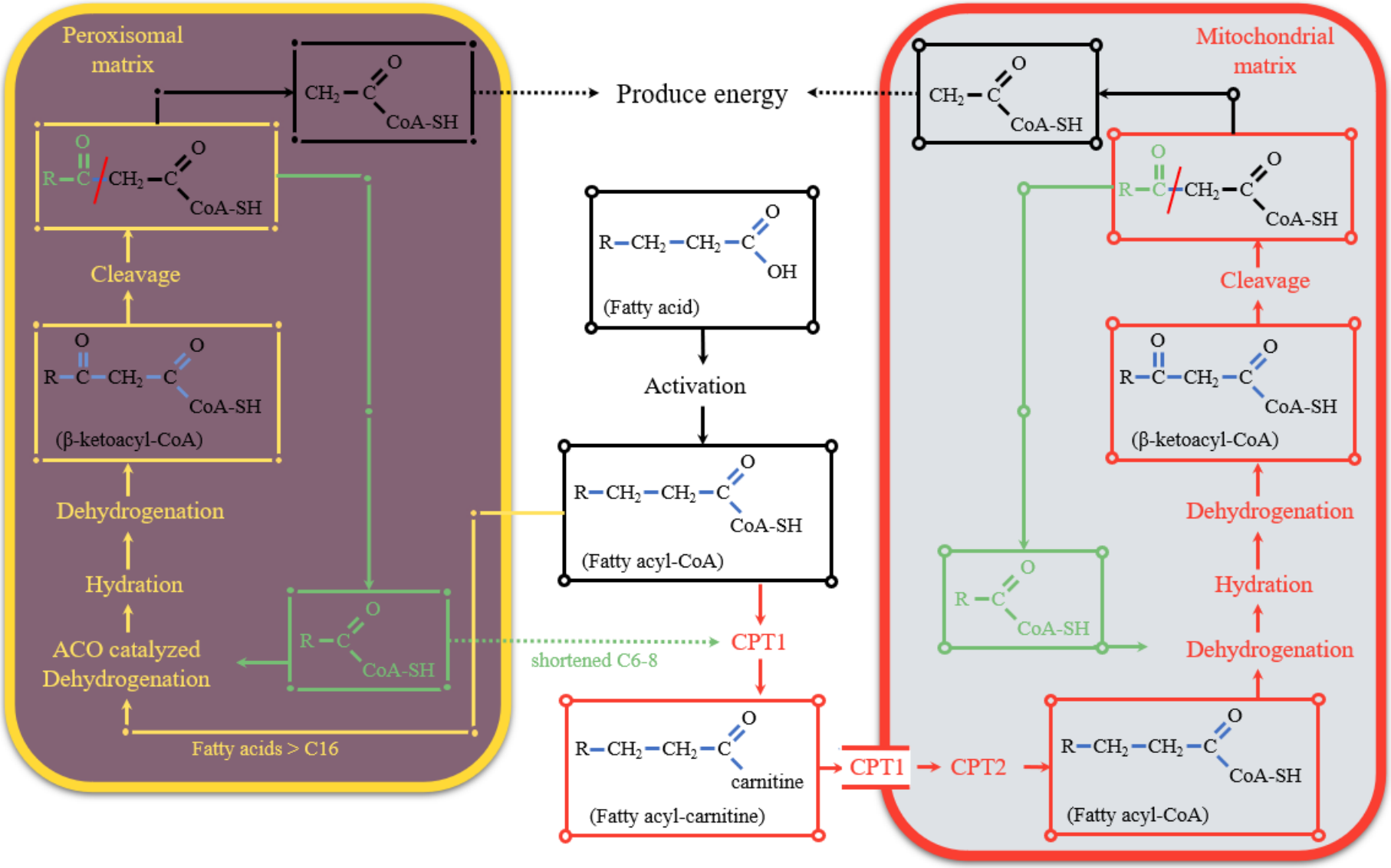


Figure 1.6 Fatty acid beta-oxidation process in mitochondria (right) and peroxisomes (left).

1.3.3.3 Transcriptional regulation

It has been extensively reviewed that dietary PUFA have various impacts on the hepatic transcriptional regulation (Guillou *et al*., 2008), represented by the modification of the hepatic transcription factors from dietary fatty acids to regulate the lipid and fatty acid homeostasis as the feedback (Jump, 2002; 2004). Most research has focused on hepatic regulation, but PUFA is demonstrated to transcriptionally regulate in other tissues such as adipose tissue (Jones *et al*., 1996). There are few transcription factors shown to be modulated by PUFA including: peroxisome proliferators-activated receptor (PPAR); liver X receptor (LXR); hepatic nuclear factor (HNF) and sterol regulatory element-binding proteins (SREBP). Understanding the mechanisms by which PUFA interacts with transcription factors could help us understand the way to boost fatty acid production in fish. The respective genes involved are briefly introduced in Table 1.1.

Peroxisome proliferators-activated receptor

The first identified transcription factor in rodents, PPAR, is a ligand-activated transcription factor and belongs to the superfamily of the nuclear hormone receptors (Issemann and Green, 1990). The ligands include fatty acids, peroxisome proliferators and eicosanoids, among others (Kersten and Wahli, 2000). PPAR is able of activating the expression of target genes containing PPAR responsive elements in the promoter. Most target genes of PPAR are implicated in various aspects such as lipid metabolism and energy homeostasis which suggests the importance of PPAR as a critical regulator in vertebrate physiology (Kersten *et al*., 2000; Michalik *et al*., 2003).

There are three isoforms of PPAR (α, β and γ). PPARα is the predominant subtype in mammal liver and is believed to control the genes related to: LC-PUFA biosynthesis by regulating long chain fatty acid acyl-CoA synthetase (Schoonjans *et al*., 1995), desaturation by activating Δ6 desaturase gene expression (Bond *et al*., 2016), intake and transportation through stimulating the fatty acid binding protein expression (Juge-Aubry *et al*., 1997) as well as β-oxidation by controlling target genes *cpt1* and *aco* (Desvergne

and Wahli, 1999; Ferré, 2004). In mammals, *pparβ* has a broad distribution and expression pattern and it is documented to be related to preadipocyte proliferation with the cooperation of PPARγ or lipid absorption (Grimaldi, 2001; Luquet *et al*., 2005; Poirier *et al*., 2001). So far, little is known about the regulation of the PPARβ gene, with further studies required for improving our understanding. Janani and Kumari (2015) extensively reviewed about *pparγ*, which is mainly expressed in adipose tissue. As its first striking feature, *pparγ* controls the expression of genes required for the differentiation of adipose tissue by adipocyte conversion (Rosen and Spiegelman, 2001). Furthermore, activated PPARγ stimulates TAG accumulation to maintain mature adipocytes, by promoting the storage of fatty acids and the production of glycerol to form TAG (Lehrke and Lazar, 2005). Therefore, the inadequate expression of *pparγ* results in adipocyte dysfunction. Similarly, PPARs has also attracted attention within the fish research community (Leaver *et al*., 2008a). Previous research has reported that PPARα and β have similar expression in profiles compared to mammals, while *pparγ* is more widely expressed in fish visceral adipose tissues indicating its involvement in fat accumulation (Cruz-Garcia *et al*., 2009; Leaver *et al*., 2005). Fish *pparα* is activated by unsaturated fatty acid, and the high level of expression of *pparα* in liver of trout and sea bream is related to the high β-oxidation capacity. In this sense, PPAR genes are elucidated to be nutritional status related and tissue-specific (Kolditz *et al*., 2008; Leaver *et al*., 2005).

Sterol regulatory element-binding proteins

SREBP is a family of membrane-bound transcription factors synthesized as membrane proteins in the endoplasmic reticulum and regarded as a global regulator (Sato, 2010). The SREBP family consists of three isoforms: SREBP-1a, SREBP-1c and SREBP-2. Among them, SREBP-1a and SREBP-1c predominantly activate gene transcription related to fatty acid and triglyceride synthesis and SREBP-2 is generally considered to be in charge of the regulation of cholesterol metabolism (DeBose-Boyd and Ye, 2018). When mammals have overnutrition, SREBP-1c is activated based on triglyceride storage and its target genes will be regulated. In contrast, undernutrition will suppress its expression

(Shimano, 2009). Activated SREBP-1c in rodent has been shown to stimulate the transcription of target genes involved in PUFA elongation via the expression of *elovl5* (Moon *et al*., 2009; Qin *et al*., 2009), and PUFA desaturation through Δ6 and Δ5 *fads* regulation (Matsuzaka *et al*., 2002). However, PUFAs have been shown to have an inhibitory effect on SREBP-1c gene expression (Yoshikawa *et al*., 2002). The underlying mechanism might be related to the LXR gene, which is the direct up-stream gene of SREBP-1c gene as mentioned below (Repa *et al*., 2000).

Liver X receptor

Apart from SREBP, LXR is another major regulator of hepatic lipid metabolism. LXR belongs to the nuclear receptor superfamily with two identified isoforms LXRα and LXRβ. Unlike ubiquitous LXRβ, LXRα is highly expressed in liver and adipose tissues. After activation by the binding of oxysterols as ligand, LXR is able to bind on the DNA sequence with high affinity (Sampath *et al*., 2005). It has been reported that LXR affects the transcription of genes related to lipid and fatty acid synthesis such as FAS and Δ9 *fad* (Ntambi *et al*., 2002; Repa *et al*., 2000), which is consistent with the lipid accumulation in adipocytes by activated LXR (Ulven *et al*., 2005). Additionally, LXR, especially LXRα, is able to bind SREBP-1c promoter and activate SREBP-1c transcription. Therefore, SREBP-1c is also considered as the direct target gene of LXR as observed in mice (Repa *et al*., 2000; Yoshikawa *et al*., 2001). Overall, there are two pathways that regulate the hepatic lipogenesis by LXRs. First, LXR can directly regulate the transcription of target lipogenic genes through binding to their promoters, or indirectly activate SREBP-1c as the target gene to affect the lipogenic gene expression downstream through the LXR-SREBP pathway. However, Yoshikawa *et al*. (2002) and Deng *et al*. (2015) reported that PUFA could inhibit the binding of LXR to the LXR response elements in the SREBP-1c promoter and further suppress SREBP-1c gene expression. This crucial process partially explains the covered mechanism mentioned above and provide a path to understand the relationship between PUFA and lipid metabolism.

Table 1.1 Abbreviation, full name and function of all genes investigated in the present thesis.

Gene	Full Name	Gene Function
fads2d6	*D6 – Fatty acid desaturase*	Required for the synthesis of highly unsaturated fatty acids
fads2d5	*D5 – Fatty acid desaturase*	Required for the synthesis of highly unsaturated fatty acids
elovl2	*Elongation of very long chain fatty acids protein 2*	Participates in the biosynthesis of long chain poly unsaturated fatty acids. Elongation of C22
elovl5a	*Elongation of very long chain fatty acids protein 5*	Participates in the biosynthesis of long chain poly unsaturated fatty acids. Primarily the elongation of C18 and C20
elovl5b	*Elongation of very long chain fatty acids protein 5*	Participates in the biosynthesis of long chain poly unsaturated fatty acids. Primarily the elongation of C18 and C20
srebp-1	*Sterol Regulatory Element-Binding Protein 1*	Indirectly involved in cholesterol synthesis, involved in sensing cholesterol availability in the endoplasmic reticulum
srebp-2	*Sterol Regulatory Element-Binding Protein 2*	Indirectly involved in cholesterol synthesis by regulating *hmgcr*
lxr	*Liver x receptor*	Regulates cholesterol and fatty acids metabolism and the conversion of cholesterol to bile acid
pparα	*Peroxisome proliferator-activated receptor alpha*	Role in promoting hepatic fatty acid oxidation in response to fasting
pparγ	*Peroxisome proliferator-activated receptor gamma*	Activates transcriptional programs for lipid storage and lipogenesis
fas	*Fatty acid synthase*	Corresponding protein participates in the fatty acid synthesis
hmgcr	*3-hydroxy-3-methyl-glutaryl-CoA reductase*	Corresponding protein is the rate limiting enzyme in cholesterol synthesis
aco	*Acyl-CoA oxidase*	Oxidoreductase that participates in peroxisomal β-oxidation
cpt1	*Carnitine palmitoyltransferase I*	Mitochondrial enzyme that mediates the transport of long-chain fatty acids across the membrane by binding them with carnitine

1.4 Dietary lipid sources

1.4.1 Current issues of marine origin ingredients in aquafeed

The aforementioned introductions present the overview of the fast-growing aquaculture industry including important aspects such as human demand, sustainability issues, the role of aquafeed and the importance of lipid nutrition. FM contains an abundance of highly digestible, well-balanced protein for the essential amino acid requirements which also provides a high palatability for aquatic livestock (Turchini *et al*., 2019). Although it is also essential component of aquafeed, the present thesis emphasizes more from the FO perspective. For decades the major lipid source in conventional aquafeeds was FO. Owing to it being well accepted and digested, as well as the favourable nutrient composition such as EPA and DHA, FO is considered as the ideal lipid source so that it almost represents the natural food and perfectly provides all the essential nutrients for fish growth. However, all these features can be a double-edge sword, the excellent role of FO results in its over-dependence followed by other consequences. Indeed, FO is largely derived from the marine fishery of which 75 % is consumed by aquaculture (Bachis, 2017; Naylor *et al*., 2021). With the increasing scale of the aquaculture industry, the over-reliance of FO will definitely burden the natural fish stock. In addition, overexploitation is considered to be short-sighted and dangerous from an ecological sustainability perspective. Irrespective, the efficiency of the marine source has been questioned and criticised due to some illogical misunderstandings (Byelashov and Griffin, 2014), as using high volumes of finite marine resources in an insufficient way will aggravate the environmental issues and production costs through the higher aquafeed cost. This reduction will consequently cut down the n-3 nutrition supply, which obviously enlarges the gap between supply and human demand, as fish is the main provider of LC-PUFA to humans. Thus, the improvement of feed formulation in order to reduce FO reliance without compromising the nutritional value has come to the forefront.

1.4.2 Alternative lipid sources

One of the strategies is to find suitable substitutes and many efforts have been made to replace FO in aquafeed with alternatives. However, any potential candidates must meet several criteria: being a sustainable source with high production levels and available at a lower price, eco-friendly without harm to the environment and, most importantly, adequate in the supply of essential nutrients. A further barrier is the degree of replacement that can be achieved as over-substitution may induce a lack of sufficient essential nutrients. Some other factors, such as an imbalance of nutrients, antinutritional factors (ANFs) or inferior digestibility and palatability for different species, probably result in the adverse effects to impair growth, nutritional value or even health of the cultured species. Based on the above, an abundance of research has been carried out to test the potential of novel ingredients, dietary inclusion levels and even processing technology over the years (Alhazzaa *et al*., 2019; Miller *et al*., 2010; Spalvins and Blumberga, 2018). For comparative purposes, FO diets are normally set as a standard reference. The main difference between FO and other sources is their fatty acid composition. Some aquafeeds, where marine ingredients are predominantly or completely replaced, can compromise growth performance in carnivorous species. In addition, some remain unprofitable as they require costly supplements to cover nutrient deficiencies (Turchini *et al*., 2019). However, continuous breakthroughs have been made to know more about the use of alternative sources in terms of nutrient composition, suitable levels either in single or as a blend, and at specific life-stages. In the following sections, some alternative sources are discussed.

1.4.2.1 Plant-based sources

Terrestrial plant-based lipid sources, predominantly vegetable oils (VO), are considered the most common alternatives to replace FO in aquafeeds. The inclusion of VO in the fish diet is increasing because of their advantages such as higher availability and better economic value. The high availability and lower cost of VO (although not always), compared to FO, are beneficial for the aquafeed industry which in turn has prompted extensive research on their application during the past two decades. Currently, VO such

as palm, soybean, rapeseed, sunflower, linseed and olive oil are increasingly used in aquafeeds to lessen the shortage of fish oil (Salin *et al*., 2018) (Figure 1.7). Although VO contain abundant amounts of SFA, MUFA and n-3 or n-6 PUFA in some oils, they share a commonality in that they are all completely devoid of any n-3 LC-PUFA (Figure 1.8). Despite that, VO provide sufficient precursors to potentially elongate and desaturate them to LC-PUFA. Nonetheless, marine teleosts with an inadequate conversion ability are not able to utilize these for the biosynthesis of EPA and DHA. As reviewed above the consequences of EFA deficiency through replacements by VO would only be possible if the EFA requirements can be met in the VO diets.

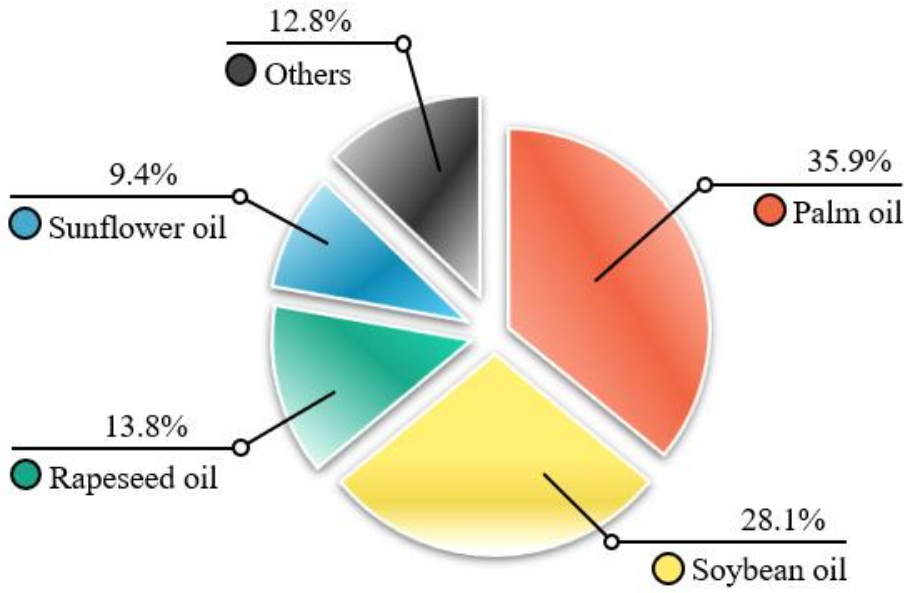


Figure 1.7 The percentage of the global production of major vegetable oils in 2021 (based on data from US Department of Agriculture).

Indeed, some studies have confirmed that using VO to replace more than half of the FO in the diets in species including salmon are feasible without compromising the growth performance. For example, feeding 10 g European seabass (*Dicentrarchus labrax*) with 60 % FO substitution by soybean, rapeseed or linseed oil for three months did not impact growth (Izquierdo *et al*., 2003). A similar result was observed in larger size of fish, where the replacement of 60 % FO by rapeseed oil resulted in a normal growth performance in

250 g rainbow trout for two months (Caballero *et al*., 2002). Furthermore, 70 % replacement by soybean and rapeseed oil was tested on 110 g sea bream for six months without any growth issues (Fountoulaki *et al*., 2009). A higher 83 % replacement by soybean oil also seemed to be feasible for 16 g seabass in a three-month trial (Martins *et al*., 2006), while in Atlantic salmon research indicated that the complete replacement by VO can be successfully achieved in freshwater salmon without impacting growth when fed for 22 months (Bell *et al*., 2004; Jordal *et al*., 2007). However, the over-inclusion of VO is very likely to trigger poor growth for some species such as in sea bream where 80 % replacement by soybean and rapeseed oil induced a reduction in growth, whereas up to 60 % replacement appeared to have no negative effect (Izquierdo *et al*., 2005; Menoyo *et al*., 2004). Likewise, 60 % but not 80 % of linseed oil replacement for 8 months in 75 g seabass did not influence growth (Montero *et al*., 2005). Therefore, 60 % replacement is not absolutely but generally regarded as preferable so that the optimal growth performance and feed utilization efficiency of fish can be maintained. In addition, Glencross and Turchini (2010) reported that replacement should be dependent on the life stage and nutritional requirements as these can vary. Thus, the research is too extensive to present here in a simple way and a comprehensive summary of VO replacement information has been reviewed in detail by Glencross (2009), Turchini *et al*. (2010) as well as Sales and Glencross (2011).

In contrast to growth performance, the fatty acid composition of the carcass or tissues tend to vary and reflect the dietary intake due to their plasticity. As mentioned, VOs are rich in their characteristic fatty acids (ALA for linseed oil; SFA for palm oil, etc) but are devoid of any n-3 LC-PUFA. As such, FO replacement by VO will directly reduce the n-3 LC-PUFA content in the fish fillet. Additionally, the high inclusion of n-6 abundant VO (such as soybean oil) is basically accompanied by an imbalance of the n-3:n-6 ratio, which will induce health issues through eicosanoid or cardiovascular disease, etc. (Montero *et al*., 2003; Torstensen and Tocher, 2010). Even though the fatty acid balance can be partially achieved by blending different VO, the key limitation of lacking LC-PUFA still exist.

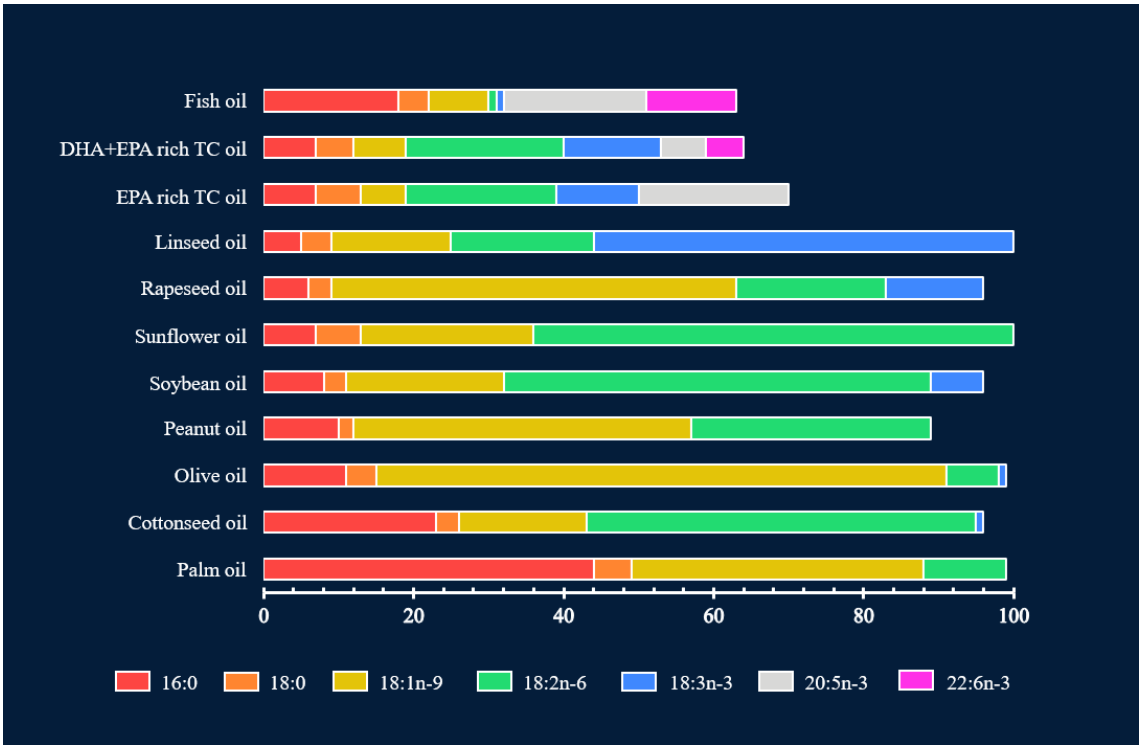


Figure 1.8 The fatty acid composition of fish oil and different alternative oil sources in percentage (Betancor *et al*., 2015a; 2016a; Glencross, 2009). TC: transgenic camelina.

1.4.2.2 Terrestrial animal-based sources

Some rendered animal fats such as that from poultry or the most widely available beef tallow, has been studied and used as alternatives in aquafeeds (Bureau, 2006; Rombenso *et al*., 2017). Although they have stable production and are more affordable than FO, they are rich in SFA and MUFA while containing low or moderate level of PUFA which could limit their application. Research has shown that the partial replacement by rendered animal fat has limited impact on growth performance in carnivorous species such as trout, sea bream, seabass and salmon (Bureau *et al*., 2008; Higgs *et al*., 2006; Monteiro *et al*., 2018; Pérez *et al*., 2014; Trushenski *et al*., 2011; Turchini *et al*., 2010), with animal fat substituting around 50 % of FO. Despite the sufficient supply of SFA and MUFA as preferred energy sources, n-3 LC-PUFA still need to be provided, and, as with VO, the lack of EPA and DHA in animal fats will also cause a decline in the nutritional value of the fillet. Trushenski and Lochmann (2009) have reviewed the information about

terrestrial animal oils and suggest that the current use of rendered animal fats in aquafeeds is expandable but more research should be performed to support further applications. Moreover, a further limitation lies within legislation and policies which raise concerns about potential consumer health risk issues by transmissible spongiform encephalitis. Although it has been proved to be safe for ultilisation if processed properly, its application is still not globally spread (Campos *et al*., 2019; Glencross *et al*., 2020)

Lipid extracts from insects and worms have also been found to be another potential alternative with PUFA interest (Rumpold and Schlüter 2013). Insect fat is very rich in SFA (Barroso *et al*., 2017), however their fatty acid profile can be altered through their diet to accumulate LC-PUFA. Nonetheless, similar to the rendered animal fats, legislative limitations exist regarding the substrates insects can be grown on, which in turn limits their application as a LC-PUFA source. The use of insect for aquaculture has been comprehensively reviewed elsewhere (Henry *et al*., 2015).

1.4.2.3 Other Marine sources

Another approach is to explore alternative marine resources that were not previously considered from the traditional wild catch fisheries for the supply of oils rich in n-3 LC-PUFA. For instance, organisms from mesopelagic fisheries or lower trophic levels could possibly be utilised. In fact, Antarctic krill (*Euphasia superba*) and calanoid copepods (*Calanus finmarchicus*) have already been investigated as sources of n-3 LC-PUFA (Betancor *et al*., 2012; Bogevik *et al*., 2010; Saleh *et al*., 2015) and approved for their nutritional properties. However, their high cost and limited production restricts their use as a primary source of n-3 LC-PUFA. Indeed, the low production volumes only allows them to serve as early-stage life diet (Sprague *et al*., 2017b) or as specialized feeds (Tocher, 2015). Even though their production is modest, they are still questioned in terms of sustainability and environmental and ecological issues for their significant role in the food chain (Hill *et al*., 2006).

Another possible source is the by-product and processing wastes from fish. Consequently, a wide range of materials can be used to produce marine oils such as non-target fisheries, discarded species and fish carcasses or tissues from both extractive fisheries and aquaculture. Its diverse origin means that the quality is variable, with different nutritional compositions and potential contaminants, making it difficult to assess both its production and utilization.

Some specific species of marine microalgae, along with other single cell microbes offer an ideal, long-term, sustainable supply of n-3 LC-PUFA for aquaculture, since they are the primary producers of these FA in the marine environment (Nagappan *et al*., 2021). Apart from the direct supply of microalgae to the marine fish, using microalgae to enrich live feeds for larval fish is also considered as a new strategy for marine source utilisation. The current involvement of microalgae in various aspects and their fatty acid compositions have been reviewed (Maltsev and Maltseva, 2021; Sajjadi *et al*., 2018; Sprague *et al*., 2017b). Some common species and strains used includes *Crypthecodinium Nannochloropsis*, *Phaeodactylum* and *Schizochytrium* sp. (Eryalcyn *et al*., 2015; Ganuza *et al*., 2008; Miller *et al*., 2007; Sørensen *et al*., 2016; Sprague *et al*., 2015). These species are selected based on features including a high n-3 LC-PUFA and oil contents, fast growth rate, high productivity and economical cost. In cases where a single species of microalgae might not be able to satisfy the fatty acid demand completely, additional strategies have emerged to incorporate with VO blend or microalgaal mixtures to give a balanced fatty acid composition (Santigosa *et al*., 2020; Sprague *et al*., 2015). Nevertheless, despite being an excellent source of n-3 LC-PUFA, some technological constraints have limited their use such as the break-down of the cell wall, absorption barrier and oxidation after extraction (Miller *et al*., 2007; Shah *et al*., 2018).

1.4.2.4 Transgenic sources

As discussed above, terrestrial plants are devoid of n-3 LC-PUFA as they lack of the necessary genes to produce them by themselves (See 1.3.3.1). By introducing these genes

from marine microalgae into plants through genetic modification (GM), plants will be capable to produce LC-PUFA (Sprague *et al*., 2017b; Tocher *et al*., 2015). So far, engineering of Camelina and Canola oilseed crops has been achieved. In this sense, Camelina has been successfully modified to produce up to 24 % EPA or 11 % EPA and 8 % DHA (Ruiz-Lopez *et al*., 2014; Walsh *et al*., 2016). The possibility of high Camelina oil and canola oil inclusion in the salmon feeds has been gradually researched (Betancor *et al*., 2015a; 2015b; 2016a; 2018; Hatlen *et al*., 2022; Ruyter *et al*., 2022). It was evident that the complete substitute of FO by GM Camelina oil not only contributes to growth performance, but also enhanced DHA biosynthesis from EPA, as well as the comparable absolute amount of EPA and DHA in salmon muscle to those fed FO (Betancor *et al*., 2016a). Currently, the potential of GM oil is being investigated in more innovative research studies, further encouraging its commercialization within the aquafeed industry (Betancor *et al*., 2016b; 2021; Napier *et al*., 2019; Napier and Betancor, 2023; Ruyter *et al*., 2019).

However, the greatest challenge, irrespective of the relatively positive nutritional composition of GM – derived oils, is the public acceptance of GM products. Therefore, the science-based evidence might be the best education to build up the trust, shape the thoughts about sustainability and support the GM food in the future society (Sprague *et al*., 2017b).

1.4.3 Limitation of the replacement

Over the past 30 years, the replacement of FO in aquafeeds has received considerable attention in order to address the urgency about increasing aquafeed demand versus finite supply. This has resulted in some major achievements being made that have enabled a range of sustainable feeds manufactured with low FO content as discussed above. However, limitations still exist regarding the complete substitution of FO, as complete and/or high replacement levels may lead to detrimental effects on fish growth as well as the reduction in the n-3 LC-PUFA nutritional value for customers. Hence, alternative

strategies, are also required in order to enhance fish performance and boost their nutritional value.

1.5 Nutritional programming

As previously mentioned, FO replacement results in a reduction in the LC-PUFA content of farmed salmon flesh due to a reduced dietary intake of these EFA, together with their poor ability of n-3 LC-PUFA biosynthesis. Therefore, an alternative approach is being explored to activate or stimulate the enzymatic machinery in order to enhance the endogenous biosynthesis ability for n-3 PUFA by nutritional regulation.

1.5.1 Concept of programming

In mammals, it is widely accepted that during certain critical developmental windows including gestation and lactation, various endogenous and exogenously sourced stimuli or cues can act as conditioning agents such as sensory stimuli, antigens, drugs, temperature or diet. These stimuli can induce permanent genetic changes in the animal's metabolism and physiology (Lucas, 1998). In addition, longer-term impacts both on the genome and phenotype can also be observed, so the process is typically regarded as "programming" (Figure 1.9). Among those stimuli, nutrition is often considered to play an especially important role. Therefore, this kind of phenomena caused by nutrients or bioactive food components is called "nutritional programming". Nutritional programming is believed to prepare the animal through adaptive changes at the cellular, molecular and biochemical level to that of a similar nutritional environment experienced in later life stages (Gluckman and Hanson, 2005; Patel and Srinivasan, 2002). For instance, the new-born epigenomic response is a reaction to both the maternal environment and the early physical and nutritional environmental in order to increase the animal's evolutionary fitness (Gluckman *et al*., 2004). Essentially, after being exposed to the experimental suboptimal diet (stimulus), the animals are then treated with a suitable diet for a certain period, followed by re-feeding with a diet similar to the stimulus diet, before several indicators are measured to reveal the outcomes of the programming. Some

studies that focus on the nutritional intervention applied to fish development has been reviewed by Hou and Fuiman (2020).

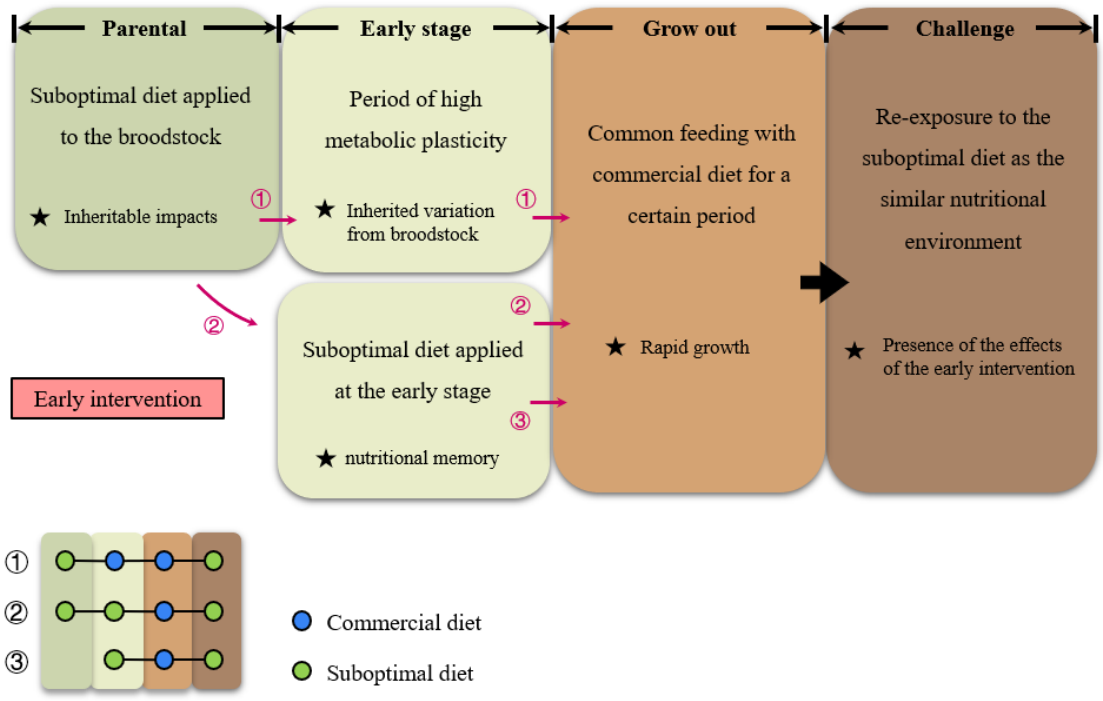


Figure 1.9 The illustration of the nutritional programming concept at various stages with different applications in mammals.

1.5.2. Early nutritional intervention

Dietary nutrients can be used to manipulate the production performance through nutritional programming. For instance, by regulating the expression of genes related to growth or modulating their metabolism to increase their nutritional value. Therefore, it is possible to selectively customize the target species for desired characteristics like more efficient growth or improved nutrient biosynthesis. Although there are comprehensive reports and reviews in mammals (mainly rodents), the knowledge about nutritional programming in fish has not been as extensively investigated to date (Lillycrop and Burdge, 2012; Martínez *et al*., 2012; Zhang, 2015). The studies performed so far have applied a stimulus diet either to broodstock fish or directly to the first feeding stage in larvae. As expected, different responses such as better carbohydrate ultilization (Fang *et*

al., 2014; Geurden *et al*., 2007; 2014) or molecular adaptions to a low FO diet (Izquierdo *et al*., 2015; Vagner *et al*., 2007; 2009; Xu *et al*., 2021) have been observed, which implies that the early developmental stage is an effective window for programming in teleosts.

The reason why nutritional programming is applied during the early stages is because during this period the embryo is vulnerable to external factors and impacts on the epigenome occur easily. The different modifications or marks on the epigenome can then either be partially erased or re-built by changes in protein or mRNA levels at the regulatory enhancer or promoter regions of target genes (Faulk and Dolinoy, 2011; Lillycrop and Burdge *et al*., 2012; Petry *et al*., 2001). The presence or absence of certain nutritional factors will impact or, more likely, fail the programming process in time, which might lead to dysregulation of gene expression and become irreversible (Gräff and Mansuy, 2009). These nutritionally acquired effects remain relatively stable once established. Nevertheless, the early critical period is not the only window as phenotypic changes in later stages of life will appear if the novel type of interventions take place for a long period of time. Programming at the adult stage is less discussed since responses are less pronounced due to the firm epigenome from early stage which requires a longer time, indicating that the window, intensity and duration are all important to impact the programming (Jiménez-Chillarón *et al*., 2012). Such modifications could occur not only originally but also trans-generationally, as some programming effects are reported to be inheritable through the altered broodstock nutrition (Turkmen *et al*., 2019a; 2019b). These traits might provide another path to program the target fish from broodstock for the adaptive responses with better fitness, such as survival and reproduction (Li *et al*., 2011).

1.5.3 Epigenetics

Traditionally, the phenotypes of mammals are believed to be determined to a large extent by genetics. However, over the past few decades, research in mammals and plants has shown that there are some additional modifications to the genome that impact their phenotypes through the effects at the cellular and physiological level. These

modifications, primarily altered by external environmental factors or internal developmental factors, are generally referred to as “epigenetics”. Epigenetics is a heritable modification of gene expression resulting from changes in the chromatin structure without specific alterations in the DNA sequence. Nutrition is one factor capable of triggering epigenetic marks, which act as important mediators of phenotypic responses to the nutritional input via gene expression. Therefore, epigenetics is considered to be the mechanism in which to perform nutritional programming (Weaver *et al*., 2004). Correspondingly, epigenetic marks probably facilitate cell division during embryonic development and persist throughout life mitotically as well as being inherited across generations (Bird, 2002; Jablonka and Raz, 2009). This further explains why nutritional programming is long-lasting, heritable and dependent on early stage intervention as mentioned above. A particular trait of epigenetics is the transcriptional impact while the DNA sequence is preserved, so all the different phenotypes do not follow the conventional theories such as Mendelian inheritance or DNA sequence mutation (Feeney *et al*., 2014), meaning that epigenetics can be beneficial or harmful (Jones and Baylin; 2002; Lyko and Brown, 2005). Mechanisms through which epigenetic marks take transcriptional effects include DNA methylation, histone modifications, chromatin remodeling and non-coding RNA (Bird, 2002; Esteller, 2007; Kouzarides, 2007; Mattick and Makunin, 2006) (Figure 1.10).

1.5.3.1 DNA methylation

The most widely investigated epigenetic mechanism is DNA methylation, which refers to the enzymatic addition of a methyl group to a cytosine residue in DNA exclusively at CpG dinucleotides. One important role of DNA methylation is to provide stable genomic imprinting for cell-type differentiation and embryonic development (Li *et al*., 1992). The modification, located on the promoters of genes, will physically block the transcription process leading to gene repression (Bell and Felsenfeld, 2000). The link between nutrition and DNA methylation is that nutrition provides the necessary substrates for DNA methylation. Some nutrients like methionine, folate, choline and vitamin B2, B6 and B12

are considered as precursors of S-Adenosyl-methionine (SAM), which is the major methyl-donor for DNA methylation. With the donors, the methyl group is transferred to DNA and maintained in the new strand during mitosis through DNA methyltransferase (DNMT) family activated by SAM (Jiménez-Chillarón *et al*., 2012). Therefore, the bioavailability of SAM from dietary nutrients largely influences DNA methylation (Feil and Fraga, 2012; McKay and Mathers, 2011). In contrast, DNA demethylation is important during embryonic development and stem cell differentiation. Generally, the inadequate activity of DNMT cannot maintain the methyl group after DNA replication and returns unmethylated (Gavin *et al*., 2013). Alternatively, DNMT can be blocked by 5-Hydroxymethylcytosine (5hmC) during DNA replication in order to stop the methylation status (Ficz *et al*., 2011).

1.5.3.2 Histone modification

The basic unit of chromatin is the nucleosome. Each nucleosome comprises double-stranded DNA and four pairs of histone proteins. The amino residues of each histone protein are called histone tails. Differing from DNA methylation, there are several modifications occurring on the histone tails such as acetylation, methylation, phosphorylation, ubiquitination, etc. Among the most studied modification is histone acetylation and deacetylation, involving the combination or removal of the acetyl group from the histone tails to adjust the electric charge of histone which then impacts the strength of the interaction with DNA. Thus, chromatin structure can be open or repressive by acetylation, which decides the accessibility of transcription and further expression (Granada *et al*., 2018). Similar to DNA methylation, the methyl group can be attached to histone as methylation to impact the transcription process. Similarly, SAM acts as the donor of the methyl group in this process as well as a regulator. The investigation from Pogribny *et al*. (2007) demonstrated the importance of altered histone modifications and indicated that the early dietary supplementation with adequate levels of methyl group donors could prevent the development of liver tumors as well as the initiation and promotion of cancer.

1.5.3.3 Chromatin remodeling

Epigenetic changes are not restricted to the DNA or histone, in fact other modifications also play important roles in the modulation of the epigenotype. Knowing that chromatin will be restructured by different modifications, the activation or repression of genes is related to this transformation termed chromatin remodeling, which essentially refers to the change of the nucleosome position or conformation leading to the assembly or disassembly of chromatin (Nakao, 2001). As an ATP-dependent process, the enzymes involved use energy derived from ATP hydrolysis to remodel (Vignali *et al*., 2000). Therefore, the chromatin can either be condensed or can expose DNA to be accessible to transcription factors (Simone, 2006). These work as important sections for a variety of cellular processes including differentiation, transcription and DNA repair and replication (Havas *et al*., 2001).

1.5.3.4 Micro RNA

Evidence shows that non-coding RNA is an important regulator of gene expression via the most researched micro-RNA (miRNA) (Carthew *et al*., 2006). Basically, the miRNA is able to inhibit protein synthesis and repress protein expression for the purpose of post-transcriptional silence or inactivation (Granada *et al*., 2018). Moreover, miRNA molecules are also considered to be epigenetic factors because of their intensive interaction with other epigenetic mechanisms, such as a significant role in controlling DNA methylation and histone modifications. In return, these epigenetic marks also modulate microRNA expression to create a well-established feedback mechanism (Choi and Friso, 2010). Several studies have shown there to be a wide range of nutrients including dietary lipids, protein, vitamin E, hormones that may alter the expression of specific miRNAs and impact the phenotypes. (Gaedicke *et al*., 2008; Jiménez-Chillarón *et al*., 2012; Parra *et al*., 2010;)

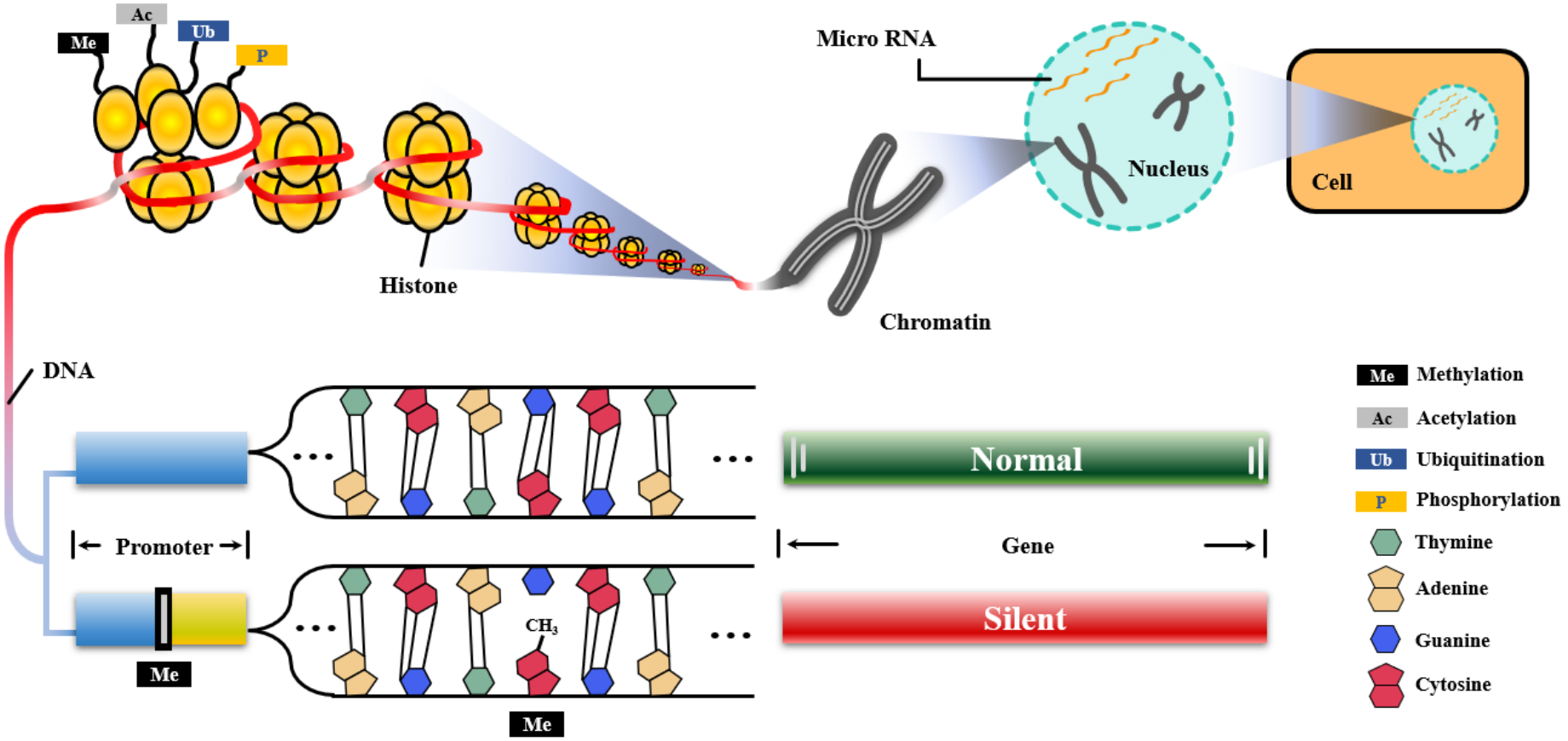


Figure 1.10 The illustration of the main elements in epigenetics including DNA methylation, histone modifications and miRNA.

1.5.4 Application in aquaculture

Increased research surrounding epigenetics will provide further insights in to how epigenetics impacts phenotype, delivering more opportunities to perform programming. In the context of aquaculture, the expected phenotype can be picked out by specific environmental conditions to improve husbandry management or productivity of profitable species as well as coping with issues such as selective breeding, disease prevention, temperature and photoperiod tolerance, salinity acceptance, sex control, nutrient utilization and so forth (Giannetto *et al*., 2013; Morán *et al*., 2013; Navarro-Martín *et al*., 2011;). Several reviews have summarized the research related to epigenetics in aquaculture proving more detail description than herein (Best *et al*., 2018; Granada *et al*., 2018; Hou and Fuiman, 2020; Jonsson and Jonsson, 2014; Moghadam *et al*., 2015).

To improve the ultilisation of dietary carbohydrate in fish, an experiment was run using zebrafish where a high carbohydrate level was included in the diet during the ontogenetic and adult stages as the challenge. The results demonstrated that the early nutritional intervention did not affect the growth but exhibited a significant decline in plasma glucose levels when high-carbohydrate diets were presented again in later life (Fang *et al*., 2014). Whereas, an experiment on rainbow trout imparted another view following the early exposure to a low protein:carbohydrate ratio diet as a short hyperglucidic–hypoproteic stimulus for trout, with the similar stimulus diet in the later life. The analysis indicated that the intestinal microbiota was affected, along with impaired glucose homeostasis suggesting that there was a long-term influence on muscle glucose metabolism from early programming (Geurden *et al*., 2014).

It has been reported that LC-PUFA can also act as a programming factor in fish. For instance, European seabass larvae fed low n-3 LC-PUFA diet (0.8 % on dry matter basis) from six-day post-hatching as dietary history displayed no difference in growth and survival. However, the DHA content in the polar lipid, Δ6 *fads* and PPAR expression were all enhanced when challenged by a similar diet after 60 days of commercial feeding

(Vagner *et al*., 2007; 2009). In another study carried out with rainbow trout fry fed a complete vegetable diet for three weeks, higher growth rates, feed intakes and efficiencies were observed when challenged with a similar plant-based diet after seven months (Geurden *et al*., 2013).

There are many detailed studies of nutritional programming about LC-PUFA on the gilthead sea bream. Izquierdo *et al*. (2015) for example, reported that high FO replacement (> 80 %) in the parental diet reduced fecundity and spawn quality along with the body size and Δ6 *fads* expression of their offspring after four months. However, when these offspring were challenged with a low marine diet later in life, a better diet utilization and growth was observed compared to progeny from broodstock fed diets with a lower substitution level (60 % replacement). Furthermore, Turkmen *et al*. (2017) replicated the earlier study of Izquierdo *et al*. (2015) but provided an additional two-month challenge after a 12-month commercial feeding period. The results indicated that the sea bream experiencing parental programming (60 % linseed oil replacement in diet) and first round challenge as history had a higher growth and feed utilization than those who did not receive any programming or challenge. Moreover, a low n-3 LC-PUFA parental diet also down-regulated lipoprotein lipase and CPT1 at the end of the trial. In another trial carried out by the same group, when four-month-old offspring was challenged by differing t levels of FO diets, the juveniles from broodstock fed 100 % FO always showed the lowest growth. In addition, 60 % of FO replaced by linseed oil in the broodstock diet increased the offspring LC-PUFA content (Turkmen *et al*., 2019b). It was further demonstrated that after programming, when challenged by a vegetable-based diet, the offspring from group with higher *fads2* expression fed a low marine diet showed better growth and better diet utilization. It was suggested that the trans-generational effects of *fads2* expression from broodstock, as well as the DNA methylation on *fads2* promoter was found to be an epigenetic mark (Turkmen *et al*., 2019a; 2020; Xu *et al*., 2021). However, differences were shown by Xu *et al*. (2019) who showed that, when challenged by low marine diet, both fish meal and FO replacement in broodstock diets negatively impacted the growth performance of offspring, but only FO replacement enhanced the *fads2* and *elovl6*

activities in the offspring.

Contrary to studies in Mediterranean fish species, studies in salmonids have used first feeding as the window in development to elicit nutritional programming. The reason for this is due to the larger size of first feeding larvae in salmonids, which allows for the use of formulated feeds with full control over the nutritional composition as compared to marine species that require live feeds. In Atlantic salmon, both diploid and triploid fry were fed either a marine diet or a vegetable diet (approximately 25 % and 3 % n-3 LC-PUFA of total fatty acids, respectively) for three weeks as a stimulus. All fish were then subsequently challenged with a vegetable diet for six weeks after feeding a commercial diet for 15 weeks. The results indicated that fish initially stimulated with the vegetable diet had a 24 % higher growth rate and 23 % better feed conversion efficiency during the challenge phase than fish fed a marine diet at first feeding. Additionally, the same fish were also reported to have higher EPA and DHA retention rates in both diploid and triploid fry (Clarkson *et al*., 2017).

Experimental Objectives

Presently, the use of the plant-based aquafeeds has reached a critical point. Atlantic salmon is able to be a net producer of n-3 LC-PUFA from precursor, while the exact production quantity remains unclear. Nutritional programming is presumed to improve the utilization of plant-based aquafeeds in salmon while maintaining the health and nutritional quality. However, application of the stimulus phase is not yet fully understood. Additionally, after long-term programming, whether a challenge is required to enhance the n-3 LC-PUFA production also needs to be addressed. Results from the present thesis will provide insights of the endogenous n-3 LC-PUFA production capability. Furthermore, the conclusions from this thesis aim to develop the nutritional programming application in salmon farming.

Therefore, the experimental objectives of the present thesis were to:

1. Determine the interaction between dietary ALA, LA levels and LC-PUFA production in Atlantic salmon and maximise the EPA and DHA endogenous production (Chapter 3).

2. Evaluate the potential impact of the plant-based stimulus in order to identify the most appropriate phase to perform the programming in Atlantic salmon (Chapter 4).

3. Assess the long-term nutritional programming effect in salmon and evaluate the effects of a similar challenge as a booster on the n-3 LC-PUFA production enhancement (Chapter 5).

CHAPTER 2

General Materials & Methods

2.1 Experimental fish and husbandry

2.1.1 Ethics statement

All of the animal experimentation performed in the present thesis were subjected to thorough ethical review carried out by the University of Stirling's (UoS) Animal Welfare and Ethical. Review Board. Approval for each experimental chapter was as follows: Chapter 3, AWERB No:16 17/118/New Non ASPA; Chapter 4, AWERB No: 18 19/045/New ASPA; Chapter 5, AWERB No: 19 20/055/New ASPA. Furthermore, all experimental procedures were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice, HMSO, London, January 1997). The fish and samples used in the present thesis originated from two different funded projects: ENDOPUFA, European Commission Aquaexcel2020 Trans-National Access, Grant no. AE040061 (Chapter 3); and NUTRIPROG, Biotechnology and Biological Sciences Research Council (BBSRC), Grant no. BB/R018812/1 (Chapter 4 and 5). The author participated in the trial design of each experimental chapter and all analyses reported in the present document were generated by the author.

2.1.2 Fish

About 3,000 Atlantic salmon eggs were used in the experimental trial presented in Chapter 3, sourced from Aquagen and transferred to the Institute of Marine Research (IMR, Matre, Norway) where they were hatched and the alevins maintained under standard rearing conditions. In Chapter 4, 2,700 eggs (388 degree-days, DD, post fertilization) were obtained from Mowi (Scotland, UK) and transferred to the UoS's Temperate Aquarium (Stirling, Scotland, UK). All eggs were hatched after 500 DD with the fish remaining in the alevin stage for a further 350 DD. In Chapter 5, Atlantic salmon eggs (369 DD post fertilization) were obtained from Stofnfiskur (Iceland) and transferred to UoS's Niall Bromage Freshwater Research Unit (NBFRU, Buckieburn, Stirlingshire, Scotland). All eggs were hatched after 500 DD and incubated to the alevin stage for a further 350 DD.

2.1.3 Experimental set up

The experiment in Chapter 3 was carried out at the facilities of the IMR. Fish from each single tank were redistributed into 12 × 1m diameter tanks (500 litres volume) in a freshwater through flow system with 200 alevins/fry per tank at a water temperature of 13 ℃ and 24:0 light:dark.

The experiment in Chapter 4 was conducted in the UoS's Temperate Aquarium facility. The incubation temperature was 7.0±0.4 °C. At 850 DD when salmon reached the fry stage with a 97.7 % survival rate, all were randomly distributed to 9 × 0.3 m^2 tanks. In the first feeding trial, the water temperature was maintained at 13.1±0.5 °C with 24:0 light: dark, the oxygen level (86 % saturation, 8-9 mg/L), pH (7.2±0.2), nitrogen dioxide (0.3±0.2 mg/L), total ammonia nitrogen (0.13±0.17 mg/L) and chloride (135.0 ±13.0 mg/L) were daily determined and controlled.

The feeding trial in Chapter 5 was carried out at NBFRU. The incubation temperature was 6.0 °C. At 884 DD prior to first exogenous feeding, all fish were distributed into 12 × 0.7 m^2 tanks for first feeding trial. The water temperature was maintained at 12.6±0.9 °C exposed to 24 hours artificial light, the oxygen level (100±7 % saturation, 8-9 mg/L), pH (7.3±0.1), nitrogen dioxide (0.14±0.09 mg/L), total ammonia nitrogen (0.40±0.27 mg/L) and chloride (131.0±40.0 mg/L) were determined and controlled daily for the feeding trial.

2.2 Experimental diets

2.2.1 Formulations

The diets were specifically formulated according to the objectives of each of the different trials and based on the minimum criteria to satisfy the nutritional requirements of Atlantic salmon with the appropriate use of pellet size requirements for each of the fish development stages (NRC, 2011). The experimental diets in Chapter 3 were

manufactured at the UoS, the dry ingredients were ground, mixed with oil and water and pelleted using a screw press cold extruder (DollyTM; Imperia & Monferrina S.p.A) as compressed feeds, whereas the diets in Chapter 4 and 5 were produced by hot extrusion at BioMar TechCentre (Brande, Denmark). Further details regarding the formulations and the nutritional composition of the diets used in the experimental trials are shown within the Materials and Methods sections of the corresponding chapters (Chapter 3, 4, 5).

2.2.1 Diet feeding

Feeds were provided to fish by automatic feeders Arvo-tec TD2000 and user interface ArvoPRO (Arvo-Tec Oy: Huutokoski, Finland) to ensure the acquisition of accurate values for feed entering the tanks. For the first feeding phase described in Chapter 3, feeds were first provided by hand so that the feeding behavior could be observed. As the fish size increased, the different pellet sizes were applied to excess by automatic feeders. The uneaten feed was collected twice a day after daily feeding by syphon pipe with filter, then dried down for correction of feed intake measurement.

2.3 Sampling procedures

Fish were starved for 24 hours prior to every sampling phase. To avoid the stress and injury prior to any procedure, sampled fish were euthanatized by the anaesthetic solution in a lethal concentration (tricaine methanesulfonate, MS-222; 400-1000 mg/L). Confirmation of death was achieved by disrupting the spinal cord.

2.3.1 Growth assessment

Fish in each tank was bulk weighed on an electronic top-loading balance to 2 decimal places and recorded. The following formulae were applied to determine the specific growth rate (SGR):

$$SGR\ (\%/day) = \frac{\ln(W_1) - \ln\ (W_0)}{t_1 - t_0} \times 100$$

Where, W_0 and W_1 are the weights of the sampled fish in grams at the time point t_0 and t_1, respectively.

2.3.2 Whole fish and tissue biochemical sampling

According to the different trial plans, after the weighing step, a pre-determined number of whole-body fish were kept in labelled sealable plastic bags following euthanasia. All samples were kept on dry ice and stored at -20 °C until further analysis. The remining fish were dissected immediately for tissue sampling using sterile disposable scalpels. The six tissues investigated in the present thesis are liver, intestine, brain, eye, muscle and gill (Figure 2.1).

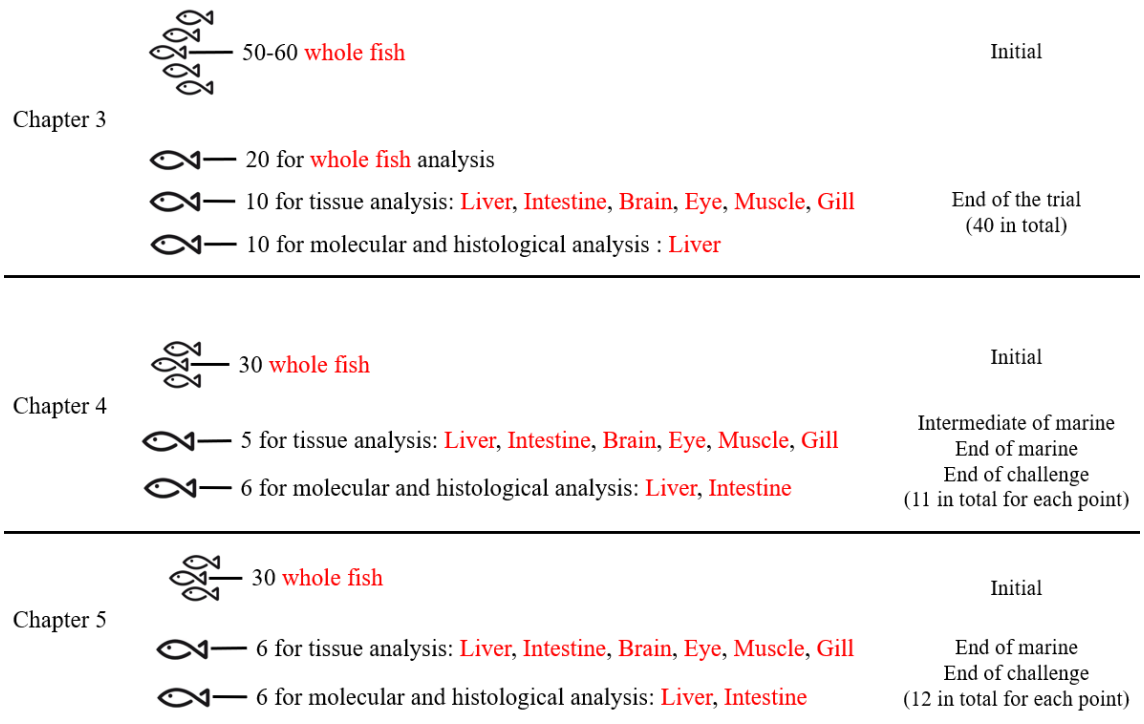


Figure 2.1 The scheme picture of all the samples collected in the present thesis.

Liver was vertically cut and split into three portions using a pre-washed (70 % ethanol) scalpel blade for the purpose of nutritional, histological and molecular analysis (See 2.3.3

and 2.3.4). The visceral lipid depots around the intestine sample were removed and any faeces present was gently squeezed out prior to the collection to ensure that the complete intestine was obtained without impurities. A piece of posterior intestine was vertically cut and collected for the purpose of histological sampling. For muscle samples, a dorsal square portion of flesh was dissected out and the skin removed. The whole gill was collected and rinsed to remove the dirt.

The samples obtained from each tank were pooled per tissue, then individually collected into six 7 mL glass vials and kept in liquid nitrogen. All bags and pots were transferred and stored at -20 ºC until further analyses.

2.3.3 Histological sampling

Before sampling, pre-labelled 50 mL histology pots containing 2 mL of 10 % buffered formalin were prepared at the UoS in advance. Following dissection (See 2.3.2), a portion of the liver and posterior intestine were collected for histological sample collection. All samples from each tank were stored in one pot, which were kept at room temperature pending analysis.

2.3.4 Molecular sampling

Before sampling, pre-labeled 2 mL cryotubes containing 1.5 mL RNALater (Sigma, Poole, UK) were prepared at the UoS in advance. After the dissection step, a portion of the liver from each fish was stored in the respective cryotube. All cryotubes were kept at 4 ºC for 24 hours before transfer to -20 ºC freezer.

2.4 Biochemical analysis

2.4.1 Proximate analysis

The methodology described below was used to determine the chemical composition of a

range of sample types including: diets, tissue samples and whole fish. Each analysis was performed based on the methods of the standard Association of Official Analytical Chemists (AOAC) methods (AOAC, 2000). Prior to analysis, all samples were pre-processed. Diet samples were ground into a powder using a FOSS Knifetec™ 1095 sample grinder (Foss Analytical AB, Högnäs, Sweden) or mortar and pestle. The whole fish samples were cut into pieces and homogenized using a commercial grade food blender mixer (Blixer® V.V., Robot-Coupe, Vincennes, France). Tissue samples were typically cut by hand and homogenized using scissors and thorough mixing.

2.4.1.1 Moisture

The moisture content of a sample was determined by gravimetric loss based on the thermal drying method. After recording the initial weight (circa. 5 g), the sample was placed into the drying oven (Gallenkamp, Cambridge, UK) at 105 ºC for 24 hours. The dried sample was taken out and placed into a desiccator to cool down for 30 min until the dry weight was constant. The dried sample was reweighed and the moisture content determined using the following calculation:

$$Moisture\ (\%) = \frac{Initial\ weight\ (g) - \ Dry\ weight\ (g)}{Initial\ weight\ (g)} \times 100$$

2.4.1.2 Ash

The ash content of a sample was determined by the gravimetric loss based on the thermal drying method. Approximately 1 g of the initial sample was weighed and placed in a pre-washed porcelain crucible. After drying in a Gallenkamp muffle furnace (Gallenkamp, UK) at 600 ºC for 24 hours, the ash sample was removed from the furnace and cooled down in a desiccator for 30 min until the dry weight was constant. The ash sample was reweighed and the ash content calculated as follows:

$$Ash\ (\%) = \frac{Ash\ weight\ (g)}{Initial\ weight\ (g)} \times 100$$

2.4.1.3 Crude protein

The crude protein content of a sample was determined using the Kjeldahl method with Kjeltec Auto-analyser (KjelROC; OPSIS LiquidLINE, Furulund, Sweden). Sample (~200 mg) were weighed on filter paper and digested with two mercury Kjeltabs and 12 mL of sulphuric acid (95 %, Aristart®, VWR Chemicals, Poole, UK) for 1 hour at 420 ºC. After cooling down until room temperature, the sample was processed by analyser for nitrogen content. Nitrogen released during sample digestion occupies approximately 16 % of protein, thus a conversion factor of 6.25 was applied. The crude protein content was calculated by the following:

$$Crude\ protein\ (\%) = \frac{nitrogen\ content \times 6.25}{Initial\ weight\ (mg)} \times 100$$

2.4.1.4 Total lipid

The total lipid from diets, whole fish and tissues was extracted based on the method of Folch *et al*. (1957). Samples around 0.5 to 1 g were weighed and placed in 50 mL Quickfit™ boiling tubes with 20 mL of chloroform-methanol (C:M; 2:1, v/v). The sample was homogenized using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). For diet samples, a further 16 mL of C:M was added after homogenization and the sample was kept overnight in a -20 ºC spark-proof freezer. However, all other samples remained on ice for 1 h before 0.88 % (w/v) potassium chloride (KCL) (5 and 9 mL for tissue and diets samples, respectively) was added before vortex-mixed to increase the water phase polarity. After standing for 5 min samples were centrifuged at 1500 rpm for 5 min before the supernatant was aspirated to remove impurities. The remaining solvent layer containing lipid was filtrated using C:M (2:1) pre-washed Whatman No. 1 filter paper (Whatman Plc., Kent, UK) and transferred to a pre-weighed 25 mL test tube denoted as “tube weight”. The solvent in test tube was evaporated with the temperature set at 40 ºC for about 50 min to keep the lipid in the tubes. The dry tube was sealed in the desiccator overnight and re-weighed as “total

weight". The lipid content was calculated as:

$$Total\ lipid\ (\%) = \frac{Total\ weight\ (g) - Tube\ weight\ (g)}{Initial\ weight\ (g)} \times 100$$

Finally, the lipid extract was re-dissolved in C:M (2:1) containing 0.01 % butylated hydroxytoluene (BHT; w/v) as an antioxidant. The extracted lipid was diluted to 10 mg/mL and transferred to 2 mL glass vial, gassed with oxygen-free nitrogen (OFN) and stored at -20 ºC fridge for further analyses.

2.4.2 Lipid analysis

2.4.2.1 Lipid class composition

Lipid class separation was performed using high-performance thin layer chromatography (HPTLC). A 20×10 cm HPTLC silica gel 60 plate (Merck KGaA, Darmstadt, Germany) was vertically soaked in 100 mL C:M (2:1, v/v) in a glass tank until the solvent reach the top of the plate, in order to remove any contamination, and then dried in desiccator overnight. The plate was set up as shown in Figure 2.2. A 10 µL syringe was pre-washed in C:M to load 1.5 µL sample (from 2.4.1.4) and standards on their respective positions. The bottom of the plate was horizontally placed into a TLC glass developing tank with 50 mL of polar lipid mix solvent until the soaked solvent reached the polar solvent mark, approximately half-way, and removed to dry in a desiccator for 15 min. A second run was repeated to the dry plate following the same way in glass tank containing 50 mL of neutral lipid mix until the solvent had reached the top mark. The make-up of the mobile solvents for both the polar and neutral lipid phases are shown in Table 2.1. After drying in the desiccator for 15 min, the sample plate was thoroughly sprayed with 3 % copper acetate and 8 % phosphoric acid then dried in a fume hood. Afterwards, the plate was heated in the oven at 160 ºC for 20 min before cooled down to room temperature and covered with aluminum foil to avoid light. The plate was scanned by TLC Scanner 3 (CAMAG, Muttenz, Switzerland) and visualized on the Wincat software package (CAMAG,

Muttenz, Switzerland).

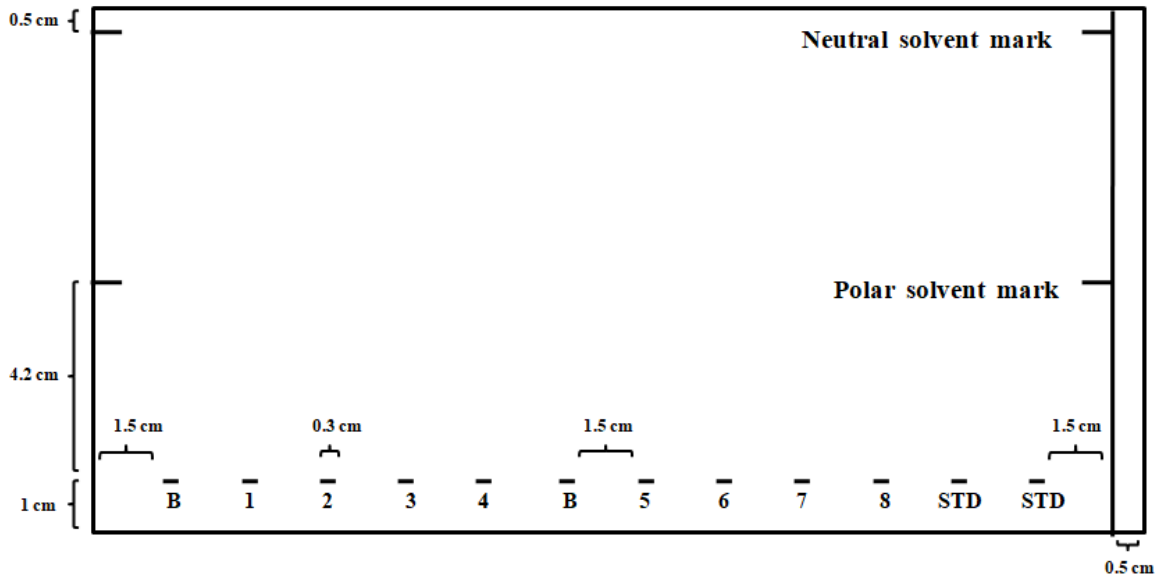


Figure 2.2 TLC plate preparation for lipid class analysis. B: Blank; STD: Standard (left: polar, right: neutral); Lines with numbers: Sample loading positions.

Table 2.1 The compositions of the mix solvent for polar and neutral lipid.

Polar		**Neutral**	
Reagent	Portion	Reagent	Portion
Methyl acetate	25.0	Isohaxane	85.0
Isopropanol	25.0	Diethyl ether	15.0
Chroloform	25.0	Glacial acetic acid	1.5
Methanol	10.0		
0.25 % (w/v) Potassium chloride	9.0		

Note: The solvents were daily prepared to ensure the freshness.

2.4.2.2 Fatty acid composition of total lipid

Fatty acid methyl esters (FAME) were catalysed from total lipid extracts through transesterification as reported by Christie (2003) then extracted and purified as described previously (Tocher and Harvie, 1988). One hundred microliters of total lipid (from 2.4.1.4) and 0.1 mL of heptadecanoic (17:0) free fatty acid standard (1 mg/mL) were mixed in the

first set of test tubes using a 100 µL Hamilton syringe, such that the 17:0 internal standard was 10 % of the total lipid). Following evaporation under a steady stream of OFN, 1 mL of toluene and 2 mL of 1 % methylating reagent (methanol: sulphuric acid=1:99) were added into the tube and vortex mixed. The test tube was gassed with OFN, sealed with a glass stopper and placed on the hot block at 50 ºC overnight (16-18 hours). After methylation, the tubes were cooled to room temperature and 2 mL of 2 % potassium bicarbonate ($KHCO_3$, w/v) and 5 mL of iso-hexane/diethyl ether (1:1, v/v) containing 0.01 % BHT added. After thorough shaking the carbon dioxide produced was released before the tubes were centrifuged at 1500 rpm for 5 min. The supernatant was transferred to the second test tube and the first tube was centrifuged again after adding 5 mL iso-hexane/diethyl ether (1:1, v/v) without BHT. The supernatant was also transferred to the second tube and the combined solvent evaporated under OFN. The FAME in the second tube was re-dissolved by adding 0.5 mL of iso-hexane and purified by using 6 mL sorbent acid washed silica solid-phase extraction cartridges (Clean-up® silica extraction columns; UCT, Bristol, Pennsylvania, USA). Cartridges were rinsed by isohexane before the sample was added, then 10 mL isohexane: diethyl ether (95:5, v/v) was used to elute the FAME. Eluted samples were centrifuged at 1500 rpm for 5 min and evaporated under OFN. Approximately 0.8 to 1.0 mL of iso-hexane was added to re-dissolve the FAME and transferred to a 2 mL glass vial. The vial was gassed with OFN and stored at -20 ºC pending analysis on a gas chromatograph.

The glass vial was loaded onto a Thermo Finnigan AS-2000 autosampler (Thermo Fisher Scientific, Milan, Italy). FAME were separated and quantified by a gas-liquid chromatography (Thermo Finnigan Trace GC, Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), 'on column' injection and flame ionisation detection. Hydrogen was used as the carrier gas at constant pressure (175 kPa) with the initial oven thermal gradient from 50 ºC to 150 ºC at 40 ºC/min, then 195 ºC at 2 ºC/min, 205 ºC at 0.5 ºC/min to a final temperature of 230 ºC at 40 ºC/min. Individual peak of FAME was identified by comparison to the peak graph of an in-house Marine fish oil (Marinol) and known standards (Restek 20-

FAME Marine Oil Standard; Thames Restek UK Ltd., Buckinghamshire, UK). The data were collected and processed using Chromcard data system for Windows (Version 2.11; Thermo Fisher Scientific Inc., Milan, Italy). The peak square was used to calculate the percentage of FAME by normalization method.

2.4.2.3 Fatty Acid composition of polar lipid

The fatty acid profile of lipid classes is based on both the lipid class separation and FAME analysis. Similarly, a 20×20 cm TLC silica gel 60 plate (Merck KGaA, Darmstadt, Germany) was vertically soaked in 100 mL C:M in a glass tank until the solvent reach the top of the plate and then dried in desiccator overnight. The plate was set up as shown in Figure 2.3. A 250 µL Hamilton syringe was pre-washed in C:M to load 200 µL sample (from 2.4.1.4) on their respective positions. To separate the polar lipid from the neutral lipid, the bottom of the plate was placed in to a TLC glass developing tank with 50 mL of neutral lipid mobile phase (see Table 2.1) until the solvent reached the top 'neutral' solvent mark. After drying in the desiccator for 15 min, the plate was sprayed with 0.1 % 2,7, dichlorofluorescein in 97 % (v/v) aqueous methanol. After drying, the plate was placed under UV illumination to identify and mark the bottom polar lipid area on the sample loading positions (shown in Figure 2.4). The silica marking the polar lipid band was scratched into a tube using a scalpel blade before 2 mL of 1 % methylating reagent was added and vortex mixed. The test tube was gassed with OFN, sealed with a glass stopper and placed on the hot block at 50 ºC for methylation overnight (16-18 hours). As with total fatty acid analysis, after methylation the tubes were cooled down to room temperature before the addition of 2 mL of 2 % $KHCO_3$ and 5 mL of iso-hexane/diethyl ether (1:1) with 0.01 % BHT. After thoroughly shaking the carbon dioxide produced was released and the tube centrifuged at 1500 rpm for 5 min. The supernatant was transferred to the second test tube and the first tube was centrifuged again following the addition of 5 mL iso-hexane/diethyl ether (1:1) without BHT. The supernatant was also transferred to the second tube and the combined solvent evaporated under OFN to dryness. FAME was re-dissolved by adding 0.8 to 1.0 mL of iso-hexane and transferred to 2 mL glass

vials, gassed with OFN and kept at -4 ºC. The polar lipid FAME analysis on a gas chromatograph followed the method described in total lipid FAME analysis (See 2.4.2.2).

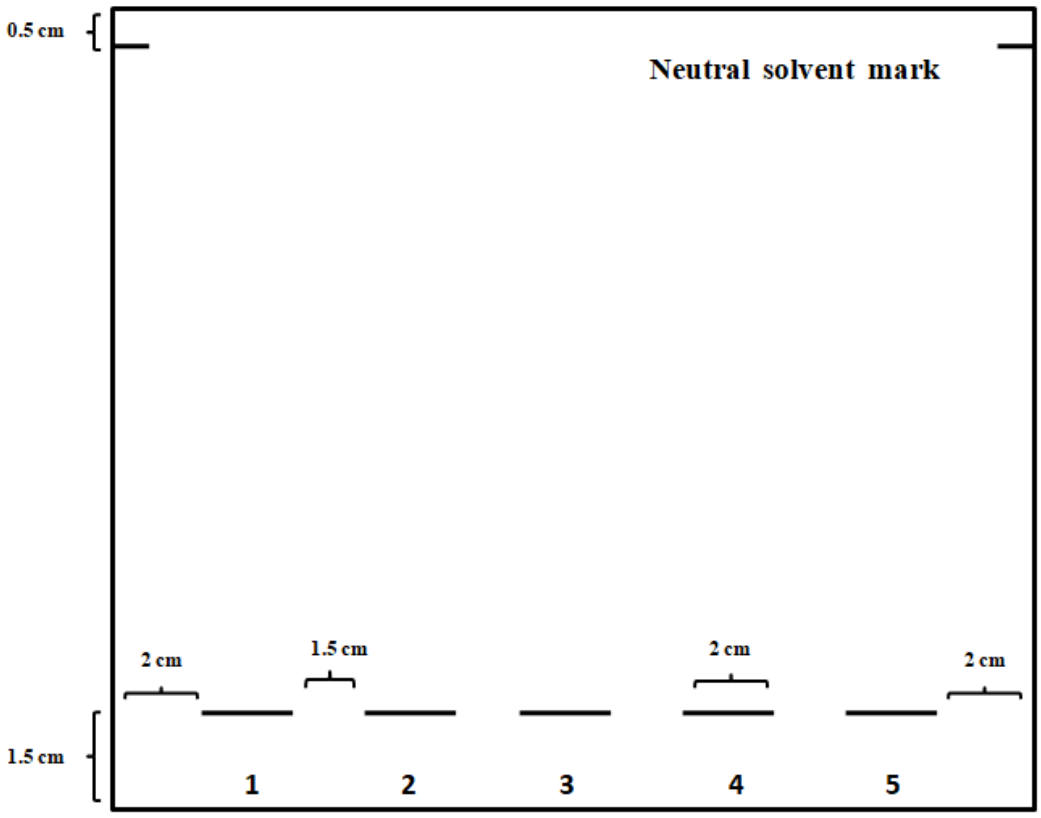


Figure 2.3 TLC plate preparation for fatty acid composition of polar lipid. Lines with numbers: Sample loading positions.

Figure 2.4 TLC plate after spraying under UV illumination with the polar lipid area mark. The polar lipid can be visualized in the red box.

2.5 Histological analysis

2.5.1 Cassette and Dehydration

All the samples in 10 % buffered formalin from each histology pot were divided into different cassettes according to tissue categories (liver or intestine) and cross cut by scalpel. Samples were placed into empty histology cassettes and rinsed consecutively in the programmable automatic tissue processor (Shandon, Citadel 2000, Thermo Scientific, Basingstoke, UK) among a series of dehydrating solvents, before finishing in wax. The machine is programmed as described in Table 2.2.

Table 2.2 Program of the dehydrating and impregnating process for automatic tissue processor.

Step	Solvent	Time (mm:ss)
1	50 % Methylated spirits	00:30
2	80 % Methylated spirits	01:30
3	100 % Methylated spirits	01:30
4	100 % Methylated spirits	01:30
5	100 % Methylated spirits	01:30
6	Absolute ethanol	01:45
7	Absolute ethanol	01:30
8	Chloroform	00:50
9	Chloroform	00:50
10	Paraffin wax	01:45
11	Paraffin wax	01:30
12	Paraffin wax	01:30

2.5.2 Embedding

After samples arrived to the paraffin step in the tissue processor, sample embedding was carried out with a Histo-embedder (Jung, Leica, Germany). The temperature setting of the Histo-embedder is shown in Table 2.3. After filling the mould with wax at the warmer area, the sample was embedded in wax on a mould by cross section facing bottom. The

mould was placed on a cold plate allowing the wax to become solid.

Table 2.3 The temperature setting of different sections for histo-embedder.

Section	Temperature (°C)
Paraffin tank	60
Mould warmer area	40
Cold plate	-5

2.5.3 Trimming and section

The trimming and sectioning for the wax blocks was performed by a microtome (Leica Rotary Microtome 2255). The thickness was set as 20 μm for trimming until the cross sections were just exposed. For sectioning, the thickness was adjusted to 5 μm and the required equipment set up at the right temperature (Table 2.4). The blocks were firstly cooled on the cold plate for sectioning. Next, the sectioned wax slice was laid flat on the water bath surface by forceps and fixed to the glass slide, followed by drying on the hot plate and incubation in a Windsor oven for at least one hour.

2.5.4 Staining and scanning

Two staining methods were used in the present thesis: haematoxylin and eosin (H&E) and periodic acid-Schiff with Alcian blue (PAS-AB). The different steps can be observed in Tables 2.5 and 2.6.

Table 2.4 The temperature setting of equipment for section.

Section	Temperature (°C)
Water bath with distilled water	45
Cold plate	-10
Hot plate	45
Windsor oven for incubation	60

Table 2.5 The procedures of H&E staining.

Step	Solvent	Time (mm:ss)
1	De-waxing xylene 1	03:00
2	De-waxing xylene 2	02:00
3	Absolute alcohol 1	02:00
4	Methylated spirits	01:00
5	Running tap water	01:00
6	Haematoxylin Z	05:00
7	Running tap water	01:00
8	1 % Acid alcohol[1]	Quick 5 dips
9	Running tap water	01:00
10	Scott tap water substitute	01:00
11	Running tap water	01:00
12	Working eosin[2]	05:00
13	Running tap water	01:00
14	Methylated spirits	01:00
15	Absolute alcohol 2	02:00
16	Absolute alcohol 3	01:00

1. 1 % acid alcohol was made by hydrochloric acid and methylated spirit
2. Working eosin was made by Putt's eosin and 1 % eosin aqueous as 1:8.

Lastly, the stained slide was rinsed in clearing xylene then fully glass mounted using Pertex (Medite GmbH, Burgdorf, Germany) for the coverslip. All the slides were scanned by Axio Scan.Z1 slide scanner (ZEISS, Cambridge, UK) and samples were visualized and photographed for further image processing.

Table 2.6 The procedures of PAS staining.

Step	Solvent	Time (mm:ss)
1	De-waxing xylene 1	03:00
2	De-waxing xylene 2	02:00
3	Absolute alcohol 1	02:00
4	Methylated spirits	01:30
5	Running tap water	02:00
6	Alcian blue solution[1]	05:00
7	Running tap water	01:00
8	1 % periodic acid[2]	05:00
9	Running tap water	05:00
10	Schiff's reagent	20:00
11	Running tap water	10:00
12	Haematoxylin Z	02:00
13	Running tap water	01:00
14	Scott tap water	01:00
15	Running tap water	01:00
16	Absolute alcohol 3	03:00

1. Alcian blue solution (pH 2.5) was made by 0.5 g Alcian blue dye dissolved in 3 mL glacial acetic acid and filter then adding 100 mL distilled water.
2. Prepared fresh daily.

2.5.5 Image processing

In the present thesis, Qupath v0.3.0 (Bankhead *et al*., 2017) and ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA.) software were used to view, capture and analyze the images. The details of the analysis about the intestinal section are illustrated in Figure 2.5

2.5.5.1 Hepatic vacuolization

After magnifying each image 20 times, six random fields (fully occupied by liver) were

screenshotted using Qupath v0.3.0. The area was measured by ImageJ from pixels and the hepatic vacuoles area marked by ImageJ from screenshot. The standard level of identifying the area occupied by intracytoplasmic vacuoles was applied to all images. The hepatic intracytoplasmic vacuolization in a single sample is the average of six results from the vacuole area divided by the chosen field area before presented as a percentage.

2.5.5.2 Intestinal circular muscle thickness

Six perpendicular lines were added to the various normal positions on the intestinal circular muscle on the screenshot. The length of the line was regarded as its thickness and measured by Qupath. The circular muscle thickness of a sample was the average of the six measurements.

2.5.5.3 Height and width of enterocyte

Six complete and well-kept villi per slide were selected for this measurement. Several lines were drawn to represent the height of the chosen enterocytes and measured by Qupath. The result of one sample was the average of all the lines drawn on six villi.

Through Qupath a 100 μm straight line was added to each of the six selected villi, in which range all the enterocytes from one side were counted. The width of the enterocyte was calculated as 100 μm divided by the enterocyte amount. The width of one sample was the average of the six results.

2.5.5.4 Goblet cell density

Six random fields containing goblet cells from each intestine image were screenshotted at 5 × magnification. The area was measured by ImageJ after the removal of margin space. The goblet cell was marked and counted by ImageJ from screenshot to obtain the cell amount. The results of goblet cell density were calculated as the cell amount divided by the area of the chosen intestinal villi and presented as amount per 10^5 μm^2.

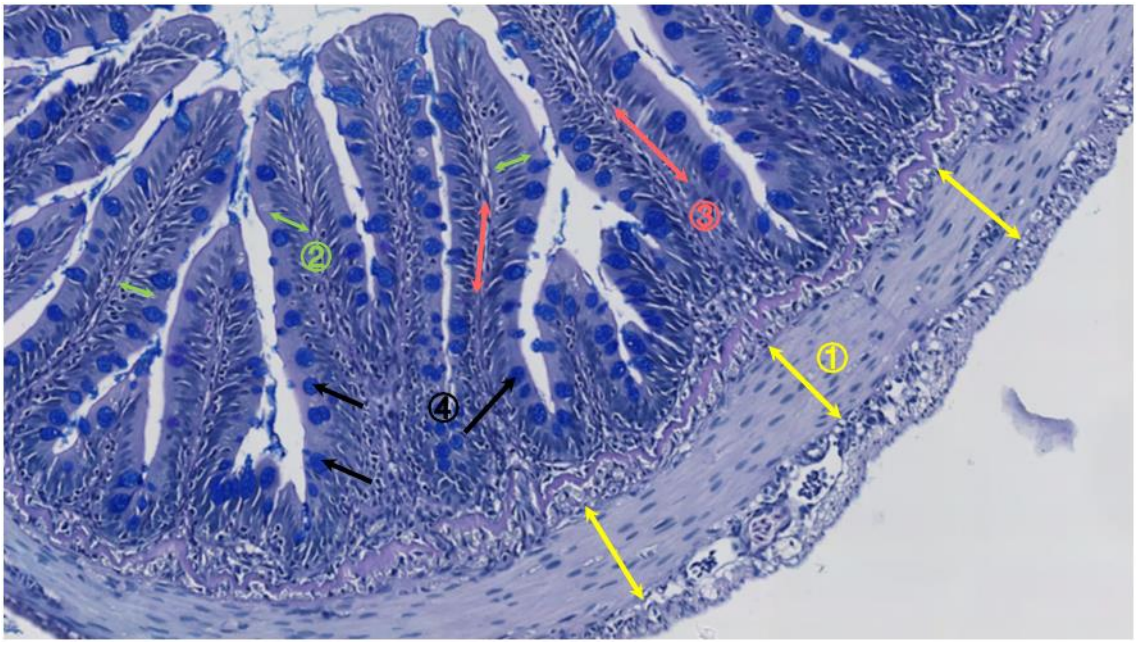


Figure 2.5 Intestinal section of salmon with PAS-Alcian Blue staining. (1) The measurements of intestinal circular muscle thickness (yellow arrows); (2) The measurements of enterocyte height (green arrows); (3) Areas of counting enterocyte amounts for the width measurements (red arrows). (4) Goblet cell (black arrows).

2.6 Molecular analysis

2.6.1 RNA extraction

Tissue samples (about 50 mg) were transferred to a tube with 1 mL of TRI-Reagent (Sigma-Aldrich, Dorset, UK) using forceps pre-washed with ethanol. After 10 min, the sample was completely homogenized using a bead tissue disruptor (BioSpec, Bartlesville, OK, USA) for 45 seconds and centrifuged at 4 ºC at 12,000 RCF for 10 min. The aqueous phase was then transferred to a 1.5 mL Eppendorf tube. After adding 100 µL of 1-bromo-3- chloropropane (BCP), the tube was shaken vigorously for 15 seconds and left to stand for 20 min before centrifuging at 4 ºC at 20000 RCF for 15 min. The supernatant containing RNA was carefully transferred by pipetting to another Eppendorf tube (maximum 300 µL, ensuring that the mid protein layer was not touched), then 150 µL RNA precipitation and isopropanol were added in order followed by a slight shaking of

the tube before being kept into -20 ºC freezer for 30 min. Afterwards, the tube was centrifuged again at 4 ºC and 20,000 RCF for 10 min, before all the liquid was slowly discarded from the tube with the RNA pellet retained inside. One millilitre of 75 % ethanol was added to rinse the RNA before vortexing to float the RNA pellet. The tube was then centrifuged at 4 ºC and 7,000 RCF for 5 min before the liquid was removed. This process was performed twice in order to thoroughly rinse the RNA pellet. The tube was then placed in the fume hood to dry the RNA pellet until it turned transparent, while the RNA free water was heated in a hot block at 50 ºC. According to the pellet, 30-50 µL heated RNA free water was added to dissolve the RNA pellet. Lastly the tube was incubated at 50 ºC in a hot block for 5 min before further analysis.

2.6.2 Quality check

RNA purity was assessed using a Nanodrop Spectrophotometer ND-1000 (Labtech Int., East Sussex, UK). The absorbance ratio at 260: 280 for pure RNA was considered adequate between 1.8 to 2.0, among which the extracted RNA was considered as high purity without protein contamination (McKenna *et al*., 2000). The lower ratio might suggest the inclusion of protein or phenol contamination arising from the extraction process, which shows high absorption near 280 nm (Wilfinger *et al*., 1997). The integrity of the RNA was then checked by running a sample of RNA on an agarose gel through electrophoresis. The 1 % agarose gel was prepared by mixing 25 mL of 0.5 × Tris-acetate-EDTA (TAE) and 0.25 g of agarose, then heating the mixture in a microwave before the addition of 0.82 µL ethidium bromide (EB) as a fluorescent dye. For sample loading, a 5 µL solution containing 250 mg RNA was prepared followed by the addition of 1 µL of loading dye and quick centrifugation. Before loading in the gel, the RNA sample was heated at 75 ºC for 5 min using a thermocycler (Eppendorf, UK). The 5 µL sample was then loaded and run at 50 V for 10 min, then 80 V for 30 min. The result on the gel was visualized under UV illumination. The presence of two clear subunits of ribosomal RNA (28s and 18s) indicated the ideal integrity of the extracted RNA (Figure 2.6).

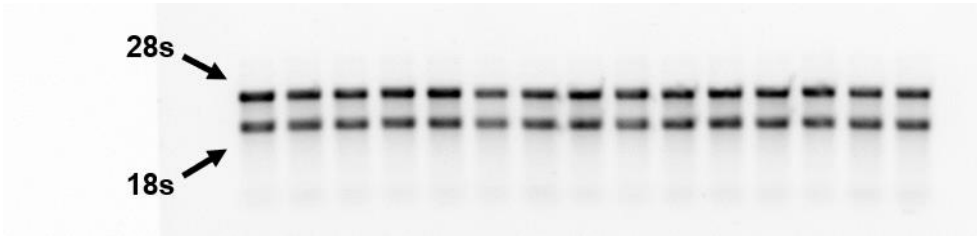


Figure 2.6 Agarose gel result showing two subunits of ribosomal RNA (28s and 18s).

2.6.3 cDNA synthesis

The complementary DNA (cDNA) was synthetized from messenger RNA via reverse transcription using transcriptase on one strand of RNA to obtain a strand of cDNA. For each tank, a known amount of RNA was taken from each sample and mixed into two pools. From each pool, 2,000 ng was collected to make up a 10 µL solution. Then, all the samples were heated in a thermocycler at 75 ºC for 5 min and cooled to 12 ºC before a quick centrifuge.

In the intervening time, the master mix was prepared from the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK). For each sample, the reagents were added in an Eppendorf tube following the products and order shown in Table 2.7 to make up a 10 µL of master mix.

Table 2.7 The composition of master mix for cDNA transcription.

Order	Reagent	Volume (µL)
1	Nuclear free water H_2O	4.2
2	10× RT buffer	2.0
2	25× dNTP Mix (100 mM)	0.8
2	10× RT Random Primer	1.5
2	0.1× Oligo dT Primer	0.5
3	Multiscribe Reverse Transcriptase	1.0

The master mix was quickly centrifuged and added to the sample tube and made up to a 20 µL reaction. A 20 µL negative control sample (non-template control, NTC) was prepared as 10 µL master mix with 10 µL nuclease free water, instead of the RNA sample, in order to check for any contamination in the master mix. In addition, a second master mix was produced to check the presence of gDNA in the sample. In this case, the reverse transcriptase (RT-) was substituted by nuclease free water. Only some selected samples of RNA were used for the RT- (approximately 10 %). The reaction was centrifuged and placed in pre-heated thermocycler at 25 ºC before continuing the cycle shown in Table 2.8. When finished, the sample was 20-fold diluted for further qPCR analysis. In addition, undiluted cDNA from all the samples were pooled in order to produce the standard curve for primer efficiency calculation.

Table 2.8 The temperature and time settings for reaction in the thermocycler.

Step	Temperature (ºC)	Time (min)
1	25	10
2	37	120
3	85	5
4	12	5

2.6.4 Quantitative real-time polymerase chain reaction

Before each Quantitative real-time polymerase chain reaction (RT-qPCR) run, the efficiency of every pair of primers was evaluated for each target gene using a standard curve. The standard curve was generated from a set of reactions with serial dilutions (1:5, 1:10, 1:15, 1:20 and 1:25) using pooled cDNA derived from the samples by plotting the threshold cycle (Ct) values. The slope of the linear regression was used to determine the efficiency using the equation: $E = (10^{-1/slope}-1) \times 100$. The efficiencies of the primers used in the present thesis were confirmed to be higher than 85 % and lower than 110 %.

RT-qPCR determines the expression changes in the mRNA level of a gene of interest (GOI) by comparison to the expression of the housekeeping gene (HK) through normalization. The Thermo Scientific Luminaris Color Higreen RT-PCR master mix kit (Thermo Scientific, Hemel Hempstead, UK) was used to prepare the master mix for qPCR, according to the manufacturer instructions. The master mix was quickly centrifuged and loaded on a 96-well microplate with the diluted cDNA sample including NTC (See 2.6.3) individually to make up a 10 µL reaction. The composition of the master mix and cDNA sample amount are listed in Table 2.9 and the primers information of all GOI and HK used in the present thesis is shown in Table 2.10.

Table 2.9 The composition of the reaction for qPCR analysis (µL).

	Reagent	HK	GOI
Master mix	Nuclear free H_2O	3.0	1.5
	Forward primer	0.5	0.5
	Reverse primer	0.5	0.5
	SYBR Green mix	5.0	5.0
cDNA sample		1.0	2.5
Total		10.0	10.0

HK, housekeeping gene; GOI, gene of interest.

The GOI and HK were run separately on different microplates with the HK run first. Each sample was loaded in duplicate on the microplate to reduce error. After a quick vortex, or spin, the microplate was loaded on the qTower^3 G real-time PCR Thermal Cycler (Analytic Jena GmbH, Jena, Germany). The condition setting for each microplate is shown in Table 2.11.

Table 2.10 Sequences of PCR primers used in the present thesis for qPCR including amplicon sizes and annealing temperatures (Tm).

Category	Gene	Forward sequence (5'→3')	Reverse sequence (3'→5')	Amplicon	Tm (ºC)	Accession no.
Fatty acid biosynthesis related	*fads2d6*	TCCTCTGGTGCGTACTTTGT	AAATCCCGTCCAGAGTCAGG	163	59	NM_001123575.2[a]
	fads2d5	GCCACTGGTTTGTATGGGTG	TTGAGGTGTCCACTGAACCA	148	59	NM_001123542.2[a]
	elovl2	GGTGCTGTGGTGGTACTACT	ACTGTTAAGAGTCGGCCCAA	190	59	NM_001136553.1[a]
	elovl5a	TGTTGCTTCATTGAATGGCCA	TCCCATCTCTCCTAGCGACA	150	59	GU238431.1[a]
	elovl5b	CTGTGCAGTCATTTGGCCAT	GGTGTCACCCCATTTGCATG	192	59	NM_001136552.1[a]
Transcription factors	*srebp1*	GCCATGCGCAGGTTGTTTCTTCA	TCTGGCCAGGACGCATCTCACACT	151	63	TC148424[b]
	srebp2	TCGCGGCCTCCTGATGATT	AGGGCTAGGTGACTGTTCTGG	147	63	TC166313[b]
	lxr	GCCGCCGCTATCTGAAATCTG	CAATCCGGCAACCAATCTGTAGG	210	58	FJ470290[a]
	pparα	TCCTGGTGGCCTACGGATC	CGTTGAATTTCATGGCGAACT	111	60	DQ294237[a]
	pparγ	CATTGTCAGCCTGTCCAGAC	TTGCAGCCCTCACAGACATG	144	60	AJ416951[a]
Lipid catabolism related	*aco*	AAAGCCTTCACCACATGGAC	TAGGACACGATGCCACTCAG	230	60	TC49531[b]
	cptI	CCTGTACCGTGGAGACCTGT	CAGCACCTCTTTGAGGAAGG	212	60	AM230810[a]
	hmgcr	CCTTCAGCCATGAACTGGAT	TCCTGTCCACAGGCAATGTA	224	60	DW561983[a]
	fas	ACCGCCAAGCTCAGTGTGC	CAGGCCCCAAAGGAGTAGC	212	60	CK876943[a]
Housekeeping genes	*hprt*	GATGATGAGCAGGGATATGAC	GCAGAGAGCCACGATATGG	165	60	BT125296.1
	ef1a	CTGCCCCTCCAGGACGTTTACAA	CACCGGGCATAGCCGATTCC	175	60	AF321836[a]
	rpl2	CTGCCCCTCCAGGACGTTTACAA	TGTTCACAGCTCGTTTACCG	112	60	XM_014137227.1[a]

GOI: *fads2d6*, delta-6 fatty acyl desaturase; *fads2d5*, delta-5 fatty acyl desaturase; *elovl2*, fatty acyl elongase 2; *elovl5a*, fatty acyl elongase 5 isoform a; *elovl5b*, fatty acyl elongase isoform b; *srebp1*, sterol regulatory element binding protein 1; *srebp2*, sterol regulatory element binding protein 2; *lxr*, liver X receptor; *pparα*, peroxisome proliferator-activated receptor alpha; *pparγ*, peroxisome proliferator-activated receptor gamma; *aco*, acyl-CoA oxidase; *cptI*, carnitine palmitoyltransferase I; *hmgcr*, 3-hydroxy-3-methyl-glutaryl-CoA reductase;

HK: *hprt*, hypoxanthine-guanine phosphoribosyltransferase; *ef1α*, elongation factor 1 alpha; *rpl2*, ribosomal protein L2; They were considered as the most stable according to the geNorm.

[a]GenBank (http://www.ncbi.nlm.nih.gov/)

[b]Atlantic salmon Gene Index (http://compbio.dfci.harvard.edu/tgi/)

Table 2.11 The PCR cycle conditions for amplication.

	Step	Temperature (ºC)	Time
	1	50	2 minutes
	2	95	10 minutes
×35 cycles	3	95	15 seconds
	4	Tm (See Table 2.10)	30 seconds
	5	72	30 seconds
	6	75-95	0.5 ºC increment

2.6.5 Calculations

In the present thesis, CT was used to calculate the relative expression of the GOI compared to all the HK. CT values obtained from PCR refers to the amplication cycle numbers at which the fluorescence distinguishes appreciably from the background fluorescence generated by the PCR. The method was generally based on the Pfaffl equation (2001) in order to obtain the relative expression ratio from the formulation below:

$$R = \frac{{E_{GOI}}^{\Delta \mathrm{CT}}}{GeoMean\left({E_{\mathrm{HK}}}^{\Delta \mathrm{CT}}\right)}$$

where, R is relative expression ratio; E is PCR efficiency+1; The control was defined in each chapter, respectively.

$$\Delta \mathrm{CT} = \mathrm{CT}_{Control} - \mathrm{CT}_{Sample}$$

$$GeoMean\left({E_{\mathrm{HK}}}^{\Delta \mathrm{CT}}\right) = \left(\prod_{i=1}^{n} {E_{\mathrm{HK}i}}^{\Delta \mathrm{CT}}\right)^{\frac{1}{n}}$$

2.7 Statistics

The statistical analysis in the present thesis was performed using SPSS 17.0 (SPSS Inc. Chicago, USA) and GraphPad Prism 8.0.2 (San Diego, CA, USA). A significant level of 5 % (P<0.05) was set for all tests performed. All data are presented as mean ± standard deviation (SD), where the sample means provide the estimated average of the whole population and SD represents the data distribution. Multivariate Principal Component Analysis (PCA) was performed to summarize the observations from various dimensions using PAST 4.0 software (Hammer *et al*., 2001). All data presented in percentage format was arcsine transformed prior to statistical analysis. It should be noted that, tank has been considered as the experimental unit (n=3) in the case of chemical analysis, whereas two pools per tank (n=6) were used for gene expression and six fish per tank (n=18) were sampled for histological evaluation. The number of replicates were selected based on Power analysis performed using GPower 3.1.9.7. Differences in sample size reflect the different width of effects among the analysis in order to obtain a power ≥0.80.

2.7.1 Normality testing and homogeneity of variance

All data were examined for the normality of distribution by Shapiro-Wilk's test to test whether the data were Gaussian distributed. Then, the homogeneity of variance between groups was examined by Levene's test.

2.7.2 Parametric test

For those variables which met the conjecture by satisfying the normality and homogeneity test at the same time, a parametric test was carried out to analyse the data.

The one-way analysis of variance (ANOVA) was used to compare the means of three or more independent groups and detect whether statistically significant differences exist. When data differed significantly, a Tukey's multiple comparison *post hoc* test was conducted to determine the cause of the differences.

The Student's t-test was performed to examine the significant differences between two groups. In the present thesis, both paired and independent t-test were applied. Paired t-test was used in Chapter 4 and independent t-test was conducted in Chapter 5.

The two-way ANOVA was performed in Chapter 5 to determine the effects of factors and their interaction among three of more independent groups. When the interaction was observed to be significant, the data was regarded to be mainly impacted by factors. When the significance was found in a single factor, it was considered as the main factor and a Tukey's multiple comparison *post hoc* test was conducted.

2.7.3 Nonparametric test

For data which did not show a normal distribution or homogeneity of variance, these were subjected to log or arcsine transformation first and examined. If the data still failed to satisfy the assumptions for parametric tests, the nonparametric method was used. A Kruskal-Wallis test was performed to detect the significant differences. When a significance was found, Dunn's multiple comparison post-hoc test was conducted.

3 CHAPTER

Endogenous production of n-3 long-chain PUFA from first feeding and the influence of dietary linoleic acid and the α-linolenic:linoleic ratio in Atlantic salmon (*Salmo salar*)

Part of the information contained in the present Chapter can be found in **Sprague** *et al*., (2019)

3.1 Abstract

Atlantic salmon (*Salmo salar*) are able to produce n-3 long-chain PUFA (LC-PUFA) such as EPA and DHA from α-linolenic acid (ALA) endogenously. The enzymes involved are also available for n-6 LC-PUFA endogenous biosynthesis including arachidonic acid (ARA) from linoleic acid (LA) as a competition with ALA for LC-PUFA. In order to quantify the endogenous production of EPA and DHA from ALA in salmon fed marine-free diets as well as to investigate the influence of different dietary LA and ALA:LA ratios on LC-PUFA production, a 22-week first feeding trial was conducted using three experimental diets formulated with linseed and sunflower oils to provide different ALA:LA ratios of approximately 3:1, 1:1 and 1:3, respectively. Endogenous n-3 LC-PUFA production was 5.9, 4.4 and 2.8 mg/g of fish, whereas n-6 LC-PUFA production was 0.2, 0.5 and 1.4 mg/g of fish when the dietary ALA:LA ratios were 3:1, 1:1 and 1:3, respectively. As the dietary ALA:LA ratio decreased, the ratio of n-3:n-6 LC-PUFA production decreased from 27.4 to 2.0, whereas the DHA:EPA ratio increased and EPA:ARA and DHA:ARA ratios subsequently decreased. When the dietary ALA:LA ratio was 1, the daily production of n-3 LC-PUFA in salmon fry/parr was 28.4 μg/g fish, with a DHA:EPA ratio of 3.4. The production of n-3 LC-PUFA was almost nine-fold that of n-6 LC-PUFA. The n-3 LC-PUFA production, EPA:ARA and DHA:ARA ratios were reduced following the decreasing dietary ALA:LA ratio but the n-6 LC-PUFA production and DHA: EPA ratio were increased. The present study shows that Atlantic salmon are capable of producing EPA and DHA from ALA, the biosynthesis capacity was evaluated quantitatively in order to better utilise the oils containing differing proportions of ALA and LA.

Key words: Arachidonic acid; Biosynthesis; Desaturation; DHA:EPA; Elongation;

3.2 Introduction

Being the key essential nutrients of the human diet, omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA), eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), provide an array of health benefits through various functions (Calder, 2014; 2018), such as a structural component of cellular membranes (Sherratt and Mason, 2018) and regulator of gene expression (Schmuth *et al*., 2014). Despite all this, many effects are not taken independently but from their antagonism of n-6 PUFA metabolism particularly arachidonic acid (ARA, 20:4n-6) (Lands, 2014). The pro-inflammatory effects of the high and imbalanced dietary n-6 PUFA content, featured in the Western-type diet, can be mitigated via blood homeostasis and the regulation of inflammation by eicosanoids and other highly biologically active derivatives produced from EPA and DHA as precursors (Simopoulos *et al*., 2006; 2011; Wang *et al*., 2014; Weylandt *et al*., 2012). In addition, dietary n-3 LC-PUFA are crucial in neural development and function (Innis, 2007; Campoy *et al*., 2012), and play a beneficial role in multiple pathological conditions including certain inflammatory diseases, some cancers, and cardiovascular disease (Delgado-Lista *et al*., 2012; Miles and Calder, 2012; Gerber, 2012; Calder, 2015).

Fish and seafood are the major n-3 LC-PUFA source from diet for human. In particular, oily fish such as Atlantic salmon (*Salmo salar*) can accumulate large amounts of n-3 LC-PUFA from food rich in EPA and DHA in the aquatic environment as well as being able to produce n-3 LC-PUFA endogenously (Bell and Tocher, 2009; Tocher, 2009; Kabeya *et al*., 2018). Thus, salmon is considered as an ideal source in supplying n-3 LC-PUFA for consumers effectively (Tur *et al*., 2012; Henriques *et al*., 2014; Sprague *et al*., 2016). However, the finite nature of the wild fisheries industry, being at or beyond their sustainable limits, can hardly meet the growing demand by the expanding population. Consequently, aquaculture now shoulders the responsibility and presently supplies more than half of all seafood and fish (FAO, 2022). Nevertheless, aquafeeds are still heavily reliant on the inclusion of fishmeal (FM) and fish oil (FO) to provide n-3 LC-PUFA.

However, FM and FO are derived from the marine fisheries which limits the expansion of the industry due to the sustainability issue (Tocher, 2015). Regarding the availability and cost of the oil perspective, plant-based vegetable oils (VO) from agriculture are typically utilized to replace FO in aquafeeds (Gatlin III *et al*., 2007; Hardy, 2010; Turchini *et al*., 2010). The replacement by VO will lower the n-3 LC-PUFA level in feeds, due to VO lacking n-3 LC-PUFA, which subsequently impacts upon the final flesh quality of farmed fish (Sprague *et al*., 2016; Tocher, 2015).

In contrast, plant-based oils are rich sources of C18 PUFA, characterized by both α-linolenic acid (ALA; 18:3n-3) and linoleic acid (LA; 18:2n-6), which are the precursors of ARA, EPA and DHA. Atlantic salmon are capable of endogenously producing ARA, EPA and DHA from these precursors with the required set of genes and enzymes (Tocher, 2010; Castro *et al*., 2016). It is notable that salmon has been reported to possess *fads2* genes, which code for specific desaturase with separate Δ6 and Δ5 activities, and fatty acyl elongase 5 (*elovl5*) that are essential for the production of EPA from ALA (Hastings *et al*., 2004; Zheng *et al*., 2005a; Monroig *et al*., 2010a). The existence of Δ6 fatty acyl desaturase (*fads2d6*), fatty acyl elongase 2 (*elovl2*) and fatty acyl elongase 4 (*elovl4*) can further produce DHA from EPA (Morais *et al*., 2009; Carmona-Antoñanzas *et al*., 2011). As a precursor, the presence of LA in the same LC-PUFA biosynthesis pathway will compete with ALA and be converted into n-6 LC-PUFA particularly ARA using the same enzymes (Bell and Tocher, 2009).

There have been several studies focusing on the LC-PUFA biosynthesis in an attempt to measure the endogenous production of EPA and DHA from different ALA and LA levels in salmon hepatocyte or at juvenile stage (Ruyter *et al*., 2003; Berge *et al*., 2009; Turchini *et al*., 2010). However, quantitative studies regarding the precise impact of the dietary ALA:LA ratio on the biosynthesis in Atlantic salmon from first feeding still needs to be explored. Thus, the main aim of the present study was to quantify the endogenous production of EPA and DHA from ALA in salmon fed from first feeding on diets that contain no EPA and DHA. In addition, this study aims to determine the influence of

dietary LA and ALA: LA ratio on LC-PUFA production.

3.3 Materials and methods

3.3.1 Ethics statement

Ethical approval for the trial was provided by the University of Stirling's (UoS) Animal Welfare and Ethical Review Board (AWERB No: 19 20/055/New ASPA), together with the Norwegian national legislation via the Norwegian Animal Welfare Act (LOV-2015-06-09-19-65) Regulations on the Use of Animals in Experiments (FOR-2017-04-05-451) that was amended to implement the requirements contained in the EU regulations for animal research (Directive 2010/63/EU). All the experimental procedures were conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice, HMSO, London, January 1997) and EU regulations (EC Directive 86/609/EEC).

3.3.2 Dietary trial

Approximately, 3,000 Atlantic salmon eggs were obtained from AquaGen (Trondheim, Norway) and transferred to the Institute of Marine Research (IMR, Matre Research Station) under standard rearing conditions after hatching until the alevin stage for the feeding trial. A triplicated feeding trial (initial weight ~0.18 g) was run for 22 weeks. Three isoenergetic (digestible energy, DE=18 $MJ.kg^{-1}$) and isoproteic (digestible protein, DP=49 %; DP:DE, 27) diets with 15 % of lipid were formulated and manufactured at the UoS (Table 3.1). These marine-free diets contained linseed and sunflower oils in different ratios to provide three differing feeds with ALA:LA ratios of approximately 3:1 (diet A), 1:1 (diet B) and 1:3 (diet C). The ground ingredients were mixed with oil and water then prepared as two batches through either a 1 or 2 mm mould using a screw press cold extruder (DollyTM; Imperia & Monferrina S.p.A). After drying overnight at 40 ºC, feeds were ground and sieved to produce pellet sizes of 0.5-0.8, 0.8-1.0, 1.0-1.5 and 1.5-2.0 mm. The reference (REF) group was fed a commercially available marine-based feed (formulation not known), typically used in salmonid production, which was intended to

act as a guide (reference) on which to base results. The fatty acid compositions of all four groups are shown in Figure 3.1, and the ratios of ALA:LA were 2.61, 0.95 and 0.36 in diets A, B and C, respectively. Concerning the plant-based diet acceptance, all alevins/fry were distributed into four tanks (1 × 1 m) with 750 fish per tank for the higher fish density to ensure a good feeding response. Feeding was performed manually for the first three-week phase in order to observe intake. When fish reached approximately 0.5 g, they were redistributed into twelve (four groups in triplicates) 3 × 1 m diameter tanks (500 litres volume). The rearing water temperature was set as 13 ºC and the tanks were exposed to 24 hours artificial light. Different pellet sizes of diet were given according to fish size by automatic feeders Arvo-tec TD2000 (Arvo-Tec Oy: Huutokoski, Finland).

3.3.3 Sampling

Before first feeding, a triplicate sample of 50–60 alevins (approximately 10 g wet weight) were euthanized by an anesthetic overdose (tricaine methanesulfonate, MS-222; 400 mg/L in hydrogen carbonate-buffered solution; Pharmaq®, UK) as initial samples. After being bulk weighed on an electronic top-loading balance to 0.1 g accuracy, the initial samples were immediately frozen in liquid nitrogen and stored at –70 ºC for further compositional analyses. At the end of the trial, forty fish per tank were euthanized in the same way as described above before weighed individually to 0.1 g accuracy. Of the fish sampled, twenty whole fish were collected as two portions of pooled ten fish (n=6 per group) and immediately frozen in liquid nitrogen and stored at –70 ºC prior to analyses. A further ten fish were dissected for tissue collection; the liver, brain, white muscle, gill, eye and intestine of every five fish were pooled respectively and immediately stored in liquid nitrogen. The last ten fish were dissected for molecular and histological analysis, part of the collected liver was stored into RNALater (Sigma, Poole, UK) and kept overnight at 4 ºC before freezing at –70 ºC for RNA extraction. The other part was placed in 2 mL 4 % buffered formalin for further histology analysis.

Table 3.1 The formulations and analysed proximate compositions of experimental feeds.

Group	**A**	**B**	**C**
Feed ingredients (%)			
Soya protein concentrate[1]	20.0	20.0	20.0
Soya protein isolate[1]	20.0	20.0	20.0
Wheat gluten[1]	20.0	20.0	20.0
Hydrolysate[2]	2.0	2.0	2.0
Casein[3]	1.7	1.7	1.7
Linseed oil[4]	13.4	9.0	4.4
Sunflower oil|[5]	0.0	4.4	9.0
Cellulose[6]	7.7	7.7	7.7
Pregelled starch[7]	7.0	7.0	7.0
Crystalline amino acids[8]	2.6	2.6	2.6
Premix[9]	0.5	0.5	0.5
$CaHPO_4$[1]	5.0	5.0	5.0
Yttrium oxide[10]	0.1	0.1	0.1
Proximate			
Dry matter (%)	94.4	94.3	96.0
Ash (%)	7.3	7.1	7.8
Crude protein (%)	50.8	49.5	50.5
Digestible protein (%)	48.9	48.9	48.9
Lipid (%)	15.7	15.5	13.8
Gross energy (KJ/g)	18.0	18.1	18.1

[1] BioMar Ltd.

[2] HP1 from Aquativ.

[3] Bulk Powders Ltd.

[4] Cold-pressed, AniForte UK Ltd.

[5] Sainsbury's Supermarkets Ltd.

[6] Microcrystalline cellulose, Blackburn Distributions Ltd.

[7] Sigma Pharmaceuticals.

[8] Includes methionine (1 %), lysine (1 %), taurine (0.5 %) and also choline (0.1 %).

[9] OVN Salmonid from DSM Nutritional Products.

[10] Stanford Materials.

3.3.4 Lipid content, class composition and fatty acid analyses

Total lipid (TL) in the feed and tissue samples was extracted by homogenization in 20 mL of chloroform-methanol (2:1, v/v) using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK) and determined based on the method of Folch *et al*. (1957). The extracted TL was prepared as a 10 mg/mL solution in chloroform: methanol (2:1) + 0.01 % (w/v) BHT for further analysis.

About 200 µl (2 mg) of total lipid was loaded on to 4 cm origins on 20×20 cm high-performance thin layer chromatography (HPTLC) plates (VWR, Lutterworth, UK) to analyse the lipid class by the method from Henderson and Tocher (1992). Soaking the plates in mixed solvent separated phospholipids (PL) and triacylglycerols (TAG) to different positions and the solvent was prepared as isohaxane: diethyl ether: glacial acetic acid (85:15:1.5). The identified PL and TAG was sprayed with 0.1 % (w/v) 2-7-dichlorofluorescein in 97 % methanol (v/v) and visualized under UV light at 240 nm (UVP® Mineralight®R-52G; UVP Inc.). They were scraped into glass tube for fatty acid analyses.

Fatty acid methyl esters (FAMEs) were prepared from TL by acid-catalysed transmethylation at 50 ºC for 16 h, and were extracted and purified as described previously (Christie 2003; Tocher and Harvie, 1988). FAMEs were separated and quantified by a gas-liquid chromatography (Thermo Finnigan Trace GC, Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), on column injection and flame ionisation detection. Hydrogen was used as the carrier gas at constant pressure (175 kPa) with the initial oven thermal gradient from 50 ºC to 150 ºC at 40 ºC.min^{-1}, then 195 ºC at 2 ºC.min^{-1}, 205 ºC at 0.5 ºC.min^{-1} to a final temperature of 230 ºC at 40 ºC.min^{-1}. Individual peak of FAME was identified by comparison to the peak graph of an in-house Marine fish oil (Marinol) and known standards (Restek 20-FAME Marine Oil Standard; Thames Restek UK Ltd., Buckinghamshire, UK). The collected data were processed by Chromcard data system

(Version 2.11; Thermo Fisher Scientific Inc., Milan, Italy). The percentage of FAME was calculated from the peak square by the normalization method. Heptadecanoic acid (17:0) was used as internal standard to calculate the absolute fatty acid content in tissues.

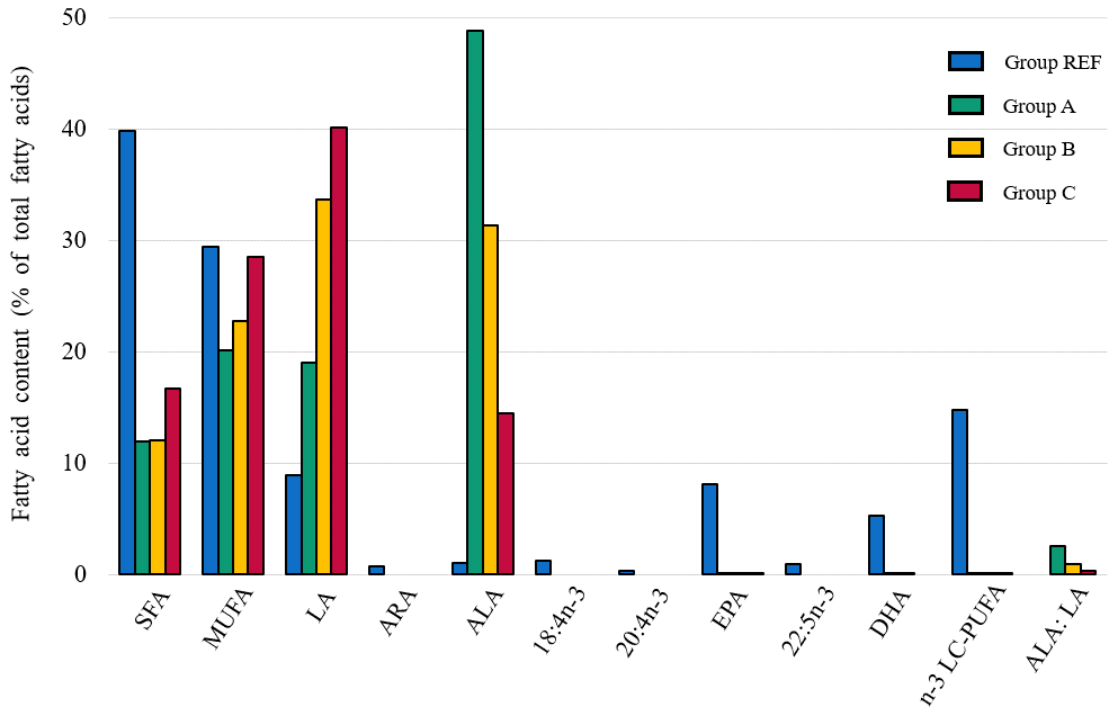


Figure 3.1 Fatty acid composition (percentage of total fatty acids) of experimental feeds.

3.3.5 Molecular analyses

The liver sample from each fish was homogenized to extract the RNA by 1 mL of TriReagent® (Sigma-Aldrich, Dorset, UK). The quantity, quality and concentration of extracted RNA was checked by spectrophotometry (Nanodrop ND-1000; Labtech Int., East Sussex, UK) and 250 ng of total RNA in 1 % agarose gel electrophoresis was used to access the integrity. The extracted RNA from ten samples per tank were pooled into two samples (five fish per sample) and 2000 ng RNA from samples were used for cDNA synthesis in a 20 µL reaction volume according to the high-capacity reverse transcription kit following the manufacturer's protocol (Applied Biosystems, Warrington, UK). The obtained cDNA was diluted 20-fold with milliQ® water (Millipore UK Ltd., Watford, UK).

Expression of genes of interest (GOI) involved in the key pathways include LC-PUFA biosynthesis (*fads2d6; fads2d5, elovl2, elovl5a, elovl5b*), transcription factors (*srebp1, srebp2, lxr, pparα, pparγ*) and lipid metabolism (*fas, hmgcr, aco, cpt1*). The expressions were determined by real-time quantitative PCR, as described in detail by Betancor *et al*., (2018), using a BiometraTOptical Thermocycler (Analytik Jena, Goettingen, Germany) in ninety-six well plates in duplicate 10 µL reaction volumes of mastermix consisted of 5 µL of Luminaris Colour HiGreen qPCR MasterMix (Thermo Scientific, Hemel Hempstead, UK), 1.0 µL of the primers corresponding to the analysed gene, 1.5 µL of molecular biology grade water and 2.5 µL of cDNA while 1 µL of cDNA and 3 µL of molecular biology grade water for housekeeping genes accordingly (detail shown in Table 2.10). A negative control without cDNA (no template control) was included to check for any contamination. Standard amplification parameters contained a DNase pre-treatment at 50 ºC for 2 min, an initial activation step at 95 ºC for 10 min, followed by thirty-five cycles: 15 s at 95 ºC, 30 s at the annealing Tm and 30 s at 72 ºC.

The results of the target gene expression by fold change were normalised and calculated following the method of Pfaffl (2001) using reference housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase (*hprt*), elongation factor 1 alpha (*ef1α*) and ribosomal protein L2 (*rpl2*) that were considered as the most stable according to the geNorm.

3.3.6 Histology analyses

Liver samples were fixed in 4 % buffered neutral-formaldehyde Shandon Citadel 2000 Automatic Tissue Processor (Thermo Scientific, Basingstoke, UK). Samples were then embedded into paraffin blocks using a Histo-embedder (Jung, Leica, Germany) and fixed on slides after sectioning into 5 µm thickness wax slices. The samples were stained with haematoxylin-eosin (H&E) and scanned by Axio Scan.Z1 slide scanner (ZEISS, Cambridge, UK) for digital images. The results were analysed using Qupath v0.3.0 (Bankhead *et al*., 2017) software. The stained sections of liver were assessed to evaluate

the cytoplasmic lipid vacuolisation and inflammation using a four graded examination scheme: 0, not observed; 1, few; 2, medium and 3, severe.

3.3.7 Statistical analyses

All data are presented as mean ± standard deviation (SD) for each group. All percentage data were subjected to arcsine square-root transformation for statistical analyses. Data were tested for normality and homogeneity using Shapiro–Wilk test and Levene's test. The differences among groups were established by one-way ANOVA. The significance of difference was set at $p<0.05$ and a Tukey's *post hoc* test for multiple comparisons of means if difference were detected. All statistical analyses were performed using SPSS software (IBM SPSS Statistics 19; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, USA).

3.4 Results

3.4.1 Growth performance

At the end of the trial, the final weight of the salmon from group A, B and C fed diets without EPA and DHA was 18.21, 18.38 and 14.83 g respectively, which are less than half of the Ref group fed the reference diet containing FO (46.3 g; Figure 3.2). Within the experimental groups, fish fed the highest content of LA in group C showed the lowest growth, although no significant difference was found when comparing to group A and B. During the trial, after the initial feeding phase, mortality was low and no difference was found in feed intake across all experimental diets.

Table 3.2 Feed intake of Atlantic salmon in the trial.

Group	REF	A	B	C
Feed intake (kg)	4.7 ± 0.0^{a}	2.5 ± 0.0^{b}	2.5 ± 0.1^{b}	2.2 ± 0.1^{b}

Data are means of triplicate measurement with standard deviations (n=3). a, b Mean values with unlike letters were significantly different between groups.

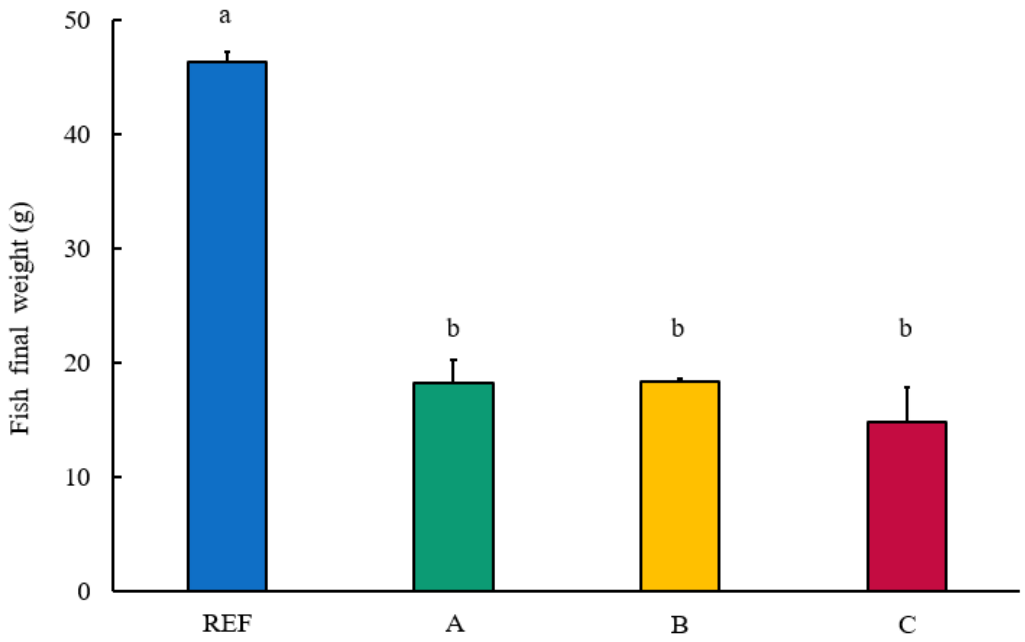


Figure 3.2 Final weights (g) of salmon after feeding the experimental and reference (REF) diets for 22 weeks (154 d). Data are means of triplicate measurement (n=3), with standard deviations represented by vertical bars. a, b Mean values with unlike letters were significantly different between diets.

3.4.2 LC-PUFA production

The initial fry sample contained 1.0 mg of total n-3 LC-PUFA (including EPA, DHA and docosapentaenoic acid, DPA) and the content per fish found at the end of the trial was 106.8 mg in group A, 80.4 mg in group B and 41.6 mg in group C, respectively (Figure 3.3). All the accumulation of n-3 LC-PUFA in the FO-free diet group was considered as endogenous production and the EPA and DHA content reduced significantly when the ALA:LA ratio decreased. Although the 3:1 ratio of ALA:LA in group A showed the highest efficiency, the production of EPA, DPA and DHA was still significantly lower than the REF group (756.7 mg). In contrast, the highest amount of n-6 LC-PUFA was found in group C which accumulated 21.2 mg, significantly higher than both groups A and B. The REF group accumulated 28.6 mg n-6 LC-PUFA in the fry (Figure 3.4). Although the difference of n-6 LC-PUFA production between REF and group C (highest n-6 LC-PUFA production) was significant (28.6 vs 21.2 mg), the values were much closer than the difference of n-3 LC-PUFA production found between REF and group A (756.7

vs 106.8 mg). The DHA:EPA ratio in the initial fry was 2:1, whereas this ratio in fry was over 2.6:1 in group A, 3.4:1 in group B and 5.3:1 in group C. The fish fed the commercial diet in REF group presented a DHA:EPA ratio of 3.2:1 (Figure 3.5).

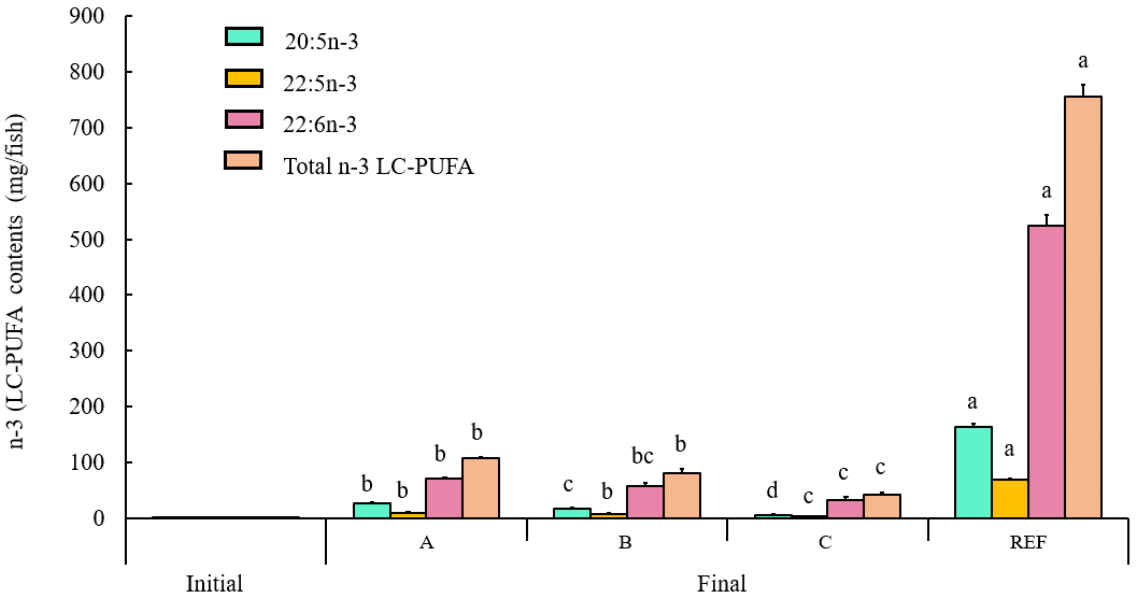


Figure 3.3 n-3 Long-chain PUFA (LC-PUFA) contents (mg/fish) of whole fish at first feeding (Initial) and after the experimental diets (A, B and C) the reference diet (REF) for 22 weeks (Final). Data are means of triplicate measurement (n=3), with standard deviations represented by vertical bars. a, b, c, d Mean values with unlike letters were significantly different between diets.

Based on the final weight, the endogenous production of n-3 LC-PUFA was 5.9, 4.4 and 2.8 mg/g per fish and 0.2, 0.5 and 1.4 mg/g of n-6 LC-PUFA per fish in group A, B and C, respectively. Correspondingly, the daily n-3 LC-PUFA production was calculated to be 38.1, 28.4 and 18.2 µg/g/d per fish and 1.4, 3.4 and 9.3 µg/g/d of n-6 LC-PUFA per fish fed diets A, B and C, respectively. The n-3:n-6 ratio decreased significantly with the reduction in the ALA:LA ratio, where the highest ratio was 27.4 in group A while the lowest ratio of 2.0 was observed in group C. In addition, the ratios of EPA:ARA and DHA:ARA shared the same pattern as that of ALA:LA. Conversely, the DHA:EPA ratio increased significantly when the ALA:LA ratio decreased (Figure 3.5).

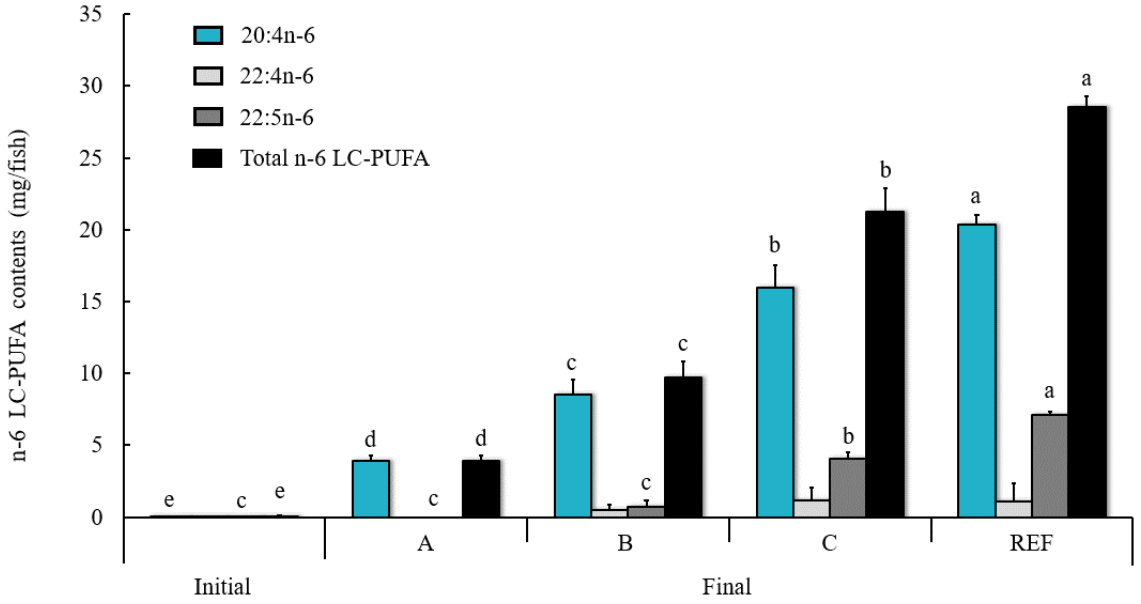


Figure 3.4 n-6 Long-chain PUFA (LC-PUFA) contents (mg/fish) of whole fish at first feeding (Initial) and after the experimental diets (A, B and C) the reference diet (REF) for 22 weeks (Final). Data are means of triplicate measurement (n=3), with standard deviations represented by vertical bars. a, b, c, d Mean values with unlike letters were significantly different between diets.

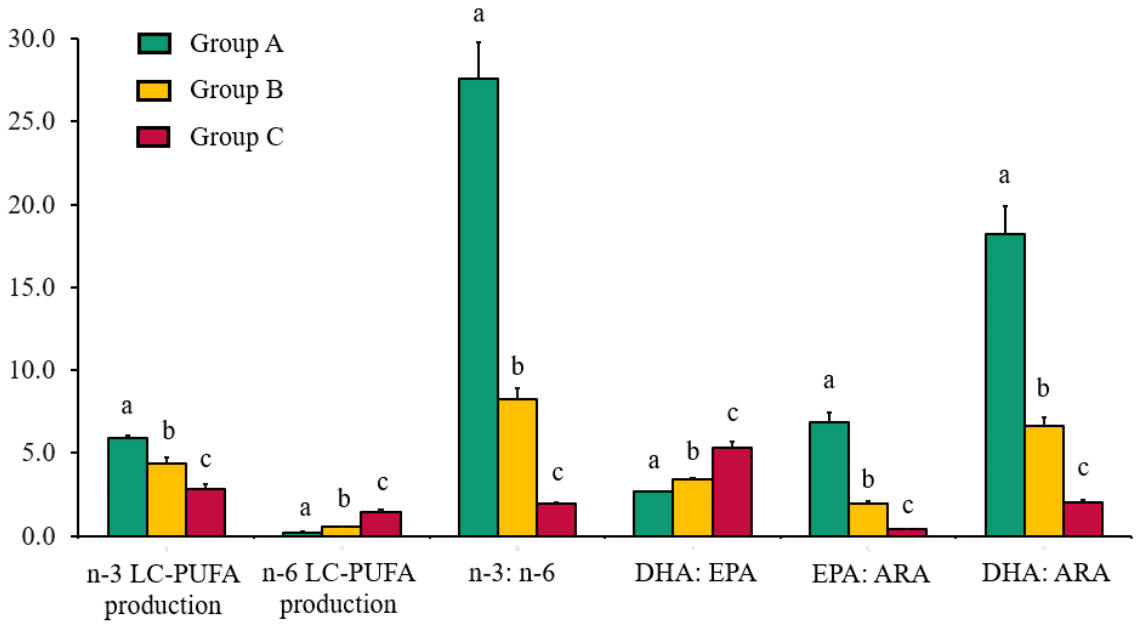


Figure 3.5 Production of n-3 and n-6 LC-PUFA (mg/g fish), n-3:n-6 LC-PUFA, DHA: EPA, EPA:ARA and DHA:ARA ratios in whole fish after feeding the experimental diets (A, B and C) for 22 weeks. Data are means of triplicate measurement (n=3), with standard deviations represented by vertical bars. a, b, c, d Mean values with unlike letters were significantly different between diets.

3.4.3 Fatty acid distribution

The distribution of LC-PUFA in the different lipid classes was studied. When comparing the percentage of n-3 and n-6 LC-PUFA in total lipid, PL and TAG, results showed that, for all groups, PL had a higher proportion of ARA, EPA and DHA than observed in both TAG and total lipid (Figure 3.6), especially it can be observed that DHA was highly incorporated in PL. In addition, when EPA and DHA content decreased from group A to C, their percentage in both PL and TAG showed declined value at the same time (Figure 3.6). Among the different tissues sampled, the DHA content in all tissues were much higher than EPA. The group with the highest production of ARA, EPA and DHA in whole fish sample was set as the reference, so the levels of these fatty acids from each group were compared to the corresponding reference value. For EPA, liver showed the highest amount in group A (243.0 mg/g) followed by the brain (182.2 mg/g), both tissues had a higher content of EPA than the whole fish. In contrast, for DHA, brain was observed to contain a much higher proportion of DHA (860.4 mg/g in average) than any other tissues including whole fish samples. The DHA content in group A from liver (570.3 mg/g) was higher than other groups. With ARA, only the liver showed a relatively higher amount in group C (186.3 mg/g) compared to the whole fish. It's notable that the EPA and DHA content in brain were comparable among the three groups (A, B and C) and higher or similar to the whole fish reference except the EPA content in group C. Besides, DHA contents in tissues were less impacted by the different diets when compared to EPA content (Figure 3.7).

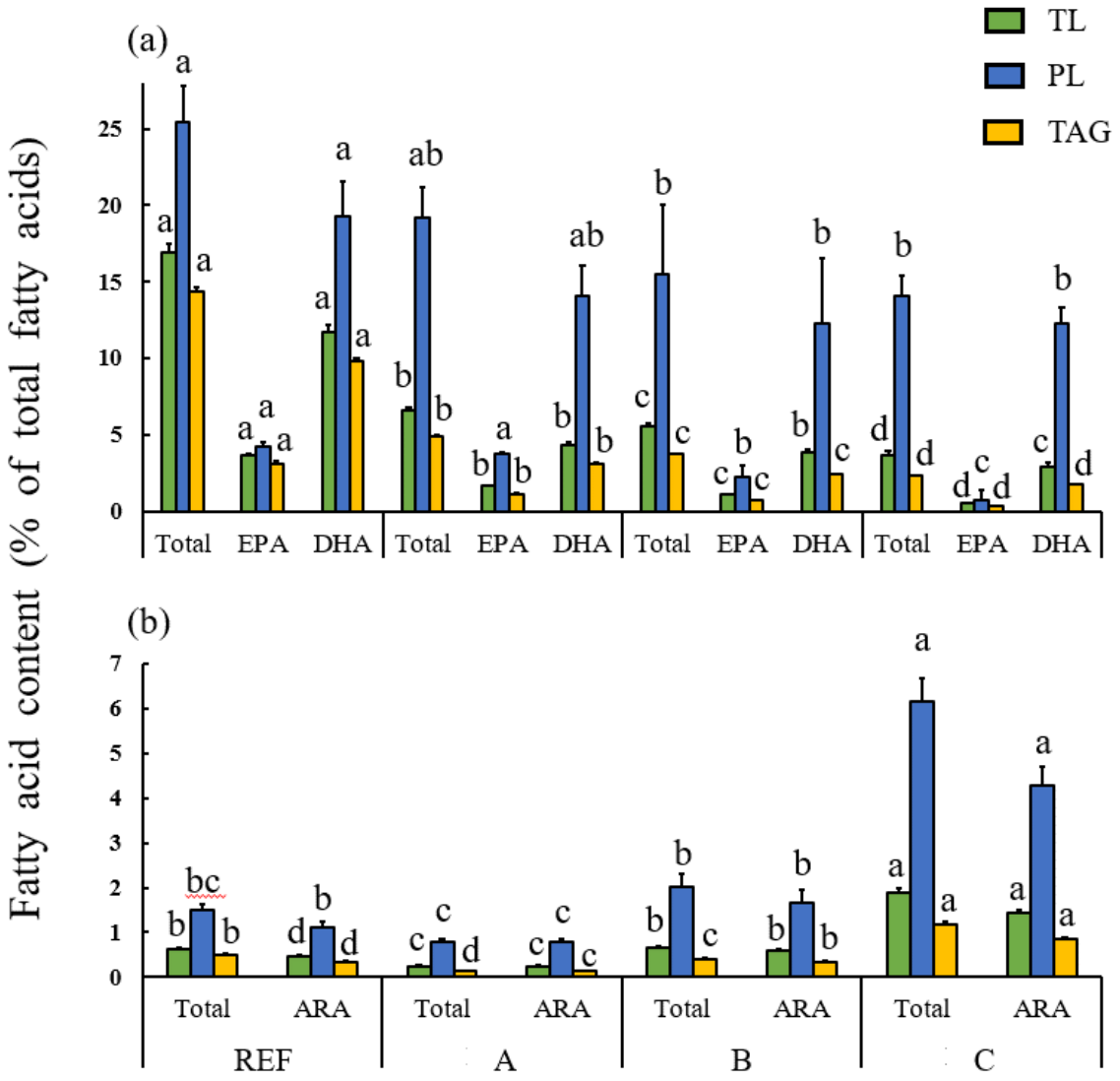


Figure 3.6 n-3 (a) and n-6 (b) LC-PUFA composition (percentage of total fatty acids) of whole fish total lipid (TL), phospholipids (PL) and triacylglycerol (TAG). Data are means of triplicate measurement (n=3), with standard deviations represented by vertical bars. a, b, c, d Mean values for each parameter with unlike letters were significantly different between diets in contents in TL, PL and TAG determined by ANOVA followed by Tukey's multiple comparison test.

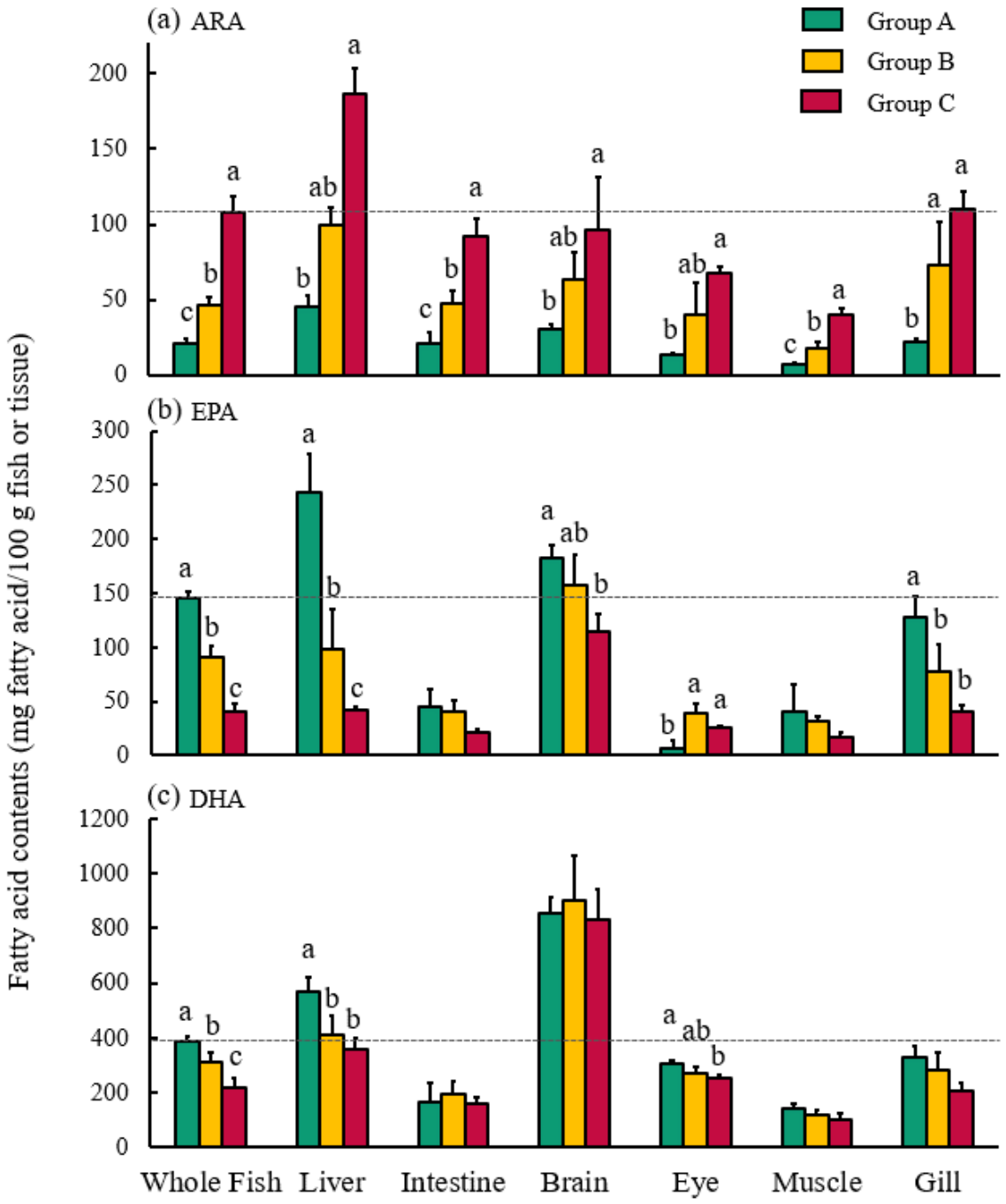


Figure 3.7 Tissue fatty acid contents (mg fatty acid/100 g of whole fish or tissue) of ARA, EPA and DHA after feeding the experimental diets A, B and C. Data are means of triplicate measurement (n=3), with standard deviations represented by vertical bars. a, b, c Mean values with unlike letters for each tissue were significantly different between diets determined by ANOVA followed by Tukey's multiple comparison test. The dotted lines in the upper panels (a) represent the ARA contents, of whole fish in fish fed diet C that gave the highest production of ARA. Similarly, the dotted line in the middle and bottom panel (b, c) represents the EPA and DHA content of whole fish in fish fed diet A that gave the highest production of EPA and DHA.

3.4.4 Liver gene expression

The quantitative real time-PCR analysis of liver samples on the key genes of LC-PUFA biosynthesis indicated a significantly higher *fads2d6*, *fads2d5* and *elovl2* expression in the plant-based diet groups than the REF group. In group B which were fed a diet with ALA: LA ratio of 1:1, the gene expressions of *fads2d6*, *fads2d5* and *elovl2* were found to be slightly, but not significantly, lower than the other groups (p>0.05). No significant difference was found with *elovl5a*, although the REF group showed slightly higher expression of *elovl5b* than the experimental treatment groups. The expression of some transcription factors was determined, demonstrating increased *srebp1* and *srebp2* when ALA:LA was decreased by the higher LA inclusion. A significantly lower expression of *pparα* and *pparγ* was found in group B. The *aco* showed similarly lowest expression in group B but *fas* expression was significantly lower in group A than others (Figure 3.8).

3.4.5 Histology

At the end of the trial, determination of the hepatic intracytoplasmic vacuolization through histological analysis showed that fish fed the REF diet had the lowest degree of hepatic lipid vacuolisation, whereas all the plant-based groups demonstrated a significantly higher degree of vacuolisation than the REF group. In particular, the reduction of the ALA:LA ratio increased the degree of vacuolisation with the highest level observed in group C (ALA:LA=1:3). The hepatic inflammation status of all groups was also evaluated. The lowest value was found in REF group, which was significantly lower than experimental groups (except group A). Again, the lower ALA:LA ratio resulted in a higher inflammation level. Consequently, group C exhibited a significantly higher level of inflammation than group A (Table 3.2).

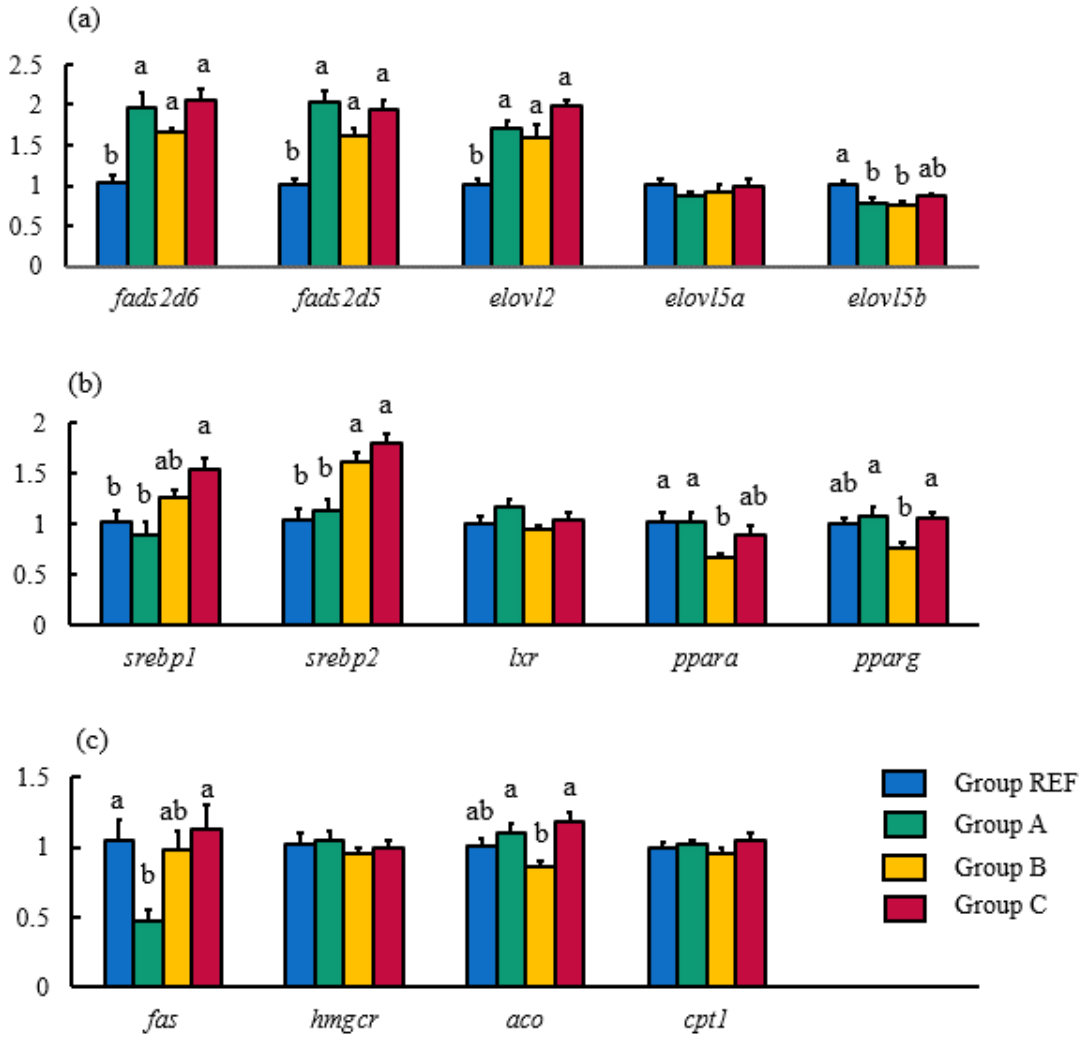


Figure 3.8 Relative expression of genes of LC-PUFA biosynthesis (a), transcription factor (b) and lipid metabolism (c) in liver of Atlantic salmon as determined by qPCR. Results are normalised expression ratios. Data are means of sextuplicate measurement (n=6), with standard deviations represented by vertical bars. a, b Mean values with unlike letters for each gene were significantly different between diets determined by ANOVA followed by Tukey's multiple comparison test.

Table 3.3 Mean scores for the lipid vacuolization and inflammation in liver of Atlantic salmon fed the experimental diets for 22 weeks.

	REF	A	B	C
Cytoplasmic lipid vacuolisation	1.28 ± 0.36^{c}	1.68 ± 0.05^{b}	1.85 ± 0.06^{ab}	2.07 ± 0.21^{a}
Inflammation	0.30 ± 0.12^{c}	0.50 ± 0.10^{bc}	0.73 ± 0.16^{ab}	0.94 ± 0.29^{a}

Data are means of sextuplicate measurement with standard deviations (n=6). Scoring was on a scale from 0 to 3, 0 = not observed, 1 = few, 2 = medium, and 3 = severe. a, b, c Mean values with unlike letters were significantly different between diets determined by ANOVA followed by Tukey's multiple comparison test.

3.5 Discussion

In the present study, salmon fry fed plant-based feeds were significantly smaller as expected, as compared to the REF group fed a diet containing FO. However, the different ALA:LA ratios had little impact on fish growth. The inclusion of the REF group was to demonstrate the difference between the FO-free and commercial diets. As the salmon post-smolts were able to grow on a diet containing no marine ingredients without major growth impacts (Crampton *et al*., 2010; Bendiksen *et al*., 2011), the present study focused on the production of LC-PUFA rather than growth performance, feed efficiency or ingredient evaluation. In this case, all the experimental feeds were formulated according to the nutritional requirement in order not to induce the essential fatty acids deficiency (NRC, 2011). The adequate inclusion levels of both ALA and LA were able to satisfy the fatty acid requirements in a diet devoid of any EPA, DHA or ARA because salmon are reported to possess the complete LC-PUFA biosynthesis pathways from the ALA and LA precursors (NRC, 2011). Although there was no EFA deficiency in the experimental groups, the inferior growth performance, as compared to the commercial feed which was solely included as a reference diet, could be partially explained by the absence of LC-PUFA since EFA requirements are more directly met by LC-PUFA inclusion (Ruyter *et al*., 2000a; 2000b). Although the general production of LC-PUFA per fish displayed differences between groups, the growth rate was able to impact LC-PUFA production as some key metabolic activities, including LC-PUFA biosynthesis, are closely associated. In this sense, data in per gram of fish could be a better way of presenting LC-PUFA production in fish and it will not be affected by the different growth rates among groups.

Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) are both reported to be a net producer of n-3 LC-PUFA when fed a diet containing low FO (Sanden *et al*., 2011; Turchini *et al*., 2011a). To evaluate the *in vivo* production of EPA and DHA, previous studies looked at calculating the gap between total fatty acid intake and the final whole fish content (Stubhaug *et al*., 2007), or by a whole-body mass balance method (Turchini *et al*., 2007). However, the accuracy of these assessments could be further improved. In

the present study, the very small amounts of EPA, ARA and DHA found initially in the alevins, together with the diets containing no marine ingredients, indicate that the majority of LC-PUFA detected in the final whole fish was the result of endogenous biosynthesis, thereby simplifying the quantitative calculation of endogenous LC-PUFA production.

The data showed that the production of n-3 LC-PUFA from ALA in salmon was 5.9, 4.4 and 2.8 mg/g per fish in group A, B and C, respectively. However, these results are, in fact, lower than the theoretical production in each group as these results were considered as the minimum amount because part of the produced LC-PUFA was very likely to be oxidised or utilised as demonstrated previously (Turchini *et al*., 2007; 2011; Emery *et al*., 2016), plus there was no LC-PUFA intake from diets that this oxidation normally prioritises and no biochemical or physiological mechanism could prevent from this oxidation (Tocher, 2003). Therefore, the total amount of the LC-PUFA production must be higher than the present data. In addition to this, the ability to biosynthesize LC-PUFA could vary by life stage, with the present results limited to the fry and parr stages. A higher level of production might be found when seawater smoltification. Indeed, it was previously reported that during the parr-smolt transformation, the hepatic LC-PUFA biosynthesis from labelled ^{14}C ALA increased in salmon indicating a higher endogenous production capacity (Bell *et al*., 1997; Tocher *et al*., 2000). Nevertheless, how age might affect the biosynthesis capacity is not fully elucidated as few data can be found within the literature. In salmon, the production activity is speculated to be lower in seawater phase, suggested to be related to the marine environment which is highly abundant in LC-PUFA for the food chain (Tocher, 2003).

Converting ALA to n-3 LC-PUFA has been shown, using isotopic labelling, to be very limited in humans especially the poor bioconversion from EPA to DHA or even incapacity (Baker *et al*., 2016; Burdge *et al*., 2005; Wood *et al*., 2015). Although little information about this measurement in fish is known, a previous feeding trial in rainbow trout of 3.2-15.6 g initial weight, and fed a diet containing 5 % fish meal and 11 % VO, found that the

daily DHA production from ALA was 0.54 µg/g/d/mg (Bell *et al*., 2001b), and the net ALA intake was about 3.3 µmol/g/d (Emery *et al*., 2013), which is different to the result shown in the present study. Although the calculation methods used in both studies were similar, in the previous trial it was assumed that this modest production rate was due to the diets, which included fish meal as a marine source and provided approximately 1.5 % n-3 LC-PUFA. As reported previously, the presence of dietary n-3 LC-PUFA such as DHA could decrease the Δ6 desaturation and limit the conversion rate from ALA (Thomassen *et al*., 2012; Betancor *et al*., 2016a).

It has been previously reported that the conversion from ALA to EPA or further to DHA was increased when the dietary LA decreased to give a higher ALA:LA ratio (Baker *et al*., 2016; Wood *et al*., 2015). The impact of the ALA:LA ratio on the endogenous LC-PUFA production in salmon has been demonstrated quantitatively in the present study. Although the ALA:LA ratio varied from 3.0 to 0.3, the ratio of n-3:n-6 production decreased greatly from 27.4 to 2.0 fold, respectively. Despite the lowest n-3 LC-PUFA content found in group C, where the ALA:LA ratio was 1:3, it still indicates that the n-3 LC-PUFA content was much greater than n-6. The competition between n-3 and n-6 biosynthesis lies in the combination of the substrates to their common enzymes. The higher value of n-3 LC-PUFA content in the present trial demonstrates the priority of n-3 for the enzymes involved in the LC-PUFA biosynthesis pathway, which was previously confirmed by enzyme activity using radiolabelled tracers in hepatocytes and preferences of individual desaturase and elongase proteins in heterologous expression assays (Bell *et al*., 1997; Monroig *et al*., 2018). Similarly, the effect of the dietary ALA:LA ratio on LC-PUFA production in Murray cod (*Maccullochella peelii peelii*) resulted in an increased dietary LA level significantly increasing n-6 LC-PUFA and reducing n-3 LC-PUFA production (Senadheera *et al*., 2011). In contrast, in rainbow trout, aside from an enhanced n-6 production, the production of EPA was reduced (slightly on DHA) by an increased dietary LA level but no significant impact was observed for n-3 LC-PUFA production (Emery *et al*., 2013; Thanuthong *et al*., 2011). Additionally, in the present trial the daily total LC-PUFA production rate (n-3 and n-6 combined) decreased with the ALA:LA ratio

from group A to C (38.1+1.4 to 18.2+9.3 µg/g/d/g fish, respectively), which suggests an inhibitory effect of the higher ALA:LA ratio on the overall LC-PUFA production pathway. This obvious reduction in LC-PUFA production was mainly due to the large variation of the n-3 production, which is in further agreement with the enzyme priority for n-3 LC-PUFA production.

To date, the optimal dietary DHA:EPA ratio and their relationship has not been fully explored for many species including salmon (Emery *et al*., 2016; Codabaccus *et al*., 2012). The natural production ratio of DHA:EPA might be an indicator, so the endogenously produced EPA and DHA in salmon was quantified in the present trial when dietary EPA and DHA input was not given. It was notable that, in the present study, DHA was always higher than EPA with the DHA:EPA ratio over one. Similarly, when rainbow trout received a DHA and EPA free diet, with the ALA:LA ratio of approximately one, the DHA:EPA ratio varied between 1.3 in the neutral lipid of whole body carcass and 4.1 in the polar lipid of liver indicating the importance of DHA (Tocher, 2015). Rather than a specific value, in the present study the DHA:EPA ratio was impacted by the dietary ALA:LA ratio with a higher LA level (lower ALA:LA ratio) could increase the DHA: EPA ratio. It has been generally observed that increased dietary levels of LA could increase DHA:EPA ratios in whole fish and/or tissues in some freshwater and salmonid species (Thanuthong *et al*., 2011; Tan *et al*., 2009; El-Husseiny *et al*., 2010; Senadheera *et al*., 2010; Wu and Chen, 2012; Tian *et al*., 2016; Paulino *et al*., 2018; Xie *et al*., 2018). However, the inclusion of EPA and DHA in the early-stage diet might make the calculation more complicated when comparing the results from those aforementioned to the present trial. The underlying mechanism is suspected as being that the higher dietary LA supply could occupy more Δ6 desaturases in the LC-PUFA biosynthesis pathway when competing against ALA, which subsequently led to the lower production of EPA from ALA. Due to the critical role in the membrane structure and function, the production of DHA tends to be physiologically maintained so it is not affected to the same extent (Emery *et al*., 2016). Research in rodents showed that the ratio of DHA:EPA was highly tissue-dependent with higher amount of EPA than DHA observed in the blood and liver

whereas DHA was dominant within the heart and brain. Furthermore, the tissue DHA:EPA ratios were relatively less impacted by the ALA content and ALA:LA ratio in diets without EPA and DHA (Talahalli *et al*., 2010).

In the present study, the focus was not only on the quantitative perspective but also investigating the traces of the endogenously produced LC-PUFA. All groups, including REF, exhibited a higher proportion of total LC-PUFA, ARA, EPA and DHA in PL than TAG, although this was more obvious in the experimental groups as compared with REF. Therefore, it has been clearly demonstrated that the endogenously produced ARA, EPA and DHA were highly preferentially incorporated into membrane PL, whereas the LC-PUFA from the diet tended to be deposited in TAG as a storage for energy consumption. These results are in accordance with that previously proposed by Tocher, 2003. To evaluate the deposition preferences of endogenously produced LC-PUFA in different tissues, the proportions in tissues were compared to whole fish as a reference, with preference defined when the content per unit mass was higher than the whole-body reference. In this case, the only noticeable trends were the higher DHA content in the brain and the LC-PUFA in the liver, reflecting the importance and key metabolic role in the biosynthesis for both tissues (Tocher, 2003; 2010). Moreover, the active role of the liver in LC-PUFA biosynthesis was confirmed by the molecular data. Gene expression data demonstrated an up-regulation in *fads2d6* and *fads2d5* and *elovl2* in the experimental groups as compared with REF, which is consistent with other studies on salmon (Monroig *et al*., 2010a; 2018). However, no significant effect was found between the different groups indicating the little impact that dietary ALA:LA ratio had on gene expression. Thus, rather than the ALA:LA ratio *per se*, the higher inclusion of total C18 PUFA and less n-3 LC-PUFA might be assumed as the reason for higher expression *fads2d6* and *fads2d5* and *elovl2* in all the experimental groups. A study conducted in rainbow trout similarly demonstrated that the expression of Δ6 desaturase and *elovl5* in liver were not related to the ALA:LA ratio, and a similar study in rabbit fish (*Siganus canaliculatus*), which possess the full set of genes required for n-3 LC-PUFA biosynthesis, reported that Δ6, Δ4 desaturase and *elovl5* were indeed affected by ALA:LA ratio but in a random

pattern (Emery *et al*., 2013; Xie *et al*., 2018). Apart from DHA, in the present trial the relatively high content of EPA in brain tissue also implied a possibility of the *in situ* production of DHA from EPA, since the total DHA in brain detected cannot be classified as the direct production or simply deposition, so the exact quantitative evaluation in the brain were inaccessible.

An increase in the transcription factors *srebp1* and *srebp2* when the ALA:LA ratio decreased was observed. Previously, experimental studies on salmon reported that lower dietary n-3 LC-PUFA increased *srebp1* and/or *srebp2* expression (Betancor *et al*., 2014a; Hixson *et al*., 2017). Subsequently, these increases in transcription factors resulted in the experimental groups being higher than REF. The lowest expression of *pparα* and *pparγ* in group B when ALA:LA was 1:1 suggests that this ratio cannot be considered best for the biosynthesis of n-3 LC-PUFA. For the fatty acid metabolism, *aco* followed the pattern found in *pparα* and *pparγ* showing the lowest expression in when the dietary ALA:LA was 1:1, which was similarly found for *fads2d6* and *fads2d5* among groups. This could be interpreted by them being the target genes of *pparα* and *pparγ* (Ferré, 2004; Kersten *et al*., 2000; Matsuzaka *et al*., 2002), which further indicates the suppression of n-3 LC-PUFA biosynthesis by an ALA:LA of 1:1. The histological analysis in the present study showed a higher degree of cytoplasmic lipid vacuolisation in experimental groups as compared to REF, suggesting that the high lipid accumulation in the liver could be correlated to high levels of C18 PUFA (Izquierdo *et al*., 2008; Betancor *et al*., 2016b). Another explanation for the deposition might be due to the lower levels of n-3 LC-PUFA. This was confirmed by the comparison among the experimental groups demonstrating that the cytoplasmic lipid vacuolisation increased when the ALA:LA ratio declined. The lowest ALA:LA ratio in group C resulted in the lowest n-3 LC-PUFA production and n-3:n-6 and then aggravated the hepatic vacuolisation. Meanwhile, the present study also found an increased inflammatory response when the ALA:LA ratio decreased. Indeed, the higher inclusion of LA provided more n-6 PUFA that is considered pro-inflammatory (Tocher, 2003). Therefore, the increased inflammatory response suggests that the higher LA inclusion with lower ALA:LA ratio may affect stress resistance, immunity against

pathogens or other health issues.

The present study focused on the evaluation of the LC-PUFA production capacity in salmon in order to provide a better understanding about the interaction between dietary ALA:LA ratio and LC-PUFA production when salmon are fed a diet without marine ingredient sources from first feeding. Currently the salmon farming industry is still reliant on FO to satisfy the n-3 LC-PUFA demand, especially for EPA and DHA. However, taking into account sustainability, it will be more ideal if the level of FO inclusion in the aquafeeds could be further reduced as aquaculture is the main consumer of FO (Ytrestøyl *et al*., 2015). At present, some VOs mainly containing C18 PUFA such as ALA and LA are widely used to meet the dietary oil requirement, but unfortunately this would mean a decrease in EPA and DHA content in salmon and a decline in nutritional value (Sprague *et al*., 2016). In addition, both algal and GM-derived sources are the imminent candidates being the supplier of EPA and DHA. However, their cost and availability currently restrict the use of these new sources, and their usability still remains uncertain and unreliable (Tocher *et al*., 2019). Based on this situation, including a high level of VO for sustainability concerns together with some degree of FO inclusion to maintain the EPA and DHA level in salmon feeds will still be the major role in salmon farming. Regarding the ALA:LA ratio provided by the different oils, the present study demonstrates the possibility of maximising the endogenous production of EPA and DHA through control of the dietary ALA:LA ratio, thereby providing some guidance on the use of oils containing differing proportions of ALA and LA.

3.6 Conclusions

When the dietary n-3:n-6 PUFA ratio was 1:1, the endogenous production of n-3 LC-PUFA in Atlantic salmon fry/parr was approximately 4.4 mg/g per fish and the ratio of produced DHA and EPA was 3.4:1. Besides, the production of n-3 LC-PUFA was proved to be almost nine times than that of n-6 LC-PUFA. The reduction of the dietary n-3:n-6 ratio could decrease the n-3 LC-PUFA production, EPA:ARA and DHA:ARA ratios but also increase n-6 LC-PUFA production and DHA:EPA ratio. In the present study, salmon fry was fed diets containing no EPA and DHA from first feeding, as salmon has complete pathways (via the Sprecher shunt), so the detection of LC-PUFA was totally from endogenous production. In this sense, the LC-PUFA biosynthesis capacity can be better evaluated quantitatively. Furthermore, knowing how dietary ALA:LA ratio affects LC-PUFA production could provide deeper insight for the better utilisation of different dietary oils.

CHAPTER 4

The effects of first feeding nutritional stimulus period and a plant-based diet challenge in Atlantic salmon (*Salmo salar*) fingerlings

4.1 Abstract

Nutritional programming could potentially be a strategy for the sustainable development of aquaculture, as it can enhance the ability of fish to adapt to feeds formulated with low levels of marine ingredients. To determine the effect of an early stimulus period and to reveal the impacts of a similar diet as challenge, the present research focused on feeding salmon a marine diet for two weeks (M), plant-based diet for one week (V1) and two weeks (V2) from first feeding, respectively, followed by a marine diet phase until 16 weeks before transferring to an additional challenge phase for 6 weeks. Salmon from the V1 group showed better growth performance. After challenge, there were generally few phenotypic impacts on the fatty acid compositions of both total and polar lipid in tissues caused by the different stimulus durations. However, fatty acid composition of tissues responded to the diets and the preferential retention of n-3 LC-PUFA in polar lipid was found especially in the brain and eye. Salmon from V1 group exhibited a significant upregulation in the key genes for LC-PUFA biosynthesis, whereas most transcription factors were downregulated in V2. Histological analysis revealed a lower goblet cell presence in V1 following challenge. Overall, the growth and molecular analysis highlights that a brief one-week stimulus will elicit positive effects on programming in salmon. However, for the refinement of nutritional programming in salmon, further investigation is recommended to provide more detailed data such as the effects under longer challenge periods.

Key words: Nutritional programming; Atlantic salmon; stimulus period; challenge; n-3 LC-PUFA; tissues;

4.2 Introduction

The traditional ingredients of aquafeeds are fish oil (FO) and fishmeal (FM) are of current concern due to the pressing urgency over their availability (Hodar *et al*., 2020; Xu *et al*., 2021). These finite marine resources are commonly replaced by a range of alternatives ingredients such as algae or land animal products. So far, plant-based proteins and oils, such as soybean, rapeseed and sunflower, are still the preferred choice owing to their higher production volumes and lower prices (Bandara, 2018). Several studies have focused on FO replacement for different teleost species (e.g. Fountoulaki *et al*., 2009; Glencross *et al*., 2003; Montero *et al*., 2005), with some studies reporting a negative effect on growth performance and health (Glencross *et al*., 2020; Hardy, 2010; Wacyk *et al*., 2012). The carbohydrate content of some plant protein sources can present some difficulties in their use due to digestibility issues in various species, as well as the presence of anti-nutritional factors that can also negatively impair digestion (Hua *et al*., 2019). On the other hand, vegetable oils can have a varied fatty acid profile, although they are generally characteristically rich in oleic acid (18:1n-9), linoleic acid (LA, 18:2n-6) and/or α-linoleic acid (ALA, 18:3n-3). One common feature among all vegetable oils is the lack of the long-chain polyunsaturated fatty acid (LC-PUFA), especially arachidonic acid (ARA; 20:4n-6), eicosapentaenoic acid (EPA; 20:5n−3) and docosahexaenoic acid (DHA; 22:6n−3). Apart from LA and ALA, EPA and DHA are also recognized as essential fatty acid (EFA) in fish for their functional importance on cell membrane structure, body immunity and neural system development, although, in general, marine fish typically have a *de novo* bioconversion deficiency (Glencross, 2009; Schmitz and Ecker 2008; Tocher, 2010). Thus, adequate levels of EPA and DHA must be supplied through the diet to avoid adverse effects on growth performance and nutritional quality (Sprague *et al*., 2016). Furthermore, the replacement level has reached a critical point where it potentially affects fish growth, health as well as feed efficiency (Herman and Schmidt, 2016). Therefore, the application of sustainable feeds using vegetable sources remain restricted.

Research in mammals have found that, during the early critical development stages like gestation and lactation, nutritional interventions can induce long-term changes on animal metabolism and physiology (Lucas, 1998). This phenomenon is termed “nutritional programming” and is believed to prepare the animal through adaptive changes at the cellular, molecular and biochemical level for a similar nutritional environment in later life stages via the underlying mechanism of epigenetics (Gluckman *et al*., 2005; Patel and Srinivasan, 2002). Nutritional factors can either partially erase or re-build the epigenetic modifications by early dietary stimulus leading to a more efficient utilisation of diets and growth as well as improved nutrient biosynthesis when a similar nutritional environment appears (Lillycrop and Burdge, 2012; Petry *et al*., 2001). Several studies have shown that rainbow trout (*Oncorhynchus mykiss*) fry showed higher growth rates, feed intake and feed efficiency when challenged by plant-based diets as well as a better utilization of carbohydrate at the digestive level after an acute nutritional stimulus with similar feeds (Geurden *et al*., 2007, 2013). Moreover, it was shown that nutritional programming improved plant-based diet efficiency in gilthead sea bream, and that the partial replacement of FO with VO in the parental diet could induce long-term molecular impacts on n-3 LC-PUFA and energy metabolism in the liver of offspring (Izquierdo *et al*., 2015; Turkmen *et al*., 2017). Although the endogenous biosynthesis of EPA and DHA from the ALA precursor is inadequate in salmon, due to the limiting enzyme delta-6-desaturase (Δ6 FAD) (Tocher, 2003), its gene expression was known to be modulated by nutritional programming in utilizing C18 fatty acids (Turkmen *et al*., 2019a; Xu *et al*., 2019). Indeed, Turkmen *et al*. (2019a) demonstrated that the pathway for DHA biosynthesis was upregulated when programmed sea bream offspring encountered a similar nutritional challenge, together with improved growth rate. This indicates a potential strategy to relieve the issue of the global n-3 LC-PUFA supply problem that dietary marine ingredient replacement brings, and hence promote the sustainability of aquafeed.

As the diet fatty acid composition can be reflected in tissues, the evaluation of the tissue fatty acid composition can help better understand the potential effects of nutritional programming from the perspective of metabolic or physiological functions. In salmon,

the high plasticity of LC-PUFA biosynthesis could be regulated by tissue n-3 LC-PUFA capacity (Leaver *et al*., 2008b). Liver is considered as the most active metabolic tissue and is an important site for LC-PUFA synthesis and lipid metabolism in fish that is highly responsive to dietary change, leading to a larger transcriptional response (Betancor *et al*., 2015b; Monroig *et al*., 2010a). Apart from liver, the intestine is also known as another active organ for LC-PUFA biosynthesis in rainbow trout (Tocher *et al*., 2004). Although less affected by fatty acid changes, the brain as a neural tissue selectively retains DHA to avoid impaired visual performance (Bell and Tocher, 1989; Bell *et al*., 1999; Betancor *et al*., 2014a). So far, the effect of nutritional programming on tissue lipid class profiles remains unclear, especially the polar lipid (PL) proportion due to its critical role in cell membrane function (Tocher *et al*., 2008). Although tissue lipid class profile is speculated to be less varied, the PL fatty acid composition might be responsive to the programming and how LC-PUFA contents in tissue PL altered is still underexplored.

In a previous study, a three-week stimulus trial was applied from first feeding to confirm the potential of nutritional programming in Atlantic salmon fry in order to tailor the ability of adult fish ability to efficiently utilize alternative diets (Clarkson *et al*., 2017). Although the concept was clearly demonstrated, 3 weeks of feeding stimulus also induced phenotypic differences including weight differences between marine and stimulated groups at the end of the stimulus. The aim of the present study was to evaluate 1) if nutritional programming changes can be triggered by a shorter (one or two weeks) period; 2) whether nutritional interventions are still prevalent after 16 weeks; and 3) whether a new challenge (booster) will enhance the biosynthesis capability of EPA and DHA. Consequently, the different lipid statuses of tissues are evaluated at different sampling points, together with selected molecular markers and histological evaluation.

4.3 Materials and methods

4.3.1 Ethics statement

Animals were handled in accordance with the Animals (Scientific Procedures) Act 1986 (ASPA) revised to transpose European Directive 2010/63/EU. In addition, all procedures and protocols carried out in the present study were subjected to an ethics review by the University of Stirling Animal Welfare and Ethical Review Board (AWERB No: 18 19/045/New ASPA) prior to the commencement of the trial.

4.3.2 Dietary trial

Two thousand and seven hundred eggs (388 degree-days post fertilization) were obtained from Mowi (Scotland, UK) and transferred to the temperate freshwater facility at the Institute of Aquaculture, University of Stirling, for the experiment. All eggs were hatched after 500 degree-days (DD) with the fish remaining in the alevin stage for a further 350 DD. The incubation temperature was 7.0±0.4°C. At 850 DD, when salmon reached the fry stage with a 97.7 % survival rate, all fish were distributed to 9 × 0.3 m^2 tanks for the first feeding trial.

The feeding trial started from first feeding (initial weight approx. 0.15 g) and continued for 22 weeks. Two types of experimental feeds were manufactured and provided by BioMar Ltd. (TechCentre, Brande, Denmark) following the formulations shown in Table 4.1, which included some mixed ingredients as is standard practice within commercial feed production. The Marine diet (M) contained fish meal and fish oil as the main protein and lipid sources, respectively, whereas the Vegetable-based diet (V) contained terrestrial plant protein and oil. Each type of diet was prepared at different pellet sizes (0.5 mm, 0.8 mm, 1.1 mm, 1.5 mm and 2.0 mm) to tailor for the different size of fish. The fatty acid compositions for all diets are shown in Table 4.1. Fish were allocated into three experimental groups in order to find out the minimum stimulus period for nutritional programming as demonstrated in Figure 4.1. At the first feeding stage termed “stimulus

phase", three groups (300 fish per tank, n=3) were fed either a vegetable-based diet for one (V1) or 2 weeks (V2) or a marine diet (M), respectively to provide stimulus for better future performance on diets with low marine ingredients as programming. Afterwards, all fish were then fed M diet until the end of 16th week (termed as marine phase) before all groups were transferred back to the V diet as a challenge for a further 6 weeks (termed challenge phase). The feeds were provided to fish by automatic feeders (Arvo-tec TD2000, Huutokoski, Finland) and user interface (ArvoPRO) to ensure the acquisition of accurate values for feed entering the tanks. Uneaten feed was collected twice a day after daily feeding using a syphon pipe with filter, before drying to enable correction of feed intake measurement. The trial was carried out in the temperate freshwater facilities of the Institute of Aquaculture using Recirculating Aquaculture System (RAS) tanks. The water volume was set as 300 litre per tank and the water temperature was maintained at 13.1±0.5 °C with 24:0 light: dark, the oxygen level (86 % saturation, 8-9 mg/L), pH (7.16±0.2), nitrogen NO_2 (0.32±0.2 mg/L), total ammonia nitrogen TAN (0.13±0.17 mg/L) and chloride (135.0±13.0 mg/L) were determined and controlled daily for the feeding trial.

4.3.3 Sampling

Samples were collected at three time points: intermediate of marine phase, end of marine phase and end of the challenge phase. At each time point, following a 24-hour fasting period, 11 individuals from each tank were randomly selected and euthanised with tricaine methanesulfonate (MS-222; 1000 mg/L in hydrogen carbonate-buffered solution). Prior to sampling, 11 sampled fish were bulk weighed on an analytical balance. Samples of liver and intestine from six fish were divided into two portions, one portion was collected in 1.5 mL RNALater (Sigma, Poole, UK) and stored overnight at 4°C before freezing at -70 °C for molecular analysis. The other portion was placed in 2 mL 4 % buffered formalin for further histology analysis. Another five fish were dissected with tissues of liver, brain, white muscle, gill, eye and intestine collected for biochemical analysis, being stored in liquid nitrogen and -70 ºC prior to analyses.

Table 4.1 The formulations, proximate and fatty acids compositions (percentage of total fatty acids) of the experimental feeds.

	Stimulus phase		**Marine phase**			**Challenge phase**	
	0.5 mm M	**0.5 mm V**	**0.8 mm M**	**1.1 mm M**	**1.5 mm M**	**1.5 mm V**	**2.0 mm V**
Feed ingredients (%)							
Fish Meals	66.7	–	53.0	53.0	49.3	5.0	5.0
Krill meal (56 %)	10.0	2.5	7.0	7.0	–	–	–
Fish Peptones	5.0	2.5	5.0	5.0	–	5.0	5.0
Plant proteins (SPC, wheat & maize gluten, pea prot.)	3.5	70.6	16.4	16.4	26.1	60.0	60.0
Starch sources	6.5	4.0	5.5	5.5	9.5	8.1	8.1
Fish Oil	4.4		9.8	9.8	8.6	–	–
Vegetal oil	–	6.7	0.5	0.5	5.1	14.1	14.1
Lecithin	0.5	3.6	0.6	0.6	0.5	0.5	0.5
Premix (vit., min., AAs, nucl., antiox., yttrium)	3.0	10.9	3.0	3.0	2.5	8.8	8.8
Proximate							
Dry matter (%)	94.5	94.5	93.4	91.5	96.9	92.9	95.2
Ash (%)	12.2	8.8	11.1	10.0	10.1	7.3	7.3
Protein (%)	60.0	56.0	55.7	53.0	52.6	50.9	51.8
Lipid (%)	13.3	13.4	17.0	16.4	17.8	20.0	16.3
Gross energy (KJ/g)	20.6	21.1	21.3	20.9	21.8	22.4	22.3
FA composition (%)							
Σ SFA[1]	29.0	17.0	28.6	28.7	20.9	10.1	10.4
Σ MUFA[2]	28.8	45.9	34.3	35.1	41.8	56.6	55.4
18:2n-6	5.7	21.5	4.6	5.9	10.3	22.7	23.6
20:3n-6	0.1	0.0	0.1	0.1	0.0	0.0	0.0
20:4n-6	0.1	0.7	0.7	0.7	0.5	0.1	0.1
22:5n-6	0.2	0.0	0.2	0.2	0.2	0.0	0.0
Σ n-6 PUFA[3]	7.1	22.1	6.2	7.3	11.4	22.8	23.8
18:3n-3	1.8	7.0	1.4	1.5	4.2	8.7	8.9
20:5n-3	11.9	3.4	10.9	10.4	8.1	0.6	0.5
22:5n-3	1.2	0.2	1.2	1.2	1.0	0.1	0.1
22:6n-3	14.1	2.5	10.5	9.7	8.2	0.8	0.7
Σ n-3 PUFA[4]	33.0	14.6	27.9	26.4	24.1	10.4	10.3
n-3 LC-PUFA[5]	27.8	6.2	23.4	22.0	17.8	1.5	1.2
DHA+EPA	26.0	5.9	21.3	20.1	16.3	1.4	1.2
DHA/EPA	1.2	0.7	1.0	0.9	1.0	1.3	1.4
n-3:n-6	4.6	0.7	4.5	3.6	2.1	0.5	0.4

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3

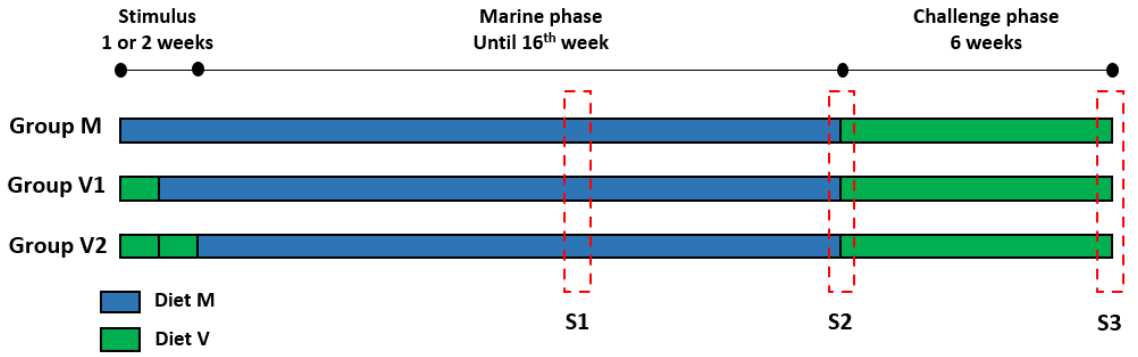


Figure 4.1 The design of the dietary experiment in Chapter 4. S1 (sample point 1): Intermediate of marine phase; S2: End of marine phase; S3: End of the challenge phase.

4.3.4 Lipid content, class composition and fatty acid analyses

Total lipid (TL) content of tissue and feed samples was extracted after homogenization in 20 or 36 mL of chloroform-methanol (2:1, v/v) respectively, using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK). The lipid content was determined gravimetrically based on the method of Folch *et al*. (1957). The extracted TL was made up to a concentration of 10 mg/mL in chloroform: methanol (2:1) + 0.01 % (w/v) BHT for further analysis.

For lipid class separation, A high-performance thin layer chromatography (HPTLC) method using 20 × 10 cm plates (VWR, Lutterworth, UK) was used for lipid class separation according to Henderson and Tocher (1992). The mobile solvent phase consisting of methyl acetate:isopropanol:chroloform:methanol:0.25 % (w/v) potassium chloride (25:25:25:10:9, by vol.) was used for polar lipid separation running to approximately half-distance of the plate, and the mobile solvent phase consisting of isohaxane:diethyl ether:glacial acetic acid (85:15:1.5) was used for neutral lipid separation running to full distance of the plate. Classes were visualised after thorough

spraying with 3 % copper acetate and 8 % phosphoric acid and placing in a drying oven at 160 ºC for 20 min. The plates were scanned by TLC Scanner 3 (CAMAG, Muttenz, Switzerland) and the data were processed using the Wincat software package (CAMAG, Muttenz, Switzerland).

Fatty acid methyl esters (FAMEs) were prepared from TL by acid-catalysed transmethylation at 50 ºC for 16 h, and purified as described previously (Christie, 2003; Tocher and Harvie, 1988). FAMEs were separated and quantified by gas-liquid chromatography (Thermo Finnigan Trace GC, Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 µm ZB-wax column (Phenomenex, Cheshire, UK), 'on column' injection and flame ionisation detection. Hydrogen was used as the carrier gas at constant pressure (175 kPa) with the initial oven thermal gradient from 50 ºC to 150 ºC at 40 ºC.min^{-1}, then 195 ºC at 2 ºC.min^{-1}, 205 ºC at 0.5 ºC.min^{-1} to a final temperature of 230 ºC at 40 ºC.min^{-1}. Individual peaks of FAME were identified by comparison to the peak graph of an in-house Marine fish oil (Marinol) standard and known standards (Restek 20-FAME Marine Oil Standard; Thames Restek UK Ltd., Buckinghamshire, UK). The data were collected and processed using Chromcard data system for Windows (Version 2.11; Thermo Fisher Scientific Inc., Milan, Italy). Heptadecanoic acid (17:0) was used as internal standard to calculate fatty acid content per gram tissue.

Fatty acids of individual lipid classes were performed by separating TL samples on 20 × 20 cm thin-layer chromatography plates (VWR, Lutterworth, UK) using the respective solvent phases described for lipid class analysis. Individual PL as well as triacylglycerol (TAG) was identified by spraying plates with 0.1 % (w/v) 2-7-dichlorofluorescein in 97 % methanol (v/v). The individual classes weremarked under UV light at 240 nm (UVP® Mineralight®R-52G; UVP Inc.) and the silica scrapped from plates into tubes and methylated as described for fatty acid analysis. Samples were extracted as described by Tocher and Harvie (1988) and analysed using the same conditions detailed above.

4.3.5 Molecular analyses

The total RNA of liver sample from six individual fish per tank was homogenised in 1 mL of TriReagent® (Sigma-Aldrich, Dorset, UK). The quantity, quality and concentration of RNA was determined by spectrophotometry (Nanodrop ND-1000; Labtech Int.), and RNA integrity was checked by 1 % agarose gel electrophoresis. The livers from each tank were pooled into two samples (three livers per pool) and 2000 ng RNA from samples were used for cDNA synthesis with a random primer in 20 µL reaction volume and high-capacity reverse transcription kit following the manufacturer's protocol (Applied Biosystems, Warrington, UK). The synthesized cDNA was diluted 20-fold with milliQ water (Thermo Fisher Scientific).

Expression of genes of interest (GOI) involved in key pathways include LC-PUFA biosynthesis (*fads2d6; fads2d5, elovl2, elovl5a, elovl5b*), transcription factors (*srebp1, srebp2, lxr, ppara, pparg*) and lipid metabolism (*fas, hmgcr, aco, cpt1*). The expressions were determined by real-time quantitative PCR in liver, as described in detail by Betancor *et al*. (2021). Results were normalised using reference housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase (*hprt*), elongation factor 1 alpha (*ef1α*) and ribosomal protein L2 (*rpl2*) that were considered as the most stable according to the geNorm (the detailed information of full name of GIO, housekeeping genes and primers are shown in Table 2.10). The qPCR was performed using a qTower^3 G real-time PCR Thermal Cycler (Analytic Jena GmbH, Jena, Germany) in 96-wellplates in duplicate 10 µL reaction volumes of mastermix containing 5 µL of Thermo Scientific Luminaris Color Higreen RT-PCR master mix kit (Thermo Scientific, Hemel Hempstead, UK), 1.0 µL of the primers corresponding to the analysed gene, 1.5 µL of molecular biology grade water and 2.5 µL of cDNA. As for housekeeping genes, 1 µL of cDNA and 3 µL of molecular biology grade water were used instead. A negative control without cDNA (no template control) was used to make sure no contaminant was present in the master mix. Standard amplification parameters contained a DNase pre-treatment at 50 °C for 2 min, an initial activation step at 95 °C for 10 min, followed by thirty-five cycles: 15 s at 95 °C, 30 s at

the annealing Tm and 30 s at 72 °C.

4.3.6 Histology analyses

Liver and intestine samples were rinsed in water and fixed in 4 % buffered neutral-formaldehyde by Shandon Citadel 2000 Automatic Tissue Processor (Thermo Scientific, Basingstoke, UK). Samples were then embedded into paraffin blocks (Histo-embedder, Jung, Leica, Germany). After being grimed the tissue was sectioned as a 5 µm thickness wax slice and fixed on the slide. All the slides were stained with haematoxylin-eosin while intestine samples were also stained with Alcian blue/Periodic Acid Schiff (ABPAS) for acidic and neutral goblet cells. The slides were then scanned and the digital images obtained from Axio Scan.Z1 slide scanner (ZEISS, Cambridge, UK) were analysed using Qupath v0.3.0 (Bankhead *et al*., 2017) and ImageJ (Research Services Branch, National Institute of Mental Health, Bethesda, Maryland, USA) software. The following histological factors were measured: intracytoplasmic lipid vacuolization; height and width of enterocyte; AB and PAS positive goblet cell density; intestinal circular muscle thickness. These data were collected by six randomly chosen fields from each tissue image at 20 × magnification. The intracytoplasmic lipid vacuolization was calculated as dividing the lipocyte area by the respective chosen area and the result was expressed as an amount per 10^5 µm^2. Goblet cell density was calculated as dividing the cell number by the area of intestinal villi from the tissue images. As for the height and width of the enterocytes and intestinal circular muscle thickness measurement, the methodology has been described elsewhere (Escaffre *et al*., 2007).

4.3.7 Statistical analyses

All data are shown as mean ± standard deviation. Percentage data were arcsine square-root transformed before statistical analyses. The results of the target gene were normalised to the housekeeping gene and calculated by the method of Pfaffl (2001). All variables were tested for normality and homogeneity of variances with Levene's test. The significance of difference was set at $p<0.05$. In the present study, the significance between

groups at the same phase was determined by one-way analysis of variance (ANOVA) followed by a Tukey's *post hoc* test for multiple comparisons of means where differences were detected. The significance between two phases for the same group was determined by a paired t-test. Multivariate principal component analysis (PCA) was conducted to define the similarity between samples with several features. All statistical analyses were performed using SPSS software (IBM SPSS Statistics 19; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, USA).

4.4 Results

4.4.1 Growth performance

Fish from all the experimental groups doubled their weights at the end of challenge phase with a final weight of over 30.0 g. At the end of the marine phase, fish from the V1 group showed a significantly higher body weight than the V2 group. No difference in SGR and feed intake after both phases was observed among groups at the end of the trial (Table 4.2).

Table 4.2 Growth performance of Atlantic salmon for each phase.

	M	V1	V2
Whole fish weight (g)			
End of stimulus phase	0.4±0.0	0.3±0.0	0.3±0.0
End of marine phase	15.2±0.2^{ab}	15.7±0.5^{a}	14.5±0.6^{b}
End of challenge phase	33.1±2.1	35.9±2.7	31.8±4.3
Growth rate (SGR %/ day)[1]			
Stimulus phase	6.3±0.2	6.0±0.3	5.9±0.1
Marine phase	4.0±0.0	4.1±0.0	4.0±0.0
Challenge phase	1.8±0.1	1.9±0.2	1.8±0.2
Feed intake (kg)			
Stimulus phase	0.03±0.0	0.03±0.0	0.03±0.0
Marine phase	2.0±0.0	2.0±0.0	2.1±0.0
Challenge phase	2.7±0.0	2.6±0.0	2.7±0.1

Data are means of triplicate measurement with standard deviations (n=3). a, b Mean values with unlike letters were significantly different between groups.

[1] The calculation of SGR will follow the formulation as: $SGR = 100 \times (\ln W_t - \ln W_i) / t$; W_t refers to final weight; W_i refers to initial weight; t refers to time.

4.4.2 Tissue lipid contents and lipid classes

During the whole trial, no difference was found on total lipid contents for any of the analysed tissues among groups at any phase (Tables 4.4-4.9). When comparing the lipid content before and after the diet change at the challenge phase, the intestine and muscle were found to have accumulated more lipid as shown by a significantly increased content (Table 4.5, 4.8). Different nutritional programming periods in the trial showed very little influence on the lipid class composition of all tissues, with no differences detected among groups. In addition, the similar composition between pre- and post-challenge samples indicate that the proportions did not vary to the challenge diet. It was noted that the polar lipid was the majority lipid class in liver and brain tissues (45 % and 53 % of TL, respectively). In contrast, triacylglycerols (TAG) dominated the majority in TL in other tissues. (Figure 4.2).

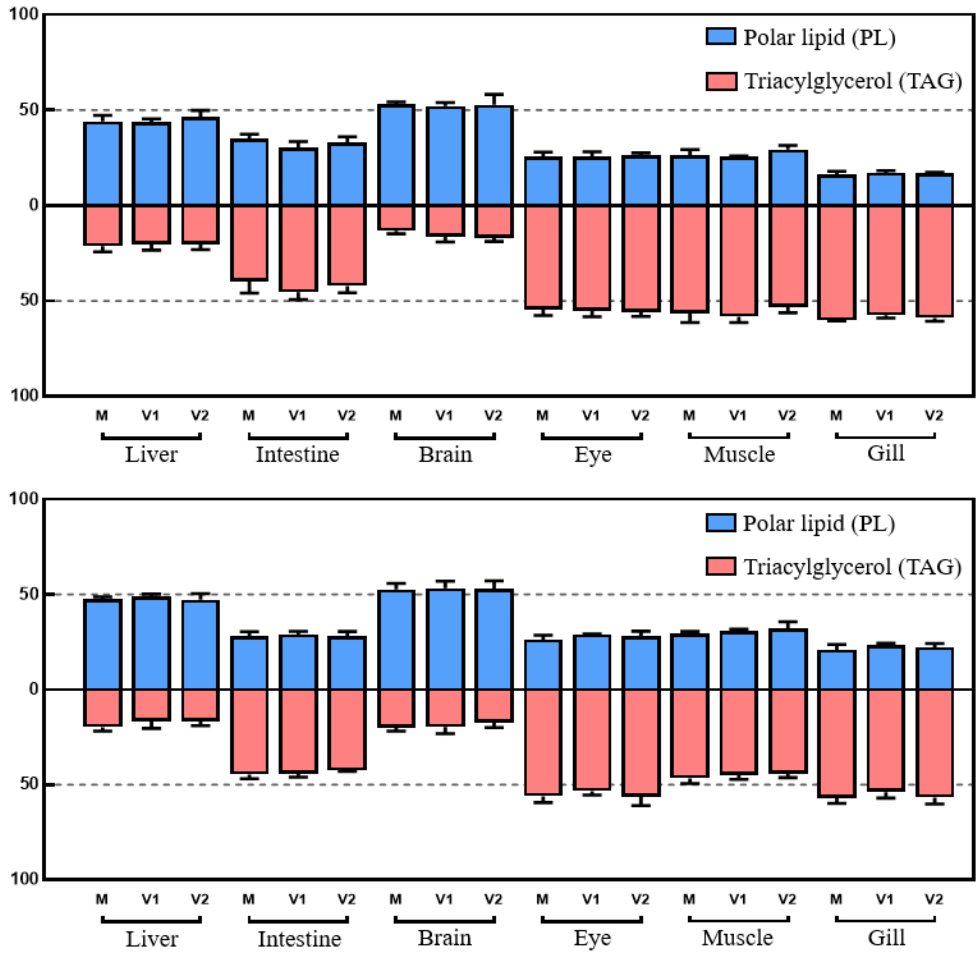


Figure 4.2 The percentage of polar lipid and triacylglycerol of different Atlantic salmon tissues among groups before (top) and after (bottom) the challenge phase. Data are means of triplicate measurement (n=3), with standard deviation represented by vertical bars.

4.4.3 Tissue total lipid fatty acid composition

At the end of the stimulus phase, the fatty acid composition of whole fish displayed significant differences among groups, especially in the V2 group. Fish from V2 showed the highest LA and n-6 PUFA levels, as a proportion of total lipid, as well as the lowest percentage of ARA, n-3 LC-PUFA n-3:n-6 ratio. Only negligible differences between fish from M and V1 group were observed, with higher LA, n-6 PUFA and lower n-3:n-6 ratio in group V1, whereas no impact was detected for n-3 PUFA (Table 4.3). In general terms, PCA of the fatty acid compositions of whole fish revealed that both M and V1 were similar, being clustered together with the M diet composition, whereas V2 was more separated by the relevance to the VO challenge diet. (Figure 4.3).

At the intermediate of the marine phase, livers from V2 fish presented a significantly higher MUFA and lower DHA level as well as a lower n-3:n-6 ratio as compared to M and V1. However, these differences were not observed after this time point. No difference in the liver fatty acid composition among groups was detected at the end of marine phase. Nevertheless, by the comparison between pre- and post-challenge, all n-6 PUFA were found to be elevated significantly by the challenge, whereas a significant drop in both EPA and DHA together with n-3:n-6 was found in all groups (Table 4.4). No differences were observed in the intestine fatty acid composition between groups at any of the three phases. Additionally, the presence of challenge significantly increased most of the n-6 PUFA and reduced all the n-3 PUFA contents in all groups (Table 4.5).

In the brain and eye, there were no differences in the fatty acid compositions between groups at any of the phases (Table 4.6, 4.7). In the brain of all groups, the challenge diet significantly increased the MUFA, LA and n-6 PUFA, while DHA, n-3 LC-PUFA and n-3:n-6 significantly decreased. However, the reduction in the proportion of n-3 LC-PUFA induced by the challenge diet in brain was less severe as compared with the other tissues (Table 4.6). The eye EPA contents for all groups was reduced by the challenge diet (Table 4.7).

In muscle, a significantly higher proportion of EPA and DHA was observed in V2 at the end of marine phase but disappeared after challenge. The challenge diet led to an increased level of LA and n-6 PUFA as well as the decreased level in EPA, DHA and n-3:n-6 in all groups significantly (Table 4.8). In contrast, fish from both stimulus groups had the lowest DHA content in the gill at the intermediate marine phase only. Furthermore, after challenge levels of LA, ARA and n-6 PUFA increased, whereas EPA and DHA as well as total n-3 LC-PUFA reduced in all groups (Table 4.9).

Table 4.3 Fatty acid compositions (percentage of total fatty acids) of Atlantic salmon whole fish after stimulus phase.

	M	**V1**	**V2**
Σ SFA[1]	26.6±0.5^{a}	25.4±0.4^{b}	20.5±0.3^{c}
Σ MUFA[2]	30.5±0.9^{b}	31.0±0.2^{b}	37.6±0.4^{a}
18:2n-6	6.1±0.2^{c}	7.6±0.2^{b}	14.1±0.1^{a}
20:3n-6	0.3±0.0^{b}	0.4±0.0^{b}	0.8±0.0^{a}
20:4n-6	1.4±0.1^{a}	1.4±0.0^{a}	1.2±0.0^{b}
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0
Σ n-6 PUFA[3]	8.8±0.3^{c}	10.4±0.2^{b}	17.7±0.2^{a}
18:3n-3	2.0±0.1^{b}	2.3±0.1^{b}	3.6±0.2^{a}
20:5n-3	7.4±0.2^{a}	7.0±0.1^{a}	4.5±0.1^{c}
22:5n-3	3.0±0.1^{a}	3.2±0.1^{a}	2.6±0.0^{b}
22:6n-3	18.0±0.9^{a}	17.1±0.1^{a}	10.6±0.2^{b}
Σ n-3 PUFA[4]	33.5±1.1^{a}	32.6±0.5^{a}	23.9±0.4^{b}
n-3 LC-PUFA[5]	29.9±1.2^{a}	28.9±0.4^{a}	19.1±0.3^{b}
DHA+EPA	25.5±1.2^{a}	24.1±0.2^{a}	15.0±0.2^{b}
DHA/EPA	2.4±0.1	2.5±0.0	2.4±0.1
n-3:n-6	3.8±0.2^{a}	3.1±0.1^{b}	1.3±0.0^{c}

Data are means of triplicate measurement with standard deviations (n=3). For each group, different superscript letters within a row denote significant differences among groups determined by one-way ANOVA with Tukey's comparison test (p<0.05). SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, 20:3n−6, 22:4n−6 and 22:5n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3, 21:5n-3 and 22:5n-3.
[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3

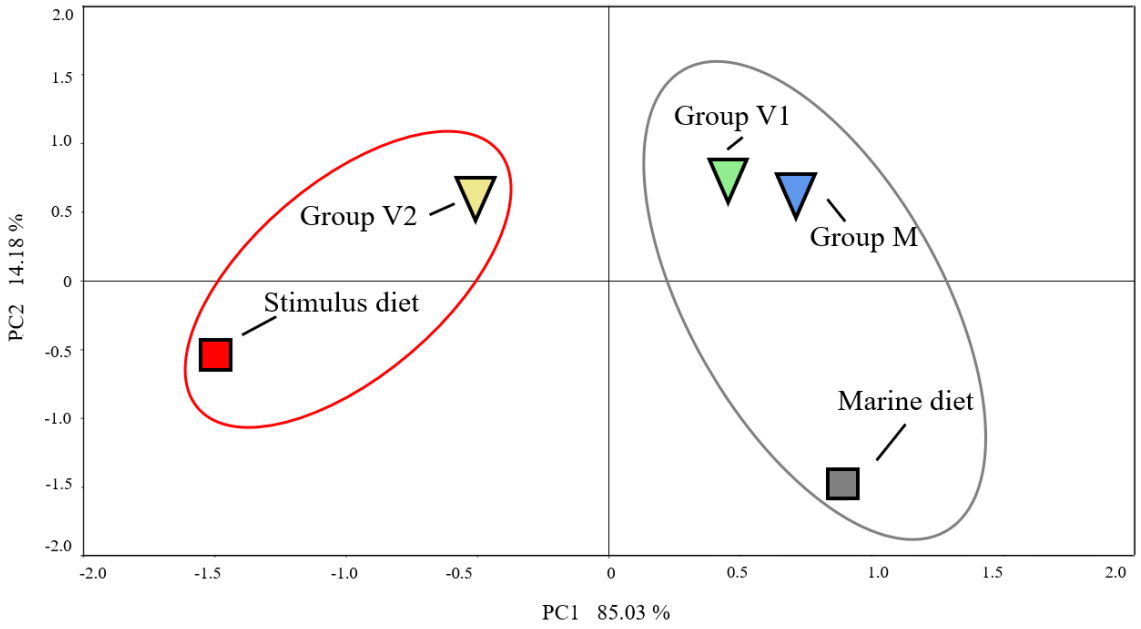


Figure 4.3 Principal component analysis (PCA) plot based on logarithmically transformed fatty acid composition of salmon whole fish and early diet after the stimulus stage.

4.4.4 Tissue polar lipid fatty acid composition

No differences in the fatty acid composition of polar lipid were found between groups for both the liver and intestine at any of the phases. All the main n-6 PUFAs increased significantly at the end of challenge phase in all groups, whereas the EPA and DHA content decreased. Besides, all these variations contributed to the lower n-3:n-6 ratio in both tissues after challenge (Table 4.4, 4.5).

Throughout the trial, no difference in the fatty acid composition of polar lipid from the brain and eye was induced by the stimulus duration. The impact of polar lipid fatty acid composition from the challenge diet on the brain and eye was less than other tissues. The proportions of ARA and DHA were significantly changed. Nonetheless, when comparing the reduction in the DHA content with other tissues, the brain and eye still displayed a relatively marked conservation. Moreover, the increase in n-6 fatty acids predominantly

impacted the n-3:n-6 ratio in both tissues. There was also an extremely high ratio of n-3:n-6 in both tissues, especially the brain tissues (Table 4.6, 4.7).

In muscle and gill, the duration of the stimulus did not show any noticeable impact at any of the phases, DHA content in the muscle of fish from V2 group at the intermediate phase. The challenge diet increased the LA and ARA as well as significantly decreased the EPA, DHA and total n-3 LC-PUFA content in both the muscle and gill. Similar to liver and intestine, there was a marked increase in the ARA content in gill resulting from the challenge diet, with an approximate three-fold increase (Table 4.8, 4.9).

Table 4.4 Fatty acid compositions (percentage of total and polar fatty acids) of Atlantic salmon liver from three groups at three phases.

	Intermediate of marine phase			End of marine phase			End of challenge phase		
Liver	**M**	**V1**	**V2**	**M**	**V1**	**V2**	**M**	**V1**	**V2**
Lipid % (wet wt.)	5.0±0.2	4.9±0.5	5.5±0.4	4.6±0.4	4.9±0.4	5.0±0.6	4.7±0.5	5.3±0.7	5.5±0.7
Total FA %									
Σ SFA[1]	23.1±0.5	23.3±1.6	21.6±0.6	23.2±0.4	22.4±0.6	22.6±0.9	19.6±1.1	20.8±0.6	20.7±0.9
Σ MUFA[2]	36.0±1.5[b]	33.7±3.6[b]	41.2±0.6[a]	33.4±2.1	36.2±2.8	35.5±3.5	43.8±3.8	40.5±3.2	40.2±3.0
18:2n-6	5.2±0.1	5.0±0.7	5.9±0.2	5.6±0.6	6.1±0.7	6.0±0.7	8.4±0.6*	8.4±0.2*	8.6±0.6*
20:3n-6	0.8±0.0	0.8±0.1	0.8±0.0	0.7±0.1	0.7±0.0	0.7±0.0	2.3±0.7*	3.1±0.3*	2.7±0.6*
20:4n-6	2.1±0.2	2.2±0.4	1.7±0.0	2.0±0.3	1.9±0.2	1.9±0.2	4.9±0.7*	5.4±0.6*	5.2±0.6*
22:5n-6	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.1	1.1±0.2*	1.3±0.1*	1.5±0.1*
Σ n-6 PUFA[3]	9.6±0.0	9.5±0.4	10.0±0.3	9.6±0.1	10.2±0.8	10.1±0.5	19.0±0.9*	20.7±0.5*	20.5±0.9*
18:3n-3	1.1±0.1	1.1±0.1	1.2±0.0	1.4±0.3	1.3±0.1	1.3±0.1	1.2±0.1	1.1±0.1	1.0±0.0
20:5n-3	3.8±0.2	4.2±0.6	3.3±0.2	4.1±0.3*	3.9±0.3*	3.9±0.5*	1.7±0.8	1.8±0.3	1.8±0.0
22:5n-3	1.0±0.1	1.0±0.1	0.8±0.0	1.1±0.1	0.9±0.1	0.9±0.1	0.9±0.3	1.0±0.2	0.7±0.2
22:6n-3	24.7±0.9[a]	25.7±1.0[a]	20.2±0.7[b]	25.7±3.4*	23.9±2.7*	24.3±2.3*	12.4±2.1	13.0±2.0	13.6±2.3
Σ n-3 PUFA[4]	31.7±1.1[a]	33.0±1.7[a]	26.6±0.8[b]	33.4±2.7*	31.0±2.9*	31.1±2.8*	17.4±3.5	17.8±2.5	18.3±2.6
n-3 LC-PUFA[5]	30.0±1.1[a]	31.4±2.0[a]	24.9±0.9[b]	31.5±3.4*	29.3±3.1*	29.5±2.9*	15.3±5.2	15.9±2.4	16.6±2.5
DHA+EPA	28.5±1.1[a]	29.9±1.7[a]	23.5±0.8[b]	29.8±3.5*	27.9±2.9*	28.1±2.8*	14.1±4.8	14.8±2.2	15.3±2.3
DHA/EPA	6.5±0.3	6.2±0.4	6.1±0.2	6.4±0.8	6.1±0.4	6.2±0.4	7.5±1.2	7.3±0.3	7.7±1.1
n-3:n-6	3.3±0.1[a]	3.5±0.5[a]	2.7±0.2[b]	3.5±0.3*	3.1±0.5*	3.1±0.6*	0.9±0.3	0.9±0.1	0.9±0.2
Polar FA %									
Σ SFA[1]	28.3±0.2	28.8±1.1	27.1±0.1	28.4±0.3	27.5±0.7	27.8±0.1	24.0±1.1	24.4±0.6	24.1±0.6
Σ MUFA[2]	21.0±0.2	20.8±1.5	22.1±0.6	19.4±0.5	20.4±0.6	19.6±0.7	26.3±2.8*	25.7±1.8*	26.3±2.4*
18:2n-6	3.0±0.1	3.1±0.2	3.4±0.1	3.2±0.0	3.4±0.1	3.4±0.1	5.8±1.0*	6.2±0.3*	5.9±0.3*
20:3n-6	0.9±0.1	0.9±0.0	0.9±0.1	0.8±0.1	0.7±0.0	0.8±0.0	3.1±1.1*	4.1±0.3*	3.5±0.8*
20:4n-6	3.2±0.2	3.1±0.2	2.9±0.1	3.1±0.1	3.1±0.5	3.1±0.1	8.9±1.2*	8.7±0.2*	9.3±0.3*
22:5n-6	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.4±0.0	0.4±0.0	2.2±0.4*	2.4±0.1*	2.4±0.1*
Σ n-6 PUFA[3]	8.7±0.2	8.6±0.3	8.6±0.3	8.3±0.1	8.1±0.1	8.1±0.3	21.6±2.4*	23.1±0.1*	23.0±1.5*
18:3n-3	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.1	0.6±0.1	0.6±0.0
20:5n-3	4.5±0.2	4.7±0.2	4.6±0.2	4.6±0.4*	4.8±0.1*	4.7±0.2*	2.6±0.2	2.5±0.1	2.4±0.1
22:5n-3	1.0±0.0	1.0±0.1	1.0±0.0	1.0±0.1	1.0±0.0	1.0±0.1	1.4±0.2	1.5±0.2	1.2±0.2
22:6n-3	34.9±0.6	34.6±0.3	35.0±0.7	36.9±0.8*	36.9±0.4*	37.3±0.2*	22.5±1.7	21.8±1.2	21.1±2.5
Σ n-3 PUFA[4]	41.5±0.5	41.4±0.4	41.7±0.8	43.7±0.4*	43.8±0.3*	44.2±0.3*	27.9±2.1	26.8±1.3	26.3±2.6
n-3 LC-PUFA[5]	40.8±0.5	40.7±0.5	40.9±0.9	42.9±0.4*	43.0±0.3*	43.4±0.2*	26.8±2.2	25.8±1.3	25.3±2.6
DHA+EPA	39.4±0.5	39.3±0.5	39.5±0.9	41.5±0.4*	41.6±0.3*	42.0±0.2*	25.1±2.0	24.0±1.2	23.5±2.4
DHA/EPA	7.7±0.4	7.4±0.3	7.6±0.2	8.0±0.8	7.8±0.2	7.9±0.3	9.0±1.3	8.4±0.6	8.8±1.4
n-3:n-6	4.8±0.2	4.8±0.2	4.8±0.3	5.3±0.1*	5.4±0.1*	5.4±0.2*	1.3±0.2	1.2±0.1	1.2±0.2

Data are means of triplicate measurement with standard deviations (n=3). For each phase, different superscript letters within a row denote significant differences among treatments.
The mark * denotes significant difference between the end of marine phase and the end of challenge phase for each treatment (p<0.05).
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;
[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.
[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.
[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.
[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.
[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 4.5 Fatty acid compositions (percentage of total and polar fatty acids) of Atlantic salmon intestine from three groups at three phases.

	Intermediate of marine phase			End of marine phase			End of challenge phase		
Intestine	**M**	**V1**	**V2**	**M**	**V1**	**V2**	**M**	**V1**	**V2**
Lipid % (wet wt.)	6.8±0.6	7.0±0.5	6.4±0.1	7.4±0.7	6.5±0.4	6.2±0.6	9.7±0.4*	9.3±0.3*	8.9±1.0*
Total FA %									
Σ SFA[1]	26.4±0.6	26.2±0.5	25.9±0.3	26.2±0.3*	25.6±0.2*	25.7±0.5*	19.8±1.6	19.4±0.5	19.4±0.4
Σ MUFA[2]	35.7±2.2	37.7±1.4	37.1±0.6	37.3±1.7	39.3±2.4	38.0±1.6	47.0±0.8*	46.9±0.9*	46.7±1.1*
18:2n-6	6.4±0.3	6.8±0.4	6.6±0.3	7.1±0.4	7.6±0.3	7.4±0.4	12.9±1.0*	13.2±0.4*	12.8±0.3*
20:3n-6	0.5±0.1	0.4±0.1	0.4±0.0	0.4±0.1	0.4±0.1	0.4±0.0	1.2±0.2*	1.3±0.1*	1.3±0.1*
20:4n-6	1.0±0.2	0.9±0.2	1.0±0.0	1.0±0.1	0.8±0.1	0.9±0.1	1.4±0.0*	1.4±0.0*	1.6±0.2*
22:5n-6	0.3±0.1	0.2±0.1	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.5±0.1	0.5±0.1	0.6±0.0
Σ n-6 PUFA[3]	9.5±0.1	9.4±0.2	9.4±0.2	9.8±0.1	10.0±0.1	10.1±0.1	18.2±1.4*	18.5±0.6*	18.4±0.2*
18:3n-3	1.8±0.2	2.0±0.2	1.9±0.1	2.1±0.2	2.3±0.1	2.2±0.2	3.2±0.2*	3.1±0.1*	3.0±0.1*
20:5n-3	3.4±0.1	3.4±0.1	3.5±0.0	3.4±0.1*	3.3±0.1*	3.4±0.1*	1.4±0.1	1.6±0.1	1.6±0.1
22:5n-3	1.2±0.1	1.2±0.1	1.2±0.1	1.2±0.0*	1.2±0.0*	1.2±0.1*	0.7±0.1	0.7±0.1	0.7±0.0
22:6n-3	18.9±2.3	16.9±1.8	17.9±0.6	16.9±2.1*	14.9±2.5*	16.2±1.7*	7.1±0.8	7.1±0.6	7.6±0.7
Σ n-3 PUFA[4]	27.4±1.7	25.7±1.2	26.6±0.5	25.6±1.6*	24.0±2.4*	25.2±1.3*	14.4±0.7	14.5±0.9	14.8±0.9
n-3 LC-PUFA[5]	24.4±2.0	22.4±1.6	23.3±0.5	22.2±2.0*	20.3±2.5*	21.6±1.6*	9.7±1.0	9.9±0.8	10.4±0.9
DHA+EPA	22.3±2.2	20.3±1.8	21.3±0.6	20.2±2.1*	18.2±2.6*	19.6±1.7*	8.5±0.8	8.7±0.8	9.2±0.8
DHA/EPA	5.5±0.9	4.9±0.7	5.2±0.2	5.1±0.7	4.5±0.8	4.8±0.5	5.0±0.5	4.6±0.2	4.8±0.2
n-3:n-6	2.9±0.1	2.7±0.2	2.8±0.1	2.6±0.2*	2.4±0.3*	2.5±0.2*	0.8±0.1	0.8±0.1	0.8±0.1
Polar FA %									
Σ SFA[1]	32.6±0.2	32.5±0.8	32.2±0.6	31.0±0.2	30.8±0.4	30.6±0.4	28.8±1.6	28.4±1.1	28.2±0.4
Σ MUFA[2]	17.6±0.4	19.1±0.9	19.2±1.8	18.6±1.1	19.1±0.8	18.4±0.5	24.3±1.9*	25.4±3.1*	25.2±1.1*
18:2n-6	3.4±0.0	3.4±0.1	3.6±0.3	2.9±0.0	3.0±0.0	3.1±0.1	7.5±0.7*	8.0±0.6*	7.9±0.2*
20:3n-6	0.9±0.1	0.8±0.0	0.8±0.1	0.8±0.1	0.7±0.0	0.8±0.0	3.3±0.6*	3.5±0.3*	3.4±0.5*
20:4n-6	1.9±0.0	1.9±0.0	2.0±0.1	2.6±0.2	2.5±0.1	2.5±0.1	6.5±0.1*	6.1±0.3*	6.4±0.4*
22:5n-6	0.7±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0	2.6±0.8*	2.2±0.4*	2.4±0.2*
Σ n-6 PUFA[3]	8.9±0.2	8.5±0.1	8.7±0.7	8.1±0.1	8.1±0.1	8.2±0.2	21.6±2.1*	21.5±1.7*	21.5±1.2*
18:3n-3	0.4±0.1	0.4±0.1	0.4±0.1	0.3±0.0	0.4±0.1	0.4±0.1	0.7±0.3	0.8±0.3	0.8±0.2
20:5n-3	3.0±0.1	3.3±0.3	3.1±0.4	3.2±0.2*	3.1±0.2*	3.2±0.0*	1.6±0.2	1.6±0.1	1.5±0.0
22:5n-3	0.8±0.0	0.9±0.0	0.8±0.1	0.8±0.0	0.8±0.1	0.8±0.0	0.7±0.0	0.7±0.0	0.7±0.0
22:6n-3	35.4±0.2	33.8±0.8	34.1±1.6	36.1±1.1*	35.9±1.0*	36.4±0.2*	19.9±1.8	19.1±2.7	19.5±0.6
Σ n-3 PUFA[4]	40.1±0.4	39.0±0.9	39.1±1.9	40.8±1.1*	40.6±0.7*	41.2±0.2*	23.5±1.6	22.8±2.7	23.2±0.7
n-3 LC-PUFA[5]	39.5±0.4	38.4±1.0	38.5±2.0	40.4±1.1*	40.1±0.7*	40.8±0.2*	22.4±1.6	21.6±2.8	22.0±0.7
DHA+EPA	38.3±0.3	37.1±1.0	37.2±1.9	39.2±1.1*	39.0±0.8*	39.6±0.2*	21.5±1.7	20.6±2.8	21.0±0.7
DHA/EPA	12.0±0.5	10.5±1.0	11.0±1.0	11.3±0.7	11.6±1.2	11.2±0.0	12.7±2.4	12.2±1.0	12.7±0.1
n-3:n-6	4.5±0.1	4.6±0.1.0	4.5±0.1	5.1±0.1*	5.0±0.2*	5.0±0.1*	1.1±0.0	1.1±0.1	1.1±0.1

Data are means of triplicate measurement with standard deviations (n=3). For each phase, different superscript letters within a row denote significant differences among treatments.
The mark * denotes significant difference between the end of marine phase and the end of challenge phase for each treatment (p<0.05).
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;
[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.
[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.
[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.
[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.
[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 4.6 Fatty acid compositions (percentage of total and polar fatty acids) of Atlantic salmon brain from three groups at three phases.

	Intermediate of marine phase			End of marine phase			End of challenge phase		
Brain	**M**	**V1**	**V2**	**M**	**V1**	**V2**	**M**	**V1**	**V2**
Lipid % (wet wt.)	7.7±0.3	7.6±0.7	7.4±0.5	7.1±0.3	7.0±0.5	6.8±0.1	7.1±0.5	8.1±0.9	6.8±0.1
Total FA %									
Σ SFA[1]	27.3±0.4	27.5±0.4	27.4±0.6	28.3±0.6	27.8±0.6	27.5±0.4	24.1±2.6	23.6±2.1	24.8±0.7
Σ MUFA[2]	34.2±1.9	33.2±3.2	32.3±1.9	30.8±0.6	31.6±1.7	32.1±0.9	37.9±3.2*	39.4±3.3*	36.3±0.8*
18:2n-6	2.5±0.3	2.5±0.4	2.4±0.2	1.9±0.2	2.1±0.3	2.3±0.4	5.2±1.6*	5.4±1.1*	3.9±0.5*
20:3n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.8±0.1*	0.8±0.0*	0.7±0.1*
20:4n-6	0.9±0.0	0.9±0.1	0.9±0.1	0.9±0.0	0.9±0.1	0.9±0.0	1.6±0.4	1.6±0.3	1.8±0.1
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.4±0.1	0.4±0.1	0.4±0.0
Σ n-6 PUFA[3]	4.1±0.4	4.2±0.5	4.0±0.6	3.3±0.3	3.6±0.3	3.8±0.4	8.9±1.7*	9.1±1.5*	7.5±0.8*
18:3n-3	0.7±0.1	0.8±0.5	0.6±0.1	0.5±0.1	0.6±0.3	0.7±0.3	1.3±0.7	1.3±0.8	0.9±0.3
20:5n-3	4.1±0.1	4.1±0.3	4.2±0.1	4.2±0.1	4.2±0.1	4.1±0.1	3.0±0.4	2.9±0.6	3.2±0.3
22:5n-3	1.8±0.1	1.9±0.1	2.0±0.1	1.7±0.0	1.7±0.1	1.7±0.0	1.3±0.2	1.2±0.2	1.4±0.1
22:6n-3	24.0±2.1	24.5±2.2	25.7±2.4	27.3±0.5*	26.4±2.1*	25.9±1.1*	19.2±2.2	18.1±1.7	21.3±1.4
Σ n-3 PUFA[4]	31.6±2.1	32.2±2.7	33.4±2.2	34.2±0.4*	33.5±1.7*	33.0±0.9*	25.8±2.5	24.4±2.2	27.5±1.3
n-3 LC-PUFA[5]	30.3±2.2	30.9±3.4	32.3±2.4	33.5±0.4*	32.7±2.2*	32.0±1.1*	23.9±2.7	22.5±2.4	26.3±2.7
DHA+EPA	28.1±2.2	28.6±3.5	29.9±2.5	31.5±0.5*	30.6±2.2*	30.0±1.2*	22.2±4.6	20.9±5.3	24.5±1.6
DHA/EPA	5.9±0.4	6.0±0.6	6.2±0.4	6.5±0.2	6.3±0.3	6.3±0.2	6.4±0.7	6.3±0.3	6.7±0.2
n-3:n-6	7.7±1.2	8.5±1.2	8.5±1.8	10.1±1.4*	9.8±2.7*	8.9±2.1*	3.1±1.1	3.1±1.7	3.8±0.9
Polar FA %									
Σ SFA[1]	30.2±1.0	31.7±1.0	30.9±1.3	30.9±0.2	30.1±0.3	30.1±0.8	28.1±0.5	27.4±0.4	27.7±1.0
Σ MUFA[2]	30.8±1.5	28.4±1.3	29.8±1.4	29.2±0.4	28.8±1.3	29.3±0.2	32.2±0.5	32.4±0.9	31.8±2.0
18:2n-6	0.4±0.0	0.5±0.1	0.5±0.0	0.4±0.1	0.5±0.1	0.5±0.1	0.9±0.1	0.9±0.2	0.9±0.2
20:3n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.4±0.0	0.4±0.1	0.4±0.1	0.7±0.3	0.7±0.3	0.7±0.1
20:4n-6	0.9±0.0	0.9±0.0	0.9±0.0	0.8±0.0	0.8±0.0	0.8±0.0	1.9±0.1*	1.9±0.1*	1.9±0.1*
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.5±0.1	0.5±0.0	0.5±0.0
Σ n-6 PUFA[3]	1.8±0.0	1.9±0.1	1.9±0.1	1.7±0.1	1.7±0.0	1.8±0.0	4.3±0.3*	4.4±0.2*	4.3±0.4*
18:3n-3	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.2±0.0
20:5n-3	3.8±0.1	3.8±0.1	3.7±0.1	3.7±0.0	3.8±0.1	3.8±0.1	3.3±0.1	3.3±0.1	3.3±0.0
22:5n-3	1.8±0.0	1.9±0.0	1.8±0.0	1.7±0.0	1.7±0.1	1.7±0.0	1.6±0.0	1.6±0.1	1.6±0.0
22:6n-3	26.1±1.3	27.2±0.1	26.7±1.1	28.1±0.5*	28.8±1.4*	28.4±0.4*	24.7±0.6	24.8±0.8	25.2±1.3
Σ n-3 PUFA[4]	32.1±1.2	33.3±0.2	32.6±1.1	33.9±0.5*	34.7±1.5*	34.3±0.5*	30.1±0.8	30.2±0.8	30.7±1.2
n-3 LC-PUFA[5]	31.9±1.2	33.1±0.2	32.4±1.1	33.7±0.5*	34.5±1.5*	34.1±0.5*	29.8±0.8	30.0±0.8	30.4±1.3
DHA+EPA	29.9±1.1	31.0±0.2	30.3±1.1	31.8±0.5*	32.6±1.5*	32.2±0.5*	27.9±0.8	28.1±0.8	28.5±1.2
DHA/EPA	7.0±0.6	7.1±0.1	7.2±0.4	7.6±0.2	7.5±0.2	7.5±0.2	7.6±0.1	7.5±0.2	7.7±0.5
n-3:n-6	17.4±0.9	17.9±1.1	17.0±0.9	20.1±1.1*	20.0±1.3*	19.2±0.6*	7.0±0.6	6.8±0.3	7.1±0.4

Data are means of triplicate measurement with standard deviations (n=3). For each phase, different superscript letters within a row denote significant differences among treatments.
The mark * denotes significant difference between the end of marine phase and the end of challenge phase for each treatment (p<0.05).
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;
[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.
[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.
[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.
[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.
[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 4.7 Fatty acid compositions (percentage of total and polar fatty acids) of Atlantic salmon eye from three groups at three phases.

	Intermediate of marine phase			End of marine phase			End of challenge phase		
Eye	**M**	**V1**	**V2**	**M**	**V1**	**V2**	**M**	**V1**	**V2**
Lipid % (wet wt.)	7.9±0.3	7.9±0.9	8.0±0.7	8.1±0.8	8.6±0.7	7.9±0.5	8.7±0.3	9.0±0.8	7.8±0.2
Total FA %									
Σ SFA[1]	25.3±0.3	25.2±0.4	24.9±0.4	24.1±0.1*	24.0±0.2*	23.7±0.4*	18.8±0.9	18.4±0.2	18.6±0.2
Σ MUFA[2]	42.4±0.6	41.8±1.0	42.7±0.3	42.2±0.4	42.1±0.2	41.3±0.1	48.6±1.1*	48.8±0.3*	49.0±0.1*
18:2n-6	7.3±0.1	7.3±0.2	7.5±0.2	8.0±0.3	8.1±0.0	8.0±0.1	12.6±1.0*	13.1±0.3*	12.9±0.3*
20:3n-6	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.2±0.1	0.2±0.0	0.9±0.1*	1.0±0.1*	0.9±0.1*
20:4n-6	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.8±0.0	0.8±0.0	0.8±0.0
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.2±0.0
Σ n-6 PUFA[3]	9.1±0.1	9.1±0.1	9.2±0.2	9.7±0.3	9.7±0.1	10.1±0.8	16.6±1.4*	17.4±0.4*	16.9±0.3*
18:3n-3	2.3±0.1	2.3±0.1	2.4±0.1	2.6±0.1	2.7±0.0	2.7±0.1	3.3±0.2	3.3±0.1	3.4±0.1
20:5n-3	3.4±0.0	3.6±0.1	3.4±0.0	3.4±0.1*	3.4±0.0*	3.5±0.0*	1.7±0.3	1.5±0.1	1.6±0.1
22:5n-3	1.3±0.0	1.4±0.0	1.3±0.0	1.3±0.0*	1.4±0.0*	1.4±0.0*	0.7±0.2	0.6±0.0	0.7±0.0
22:6n-3	12.6±0.7	13.0±1.0	12.5±0.3	12.9±0.6*	12.8±0.3*	13.4±0.4*	7.4±1.2	7.4±0.4	7.2±0.2
Σ n-3 PUFA[4]	22.1±0.8	22.7±1.1	21.9±0.5	22.7±0.6*	22.9±0.2*	23.5±0.2*	15.5±1.5	15.0±0.5	15.1±0.1
n-3 LC-PUFA[5]	18.2±0.8	18.7±1.2	18.2±0.3	18.5±0.7*	18.6±0.2*	18.8±1.1*	10.4±1.8	10.0±0.6	10.0±0.2
DHA+EPA	16.0±0.7	16.5±1.1	15.9±0.3	16.3±0.7*	16.2±0.3*	16.3±0.8*	9.1±1.6	8.9±0.5	8.8±0.2
DHA/EPA	3.7±0.2	3.6±0.2	3.6±0.1	3.8±0.1	3.7±0.1	3.8±0.1	4.4±0.2	4.9±0.1	4.5±0.2
n-3:n-6	2.4±0.1	2.5±0.1	2.4±0.1	2.3±0.1*	2.4±0.0*	2.3±0.2*	0.9±0.2	0.9±0.0	0.9±0.0
Polar FA %									
Σ SFA[1]	29.7±0.1	29.2±0.5	29.3±0.8	27.1±0.4	26.8±0.4	26.6±0.2	25.0±0.2	24.8±0.1	24.3±0.6
Σ MUFA[2]	23.2±1.4	23.3±1.0	23.5±1.1	22.6±1.1	22.8±1.0	22.2±0.4	24.7±0.5	24.5±0.7	26.9±0.8
18:2n-6	2.5±0.4	1.9±0.2	2.1±0.3	1.5±0.2	1.7±0.4	1.4±0.1	3.4±0.7*	3.4±0.3*	3.5±0.2*
20:3n-6	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.0	1.6±0.4*	1.7±0.1*	1.6±0.2*
20:4n-6	1.4±0.0	1.4±0.0	1.4±0.0	1.5±0.1	1.5±0.0	1.5±0.1	3.4±0.4*	3.4±0.2*	3.3±0.1*
22:5n-6	0.4±0.0	0.4±0.0	0.4±0.0	0.7±0.2	0.7±0.2	0.8±0.2	1.1±0.3	1.2±0.1	1.1±0.1
Σ n-6 PUFA[3]	4.9±0.2	4.3±0.1	4.6±0.4	4.0±0.2	4.2±0.4	3.9±0.3	10.2±2.0*	10.5±0.6*	10.4±0.6*
18:3n-3	0.6±0.1	0.5±0.0	0.5±0.0	0.5±0.1	0.5±0.1	0.5±0.1	0.7±0.1	0.7±0.1	0.7±0.0
20:5n-3	3.8±0.1	4.1±0.1	4.1±0.3	3.7±0.2	3.8±0.2	3.8±0.1	3.5±0.2	3.3±0.1	3.4±0.0
22:5n-3	1.2±0.0	1.3±0.1	1.3±0.1	1.2±0.0	1.2±0.1	1.2±0.0	1.2±0.1	1.1±0.0	1.1±0.0
22:6n-3	34.9±1.5	35.6±1.2	34.9±0.9	37.6±0.9*	37.5±1.6*	38.6±0.6*	32.0±1.6	32.6±1.4	30.3±0.3
Σ n-3 PUFA[4]	41.2±1.7	42.1±1.0	41.5±1.1	43.5±1.0*	43.6±1.3*	44.5±0.5*	38.0±1.7	38.3±1.1	36.2±0.3
n-3 LC-PUFA[5]	40.3±1.8	41.3±1.1	40.7±1.2	42.6±0.7*	42.8±1.5*	43.8±0.6*	35.1±1.9	35.4±1.2	35.2±0.3
DHA+EPA	38.8±1.7	39.7±1.1	39.0±1.1	41.1±0.7*	41.3±1.5*	42.3±0.5*	33.5±1.8	34.0±1.3	33.7±0.3
DHA/EPA	9.1±0.1	8.7±0.5	8.5±0.4	10.1±0.5	9.9±0.8	10.3±0.5	12.8±0.4	14.2±1.1	12.5±0.4
n-3:n-6	8.4±0.8	9.9±0.3	9.1±0.7	10.9±0.7*	10.6±1.2*	11.3±1.0*	3.7±0.3	3.6±0.3	3.4±0.2

Data are means of triplicate measurement with standard deviations (n=3). For each phase, different superscript letters within a row denote significant differences among treatments.
The mark * denotes significant difference between the end of marine phase and the end of challenge phase for each treatment (p<0.05).
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;
[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.
[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.
[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.
[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.
[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 4.8 Fatty acid compositions (percentage of total and polar fatty acids) of Atlantic salmon muscle from three groups at three phases.

	Intermediate of marine phase			End of marine phase			End of challenge phase		
Muscle	**M**	**V1**	**V2**	**M**	**V1**	**V2**	**M**	**V1**	**V2**
Lipid % (wet wt.)	5.3±0.3	4.4±0.6	4.4±0.6	4.7±0.4	4.6±0.2	3.8±0.4	6.9±0.6*	6.5±0.5*	6.1±0.4*
Total FA %									
Σ SFA[1]	25.9±0.6	25.6±0.8	25.5±0.7	22.7±1.3*	22.0±1.2*	22.2±0.6*	18.2±0.4	17.7±0.1	17.8±0.2
Σ MUFA[2]	41.5±0.9	40.0±1.6	41.1±0.4	42.5±0.4	42.5±0.6	41.2±1.1	47.9±1.2*	48.3±0.1*	47.7±0.2*
18:2n-6	7.2±0.2	7.3±0.1	7.5±0.1	8.4±0.2	8.5±0.0	8.3±0.1	13.4±0.5*	13.9±0.2*	13.7±0.1*
20:3n-6	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	1.1±0.2*	1.2±0.1*	1.2±0.1*
20:4n-6	0.5±0.0	0.6±0.1	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.8±0.1	0.8±0.0	0.8±0.0
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.3±0.0	0.3±0.0	0.3±0.0
Σ n-6 PUFA[3]	9.0±0.2	9.2±0.1	9.3±0.1	10.2±0.2	10.2±0.1	10.1±0.1	17.7±1.0*	18.5±0.3*	18.2±0.3*
18:3n-3	2.2±0.1	2.3±0.1	2.3±0.1	2.8±0.1	2.8±0.0	2.8±0.1	3.5±0.1	3.5±0.1	3.5±0.1
20:5n-3	3.8±0.2	4.1±0.4	4.0±0.1	3.7±0.1^{b}*	4.0±0.1^{a}*	4.1±0.1^{a}*	1.7±0.0	1.6±0.1	1.8±0.0
22:5n-3	1.6±0.1	1.6±0.1	1.1±0.9	1.5±0.0	1.5±0.0	1.5±0.1	0.8±0.1	0.7±0.0	0.7±0.0
22:6n-3	12.1±1.0	13.4±1.7	12.4±0.4	12.7±0.8^{b}*	12.9±0.5^{b}*	14.2±0.5^{a}*	7.3±0.6	7.0±0.2	7.4±0.1
Σ n-3 PUFA[4]	22.4±1.3	24.1±2.3	22.9±0.6	23.3±0.9^{b}*	23.8±0.4^{b}*	25.2±0.5^{a}*	15.8±1.2	15.2±0.2	15.9±0.2
n-3 LC-PUFA[5]	18.4±1.2	20.1±2.3	18.5±1.1	18.9±0.8^{b}*	19.3±0.5^{b}*	20.7±0.5^{a}*	10.3±1.0	9.9±0.3	10.6±0.2
DHA+EPA	15.9±1.1	17.5±2.1	16.4±0.4	16.4±0.9*	16.9±0.5*	18.3±0.6*	8.9±1.0	8.6±0.2	9.3±0.1
DHA/EPA	3.2±0.1	3.2±0.1	3.1±0.1	3.4±0.1	3.2±0.2	3.5±0.1	4.3±0.6	4.4±0.4	4.1±0.0
n-3:n-6	2.5±0.1	2.6±0.2	2.5±0.1	2.3±0.0*	2.3±0.0*	2.5±0.0*	0.9±0.1	0.8±0.0	0.9±0.0
Polar FA %									
Σ SFA[1]	26.5±0.1	26.2±0.3	26.0±0.1	23.6±0.4*	23.5±0.4*	24.1±0.7*	20.1±0.6	19.8±0.2	20.1±0.3
Σ MUFA[2]	20.1±0.3	20.0±0.6	19.3±0.1	18.4±0.5	17.6±0.4	17.3±0.1	24.2±1.9*	24.6±0.2*	24.3±0.3*
18:2n-6	4.1±0.0	4.2±0.3	4.1±0.1	3.9±0.0	3.9±0.2	3.7±0.0	8.9±0.9*	9.5±0.3*	9.1±0.1*
20:3n-6	0.7±0.1	0.6±0.0	0.6±0.0	0.5±0.0	0.5±0.0	0.5±0.0	2.7±0.4*	2.9±0.2*	2.8±0.2*
20:4n-6	1.4±0.0	1.4±0.0	1.4±0.0	1.4±0.1	1.2±0.0	1.3±0.2	3.3±0.2*	3.2±0.2*	3.2±0.2*
22:5n-6	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0	0.6±0.0	1.4±0.1	1.4±0.1	1.4±0.1
Σ n-6 PUFA[3]	7.4±0.0	7.4±0.4	7.2±0.3	7.0±0.1	6.8±0.3	6.7±0.2	18.2±1.9*	18.9±0.8*	18.3±0.6*
18:3n-3	1.5±0.0	1.5±0.1	1.5±0.1	1.7±0.1	1.7±0.1	1.6±0.0	2.7±0.1	3.0±0.0	2.9±0.1
20:5n-3	7.3±0.2	7.4±0.2	7.6±0.2	7.3±0.1*	7.5±0.2*	7.3±0.1*	4.2±0.5	4.0±0.2	4.2±0.1
22:5n-3	2.2±0.1	2.2±0.0	2.2±0.0	2.0±0.0	2.1±0.1	2.0±0.1	1.5±0.1	1.4±0.1	1.5±0.0
22:6n-3	32.7±0.3^{b}	33.0±0.6^{ab}	33.9±0.3^{a}	36.1±0.8*	37.1±0.8*	37.4±0.7*	24.8±0.8	24.2±0.8	24.8±0.7
Σ n-3 PUFA[4]	45.5±0.3^{b}	45.9±0.6^{b}	47.1±0.3^{a}	48.9±0.8*	50.1±0.4*	49.8±0.7*	35.5±3.2	34.9±1.0	35.4±0.7
n-3 LC-PUFA[5]	43.1±0.3^{b}	43.5±0.7^{ab}	44.6±0.3^{a}	46.4±0.8*	47.6±0.5*	47.5±0.7*	31.3±3.4	30.4±1.1	31.2±0.7
DHA+EPA	40.0±0.4^{b}	40.4±0.7^{ab}	41.5±0.3^{a}	43.5±0.8*	44.6±0.6*	44.7±0.7*	29.1±3.3	28.3±1.0	28.9±0.7
DHA/EPA	4.5±0.1	4.5±0.1	4.5±0.1	4.9±0.2	4.9±0.2	5.2±0.1	5.8±0.0	6.0±0.1	6.0±0.3
n-3:n-6	6.2±0.0	6.2±0.4	6.6±0.3	7.0±0.2*	7.4±0.4*	7.4±0.3*	2.0±0.3	1.9±0.1	1.9±0.1

Data are means of triplicate measurement with standard deviations (n=3). For each phase, different superscript letters within a row denote significant differences among treatments.
The mark * denotes significant difference between the end of marine phase and the end of challenge phase for each treatment (p<0.05).
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;
[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.
[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.
[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.
[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.
[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 4.9 Fatty acid compositions (percentage of total and polar fatty acids) of Atlantic salmon gill from three groups at three phases.

	Intermediate of marine phase			End of marine phase			End of challenge phase		
Gill	**M**	**V1**	**V2**	**M**	**V1**	**V2**	**M**	**V1**	**V2**
Lipid % (wet wt.)	9.9±0.4	8.7±0.6	9.8±0.5	10.4±0.9	10.4±1.3	9.1±0.4	10.6±1.0	10.4±0.7	10.3±0.5
Total FA %									
Σ SFA[1]	26.7±0.3	27.6±0.3	27.4±0.3	24.9±0.4*	24.6±0.4*	24.8±0.3*	20.1±0.2	19.3±0.4	19.3±0.2
Σ MUFA[2]	42.1±0.4[b]	43.0±0.5[ab]	43.5±0.1[a]	42.3±0.2	42.2±0.2	41.7±0.5	48.4±0.1*	48.6±0.7*	48.5±0.5*
18:2n-6	7.3±0.1	7.3±0.1	7.3±0.2	8.2±0.3	8.1±0.1	8.1±0.1	12.6±0.4*	13.1±0.2*	12.9±0.3*
20:3n-6	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.9±0.1*	0.9±0.1*	0.9±0.1*
20:4n-6	0.7±0.0	0.7±0.0	0.6±0.0	0.6±0.0	0.7±0.0	0.7±0.0	1.1±0.0*	1.1±0.0*	1.1±0.1*
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.1	0.3±0.1	0.3±0.0	0.3±0.0
Σ n-6 PUFA[3]	9.1±0.0	9.1±0.1	9.1±0.2	10.0±0.2	10.0±0.1	10.0±0.1	16.7±0.9*	17.3±0.3*	17.1±0.5*
18:3n-3	2.3±0.0	2.2±0.0	2.2±0.0	2.7±0.2	2.7±0.1	2.6±0.1	3.3±0.0	3.4±0.0	3.3±0.1
20:5n-3	3.5±0.2	3.3±0.1	3.3±0.1	3.5±0.1*	3.6±0.1*	3.6±0.2*	1.6±0.1	1.6±0.1	1.7±0.1
22:5n-3	1.4±0.1	1.2±0.1	1.2±0.1	1.4±0.0*	1.4±0.0*	1.4±0.0*	0.7±0.1	0.7±0.1	0.7±0.1
22:6n-3	11.0±0.4[a]	9.8±0.4[b]	9.6±0.3[b]	11.3±0.1*	11.4±0.2*	11.8±0.4*	6.3±0.4	6.3±0.5	6.3±0.4
Σ n-3 PUFA[4]	20.8±0.7[a]	19.0±0.6[b]	18.8±0.4[b]	21.5±0.1*	21.8±0.3*	22.1±0.7*	14.2±0.7	14.2±0.7	14.4±0.7
n-3 LC-PUFA[5]	16.8±0.6[a]	15.2±0.6[b]	14.9±0.4[b]	17.1±0.2*	17.4±0.2*	17.7±0.6*	9.2±0.6	9.1±0.7	9.4±0.6
DHA+EPA	14.6±0.5[a]	13.1±0.5[b]	12.9±0.3[b]	14.8±0.2*	15.0±0.2*	15.4±0.6*	7.9±0.5	7.9±0.6	8.1±0.5
DHA/EPA	3.1±0.1	3.0±0.1	2.9±0.1	3.2±0.1	3.2±0.1	3.3±0.0	3.8±0.1	3.8±0.0	3.7±0.1
n-3:n-6	2.3±0.1	2.1±0.1	2.1±0.1	2.1±0.0*	2.2±0.0*	2.2±0.0*	0.9±0.1	0.8±0.1	0.8±0.1
Polar FA %									
Σ SFA[1]	32.3±0.3	32.8±1.0	31.9±0.6	29.9±0.2	30.3±0.2	30.4±0.9	27.6±0.3	27.2±0.6	27.7±0.6
Σ MUFA[2]	26.9±0.5	27.6±2.5	26.5±0.2	25.7±0.5	26.1±0.6	25.5±0.5	27.5±0.5	26.8±0.3	27.3±1.3
18:2n-6	2.9±0.1	3.0±0.2	2.9±0.1	2.7±0.1	2.8±0.1	2.7±0.1	5.1±0.2*	5.9±0.2*	5.2±0.5*
20:3n-6	0.5±0.0	0.5±0.0	0.5±0.0	0.4±0.0	0.4±0.0	0.4±0.0	2.5±0.4*	2.9±0.1*	2.5±0.5*
20:4n-6	2.4±0.1	2.2±0.1	2.4±0.1	2.7±0.0	2.6±0.0	2.7±0.1	6.6±0.2*	7.0±0.3*	7.1±0.9*
22:5n-6	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.2	0.5±0.0	0.5±0.0	1.6±0.2	1.8±0.1	1.7±0.2
Σ n-6 PUFA[3]	6.9±0.3	6.8±0.2	6.8±0.4	7.1±0.6	6.6±0.1	6.9±0.1	17.0±1.3*	19.2±0.5*	17.6±2.6*
18:3n-3	0.5±0.0	0.6±0.1	0.6±0.1	0.6±0.1	0.6±0.1	0.6±0.1	0.8±0.1	0.8±0.1	0.8±0.0
20:5n-3	4.0±0.1	4.2±0.2	4.6±0.3	4.3±0.2*	4.0±0.2*	4.1±0.2*	2.3±0.3	2.2±0.0	2.2±0.1
22:5n-3	1.0±0.0	1.0±0.1	1.0±0.1	0.8±0.1	0.8±0.1	0.8±0.0	0.9±0.0	0.8±0.0	0.9±0.0
22:6n-3	25.4±0.7	24.7±1.9	25.7±0.6	27.0±0.5*	26.8±0.7*	26.9±0.8*	18.8±0.9	18.4±0.1	18.6±0.8
Σ n-3 PUFA[4]	31.5±0.6	31.2±2.0	32.6±0.3	33.2±0.2*	32.7±0.8*	32.7±0.6*	23.3±1.3	22.9±0.1	23.0±0.9
n-3 LC-PUFA[5]	30.7±0.7	30.2±2.1	31.7±0.3	32.4±0.2*	31.8±0.8*	31.9±0.7*	22.2±1.2	21.7±0.1	21.9±1.0
DHA+EPA	29.4±0.8	28.9±2.0	30.3±0.2	31.3±0.3*	30.8±0.7*	30.9±0.7*	21.1±1.2	20.6±0.1	20.8±0.9
DHA/EPA	6.3±0.1	5.9±0.5	5.7±0.5	6.3±0.4	6.7±0.5	6.6±0.5	8.3±0.6*	8.4±0.2*	8.3±0.2*
n-3:n-6	4.6±0.3	4.7±0.5	4.8±0.3	4.7±0.4*	5.0±0.2*	4.7±0.1*	1.4±0.2	1.2±0.0	1.3±0.3

Data are means of triplicate measurement with standard deviations (n=3). For each phase, different superscript letters within a row denote significant differences among treatments.
The mark * denotes significant difference between the end of marine phase and the end of challenge phase for each treatment (p<0.05).
SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;
[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.
[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.
[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.
[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.
[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

4.4.5 Liver gene expression

In terms of LC-PUFA biosynthetic genes, at the end of marine phase, no difference was found in the hepatic expression of *fadsd5*, *fadsd6*, *elovl2, elovl5a* or *elovl5b*. However, after challenge, both *fads* and *elovl* expressions in V1 was upregulated, becoming the highest among groups. In the M group, expression levels were intermediate, whereas significantly lower expressions were observed in V2 (Figure 4.4 a-e).

As for transcription factors, both *srebp1* and *srebp2* showed a similar pattern of expression. In this sense, V2 fish displayed the highest *srebp1* and *srebp2* expression levels at the end of marine phase but showed a significant downregulation when the trial ended. The expression of *lxr* followed *srebp* in that M and V1 were comparable while V2 was the lowest. The expression of *pparα* and *pparγ* was found to be the lowest in V2 at both the marine and challenge phases (Figure 4.4 f-j).

Conversely, the regulation of lipid metabolism related genes shared a similar pattern. Before the challenge, V2 showed the highest expression of *fas*, *hmgcr*, *aco* and *cpt1* at the end of marine phase. However, after the challenge, all of these genes displayed an opposite trend so that V2 was significantly lower than M group with intermediate levels of expression observed in V1. Although not significant, the expression of *hmgcr* exhibited a similar pattern (p=0.07) (Figure 4.4 k-n). The overview of the gene expression alternation is shown in the heat map in Figure 4.5.

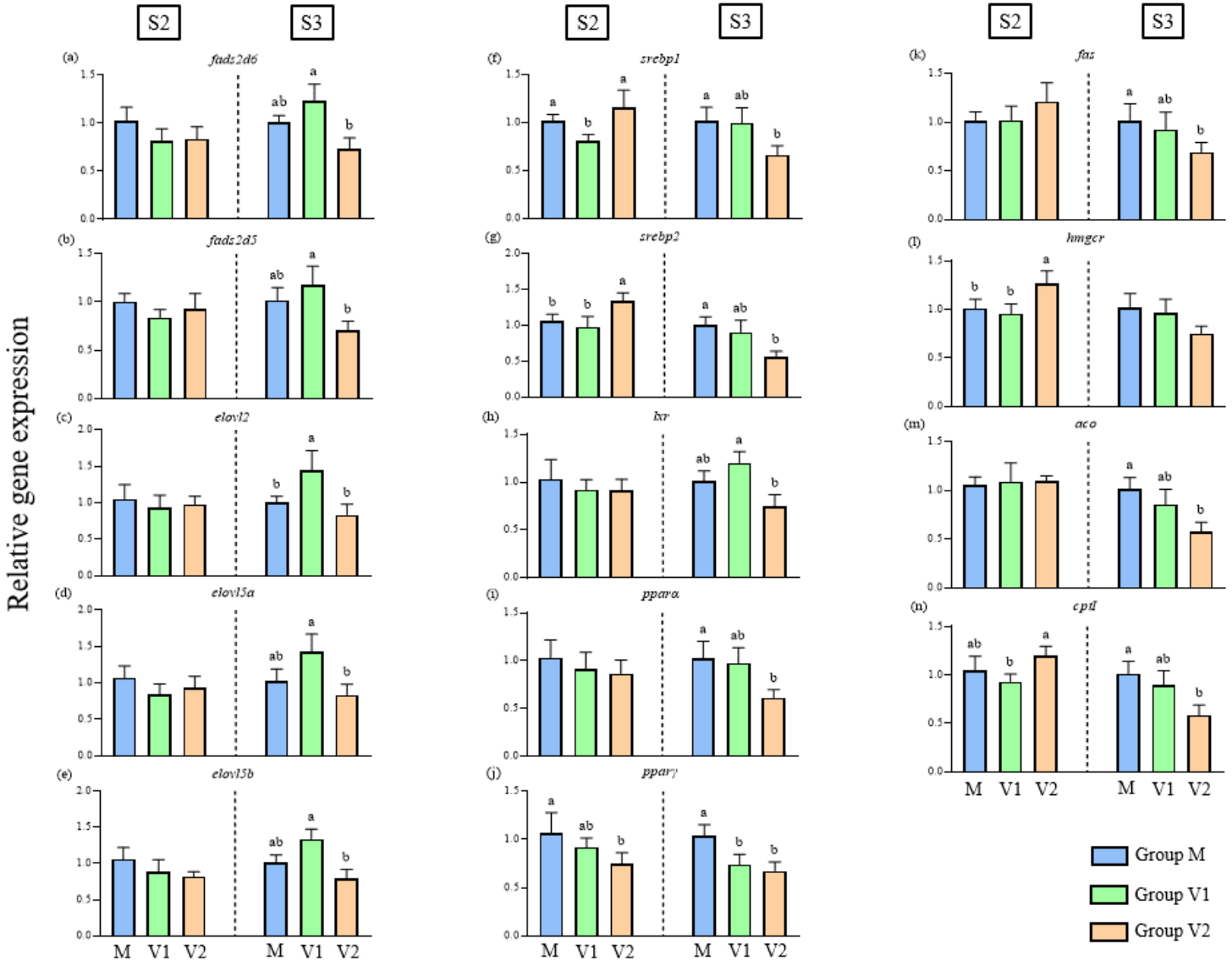


Figure 4.4 Relative expression of genes of long-chain PUFA biosynthesis (left), transcription factors (mid) and lipid metabolism (right) in liver of Atlantic salmon. Results are normalised expression ratios. Data are means of sextuplicate measurement (n=6), with standard deviation represented by vertical bars. The lowercase letters denote significant difference among groups at individual phase (p<0.05).

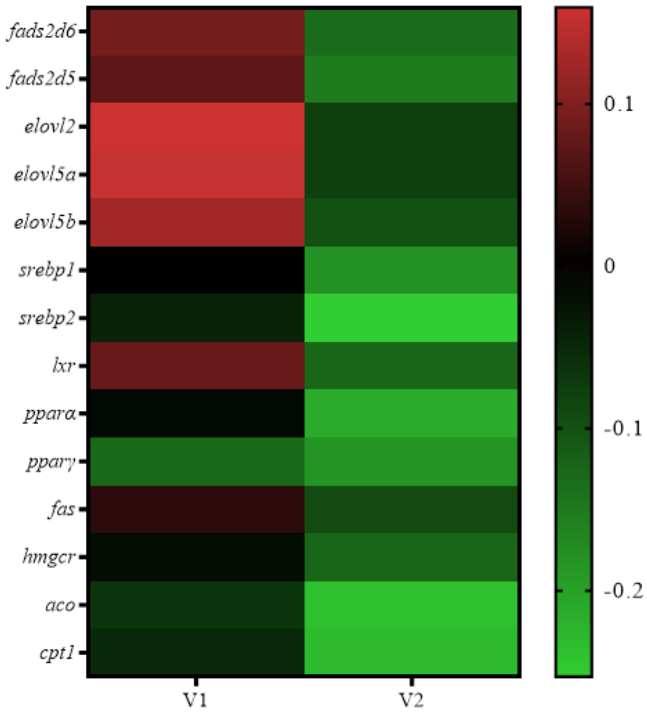


Figure 4.5 Heatmap of the fourteen target genes analysed based on qPCR gene data. M group was chosen as control group. Columns represent mean data values of the three different dietary groups analysed in the liver and rows represent single genes. Expression level of each gene was log10 transferred. Means are depicted by a colour scale, indicating low (green), neutral (black) or high (red) relative expression levels, as indicated by the colour bar on the right.

4.4.6 Histology

Histological analysis of the liver revealed that the length of the stimulus altered the hepatic intracytoplasmic lipid vacuolization, with the amount of vacuolization significantly differing between groups. Consequently, V2 group was significantly higher than the M group with V1 demonstrating intermediary levels in both phases (Figure 4.6 a, f, g). At the end of the challenge phase, fish which had a stimulus applied had a higher intestinal muscular thickness than those in the M group (Figure 4.6 b). Stimulus had no major impact on the width and height of the enterocyte at neither the marine or challenge phase (Figure 4.6 c, 4.6 d). In the intestine, the challenge diet resulted in a significantly lower density of goblet cells in V1 when compared with M group, while V2 showed intermediary values (Figure 4.6 e, h, i).

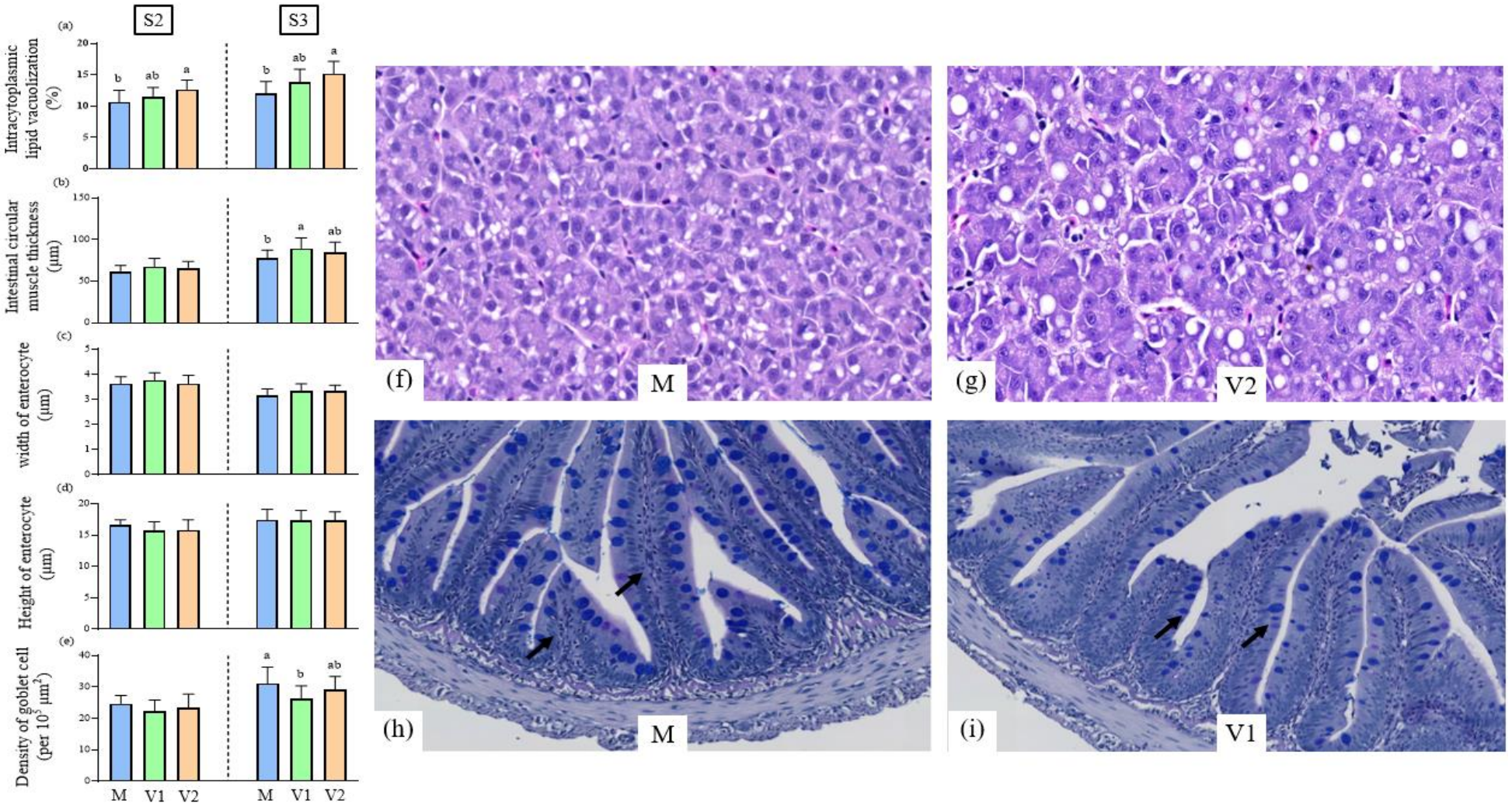


Figure 4.6 The results of histology analysis in liver and anterior intestine of Atlantic salmon. Data are means of triplicate measurement (n=3), with standard deviation represented by vertical bars. a, b Mean values with unlike letters were significantly different between groups at individual phase by one-way ANOVA (a-e). Histological analysis of vacuolization and goblet cells in Atlantic salmon for different groups at the end of the challenge were shown in f-i. Upper (f, g) shows the results from M and V2, respectively, and lower (h, i) shows the results from M and V1, respectively.

4.5 Discussion

In the present trial, the concept of "nutritional programming" was introduced in order to improve the utilization of plant-based feeds while maintaining fish performance and quality. In previous studies, nutritional programming was shown to enhance *fads2* expression and stimulate the fatty acid desaturation pathways of n-3 LC-PUFA without compromising growth performance in seabass juveniles (*Dicentrarchus labrax*) (Vagner *et al*., 2009), improve the growth of the offspring from high *fads2* expression parents and utilization of low n-3 LC-PUFA diet in gilthead sea bream when challenged by high VO diet (Izquierdo *et al*., 2015; Turkmen *et al*., 2019a; 2020) and alter numerous metabolic processes in salmon to potentially cope with the presence of ANFs in plant-based diets (Vera *et al*., 2017). In the present study, salmon from all groups had a similar body weight throughout the entire stimulus phase, which indicates that an early nutritional intervention for one or two weeks will not induce any growth change as expected. The fish weight gain in V1 group was higher than those in V2 group after "grow-out" marine phase (15.5 g v.s. 14.3 g, respectively) and challenge phase (20.1 g v.s. 17.3 g, respectively). Therefore, accompanied by the SGR data, V1 was shown to have a better growth performance than the others groups, although this was not significant at the challenge phase. Compared to the previous study by Clarkson *et al*. (2017), who ran a three-week trial, the growth at stimulus and marine phase for all groups did not decrease in the present study and, in-fact, still increased in V1 fish following the challenge. This better growth performance suggested that the one-week stimulus period had a positive effect at the challenge phase. A series of experiments looking at stimulus duration on carbohydrate programming in Nile tilapia (*Oreochromis niloticus*) demonstrated that low-protein/high-carbohydrate stimulus for four weeks decreased the growth performance (Kumkhong *et al*., 2020). Nevertheless, when the stimulus was shortened to one week, it was found to be sufficient enough to induce hyperglucidic programing without impacting growth (Srisakultiew *et al*., 2022). Nutrition memory has been described as being able to alter the plasticity of organ physiology such as olfaction, which is believed to be important for guiding feeding behavior in salmonids (Hara, 2006). The early exposure of the vegetable-based diet may

have triggered olfaction imprinting, leading to a better acceptance of the future challenge diet and, subsequently, greater growth.

High dietary levels of terrestrial sources have been reported to influence the lipid deposition in the different tissues in fish (Bell *et al*., 2001a; Betancor *et al*., 2016a). In the present study, both the intestine and muscle showed an increased lipid content following the challenge. One possible explanation in the case of intestine is that this tissue is the major site for lipid absorption and digestion (Krogdahl *et al*., 1999), with similar results being reported in both rainbow trout and gilthead sea bream, where fish oil replacement increased the intestinal fat accumulation (Caballero *et al*., 2002; Santigosa *et al*., 2011). The increased plant-based lipid source included within the diet could also account for the higher muscle lipid deposition observed. Other studies performed in salmon, gilthead sea bream and golden pompano (*Trachinotus ovatus*) reported similar effects (Cruz-Garcia *et al*., 2011; Guo *et al*., 2021; Torstensen *et al*., 2011), indicating that a lack of n-3 LC-PUFA will potentially result in fat accumulation in muscle. All other tissues studied (brain, eye and gill) are not known as sites for lipid deposition, thus the stimulus and challenge did not show any significant impact except on liver. Notably in liver, although lipid deposition was similar between all groups from a biochemical perspective, the histological examination of the liver revealed that the greater duration of the stimulus diet induced a higher level of hepatic intracytoplasmic lipid vacuolization after challenge. The present result is consistent with previous research in red drum (*Sciaenops ocellatus*) and gilthead sea bream showed that the inclusion of plant-based oil in diets will impact upon liver histology through the excessive presence of lipid droplets (Riera-Heredia *et al*., 2020; Tucker *et al*., 1997). This may be explained by the involvement of transcription factors in regulating lipid metabolism. The enzyme PPARα is of the nuclear receptor family (Mandard *et al*., 2004), and CPT1 is known as the key enzyme regulator of mitochondrial β-oxidation in mitochondrial matrix, both of which play an intermediary role in lipid metabolism (Kerner and Hoppel, 2000). In the present study, *pparα* and its target gene *cpt1* both showed the same trend of a lower expression in V2 at the end of trial. Based on the previous study showing FO replacement

by VO reduced *pparα* expression (Morais *et al*., 2011b), one reason for V2 being the lowest is suggested to be linked to the length of the early intervention of plant-based diet, especially as the impact was more obvious following the challenge which finally led to the higher level of hepatic intracytoplasmic lipid vacuolization. Although PPARγ is believed to promote fat accumulation due to the high expression in adipose tissue, the higher hepatic lipid vacuolization might be irrelevant as its expression was lower in both stimulus groups after challenge. As the sensor of fatty acid and cholesterol metabolism balance, *srebp* is the target gene of *lxr,* and its transcription can be activated for its vital role in cholesterol biosynthesis and lipogenesis (Horton *et al*., 2003; Tobin *et al*., 2002). However, the negative correlation of hepatic intracytoplasmic lipid vacuolization and the expression of *srebp1* after challenge is opposite to that previously found by Minghetti *et al*. (2011), arguing that SREBP-1 in the liver has positive role in lipogenesis. The reason for this remains unclear at present and the liver fat content was not statistically different among groups, suggesting that a longer challenge period is perhaps required for a more obvious biochemical change to occur.

Another important factor associated with V-derived diets in nutritional programming is the alternation of the fatty acid composition and its impact among tissues after later challenge, since the diet composition will generally be reflected accordingly. Firstly, the fatty acid composition of whole fish after stimulus demonstrated that a one-week stimulus would only induce minor impacts as compared to M fed fish. The only notable differences were a higher EPA content and n-3:n-6 ratio in the M group. However, the differences between M and V2 were so considerable that most fatty acids of interest were all found to be significantly lower in V2, which indicate an obvious phenotypic outcome after a two-week stimulus.

Fatty acid compositions in TL and PL were both taken into consideration to evaluate the effect on tissues. In general, the composition of TL reflects the nutrition available to consumers, whereas the composition of PL relates to the physiological and metabolic functions in tissues, although both TL and PL can be affected by diet (Bell *et al*., 1996).

In the present study, the fatty acid compositions of TL and PL in both liver and intestine were clearly regulated by the diet. Since the diets used in challenge phase contained less than 1 % of DHA, so the drops of DHA content during the challenge phase must be significant. Generally, liver and intestine are the primary tissues for LC-PUFA synthesis in salmonids, particularly the liver (Bell *et al*., 2003; Tocher *et al*., 2006). The diet in the challenge phase contained a very small amount of ARA, however, a marked increase in ARA following challenge was found in both the liver and intestine. The high accumulation of ARA is speculated to activate the LC-PUFA biosynthesis pathway as demonstrated previously (Bell and Tocher., 2009; Tocher, 2003). Indeed, the sufficient supply of 18:2n-6 from V diet acted as precursor for 20:3n-6 and then ARA largely due to endogenous biosynthesis via desaturases and elongases. However, not only ARA follow this pathway but both DHA and EPA showed a similar trend from their precursor, 18:3n-3 (Tocher, 2010). Despite the dietary supply of the 18:3n-3 was adequate as a precursor during the challenge phase, no accumulation of 18:3n-3 found in liver might also be the evidence of the active LC-PUFA biosynthesis as well. This biosynthetic activity was also confirmed by mRNA levels. Before the challenge, all groups displayed a similar level of expression of *fads* and *elovl*, whereas after the challenge only the V1 fish showed a constant upregulation of all transcripts analysed in the liver. These results agree with other studies where upregulation of desaturases and elongases was observed in fish fed low dietary contents of n-3 LC-PUFA (Izquierdo *et al*., 2008; Tocher *et al*., 2002; 2003). Curiously, expressions in V2 fish did not show the same trend and were still low, which could infer that one week of stimulus is sufficient in generating a long-term response. In addition, the expressions of *fads* and *elovl* were in line with *lxr* and *srebp1* expression, since both *lxr* and *srebp1* were able to regulate the target genes involved in PUFA elongation via the expression of *elovl5* (Moon *et al*., 2009; Qin *et al*., 2009), and PUFA desaturation through Δ6 and Δ5 *fads* regulation (Matsuzaka *et al*., 2002; Zheng *et al*., 2009). Nonetheless, dietary n-3 LC-PUFA levels cannot be compensated by biosynthesis, which may explain the dramatic decrease in both EPA and DHA from TL and PL in liver and intestine samples. The highly accumulated ARA, along with the high proportion of EPA and DHA in the PL, were consistent with previous research as

endogenously produced n-3 LC-PUFA are expected to be highly incorporated into the membrane of phospholipids for the essential functions irrespective of the type of diet (Tocher, 2003; Sargent *et al*., 2002; Sprague *et al*., 2019). A correlation between molecular variation and stimulus duration was observed, but their interaction with fatty acid composition still requires investigation. It is postulated that a further significant influence might appear if the challenge phase was prolonged.

On the contrary, the n-3 LC-PUFA content of the brain tissue showed a relative resistance when encountering the plant-based diet challenge compared to other tissues. This finding is consistent with that of previous studies (Betancor *et al*., 2021; Rombenso *et al*., 2015). Indeed, less plasticity of the brain fatty acid composition from dietary changes is known for its functional and physiological performance (Benedito-Palos *et al*., 2010; Rombenso *et al*., 2016). The deficiency of dietary DHA has been demonstrated to induce visual impairment in juvenile herring (*Clupea harengus*) (Bell *et al*., 1995) and gilthead sea bream (Benítez-Santana *et al*., 2007), which consequently lessened growth and survival. In the present study, the DHA content in the PL fraction decreased from roughly 28 % to 25 % after challenge. Such conservation in the PL compared with other tissues can be considered as selective retention of DHA, in order to tightly regulate membrane functionality and integrity in neural tissues (Turchini *et al*., 2011b). The significantly higher n-3:n-6 ratio among tissues further demonstrates that the brain has a higher reliance on n-3 LC-PUFA. However, the early programming duration was shown to be irrelevant.

Surprisingly, despite being neural tissues, the lipid class compositions between the brain and eye showed no similarity and was not varied in the present study. The eye had a higher proportion of TAG, more than 50 %, but the PL content was only around 25 % which is opposite to that observed in the brain, TAG:PL ≈ 20 %: 55 %, respectively. The present results are consistent with those of Brodtkorb *et al*. (1997) who reported TAG as the predominating lipid class in salmon. Therefore, the fatty acid composition of the diet should be reflected in the TL more precisely rather than PL so that, when the challenge occurred, the major impacts in PL were found to be less impacted than TL. Nonetheless,

the abundance of DHA in PL indicates its vital role in retina development and predatory systems (Bell *et al*., 1995; Sargent, 1995), which was previously shown to be independent from diet (Brodtkorb *et al*., 1997). The current challenge still resulted in a reduced PL DHA content. However, as with the brain, this decline was also less responsive in order to maintain normal behaviour to avoid impaired function. The retina also had the second highest n-3:n-6 ratio among tissues which further suggests its dependence of n-3 LC-PUFA. Additionally, Slighter reduction in PL than TL further suggested that DHA in PL is selectively stored. However, as a neural tissue, the high TAG content within the eye is not fully investigated yet. On the other hand, energy requirement is largely provided via β-oxidation by adipose tissue such as muscle (Tocher, 2003), so the muscle is regarded as an important TAG storage site and its high TAG proportion is able to meet the demand for growth and swimming etc. The lipid class analysis performed in the present study confirms this by the high percentage of TAG observed at both phases. Thus, after challenge, dietary changes of fatty acid composition are subsequently reflected in muscle, which led to the reduced nutritional value. The high TAG level was assumed to be the reason for the considerable difference in DHA contents between the TL and PL.

Generally, the gills are structurally oriented for their main purpose as an osmoregulatory tissue as well as blocking direct interaction with their external environment (Bin *et al*., 2015). The high content of TAG measured in the gills in the present study might be linked to its energy demanding functions. Similarly, the fatty acid composition of gill TL generally reflected that of the diet, whereas the n-3 LC-PUFA in PL preferred to be conserved to maintain membrane structure and function when these fatty acids are absent in diet (Betancor *et al*., 2014a; Martín *et al*., 2011). Similar to the liver and intestine PL, a significant boost of ARA content in gill PL was noticed after being subjected to the challenge, which is in agreement with the research by Miao *et al*. (2022). Since the inclusion of ARA was low in diet, the increase observed was most likely the result of biosynthesis and body conservation, even if the gill was not the main site. Indeed, ARA has been reported to be the most important precursor of eicosanoids that plays an essential role in dealing with inflammation and stress (Bell *et al*., 1996). The dietary fatty acid

alterations are recognised to induce inflammatory reaction by regulating inflammatory mediators such as cytokines (Calder, 2006; Montero *et al*., 2010; Rowley *et al*., 1995). Therefore, the elevated content of ARA in the gill hint at the nutritional stress elicited by the VO challenge (Bell and Sargent, 2003). On the other hand, the significantly reduced level of EPA contributed to a higher ARA:EPA ratio, which aided the secretion of a high activity of pro-inflammatory eicosanoids (Holen *et al*., 2015). However, the early stimulus had no effect on outcomes as no difference was observed between groups.

As an important organ for nutrient absorption, the intestinal circular muscle thickness is an indicator used to assess function efficiency and intestinal health. The increasing thickness can expand the absorptive area and subsequently improve digestion and absorption for a better nutrient utilization. (Yu *et al*., 2020). In the present study, salmon experiencing a V-stimulus, especially for one week, were shown to have a higher muscular thickness during the challenge phase, which indicated the positive effects provided by the early intervention when fed the challenge diet. From an immunity perspective, apart from the aforementioned ARA boost in the intestine, the goblet cells are specialized columnar epithelial cells that are fully involved in digestion and provide innate protection against chemical substances though the secretion of mucin (Allen *et al*., 1986; Kim *et al*., 2010; Deplancke and Gaskins, 2001; Domeneghini *et al*., 2005). Therefore, increased goblet density is related to the inflammation response and has positive effects on immunocompetence (Molinari *et al*., 2020; Tomás-Almenar *et al*., 2020). In the present study, the higher density of goblet cells in M and V2, as compared with V1, may be the result of some form of inflammatory response in the intestine, albeit no inflammatory infiltrates were observed in the current study. This could potentially lead to a lower feed efficiency and inferior growth performance in the future.

4.6 Conclusions

Nutritional programming could have a radical impact on the sustainability of aquafeed raw material utilization by salmon. The present study sought to provide an insight about whether the duration of early nutrition stimulus and the later challenge significantly impact the programming. It was clearly demonstrated that a one-week stimulus help maintain the growth performance similar to marine group and adapt salmon to a better acceptance of plant-based diet. Early stimulus and different duration periods had little or no effect on the fatty acid composition of both total and polar lipid in several tissues. In addition, the changes of fatty acid compositions in liver and brain by the V-feed challenge suggested the potential n-3 LC-PUFA biosynthesis as well as selective retention in polar lipid for their structural functions. Furthermore, molecular analysis indicates that, after challenge, several LC-PUFA biosynthetic enzymes are upregulated in fish experiencing a one-week stimulus which supports this hypothesis. However, fish that experienced the two-week stimulus displayed a downregulation of most LC-PUFA biosynthetic enzymes as well as transcription factors. Taking everything into account, the current data suggests that a one-week stimulus will work better as programming. However, considering the relatively short challenge phase, future trials should seek to fully define the impact of early stimulus duration that longer challenges have on this aspect or the role that a "booster" challenge could have in the longer term.

5 CHAPTER

Early nutritional intervention from first feeding elicits long-term impacts on performance in Atlantic salmon (*Salmo salar*) experienced a plant-based diet challenge

5.1 Abstract

As part of the development of sustainable aquaculture, nutritional programming is regarded as a key strategy to adapt fish to the high vegetable inclusion used in diets. To investigate the effect of nutritional programming from first feeding in salmon, a nine-month trial was run in three stages. Salmon (0.15 g) were fed either a marine or vegetable diet for three weeks as a stimulus before all fish were fed a marine diet until month eight when both a marine or vegetable challenge was applied separately for each stimulus group for one month. After challenging, salmon fed the vegetable stimulus showed similar growth to marine groups. The fatty acid composition, total and polar lipid, of most tissues were directly impacted by the challenge. Additionally, the early vegetable stimulus plus challenge significantly upregulated the key genes for LC-PUFA biosynthesis and transcription factors. Histological analysis indicated that challenge mainly resulted in a higher hepatic lipid deposition and shortened intestinal circular muscle thickness as well as higher goblet cell presence. Salmon experienced early programming had a reduced peroxidation index in muscle and lower ARA:EPA ratio. In summary, the present trial indicated that the use of early intervention and vegetable-based challenge as a booster could be considered as a sustainable strategy although further investigation is required.

Key words: Nutrition programming; Atlantic salmon; stimulus; challenge; n-3 LC-PUFA; tissues;

5.2 Introduction

More than half the global fish consumption relies heavily on aquaculture production which is rapidly developing (FAO, 2022). The limited supply of important ingredients in aquafeeds, fish oil (FO) and fishmeal, from the wild capture fisheries requires the search for cheaper and more available alternatives to reduce the marine source input in farming (Tocher *et al*., 2019). Although there are various resources being examined such as marine algae (Sprague *et al*., 2015), and land animal oil (Pérez *et al*., 2014), terrestrial vegetable oils are the most widely-used candidates for FO replacement. The over reduction of marine ingredients in aquafeeds replaced by plant-derived sources remains a risk for many species, potentially leading to poor growth in European sea bass (*Dicentrarchus labrax* L.) (Mourente and Bell, 2006), immunity issues in gilthead sea bream (*Sparus aurata*) (Montero *et al*., 2010), impaired resistance to pathogen in salmon (Bransden *et al*., 2003) as well as the inferior nutritional value for human as final consumer (Sprague *et al*., 2016). The critical role of omega-3 long-chain polyunsaturated fatty acid (n-3 LC-PUFA), especially eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), for the development of brain, retina and synaptic membranes in nerve cells in marine larvae has been demonstrated previously (Sargent *et al*., 1999; Mourente, 2003; Benítez *et al*., 2007; Glencross, 2009). These fatty acids are absent in plant-based oils, whereas they contain abundant amounts of C18 fatty acids, like linoleic acid (LA, 18:2n-6) and α-linoleic acid (ALA, 18:3n-3). Despite the fact that marine fish have the enzymatic machinery to convert the precursors LA and ALA to EPA and DHA (Mourente and Tocher, 1994; Sargent *et al*., 2002; Monroig *et al*., 2011a), they display a poor capacity to do so the bioconversion can hardly self-satisfy the demand of n-3 LC-PUFA being essential fatty acids in marine fish (Tocher, 2010; Monroig *et al*., 2018; Sprague *et al*., 2019; Xie *et al*., 2021). Thus, the fatty acid composition of terrestrial oils restricts their high inclusion in aquafeeds, which dampers the utilisation of sustainable feeds.

As a strategy to enhance the feed utilization from another perspective, early nutritional intervention has been put forward so that fish can use the vegetable oil diet with less

negative effects. This concept has been widely studied in mammals (Gluckman and Hanson, 2008; Lucas, 1998). It is reported that influences of environmental factors, including nutrition, at the early critical developmental stages may have long-term consequences on phenotypes and physiological functions in later life (Lillycrop and Burdge, 2012; Burdge *et al*., 2007). The intervention in the plasticity period will get the body ready to future presence of similar factors previously encountered (Gluckman *et al*., 2005). This phenomenon, termed nutritional programming, may account for some fish adaptions at cellular, molecular or biochemical levels in being adequately prepared for the sustainable diets. For instance, after a diet deficient in n-3 LC-PUFA was applied to European sea bass larvae stage as a stimulus, when encountering the similar diet during the juvenile stage an increased gene expression of fatty acid desaturase 2 (*fads2*) was found compared to fish that had no "programming" experience before, accompanied by a higher DHA content in polar lipid (PL) (Vagner *et al*., 2007). It has also been reported that Atlantic salmon was able to receive the conditioning process. Fry first fed a low FO diet for three weeks were observed to have better growth performance and feed efficiency as well as demonstrating a net production of both EPA and DHA when they were returned to a complete plant-based diet (Clarkson *et al*., 2017). These phenotypic changes were accompanied with numerous metabolic process changes and improved nutrient utilization (Vera *et al*., 2017). Beyond that, early programming has been used for different purposes. Feeding a plant-based diet in early life stages improved the utilisation of carbohydrate and soybean meal in zebra fish (*Danio rerio*) (Rocha, *et al*., 2015; Perera and Yúfera, 2016), increased the growth and feed efficiency in rainbow trout (*Onchorhynchus mykiss*) (Geurden *et al*., 2013) or enhanced the diet acceptance by flavor preferences and less sensitivity to stress responses in rainbow trout (Balasubramanian *et al*., 2016). The concept of early-programming and related research provides a novel access customizing fish through specific modifications at developmental windows to enhance the acceptance of terrestrial-derived aquaculture.

Previously, a stimulus of one or two weeks did not elicit a strong nutritional response, when challenged with vegetable feeds later in life. Although growth performance and

several LC-PUFA biosynthesis regulators might have shown some differences, the phenotypical impact on in tissue fatty acids composition was minimal (Chapter 4). In addition, the potential for applying nutritional programming to Atlantic salmon fry was confirmed through a three-week stimulus from first feeding, by which juveniles were better adapted to reacting to similar plant-based diets in a subsequent challenge (Clarkson *et al*., 2017). The overall objectives of the present trial were to examine whether nutritional programming, triggered by stimulus, is still prevalent after a long-term grow-out phase as well as investigating the impacts of nutritional programming in salmon compared to the fish without experiencing stimulus. In addition, it was evaluated that whether a new challenge is a booster for programming outcomes so that the tissue net production of n-3 LC-PUFA can be enhanced as limited studies have examined the n-3 LC-PUFA status of different tissues when fish are challenged after programming. Thus, fish tissues were specifically emphasized to explore the fatty acid composition in PL, lipid-related gene modifications in liver and histological analyses in intestine in order to provide more insights about salmon programming.

5.3 Materials and methods

5.3.1 Ethics statement

The feeding trial was carried out at the Niall Bromage Freshwater Research Facility (NBFRF), University of Stirling with all experimental procedures conducted in compliance with the Animals Scientific Procedures Act 1986 (Home Office Code of Practice, HMSO, London, January 1997) under project licence PPL70/7916 'Environmental Regulation of Fish Physiology) in accordance with EU regulation (EC Directive 86/609/EEC). All the procedures and protocols were subjected to an ethics review carried out by the University of Stirling Animal Welfare and Ethical Review Board (AWERB No: 19 20/055/New ASPA) before any work being performed.

5.3.2 Dietary trial

Salmon eggs (369 degree-days post fertilization) were obtained from Stofnfiskur, Iceland and transferred to NBFRF, University of Stirling for the experiment. One hundred percent of the eggs were hatched after 500 degree-days (DD). The remaining fish were incubated in the alevin stage for a further 350 degree-days. The incubation temperature was 6.0 ºC. At 884 DD prior to first exogenous feeding, all fish were distributed into 12 × 0.3 m^2 tanks for the further first feeding trial.

A triplicated feeding trial was run in Atlantic salmon from first feeding (initial weight approximately 0.15 g) for nine months. Two types of experimental feeds were used in the trial: the diet containing fish meal and fish oil as the main protein and lipid source was named as Marine diet (M), whereas the diet containing terrestrial plant protein and oil was called Vegetable-based diet (V). All the feeds were manufactured and provided by BioMar Ltd. (TechCentre, Brande, Denmark), with some ingredients included pre-mixed as is common practice within the commercial feed production sector. Various pellet sizes for both types of diet (0.5, 0.8, 1.1, 1.5, 2.0 and 3.0 mm for diet M; 0.5 and 3.0 mm for diet V) was prepared according to the formulations to tailor for growing fish. Feed formulation and fatty acid compositions for all diets are shown in Table 5.1. Fish were initially kept in temperature-controlled recirculating aquaculture systems (RAS) and allocated into two experimental tanks (1500 fish per tank). The trial consisted of three phases: first stage was “stimulus phase”, which lasted three weeks, according to previous studies (Clarkson *et al*., 2017). During this phase one of the tanks was fed a marine diet (M group), whereas the other was fed a vegetable-based diet (V group) for three weeks to provide a stimulus in order to program the stock for better future performance on diets with low marine ingredients. The second stage was an intermediate “grow-out marine phase”, where all fish were fed M diet for 8 months. Up to a weight of approximately 0.5 g, the feeding was conducted in these two tanks to ensure good welfare and to promote feed intake with high fish density. Afterwards, the fish from each tank were redistributed into six tanks (twelve tanks in total; n=6 per stimulus). The third stage was “challenge

phase": one month prior to the end, six tanks were transferred to V diet (three tanks from M group, three tanks from V group) as a challenge, the remaining six tanks were maintained on M diet without challenge. The groups were named accordingly to the stimulus and challenge phase diet (MM, MV, VM, VV, illustrated in Figure 5.1). The feeds were administered with Arvo-tec TD2000 (Huutokoski, Finland) feeders and user interface (ArvoPRO) to ensure the acquisition of accurate values for feed entering the tanks. Uneaten feed was collected twice a day after daily feeding by syphoning with a pipe with filter, before drying the collected waste feed for correction of feed intake measurement. The water volume was set as 300 litre per tank, water temperature was maintained at 12.6±0.9 ºC and photoperiod was 24 hours artificial light, the oxygen level (90 % saturation, 8-9 mg/L), pH (7.29±0.1), nitrogen dioxide (0.14±0.09 mg/L), total ammonia nitrogen (0.40±0.27 mg/L) and chloride (131.0±27.0 mg/L) were all determined and controlled daily for the feeding trial.

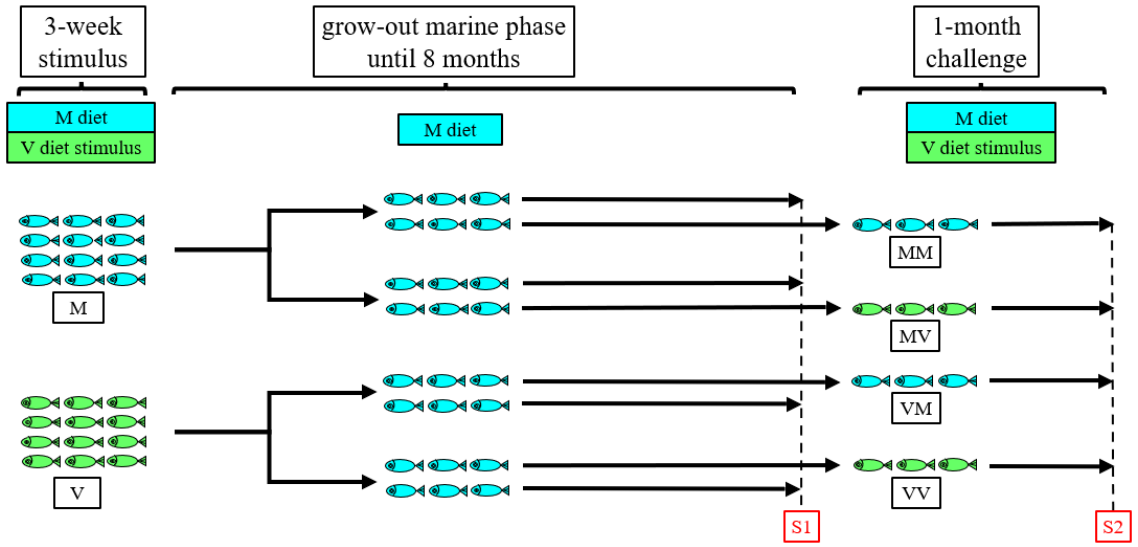


Figure 5.1 The design of the dietary experiment in Chapter 5. S1 (sample point 1): End of marine phase; S2 (sample point 2): End of challenge phase.

Table 5.1 The formulations, proximate and fatty acids compositions (percentage of total fatty acids) of the experimental feeds.

	Stimulus phase		**Marine phase**				**Challenge phase**	
	0.5 mm M	**0.5 mm V**	**0.8 mm M**	**1.1 mm M**	**1.5 mm M**	**2.0 mm M**	**3.0 mm M**	**3.0 mm V**
Feed ingredients (%)								
Fish Meals	74.2	4.3	59.2	59.2	40.0	47.0	42.4	5.0
Qrill High Protein	/	1.5	/	/	/	/	/	/
Krill meal (56 %)	10.0	/	7.0	7.0	/	/	/	/
Fish Peptones	5.0	2.5	3.0	3.0	/	/	/	5.0
Soya SPC	/	7.2	/	/	15.0	13.0	13.0	11.2
Wheat & maize gluten	/	28.5	10.0	10.0	14.4	11.8	8.0	16.0
Pea protein	/	37.0	/	/	5.0	/	3.1	25.0
Starch sources	5.6	4.0	7.5	7.5	9.5	9.5	12.9	8.1
Fish Oil	4.1	/	10.2	10.2	8.6	7.8	8.0	/
Vegetable oil	/	4.9	/	/	5.1	7.6	9.0	17.4
Lecithin	0.5	4.0	0.5	0.5	0.5	0.8	0.5	0.5
Premix (vit., min., AAs, nucl., antiox., yttrium)	3.7	10.0	4.6	4.4	3.6	4.3	3.0	10.7
Water change	-3.3	-3.9	-2.0	-1.4	-1.7	-1.9	0.1	1.0
Proximate								
Dry matter (%)	93.1	93.3	93.3	91.4	94.0	93.7	91.3	92.3
Ash (%)	13.3	10.9	12.1	11.1	10.4	10.4	9.3	9.7
Protein (%)	59.5	57.5	55.5	54.6	50.3	50.1	45.9	46.7
Lipid (%)	14.1	12.2	17.3	18.2	20.4	21.6	20.2	21.7
Gross energy (KJ/g)	20.3	21.0	21.1	21.1	21.6	22.0	21.7	22.4
FA composition (%)								
Σ SFA[1]	30.0	15.6	33.8	33.4	24.3	21.0	24.0	8.6
Σ MUFA[2]	31.2	43.7	30.4	29.4	39.2	44.4	39.7	60.5
18:2n-6	8.2	32.3	5.5	4.8	11.0	12.0	11.1	21.8
20:3n-6	0.0	0.0	0.1	0.1	0.0	0.0	0.0	0.0
20:4n-6	0.7	0.0	0.9	1.0	0.6	0.5	0.6	0.0
22:5n-6	0.3	0.0	0.2	0.3	0.2	0.1	0.2	0.0
Σ n-6 PUFA[3]	9.5	32.4	7.1	6.5	12.0	12.9	12.1	21.8
18:3n-3	1.5	6.7	1.0	1.0	3.5	4.2	3.4	8.6
20:5n-3	10.7	0.6	11.5	12.6	8.5	6.9	8.4	0.2
22:5n-3	0.9	0.0	1.6	1.7	1.2	0.9	1.2	0.0
22:6n-3	12.0	0.8	8.2	8.6	6.6	5.8	6.5	0.3
Σ n-3 PUFA[4]	27.7	8.3	25.5	27.3	22.1	19.7	21.8	9.2
n-3 LC-PUFA[5]	24.1	1.4	21.9	23.6	16.7	14.0	16.5	0.5
DHA+EPA	22.7	1.4	19.7	21.2	15.1	12.7	14.9	0.5
DHA/EPA	1.1	1.3	0.7	0.7	0.8	0.8	0.8	1.7
n-3:n-6	2.9	0.3	3.6	4.2	1.8	1.5	1.8	0.4

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3

5.3.3 Sampling

At the end of the marine phase and the challenge phase, fish samples were collected. Two time points were marked as S1 (End of the marine phase) and S2 (End of the challenge phase). After a 24-hour fasting period from last feed, twelve fish from each tank were randomly selected and euthanised with tricaine methanesulfonate (MS-222; 1000 ppm in hydrogen carbonate-buffered solution) and weighed on an electronic top-loading balance to 0.1 g accuracy individually. Six fish were dissected for tissue biochemical sample collection, with the liver, brain, white muscle, gill, eye and intestine collected and stored in liquid nitrogen immediately. A further six fish were killed and each single liver and intestine was divided into two portions, one portion was collected in 1.5 mL RNALater (Sigma, Poole, UK) and stored overnight at 4 ºC before freezing at -70 ºC for RNA extraction and gene expression. The other portion was placed in 2 mL 4 % buffered formalin for further histology analysis.

5.3.4 Lipid content, lipid class and fatty acid analyses

Total lipid (TL) content of tissue and feed samples was extracted after homogenization in 20 or 36 mL of chloroform-methanol (2:1, v/v) respectively, using an Ultra-Turrax tissue disrupter (Fisher Scientific, Loughborough, UK) and determined gravimetrically based on the method of Folch *et al*. (1957). The extracted TL was made up to a concentration of 10 mg/mL in chloroform: methanol (2:1) + 0.01 % (w/v) BHT for further analysis.

Lipid class separation was performed according to Henderson and Tocher (1992) by loading extracted TL on a 20 × 10 cm high-performance thin layer chromatography (HPTLC) plate (VWR, Lutterworth, UK). Classes were separated by running plates in two different mobile solvent phases consisting of methyl acetate:isopropanol: chloroform:methanol:0.25 % (w/v) potassium chloride (25:25:25:10:9, by vol.) to half distance, and isohexane:diethyl ether:glacial acetic acid (85:15:1.5) to full distance for separation of polar and neutral lipids, respectfully. Lipid classes were visualised by thoroughly spraying with 3 % copper acetate and 8 % phosphoric acid. The plates were

dried in the oven before being scanned by TLC Scanner 3 (CAMAG, Muttenz, Switzerland) and the classes were visualized on Wincat software package (CAMAG, Muttenz, Switzerland).

Fatty acid methyl esters (FAMEs) were prepared from TL by acid-catalysed transmethylation at 50 ºC for 16 h, and were extracted and purified as described previously (Christie 2003; Tocher and Harvie, 1988). FAMEs were separated and quantified using a gas-liquid chromatography (Thermo Finnigan Trace GC, Thermo Scientific, Milan, Italy) equipped with a 30 m × 0.32 mm i.d. × 0.25 μm ZB-wax column (Phenomenex, Cheshire, UK), on column injection and flame ionisation detection. Hydrogen was used as the carrier gas at constant pressure (175 kPa) with the initial oven thermal gradient from 50 ºC to 150 ºC at 40 ºC.min^{-1}, then 195 ºC at 2 ºC.min^{-1}, 205 ºC at 0.5 ºC.min^{-1} to a final temperature of 230 ºC at 40 ºC.min^{-1}. Individual FAME peak was identified by comparison to an in-house Marine fish oil (Marinol) and known standards (Restek 20-FAME Marine Oil Standard; Thames Restek UK Ltd., Buckinghamshire, UK). The data were collected and processed using Chromcard data system for Windows (Version 2.11; Thermo Fisher Scientific Inc., Milan, Italy). Heptadecanoic acid (17:0) was used as internal standard to calculate fatty acid content per gram tissue.

Fatty acids of individual lipid classes were performed by separating TL sample on 20 × 20 cm thin-layer chromatography plates (VWR, Lutterworth, UK) using the mobile phases described for lipid class analysis. Individual phospholipids (PL) as well as triacylglycerol (TAG) was identified by spraying plates with 0.1 % (w/v) 2-7-dichlorofluorescein in 97 % methanol (v/v) and visualising under UV light at 240 nm (UVP® Mineralight®R-52G; UVP Inc.). Individual classes were scrapped from plates and placed into tubes and methylated as described for fatty acid analysis. Samples were extracted as described by Tocher and Harvie (1988) and analysed by GC using the same conditions detailed above.

5.3.5 Nutritional indices

Considering the fillet lipid quality, some nutritional indices were determined from the results of total fatty acid composition in muscle. The evaluated indices were calculated following the following formulae: The index of atherogenic (AI) = (12:0 + 4 * 14:0 + 16:0) / (Σ Unsaturated FA) (Ulbricht and Southgate, 1991); The index of thrombogenic (TI) = (14:0 + 16:0 + 18:0) / (0.5* Σ 18:1) + (0.5* Σ Monounsaturated FA) + (0.5* Σ n-6 PUFA) + (3* Σ n-3 PUFA) + (Σ n-3 PUFA / Σ n-6 PUFA) (Ulbricht and Southgate, 1991); The index of peroxidation (PI) = 0.025 × (Σ monoenoic fatty acids) + 1 × (Σ dienoic fatty acids) + 2 × (Σ trienoic fatty acids) + 4 × (Σ tetraenoic fatty acids) + 6 × (Σ pentaenoic fatty acids) + 8 × (Σ hexaenoic fatty acids) (Hulbert *et al*., 2007).

5.3.6 Molecular analyses

The total RNA of liver sample from each fish per tank was extracted and homogenised in 1 mL of TriReagent® (Sigma-Aldrich, Dorset, UK). The quantity, quality and concentration of RNA was determined by spectrophotometry (Nanodrop ND-1000; Labtech Int.) and RNA integrity was checked by using 250 ng total RNA in a 1 % agarose gel electrophoresis. The livers from each tank were pooled into two samples (three livers per pool) and 2000 ng RNA from samples were used for cDNA synthesis with a random primer in 20 µL reaction volume and high-capacity reverse transcription kit following the manufacturer's protocol (Applied Biosystems, Warrington, UK). The obtained cDNA was diluted 20-fold with milliQ water (Thermo Fisher Scientific).

Expression of genes of interest (GOI) involved in the key pathways include LC-PUFA biosynthesis (*fads2d6; fads2d5, elovl2, elovl5a, elovl5b*), transcription factors (*srebp1, srebp2, lxr, pparα, pparγ*) and lipid metabolism (*fas, hmgcr, aco, cpt1*). The expressions were determined by real-time quantitative PCR, as described in detail by Betancor *et al*. (2021) using a qTower^3 G real-time PCR Thermal Cycler (Analytic Jena GmbH, Jena, Germany) in ninety-six well plates in duplicate 10 µL reaction volumes of mastermix containing 5 µL of Thermo Scientific Luminaris Color Higreen RT-PCR master mix kit

(Thermo Scientific, Hemel Hempstead, UK), 1.0 µL of the primers corresponding to the analysed gene, 1.5 µL of molecular biology grade water and 2.5 µL of cDNA. Meanwhile, 1 µL of cDNA and 3 µL of molecular biology grade water were prepared instead for housekeeping genes accordingly (details shown in Table 2.10). A negative control without cDNA (no template control) was included to ensure no contamination was present in the master mix. Standard amplification parameters contained a DNase pre-treatment at 50 ºC for 2 min, an initial activation step at 95 ºC for 10 min, followed by thirty-five cycles: 15 s at 95 ºC, 30 s at the annealing Tm and 30 s at 72 ºC.

Results were normalised using reference housekeeping genes, hypoxanthine-guanine phosphoribosyltransferase (*hprt*), elongation factor 1 alpha (*ef1α*) and ribosomal protein L2 (*rpl2*) that were considered as the most stable according to the geNorm.

5.3.7 Histology analyses

Liver and intestine samples were rinsed in water and fixed in 4 % buffered neutral-formaldehyde by Shandon Citadel 2000 Automatic Tissue Processor (Thermo Scientific, Basingstoke, UK). Samples were then embedded into paraffin blocks (Histo-embedder, Jung, Leica, Germany) and fixed on the slide after being sectioned as a 5 µm thickness wax slice. Both liver and intestine samples were stained with haematoxylin-eosin (H&E) while intestine samples were also stained with Alcian blue/Periodic Acid Schiff (ABPAS) for acidic and neutral goblet cells. The slides were then scanned and the digital images obtained from Axio Scan.Z1 slide scanner (ZEISS, Cambridge, UK) were analysed using Qupath v0.3.0 (Bankhead *et al*., 2017) and ImageJ (National Institutes of Health, USA) software. The stained sections of liver were assessed for intracytoplasmic lipid vacuolization, the stained sections of intestine were examined for height and width of simple columnar epithelial cells; AB and PAS positive goblet cell density; intestinal wall thickness. These data were collected by six randomly chosen fields from each tissue image at 20 × magnification. The results of intracytoplasmic lipid vacuolization presented by a percentage were calculated using lipocyte area divided by their chosen field area

respectively. The results of goblet cell density presented as an amount per 10^5 μm^2 were calculated as cell number divided by the area of the chosen intestinal villi. As for the height and width of the enterocytes and intestinal circular muscle thickness measurement, the methodology has been described previously (Escaffre *et al*., 2007).

5.3.8 Statistical analyses

All mean values and standard deviation (SD) were calculated (n=3, n=6 in molecular analysis) for each group. All the percentage data were arcsine square-root transformed for statistical analyses. The results of the target gene expression by fold change were normalised to the housekeeping gene and calculated following the method of Pfaffl (2001). Data were tested for normality and homogeneity using Shapiro–Wilk test and Levene's test. Results obtained at marine phase was treated by means of an unpaired two-tailed Student's t test. Two-way ANOVA was performed for the effects of factors including stimulus (M group stimulus and V group stimulus), challenge (with and without), and their interaction. The differences among groups were established by one-way ANOVA. The significance of difference was set at $p<0.05$ and a Tukey's *post hoc* test for multiple comparisons of means if differences were detected. Part of the histological results were analysed using a Kolmogorov-Smirnov test for non-parametric comparison. Multivariate principal component analysis (PCA) was conducted to define the similarity between samples with various of features. All statistical analyses were performed using SPSS software (IBM SPSS Statistics 19; SPSS Inc., Chicago, IL, USA) and GraphPad Prism 8 (GraphPad Software, USA).

5.4 Results

5.4.1 Growth performance

The body weight of the fry showed no difference after the three-week stimulus, when both groups doubled their weight from approximately 0.15 g to 0.36 g. The stimulus did not significantly affect the body weight at the end of the marine phase as well. After the

challenge, the salmon from all the groups had the similar final weight. Among them VM had the highest body weight, albeit this was not significant (p=0.06). Similarly, no significant differences in feed intake were found between groups at any timepoint (Table 5.2).

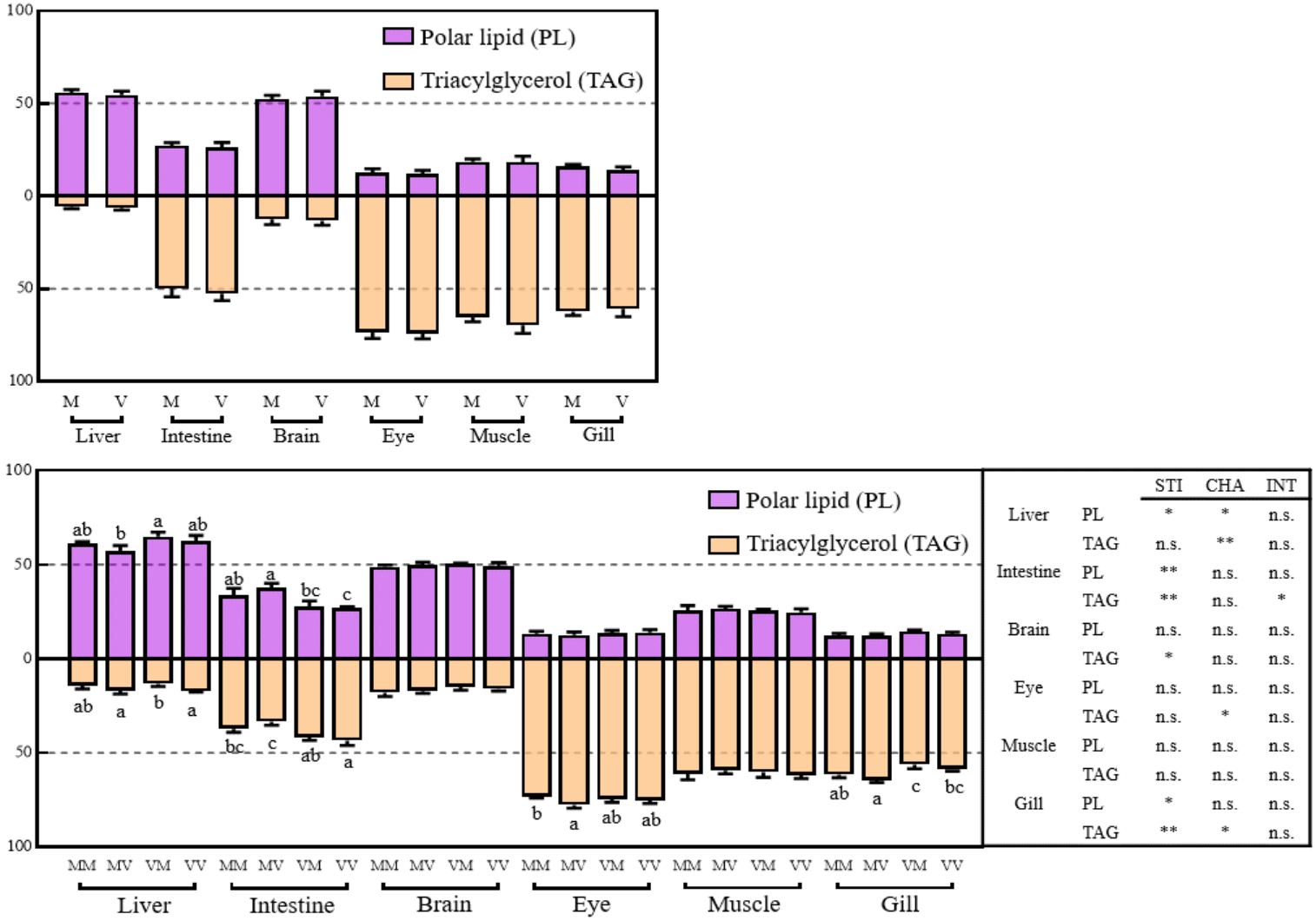
No differences were observed in the specific growth rate (SGR) during the stimulus phase. Nonetheless, the higher SGR in MM and VM compared with others denoted the main effect from challenge. In this sense, the two-way ANOVA analysis of both body weight and SGR highlighted that they were remarkably affected by challenge. No interaction between stimulus and challenge was found (Table 5.2).

Table 5.2 Growth performance of Atlantic salmon for each phase.

	M-M	**M-V**	**V-M**	**V-V**	***P***		
Whole fish weight (g)					**STI**	**CHA**	**INT**
End of stimulus phase	0.4 (M)		0.4 (V)		/	/	/
End of marine phase	100.1±1.6 (M)		100.8±2.8 (V)		/	/	/
End of challenge phase	116.5±1.3	114.0±2.6	123.4±5.3	116.1±3.9	n.s.	*	n.s.
SGR (%/ day) [1]							
Stimulus phase	4.5 (M)		4.2 (V)		/	/	/
Marine phase	2.5±0.0 (M)		2.5±0.0 (V)		/	/	/
Challenge phase	0.6 ± 0.0^{ab}	0.5 ± 0.1^{b}	0.8 ± 0.1^{a}	0.5 ± 0.1^{b}	n.s.	*	n.s.
Total feed intake (kg)							
Stimulus phase	0.2 (M)		0.3 (V)		/	/	/
Marine phase	9.8±0.8 (M)		9.7±0.6 (V)		/	/	/
Challenge phase	1.8±0.1	1.7±0.1	2.0±0.1	1.8±0.2	/	/	/

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. * p<0.05. ** p<0.01.

[1] SGR: Specific growth rate; The calculation will follow the formulation as: SGR = 100 × ($\ln W_t$ - $\ln W_i$) /t; W_t refers to final weight; W_i refers to initial weight; t refers to time.



		STI	CHA	INT
Liver	PL	*	*	n.s.
	TAG	n.s.	**	n.s.
Intestine	PL	**	n.s.	n.s.
	TAG	**	n.s.	*
Brain	PL	n.s.	n.s.	n.s.
	TAG	*	n.s.	n.s.
Eye	PL	n.s.	n.s.	n.s.
	TAG	n.s.	*	n.s.
Muscle	PL	n.s.	n.s.	n.s.
	TAG	n.s.	n.s.	n.s.
Gill	PL	*	n.s.	n.s.
	TAG	**	*	n.s.

Figure 5.2 The percentage of polar lipid and triacylglycerol of different Atlantic salmon tissues among groups after marine phase (top) and challenge phase (bottom). Data are means of triplicate measurement (n=3), with standard deviation represented by vertical bars. The mark § denotes significant difference between groups at the end of marine phase. The lowercase letters denote significant difference among groups at the end of the challenge phase ($p<0.05$). STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. ∗ $p<0.05$. ∗∗ $p<0.01$.

5.4.2 Tissue lipid content and lipid class

Tissues from salmon with different nutrition histories displayed no difference in total lipid content (Table 5.4-5.9) or lipid class composition before the challenge (Figure 5.2). At the end of the challenge phase, intestine showed differences in lipid content. The significant higher level in intestine was found in the VV group (Table 5.5). The lipid class composition showed some significant differences in all tissues excepting brain and muscle. In the liver, PL content was approximately 60 %, with higher PL levels found in VM than MV due to the stimulus and challenge, respectively. The PL in the intestine presented a higher value in the M-stimulus group regardless of the challenge (MM and MV), this significant difference was explained by the stimulus. Regarding the eye, the higher content of TAG in V-challenged groups compared with M-challenged groups showed the major effect from challenge. There was a significance in the gill TAG resulting in the following order with MV > MM > VV > VM, which was mainly impacted by stimulus (p<0.01). In the brain, PL also accounted for about 50 %, but no significant differences were found. The major lipid class in muscle, eye and gill was PL (Figure 5.2).

5.4.3 Tissue total lipid fatty acid composition

After the stimulus phase different fatty acid compositions were observed between fish from the two stimulus groups. In M group showed a higher percentage of SFA and most n-3 LC-PUFAs as well as n-3:n-6. In contrast, the proportion of monounsaturated fatty acid (MUFA), and short chain n-6 and n-3 PUFAs, specifically 18:2n-6 and 18:3n-3 were higher in V group (Table 5.3).

At the marine phase, the fatty acid composition did not vary greatly. In liver, a higher amount of MUFA in the V group, and n-3 LC-PUFA in the M group was found (Table 5.4). No differences were found in intestine, brain and eye (Table 5.5, 5.6, 5.7). For the muscle and gill, no differences were observed in the fatty acid profile of total lipid apart from a higher content of MUFA in the V group (Table 5.8, 5.9).

Table 5.3 Fatty acid compositions (percentage of total fatty acids) of Atlantic salmon whole fish after stimulus phase.

	M	**V**
Σ SFA[1]	26.6±0.9§	20.8±0.3
Σ MUFA[2]	29.8±0.9	35.9±0.9§
18:2n-6	6.5±0.4	18.4±0.4§
20:3n-6	0.3±0.0	1.8±0.1§
20:4n-6	1.3±0.0	1.7±0.1§
22:5n-6	0.3±0.0	0.8±0.0§
Σ n-6 PUFA[3]	9.3±0.5	25.7±0.6§
18:3n-3	1.3±0.2	2.5±0.1§
20:5n-3	6.6±0.1§	2.4±0.1
22:5n-3	2.6±0.1§	1.6±0.1
22:6n-3	21.1±0.5§	9.3±0.5
Σ n-3 PUFA[4]	33.6±0.5§	17.6±0.6
n-3 LC-PUFA[5]	31.4±0.6§	14.2±0.7
DHA+EPA	27.7±0.6§	11.7±0.6
DHA/EPA	3.2±0.0	3.9±0.3§
n-3:n-6	3.6±0.2§	0.7±0.0

Data are means of triplicate measurement with standard deviations (n=3). The mark **§** within a row denote significant difference between groups at the end of marine phase determined by Student's t-test ($p<0.05$).

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, 20:3n−6, 22:4n−6 and 22:5n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3, 21:5n-3 and 22:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3

The challenge phase did elicit some changes among the total lipid fatty acid profile in all the tested tissues. In the liver, fish fed V diet during the challenge (MV and VV) displayed higher amounts of LA, 20:3n-6 and total n-6, together with lower EPA and 22:5n-3 (Table 5.4). Two-way ANOVA indicated that the challenge was the main driver of differences in this tissue. DHA, n-3 LC-PUFA and n-3:n-6 ratio were found to be significantly higher

in MM followed by VM and VV, due to the challenge and the interaction (Table 5.4). In the intestine, fish from MV and VV groups showed the highest amount of LA, 20:3n-6 and total n-6, whereas EPA, 22:5n-3, DHA, n-3 LC-PUFA and n-3:n-6 ratio were found to be higher in MM and VM, both indicated the significant effect from the challenge (Table 5.5). Fewer differences were observed in brain, specifically, 20:3n-6 and 20:4n-6 (ARA) levels were found to be impacted by the challenge. The interaction between the two factors (stimulus*challenge) was shown to influence on LA, n-6 and the ratio of n-3:n-6 subsequently (Table 5.6). In the eye, the challenge mainly led to differences on LA and all the n-3-LC PUFA (Table 5.7). As for the muscle, both stimulus and challenge contributed to the highest LA and total n-6 content in VV group, whereas the interaction led to the lowest EPA. The proportion of SFA, LA and 20:3n-6 in gill were significantly different by the challenge. The total n-6 content was impacted by the interaction but the significant differences of EPA content and n-3:n-6 ratio among group were solely induced by stimulus (Table 5.9).

5.4.4 Tissues polar lipid fatty acid composition

At the end of marine phase, polar fatty acid composition in liver, intestine and eye did not show any difference among groups (Table 5.4, 5.5, 5.7). (Table 5.6). A significantly higher content of EPA in M groups was detected in muscle (Table 5.8). In gill, higher amounts of LA and ALA was found in V group (Table 5.9).

The fatty acid compositions of liver PL and TL were similar with the higher content of MUFA and some n-6 series fatty acids, together with the lower content of SFA, most n-3 fatty acids and related indicators found in MV and VV. These were all significantly impacted by challenge solely (Table 5.4). Both factors and their interaction triggered the significant difference in LA, 20:3n-6 and total n-6 in intestine PL and the VV group was significantly higher than other groups, while all the significances from n-3 related elements were induced by challenge and displayed the significantly lower value in MV and VV group (Table 5.5). A similar pattern was found between the brain and eye, with

the significantly lower content in MM and VM being: LA, 20:3n-6 and total n-6 in both tissues. Additionally, in brain and eye, the higher ratio of n-3:n-6 in MM and VM was observed as well as the significantly lower EPA content in VV group. These were significantly affected by challenge solely (Table 5.6, 5.7). The muscle was largely influenced by challenge. It mainly elicited the significantly lower LA and 20:3n-6 in MM and VM. Moreover, the challenge phase also resulted in the significantly lower EPA and 22:5n-3 in VV group and the subsequent n-3:n-6 (Table 5.8). Regarding the gill, both factors and their interaction induced the significance of ARA and total n-6, likewise most n-3 indicators were impacted by both stimulus and interaction. Notably, the content of EPA and DHA along with n-3 LC-PUFA and n-3:n-6 were significantly lower in VV group (Table 5.9).

The fatty acid composition of all tissues in TL and PL at the end of the challenge were analysed by PCA. The result plot clearly showed that liver, brain and other four tissues were clustered in three different groups according to the fatty acid features in TL. Most tissues were affected by the challenge rather than the stimulus. In other tissues, apart from the liver and brain, the fatty acid compositions of TL and PL were separately clustered (Figure 5.3).

Table 5.4 Lipid content and fatty acid compositions (percentage of total and polar fatty acids) from liver of Atlantic salmon after different groups at two stages.

	End of marine		End of challenge				*P*		
Liver	**M**	**V**	**M-M**	**M-V**	**V-M**	**V-V**	**STI**	**CHA**	**INT**
Lipid % (wet wt.)	4.2±0.1	4.5±0.3	3.6±0.1	3.8±0.4	3.6±0.1	3.9±0.3	n.s.	n.s.	n.s.
Total FA %									
Σ SFA[1]	23.9±0.6	23.4±0.4	27.3±0.5^{a}	23.6±0.1^{b}	27.2±1.5^{a}	23.5±1.1^{b}	n.s.	**	n.s.
Σ MUFA[2]	27.5±1.2	29.8±1.7§	23.6±1.3^{c}	30.4±1.5^{a}	26.5±1.2^{b}	27.9±1.2^{ab}	n.s.	**	**
18:2n-6	5.5±0.1	5.5±0.4	6.0±0.2^{b}	9.1±0.3^{a}	6.4±0.4^{b}	9.5±0.4^{a}	n.s.	**	n.s.
20:3n-6	0.9±0.1	0.9±0.1	0.6±0.0^{b}	2.5±0.2^{a}	0.6±0.1^{b}	2.4±0.2^{a}	n.s.	**	n.s.
20:4n-6	2.8±0.2	2.6±0.3	2.6±0.1	3.3±0.5	2.6±0.3	2.8±0.5	n.s.	n.s.	n.s.
22:5n-6	0.4±0.1	0.4±0.0	0.3±0.0	0.3±0.1	0.3±0.0	0.3±0.1	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	11.0±0.3	11.0±0.1	10.4±0.2^{b}	16.7±1.1^{a}	10.9±0.1^{b}	16.5±0.6^{a}	n.s.	**	n.s.
18:3n-3	0.9±0.3	1.0±0.1	1.6±0.1	2.0±0.3	1.7±0.2	1.9±0.0	n.s.	*	n.s.
20:5n-3	5.5±0.4	5.4±0.2	5.3±0.2^{a}	3.3±0.2^{b}	5.6±0.2^{a}	3.4±0.5^{b}	n.s.	**	n.s.
22:5n-3	1.6±0.2	1.5±0.1	1.8±0.1^{a}	1.2±0.1^{b}	1.7±0.2^{a}	1.7±0.2^{a}	n.s.	**	**
22:6n-3	28.3±1.1	26.6±1.7	28.4±0.8^{a}	20.9±1.4^{c}	24.7±0.7^{b}	24.0±0.0^{b}	n.s.	**	**
Σ n-3 PUFA[4]	37.3±1.0§	35.6±1.7	38.5±0.9^{a}	29.0±1.1^{d}	35.2±0.7^{b}	32.5±0.6^{c}	n.s.	**	**
n-3 LC-PUFA[5]	36.1±1.0§	34.2±1.8	36.5±1.0^{a}	26.5±1.3^{d}	33.0±0.9^{b}	30.1±0.7^{c}	n.s.	**	**
DHA+EPA	33.7±0.9	31.9±1.8	33.7±1.0^{a}	24.3±1.6^{d}	30.3±0.6^{b}	27.4±0.5^{c}	n.s.	**	**
DHA/EPA	5.2±0.5	5.0±0.3	5.3±0.1^{bc}	6.3±0.2^{ab}	4.4±0.2^{c}	7.1±1.2^{a}	n.s.	**	*
n-3:n-6	3.4±0.2	3.2±0.2	3.7±0.2^{a}	1.7±0.1^{c}	3.2±0.1^{b}	1.9±0.1^{c}	n.s.	**	**
Polar FA %									
Σ SFA[1]	27.0±0.6	26.8±0.3	31.4±0.9^{a}	27.2±0.5^{c}	30.4±1.4^{ab}	28.1±1.1^{bc}	n.s.	**	n.s.
Σ MUFA[2]	21.1±0.8	21.7±1.0	19.0±0.3^{b}	23.4±0.7^{a}	19.1±1.3^{b}	21.7±1.2^{a}	n.s.	**	n.s.
18:2n-6	4.5±0.1	4.5±0.2	5.1±0.1^{b}	8.0±0.4^{a}	5.1±0.2^{b}	7.8±0.4^{a}	n.s.	**	n.s.
20:3n-6	1.1±0.1	1.1±0.1	0.5±0.0^{b}	3.1±0.3^{a}	0.6±0.1^{b}	2.4±0.9^{a}	n.s.	**	n.s.
20:4n-6	3.4±0.2	3.3±0.3	2.9±0.1	3.8±0.8	3.2±0.1	3.8±0.5	n.s.	*	n.s.
22:5n-6	0.5±0.0	0.5±0.0	0.3±0.0	0.4±0.1	0.3±0.0	0.4±0.1	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	11.0±0.1	10.9±0.2	9.7±0.1^{b}	16.2±1.2^{a}	10.2±0.4^{b}	15.4±1.8^{a}	n.s.	**	n.s.
18:3n-3	0.6±0.0	0.6±0.1	1.2±0.1	1.3±0.1	1.2±0.1	1.3±0.1	n.s.	n.s.	n.s.
20:5n-3	6.2±0.4	6.2±0.3	5.5±0.1^{a}	3.8±0.5^{b}	5.8±0.3^{a}	4.0±0.7^{b}	n.s.	**	n.s.
22:5n-3	1.5±0.1	1.5±0.1	1.7±0.1^{ab}	1.2±0.2^{b}	1.8±0.1^{a}	1.6±0.3^{ab}	n.s.	**	n.s.
22:6n-3	31.6±0.9	31.2±0.8	30.1±0.6^{a}	25.4±0.6^{b}	30.2±0.6^{a}	27.9±1.0^{ab}	n.s.	**	n.s.
Σ n-3 PUFA[4]	40.7±0.7	40.4±0.8	39.5±0.6^{a}	32.9±1.0^{b}	39.9±0.2^{a}	35.6±1.9^{ab}	n.s.	**	n.s.
n-3 LC-PUFA[5]	39.9±0.7	39.5±0.9	38.0±0.6^{a}	31.3±1.1^{b}	38.5±0.1^{a}	34.0±1.9^{ab}	n.s.	**	n.s.
DHA+EPA	37.8±0.7	37.5±0.8	35.6±0.6^{a}	29.3±0.9^{b}	36.0±0.3^{a}	32.0±1.7^{ab}	n.s.	**	n.s.
DHA/EPA	5.1±0.4	5.0±0.3	5.5±0.2^{ab}	6.7±0.8^{a}	5.2±0.4^{b}	6.9±0.9^{a}	n.s.	**	n.s.
n-3:n-6	3.7±0.1	3.7±0.1	4.1±0.0^{a}	2.0±0.2^{b}	3.9±0.1^{a}	2.3±0.4^{b}	n.s.	**	n.s.

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). The mark § within a row denotes significant difference between groups at the end of marine phase determined by Student's t-test (p<0.05).

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. * p<0.05. ** p<0.01.

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 5.5 Lipid content and fatty acid compositions (percentage of total and polar fatty acids) from intestine of Atlantic salmon after different groups at two stages.

	End of marine		End of challenge				*P*		
Intestine	**M**	**V**	**M-M**	**M-V**	**V-M**	**V-V**	**STI**	**CHA**	**INT**
Lipid % (wet wt.)	7.7±0.7	7.3±0.5	6.0±0.2^{b}	6.1±0.5^{b}	6.6±0.7^{b}	8.7±0.4^{a}	**	**	*
Total FA %									
Σ SFA[1]	25.2±0.5	24.9±0.1	24.4±0.3^{a}	23.1±1.7^{ab}	23.7±0.7^{ab}	21.5±0.9^{b}	n.s.	*	n.s.
Σ MUFA[2]	37.9±0.7	38.6±2.1	37.7±0.2	39.3±5.2	38.8±2.0	42.3±1.6	n.s.	n.s.	n.s.
18:2n-6	8.9±0.2	8.8±0.3	9.8±0.2^{b}	11.5±0.6^{a}	10.0±0.3^{b}	12.1±0.5^{a}	n.s.	**	n.s.
20:3n-6	0.4±0.0	0.4±0.1	0.4±0.0^{b}	1.1±0.2^{a}	0.4±0.1^{b}	0.9±0.1^{a}	n.s.	**	n.s.
20:4n-6	1.0±0.1	1.0±0.2	1.3±0.0	1.5±0.8	1.2±0.2	1.2±0.2	n.s.	n.s.	n.s.
22:5n-6	0.3±0.0	0.3±0.0	0.2±0.0	0.2±0.1	0.2±0.0	0.2±0.0	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	11.9±0.5	12.1±0.3	12.8±0.2^{b}	15.7±2.0^{a}	13.0±0.1^{b}	15.7±0.5^{a}	n.s.	**	n.s.
18:3n-3	2.3±0.1	2.3±0.2	2.7±0.1	2.8±0.6	2.8±0.2	3.2±0.3	n.s.	n.s.	n.s.
20:5n-3	3.6±0.1	3.5±0.2	3.6±0.1^{a}	2.8±0.2^{b}	3.5±0.0^{a}	2.6±0.1^{b}	n.s.	**	n.s.
22:5n-3	1.6±0.0	1.5±0.1	1.4±0.1	1.2±0.1	1.4±0.1	1.2±0.1	n.s.	*	n.s.
22:6n-3	14.2±0.7	14.1±2.1	15.0±0.1^{a}	12.7±1.3^{ab}	14.3±1.7^{a}	10.7±1.3^{b}	n.s.	*	n.s.
Σ n-3 PUFA[4]	23.7±0.6	23.4±1.9	24.3±0.1^{a}	21.2±1.5^{ab}	23.7±1.3^{a}	19.6±1.1^{b}	n.s.	**	n.s.
n-3 LC-PUFA[5]	20.2±0.7	20.0±2.2	20.7±0.1^{a}	17.5±1.3^{ab}	20.0±1.6^{ab}	15.4±1.4^{b}	n.s.	**	n.s.
DHA+EPA	17.8±0.7	17.6±2.3	18.6±0.0	15.5±1.4	17.7±1.7	13.3±1.4	n.s.	*	n.s.
DHA/EPA	4.0±0.2	4.0±0.5	4.2±0.1	4.5±0.5	4.1±0.5	4.0±0.3	n.s.	n.s.	n.s.
n-3:n-6	2.0±0.1	1.9±0.1	1.9±0.0^{a}	1.4±0.1^{b}	1.8±0.1^{a}	1.2±0.1^{b}	n.s.	**	n.s.
Polar FA %									
Σ SFA[1]	31.6±0.4	31.4±0.5	32.1±0.6^{a}	30.7±0.3^{a}	31.0±0.6^{a}	28.9±0.7^{b}	**	**	n.s.
Σ MUFA[2]	18.6±0.7	18.7±1.1	20.6±1.3^{ab}	22.9±1.3^{a}	20.0±0.7^{b}	22.2±0.4^{ab}	n.s.	**	n.s.
18:2n-6	4.9±0.1	4.9±0.2	5.6±0.2^{c}	8.3±0.5^{b}	5.4±0.5^{c}	10.2±0.4^{a}	*	**	**
20:3n-6	1.0±0.1	1.0±0.1	0.6±0.1^{c}	1.8±0.1^{b}	0.7±0.1^{c}	3.0±0.4^{a}	**	**	**
20:4n-6	2.7±0.0	2.6±0.1	2.8±0.3^{b}	3.0±0.3^{b}	2.9±0.1^{b}	3.8±0.5^{a}	n.s.	*	**
22:5n-6	0.6±0.0	0.6±0.0	0.4±0.0	0.5±0.1	0.5±0.0	0.7±0.2	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	11.8±0.2	12.1±0.2	10.6±0.4^{c}	15.0±0.9^{b}	10.6±0.8^{c}	19.0±1.6^{a}	*	**	*
18:3n-3	0.4±0.0	0.4±0.0	0.8±0.1^{ab}	1.1±0.3^{a}	0.7±0.1^{b}	1.0±0.1^{a}	n.s.	**	n.s.
20:5n-3	4.0±0.4	3.9±0.2	4.8±0.3^{a}	3.2±0.3^{b}	4.7±0.4^{a}	2.8±0.5^{b}	n.s.	**	n.s.
22:5n-3	1.2±0.1	1.2±0.1	1.2±0.1^{a}	0.9±0.1^{b}	1.3±0.2^{a}	0.9±0.1^{b}	n.s.	**	n.s.
22:6n-3	30.9±1.0	30.8±1.4	28.2±1.5^{a}	24.3±1.8^{b}	30.0±0.3^{a}	23.5±1.7^{b}	n.s.	**	n.s.
Σ n-3 PUFA[4]	37.0±0.7	36.9±1.6	35.7±1.7^{a}	30.5±1.8^{b}	37.3±0.7^{a}	28.8±2.5^{b}	n.s.	**	n.s.
n-3 LC-PUFA[5]	36.5±0.7	36.3±1.6	34.7±1.8^{a}	29.1±2.1^{b}	36.4±0.6^{a}	27.6±2.4^{b}	n.s.	**	n.s.
DHA+EPA	34.9±0.8	34.7±1.5	33.0±1.7^{a}	27.6±2.0^{b}	34.7±0.4^{a}	26.3±2.2^{b}	n.s.	**	n.s.
DHA/EPA	7.7±0.8	7.8±0.4	5.9±0.2^{c}	7.6±0.5^{ab}	6.4±0.6^{bc}	8.5±1.1^{a}	n.s.	**	n.s.
n-3:n-6	3.1±0.1	3.0±0.1	3.4±0.2^{a}	2.0±0.2^{b}	3.5±0.3^{a}	1.5±0.3^{b}	n.s.	**	*

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). The mark § within a row denotes significant difference between groups at the end of marine phase determined by Student's t-test (p<0.05).

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. * p<0.05. ** p<0.01.

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 5.6 Lipid content and fatty acid compositions (percentage of total and polar fatty acids) from brain of Atlantic salmon after different groups at two stages.

	End of marine		End of challenge				*P*		
Brain	**M**	**V**	**M-M**	**M-V**	**V-M**	**V-V**	**STI**	**CHA**	**INT**
Lipid % (wet wt.)	6.5±0.6	6.4±0.4	7.2±0.4	7.0±0.3	7.2±0.5	6.8±0.5	n.s.	n.s.	n.s.
Total FA %									
Σ SFA[1]	26.6±0.7	27.1±0.4	25.0±0.3	24.7±0.5	25.8±1.0	25.6±1.0	n.s.	n.s.	n.s.
Σ MUFA[2]	33.0±1.9	32.2±1.2	38.0±1.5	36.5±1.5	37.0±1.6	36.6±2.2	n.s.	n.s.	n.s.
18:2n-6	1.9±0.5	2.1±0.6	4.4±0.9^{a}	2.4±0.4^{b}	2.2±0.8^{b}	3.2±0.5^{ab}	n.s.	n.s.	**
20:3n-6	0.2±0.0	0.2±0.0	0.2±0.0^{b}	0.3±0.1^{a}	0.2±0.0^{b}	0.4±0.1^{a}	n.s.	**	n.s.
20:4n-6	1.1±0.1	1.1±0.1	0.9±0.1	1.1±0.1	1.0±0.1	1.1±0.1	n.s.	n.s.	n.s.
22:5n-6	0.1±0.0	0.1±0.0	0.1±0.1	0.1±0.0	0.1±0.1	0.1±0.1	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	3.6±0.5	4.0±0.6	6.1±1.0^{a}	4.4±0.4^{ab}	3.7±1.0^{b}	5.3±0.7^{ab}	n.s.	n.s.	**
18:3n-3	0.6±0.4	0.6±0.2	1.1±0.6	0.8±0.2	0.6±0.3	0.9±0.2	n.s.	n.s.	n.s.
20:5n-3	4.6±0.4	4.7±0.1	4.5±0.4	4.6±0.0	4.8±0.1	4.5±0.1	n.s.	n.s.	n.s.
22:5n-3	1.9±0.1	1.9±0.0	1.7±0.1	1.7±0.0	1.6±0.0	1.5±0.0	n.s.	n.s.	n.s.
22:6n-3	25.8±1.8	26.0±1.4	18.8±2.0	21.6±1.1	20.3±2.2	19.8±1.5	n.s.	n.s.	n.s.
Σ n-3 PUFA[4]	33.6±1.7	33.7±1.3	27.0±1.5	29.4±0.8	28.0±1.9	27.5±1.4	n.s.	n.s.	n.s.
n-3 LC-PUFA[5]	32.7±2.1	32.9±1.5	25.4±2.3	28.3±1.1	27.1±2.3	26.3±1.5	n.s.	n.s.	n.s.
DHA+EPA	30.4±2.1	30.6±1.6	23.3±2.3	26.1±1.2	25.1±2.3	24.3±1.5	n.s.	n.s.	n.s.
DHA/EPA	5.6±0.3	5.6±0.2	4.2±0.3	4.7±0.2	4.2±0.4	4.4±0.4	n.s.	n.s.	n.s.
n-3:n-6	9.5±1.7	8.7±1.4	4.5±0.9	6.7±0.6	7.9±2.4	5.3±0.8	n.s.	n.s.	*
Polar FA %									
Σ SFA[1]	28.9±0.8	29.8±0.7	26.8±1.0	27.0±0.4	27.3±0.4	27.5±1.2	n.s.	n.s.	n.s.
Σ MUFA[2]	32.6±1.2	31.3±0.8	36.7±2.8	36.2±0.2	36.6±0.5	35.8±2.5	n.s.	n.s.	n.s.
18:2n-6	0.6±0.1	0.5±0.0	0.7±0.0^{b}	0.9±0.1^{a}	0.6±0.1^{b}	0.9±0.1^{a}	n.s.	**	n.s.
20:3n-6	0.2±0.0	0.2±0.0	0.1±0.0^{b}	0.3±0.1^{a}	0.1±0.0^{b}	0.3±0.1^{a}	n.s.	**	n.s.
20:4n-6	0.9±0.0	1.0±0.0	0.9±0.0	1.0±0.1	0.9±0.1	1.0±0.1	n.s.	n.s.	n.s.
22:5n-6	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	0.1±0.0	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	2.1±0.1	2.1±0.0	2.2±0.0^{b}	2.6±0.3^{a}	2.1±0.3^{b}	2.7±0.2^{a}	n.s.	**	n.s.
18:3n-3	0.1±0.0	0.1±0.0	0.2±0.0	0.2±0.0	0.2±0.0	0.2±0.0	n.s.	n.s.	n.s.
20:5n-3	4.0±0.1	4.0±0.0	4.4±0.1^{a}	4.1±0.1^{ab}	4.2±0.2^{ab}	4.0±0.1^{b}	n.s.	*	n.s.
22:5n-3	1.7±0.1	1.7±0.0	1.6±0.1	1.6±0.0	1.6±0.1	1.5±0.1	n.s.	n.s.	n.s.
22:6n-3	24.5±0.8	25.3±0.7	21.7±2.2	21.8±0.4	21.4±1.0	22.0±1.7	n.s.	n.s.	n.s.
Σ n-3 PUFA[4]	30.6±0.8	31.4±0.7	28.2±2.2	28.1±0.6	27.7±1.1	28.1±1.7	n.s.	n.s.	n.s.
n-3 LC-PUFA[5]	30.4±0.8	31.3±0.7	28.0±2.2	27.8±0.6	27.5±1.0	27.9±1.7	n.s.	n.s.	n.s.
DHA+EPA	28.5±0.8	29.4±0.7	26.0±2.1	25.9±0.6	25.6±0.9	25.9±1.6	n.s.	n.s.	n.s.
DHA/EPA	6.1±0.3	6.3±0.2	5.0±0.6	5.3±0.1	5.1±0.4	5.5±0.5	n.s.	n.s.	n.s.
n-3:n-6	14.9±1.1	15.1±0.4	13.0±0.9^{a}	10.9±1.4^{b}	13.6±1.3^{a}	10.4±0.3^{b}	n.s.	*	n.s.

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). The mark § within a row denotes significant difference between groups at the end of marine phase determined by Student's t-test (p<0.05).

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. * p<0.05. ** p<0.01.

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 5.7 Lipid content and fatty acid compositions (percentage of total and polar fatty acids) from eye of Atlantic salmon after different groups at two stages.

	End of marine		End of challenge				*P*		
Eye	**M**	**V**	**M-M**	**M-V**	**V-M**	**V-V**	**STI**	**CHA**	**INT**
Lipid % (wet wt.)	11.0±1.0	10.8±0.6	12.9±0.7	14.1±0.7	12.1±0.5	13.1±0.5	*	*	n.s.
Total FA %									
Σ SFA[1]	23.7±0.3	23.6±0.3	23.8±0.4^{a}	22.8±0.4^{b}	22.5±0.1^{bc}	21.7±0.3^{c}	**	**	n.s.
Σ MUFA[2]	43.5±0.5	43.8±0.4	44.6±0.2^{b}	46.1±0.9^{a}	45.4±0.3^{ab}	46.5±0.5^{a}	n.s.	**	n.s.
18:2n-6	9.3±0.2	9.2±0.1	10.3±0.2^{b}	11.1±0.5^{a}	10.3±0.1^{b}	11.2±0.5^{a}	n.s.	**	n.s.
20:3n-6	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.2±0.0	0.3±0.1	n.s.	n.s.	n.s.
20:4n-6	0.6±0.0	0.6±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.5±0.0	n.s.	n.s.	n.s.
22:5n-6	0.2±0.0	0.2±0.0	0.1±0.1	0.1±0.0	0.1±0.0	0.1±0.0	**	n.s.	n.s.
Σ n-6 PUFA[3]	11.4±0.2	11.3±0.1	12.1±0.2^{c}	13.1±0.6^{ab}	12.2±0.1^{bc}	13.2±0.4^{a}	n.s.	**	n.s.
18:3n-3	2.6±0.1	2.6±0.0	3.3±0.1	3.4±0.1	3.2±0.1	3.4±0.1	n.s.	n.s.	n.s.
20:5n-3	3.3±0.1	3.2±0.1	3.1±0.0^{a}	2.7±0.2^{b}	3.0±0.1^{a}	2.7±0.1^{b}	n.s.	**	n.s.
22:5n-3	1.6±0.0	1.6±0.0	1.3±0.0	1.2±0.1	1.4±0.0	1.3±0.1	n.s.	n.s.	n.s.
22:6n-3	10.5±0.4	10.7±0.3	8.7±0.1	7.7±0.8	9.0±0.4	8.3±0.7	n.s.	*	n.s.
Σ n-3 PUFA[4]	20.2±0.4	20.1±0.4	18.4±0.3	17.0±1.0	18.9±0.5	17.6±0.9	n.s.	*	n.s.
n-3 LC-PUFA[5]	16.2±0.4	16.2±0.4	13.9±0.2	12.4±1.1	14.4±0.5	13.1±0.9	n.s.	*	n.s.
DHA+EPA	13.8±0.4	13.8±0.4	11.7±0.1	10.4±1.0	12.1±0.5	11.0±0.8	n.s.	*	n.s.
DHA/EPA	3.2±0.1	3.4±0.1	2.8±0.0	2.8±0.1	3.0±0.1	3.0±0.2	*	n.s.	n.s.
n-3:n-6	1.8±0.0	1.8±0.0	1.5±0.0^{a}	1.3±0.1^{b}	1.5±0.0^{a}	1.3±0.1^{b}	n.s.	**	n.s.
Polar FA %									
Σ SFA[1]	28.0±0.6	28.7±0.9	28.1±0.8^{a}	28.1±0.7^{a}	26.6±0.4^{ab}	26.4±0.7^{b}	**	n.s.	n.s.
Σ MUFA[2]	25.7±2.1	25.3±1.2	28.5±2.6	28.7±2.4	32.3±1.9	29.4±2.0	n.s.	n.s.	n.s.
18:2n-6	2.2±0.2	2.3±0.2	2.6±0.4^{b}	4.1±0.6^{a}	2.4±0.0^{b}	3.7±0.2^{a}	n.s.	**	n.s.
20:3n-6	0.5±0.2	0.5±0.1	0.3±0.0^{b}	0.7±0.1^{a}	0.2±0.0^{b}	1.1±0.5^{a}	n.s.	**	n.s.
20:4n-6	1.7±0.1	1.8±0.1	1.6±0.0	1.7±0.1	1.5±0.0	1.7±0.1	n.s.	n.s.	n.s.
22:5n-6	0.4±0.0	0.4±0.0	0.3±0.0	0.3±0.0	0.3±0.0	0.4±0.0	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	5.3±0.5	5.5±0.4	5.2±0.4^{b}	7.4±1.0^{a}	4.9±0.1^{b}	7.5±0.7^{a}	n.s.	**	n.s.
18:3n-3	0.5±0.1	0.5±0.0	0.8±0.2^{ab}	1.0±0.2^{a}	0.6±0.0^{b}	0.9±0.0^{ab}	n.s.	**	n.s.
20:5n-3	4.0±0.3	4.2±0.1	4.3±0.1^{a}	3.7±0.5^{ab}	4.0±0.3^{ab}	3.4±0.2^{b}	n.s.	**	n.s.
22:5n-3	1.3±0.1	1.4±0.0	1.4±0.1	1.2±0.1	1.3±0.1	1.2±0.0	n.s.	n.s.	n.s.
22:6n-3	33.1±1.2	32.5±1.4	29.5±2.0	27.7±1.4	28.1±1.4	29.2±2.1	n.s.	n.s.	n.s.
Σ n-3 PUFA[4]	39.5±1.6	39.1±1.2	36.7±1.9	34.4±1.9	34.7±1.6	35.4±2.1	n.s.	n.s.	n.s.
n-3 LC-PUFA[5]	38.8±1.5	38.4±1.3	35.6±1.9	33.1±1.9	33.8±1.6	34.2±2.1	n.s.	n.s.	n.s.
DHA+EPA	37.2±1.4	36.7±1.3	33.8±2.0	31.4±1.8	32.2±1.5	32.6±2.1	n.s.	n.s.	n.s.
DHA/EPA	8.3±0.4	7.8±0.6	6.9±0.6	7.6±0.8	7.0±0.6	8.5±0.7	n.s.	*	n.s.
n-3:n-6	7.4±0.5	7.2±0.6	7.0±0.6^{a}	4.7±0.7^{b}	7.0±0.3^{a}	4.8±0.6^{b}	n.s.	**	n.s.

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). The mark § within a row denotes significant difference between groups at the end of marine phase determined by Student's t-test (p<0.05).

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. * p<0.05. ** p<0.01.

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 5.8 Lipid content and fatty acid compositions (percentage of total and polar fatty acids) from muscle of Atlantic salmon after different groups at two stages.

	End of marine		End of challenge				*P*		
Muscle	**M**	**V**	**M-M**	**M-V**	**V-M**	**V-V**	**STI**	**CHA**	**INT**
Lipid % (wet wt.)	4.8±0.5	5.5±0.7	4.4±0.5	4.1±0.6	4.5±0.6	4.9±0.6	n.s.	n.s.	n.s.
Total FA %									
Σ SFA[1]	23.3±0.2	23.4±0.1	23.5±0.6^{a}	22.8±0.1^{ab}	23.2±0.4^{a}	21.7±0.9^{b}	n.s.	∗	n.s.
Σ MUFA[2]	41.6±0.6	42.4±0.4§	42.3±0.9	42.9±0.9	42.2±0.1	43.9±0.7	n.s.	n.s.	n.s.
18:2n-6	9.5±0.2	9.6±0.1	10.5±0.2^{b}	10.7±0.4^{b}	10.8±0.4^{b}	11.7±0.4^{a}	∗	∗	n.s.
20:3n-6	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.0	0.3±0.0	0.4±0.1	n.s.	n.s.	n.s.
20:4n-6	0.6±0.0	0.6±0.0	0.5±0.0	0.6±0.0	0.5±0.0	0.5±0.0	n.s.	n.s.	n.s.
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.1	0.1±0.1	0.2±0.0	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	11.7±0.1	11.8±0.1	12.3±0.2^{b}	12.6±0.3^{ab}	12.6±0.5^{ab}	13.9±0.8^{a}	∗	∗	n.s.
18:3n-3	2.8±0.1	2.9±0.0	3.4±0.1	3.3±0.1	3.5±0.1	3.6±0.1	n.s.	n.s.	n.s.
20:5n-3	3.8±0.2	3.5±0.1	3.3±0.1^{a}	3.2±0.3^{a}	3.4±0.1^{a}	2.9±0.2^{b}	n.s.	n.s.	∗
22:5n-3	1.7±0.0	1.6±0.0	1.4±0.1	1.4±0.1	1.4±0.0	1.3±0.1	n.s.	n.s.	n.s.
22:6n-3	11.7±0.6	11.1±0.5	10.8±1.0	10.8±1.1	10.6±0.3	10.0±0.7	n.s.	n.s.	n.s.
Σ n-3 PUFA[4]	22.2±0.7	21.3±0.5	20.8±1.1	20.6±1.3	21.0±0.1	19.7±0.8	n.s.	n.s.	n.s.
n-3 LC-PUFA[5]	18.0±0.8	17.1±0.6	16.3±1.1	16.2±1.5	16.3±0.2	14.9±0.9	n.s.	n.s.	n.s.
DHA+EPA	15.4±0.7	14.6±0.6	14.1±1.0	14.0±1.4	14.0±0.3	12.8±0.8	n.s.	n.s.	n.s.
DHA/EPA	3.1±0.1	3.2±0.1	3.3±0.3	3.3±0.1	3.1±0.1	3.5±0.3	n.s.	n.s.	n.s.
n-3:n-6	1.9±0.1	1.8±0.1	1.7±0.1	1.6±0.1	1.7±0.1	1.4±0.1	n.s.	n.s.	n.s.
Polar FA %									
Σ SFA[1]	24.0±0.1	24.1±0.4	24.7±1.0	24.2±0.5	24.4±0.3	23.1±0.6	n.s.	∗	n.s.
Σ MUFA[2]	19.5±0.4	19.9±0.6	14.6±0.5	15.4±1.2	15.2±0.3	16.8±1.3	n.s.	n.s.	n.s.
18:2n-6	5.3±0.1	5.2±0.1	3.6±0.2^{b}	4.3±0.5^{ab}	3.7±0.1^{b}	5.2±0.7^{a}	n.s.	∗∗	n.s.
20:3n-6	0.6±0.0	0.7±0.0	0.4±0.0^{b}	0.7±0.1^{a}	0.4±0.0^{b}	0.9±0.2^{a}	n.s.	∗∗	n.s.
20:4n-6	1.6±0.0	1.6±0.0	1.4±0.0	1.6±0.1	1.5±0.1	1.7±0.1	n.s.	n.s.	n.s.
22:5n-6	0.6±0.0	0.6±0.0	0.5±0.0	0.5±0.0	0.5±0.0	0.6±0.0	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	9.1±0.1	9.0±0.1	6.5±0.2^{b}	7.7±0.9^{ab}	6.8±0.2^{b}	9.1±1.3^{a}	n.s.	∗∗	n.s.
18:3n-3	1.9±0.0	1.8±0.0	1.8±0.1^{b}	2.0±0.1^{ab}	1.9±0.0^{b}	2.3±0.2^{a}	∗	∗∗	n.s.
20:5n-3	8.1±0.1§	7.8±0.1	7.5±0.5^{ab}	7.4±0.5^{a}	7.8±0.3^{a}	6.5±0.5^{b}	n.s.	∗	n.s.
22:5n-3	2.5±0.0	2.4±0.1	2.3±0.1^{ab}	2.2±0.1^{a}	2.4±0.1^{a}	2.0±0.2^{b}	n.s.	∗∗	n.s.
22:6n-3	32.2±0.3	32.4±0.4	40.5±0.4	39.0±1.3	39.5±0.6	37.9±2.0	n.s.	n.s.	n.s.
Σ n-3 PUFA[4]	46.5±0.3	46.2±0.5	53.6±0.8	52.1±1.6	53.1±0.5	50.5±2.3	n.s.	∗	n.s.
n-3 LC-PUFA[5]	43.8±0.4	43.6±0.5	51.3±0.7	49.5±1.8	50.7±0.6	47.4±2.6	n.s.	∗	n.s.
DHA+EPA	40.3±0.3	40.2±0.5	48.0±0.6	46.3±1.8	47.4±0.5	44.5±2.4	n.s.	∗	n.s.
DHA/EPA	4.0±0.0	4.2±0.1	5.4±0.4	5.3±0.2	5.0±0.2	5.8±0.2	n.s.	n.s.	∗
n-3:n-6	5.1±0.1	5.1±0.1	8.2±0.2^{a}	6.8±0.9^{ab}	7.9±0.4^{a}	5.6±1.0^{b}	n.s.	∗∗	n.s.

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). The mark § within a row denotes significant difference between groups at the end of marine phase determined by Student's t-test (p<0.05).

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. ∗ p<0.05. ∗∗ p<0.01.

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

Table 5.9 Lipid content and fatty acid compositions (percentage of total and polar fatty acids) from gill of Atlantic salmon after different groups at two stages.

	End of marine		End of challenge				*P*		
Gill	**M**	**V**	**M-M**	**M-V**	**V-M**	**V-V**	**STI**	**CHA**	**INT**
Lipid % (wet wt.)	11.6±0.6	11.2±0.3	7.2±0.5	7.5±0.3	6.4±0.4	7.0±0.8	n.s.	n.s.	n.s.
Total FA %									
Σ SFA[1]	25.2±0.4	24.6±0.4	24.9±0.1^{ab}	24.4±0.3^{ab}	25.5±0.4^{a}	23.9±0.8^{b}	n.s.	**	n.s.
Σ MUFA[2]	41.3±0.4	42.4±0.4§	42.9±0.8	43.4±0.3	43.6±1.7	44.3±0.4	n.s.	n.s.	n.s.
18:2n-6	9.0±0.2	9.2±0.2	10.0±0.1^{b}	10.3±0.1^{ab}	10.0±0.2^{b}	10.9±0.5^{a}	n.s.	*	n.s.
20:3n-6	0.3±0.0	0.3±0.0	0.2±0.0	0.3±0.1	0.2±0.0	0.4±0.1	n.s.	n.s.	n.s.
20:4n-6	0.8±0.0	0.8±0.0	0.8±0.1	0.9±0.1	0.9±0.1	0.9±0.0	n.s.	n.s.	n.s.
22:5n-6	0.2±0.0	0.2±0.0	0.2±0.0	0.1±0.1	0.1±0.1	0.1±0.1	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	11.4±0.2	11.5±0.2	12.1±0.0^{c}	12.5±0.1^{b}	12.1±0.1^{bc}	13.3±0.6^{a}	n.s.	**	*
18:3n-3	2.6±0.1	2.7±0.1	3.1±0.0	3.1±0.0	3.0±0.0	3.2±0.2	n.s.	n.s.	n.s.
20:5n-3	3.7±0.1	3.6±0.1	3.3±0.1^{a}	3.2±0.2^{a}	3.1±0.3^{a}	2.8±0.1^{b}	*	n.s.	n.s.
22:5n-3	1.6±0.0	1.5±0.0	1.3±0.1	1.3±0.1	1.2±0.1	1.1±0.1	n.s.	n.s.	n.s.
22:6n-3	10.8±0.2	10.6±0.4	9.4±0.7	9.2±0.4	8.6±0.8	8.5±0.2	n.s.	n.s.	n.s.
Σ n-3 PUFA[4]	20.9±0.3	20.7±0.5	19.1±0.8	18.8±0.6	17.7±1.4	17.6±0.4	n.s.	n.s.	n.s.
n-3 LC-PUFA[5]	16.9±0.3	16.6±0.5	14.8±0.8	14.5±0.5	13.6±1.4	13.2±0.3	n.s.	n.s.	n.s.
DHA+EPA	14.5±0.3	14.2±0.5	12.7±0.8	12.5±0.5	11.6±1.4	11.3±0.3	n.s.	n.s.	n.s.
DHA/EPA	2.9±0.0	3.0±0.0	2.9±0.1	2.9±0.2	2.8±0.2	3.0±0.0	n.s.	n.s.	n.s.
n-3:n-6	1.8±0.0	1.8±0.0	1.6±0.1	1.5±0.0	1.5±0.2	1.3±0.1	*	n.s.	n.s.
Polar FA %									
Σ SFA[1]	26.5±0.5	26.2±0.4	32.1±1.1^{ab}	31.2±1.1^{b}	34.0±0.6^{a}	31.6±1.3^{ab}	n.s.	*	n.s.
Σ MUFA[2]	27.3±0.4	27.0±1.2	30.9±1.6^{b}	30.1±1.3^{b}	33.1±1.4^{a}	34.6±1.9^{a}	*	n.s.	n.s.
18:2n-6	4.1±0.1	4.5±0.1§	4.3±0.5^{b}	4.6±0.2^{b}	4.6±0.8^{b}	6.1±0.1^{a}	*	*	n.s.
20:3n-6	0.6±0.0	0.6±0.1	0.4±0.1^{b}	0.9±0.2^{a}	0.3±0.0^{b}	1.2±0.3^{a}	n.s.	**	n.s.
20:4n-6	3.8±0.0	3.7±0.1	2.4±0.3^{ab}	2.7±0.4^{a}	1.9±0.3^{b}	2.4±0.2^{ab}	*	*	*
22:5n-6	0.5±0.0	0.5±0.0	0.3±0.0	0.4±0.1	0.3±0.1	0.4±0.0	n.s.	n.s.	n.s.
Σ n-6 PUFA[3]	9.8±0.1	9.9±0.2	8.2±0.3^{bc}	9.2±0.6^{b}	7.8±0.3^{c}	11.0±0.5^{a}	*	**	**
18:3n-3	0.7±0.0	1.3±0.0§	1.1±0.2	1.0±0.0	1.1±0.3	1.3±0.3	n.s.	n.s.	n.s.
20:5n-3	5.4±0.2	5.5±0.1	4.2±0.6^{a}	3.7±0.5^{ab}	3.2±0.7^{ab}	2.7±0.1^{b}	**	n.s.	n.s.
22:5n-3	1.3±0.1	1.4±0.1	0.9±0.1	0.8±0.1	0.8±0.1	0.7±0.0	n.s.	n.s.	n.s.
22:6n-3	25.5±0.4	25.8±1.0	18.5±1.8^{ab}	20.8±1.0^{a}	17.1±1.7^{bc}	14.7±0.6^{c}	**	n.s.	*
Σ n-3 PUFA[4]	33.9±0.6	34.7±1.1	25.5±2.3^{ab}	27.1±1.5^{a}	22.9±1.6^{bc}	20.3±0.4^{c}	**	n.s.	*
n-3 LC-PUFA[5]	32.6±0.6	33.1±1.1	24.0±2.4^{a}	25.8±1.5^{a}	21.4±1.9^{ab}	18.5±0.5^{b}	**	n.s.	*
DHA+EPA	30.9±0.5	31.3±1.1	22.6±2.4^{a}	24.5±1.5^{a}	20.2±1.8^{ab}	17.4±0.5^{b}	**	n.s.	*
DHA/EPA	4.7±0.2	4.7±0.1	4.5±0.3^{b}	5.6±0.5^{a}	5.6±0.6^{a}	5.5±0.4^{a}	n.s.	n.s.	n.s.
n-3:n-6	3.5±0.0	3.5±0.1	3.1±0.3^{a}	2.9±0.1^{a}	3.0±0.3^{a}	1.8±0.1^{b}	**	**	**

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). The mark § within a row denotes significant difference between groups at the end of marine phase determined by Student's t-test (p<0.05).

SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid;

STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. * p<0.05. ** p<0.01.

[1] Contains 15:0, 16:0, 18:0, 20:0, 22:0 and 24:0.

[2] Contains 16:1n−9, 18:1n−9, 20:1n-11, 20:1n-9, 22:1n-11, 22:1n-9 and 24:1n−9.

[3] Contains 18:3n-6, 20:2n−6, and 22:4n−6.

[4] Contains 18:4n-3, 20:3n-3, 20:4n-3 and 21:5n-3.

[5] n-3 LC-PUFA, the sum of 20:4n-3, 20:5n-3 22:5n-3 and 22:6n-3.

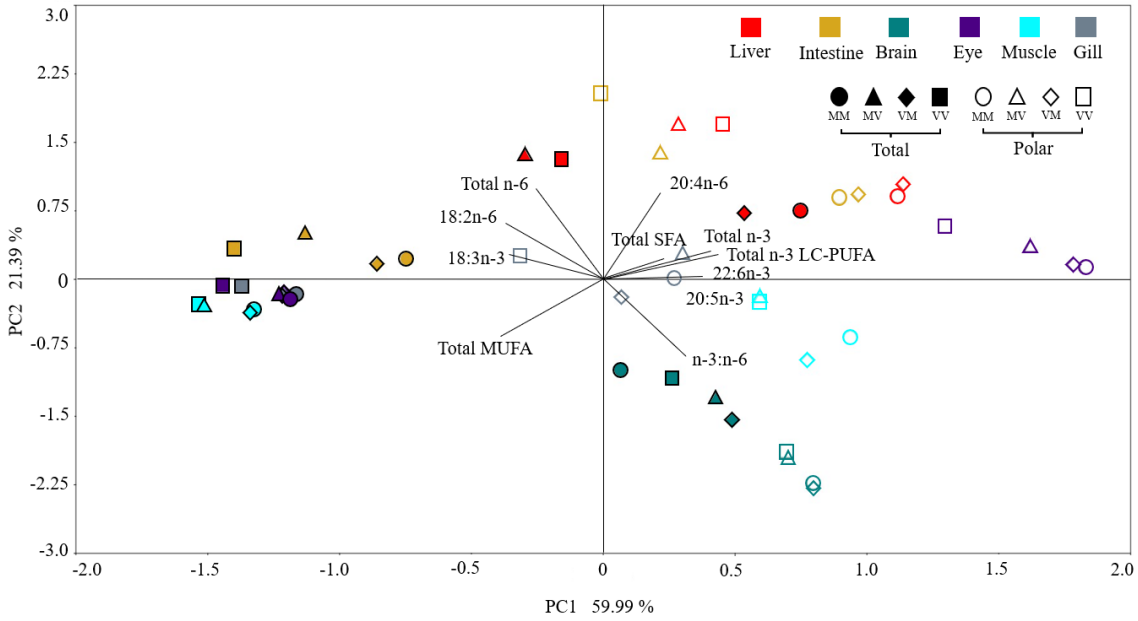


Figure 5.3 Principal component analysis (PCA) plot based on logarithmically transformed fatty acid composition of total lipid and polar lipid from six tissues in salmon after the challenge phase.

5.4.5 Flesh lipid quality

Several indices of muscle quality were assessed at the end of both marine and challenge stages. At the marine phase similar AI level were found in salmon fillets from two groups, whereas a higher TI was observed in the V group. Additionally, M group showed a higher PI (Table 5.10).

While at the end of the challenge phase, the differences showed the opposite trend. The value of TI and PI did not differ among the four groups, whereas the AI value was in M-challenged group than V-challenged group (Table 5.10).

Table 5.10 The lipid quality indices of Atlantic salmon muscle from different groups at two phases.

	M-M	M-V	V-M	V-V	*P*		
End of marine phase					**STI**	**CHA**	**INT**
AI	0.37±0.01 (M)		0.38±0.01 (V)		/	/	/
TI	0.20±0.00 (M)		0.21±0.00 (V) §		/	/	/
PI	157.11±5.73 (M) §		150.44±4.42 (V)		/	/	/
End of challenge phase							
AI	0.39±0.02^{a}	0.37±0.01^{ab}	0.39±0.01^{a}	0.34±0.02^{b}	n.s.	∗	n.s.
TI	0.21±0.01	0.21±0.01	0.21±0.00	0.20±0.01	n.s.	n.s.	n.s.
PI	144.87±8.71	144.59±10.5	145.33±0.97	137.87±6.44	n.s.	n.s.	n.s.

Data are means of triplicate measurement with standard deviations (n=3). Different superscript letters within a row denote significant differences among groups at the end of the challenge phase determined by one-way ANOVA with Tukey's comparison test (p<0.05). The mark **§** within a row denote significant difference between groups at the end of marine phase determined by Student's t-test (p<0.05).

AI: Atherogenic index; TI: Thrombogenic index; PI: Peroxidation index; STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. ∗ p<0.05. ∗∗ p<0.01.

5.4.6 Liver gene expression

No differences were observed in most of the evaluated genes in the liver before the challenge (Figure 5.4). The exception to this was *fas*, which was up-regulated in the M group (Figure 5.4 k).

After the challenge phase, Figure 5.5 shows an overview of the varied regulations of gene expression in different groups. In this sense, for genes involved in LC-PUFA biosynthesis, both desaturases were highly expressed in the V-challenged group, specifically VV showed the highest expression, indicating that challenge had a significant influence on the desaturases. Additionally, in *fads2d5* the factor stimulus also led to differences, as well as an interaction of stimulus and challenge (Figure 5.4 a, b). The higher expression of *elovl5b* in VV could be explained by the stimulus effect (p>0.05) (Figure 5.4 e). VV group also showed the higher relative expression for *srebp1*, indicating the significant effect of both stimulus and challenge (Figure 5.4 f). The higher expression of *lxr* and

pparα in V-stimulus groups was due to the stimulus (Figure 5.4 h, i), whereas the interaction of stimulus and challenge explained differences observed in *pparγ* (Figure 5.4 j). Furthermore, the highest level of *fas* was observed in VV and the interaction had a major impact (Figure 5.4 k). As for *hmgcr*, *aco* and *cpt1*, both V-stimulus groups showed higher expression compared with the M-stimulus groups but were not statistically different with the exception of *aco*, on which stimulus had a significant effect (Figure 5.4 l-n).

5.4.7 Histology

After the long-term grow out marine phase, a significant higher percentage of intracytoplasmic lipid vacuolization in liver was observed in the V-stimulus group as compared with the M-stimulus group (Figure 5.6 a). No differences were observed in any of the other parameters evaluated at this stage.

Concerning the end of challenge, MV and VV exhibited a higher percentage of intracytoplasmic lipid vacuolization than M-challenged groups. The significant effect was confirmed by two-way ANOVA (Figure 5.6 a, f, g). Conversely, in intestinal tissue the thicker circular muscle in MM and VM was found due to the major influence of challenge (Figure 5.6 b). Results also suggested that both stimulus and challenge did not lead to any change to enterocytes (Figure 5.6 c, d). However, the results of goblet cell density followed the order VV = MV > VM = MM, indicating that both stimulus and challenge had major effect on goblet cell density among groups (Figure 5.6 e, h, i).

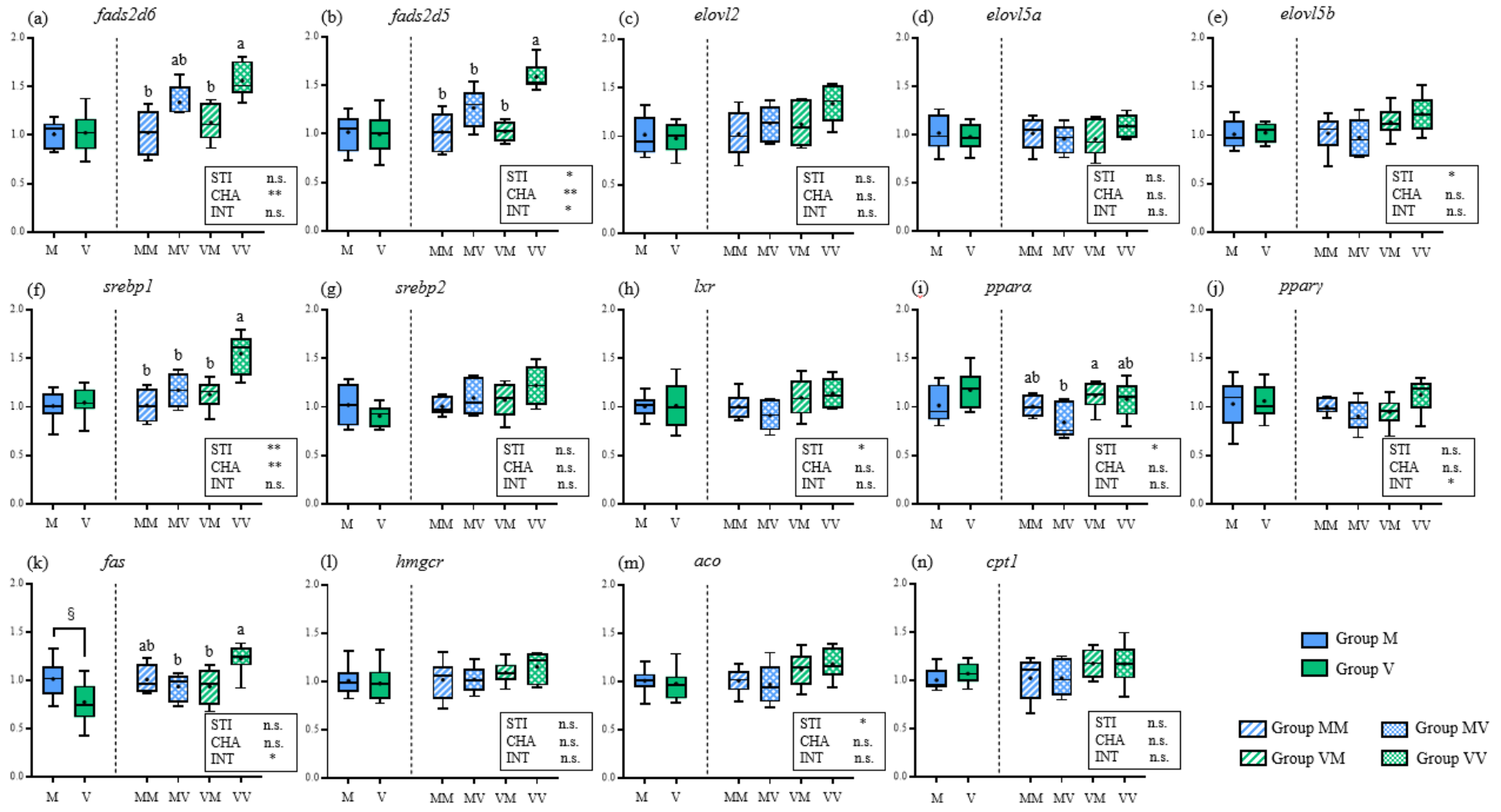


Figure 5.4 Relative expression of genes for long-chain PUFA biosynthesis (top), transcription factors (mid) and lipid metabolism (bottom) in liver of Atlantic salmon. Results are normalised expression ratios. Data are means of sextuplicate measurement (n=3), with standard deviation represented by vertical bars. The mark § and lowercase letters denotes significant difference between groups at the end of marine phase (left side of dotted line) and among the groups at the end of the challenge phase separately (right side of dotted line) (p<0.05). STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. ∗ p<0.05. ∗∗ p<0.01

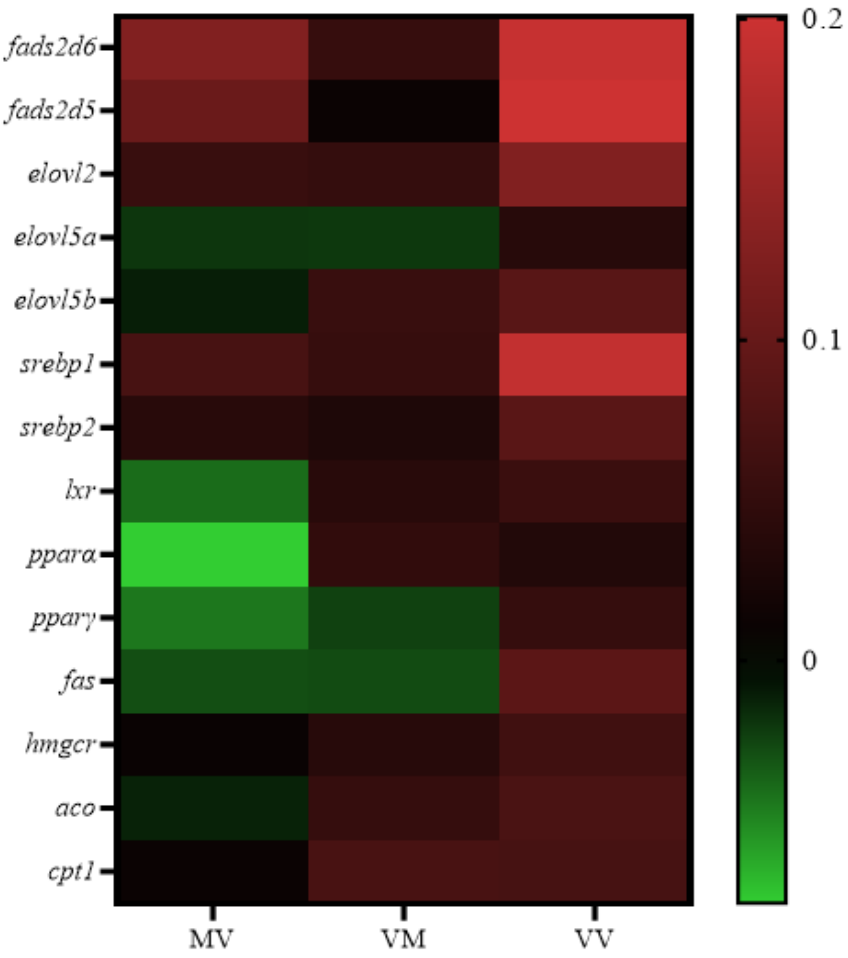


Figure 5.5 Heatmap of the fourteen target genes analysed based on qPCR gene data. MM was chosen as control group. Columns represent mean data values of the three different dietary groups analysed in the liver and rows represent single genes. Expression level of each gene was log10 transferred. Means are depicted by a colour scale, indicating low (green), neutral (black) or high (red) relative expression levels, as indicated by the colour bar on the right.

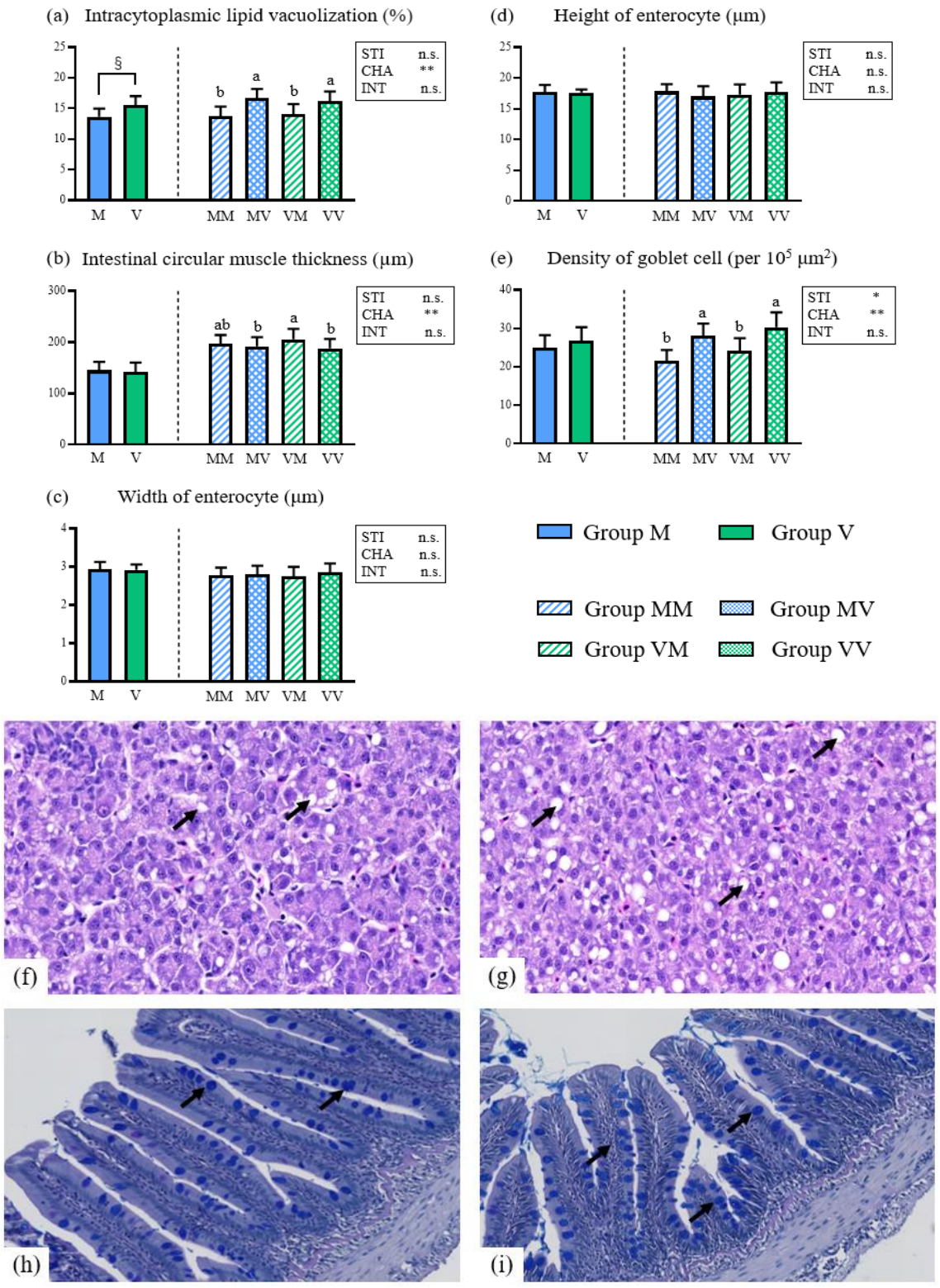


Figure 5.6 Histological analysis of liver and intestine in Atlantic salmon. Data are means of triplicate measurement (n=3), with standard deviation represented by vertical bars. The mark § and lowercase letters denotes significant difference between groups at the end of marine phase (left side of dotted line) and among the groups at the end of the challenge phase separately (right side of dotted line) (p<0.05). STI, stimulus; CHA, challenge; INT, Interaction (STI×CHA); n.s. not significant. ∗ p<0.05. ∗∗ p<0.01. Histological analysis of vacuolization and goblet cells in Atlantic salmon for different groups at the end of the challenge were shown in f-i. Among them, f and h showed the results from MM, while g and i showed the results from VV, respectively.

5.5 Discussion

From an aquaculture nutrition perspective, the expansion of the industry requires the use of alternative dietary sources with better availability to replace FO. However, its maximum utilization conflicts with the key role of n-3 LC-PUFA (especially EPA and DHA) as essential fatty acids from marine-derived aquafeed to carnivorous fish including salmon (Tocher *et al*., 2019). The root cause is the lack or insufficient ability to synthesize these fatty acids from their precursors, thereby constraining substitution (Nyunoya *et al*., 2021). In the present study, nutritional programming was introduced to improve the utilization of terrestrial-based diets. All the fry gained weight to 0.36 g regardless of the diet, suggesting that stimulus will not induce the phenotypic changes when a three-week stimulus is applied. Despite all this, the lower SGR of V than M (4.2 vs 4.5) still predicted the potential risk of a lower growth performance in V stimulated groups, similarly to what was observed in Clarkson *et al*. (2017). The current diet formulation so far could be considered as practical for a stimulus period up to three weeks. After eight months, the final fish weight did not show differences, which means all fish were able to accept both stimulus and commercial diet. As demonstrated recently, the lack of dietary n-3 LC-PUFA in rainbow trout from the high presence of plant-based ingredients could modify the olfactory sensing system and lead to poor feed intake and growth consequently (Heraud *et al*., 2022; Roy *et al*., 2020). The diet acceptance in this trial, supported by feed intake results (data not shown) in turn implied the well-developed feeding behaviour, thereby suggesting that the sufficient amount of n-3 LC-PUFA in the salmon fry from feed or, more possibly, endogenous production. It might be explained that at very early stages, the n-3 LC-PUFA biosynthesis was higher than adult stages as its capacity and plasticity was life-stage related (Gillard *et al*., 2018), this would be the critical window for the early nutritional intervention. Nonetheless, at the end of the challenge phase, both M-challenged groups had higher body weight and SGR potentially indicating that fish did not accustom themselves to the dietary change in such a short period. However, when comparing MM to VM and MV to VV, the effect of early programming was highlighted by the higher growth in groups experiencing stimulus although this was not statistically

significant (p=0.06). Similar results were previously reported in rainbow trout arguing that the early plant-based diet promised a better growth when challenged after seven months (Geurden *et al*., 2013). In the present trial, salmon was shown to be able to "recognize" their first feeding diet after eight months, which was consistent with the finding that the adaptions through epigenetics are long-lasting (Kaliman, 2019). Potentially, significant difference in growth might appear if the challenge phase was extended.

Applying dietary stimulus to the salmon fry involves the change of the whole fish fatty acid composition. The general known idea is that fatty acid composition, to a large extent, reflects the dietary fatty acid profile (Fernandez-Jover *et al*., 2011). The differences found in whole fish after stimulus between the two experimental groups confirmed that idea by the presence of higher MUFA, LA and ALA amount in V-stimulus group since these fatty acids are regarded as terrestrial source feature (Turchini *et al*., 2010). Noticeably, 20:3n-6, ARA and 22:5n-6 were not present in the V diet, but their amounts were higher in the V group after stimulus. Although n-3 LC-PUFA content in group V was still significantly lower, it cannot be ignored that the supplies of n-3 LC-PUFA in two diets were 24:1 (M:V), but its content in whole fish from both groups were 31:14 (M:V). With the minor supply, the decent n-3 LC-PUFA value in whole fish from group V suggested its effective endogenous production. In addition, it was previously demonstrated that salmon will modify the LC-PUFA biosynthetic genes in response to the dietary LC-PUFA change (Tocher, 2010; Leaver *et al*., 2008b). Given the lower n-3 LC-PUFA supply in the diet of the V group, the content of EPA, 22:5n-3 as well as DHA suggested its upregulation of the biosynthetic genes and which may explain how the V group retained its biosynthetic ability as a long-term imprint by nutritional programming.

Both stimulus and challenge exerted various effects on several tissues from different respects. Regarding the liver lipid content, no statistical difference during the whole trial was observed among groups. However, the histological analysis evaluated the intracytoplasmic lipid vacuolization and revealed that this was higher in V group before

the challenge and remained higher in V-challenged rather than M-challenged fish at the end. Both findings could be explained by previous studies where the high levels of C18 fatty acids from the inclusion of vegetable oil could result in an increased lipid deposition in liver due to the enhanced synthesis and accumulation of lipids driven by the gene expression modifications. Its long-lasting effect was able to be detected even after the seven-month marine diet (Betancor *et al*., 2016b, 2017; Peng *et al*., 2014). This can be observed as well at the end of the present trial, the percentage of intracytoplasmic lipid vacuolization in V-challenged groups were still higher than M-challenged ones, irrespective of stimulus. In this sense, it can be concluded that the stimulus had minor effect on the percentage of vacuolization, but the challenge directly pronounced the impacts in terms of significance at the end. In liver, the fatty acid composition of TL reflected the diet condition. The differences in MUFA and n-3 LC-PUFA from the stimulus diet persisted after a long-term marine diet "wash out". In addition, only DHA was regulated by challenge and interaction when other fatty acids showed dietary characteristics by challenge alone. Interestingly, although the DHA value cannot be comparable to MM group, it was still notable that the similar DHA values observed in VM and VV groups indicated the better acceptance when facing similar suboptimal diet as a challenge. The lowest DHA value observed in the MV group confirmed the benefit that the experienced stimulus brought at an early stage. The PL performs a critical role in structural functions as well as in transportation and enzymes in vertebrate cell membrane (Sargent *et al*., 2002; Tocher *et al*., 2008). Fittingly, the high proportion of PL in the liver (~60 %) suggests the functional importance of this tissue. Therefore, both PL and TL showed similar fatty acid profiles as illustrated by PCA analysis. The fatty acid composition of PL was found to be altered by dietary fatty acids to purely meet the functional demands in tissues (Betancor *et al*., 2014a). Like TL, when challenge presented an overall effect on liver fatty acid composition, the less impact of DHA retention in PL with a moderate content in VV group compared to MV still suggested the benefit of nutritional programming. Because of the important role of DHA in physiological function for cell membrane (Morais *et al*., 2011a), this selective retention could be due to the elevated endogenous production that modified by the gene expression.

The expression of *fads2*, the rate-limiting enzyme in the first step of LC-PUFA biosynthesis restricts the ability to desaturate the precursors LA and ALA to n-6 and n-3 LC-PUFA, respectively (Turkmen *et al*.,2019). Knowing that the V diet inclusion is generally accompanied by molecular modifications of desaturases and elongases (González-Rovira *et al*., 2009; Torstensen and Tocher, 2010), the higher expression of *fads2d6* and *fads2d5* observed in V challenge groups and *elovl5b* in V stimulus groups was as expected and all the biosynthetic genes showed the highest expression in the VV group, although not all were significant. Additionally, the higher content of the intermediary product 20:3n-6 and ARA may be related to this improved biosynthesis within the liver. The transcription factors especially *srebp1, lx*r and *pparα*, were significantly impacted by the stimulus diet, their higher expressions in V-stimulus groups could also imply the effects of early intervention on intermediary metabolism. Some studies showed that *srebp1* could be activated by *lxr* through the "*lxr-srebp1* pathway" and plays an essential role in fatty acid synthesis regulation (Horton *et al*.,2002; Lee *et al*., 2015). Their interaction is able to regulate transcriptional activation of the target genes *fads2d6* and *fads2d5* (Minghetti *et al*., 2011; Betancor *et al*., 2014b), which could be partially reflected by the similar pattern between *srebp1*, *fads2d5* and *fads2d6* in the present research and in accordance with a previous study (Zheng *et al*., 2005b). Because n-3 LC-PUFA content, mainly DHA and EPA, downregulates the expression of some lipogenic genes including *srebp1* as well as its target genes *fads2d5* and *fads2d6* (Minghetti *et al*., 2011; Datsomor *et al*., 2019), it is highly likely that their higher expression corresponds to the lower n-3 LC-PUFA levels in MV and VV. Besides, as another target gene of *srebp1*, *fas* also displayed the highest expression in VV implying that the challenge after the early stimulus contributed to a better fatty acid biosynthesis. As shown by the heat map, most genes of interest were upregulated in VV. This seems to indicate that the terrestrial source programming and a future challenge with similar diet would be the best "combination" to boost the potential of n-3 LC-PUFA biosynthesis in liver. In fatty acid catabolism, *pparα* has proved to be critical in peroxisomal and mitochondrial β-oxidation by regulating the expression of its target genes *aco* and *cpt1*

(Yoon, 2009). In the present study, the stimulus had significant impact on the expression of *pparα*. Therefore, as a response, both *aco* and *cpt1* along with *pparα* showed higher expression in V-stimulus groups even though they were not significant. This indicated that the stimulus had potential to promote lipolysis when encountering the V diet again at a later timepoint. Although no significant differences were found (p=0.08), the higher *pparγ* expression in VV might be potentially associated with the high intracytoplasmic lipid vacuolization due to its role in lipid accumulation in adipose tissues (Walczak *et al*., 2002; Xu *et al*., 2018).

It was assumed that the lipid accumulation observed in the intestine was related to the tissue phospholipids (Olsen *et al*., 1999; Tocher *et al*., 2008). Investigations of linseed oil inclusion diet in Arctic char (*Salvelinus alpinus L*.) demonstrated that the lack of *de novo* phospholipid biosynthesis resulted in insufficient lipoprotein biosynthesis to transport absorbed lipids (Olsen *et al*., 2000). The only notable lipid accumulation among all groups was in VV which suggests that the plant-based diet intervention in both stimulus and challenge, as a combined effect, might fail to meet the fatty acid demand for lipoprotein biosynthesis. Indeed, palmitic acid (16:0) was reported to maintain the lipoprotein synthesis in PL, the insufficient supply of 16:0 will keep the lipids stored in enterocytes (Olsen *et al*., 2003). The V diet used in the stimulus and challenge phases contained lower 16:0 than M diet (12 % vs 20 % and 6 % vs 16 % respectively), as less marine source was included, should be taken into consideration. The histological analysis of intestinal morphology was adopted to assess the health, function condition and organ status through indicators such as the height and width of enterocyte or the muscular thickness (Swatson *et al*., 2002). Neither early stimulus nor later challenge induced damages towards the integrity of the intestinal structure as no differences were observed compared with MM. On the other hand, muscular thickness was mainly reduced in VV group, which seemingly indicates that nutrient absorption and digestion efficiency were negatively influenced (Yu *et al*., 2020). In agreement, shortened thickness of the villi was previously reported in Japanese seabass (*Lateolabrax japonicus*) when marine sources were replaced in diet (Zhang *et al*., 2018). Goblet cells are believed to secrete mucus as

a protective layer and act as an integral structural component in the intestine (Deplancke and Gaskins, 2001; Castro and Tafalla, 2015). The higher goblet cell density observed in V-challenged groups could be regarded as an inflammation sign, as the high replacement with terrestrial sources in diet could have inflammatory potential as found previously (Venold *et al*., 2012). The lowest content of n-3 LC-PUFA in both TL and PL was found in the V-challenged groups, this together with some anti-nutritional factors was speculated to be responsible for the inflammation in the present trial as reviewed by Teitelbaum and Walker, (2001). When comparing to the liver, although TL fatty acid composition was different, the similar composition in PL suggests their common function of PUFA biosynthesis (Tocher *et al*., 2006). However, early stimulus appears to be irrelevant.

Brain acts as an essential role for cognition, learning and consciousness in fish (Fernö *et al*., 2020), the importance may require the brain to be more conserved than other tissues for a stable status. At the end of the trial, unlike other tissues, fatty acid compositions among groups were hardly varied especially in PL. This minor influence suggested that the brain of salmon was impacted less by the external environment including nutrition. Moreover, the PCA illustrated the high similarity in fatty acid compositions between PL and TL in brain, being characterized by its high n-3:n-6 (Tocher and Harvie, 1988). This corresponded to the high PL content in brain lipid class analysis, which further proved its functional role rather than lipid storage. In addition, PCA analysis indicated that brain had a high amount of n-3 LC-PUFA in TL from all the groups. Indeed, as a neural tissue, brain is expected to be rich in n-3 LC-PUFA to meet the demands for function maintenance such as the formation of the structure of the cell membranes and visual development (Betancor *et al*., 2014a; Salem *et al*., 2001; Zhang *et al*., 2011). The selective uptake from diet and retention were reported previously even when fed a n-3 deficient diet for a short term (Moriguchi *et al*., 2001). Therefore, if the vegetable challenge diet was applied longer, some potential influence may probably appear in the brain fatty acid composition. Another neural tissue evaluated in the present study was the eye where, similar to brain, its fatty acid composition appeared less affected by dietary change. However, in contrasts with other tissues, the PCA clustered the TL and PL separately mainly because of the gap

of MUFA and DHA. The lipid class analysis of eye demonstrated that the TAG represented approximately 75 % of the TL. Therefore, the TL, which was assumably represented by TAG, reflected the dietary change, while the PL only mirrored the structure and functional aspect (Osmond *et al*., 2021). Indeed, in TL, DHA was impacted by the challenge and only accounted for about 8.5 %, whereas in PL the DHA content was approximately 28.5 % regardless of the effect from stimulus and challenge. This highlights the importance of DHA in the eye as it will be prioritized for optimizing the integrity of the retina and maintaining visual acuity (Osmond *et al*., 2021). However, being a neural tissue like brain, the higher level of TAG rather than PL in eye was somewhat unexpected as it is not known as a lipid storage organ. Apart from salmon, similar results were also observed in rainbow trout, turbot (*Scophthalmus maximus*) and redfish (*Sebastes marinus*) (Tocher and Harvie, 1988; Brodtkorb *et al*., 1997; Geurden *et al*., 1998; Stoknes *et al*., 2004). This might be related to the high energy consumption demand for visual development or function. To date, the reason behind this particular composition still requires further investigation.

The result of the PCA demonstrated that considering TL fatty acid composition, muscle and gill were clustered by a high similarity. Surprisingly, the high TAG in muscle usually reflects the dietary fatty acid composition, but the present research showed static DHA and other n-3 LC-PUFA content, indicating that these were not manipulated by diet, which inferred the selective retention of DHA as well in muscle. The differences between TL and PL in muscle were due to the different MUFA and DHA contents. High levels of MUFA could be explained by the high TAG content for the purpose of energy metabolism and storage (Miller *et al*., 2006), while endogenously produced DHA prefer to incorporate with membrane PL for the fluidity and selective metabolism (Morais *et al*., 2011a; Tocher *et al*., 2003). On the other hand, the indices evaluating the quality of muscle showed some benefit from nutritional programming. Lower marine sources and higher vegetable inclusion minimized the 16:0 and introduced more PUFA, which contributed to the lower risk of atherogenicity and peroxidation in the muscle, especially in the VV group.

Unlike muscle, instead of being structurally orientated for lipid energy storage, the gills are the major site of oxygen exchange and the osmoregulatory organ in teleost and act as a boundary of internal and external environment (Sellner and Hazel, 1982; Bin *et al*., 2015). Even though a higher proportion of TAG (~60 %) was observed in gill TL while PL only accounted for 13 %, a greater number of differences in PL fatty acid profiles, particularly n-3, among groups were observed. In the present study, fish experiencing the early V stimulus turned out to have lower n-3 LC-PUFA in PL, but earlier research in sea bream showed that diets lacking in n-3 LC-PUFA was not reflected in gill PL but rather the neutral lipid, suggesting that this conservation strategy could maintain membrane structure and function (Martín *et al*., 2011). This inconsistency between studies might be due to the species-specificity and early dietary stimulus. The salmon study suggests that the ARA:EPA in gill PL will be affected by dietary composition for its potential physiological change, the ratio was also assumed to alter eicosanoid metabolism such as the production of prostaglandin for better electrolyte balance and environmental adaptions like smoltification (Bell *et al*., 1997; Tocher *et al*., 2000; Van Praag *et al*., 1987). Therefore, the higher ratio might be beneficial for the later seawater phase (challenge phase sampling was followed by transfer of the fish to marine water). The present study showed a stable content of ARA in the gill but EPA fluctuated among groups, which implied the higher priority of ARA and highlighted the improved ability for future adaption after early V stimulus, especially in the VV group. However, the detailed insight of the mechanism remains unclear at present.

5.6 Conclusions

When facing later vegetable challenge, the salmon in VV group with the application of an early vegetable source stimulus was found to have the similar growth with MM. Applying a stimulus had marginal effect on the fatty acid composition of tissues, whereas the following vegetable-based challenge in VV group resulted in some adverse effects such as lower n-3 LC-PUFA content and higher intracytoplasmic lipid vacuolization in liver. The biosynthesis of n-3 LC-PUFA in liver was elevated by both V stimulus and V challenge. In addition, the better fillet lipid quality was available and the better adaption of gill for the future smoltification could be expected. Meanwhile, the inflammation status in intestinal tissues and possible alterations in the normal functioning of the brain should be considered if the challenge is to persist for longer. In summary, the application of nutritional programming and a vegetable-based challenge as a booster could be considered as a sustainable strategy, some adverse impacts and challenge should be further investigated and refined in order to improve the utilization of terrestrial ingredients.

6 CHAPTER

General Discussion

1. Can Atlantic salmon fry produce LC-PUFA from first feeding?

As the important nutrients for the development in fish, the n-6 and n-3 LC-PUFA, namely ARA, EPA and DHA, provide health benefits through cellular and physiological aspects as well as ensure the functions of tissues considered essential (Tocher *et al*., 2019). Although some species are able to produce n-3 LC-PUFA endogenously, this biosynthesis is generally inadequate to satisfy the demands. Thus, traditionally n-3 LC-PUFA were highly provided by fishmeal and fish oil as the basic ingredient in aquafeeds. Atlantic salmon are known to possess the full set of genes and enzymes required for the endogenous biosynthesis of n-3 and n-6 LC-PUFA from their precursors ALA and LA (Monroig *et al*., 2011a; Castro *et al*., 2016). Indeed, previous reports also have shown that the endogenous production of n-3 LC-PUFA could be achieved in juvenile salmon (Mock *et al*., 2019). Nonetheless, whether salmon is able to produce LC-PUFA from first feeding and how much LC-PUFA can be synthesized are questioned.

The present thesis quantitatively explored the endogenous production of LC-PUFA in salmon fry by feeding FO and FM-free diets from first feeding (Chapter 3), when dietary ALA and LA levels are far above the reported requirement levels to satisfy the EFA demand. Being precursors for LC-PUFA biosynthesis, ALA and LA ratio had significant impact on the LC-PUFA production. On a per fish basis, endogenous production of n-3 LC-PUFA was at least 5.9, 4.4 and 2.8 mg/g fish, and that of n-6 LC-PUFA was at least 0.2, 0.5 and 1.4 mg/g fish in salmon fed diets with ALA:LA ratios of 3:1, 1:1 and 1:3, respectively. There are more interpretations to these data. As the diets contained essentially no LC-PUFA, so all the LC-PUFAs detected in fish were believed to be obtained from endogenous biosynthesis. The actual productions were supposed to be higher than the present data owing to the oxidation and utilisation of the produced LC-PUFA as demonstrated previously (Turchini *et al*., 2011; Emery *et al*., 2016). In addition to this, the salmon in the present trial was at fry or parr stages, which these results can reflect only. It has been previously reported that the ability to biosynthesize LC-PUFA could vary by life stage and a higher level of hepatic LC-PUFA biosynthesis was found

during the parr-smolt transformation (Bell *et al*., 1997; Tocher *et al*., 2000). Obviously the higher *fads2* and *elovl2* expressions in the experimental groups than control group suggested the enhanced LC-PUFA biosynthesis ability.

Apart from the direct evidence, in Chapter 4, the salmon fry fed a plant-based diet for one week had similar n-3 LC-PUFA content to the fry fed a diet rich in marine ingredient after early stimulus, which also implied the endogenous biosynthesis of LC-PUFA. Furthermore, the content of 22:5n-3 could be used as an indicator because it is an intermediate product of DHA biosynthesis and its presence indicates the existence of endogenous biosynthesis. However, comparing to two-week stimulus, one more week of marine diet in one-week stimulus group should be fully taken into account for its higher n-3 LC-PUFA content. Furthermore, it would be of potential interest to explore other methods that reveal or calculate the fatty acid biosynthesis pathways such as radioisotopic labelling, calculation of the gap between total fatty acid intake and final whole body, or whole-body mass balance (Ghioni *et al*., 1999; Stubhaug *et al*., 2007; Turchini *et al*., 2007). However, these methods are either expensive, not commonly used, dependent upon operator competence, or inaccurate. Therefore, the present trial provides an alternative solution for accurately determining the biosynthesis of n-3 LC-PUFA.

2. How does the dietary n-3 and n-6 ratio impact the n-3 LC-PUFA biosynthesis in salmon fry?

Atlantic salmon is the ideal dietary source of n-3 LC-PUFA for human consumers (Sprague *et al*., 2016). However, limited marine sources from fisheries has led to the increasing replacement of FM and FO in salmon aquafeeds by terrestrial plant sources, which lack n-3 LC-PUFA but are generally abundant in n-6 short chain PUFA. Consequently, the lower dietary n-3 LC-PUFA reduces the accumulation of these nutrients in salmon. Another factor that matters is the altered n-3:n-6 ratio as it is believed that n-3:n-6 ratio plays a critical role in inflammatory responses and lipid metabolism regulation including n-3 LC-PUFA biosynthesis (Tocher *et al*., 2015). Some studies have

demonstrated the impacts of increasing dietary n-6 fatty acid on the n-3 LC-PUFA content in salmon (Sissener *et al*., 2020; Hundal *et al*., 2021). In the present thesis, it has been proved that the plant-based diets with ALA and LA in different ratios significantly affect the n-3 and n-6 LC-PUFA production and their ratio (Chapter 3). In this sense, when the ratio decreased from 3:1 to 1:3, the production of n-3 and n-6 LC-PUFA was reduced from 5.9 to 2.8 mg/g fish and increased from 0.2 to 1.4 mg/g fish in wet weight, respectively. Accordingly, the ratio of the endogenous biosynthesis of n-3:n-6 LC-PUFA was markedly reduced from 27.4 to 2.0. Indeed, ALA and LA, compete for the same enzymes for the biosynthesis of EPA, DHA and ARA (Norambuena *et al*., 2015). Therefore, it would be expected that a decrease in dietary ALA: LA ratio would lead to a lower n-3 LC-PUFA production, as observed in Chapter 3.

Apart from the overall n-3 LC-PUFA production, the dietary n-3:n-6 ratio also impacts the ratio of EPA and DHA production. In Chapter 3, fish received no external source of EPA and DHA. This triggered endogenous biosynthesis which may reflect the beneficial and natural ratio of both LC-PUFA, what in turn can provide a deeper insight of the optimal dietary ratio of DHA: EPA as it is not fully understood in salmon (Emery *et al*., 2016). However, quantitative research revealed that DHA is higher than EPA indicating its importance in membrane and neural function (Farooqui, 2009). Nonetheless, this value is not fixed but can be impacted by dietary n-3:n-6 ratio as well. The results observed in Chapter 3, that DHA: EPA ratio had a negative correlation to the dietary n-3:n-6 ratio, were consistent with previous studies in rainbow trout and rabbitfish (*Siganus canaliculatus*) (Thanuthong *et al*., 2011; Xie *et al*., 2018). The explanation perhaps is due to the high conservation or selective retention of DHA because of its forementioned critical role. On the other hand, when high dietary levels of LA are supplied, there is an increased production of n-6 LC-PUFA by Δ6 desaturases what reduces further production of EPA and DHA from ALA (Emery *et al*., 2016). In agreement, in Chapter 3, when the n-3:n-6 decreased, DHA levels in fish was maintained whereas EPA levels were reduced, which consequently led to an increased DHA: EPA ratio.

3. What is the preferable stimulus duration of plant-based diet for nutritional programming in Atlantic salmon?

Nutrients are believed to act as conditioning stimuli during certain critical developmental windows and induce permanent genetic changes in metabolism and physiology (Lucas, 1998). Not only the type of stimulus, but its duration has been proved critical to induce early programming in marine teleost (Izquierdo *et al*., 2015; Turkmen *et al*., 2019a). In Atlantic salmon, nutritional programming has been proved to be effective through a three-week stimulus from first feeding, but some undesirable phenotypic differences after stimulus were observed, namely reduced growth (Clarkson *et al*., 2017). Since the stimulus duration was speculated to be the major factor, the present thesis compared two shorter durations (one-week vs. two-weeks, Chapter 4) to evaluate whether the duration can be shortened while eliciting nutritional programming without impacting on fish growth. The obtained results showed that shorter stimulus did not impact fish growth performance at the end of the stimulus phase (data not shown). However, the fish weight was higher in the one-week stimulus group after the marine phase although this difference disappeared at the end of the trial. Similar results were reported in gilthead sea bream where 14 days of plant-based diet from first feeding compromised the growth at the end of trial while 10 days of stimulus was proved to be tolerable (Perera and Yúfera, 2017). Another series of research on early carbohydrate programming in Nile tilapia (*Oreochromis niloticus*) demonstrated that low-protein/high-carbohydrate stimulus for four weeks significantly compromised the growth performance (Kumkhong *et al*., 2020). The following study using one week stimulus proved that the shortened duration as one week was sufficient to induce the hyperglucidic programing but without growth reduction (Srisakultiew *et al*., 2022).

Another concerning alteration found due to the different stimulus durations was the fatty acid composition. In the present thesis, the fish that received two-week (Chapter 4) or three-week (Chapter 5) stimulus presented significant lower ARA, EPA and DHA content

as well as n-3:n-6 ratio compared to fish fed commercial diet during stimulus, while their decline in fish experienced one-week stimulus were milder. In this sense, even though the compensation of the LC-PUFA production occurred afterwards, stimulus duration over two weeks was still assumed to induce the phenotypic change in fatty acid composition, whereas one-week stimulus impacted less. Therefore, from both a growth and a fatty acid perspective, it was further hypothesized that one-week stimulus could be a more acceptable duration if the programming was elicited.

The qPCR analysis in the present thesis (Chapter 4) showed a constant upregulation of *fads* and *elovl* in liver of one-week stimulus fish at the end of the challenge phase, which was in agreement with previous studies where fish are fed low dietary contents of n-3 LC-PUFA (Tocher *et al*., 2002; Izquierdo *et al*., 2008). In the other hand, the expression level of most transcription factors (except *pparγ*) between marine control diet and one-week stimulus groups were close at the end of the challenge phase, which implied that the one week of stimulus suffices to generate a long-term response to improve the n-3 LC-PUFA biosynthesis when challenge presented. As for two-week stimulus, the lowest expression of genes involved in LC-PUFA biosynthesis, transcription factors and lipid metabolism at the end of trial suggested that two weeks of stimulus might not be beneficial for the endogenous production of n-3 LC-PUFA, but the fatty acid composition in tissues have not displayed any significant phenotypic difference yet. Again, one more week of the marine diet for one-week stimulus should not be ignored.

To sum up, according to the results mentioned, fish did not show negative growth after one-week stimulus phase and have slight benefit on growth performance afterwards. However, the fatty acid data after stimulus was not convincing enough to judge whether the programming was properly elicited. In addition, the discrete fatty acid composition change in tissues by challenge cannot confirm the effect of stimulus durations. Further molecular analysis after challenge only proved the positive effects in one-week stimulus groups at transcriptional level (Chapter 4). Taking all these results together, it seems that one week stimulus might work better as the key metabolic processes, such as LC-PUFA

biosynthesis and lipid metabolism, were not altered. However, to ensure the presence of programming effects for further investigations, three-week stimulus was still chosen from previous results (Clarkson *et al*., 2017) for the long-term research (Chapter 5).

4. Does nutritional programming affect the performance of salmon in a long-term perspective?

Conducting a long-term trial could provide empirical evidence about impact and application of nutritional programming. In Chapter 5, three experimental groups fed a plant-based diet either during early stage (VM) or later challenge (MV) or both (VV) were compared to the control group fed a marine diet full-time (MM). Therefore, through comparison, the effects of the early nutritional intervention were explored. Additionally, whether a challenge with similar suboptimal diet was required as a booster for LC-PUFA production was evaluated. After the three-week stimulus stage, all the fish showed similar weight, suggesting that the early plant-based diet did not compromise growth in the present study. This contravenes the results obtained by Clarkson *et al*. (2017) using three weeks as the stimulus period, where higher growth in fish fed a marine-based diet during the stimulus. However, fish fed the stimulus diet displayed much lower levels of EPA and DHA in whole fish than those fed the marine-based diets after stimulus in the present study, which is fully expected.

At the end of the trial, the individual effects of the early stimulus and challenge as a booster could be identified. In the present thesis, under the same challenge background, plant-stimulus groups always had the higher body weight even though the difference was not statistically significant. However, among all the groups, the lower SGR was always observed in the challenged groups. The results indicated the greater influence of challenge than stimulus on growth performance what could be due to the sudden dietary change meaning that fish could not adapt in such a short period.

The molecular analysis confirmed that the expressions of most genes before challenge

were similar among groups. At the end of the trial, it was observed that *fads2d6* and *fads2d5* shared a similar trend: V-challenged groups (MV, VV) always showed higher expression than the M-challenged one (MM, VM), especially significant if the fish experienced stimulus (VV). The challenge-caused differences in expression could be explained as a response to the reduced dietary EPA and DHA levels, which was in agreement with previous studies in salmon (Monroig *et al*., 2010a). Whereas, the stimulus-evoked difference in expression was highly likely a consequence of epigenome modification. As previously reported, LC-PUFA supplementation during early developmental stages can impact metabolism by epigenetic mechanisms such as DNA methylation (Lillycrop and Burdge, 2018). In this sense, research in gilthead sea bream demonstrated that parental diet had a significant effect on CpG2 and CpG3 methylation (Turkmen *et al*., 2019b). However, although parental feeding with a diet deficient in n-3 LC-PUFA resulted in a higher methylation level, no difference in *fads2* mRNA levels was found (Turkmen *et al*., 2019b). Although the *elovl* series did not show any statistical difference, it still can be found that when fish received both stimulus and challenge, the genes encoding for the *fads* and *elovl* were higher expressed insignificantly.

Even though *srebp* is the target gene of *lxr*, they did not display exactly the same results. On the other hand, in the present thesis, *fads* and *srebp1* expression levels showed the same trend, supporting the previous finding that *fads* is a target gene of *srebp1* (Matsuzaka *et al*., 2002). According to the heat map, most of the studied genes were up-regulated in fish from VV group (albeit not significantly for every gene) which suggested the potential of nutritional programming to regulate lipid and fatty acid metabolism.

5. Do lipid deposition and lipid class in tissues get impacted by nutritional programming?

Different tissues (liver, intestine, brain, eye, muscle and gill) were examined in the present thesis. The comparison of lipid content in tissues was conducted in two dimensions in Chapter 4. Firstly, after challenge the lipid content in intestine and muscle was

significantly increased, indicating that low dietary n-3 LC-PUFA, as found in the challenge diet, could lead to the rapid fat deposition in these tissues as previously observed in several teleost species (Caballero *et al*., 2003; Tocher, 2003). Secondly, for each timepoint, the present data could not fully confirm the relevance between different stimulus durations and lipid deposition in tissues as no significant difference among groups was detected. However, the long-term trial (Chapter 5) showed different results: muscle had no reaction to the dietary change but fish which experienced V-stimulus and V-challenge had significant higher lipid content in intestine after the plant-based challenge, which was quite surprising. Notably, as indicated by histological analysis, higher hepatic vacuolization was found in V-challenged groups at the end of the trial and the early V-stimulus group (Chapter 5). The reason behind this could be the epigenetic modification of lipid metabolism genes due to the early exposure to the suboptimal diet. This hypothesis is reinforced by the positive correlation between vacuolisation and stimulus duration observed in Chapter 4. Interestingly, in Chapter 5, the slightly higher expression of *pparα* was observed after both marine and challenge phases in plant-based stimulus groups, indicating the effect of early stimulus. It has been proved that *pparα* is critical in β-oxidation by regulating the expression of its target genes *aco* for peroxisomal and *cpt1* for mitochondrial process during fatty acid catabolism (Yoon, 2009). Therefore, the higher *cpt1* (albeit not significant), *aco* and *pparα* in fish from the stimulus group could indicate the potential for better lipolysis when encountering the suboptimal diet again later on as a challenge. Thus, considering their roles in lipid metabolism, the comparable lipid content in liver and different hepatic vacuolization levels among groups from current analysis might be the comprehensive results. Although there were no differences in the hepatic lipid content, it is hypothesized that if the challenge phase lasts longer, differences might have arisen.

Lipid class composition is known to be tissue-specific based on their functions, such as PL is essential for membrane function, and TAG acts as the energy storage (Stoknes *et al*., 2004). Therefore, the lipid class composition was believed to be conserved and independent from external impacts. Indeed, the effects of programming on the lipid class

in the present thesis indicated that it was static regardless of the dietary background. Fish from all trials presented similar lipid class composition for each tissue, where PL predominated in liver and brain while other tissues had higher TAG (Chapters 4, 5). Although there were some statistical differences among groups in the long-term trial (Chapter 5), the proportions of TAG and PL did not indicate any logical evidence about the effect of nutritional programming. Similarly, the early stimulus duration was not proved to impact the tissues lipid class composition (Chapter 4).

6. What is the effect of nutritional programming on the n-3 LC-PUFA content in tissues?

In the present thesis, of particular interest was that the tissue fatty acid composition in was significantly affected by the dietary history. At the end of the long-term trial (Chapter 5), fish fed only marine-based diet (MM) had the highest liver n-3 LC-PUFA content, as expected. Although the fish fed a plant-based stimulus (VM, VV) did not reach the same level, they contained the second-best DHA content, superior to that of fish challenged but not early stimulated (MV) by the plant-based diet, indicating production of these FA when the challenge diet was provided. However, unlike DHA, fish given the vegetable diet during both stimulus and challenge phases (VV) were not able to maintain the levels of EPA in liver. This significant decline in EPA could indicate the active elongation of this fatty acid to produce DHA, which is supported by the higher DPA contents in VV. Both aspects contributed to the higher DHA:EPA in VV group. Indeed, Chapter 3 demonstrated that the ability to biosynthesize EPA and DHA in salmon was differently affected with a preferential maintenance for DHA when dietary n-3:n-6 ratio was decreased from first feeding. This might be the reason why, in Chapter 4, an increased DHA:EPA ratio was observed after the plant-based challenge. Together this might explain why salmon fed a vegetable-based diet during stimulus had a better DHA biosynthesis capacity when a challenge was applied later on. On the other hand, fish fed a plant-based diet during stimulus and challenge phases (VV) displayed the lowest n-3 LC-PUFA content in intestine, indicating the ineffective biosynthesis or retention of n-3 LC-PUFA by

nutritional programming and this might be further correlated to the higher lipid content and n-3:n-6 imbalance (Chapter 5). Brain and eye are related to neurological function and visual acuity, and it is known that an adequate supply of n-3 LC-PUFA is required for their development in vertebrates (Osmond *et al*., 2021). Consequently, their lipid classes and fatty acid compositions were proposed to be more conserved and resistant to dietary change in order to maintain their normal functions. Nonetheless, when no dietary n-3 LC-PUFA was supplied, the endogenous production of DHA was still considerable especially in brain regardless of the group, so nutritional programming was not expected to influence brain fatty acid composition (Chapters 3, 5).

Another interesting finding from Chapter 3 is that all the ARA, EPA and DHA endogenously produced were preferentially incorporated into membrane PL rather than TAG in whole fish. This was also observed in tissues, as the content of these fatty acids were higher in PL than TL. Correspondingly, the diets in the challenge phase also had an impact on the fatty acid composition in PL. An exception were brain and eye which were not affected by dietary change, whereas in most tissues the n-3 LC-PUFA content in challenged groups had the significantly lower value (Chapter 4, 5). Remarkably, the high n-3 LC-PUFA content in TL and PL of muscle did not show any significant decline by nutritional programming and later challenge like other tissues, which could support the feasibility of nutritional programming technique on large-scale salmon farming (Chapter 5). On the other hand, different stimulus durations did not have any impact on the tissue fatty acid composition either (Chapter 4).

Additionally, more attention should be paid to the nutritional value of fish flesh. Several recommendations exist about the optimal intake dose of EPA+DHA required to promote cardiac health and prevent cancer or neural dysplasia, ranging from, 250 to 500 mg/person/day (Aranceta and Pérez-Rodrigo, 2012; Richter *et al*., 2016). However, the global mean consumption of n-3 LC-PUFA is about 170 mg/person/day, which indicates that the present annual supply of n-3 LC-PUFA is unable to satisfy all the human requirements (Micha *et al*., 2014; Tocher, 2015). The three experimental chapters from

the present thesis provided absolute amounts of n-3 LC-PUFA in salmon fillets in order to offer some information and guidance to satisfy the demand. Nevertheless, within the ENDOPUFA study (Chapter 3) the endogenous content of n-3 LC-PUFA was lower with the increasing inclusion of LA, whereas nutritional programming with a longer plant-based stimulus may slightly induce a lower n-3 LC-PUFA deposition (Chapter 4). Although these amounts per unit were still lower than the recommended intake levels, what should be considered is that the sampled fish were still below harvest size, being ~20g and ~35g in Chapter 3 and 4, respectively. The similar amount of n-3 LC-PUFA per unit in the MM and VV fed fish suggests that the potential strategy of nutritional programming can be considered as a practical application within aquaculture in contributing towards the development and supply of n-3 LC-PUFA for human consumers. Consequently, adequate levels of n-3 LC-PUFA in the flesh could be achieved by using less marine ingredients.

In summary, it can be speculated that higher ALA:LA of plant source oil inclusion will be more beneficial for n-3 LC-PUFA biosynthesis combined with a shorter stimulus period for early programming. Furthermore, the presence of a challenge diet will act as a promoter for comparable fillet nutritional values. Nevertheless, as since the fish studied in the present thesis were not of commercial size, the longer impact or different stimulus types of nutritional programming needs to be studied in depth.

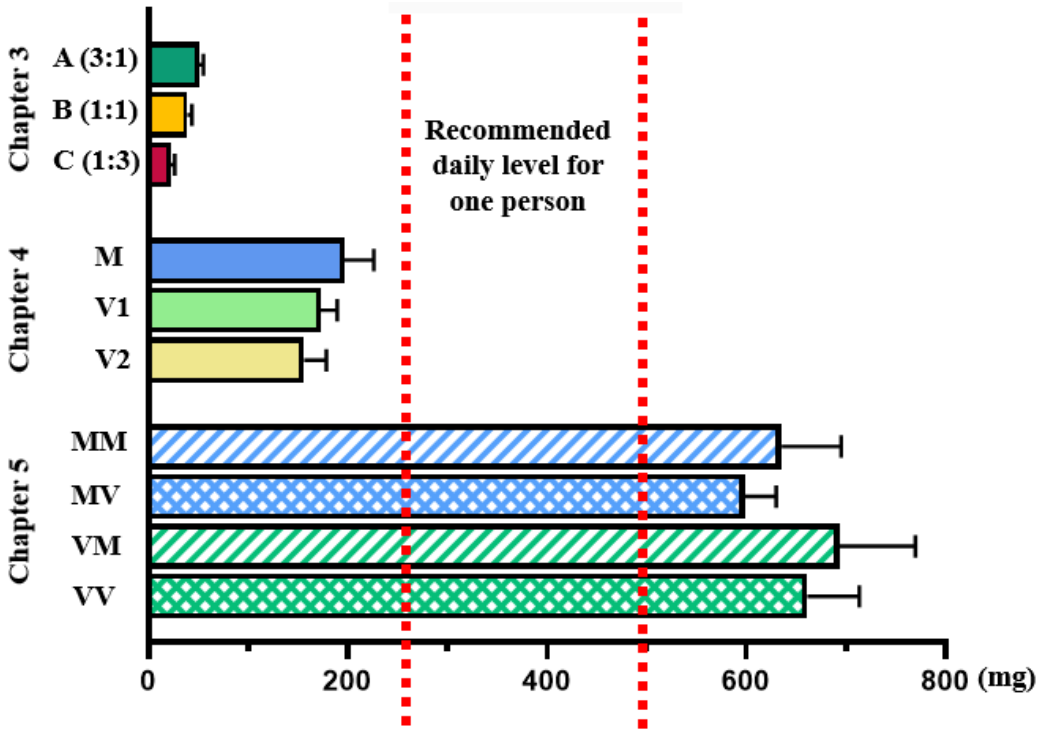


Figure 6.1 The absolute amount of n-3 LC-PUFA (mg) in different fish fillets analysed from three chapters within the present thesis. The area between red lines donates the recommended daily intake of n-3 LC-PUFA for one adult.

Future prospects

Findings presented in this thesis confirm that the salmon is capable to endogenous biosynthesize LC-PUFA from first feeding and further highlight the interaction between production of LC-PUFA and ALA:LA ratios in diet from Chapter 3. However, feeding diets containing only ALA and LA but no EPA and DHA accompanied by other consequences such as excessive deposition in liver (Peng *et al*., 2014), morphological change in intestine (Torrecillas *et al*., 2017) and affect immunity (Montero *et al*., 2010). Accordingly, it will be interesting to recruit the analysis from these aspects in the future research. For example, the lipid content in liver as well as other tissues would be valuable, as it could help to better understand the lipid metabolism between different ALA:LA ratios together with the molecular and histological results. Differences were observed in hepatic intracytoplasmic vacuolization, similarly the dietary ALA:LA ratio might also

alter the intestinal muscular thickness and villi or enterocyte status. So, sampling of intestine for histological analysis may contribute to better focus on the intestine health. This inspires the investigation of the nutritional immunology as dietary fatty acid composition will influence immune function by changing the membrane of immune cell (Calder, 2006). The correlation between eicosanoid production and EPA:ARA ratio, the expression of PGE_2 synthesis related genes and the concentration of PGE_2 in plasma can be explored. Rather than production only, different dietary ALA:LA ratios and other PUFA related perspectives can be connected for more comprehensive understanding through these analyses. Moreover, an extension of the trail can be an improvement. Another period that swapping diets among the current groups or including another higher or lower ratio diet could be followed after 22 weeks, this would help to identify if the LC-PUFA biosynthesis ability can be further impacted. As concluded, fish size or stage will influence the endogenous production, therefore, some additional samplings at different timepoints could be further examined to determine and explain how endogenous biosynthesis varies to the salmon size by the regression analysis, etc. Another remarkable factor is the type of vegetable oil used in the present research. As mentioned, different vegetable oils have their own unique fatty acid compositions or features, the programming impact and outcome might presumably vary according to the oil types. So, other vegetable oils or alternative oil sources could be considered and introduced into the future trial for the comprehensive understanding. Besides, Fish oil restore is also a potential topic after the present trial, it will indicate the duration for each experimental group to restore the n-3 LC-PUFA content as control group when n-3 LC-PUFA biosynthesis ability are enhanced previously.

In this PhD project, analyses were oriented for an improvement, refinement and deeper exploration of nutritional programming as a strategy. The findings reported in Chapter 4 were focusing on the effects of different durations of early stimulus on growth, fatty acid composition and molecular aspects. As it was shown, not only the results emphasized on the plant-based challenge, but also the significant alterations by stimulus durations were noticed. However, different durations mainly showed the effects from the molecular

aspect instead of the biochemical fatty acid composition of tissues. As suggested by Clarkson *et al*. (2017), three weeks of stimulus might be too long in Atlantic salmon. So, a better physiological programming in the present thesis was supposed to be shorter. Taking into account that the adaptations were very likely to be subtle, the challenge phase set as 6 only weeks was possible to limit the discovery of expected outcomes. The current evaluation of the programming effects can only prove the availability after 6-week challenge, some further positive or negative responses might require longer exposure to appear. Presumably, a longer challenge phase would be an improvement of the design and more conclusive. However, considering the different stimulus types, grow-out durations and species, similar investigations were run with the different challenge durations (Srisakultiew *et al*., 2022; Turkmen *et al*., 2019a), the proper challenge duration remain to be investigated in the future.

Results from Chapter 5 further highlight the long-term effects of nutritional programming through an 8-month trial. Similar to Chapter 4, a short challenge may conceal the facts, especially some potential trends were able to be discovered. Therefore, a longer challenge was further suggested. For something different, the present analyses were restricted to hepatic molecular study, intestine is known as another active organ for LC-PUFA biosynthesis in rainbow trout (Tocher *et al*., 2004). Combined with liver, investigating the gene expression of LC-PUFA biosynthesis in intestine would provide a better picture of the programming on salmon. Besides, some genes related to fish health can be focused or supplemented as well in both Chapter 4 and 5 (Xu *et al*., 2021; Liu *et al*., 2022).

To date, the current research has focused on measuring the expression of genes of interest. This was expected to provide some information in demonstrating the how and why n-3 LC-PUFA contents are affected by nutritional programming. However, the levels of metabolites (LC-PUFA) were not directly related to the expression patterns of the evaluated genes, which raises the question as to whether there is post transcriptional regulation of these enzymes. Several studies have been performed *in vitro* regarding the characterization of salmon desaturases and elongases, showing that there was a strong

correlation between enzyme activity and expression (Zheng *et al*., 2005a). This appears to indicate that the activity might not be sufficient to produce significant levels of n-3 LC-PUFA. Therefore, it might be interesting to further research the transcription and translation process through enzyme activities studies.

In the present thesis, the nutritional programming trial only covered the freshwater phase. Whether the concept of nutritional programming could be further employed to the seawater phase remains unstudied. As previously reported, during seawater phases the environmental temperature change can modify the fatty acid composition of fish, especially membrane PL, and change the content of EPA and DHA. Thus, the seawater transfer will result in different nutritional demands, metabolic changes and physiological adaptations (Rosenlund *et al*., 2016). In addition, salinity has also been shown to affect both the composition of fatty acid and amino acids (Tocher *et al*., 1995; Duan *et al*., 2021). Currently no footprint can be found in the seawater research of nutritional programming area. To aim this, it would be attractive to explore the optimal stimulus diet, duration, challenge timing, interaction with freshwater phase, etc. The information collected from further trial will remarkably promote the nutritional programming application in salmon farming.

Known as the underlying mechanism, epigenetics research is a way to validate nutritional programming. The present thesis mainly focussed on the biochemical effects of programming on salmon juvenile, as well as some the molecular pathways involved. Nonetheless, what leads to the altered gene expression is yet to be investigated. In this regard, a next step will be to conduct epigenetics analysis. Reduced representation bisulfite sequencing (RRBS) has been employed in DNA methylation research. Bisulfite group can convert unmethylated cytosines into uracil residues while the methylated cytosines remained. With restriction enzymes digestion and sequencing, the DNA methylation pattern in high CpG content regions of the genome is able to be analysed (Harris *et al*., 2010). This effective tool reveals the modifications to the genome of fish through nutritional programming. It has been proved that in sea bream, methylation in the

promoter of *fads2* of the offspring was correlated with the parental *fads2* expression levels and type of the diet in broodstock (Turkmen *et al*., 2019b). In the future work, it would be interesting to include RRBS as it could provide more reliable and in-depth evidence of how salmon response to the dietary changes fundamentally. Thus, together with the obtained information from the present thesis, the nutritional programming in Atlantic salmon at first-feeding stage that tailor adult fish capable to use plant-based diets efficiently can be better refined and further investigated.

7 CHAPTER

General Conclusions

1. Salmon fed diets containing no n-3 LC-PUFA from the first feeding exhibited a significantly lower weight gain than those fed a commercial diet, while the ALA:LA ratio had no impact on growth.

2. When a dietary n-3:n-6 ratio of 1:1 was used, the production of n-3 LC-PUFA was at least 4.4 mg/g fish with a DHA:EPA ratio of 3.4:1, with the production of n-3 LC-PUFA exceeding that of n-6 LC-PUFA by almost 9-fold.

3. The decrease in the dietary n-3:n-6 ratio resulted in a reduction of n-3 LC-PUFA production, DHA:ARA and EPA:ARA, as well as an increase of n-6 LC-PUFA production and DHA:EPA.

4. An early plant-based intervention of one week did not impact the growth performance and help salmon adapt and better accept a sustainable diet. However, different durations had little or no effect on the fatty acid composition of both total and polar lipid in several tissues.

5. Salmon fed for two weeks with a terrestrial-based diet during stimulus displayed a downregulation of most LC-PUFA biosynthetic enzymes and transcription factors, indicating a better effect of one-week rather than two-week stimulus.

6. Compared to the marine control group, the combined plant-based stimulus and challenge group (VV) did not reduce salmon growth but resulted in a lower n-3 LC-PUFA content and higher intracytoplasmic lipid vacuolization in liver.

7. Applying challenge after stimulus elevated the expression n-3 LC-PUFA biosynthesis genes in liver, which suggests that a plant-based challenge could act as a booster for early programming effects.

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Presentations and Grants

Presentations

Institute of Aquaculture PhD Conference (April 2018, Stirling, UK)
Poster presentation title: Investigating the potential of nutritional programming to improve the utilization of sustainable feeds in Atlantic salmon (*Salmo salar* L.)

Institute of Aquaculture PhD Symposium (October 2019, Stirling, UK)
Oral and poster presentation title: Can Atlantic salmon (*Salmo salar* L.) produce long chain polyunsaturated fatty acids?

Young Lipid Scientist Award Presentations (June 2019, London, UK)
Oral presentation title: Can Atlantic salmon (*Salmo salar* L.) produce long chain polyunsaturated fatty acids? (Shortlisted)

Grants

Rank Prize Fund (July 2021)
Awards for final year PhD students in Nutrition

Publications

Sprague, M., **Gong, X**., Betancor, M.B., Olsen, R.E., Torrissen, O., Glencross, B.D. and Tocher, D.R., 2019. Endogenous production of n-3 long-chain PUFA from first feeding and the influence of dietary linoleic acid and the α-linolenic: linoleic ratio in Atlantic salmon (*Salmo salar*). British Journal of Nutrition, 122(10), pp.1091-1102.

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Betancor, M.B., Sprague, M., González-Silvera, D., Ortega, A., de la Gándara, F., **Gong, X**., Napier, J.A., Tocher, D.R. and Mourente, G., 2022. Oils Derived from GM Crops as Sustainable Solutions to the Supply of Long-Chain Omega-3 for On-Growing Atlantic Bluefin Tuna (*Thunnus thynnus* L.). Fishes, 7(6), p.366.