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High and low fat diets, leucine and DHA influence growth, gene expression and fatty acid composition of different tissues in Atlantic salmon (*Salmo salar L*)

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Master of Science in Aquaculture

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Abbreviations

ATP	Adenosine triphosphate
BCAA	Branched-chain amino acids
BHT	Butylated hydroxytolu-ene
cRNA	copy ribonucleic acid
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
DTT	Dithiothreitol
EPA	Eicosapentaenoic acid
FCR	Feed conversion rate
GC	Gas chromatography
HSI	Hepatosomatic index
HUFA	Highly unsaturated fatty acids
IUGR	Intrauterine growth-retarded
K factor	Condition factor
MUFA	Monounsaturated fatty acids
PCR	Polymerase chain reaction
PUFA	Polyunsaturated fatty acids
RNA	Ribonucleic acid
RPM	rounds per minute
SFA	Saturated fatty acids
SGR	Specific growth rate
TCA	Tricarboxylic acid cycle
TG	Triglycerides

Abstract (250-350 words)

The main goal of this master thesis project was to examine how protein to lipid ratio in the diets, and DHA and leucine in combination, interact and affect the energy utilization for growth and lipid and protein deposition in Atlantic salmon (*Salmo salar L*) in the early freshwater phase. Leucine was chosen because it is known to be higher in certain plant meal ingredients than in fish meals and DHA was chosen because it is found in fish oil and not existent in plant oils and therefore reduced in modern fish diets. Both leucine and DHA are known to influence the lipid metabolism in animals. Atlantic salmon was fed 6 different diets supplemented with leucine and DHA with a high- or normal-fat dietary content. Whole body, in addition to tissue samples of liver, muscle and adipose were analyzed for fatty acid composition. A microarray analysis was performed on liver, muscle and adipose tissues to study the different gene expressions related to the lipid metabolism in the fish.
The fish grew from 4 g to 22 grams during the trial and the fat, protein, leucine or DHA levels in the diet did not influence the growth. The protein to fat ratio in the feed affected however the whole body protein and fat contents of the fish, with higher protein and less fat in the low fat dietary groups. Dietary leucine did not increase protein and muscle growth, but affected the liver fat deposition negatively in combination with high fat diet.

Formålet med masteroppgaven var å undersøke hvordan protein:lipid ratioen i diettene og kombinasjonen av DHA og leucin interagerer og påvirker anvendelsen av energi til vekst og lipid- og proteinlagring i Atlantisk laks (*Salmo salar L*) i tidlig ferskvannsfase. Leucin ble valgt fordi den er kjent for å finnes i større mengder i noen plantemel ingredienser enn i fiskemel, og DHA ble valgt fordi den finnes i fiskeolje og ikke i planteoljer og er derfor redusert i moderne fiskedietter. Både leucin og DHA er kjent for å påvirke lipidmetabolismen i dyr. Atlantisk laks ble fôret 6 dietter med leucin og DHA supplement og høyt og lavt fettinnhold. Helkropp, i tillegg til at vev av lever, muskel og fett ble analysert for fettsyrekomposisjon. En microarrayanalyse ble gjennomført på vev av lever, muskel og fett for å studere de ulike genuttrykkene relatert til fettsyremetabolismen i fisken. Fisken vokste fra 4 gram til 22 gram i løpet av fôringsforsøket og fett-, protein-, leucin- eller DHA-nivåene i dietten påvirket ikke tilveksten. Protein:lipid ratioen i fôret påvirket protein i helkropp og fettinnhold i fisken, med høyere protein og mindre fett i lavfett gruppene. Leucin økte ikke protein- og muskelvekst, men påvirket fettlagring negativt i kombinasjon med høyfett-dietten.

Introduction

The production of fish oil and fish meal have been quite stable for the last 10 years, as seen in Figure 1, while at the same time the aquaculture production has increased rapidly, and thereby also increased the requirement for feed ingredients (PwC, 2017). The Norwegian government has set a goal of producing 5 million tons of sustainable seafood in 2050, while being challenged with combating salmon lice and the increased requirement of feed ingredients (PwC, 2017). PwC developed three different scenarios of the Norwegian aquaculture production towards 2050, represented in Figure 2, where only the optimistic scenario can reach the 5 million tons of sustainable seafood production. Many factors will contribute in determining the production growth towards 2050, among them development of technological equipment, methods to combat salmon lice and aquaculture feeds, while working towards a Norwegian sustainable aquaculture industry (PwC, 2017).

The diet of Atlantic salmon (*Salmo salar L*) has gone through a shift from marine ingredients towards plant-based ingredients (Jin et al., 2018). Fish meal and fish oil are today still used in the fish diet, but in a smaller scale than before, due to its scarce availability and its high costs. The replacement of fish meal and fish oil with feed resources of vegetable origin has led to reduced concentrations of Docosahexaenoic acid (DHA) and Eicosapentaenoic acid (EPA), which are important for optimal growth and health in salmon. The increased use of plant-based ingredients in salmon feed presents challenges due to the high content of n-6 and n-9 fatty acids. This increased use of vegetable oils, combined with the reduction of marine ingredients with high LC PUFA n-3 fatty acids, can lead to a disturbance of the n-6:n-3 fatty acid composition in the fish, which can affect the fish health negatively.

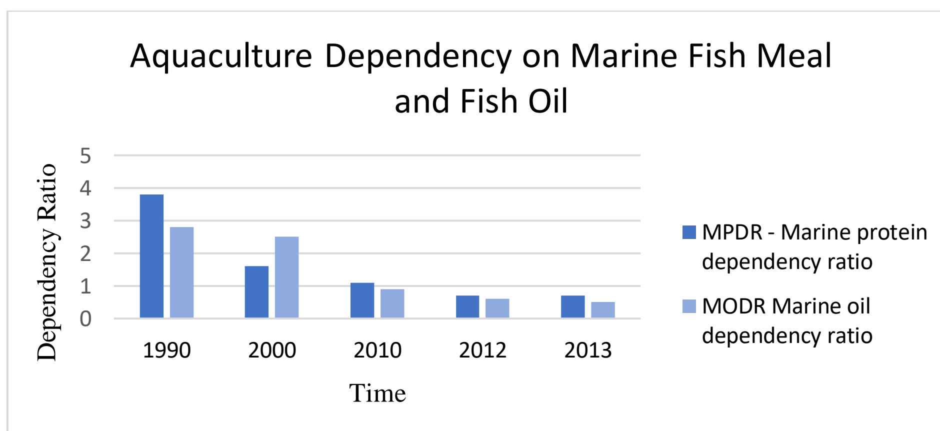

Figure 1. Aquaculture Dependency on Marine Fish Meal and Fish Oil. An overview of the dependency on marine protein- and oil of pelagic fish species in the Norwegian aquaculture industry from 1990 -2013 presented in marine protein dependency ratio (MDPR) and marine oil dependency ratio (MODR). This figure is recreated based on Figure 6 by Ytrestøyl et al., (2015).

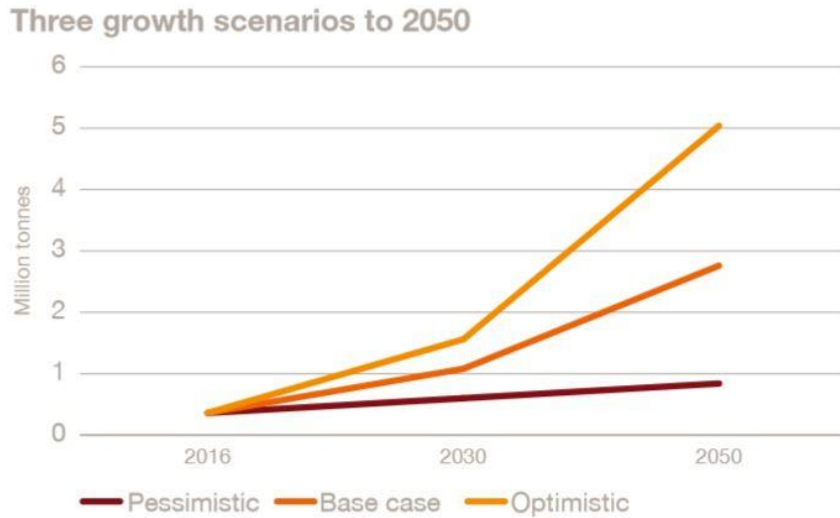

Figure 2. Three scenarios of the development in the Norwegian aquaculture industry towards 2050 by PwC. Pessimistic scenario in dark red color, base case in orange color and optimistic case in yellow color. Figure taken from PwC.(2017), page 9.

Lipids and Fatty Acids

Fat is the main source of energy in salmon feed, followed by proteins and carbohydrates. Lipids can be divided into two groups, polar lipids like phospholipids in the cell membranes and neutral lipids such as triglycerides, which is the main form of stored fats in salmon (Tocher, D., R., 2003). Lipids in the form of fatty acids can be divided into saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Fatty acids can also be divided into essential and non-essential fatty acids, where essential fatty acids are fatty acids that cannot be synthesized in vivo, but are needed for metabolic purposes, and must be supplemented through the diet. In salmon, the C18:3n-3 and C18:2n-6, make up the essential fatty acids, while EPA (C20:5n-3) and DHA (C22:6n-3) are supplemented through the diet due to low capacity of the Δ5 desaturase enzyme.

Fatty acid synthesis

The liver is an important organ in lipid metabolism regulation (Jin et al., 2018), and the fatty acid composition in fish tissues is influenced both by dietary fatty acid composition and enzyme capacity (Monroig et al., 2018). Non-essential fatty acids synthesis starts in the mitochondria when acetyl-coA is converted into malonyl-CoA by acetyl-CoA carboxylase (Bou, 2016). Malonyl-CoA and acetyl-CoA goes through several condensations before the fatty acid synthase (FAS) help generate the SFAs C16:0 and C18:0 (Bou et al., 2016; Bou, 2016). These saturated fatty acids (SFA) can further become monounsaturated fatty acids (MUFAs), C16:1n-7 and C18:1n-9, with help from microsomal Δ9 stearoyl-CoA desaturase

(Bou, 2016). Elongation of these fatty acids by fatty acyl elongases and desaturation by fatty acyl desaturase results in n-7 and n-9 polyunsaturated fatty acids (PUFAs) (Bou, 2016).
The importance of PUFAs and their metabolites is well-recognized in processes in cells and tissues of animals, and in regulating the viscosity of the phospholipid cell membranes (Murzina et al., 2016). In adult salmon, the PUFAs of the n-3 and n-6 groups can only be synthesized from the dietary C18:3n-3 and C18:2n-6 PUFAs due to a lack of certain desaturases (Tocher et al., 1998, (Bou, 2016). However, in salmon fingerlings and juveniles, the capacity of elongation and desaturation of the LC PUFAs is somewhat still unknown (Jin et al., 2018). Salmon can convert α-linolenic acid (C18:3n-3) to the PUFAs EPA and DHA through reactions of desaturation (the formation of a double bond), elongation (the lengthening of the carbon chain) and β-oxidation (shortening of the carbon chain), but they are not very efficient (Kjær et al., 2016;Ruyter et al., 2000).

The role of Lipids in Gene Regulation in Atlantic Salmon

Several fatty acids affect gene regulation either by up- or down- regulation of gene expressions. The n-3 PUFA synthesis is affected by the presence of the substrates C18:2n-6 and C18:3n-3 (Harnack et al., 2009), which affects what types of LC PUFAS are being synthesized. DHA and EPA are shown to affect the n-3 HUFA synthesis genes by increasing the *elovl2* elongase gene expression and DHA decreasing the expression of the desaturase genes *Δ5fad, Δ6fad_a, Δ6fad_b* and elongase gene *elovl5b* in hepatocytes of Atlantic salmon Kjær et al., 2016). These enzymes have different substrate specificities, and this will help determine which fatty acids of the n-3 group that are being synthesized.
Salmon has a low conversion rate of C18:3n-3 to EPA and DHA due to low Δ5 desaturase activity, and consequently the diet is enriched with these fatty acids. The desaturation reactions of PUFAs are catalyzed by fatty acyl desaturases called fads and the elongation reactions are catalyzed by elongation of very long-chain fatty acid proteins (elovl). In salmon the fads2 (Δ6 and Δ5 desaturases) are present with the elovl5 and elovl2 elongases (Hamid et al., 2016), but due to low the conversion of C18:3n-3 to EPA and DHA is not very efficient, and consequently the diet is enriched with these fatty acids.
The two elongases elovl5b and elovl2 differ in their elongation activity of the three PUFA groups C18, C20 and C22, where the elovl5b elongates C18 to C20 PUFA rather than C22, while elovl2 elongates C20 to C22 PUFA more frequently than C18 (Morais et al., 2009). A connection between increased transcripts of elovl5b and elovl2 in liver tissue as a result of a

diet with vegetable oil was also explained (Morais et al., 2009). In addition to this, Kjær et al., 2016, showed that efficient conversion of n-3 fatty acids is affected by differential expression of gene copies, rhythmic gene expressions and the finite ability to affect the gene expression, emphasizing the complexity of the regulation and conversion of n-3 fatty acids in salmon.

Lipid Deposition in Atlantic Salmon

A balanced diet with an optimal dietary composition of nutrients is the basis for metabolic homeostasis and good health in Atlantic salmon. An unbalanced diet can cause a disturbance of the homeostasis and the fish metabolism and can ultimately affect the lipid storage pattern (NIFES., 2015). Salmon stores lipids mainly in the muscle tissue and viscera (NIFES., 2015). Todorčević et al (2008) showed that increasing the dietary levels of the n-3 highly unsaturated fatty acids (HUFAs) DHA and EPA reduced the fat percentage in white adipose tissue of Atlantic salmon and increased the fatty acid β-oxidation capacity. The study also found that high levels of dietary DHA and EPA caused oxidative stress, mitochondrial malfunction and finally apoptosis of white adipose tissue, resulting in a reduction of fat percentage.

Amino acids

Amino acids are the building blocks of proteins and consist of an amino group ($-NH_2$), a carboxyl group (-COOH) and a functional group (-R), which is unique to each amino acid. The -R group provides the amino acids with specific characteristics, which make them important metabolites in biological processes such as growth, gene expression and reproduction (Wu, G., (2014). Some amino acids, such as methionine and arginine, are known to influence the lipid metabolism in salmon by increasing the lipid storage in the liver and improving the utilization of fatty acids as an energy source respectively (NIFES, 2015).

Branched-chain Amino Acids

In humans, the branched-chain amino acid (BCAA) metabolism in tissues start with deamination by BCAT, which produces branched-chain keto acids (Platell et al., 2000). These acids can become part of proteins or function as energy substrate in organs such as muscle and liver. In the latter scenario, the branched-chain α-keto acid dehydrogenase (BCKDH) performs the oxidative decarboxylation of the branched-chain keto acids, turning them into substrates for the tricarboxylic acid cycle (TCA cycle). The function and importance of the branched-chain amino acids (BCAAs), isoleucine, leucine and valine, on the lipid metabolism

in salmon are not fully understood, but several animal and human studies have reported both negative and positive health effects of a dietary BCAA.

In Zucker fatty rats fed a low-fat diet the circulating BCAA level was increased compared to Zucker lean rats on the same diet, and the following increase of BCAA oxidation disturbed the lipid oxidation in skeletal muscle tissue, ultimately resulting in obesity-related insulin resistance (White et al., 2016). Oppositely, a restriction of the dietary BCAAs in the same rats reversed the negative impact of the BCAAs, and improved muscle insulin sensitivity by enhancing the lipid oxidation (White et al., 2016). A reduction of dietary BCAAs also reduced the accumulation of visceral adipose tissue and maintained insulin sensitivity (White et al., 2016).

A similar restoration of metabolic health by reducing dietary BCAAs was observed in obese mice through normalization of body weight, a reduction in fat mass and improved glucose tolerance and insulin sensitivity (Cummings et al., 2017). The expression of several genes of lipogenic nature involved in the lipid metabolism was repressed by a reduced BCAA level and a reduction of the liver lipid droplet size was observed, suggesting a possible improvement of liver health too (Cummings et al., 2017).

Leucine has been reported to improve the bodyweight loss and the reduced feed intake in intrauterine growth-retarded (IUGR) weaning piglets (Weipeng et al., 2017). In addition to this, leucine improved the liver health by increasing ATP production and liver weight (Weipeng et al., 2017). In a study of BCAA effect on Indian major carp (*Cirrhinus mrigala)*, whole-body fat level increased when subjected to increasing dietary leucine (Ahmed & Khan., 2006). The study also reported reduced growth and FCR when subjected to excess BCAA levels.

An optimal dietary level of leucine (1,72 %) in juvenile Blunt snout bream (*Megalobrama amblycephala*) reduced plasma glucose level by activating both the insulin signaling and glycolysis pathway and stimulated the lipid and triglyceride (TG) synthesis (Liang et al., 2019). Oppositely, an excess level of dietary leucine inhibited the insulin signaling pathway and upregulated the genes involved in the gluconeogenesis (Liang et al., 2019).

Master Thesis Project

The main goal of this master thesis project was to examine how protein to lipid ratio in the diets and DHA and leucine in combination interact and affect the energy utilization for growth and lipid and protein deposition in Atlantic salmon in the early freshwater phase. Leucine is chosen, since this branched-chain amino acid is known to be higher in certain plant meal ingredients than in fish meals, for instance peas and lupin that are commonly used as meal ingredients in commercial fish diets. Leucine is also known to interact with lipid metabolism in mammalian species. DHA is chosen since this fatty acid is found in fish oil and not existent in plant oils and therefore reduced in modern fish diets. DHA is also known to influence lipid deposition in Atlantic salmon. For this purpose, Atlantic salmon was fed 6 different diets supplemented with leucine and DHA with a high- or normal-fat dietary content from 4-22 gram body weight. Whole body and tissue samples of liver, muscle and adipose from these fish were analyzed for fatty acid composition and a microarray analysis was performed on liver, muscle and adipose tissues to study the different gene expressions related to the lipid metabolism in the fish.

Materials and Methods

Project Description

The feeding trial was carried out at Nofima Reaseach Station in Sunndalsøra for approximately 8 weeks in the spring of 2018, during the freshwater phase of Atlantic salmon. The experimental fish were distributed between 18 tanks with 100 individuals in each tank. The fish were fed 5 different diets with a high or low level of fat. The diets further contained normal or high levels of leucine and were with or without added DHA, as seen in Table 1. A control diet was implemented and used as a reference diet. At the beginning of the project, the fish were bulk weighed with a mean start weight of ~ 4,5 grams. The tanks used were start-feeding tanks with a tank diameter of 0,5 meters and the oxygen saturation was kept above 80% at 12-13ºC. The diets used for this project had a pellet size of roughly 1,5 mm and was produced by Nofima Bergen. An automatic belt feeder ensured regularly distributed feeding of the fish and the diets were introduced in triplicate tanks. At the end of the feeding trial the fish were bulk weighed, counted and tissue samples of liver, adipose and muscle were collected along with whole body samples for microarray and fatty acid analysis.

Table 1. Dietary composition in % of the 6 fish feeds used in the feeding trial. NL,NF = normal-leucine, normal-fat diet, HL,NF = high-leucine, normal-fat diet, HL,NF+DHA = high-leucine, normal-fat +DHA diet, NL,HF = normal-leucine, high-fat diet, HL,HF = high-leucine, high-fat diet and HL,HF+DHA = high-leucine, high-fat +DHA diet.

Dietary Composition	Diet					
	NL,NF	HL,NF	HL,NF+DHA	NL,HF	HL,HF	HL,HF+DHA
Fish meal 29/17	60,00	56,76	56,76	53,74	50,83	50,83
Wheat 6/18	11,40	10,78	10,78	10,21	9,66	9,66
Wheat gluten 4/18	10,00	9,46	9,46	8,96	8,47	8,47
Soy protein concentrate 27/17	2,00	1,89	1,89	1,79	1,69	1,69
Leucine	0,00	4,15	4,15	0,00	3,75	3,75
Betafin T5/17	0,50	0,50	0,50	0,50	0,50	0,50
Lecithin 7/17	0,50	0,50	0,50	0,50	0,50	0,50
Fish oil 5/18 (30%)	9,10	9,55	4,80	9,90	10,30	5,30
Rapeseed oil 31/17 (70%)	2,20	2,10	3,90	10,10	10,00	12,00
Vitamin mix T1/18	2,00	2,00	2,00	2,00	2,00	2,00
MSP T13/17	1,70	1,70	1,70	1,70	1,70	1,70
Astaxanthin 10% T6/18	0,01	0,01	0,01	0,01	0,01	0,01
Mineral mix P43/17	0,59	0,59	0,59	0,59	0,59	0,59
DHA concentrate P2/17			2,95			3,00

Table 1 shows the dietary composition (%) of the 6 feeds used in the feeding trial.

Table 2. Chemical composition in % of the 6 feeds used in the feeding trial. NL,NF = normal-leucine, normal-fat diet, HL,NF = high-leucine, normal-fat diet, HL,NF+DHA = high-leucine, normal-fat +DHA diet, NL,HF = normal-leucine, high-fat diet, HL,HF = high-leucine, high-fat diet and HL,HF+DHA = high-leucine, high-fat +DHA diet.

Chemical composition in the feed (% in diet)	Diet					
	NL,NF	HL,NF	HL,NF+DHA	NL,HF	HL,HF	HL,HF+DHA
Protein	51,97	53,34	53,34	46,60	47,85	47,85
Lipid	18,01	18,01	18,01	26,04	26,02	26,02
Starch	6,63	6,27	6,27	5,94	5,62	5,62
Ash	11,26	10,75	10,75	10,27	9,80	9,80
Water	7,05	6,67	6,67	6,32	5,98	5,98
Sum	94,94	95,05	95,05	95,17	95,27	95,27
Energy MJ/kg	20,69	20,96	20,96	22,48	22,72	22,72
Leucine, % in diet	3,73	7,68	7,68	3,34	6,91	6,91
Leucine, % and CP	7,17	14,39	14,39	7,16	14,44	14,44
EPA, % of diet	1,08	1,08	1,08	1,09	1,09	1,08
DHA, % of diet	1,43	1,42	2,82	1,41	1,41	2,82
Sum EPA + DHA, % of diet	2,50	2,50	3,91	2,50	2,50	3,90
Sum EPA + DHA, % of fat	13,89	13,91	21,69	9,59	9,61	14,99
Kg per diet	50,00	50,00	50,00	50,00	50,00	50,00

Table 2 shows the chemical composition (%) of the 6 feeds used in the feeding trial. The different feeds were designed isoenergic, but with different protein:lipid ratios. The three low-fat diets contained approximately 53% protein and 18 % lipid, while the three high-fat diets contained approximately 47% protein and 26% lipid. Leucine was added at 7 and 14% in both the high- and low-fat diets, while DHA was supplemented in 1,4 and 2,8 % respectively.

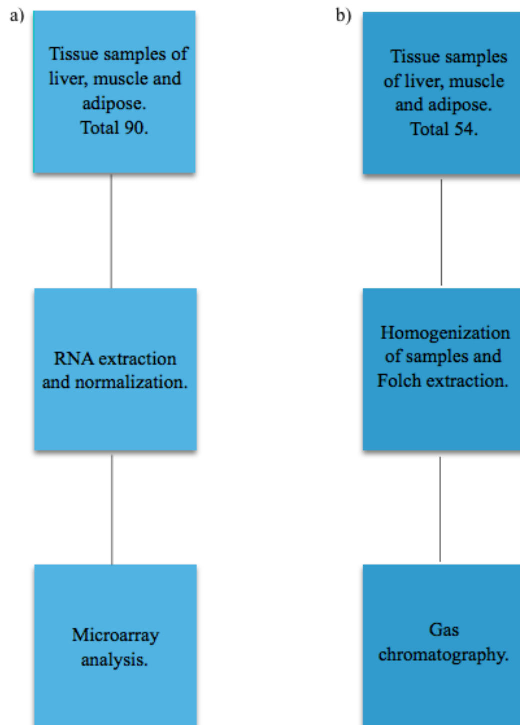

Figure 3. Flow chart of master thesis laboratory work. a) Samples of liver, muscle and adipose tissues of Atlantic salmon were used for RNA extraction (30 samples per tissue type). The samples were normalized to approximately 100 ng/µl before the samples were analyzed with microarray. b) Samples of liver, muscle and adipose (18 samples per tissue type, 6 dietary groups, a pooled sample of tissues and organs from 5 fish per tank and triplicate tanks) tissues were homogenized and extracted by the Folch method and analyzed for fatty acid composition by gas chromatography.

1. Growth Data

The growth data collected by Nofima personnel at Sunndalsøra at the end of the feeding trial included bulk weight of whole-body weight (grams), liver weight (grams) and length (centimeter). From these data, specific growth rate (SGR), hepatosomatic index (HSI), and condition factor (K factor) were calculated. Some of the experimental fish was also weighed individually and used for analysis purposes.

SGR was calculated using the following equation:

$$\text{SGR \%} = \left(\left(\frac{W_2}{W_1}\right)^{\frac{1}{days}} - 1\right)100$$

W_2 = body weight at the end of the trial (g)
W_1 = body weight at the beginning of the trial (g) = 4,5 g

HSI was calculated using the following equation:

$$\text{HSI} = \frac{Liver\ weight\ (g)}{Body\ weight\ (g)} * 100$$

K factor was calculated using the following equation:

$$\text{K factor} = \frac{Body\ weight\ (g) * 100}{Length\ (cm)^3}$$

2. RNA Extraction and Microarray Analysis

2.1 Tissue RNA Extraction

Introduction to RNA extraction

RNA extraction is a method commonly used in molecular biology to obtain RNA that can be used as starting material for further research purposes, and the RNA extraction is therefore a critical step in obtaining a sufficient amount of RNA (Thermofisher 2012). RNA is very unstable and will quickly degrade due to presence of RNases in the environment (Thermofisher 2012). The method used for RNA extraction in this project involved cell lysis followed by stabilization of RNA in lysis buffer, removal of contaminants by washing and final RNA elution (RNeasy Mini Handbook). Different tissues tend to vary in content of total amount of RNA. Liver tissues are known to contain more RNA than adipose and muscle tissues, which tend to be low in RNA. Because of the specific characteristics of and the content of total RNA in the respective tissues, the protocol was adapted to each tissue to facilitate optimal RNA extraction.

For RNA extraction from liver, adipose and muscle tissue in Atlantic salmon, the RNeasy Mini Kit was used, as it is known for being capable to extract RNA from small samples of animal tissues, and the extraction was performed according to the manufacturer's

recommendations (Qiagen 2012, RNeasy Mini Handbook 2012). The spin column used contained a silica membrane with specialized binding properties, which can bind RNA longer than 200 nucleotides in the presence of a high-salt buffer (RNeasy Mini Handbook 2012). Samples were lysed and homogenized with buffer, which inactivates RNases and prevents degraded RNA (RNeasy Mini Handbook 2012). Ethanol was added to facilitate binding conditions between the silica membrane and RNA, and residue was washed away (RNeasy Mini Handbook 2012). The RNA was eluded with RNase-free water and the eluate consisted mainly of mRNA as other types of RNA tend to be smaller than 200 nucleotides and are removed with other substances during washing (RNeasy Mini Handbook 2012).

Sample preparation

RNA extraction was performed on 90 samples, 30 from each respective tissue; liver, muscle and adipose. A solution containing 0,04 M of Dithiothreitol (DTT) in Buffer RLT was prepared in 350 μl for liver samples and 600 μl for muscle and adipose samples. Buffer RLT was used because of its content of guanidine thiocyanate, which facilitates efficient lysis of the tissue cells and denatures proteins, such as RNases (RNeasy Mini Handbook 2012). Zirkonium oxide beads (2.8 mm) were added to the buffer solution; two for liver and adipose tissues to help with the homogenization. For muscle tissues, 4 beads were used to ensure proper homogenization. A piece of frozen tissue was cut off and weighed before being added to the buffer solution containing beads. The weight for liver tissue used was 15-20 mg, for muscle tissue 25-30 mg and for adipose tissue 50 mg. These amounts of tissues were used to obtain optimal RNA elution and to avoid clogging of the spin column in subsequent steps (RNeasy Mini Handbook 2012). Each scalpel blade was used only on samples from the same tank. The samples were stored on ice before further preparation.

Homogenization of tissue samples

Both homogenization and centrifugation of the samples were performed in a fume hood due to the strong odor of DDT in the buffer solution. The tissue samples were homogenized in a Precellys 24 homogenizer, for 2x30 seconds (Berlin Technologies). After homogenization, the lysates were centrifuged in an Eppendorf Centrifuge 5424 at maximum speed for one minute (VWR, Norway). The lysates containing liver and muscle were placed in an incubator at 40ºC for 25 minutes, while lysates of adipose tissue were incubated for 15 minutes.

Elution of RNA

To the lysate, 350 μl ethanol (70%) was added and mixed thoroughly. From the sample, 700 μl was transferred to a spin column placed in a 2 mL collection tube. The sample was centrifuged for 30 seconds at 8000 x g. For muscle and adipose tissue samples, the remaining volume of lysate was transferred to the spin column and centrifuged again for 30 seconds at 8000 x g. The liquid residue in the collection tube was discarded and the column was placed back into the collection tube. To the column, 700 μl Buffer RW1 was added and centrifuged for 30 seconds at 8000 x g to wash the column membrane. The buffer contains guanidine salt and ethanol that remove carbohydrates, fatty acids, proteins and other substances, while RNA molecules larger than 200 bases remain bound to the silica membrane in the spin column (RNeasy Mini Handbook 2012). To the column, 500 μl Buffer RPE was added and was centrifuged for 30 seconds at 8000 x g to wash the column membrane. The buffer ensures that salt residues from previous washing steps are removed (RNeasy Mini Handbook 2012). A second washing step with 500 μl of Buffer RPE was carried out and centrifuged for two minutes to ensure that any ethanol residue was removed before RNA elution (RNeasy Mini Handbook 2012). The flow-through was discarded before the samples were centrifuged at full speed for one minute to remove residual liquids. The spin column was placed in a new 1,5 mL collection tube and 30 μl RNase-free water was added. The sample was centrifuged for one minute at 8000 x g to eluate the RNA.

Quantification of RNA

The concentration of total RNA in the eluates was measured by NanoDrop 1000 Spectrometer (Thermo Scientific, Waltham, MS,USA) according to the manufacturer`s protocol and guidelines to ensure sufficient concentrations for normalization and microarray analysis. The sample quality was further determined in an Agilent 2100 Bioanalyzer bioanalyzer by laboratory technicians at Nofima, where the samples with a RIN number around 8 was accepted for the microarray analysis. All reagents and equipment used for the microarray analysis were from Agilent Technologies.

2.2 Agilent One-color Microarray

Introduction to One-color Microarray

The RNA in the samples are synthesized to cDNA using RNA polymerase (*Agilent Technologies 2015)*. The RNA polymerase is added to the amplified cDNA to convert RNA

to cRNA. Cy3 is attached to the cytocines before the cRNA is purified. The samples are then hybridized for 17 hours. The samples go through a wash, are scanned and finally the gene expressions are registered for each sample. The following protocol and laboratory procedure were performed with help from the Agilent One-color Microarray pamphlet and a laboratory technician at Nofima.

Preparation of labeling reaction

RNA eluates (90 samples) were normalized to approximately 100 ng/μl and added to PCR tubes. The Thermo cycler was started and the program "AgilentL" was used for following incubation steps. The heating block was set to 80ºC. T7 master mix was prepared by adding 72 μl of T7 Promoter Primer to 90 μl nuclease-fee water in an Eppendorf tube, and the solution was mixed by pipetting.

The T7 master mix was added to the tubes containing RNA, and the amount added to the samples was calculated through a program of Agilent One-color Microarray by laboratory technicians at Nofima. The samples were incubated in the Thermo cycler for 10 minutes at 65ºC for dissociation of primer and template. The samples were incubated on ice for 5 minutes, while preparing the cDNA Master Mix. The First Strand Buffer was heated on the heating block for 3-4 minutes at 80ºC. To an Eppendorf tube 180 μl of 5X First strand Buffer was added along with 90 μl 0,1 M DTT, 45 μl 10 mM dNTP mix and 108 μl of AffinityScript RNase Block Mix. The cDNA Master Mix was mixed by pipetting. To each sample, 2,3 μl of cDNA mix was added before the samples were spun down, vortexed and spun down again quickly. The samples were incubated at 40ºC in the Thermo cycler for 2 hours followed by 15 minutes at 70ºC. The program was paused, and the samples were incubated on ice for 5 minutes. The samples were spun down in a microcentrifuge. The Transcription master mix was prepared by adding 67,5 μl of nuclease-free water to an Eppendorf tube along with 288 μl 5X Transcription Buffer, 54 μl 0,1 M DTT, 90 μl NTP mix, 18,9 μl T7 RNA Polymerase Blend and 21,6 μl Cyanine 3-CTP. To the samples 3 μl of the Transcription Master Mix was added. The samples were mixed by pipetting and spun down in a microcentrifuge. The samples were incubated in the Thermo cycler at 40ºC for 2 hours.

Purification of the labeled and amplified RNA

The samples were transferred to 1,5 mL tubes and 84 μl of nuclease-free water was added, followed by 350 μl Buffer RLT. The samples were mixed by pipetting. To the sample

solutions 250 μl of ethanol (purity 96-100%) was added and mixed by pipetting. The sample solutions (700 μl) were transferred to a spin column with a collection tube. The samples were centrifuged for 30 seconds at 13 000 RPM at 4 ºC. The flow-through and collection tube were discarded. The spin columns were placed in new collection tubes and 500 μl Buffer RPE was added. The samples were centrifuged at 13 000 RPM and the flow-through was discarded before the spin column was put back into the collection tube.
To the spin column 500 μl Buffer RLT was added and centrifuged for 60 seconds at 13 000 RPM at 4 ºC. The flow-through along with the collection tube was discarded. The spin columns were placed in new 1,5 mL Eppendorf tubes and 30 µl of nuclease-free water was added directly onto the filter membrane. The samples were incubated at room temperature for 60 seconds and centrifuged for 30 seconds at 13 000 RPM to elute the labelled RNA.
The samples were quantified using a NanoDrop spectrometer according to manufacturer's protocol and guidelines. Samples with activity over 8,0 pmol Cy3 per µg of cRNA was used further.

Hybridization

The process where the cDNA bind to the probes on the slide is called hybridization. The hybridization site can be visualized through fluorescence intensity signal, which is detected and registered by the computer and then identified.

Preparation of the 10x blocking reagent

A volume of 500 μl RNase-free water was added to lyophilized 10X Blocking Agent from the Agilent Gene Expression Hybridization Kit. The tube was vortexed quickly. To further dissolve the pellet, the mix was heated for 4-5 minutes at 37ºC in a water bath. The solution was centrifuged for 5-10 seconds to spin down any residues clinging to the tube wall.

Preparation of hybridization samples

The CY3-labelled RNA samples were normalized with nuclease free water in 8-strips PCR tubes according to their original concentration. To each sample 11 μl of 10x Blocking agent was added, followed by 2,2 μl of 25x Fragmentation Buffer. The samples were then spun down, vortexed and spun down again. The samples were incubated for 30 minutes in a Thermo Cycler at 60ºC. the samples were incubated on ice for one minute. To labelled Eppendorf tubes 55 μl of x GEx Hybridization Buffer HI-RPM was added in a fume hood and

the samples were transferred into the buffer and mixed without bubbles. The samples were centrifuged at 13 000 RPM for one minute in a fume hood. The samples were placed on ice.

Preparation of the hybridization assembly

The hybridization oven was set to 65ºC. Gasket slides were loaded onto Agilent SureHyb chambers, one in each, with the Agilent-label facing up. The samples were loaded onto the gasket slides, making sure the sample material and the pipette did not interfere with the gasket walls. An array with the active side (the side marked Agilent) facing down was placed on top of the gasket slide. The SureHyp chamber cover was placed on top of the chamber and the clamp was put on and tightened firmly. The chambers were vertically rotated to make sure no air bubbles were stuck in corners or along the sides of the gaskets between the two glass slides. The chambers were placed in a rotisserie in the hybridization oven making sure the chambers were balanced. The rotisserie speed was set to 10 rpm and the samples were incubated for 17 hours at 65ºC.

Microarray wash

The purpose of the microarray is to remove excess fluorescent probe material from the surface of the cDNA and long oligonucleotide.

One glass container with a slide rack was filled with Wash Buffer 1. Two plastic containers were filled with Wash Buffer 1 and Wash Buffer 2 respectively. The slide chambers were taken apart and the double-slides were put in Wash Buffer 1 in the glass container. The slides were taken apart with a plastic tweezer and the slide without the gaskets were placed in Wash Buffer 1 in a plastic rack for one minute while lifting the rack up and down in the Buffer solution. After one minute, the plastic rack with the slides was transferred to Wash Buffer 2, which was incubated at 37ºC overnight the day before, where it was lifted up and down for one minute. The slides were placed in a slide centrifuge for a few seconds before they were loaded onto slide holders with the active side (Agilent-label) facing up against the transparent plastic covers. The samples were scanned with a GenePix 4,100A (Molecular Devices, Sunnyvale, CA,USA) (Iliev et al., 2019). The software GenePix was used for spot-grid alignment, feature extraction and quantification. The spot quality was assessed with help from GenePix flags. Filtration of low-quality spots was carried out, and Lowess normalization of log2-expression ratios was performed. Features with > 2-fold change in both samples per treatment were selected as differentially expressed (Iliev et al., 2019).

3. Fatty Acid Extraction

Introduction

The extraction of lipids from animal tissues for analysis purposes requires removal of non-lipid substances to achieve lipid samples of high quality. A commonly used method for the extraction of lipids is the Folch method (Folch et al., 1975). The Folch method utilizes a solution of chloroform and methanol in a 2:1 ratio to extract and separate the lipids from the non-lipid substances in the sample. A separation of the sample material results in two separate phases where the upper phase contains water-soluble substances along with chloroform, methanol and water in a ratio of 3:48:47, while the lower phase consists of chloroform, methanol and water in the ratio of 86:14:1 respectively (Folch et al., 1975). Chloroform is known as a solvent for lipids and the lipids are therefore found in the lower phase. Following filtering of the biphasic product through a cotton filter facilitates removal of proteins from the sample. The lipids in the chloroform:methanol:water mixture can be used further for total fat analysis and fatty acid composition. For total fat analysis, part of the lower phase containing the lipids can be heated to remove excess chloroform and water, and the weight of the product is used to calculate total fat percentage. For an analysis of fatty acid composition of the tissues, the samples are dried in a nitrogen overflow, trans-esterified in the presence of benzene and H_2SO_4 in MeOH and separated into a biphasic product where the lower phase is dried in a nitrogen overflow. The samples are dissolved in hexane and placed in a gas chromatograph (CG) for analysis. C20:3 was used as an internal standard for the GC analysis because of its known concentration and absence in the sample material. The GC sends the sample through a column using a carrier gas (helium), and both the column passing rate (based on the characteristics of the sample material) and the stationary phase interaction where the components are separated and given a unique retention time are crucial to the analysis of the sample material. From a GC analysis the fatty acids are identified based on their retention time and quantified by their peak area. The materials and methods used in the following paragraphs were based on the protocol for fatty acid extraction and analysis described by (Mason & Waller 1964);(Hoshi et al., 1973).

In addition to fatty acid extraction and analysis of liver, muscle and adipose tissue, a whole-body lipid composition of the fish was obtained by laboratory personnel at Nofima and these data are also included in the results of this master thesis.

Homogenization of Muscle, Liver and Adipose Tissue for Foch Extraction

From each tissue type 18 samples were prepared, where 5 samples were pooled to make up 1 sample. Tissue samples of muscle and liver were homogenized separately using dry ice and a blender. Each muscle sample comprised two small fillets, while each liver sample contained 4 livers. Adipose tissue samples were cut into pieces manually with a knife because of its soft consistency and was not homogenized in a blender. To prevent melting of the samples and obtain a fine powder, the lid and blender were kept cold with dry ice throughout the homogenization process. The samples were homogenized roughly and quickly, for approximately 30 seconds -1 minute, before the homogenate was placed into an open plastic bag overnight in the freezer to remove the CO_2.

Folch Extraction

Fatty acid extraction was performed according to the Folch method in a fume hood (Folch et al., 1975). Liver, muscle and adipose tissue sample homogenates were measured to approximately 2 grams in a 100 mL Erlenmeyer flask. To the flasks containing the samples, 6 mL 0,9% NaCl was added and the content was swirled gently to prevent the tissues from sticking to the bottom of the flask. A mixture containing chloroform and methanol in a 2:1 ratio and 0,7 mg/L butylated hydroxytolu-ene (BHT) was added in a volume of 50 mL. The contents were homogenized for one minute. To the homogenized sample, 6 mL 0,9% NaCl was added and the mixture was homogenized for an additional 5 seconds. The samples were left in the fume hood to separate into a biphasic product. The homogenized product was filtered through cotton placed inside a glass funnel and into a 100 mL glass cylinder. The cylinders were capped and placed in a freezer overnight. A water-vacuum pump pipette was used to remove the upper phase from the lower phase. The lower phase containing the lipid fraction was divided in half where the first part was used for an analysis of total fat, while the second part was used for fatty acid composition analysis.

Total fat Analysis

A silver paper cup was measured before 20 mL of the lower chloroform phase containing the lipid fraction was transferred to it. The silver paper cup was placed on a heating plate to evaporate the chloroform and water until only the lipids remained. The cup containing the sample was transferred to a dryer at 105 ºC for 20 minutes to remove water residue. The weight of the silver cup was measured and % of total fat was calculated according to the following equation using 100mL of chloroform/methanol:

$$\% \text{ fat} = \frac{fat(g) * 100}{\frac{I * U}{37,5}}$$

I = weight of the sample (g)

U = Chloroform phase (20 mL)

37,5 = total volume of solvent (chloroform in extract solution =50*33,4 mL. after separation the chloroform phase contains some methanol and water, changing the volume to: $\frac{100}{89} * 33,3\ mL = 37,5\ mL$).

Fatty Acid Composition by Gas Chromatography

The remaining volume of the lower phase was transferred to glass tubes and evaporated at 60ºC under nitrogen overflow until dryness.

Benzene (2 mL) and 4 mL 2% H_2SO_4 in MeOH were added to the tubes and the content was mixed. The fatty acids in the samples will undergo transesterification due to the heating in excess methanol with H_2SO_4 as a catalyst for the reaction. The tubes were capped and incubated at room temperature overnight. To the samples 2 mL hexane and 3 mL 6% $NaHCO_3$ were added and the samples were separated into a biphasic product. The upper phase was transferred to a new glass tube and heated in a nitrogen overflow at 60ºC until dryness. To prepare the samples for GC, the samples were added 6 drops of hexane and the content was mixed gently before being transferred to 300 µL GC vials. C23:0 was used as standard sample for the GC analysis and the samples were placed in the gas chromatograph (Hewlett Packard 6890). The temperature program used was as follows: starting temperature: 50ºC, temperature increase by 4 ºC per minute to 170ºC, temperature increase 0,5 ºC per minute up to 200ºC, and finally temperature increase of 10ºC per minute up to 300 ºC. The sample material is then detected and identified electronically. Each fatty acid was presented as percentage of the total fatty acid content in the sample, and the amount of fatty acids per gram of tissue was calculated using the standard C23:0.

Statistical analysis

For the statistical analysis of the fatty acid composition in the tissues of liver, muscle, adipose and whole-body an Analysis of Variance (ANOVA analysis) was used to determine if the results were significant. The significance level used was 5%, resulting in non-significant results for all p-values above 0,05.

The statistical model used for the ANOVA analysis:
$y_{ij} = \mu_i + \varepsilon_{ij}$
i = number of treatments (6 diets)
j = observation number (n=3)
μ_i = mean of treatment i
ε_{ij}= random error component
$\varepsilon_{ij} = \mu_i$ errors have mean zero

To further distinguish between which dietary fish groups were significantly different from each other, a Tuckey test (Tuckey`s Honest Significant Difference Test) was used. The Tuckey test compares all the means with each other and figures out which ones are significantly different from each other at a significance level of 5%, as used in the ANOVA analysis. The Tuckey test assumes that the observations are independent within and among groups and that the groups are normally distributed. It also assumes equal within-group variation of the groups associated with each mean in the test. The statistical program R commander (Version 3.6.1) was used for the ANOVA analysis and Tuckey test.

The Statistical model used for the Tuckey test:

$$HSD = \frac{M_i - M_j}{\sqrt{\frac{MS_w}{n_h}}}$$

$M_i - M_j$ = the difference between a pair of means (the largest is M_i)
MS_w = the mean square within
n = number in the group

Results

1. Growth

During the feeding trial, the fish grew from approximately 4,5 grams to 22 grams, and no significant differences in specific growth rates, final weights, lengths of condition factors between the six dietary groups were found, as seen in Table 3. The hepatosomatic index (HSI) was significantly higher in fish fed the HLHF diet than in fish from the two groups NLNF and HLNF ($p<0,05$). The HLHF fish had an HSI mean value of 1,8 as opposed to NLNF and HLNF fish, both with a HSI value of 1,6.

Table 3. Mean and standard error of the mean (SEM) values of body weight (g), Condition factor (K factor), Hepatosomatic index (HSI), length (cm), Specific growth rate (SGR %) and p-value from ANOVA analysis for Atlantic salmon are presented in table 3. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA

	Dietary groups						
	NL,NF	HL,NF	HL,NF+DHA	NL,HF	HL,HF	HL,HF+DHA	P-value
Body weight	21,9 ± 0,32	22,4 ± 0,65	22,7 ± 0,07	22,8 ± 0,16	21,9 ± 0,47	21,7 ± 0,52	0,31
SGR	3,4 ± 0,03	3,4 ± 0,06	3,4 ± 0,01	3,45 ± 0,01	3,4 ± 0,04	3,0 ± 0,05	0,32
HSI	1,6 ± 0,04	1,6 ± 0,04	1,7 ± 0,04	1,7 ± 0,06	1,8 ± 0,04	1,7 ± 0,04	0,01
Length	11,8 ± 0,04	11,9 ± 0,07	12,2 ± 0,07	11,9 ± 0,15	11,8 ± 0,15	11,8 ± 0,20	0,30
K factor	1,4 ± 0,01	1,4 ± 0,02	1,4 ± 0,01	1,4 ± 0,01	1,4 ± 0,01	1,4 ± 0,02	0,26

2. Whole-body and Organ Total Lipid Composition and Whole Body Protein Composition

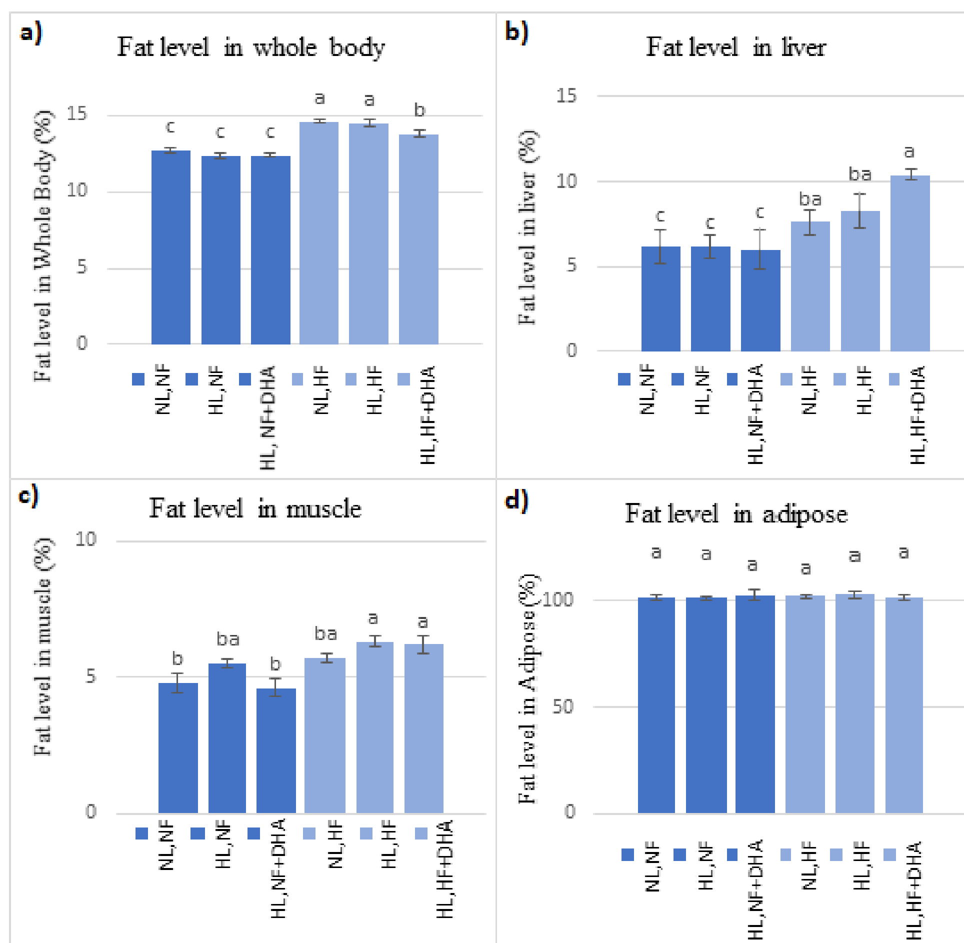

Figure 4. Fat level (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat+DHA.Fish

fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

All three normal-fat dietary groups resulted in fish with a lower fat content in whole body than fish from the three high-fat diets ($p<0,05$) as shown in Figure 4a. DHA led to a reduced fat content in the high-fat diet fish with DHA ($p<0,05$). In liver tissue, the fish fed the HL,HF+DHA diet showed a tendency of higher fat level than the fish fed the normal-fat diets ($p<0,05$) as shown in Figure 4b. In the muscle tissue of fish from the two dietary groups high-leucine, high-fat and DHA, the fat level was higher than in the two normal-fat diets NLNF and HLNF+DHA fish ($p<0,05$) shown in Figure 4c. Fhe fish from the low-fat diets seem to respond to leucine alone by increasing the fat level in muscle tissue, while DHA has a reducing effect on the muscle fat level. Little effect of DHA is observed in the high-fat group (Figure 4c). There was no difference in adipose tissue fat level between fish in the dietary groups as shown in Figure 4d.

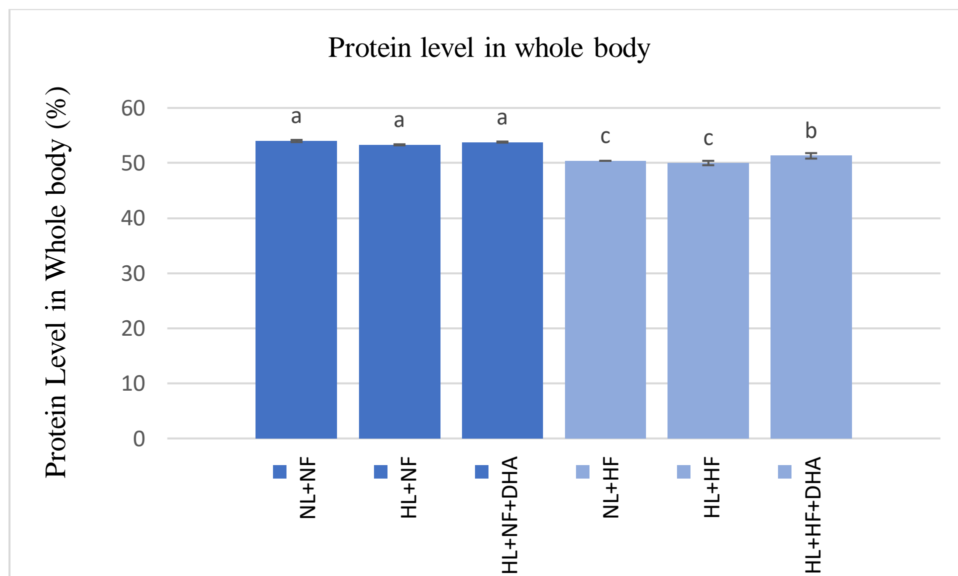

Figure 5. Protein level (%) in the whole body of Atlantic salmon. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA.Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color. The data are represented in mean ± SEM values, n-3.

The fish in all the low-fat dietary groups has significantly higher protein levels in whole body than fish fed the high-fat diets. In the low_fat groups, supplementation of leucine and DHA did not influence the protein level, while in the high-fat groups, DHA supplementation led to significantly higher protein level in whole body ($p<0,05$), shown in Figure 5.

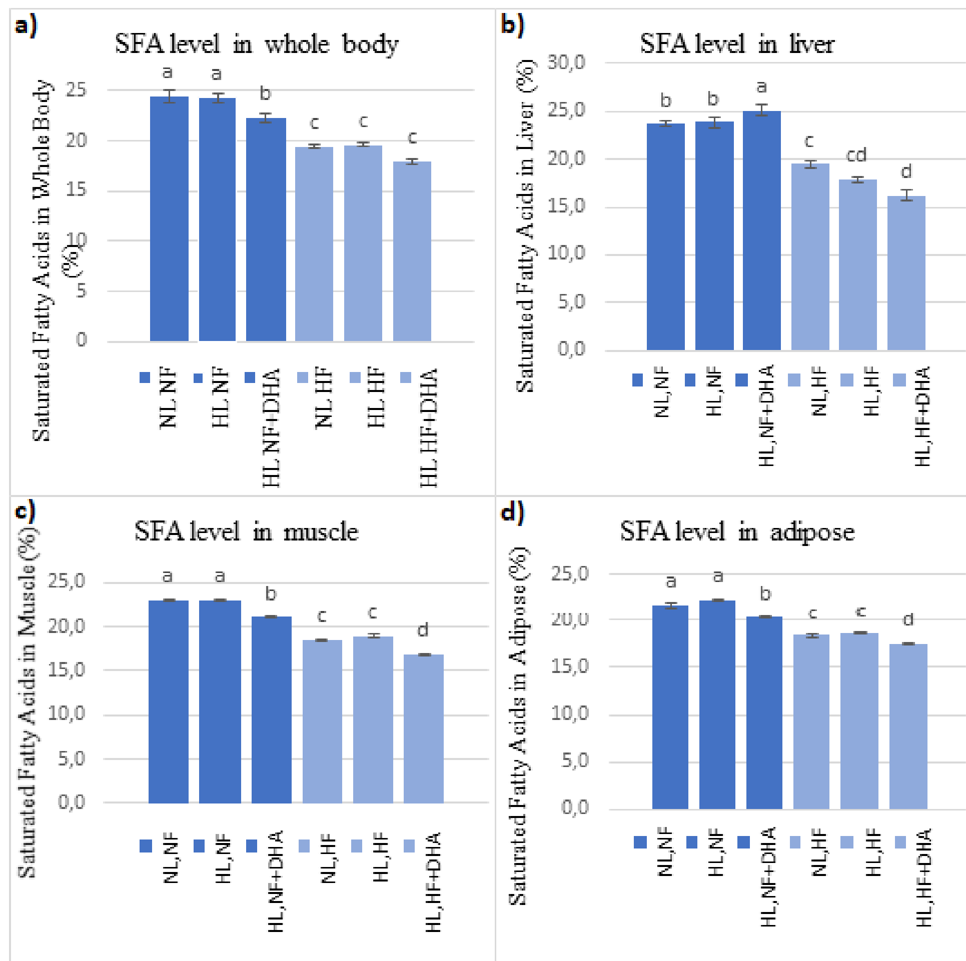

Figure 6. Saturated Fatty Acids (SFAs) (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA.Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

All three normal-fat diets resulted in fish with a higher percentage of whole body saturated fatty acids (SFA) than the high-fat diet fish ($p< 0,01$) as shown in Figure 6a. Of the normal-fat diet groups, DHA reduced the whole body SFA percentage significantly ($p<0,05$). No significant difference in whole body SFA content between fish of the high-fat diets was detected. In liver tissue, the SFA (%) was higher in the normal-fat fish than in the high-fat fish ($p<0,05$) shown in Figure 6b. Among the high-fat groups, dietary DHA showed a reducing trend of the liver SFA percentage. In muscle tissue, the SFA (%) of the two normal-fat diets NLNF and HLNF was higher than in the high-fat fish ($p<0,05$) as shown in Figure 6c.

DHA had a reducing effect on the muscle SFA (%) resulting in lower SFA (%) in both HL,NF+DHA and HL,HF+DHA fish compared to the other normal-fat and high-fat fish respectively ($p<0,05$). The SFA percentage in adipose tissue was almost identical to the response in muscle tissue as shown in Figure 6d, with a reduced SFA level in both the normal- fat and high-fat groups with dietary DHA ($p<0,05$).

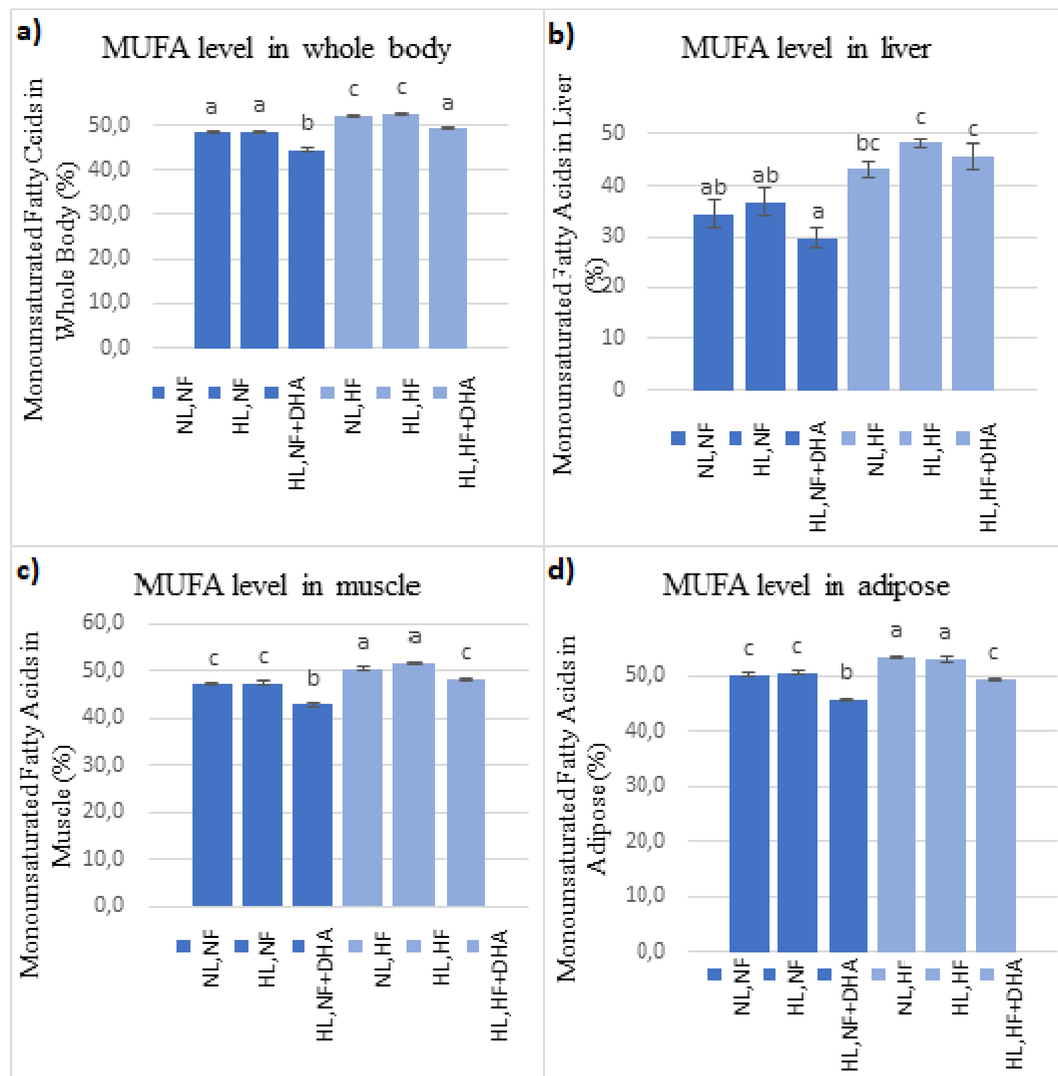

Figure 7. Monounsaturated Fatty Acids (MUFAs) (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA. Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

Fish fed the two high-fat diets NL,HF and HL,HF contained higher percentage of whole body monounsaturated fatty acids than all three normal-fat groups ($p<0,01$) as shown in Figure 7a. Among the high-fat diets, leucine and DHA reduced the whole body MUFA percentage considerably in the HLHF+DHA fish ($p<0,05$). The same reduction as a consequence of

added DHA was seen in whole body MUFA percentage among the fish on the normal-fat diets ($p<0,05$). The lowest percentage of monounsaturated fatty acids was found in the fish fed the HL,NF+DHA diet ($p<0,01$).

In liver tissue of fish fed the two high-fat diets +DHA there was a higher percentage of MUFA than in all three normal-fat groups ($p<0,05$) as seen in Figure 7b. Fish fed the NL,HF diet had higher content of MUFAs in liver than the HL,NF+DHA fish ($p<0,05$).

The HL,NF+DHA diet gave fish with the lowest percentage of muscle MUFA of the dietary groups ($p<0,05$) as seen in Figure 7c. The NL,NF and HL,NF diets both resulted in fish with lower levels of MUFA than HL,HF and NL,HF fish ($p<0,05$). HL,HF+DHA fish gave fish with the lowest level of MUFA among the high-fat diet fish ($p<0,05$).

Fish fed the HL,NF+DHA diet contained less adipose tissue monounsaturated fatty acids than all other groups ($p<0,05$). A reduction of MUFA percentage in both high- and low-fat groups with DHA was observed as a result of dietary DHA ($p<0,05$).

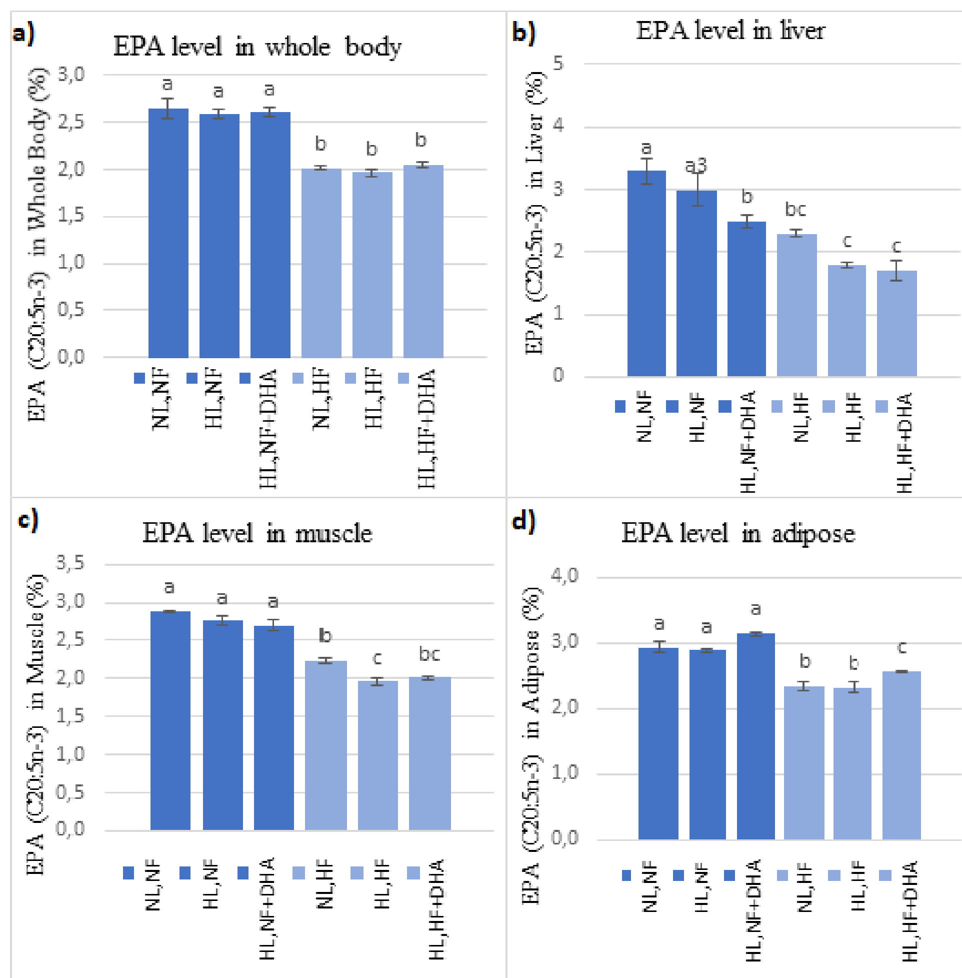

Figure 8. EPA (C20:5n-3) Eicosapentaenoic Acid (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high

leucine, high fat +DHA.Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

The three normal-fat groups were all higher in whole body EPA percentage than the three high-fat groups ($p<0,01$) as shown in Figure 8a. There was no significant difference in whole body EPA percentage between fish from the normal-fat diets, nor between the high-fat diet fish.
The liver EPA percentage of fish fed the NL,NF diet was higher than in fish from the high-fat groups and HL,NF+DHA group ($p<0,05$) as seen in Figure 8b. This indicates a reducing response of EPA % in whole body to dietary leucine and DHA. Fish from the HL,HF+DHA group had a lower level of liver EPA than fish from all three normal-fat diets ($p<0,05$). Fish fed the HL+HF diet had lower EPA content than fish from the HL+NF group ($p<0,05$).

All normal-fat diets resulted in fish with a higher muscle EPA percentage than the fish fed the high-fat diets ($p<0,05$) as seen in Figure 8c. The NL,HF diet gave the highest muscle EPA percentage of the high-fat groups ($p<0,05$). No significant difference was observed between the fish fed the normal-fat diets.

Fish fed the NL,NF and HL,NF+DHA diets had a higher EPA percentage in adipose tissue than the fish fed the three high-fat diets ($p<0,05$) as shown in Figure 8d. HL,HF+DHA fish had a lower percentage of EPA than HL,NF fish ($p<0,05$). HL,NF fish had a higher percentage of EPA than fish from the two dietary groups HL,HF and NL,HF ($p<0,05$). Fish fed HL,NF+DHA have a higher content of EPA than HL,NF fish ($p<0,05$).

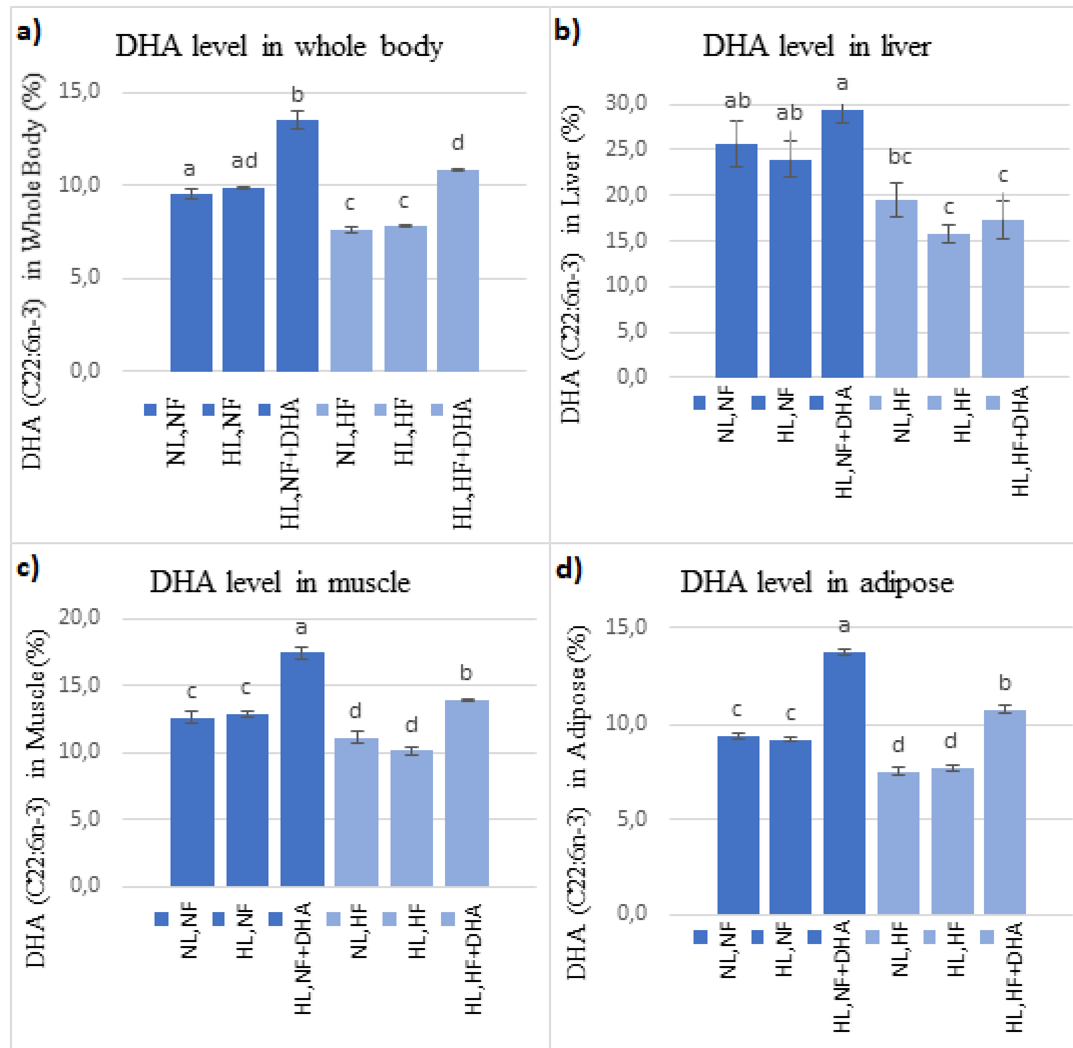

Figure 9. DHA (C22:6n-3) Docosahexaenoic Acid (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA.Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

The dietary group HL,NF+DHA displayed fish with a higher percentage of whole body DHA than all other dietary groups ($p<0,01$) as shown in Figure 9a. The two normal-fat diets, NL,NF and HL,NF, both gave fish with higher percentage of whole body DHA than the two high-fat diets HL,HF and NL,HF ($p<0,05$). The diet HL,HF+DHA gave fish with a higher whole body DHA percentage than all other dietary groups, except from HL,NF+DHA ($p<0,05$).

Fish from the HL,NF+DHA group had a higher percentage of liver DHA than fish from the high-fat diets ($p<0,05$) as seen in Figure 9b. Fish fed the HL,HF diet had a lower liver DHA percentage than fish fed the NL,NF diet ($p<0,05$).

DHA percentage in muscle tissue was higher in fish fed the HLNF+DHA diet than in all high-fat groups (p<0,05). Fish fed the HLHF+DHA diet had a higher DHA percentage than fish fed the NLNF, HLNF and NLHF diets (p<0,05). Fish fed the HLHF diet were lower in muscle DHA percentage than fish fed the HLNF, NFHF and NLNF diets (p<0,05). Fish fed the NLHF diet had a lower DHA percentage than NLNF and HLNF fish (p<0,05). Increasing tendency of DHA percentage in fish fed added leucine and DHA in muscle tissue. Fish fed the HL,NF+DHA diet had a higher DHA percentage in the adipose tissue than all other dietary groups (p<0,05). The dietary fish group with the second highest percentage of DHA is the HL,HF+DHA group (p<0,05). Both dietary groups, NL,NF and HL,NF, gave fish with more adipose tissue DHA than fish fed the NL,HF and HL,HF diets (p<0,05).

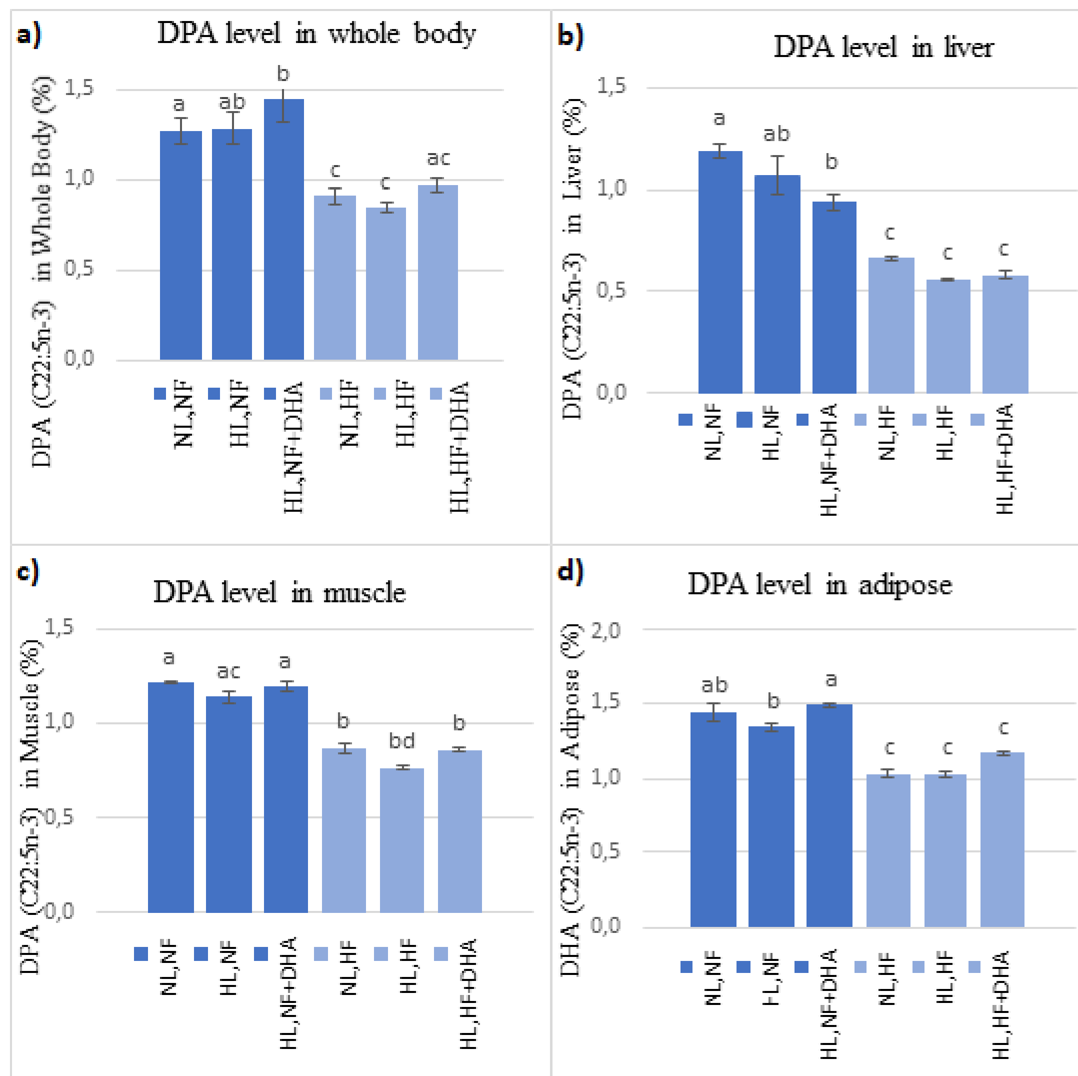

Figure 10. DPA (C22:5n-3) Docosapentaenoic Acid (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA.Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

According to Figure 10a, fish fed the HL,NF+DHA diet had a higher percentage of whole body DPA (C22:5n-3) than all other fish groups, except for NLNF (p<0,05). Within the high-fat diets, the whole body DPA percentage of the fish did not differ significantly. An increase of whole body DPA (%) as a result of dietary leucine and DHA (p<0,05). The fish fed the normal-fat diets all had a higher liver DPA percentage than the fish from the high-fat groups (p<0,05) as seen in Figure 10b. Within the normal-fat fed fish, the HL,NF+DHA fish had a lower percentage of liver DPA level than fish fed the standard diet (p<0,05). Fish fed the normal-fat diets had a higher muscle DPA percentage than fish fed the high-fat diets (p<0,05) as shown in Figure 10c. No significant difference in DPA percentage was observed between the normal-fat groups, nor between the high-fat groups. Fish fed the diets NL,NF, HL,NF and HL,NF+DHA all had a higher DPA percentage in the adipose tissue than fish fed the three high-fat diets (p<0,05). Fish fed HL,NF+DHA had higher DPA percentage than fish fed HL,NF (p<0,05).

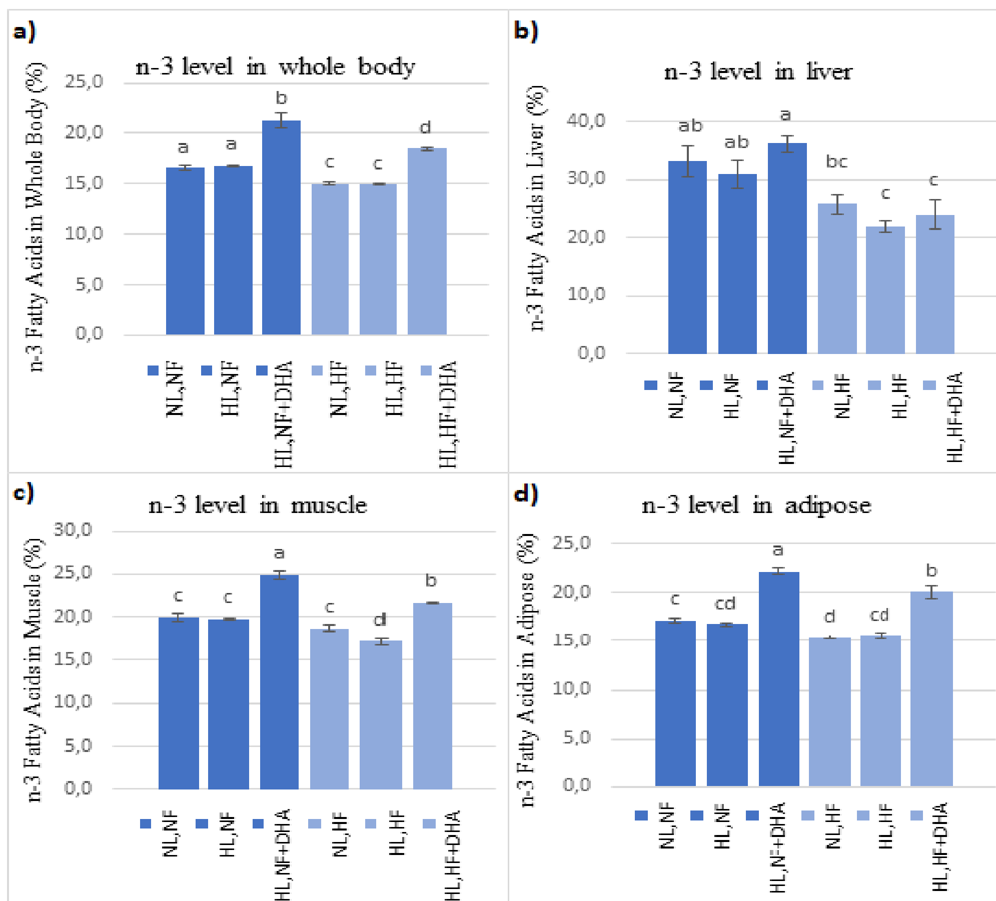

Figure 11. n-3 Fatty Acids (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA.Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

According to figure 11a, fish fed the HL,NF+DHA diet contained the highest percentage of whole body n-3 fatty acids ($p<0,01$). Within the high-fat diets, the fish on the HL,HF+DHA diet had the highest whole body n-3 fatty acid percentage ($p<0,01$). Increasing tendency of added leucine and DHA. Fish on the HL,NF+DHA diet had a higher percentage of n-3 fatty acids in the liver than all fish from the high-fat groups ($p<0,05$) as seen in Figure 11b. HL,HF fish had a lower n-3 percentage than the fish from the NL,NF group ($p<0,05$).

Fish fed HL,NF+DHA had the highest percentage of muscle tissue n-3 fatty acids of all the groups ($p<0,05$) as showed in Figure 11c. HL,HF+DHA fish contained more muscle n-3 fatty acids than the other dietary fish groups, except from the HL,NF+DHA group ($p<0,05$). Fish on the HL,HF diet contained less n-3 fatty acids than the NL,NF and HL,NF fish groups ($p<0,05$). Fish from the high-fat diets contained more n-6 fatty acids in their muscle tissue than fish fed the normal-fat diets ($p<0,05$) as seen in Figure 11c. Of the high-fat diet fish, HL,HF+DHA fish had more muscle tissue n-6 fatty acids than the other two high-fat diets ($p<0,05$). The fish fed HL,NF+DHA contained more n-6 fatty acids in their muscle tissue than the other two normal-fat groups ($p<0,05$).

Of the three normal-fat diets, HL,NF+DHA resulted in fish with the highest percentage of adipose tissue n-3 fatty acids ($p<0,05$). Among the fish on the high-fat diets, fish fed HL,HF+DHA had a higher percentage of n-3 fatty acids than the other groups ($p<0,05$). Fish on the HL,NF+DHA diet had the highest percentage of n-3 fatty acids in their adipose tissue, closely followed by fish fed the HL,HF+DHA diet ($p<0,05$). The NL,NF fish group had a higher n-3 fatty acid percentage in their adipose tissue than the NL,HF group ($p<0,05$).

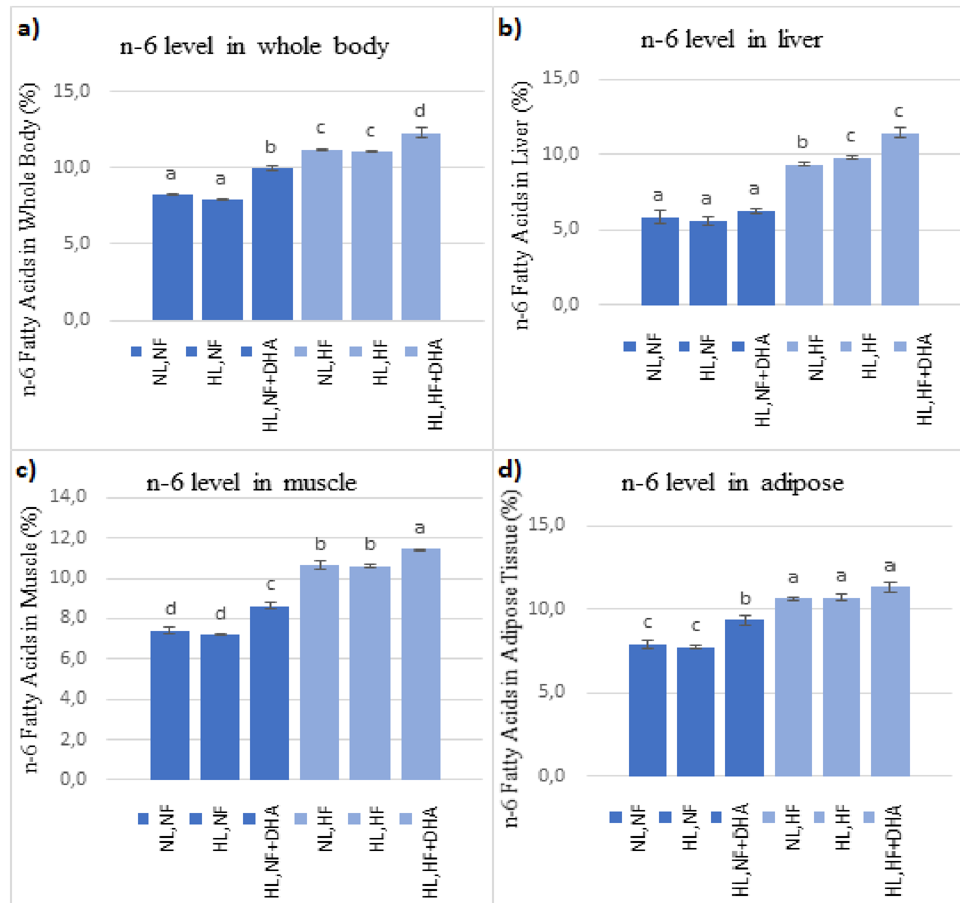

Figure 12. n-6 Fatty Acids (%) in tissues of a) whole body, b) liver, c) muscle and d) adipose. NL, NF = normal leucine, normal fat, HL,NF = high leucine, normal fat, HL,NF+DHA = high leucine, normal fat +DHA, NL,HF = normal leucine, high fat, HL,HF = high leucine, high fat and HL,HF+DHA = high leucine, high fat +DHA.Fish fed normal-fat diets are represented in dark blue color, while fish fed a high-fat diet are represented in a light blue color.

According to figure 12a, the three high-fat diets resulted in fish with a higher percentage of whole body n-6 fatty acids than the normal-fat diets ($p<0,01$). Among the normal-fat diets HL,NF+DHA gave fish with the highest percentage of n-6 fatty acids in whole body ($p<0,01$). Within the high-fat diets, the HL,HF+DHA diet had fish with the highest n-6 fatty acid percentage in whole body ($p<0,01$).

Fish fed the HL,HF+DHA diet have a higher liver percentage of n-6 fatty acids than fish from all other groups ($p<0,05$) as seen in Figure 12b. HL,HF and NL,HF fish had higher n-6 fatty acids percentage than HL,NF and NL,NF fish ($p<0,05$). NL,HF and HL+HF fish had higher liver n-6 fatty acid percentage than HL,NF+DHA fish ($p<0,05$).

Of the fish fed the normal-fat diets, the HL,NF+DHA diet resulted in fish with the highest percentage of n-6 fatty acids in the adipose tissue ($p<0,05$), while there was no significant difference in n-6 fatty acids percentage between fish fed the other two normal-fat diets shown

in Figure 12d. No significant difference was observed between fish fed the three high-fat groups. Fish fed the three high-fat diets all contain higher percentage of n-6 fatty acids than fish from the three normal-fat diets ($p<0,05$).

3. Differentially Expressed genes

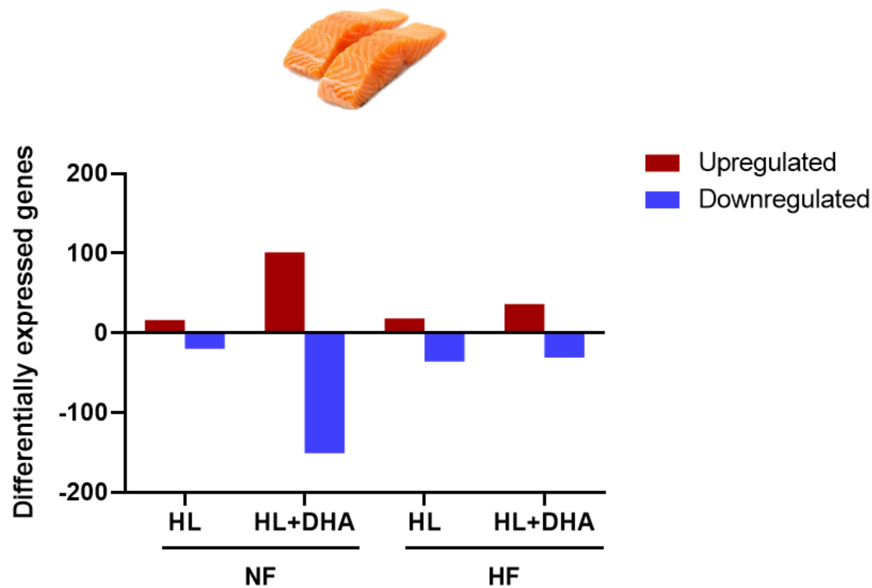

Figure 13. Differentially Expressed Genes in the Muscle tissue of Atlantic Salmon. HLNF = high leucine, normal fat, HLNF+DHA = high leucine, normal fat +DHA (the gene expression is calculated relative to NLNF group, set to 0), HLHF = high leucine, high fat and HLHF+DHA = high leucine, high fat+DHA (the gene expression is calculated relative to the NLHF group (set to 0). The number of expressed genes are marked by a red color, in which the genes are upregulated, while the genes marked in blue are downregulated.

In the muscle of normal fat groups, relative to the control group, addition of leucine alone only resulted in a small number of genes up- (<20 genes) or down- (20<genes) regulated. However, the combination of leucine and DHA led to up- (more than 100 genes) or down regulation (more than 150 genes) of a relatively high number of genes in the high fat groups. Supplementation of leucine alone or the combination of leucine and DHA only resulted in a relatively small number of genes up- or down regulated relative to the high fat group control.

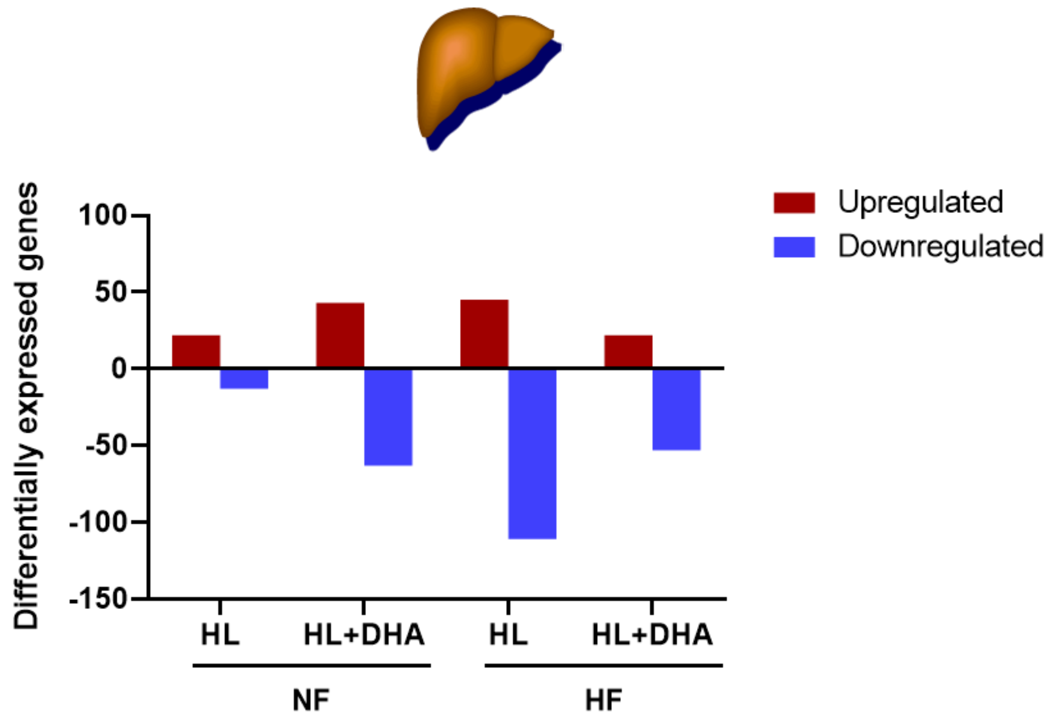

Figure 14. Differentially Expressed Genes in the Liver tissue of Atlantic Salmon. HLNF = high leucine, normal fat, HLNF+DHA = high leucine, normal fat +DHA, HLHF = high leucine, high fat and HLHF+DHA = high leucine, high fat+DHA. The number of expressed genes are marked by a red color, in which the genes are upregulated, while the genes marked in blue are downregulated.

In the liver of normal fat groups, relative to the control group, addition of leucine alone only resulted in a small number of genes up- (<20 genes) or down- (10<genes) regulated. However, the combination of leucine and DHA led to up- (less than 50 genes) or down regulation (more than 50 genes). The supplementation of leucine has much more effects in the liver of fish fed the high fat diets, with more than 100 genes down regulated and approximately 50 genes up-regulated. With further supplementation of DHA less genes were differentially expressed than with leucine alone relative to the high fat group control.

Discussion

Growth

Our study showed no significant differences in final weights or specific growth rates between fish from the 6 dietary groups. Some previous studies however, have shown that reducing the protein:lipid ratio (high fat diets) leads to increased growth in Atlantic salmon (Hillestad et al., 1994), while other studies by Dessen, (2019) have shown that a higher dietary protein:lipid ratio (larger than 1,2) increases the whole body weight gain compared to an isoenergic lower protein:lipid ratio in certain life stages. The diets used in our study were

isoenergetic although the protein:fat ratios were different in the dietary groups and this probably shows that the fish has the same prerequisite for growth in terms of dietary energy in this juvenile life stage. The reason for no effect on growth by increasing fat content may also be due to the fact that the need for protein in the diets of juvenile fish are higher since protein is needed in high extent for growth and metabolic purposes in the early life stages, as opposed to lipids which become increasingly important when the fish reaches maturation and is of a larger size.

Protein and fat level whole body:

The protein:fat ratio in the feed in our trial affected however the whole body protein and fat contents of the fish. The relative level of whole body protein was lower and the relative level of fat was higher in all the high-fat groups than in the low fat groups. This agrees with previous studies showing the increasing dietary fat level increases the fat content of whole body (Hillestad et al., 1994, Dessen 2019). Our results further showed that the whole body fat level in the high-fat experimental group was reduced by dietary DHA, which is in accordance with increased body protein:fat ratio resulting in leaner fish when fed high dietary level of DHA, as reported by Todorčević et al, (2009). However, in our study, dietary leucine did not lead to increased protein level in whole body, thus indicating that leucine does not influence protein synthesis in salmon. This is not in agreement with a study showing that modest levels of intracellular leucine increase the protein synthesis in skeletal muscle of humans (Norton and Layman., 2006), (Duan, et al., 2015) and is for this reason sold as a supplement among athletes and gym enthusiasts to increase muscle cell size. However, there were increased liver HSI in the high-fat group supplemented with leucine, which may either indicate increased protein or increased fat content. Whether this is due to increased protein synthesis in liver needs further inspection of the microarray data to examine if the genes related to the protein synthesis are affected in the high-leucine fish group.

Lipid composition of tissues

In terms of the total fat percentage in the different tissues, a trend of dietary DHA effect in muscle tissue of the normal-fat groups was observed where the total fat percentage was reduced. The reducing tendency caused by DHA in the total fat content of the muscle tissue is further supported by the high number of gene expressions that are either down-or upregulated in the muscle tissue of the normal-fat with DHA fish group. The high number of genes differently expressed relative to the group not supplemented with DHA is a strong indication

of effects of DHA in muscle, resulting in high metabolic activity in the muscle tissue. This is in agreement with a recent study by (Horn et al., 2019). This reduction is in accordance with Todorčević, et al. (2009) where DHA is showed to reduce the fat level in whole body of Atlantic salmon. Also, Bou et al (2016) shows that a reduction in EPA and DHA results in accumulated fat in the viscera and liver, emphasizing the importance of dietary DHA.

The combination of dietary leucine and DHA showed a strong trend of increasing the total fat content in the liver tissue of fish from the high-fat group. The increasing tendency caused by the both the dietary leucine and DHA in the total fat content of the liver tissue is further supported by the high number of differently expressed genes in this group relative to the high-fat control fish group. The number of genes affected is a strong indication of influence on metabolic activity in the liver tissue. The high fat percentage (approximately 10%) in liver in this group may be interpreted as an early sign of development towards fatty liver with the combination of high fat diet with excess leucine and DHA. A fatty liver is the first sign of metabolic nutritional imbalance in salmon (Sissener et al., 2016), where both a lack of or an excess of nutrients reduces the liver health considerably. An excess of leucine can result in the excess of amino acids being converted to fat and fatty acids through de novo lipogenesis as described by Bou, et al., (2016). Another finding supporting this assumption is that the relative level of 18:1n-9 fatty acid in liver tissue is increased in the high-fat groups compared to the normal-fat groups, in particular when leucine is supplemented, which could also be an indication of a development of fatty liver, due to increased de novo synthesis of lipids, since 18:1n-9 is a product of this synthesis (in addition to coming from the diet). Another interesting aspect of the liver tissue is the low increase in DHA % in the high-fat groups when DHA is supplemented in the diet. A possible explanation could be that the novo synthesis of 18:1n-9 lead to a thinning out effect of the tissue DHA and thereby the expected increase in DHA with increased level of this in the diet is not observed for the liver, but only in muscle. Further studies of gene expressions from microarray data are needed to examine this, if de novo lipogenesis is increased by leucine in liver.
However our finding that leucine seem to increase fat deposition in liver of fish fed high fat diets is in contradiction with findings in humans an improvement of liver health through the reduction in liver lipid droplet size has been suggested as a result of a reduction of dietary branched-chain amino acids (BCAAs) in obese mice (Cummings., 2017). Oppositely, a supplement of dietary leucine has also been indicated as a positive influence on ATP production in liver and subsequently increased liver weight in intrauterine growth-retarded

weaning piglets (Weipeng, S. et al., 2017). These results substantiate that leucine has an effect on liver tissue health, although its metabolic influence is not yet fully understood, but the effect is probably dose depend and the effect also varies in different tissues.

Adding dietary leucine alone seems to reduce the EPA percentage in liver tissue of fish from the normal-fat and high-fat groups. As in the case of DHA percentage reduction in liver, this could possibly be a sign of increased de novo synthesis of other lipids and a thinning effect on EPA. As the C18:1n-9 increases from 17,5-18,9% in the normal-fat groups to 25,8-31,0% in the high-fat groups, this reduces the liver EPA in the high-fat groups. The reduction of liver EPA can ultimately affect the liver health as EPA is known to prevent the development of fatty liver (Tanaka et al 2010). In humans, some amino acids including leucine, are also shown to induce the de novo lipogenesis through the insulin signaling pathway and induce insulin resistance (Charidemou et al 2019).

Summary

The fish grew from 4 g to 22 grams during the trial and the fat, protein, leucine or DHA levels in the diet did not influence the growth. The protein to fat ratio in the feed affected however the whole body protein and fat contents of the fish, with higher protein and less fat in the low fat dietary groups. Dietary leucine did not increase protein and muscle growth, but affected the liver fat deposition negatively in combination with high fat diet. The increased fat deposition in liver by leucine may be due to increased de novo synthesis of fatty acids leading to increased relative level of 18:1n-9 and reduced relative levels of the omega-3 fatty acids EPA and DHA in liver. DHA has a reducing effect on the lipid deposition in general and led to reduced fat percentage in muscle tissue in fish in the low fat group, leucine had less effect in muscle. DHA influenced a large number of genes in the muscle in the low fat dietary groups and leucine influenced a large number of genes in the high fat groups, showing that these ingredients have different effects in different tissues.

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Appendix

Table A1. Fatty acid composition (%) in whole-body Atlantic salmon fed different levels of fat, leucine and DHA. *The mean and standard error of the mean (SEM) values are shown for each fatty acid along with the p-value for each fatty acid (n=3).*

Fatty acids	Diets						
	NL,NF	HL,NF	HL,NF+DHA	NL,HF	HL,HF	HL,HF+DHA	p-value
Folch	12,7 ± 0,14	12,4 ± 0,18	12,4 ± 0,11	14,6 ± 0,12	14,5 ± 0,26	13,8 ± 0,23	<0,01
C 14:0	4,9 ± 0,09	4,8 ± 0,13	3,5 ± 0,11	3,6 ± 0,03	3,8 ± 0,04	2,7 ± 0,04	<0,01
C 15:0	0,3 ± 0,05	0,3 ± 0,05	0,2 ± 0,04	0,3 ± 0,06	0,3 ± 0,03	0,2 ± 0,01	0,71
C 16:0	15,2 ± 0,26	15,3 ± 0,33	13,4 ± 0,44	12,1 ± 0,10	12,4 ± 0,15	10,8 ± 0,17	<0,01
C 17:0	0,3 ± 0,02	0,2 ± 0,02	0,2 ± 0,03	0,4 ± 0,17	0,2 ± 0,01	0,2 ± 0,00	0,43
C 18:0	3,3 ± 0,09	3,3 ± 0,06	3,5 ± 0,17	2,7 ± 0,05	2,7 ± 0,06	2,9 ± 0,09	0,00
C 20:0	0,3 ± 0,03	0,3 ± 0,04	0,4 ± 0,04	0,4 ± 0,03	0,3 ± 0,02	0,4 ± 0,06	0,05
ΣSFA[1]	24,4 ± 0,59	24,3 ± 0,42	22,3 ± 0,53	19,5 ± 0,14	19,6 ± 0,17	18,0 ± 0,29	<0,01
C 14:1n-5	0,2 ± 0,03	0,2 ± 0,03	0,1 ± 0,02	0,2 ± 0,06	0,2 ± 0,02	0,1 ± 0,01	0,61
C 16:1n-5	0,2 ± 0,02	0,1 ± 0,01	0,1 ± 0,02	0,1 ± 0,02	0,1 ± 0,02	0,1 ± 0,01	0,54
C 16:1n-7	3,9 ± 0,02	4,0 ± 0,07	2,9 ± 0,02	2,8 ± 0,02	2,8 ± 0,10	2,1 ± 0,04	<0,01
C 16:1n-9	0,3 ± 0,00	0,3 ± 0,01	0,3 ± 0,03	0,3 ± 0,01	0,3 ± 0,00	0,3 ± 0,01	0,01
C 17:1n-7	0,2 ± 0,01	0,2 ± 0,02	0,2 ± 0,02	0,2 ± 0,01	0,2 ± 0,01	0,2 ± 0,01	0,03
C 18:1n-7	2,5 ± 0,01	2,5 ± 0,03	2,4 ± 0,01	2,7 ± 0,02	2,7 ± 0,02	2,7 ± 0,02	<0,01
C 18:1n-9	18,6 ± 0,15	18,9 ± 0,15	20,8 ± 0,57	27,4 ± 0,36	27,5 ± 0,10	29,1 ± 0,21	<0,01
C 18:1n-11	1,9 ± 0,01	1,6 ± 0,16	1,3 ± 0,01	1,3 ± 0,01	1,3 ± 0,02	0,9 ± 0,00	<0,01
C 20:1n-7	0,3 ± 0,02	0,3 ± 0,06	0,4 ± 0,05	0,3 ± 0,05	0,2 ± 0,01	0,3 ± 0,03	0,40
C 20:1n-9	7,1 ± 0,08	7,2 ± 0,08	5,5 ± 0,05	6,1 ± 0,04	6,4 ± 0,06	5,0 ± 0,04	<0,01
C 20:1n-11	1,4 ± 0,02	1,4 ± 0,03	1,1 ± 0,06	1,3 ± 0,01	1,2 ± 0,01	0,9 ± 0,02	<0,01
C 22:1n-7	0,8 ± 0,01	0,8 ± 0,07	0,8 ± 0,05	0,8 ± 0,05	0,7 ± 0,01	0,7 ± 0,06	0,29
C 22:1n-9	0,8 ± 0,04	0,8 ± 0,06	0,8 ± 0,06	0,7 ± 0,04	0,6 ± 0,11	0,7 ± 0,05	0,16
C 22:1n-11	9,1 ± 0,13	9,4 ± 0,12	6,5 ± 0,03	7,1 ± 0,02	7,4 ± 0,02	5,1 ± 0,08	<0,01
C 24:1n-9	1,0 ± 0,08	1,1 ± 0,10	1,2 ± 0,05	0,9 ± 0,05	0,9 ± 0,08	1,0 ± 0,01	0,10
ΣMUFA	48,4 ± 0,37	48,6 ± 0,09	44,5 ± 0,38	52,2 ± 0,28	52,5 ± 0,14	49,2 ± 0,21	<0,01
C 18:3n-3	1,6 ± 0,01	1,6 ± 0,02	2,2 ± 0,01	3,0 ± 0,03	3,0 ± 0,02	3,5 ± 0,02	<0,01
C 20:3n-3	0,3	0,2	0,4 ± 0,11	0,4 ± 0,07	0,3 ± 0,02	0,5 ± 0,03	0,38
C 20:4n-3	1,4 ± 0,01	1,4 ± 0,07	1,2 ± 0,03	1,0 ± 0,02	1,0 ± 0,01	0,8 ± 0,03	<0,01
C 20:5n-3	2,7 ± 0,11	2,6 ± 0,05	2,6 ± 0,05	2,0 ± 0,03	2,0 ± 0,03	2,0 ± 0,03	<0,01
C 22:5n-3	1,3 ± 0,07	1,3 ± 0,09	1,4 ± 0,12	0,9 ± 0,05	0,8 ± 0,03	1,0 ± 0,04	0,00
C 22:6n-3	9,5 ± 0,26	9,9 ± 0,06	13,5 ± 0,48	7,6 ± 0,15	7,8 ± 0,04	10,8 ± 0,08	<0,01
Σn-3	16,5 ± 0,20	16,7 ± 0,12	21,3 ± 0,67	15,0 ± 0,17	14,9 ± 0,07	18,5 ± 0,13	<0,01
C 16:2n-6	0,4 ± 0,01	0,3 ± 0,01	0,3 ± 0,04	0,3 ± 0,01	0,3 ± 0,01	0,2 ± 0,01	0,00
C 18:2n-6	6,1 ± 0,07	6,0 ± 0,09	7,4 ± 0,06	9,0 ± 0,09	9,0 ± 0,08	9,9 ± 0,05	<0,01
C 18:3n-6	0,3 ± 0,01	0,2 ± 0,02	0,3 ± 0,03	0,3 ± 0,01	0,3 ± 0,01	0,3 ± 0,07	0,27
C 20:2n-6	0,6 ± 0,02	0,6 ± 0,02	0,9 ± 0,06	0,8 ± 0,04	0,8 ± 0,02	1,0 ± 0,05	0,00

20:3n-6	0,4 ± 0,02	0,3 ± 0,03	0,4 ± 0,05	0,5 ± 0,06	0,3 ± 0,02	0,4 ± 0,03	0,39
C20:4n-6	0,4 ± 0,01	0,4 ± 0,06	0,7 ± 0,03	0,3 ± 0,03	0,3 ± 0,02	0,5 ± 0,20	0,05
Σn-6	8,2 ± 0,06	7,9 ± 0,05	9,9 ± 0,17	11,2 ± 0,04	11,0 ± 0,04	12,3 ± 0,33	<0,01
C 16:3n-4	0,2 ± 0,01	0,2 ± 0,01	0,2 ± 0,03	0,2 ± 0,02	0,2 ± 0,01	0,1 ± 0,00	0,04
Σtotal	99,4 ± 0,15	99,2 ± 0,12	99,6 ± 0,07	99,3 ± 0,22	99,5 ± 0,09	99,1 ± 0,06	0,15
ΣEPA/DHA	12,1 ± 0,34	12,5 ± 0,06	16,1 ± 0,51	9,6 ± 0,14	9,7 ± 0,08	12,9 ± 0,05	<0,01

[1] Includes C24:0

Table A2. Fatty acid composition (%) in liver tissue of Atlantic salmon fed different levels of fat, leucine and DHA. *The mean and standard error of the mean (SEM) values are shown for each fatty acid along with the p-value for each fatty acid (n=3).*

Fatty acids	Diets						
	NL,NF	HL,NF	HL,NF+DHA	NL,HF	HL,HF	HL,HF+DHA	p-value
Folch	6,2 ± 0,99	6,2 ± 0,71	6,0 ± 1,14	7,6 ± 0,69	8,3 ± 1,01	10,4 ± 0,34	0,0223
C14:0	1,8 ± 0,11	2,0 ± 0,14	1,7 ± 0,06	1,9 ± 0,16	2,0 ± 0,10	1,4 ± 0,09	0,0355
C15:0	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,04	0,2 ± 0,02	0,1 ± 0,05	0,2 ± 0,02	0,334
C16:0	16,3 ± 0,42	16,0 ± 0,42	15,9 ± 0,63	12,0 ± 0,02	10,5 ± 0,10	8,7 ± 0,69	<0,01
C17:0	0,3 ± 0,06	0,2 ± 0,02	0,2 ± 0,02	0,2 ± 0,02	0,2 ± 0,01	0,2 ± 0,01	0,735
C18:0	4,5 ± 0,11	4,9 ± 0,11	5,5 ± 0,18	4,3 ± 0,18	4,2 ± 0,30	4,0 ± 0,35	0,00484
C20:0	0,1 ± 0,01	0,1 ± 0,00	0,2 ± 0,02	0,3 ± 0,09	0,2 ± 0,01	0,2 ± 0,03	0,334
C22:0	0,2 ± 0,02	0,1 ± 0,02	0,2 ± 0,02	0,4 ± 0,02	0,4 ± 0,01	0,7 ± 0,05	<0,01
C24:0	0,4 ± 0,02	0,4 ± 0,04	1,3 ± 0,06	0,2 ± 0,01	0,2 ± 0,01	0,6 ± 0,05	<0,01
ΣSFA	23,7 ± 0,31	23,8 ± 0,52	25,1 ± 0,55	19,5 ± 0,33	17,8 ± 0,27	16,2 ± 0,57	<0,01
C14:1n-5	0,1 ± 0,01	0,1 ± 0,01	0,1 ± 0,01	0,1 ± 0,01	0,1 ± 0,01	0,1 ± 0,01	0,136
C16:1n-5	0,2 ± 0,04	0,2 ± 0,04	0,2 ± 0,04	0,2 ± 0,04	0,2 ± 0,02	0,1 ± 0,03	0,597
C16:1n-9	2,2 ± 0,27	2,6 ± 0,26	1,8 ± 0,12	1,8 ± 0,15	2,1 ± 0,07	1,6 ± 0,04	0,0164
C17:1n-7	0,2 ± 0,00	0,2 ± 0,02	0,2 ± 0,01	0,2 ± 0,01	0,2 ± 0,01	0,2 ± 0,01	0,598
C18:1n-7	1,9 ± 0,11	2,0 ± 0,12	1,7 ± 0,09	2,6 ± 0,22	2,6 ± 0,01	2,2 ± 0,33	0,0161
C18:1n-9	17,5 ± 1,36	18,9 ± 1,72	17,7 ± 0,19	25,8 ± 1,97	29,9 ± 1,22	31,0 ± 2,42	0,000101
C18:1n-11	2,3 ± 0,01	2,4 ± 0,15	1,4 ± 0,10	2,4 ± 0,01	2,9	1,1 ± 0,96	0,0903
C20:1n-7	0,1 ± 0,00	0,1 ± 0,01	0,1 ± 0,01	0,1 ± 0,01	0,2 ± 0,00	0,2 ± 0,02	0,0182
C20:1n-9	4,6 ± 0,36	4,9 ± 0,39	3,6 ± 0,43	5,7 ± 0,20	6,9 ± 0,07	5,9 ± 0,58	0,000901
C20:1n-11	0,4 ± 0,07	0,3 ± 0,01	0,2 ± 0,01	0,5 ± 0,08	0,4 ± 0,03	0,3 ± 0,01	0,0401
C22:1n-7	0,6 ± 0,05	0,5 ± 0,01	0,5 ± 0,01	0,7 ± 0,00	0,7 ± 0,01	0,6 ± 0,10	0,0405
C22:1n-9	0,4 ± 0,03	0,4 ± 0,04	0,3 ± 0,05	0,4 ± 0,02	0,5 ± 0,01	0,4 ± 0,06	0,245
C22:1n-11	2,9 ± 0,39	3,0 ± 0,29	1,7 ± 0,31	2,6 ± 0,23	2,8 ± 0,12	1,7 ± 0,33	0,0227
C24:1n-9	1,2 ± 0,08	1,2 ± 0,03	0,9 ± 0,25	1,0 ± 0,07	0,9 ± 0,04	0,7 ± 0,10	0,0758
ΣMUFA[1]	34,6 ± 2,55	37,0 ± 2,88	29,9 ± 1,87	43,3 ± 1,56	48,3 ± 0,83	45,7 ± 2,70	0,00047
C18:3n3	0,8 ± 0,13	0,7 ± 0,02	0,9 ± 0,05	1,7 ± 0,04	1,8 ± 0,03	2,6 ± 0,09	<0,01
C20:3n-3	1,3 ± 0,12	1,2 ± 0,17	2,0 ± 0,06	1,1 ± 0,12	0,9 ± 0,06	1,2 ± 0,13	0,000607
C20:4n-3	0,7 ± 0,03	0,7 ± 0,18	0,5 ± 0,06	0,8 ± 0,01	0,8 ± 0,01	0,6 ± 0,05	0,163
C20:5n-3	3,3 ± 0,21	3,0 ± 0,26	2,5 ± 0,10	2,3 ± 0,06	1,8 ± 0,04	1,7 ± 0,15	<0,01

C22:5n-3	1,2 ± 0,04	1,1 ± 0,09	0,9 ± 0,04	0,7 ± 0,01	0,6 ± 0,01	0,6 ± 0,02	<0,01
C22:6n-3	25,6 ± 2,43	24,0 ± 2,06	29,4 ± 1,35	19,5 ± 1,92	15,8 ± 0,91	17,3 ± 2,15	0,00168
Σn-3	33,1 ± 2,65	30,9 ± 2,32	36,2 ± 1,45	25,7 ± 1,75	21,8 ± 0,99	24,0 ± 2,43	0,00182
C16:2n-6	0,1 ± 0,02	0,1 ± 0,01	0,2 ± 0,11	0,1 ± 0,00	0,1 ± 0,01	0,1 ± 0,00	0,298
C18:2 n-6	3,7 ± 0,43	3,5 ± 0,13	4,1 ± 0,16	6,5 ± 0,17	6,9 ± 0,06	8,3 ± 0,23	<0,01
C18:3n-6	0,2 ± 0,01	0,2	0,2 ± 0,03	0,2 ± 0,04	0,1 ± 0,02	0,1 ± 0,04	0,682
C20:2n-6	0,9 ± 0,05	0,9 ± 0,02	1,1 ± 0,06	1,6 ± 0,07	1,8 ± 0,04	2,3 ± 0,13	<0,01
C20:3n-6	1,1 ± 0,06	1,0 ± 0,07	0,8 ± 0,11	1,0 ± 0,03	0,9 ± 0,02	0,6 ± 0,02	0,00116
Σn-6	5,9 ± 0,46	5,6 ± 0,25	6,2 ± 0,19	9,4 ± 0,16	9,8 ± 0,11	11,4 ± 0,32	<0,01
Σtotal[2]	99,6 ± 0,06	99,4 ± 0,36	99,2 ± 0,06	99,7 ± 0,09	99,6 ± 0,05	99,1 ± 0,19	0,147
Σ EPA/DHA	29,0 ± 2,64	27,0 ± 2,27	31,9 ± 1,45	21,7 ± 1,98	17,6 ± 0,94	19,0 ± 2,29	0,00142

[1] Includes C16:1 n-7
[2] Includes C16:3 n-4

Table A3. Fatty acid composition (%) in muscle tissue of Atlantic salmon fed different levels of fat, leucine and DHA. *The mean and standard error of the mean (SEM) values are shown for each fatty acid along with the p-value for each fatty acid (n=3).*

Fatty acids	Diets						
	NL,NF	HL,NF	HL,NF+DHA	NL,HF	HL,HF	HL,HF+DHA	p-value
Folch	4,8 ± 0,36	5,5 ± 0,19	4,6 ± 0,34	5,7 ± 0,15	6,3 ± 0,19	6,2 ± 0,32	0,00435
C14:0	4,2 ± 0,17	4,2 ± 0,09	2,9 ± 0,20	3,0 ± 0,08	3,0 ± 0,09	2,1 ± 0,15	<0,01
C15:0	0,4 ± 0,05	0,3 ± 0,06	0,3 ± 0,00	0,3 ± 0,00	0,3 ± 0,01	0,2 ± 0,02	0,01
C16:0	14,6 ± 0,13	14,7 ± 0,06	13,1 ± 0,05	11,9 ± 0,12	12,0 ± 0,06	10,7 ± 0,10	<0,01
C17:0	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,01	0,2 ± 0,00	0,00
C18:0	3,0 ± 0,03	3,1 ± 0,07	3,3 ± 0,02	2,5 ± 0,08	2,7 ± 0,19	2,7 ± 0,09	0,00
C20:0	0,2 ± 0,01	0,2 ± 0,00	0,3 ± 0,00	0,3 ± 0,00	0,3 ± 0,06	0,3 ± 0,01	0,00
C22:0	0,2 ± 0,00	0,3 ± 0,12	0,2 ± 0,00	0,2 ± 0,01	0,3 ± 0,00	0,3 ± 0,00	0,06
C24:0	0,2 ± 0,03	0,2 ± 0,04	0,8 ± 0,02	0,2	0,2 ± 0,01	0,3 ± 0,24	0,01
ΣSFA	23,1 ± 0,08	23,1 ± 0,04	21,1 ± 0,14	18,5 ± 0,14	19,0 ± 0,15	16,8 ± 0,07	<0,01
C14:1n-5	0,2 ± 0,01	0,2 ± 0,03	0,1 ± 0,00	0,1 ± 0,00	0,1 ± 0,00	0,1 ± 0,00	0,00
C16:1n-7	0,2 ± 0,04	0,1 ± 0,04	0,1 ± 0,03	0,2 ± 0,00	0,2 ± 0,01	0,1 ± 0,00	0,74
C16:1n-9	3,6 ± 0,10	3,7 ± 0,08	2,6 ± 0,04	2,5 ± 0,05	2,5 ± 0,05	2,0 ± 0,16	<0,01
C17:1n-7	0,2 ± 0,02	0,2 ± 0,02	0,2 ± 0,01	0,2 ± 0,01	0,2 ± 0,00	0,1 ± 0,01	0,01
C18:1n-7	2,4 ± 0,03	2,4 ± 0,02	2,4 ± 0,02	2,6 ± 0,03	2,6 ± 0,00	2,6 ± 0,02	<0,01
C18:1n-9	19,7 ± 0,74	19,4 ± 0,54	21,4 ± 0,66	27,7 ± 0,13	28,3 ± 0,50	30,2 ± 0,30	<0,01
C18:1n-11	2,0	1,9 ± 0,02	1,3 ± 0,07	0,7 ± 0,11	0,6 ± 0,03		0,00
C20:1n-7	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,01	0,1 ± 0,00	0,00
C20:1n-9	7,2 ± 0,04	7,2 ± 0,07	5,6 ± 0,03	6,1 ± 0,13	6,5 ± 0,04	5,0 ± 0,03	<0,01
C20:1n-11	1,4 ± 0,03	1,3 ± 0,02	1,0 ± 0,03	1,2 ± 0,03	1,2 ± 0,03	0,9 ± 0,01	<0,01
C22:1n-7	0,8 ± 0,01	0,8 ± 0,00	0,6 ± 0,10	0,7 ± 0,03	0,7 ± 0,01	0,6 ± 0,01	0,03
C22:1n-9	0,6 ± 0,10	0,6 ± 0,11	0,6 ± 0,00	0,6 ± 0,01	0,6 ± 0,01	0,6 ± 0,00	0,94
C22:1n-11	9,2 ± 0,06	9,2 ± 0,08	6,4 ± 0,02	7,0 ± 0,09	7,4 ± 0,07	5,0 ± 0,07	<0,01

C24:1n-9	0,9 ± 0,02	0,9 ± 0,00	0,9 ± 0,02	0,7 ± 0,00	0,8 ± 0,02	0,8 ± 0,01	<0,01
ΣMUFA[1]	47,2 ± 0,31	47,4 ± 0,41	43,0 ± 0,35	50,4 ± 0,49	51,7 ± 0,15	48,2 ± 0,11	<0,01
C18:3n-3	1,7 ± 0,01	1,6 ± 0,02	2,3 ± 0,02	3,3 ± 0,10	3,2 ± 0,02	3,7 ± 0,08	<0,01
C20:3n-3	0,4 ± 0,01	0,4 ± 0,00	0,7 ± 0,00	0,3 ± 0,01	0,3 ± 0,00	0,5 ± 0,01	<0,01
C20:4n-3	1,1 ± 0,01	1,1 ± 0,02	0,8 ± 0,01	0,8 ± 0,01	0,8 ± 0,03	0,6 ± 0,02	<0,01
C20:5n-3	2,9 ± 0,02	2,8 ± 0,06	2,7 ± 0,07	2,2 ± 0,04	2,0 ± 0,04	2,0 ± 0,02	<0,01
C22:5n-3	1,2 ± 0,01	1,1 ± 0,03	1,2 ± 0,03	0,9 ± 0,03	0,8 ± 0,01	0,9 ± 0,01	<0,01
C22:6n-3	12,7 ± 0,46	12,9 ± 0,17	17,5 ± 0,47	11,1 ± 0,40	10,2 ± 0,27	14,0 ± 0,12	<0,01
Σn-3	20,0 ± 0,46	19,8 ± 0,12	25,0 ± 0,47	18,6 ± 0,37	17,2 ± 0,30	21,7 ± 0,09	<0,01
C16:2n-6	0,3 ± 0,01	0,3 ± 0,00	0,2 ± 0,01	0,2 ± 0,01	0,2 ± 0,02	0,2 ± 0,01	<0,01
C18:2n-6	6,0 ± 0,14	5,8 ± 0,04	7,2 ± 0,16	9,1 ± 0,21	9,0 ± 0,08	9,9 ± 0,04	<0,01
C18:3n-6	0,2 ± 0,02	0,2 ± 0,02	0,2 ± 0,05	0,2 ± 0,02	0,2 ± 0,02	0,2 ± 0,02	0,74
C20:2n-6	0,6 ± 0,01	0,6 ± 0,01	0,8 ± 0,02	0,7 ± 0,02	0,8 ± 0,01	0,9 ± 0,02	<0,01
C20:3n-6	0,3 ± 0,00	0,3 ± 0,01	0,3 ± 0,04	0,4 ± 0,02	0,4 ± 0,00	0,3 ± 0,02	0,15
Σn-6	7,4 ± 0,17	7,2 ± 0,04	8,6 ± 0,19	10,6 ± 0,19	10,6 ± 0,08	11,4 ± 0,02	<0,01
C16:3n-4	0,2 ± 0,01	0,2 ± 0,01	0,1 ± 0,02	0,1 ± 0,01	0,1 ± 0,01	0,1 ± 0,01	0,00
Σtotal	99,5 ± 0,06	99,4 ± 0,19	99,4 ± 0,12	99,8 ± 0,11	99,8 ± 0,10	99,3 ± 0,10	0,05
Σ EPA/DHA	15,5 ± 0,46	15,6 ± 0,20	20,2 ± 0,54	13,4 ± 0,43	12,1 ± 0,30	16,0 ± 0,14	<0,01

[1] Includes C16:1n-5

Table A4. Fatty acid composition (%) in adipose tissue of Atlantic salmon fed different levels of fat, leucine and DHA. *The mean and standard error of the mean (SEM) values are shown for each fatty acid along with the p-value for each fatty acid (n=3).*

Fatty acids	Diets						
	NL,NF	HL,NF	HL,NF+DHA	NL,HF	HL,HF	HL,HF+DHA	p-value
Folch	102,9 ± 1,57	100,2 ± 0,39	103,1 ± 0,59	100,9 ± 0,24	101,3 ± 1,56	103,9 ± 2,20	0,355
C14:0	4,7 ± 0,05	4,8 ± 0,04	3,6 ± 0,03	3,7 ± 0,03	3,7 ± 0,05	3,1 ± 0,02	<0,01
C15:0	0,3 ± 0,00	0,3 ± 0,00	0,3 ± 0,01	0,3 ± 0,02	0,3 ± 0,01	0,2 ± 0,00	<0,01
C16:0	13,4 ± 0,14	13,6 ± 0,10	12,0 ± 0,08	11,1 ± 0,09	11,3 ± 0,04	10,6 ± 0,35	<0,01
C17:0	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,01	0,2 ± 0,01	0,2 ± 0,00	0,3 ± 0,08	0,831
C18:0	2,8 ± 0,14	3,0 ± 0,08	3,2 ± 0,03	2,6 ± 0,21	2,4 ± 0,06	2,5 ± 0,04	0,00299
C20:0	0,2 ± 0,01	0,2 ± 0,00	0,3 ± 0,00	0,3 ± 0,00	0,3 ± 0,00	0,3 ± 0,01	<0,01
ΣSFA[1]	21,7 ± 0,28	22,3 ± 0,08	20,4 ± 0,04	18,4 ± 0,23	18,6 ± 0,06	17,5 ± 0,15	<0,01
C14:1n-5	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,03	0,1 ± 0,00	0,2 ± 0,00	0,2 ± 0,08	0,792
C16:1n-5	0,2 ± 0,00	0,2 ± 0,03	0,2 ± 0,03	0,2 ± 0,04	0,2 ± 0,00	0,2 ± 0,03	0,757
C16:1n-9	4,3 ± 0,04	4,4 ± 0,06	3,6 ± 0,05	3,3 ± 0,03	3,4 ± 0,05	3,1 ± 0,10	<0,01
C17:1n-7	0,2 ± 0,00	0,2 ± 0,01	0,2 ± 0,01	0,3 ± 0,13	0,2 ± 0,00	0,1 ± 0,00	0,418
C18:1n-7	2,7 ± 0,02	2,7 ± 0,05	2,7 ± 0,02	3,0 ± 0,13	2,9 ± 0,03	2,8 ± 0,04	0,0487
C18:1n-9	19,2 ± 0,26	19,6 ± 0,10	21,8 ± 0,18	27,7 ± 0,19	27,4 ± 0,30	28,2 ± 0,14	<0,01
C18:1n-11	1,8 ± 0,03	1,8 ± 0,01	1,1 ± 0,09	1,3 ± 0,05	1,2 ± 0,07	0,9 ± 0,03	<0,01
C20:1n-7	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,01	0,2 ± 0,08	0,2 ± 0,00	0,2 ± 0,00	0,636

C20:1n-9	7,7 ± 0,00	7,7 ± 0,04	5,8 ± 0,06	6,4 ± 0,06	6,6 ± 0,10	5,2 ± 0,05	<0,01
C20:1n-11	1,6 ± 0,04	1,6 ± 0,02	1,3 ± 0,09	1,4 ± 0,01	1,4 ± 0,02	1,1 ± 0,03	<0,01
C22:1n-7	0,9 ± 0,00	0,9 ± 0,06	0,8 ± 0,01	0,8 ± 0,01	0,8 ± 0,01	0,7 ± 0,01	0,00373
C22:1n-9	0,6 ± 0,12	0,8 ± 0,03	0,6 ± 0,00	0,6 ± 0,01	0,7 ± 0,01	0,6 ± 0,01	0,249
C22:1n-11	9,6 ± 0,09	9,6 ± 0,21	6,7 ± 0,06	7,2 ± 0,05	7,4 ± 0,11	5,3 ± 0,06	<0,01
C24:1n-9	0,8 ± 0,01	0,7 ± 0,01	0,8 ± 0,01	0,7 ± 0,07	0,6 ± 0,01	0,6 ± 0,00	0,022
ΣMUFA[2]	50,3 ± 0,42	50,6 ± 0,31	45,9 ± 0,05	53,4 ± 0,07	53,0 ± 0,43	49,4 ± 0,25	<0,01
C18:3n-3	1,9 ± 0,03	1,7 ± 0,00	2,3 ± 0,03	3,2 ± 0,05	3,1 ± 0,04	3,5 ± 0,05	<0,01
C20:3n-3	0,3 ± 0,00	0,4 ± 0,07	0,5 ± 0,00	0,2 ± 0,00	0,4 ± 0,21	0,4 ± 0,01	0,354
C20:4n-3	1,1 ± 0,01	1,1 ± 0,02	0,8 ± 0,03	0,8 ± 0,01	0,8 ± 0,01	0,6 ± 0,03	<0,01
C20:5n-3	2,9 ± 0,08	2,9 ± 0,03	3,1 ± 0,03	2,3 ± 0,06	2,3 ± 0,08	2,6 ± 0,01	<0,01
C22:5n-3	1,4 ± 0,06	1,3 ± 0,02	1,5 ± 0,01	1,0 ± 0,02	1,0 ± 0,02	1,2 ± 0,01	<0,01
C22:6n-3	9,4 ± 0,16	9,2 ± 0,11	13,7 ± 0,14	7,5 ± 0,18	7,7 ± 0,16	10,8 ± 0,19	<0,01
Σn-3	17,1 ± 0,23	16,8 ± 0,18	22,2 ± 0,31	15,4 ± 0,21	15,6 ± 0,36	20,1 ± 0,63	<0,01
C16:2n-6	0,4 ± 0,01	0,4 ± 0,01	0,3 ± 0,00	0,4 ± 0,06	0,3 ± 0,01	0,3 ± 0,02	0,0767
C18:2n-6	6,5 ± 0,22	6,3 ± 0,07	7,8 ± 0,30	9,0 ± 0,13	9,0 ± 0,21	9,7 ± 0,23	<0,01
C18:3n-6	0,2 ± 0,00	0,2 ± 0,01	0,2 ± 0,02	0,2 ± 0,00	0,2 ± 0,00	0,2 ± 0,01	0,0382
C20:2n-6	0,6 ± 0,01	0,6 ± 0,01	0,7 ± 0,01	0,8 ± 0,05	0,8 ± 0,04	0,8 ± 0,01	<0,01
C20:3n-6	0,3 ± 0,01	0,3 ± 0,01	0,3 ± 0,01	0,3 ± 0,00	0,3 ± 0,00	0,3 ± 0,07	0,605
Σn-6	7,9 ± 0,24	7,7 ± 0,09	9,4 ± 0,31	10,6 ± 0,12	10,7 ± 0,18	11,3 ± 0,27	<0,01
C16:3n-4	0,3 ± 0,00	0,3 ± 0,01	0,2 ± 0,00	0,2 ± 0,02	0,2 ± 0,01	0,2 ± 0,00	0,0472
Σtotal	99,3 ± 0,19	99,6 ± 0,10	99,8 ± 0,15	99,7 ± 0,11	99,8 ± 0,10	99,8 ± 0,04	0,185
ΣEPA/DHA	12,3 ± 0,22	12,1 ± 0,13	16,8 ± 0,15	9,9 ± 0,24	10,0 ± 0,23	13,4 ± 0,19	<0,01

[1] Includes C22:0 and C24:0

[2] Includes C16:1n-7

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