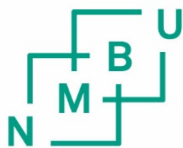

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Fatty acid composition and gene expression in the third generation of Atlantic salmon families selected for high and low capacity of omega3 fatty acid synthesis

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ABBRIVIATIONS

ALA	α-linolenic acid
ACO	Acyl-CoA oxidase and its gene
CO	Canola oil
ChREBPs	Carbohydrate response element binding proteins
cDNA	complementary DNA
DHA	Docosahexaenoic acid
DM	Dry matter
D5d	Delta/Δ-5 desaturase gene
D6dA	Delta/Δ-6 desaturase gene A
D6dB	Delta/Δ-6 desaturase gene B
D6dC	Delta/Δ-6 desaturase gene C
dsDNA	Double-stranded DNA.
EFA	Essential fatty acid
ELOVL	Elongase of very long chain fatty acid
Elovl2	ELOVL2 gene
Elovl5a	ELOVL5 gene a
Elovl5b	ELOVL5 gene b
EO	Echium oil
EPA	Eicosapentaenoic acid
FAD	Flavin adenine dinucleotide
Fad	Fatty acid desaturase
FO	Fish oil
GC	Gas chromatography
HUFA	Highly unsaturated fatty acid
LC-PUFA	Long chain polyunsaturated fatty acid
LA	Linoleic acid
LXRs	Liver X receptors
MUFA	Monounsaturated fatty acid
mRNA	messenger RNA
NAD^+	Nicotinamide adenine dinucleotide
OA	Oleic acid
PPARs	Peroxisome proliferator-activated receptors
PUFA	Polyunsaturated fatty acid
qPCR	Real time quantitative PCR
RO	Rapeseed oil
RXRs	Retinoid X receptor
SREBPs	Sterol regulatory element binding proteins
SFA	Saturated fatty acid
SE	Standard error mean
TGs	Triacylglycerols
TxA2	Thromboxane A2
VO	Vegetable oil

ABSTRACT

The major aim of this thesis was to study offspring (the third generation) of Atlantic salmon selected by high and low EPA and DHA synthesis capacity (according to high and low d6dB expression level in liver) have differences in total fat content and fatty acid composition, and also difference in gene expression of genes related to n-3 HUFA synthesis pathway in tissues (liver, intestine and muscle).

The total fat contents in each tissue were analyzed by Folch method and showed no significant difference between two genetic groups. Fatty acid composition was analyzed by GC with methylation method. Liver plus group (offspring of high EPA and DHA synthesis capacity parents) showed significantly higher percentage of EPA+DHA than liver minus group (offspring of low EPA and DHA synthesis capacity parents) and also higher than intestine and muscle. In muscle and intestine, there was no significant difference of EPA+DHA percentage between two genetic groups.

The gene expression was analyzed by qPCR and the relative expression ratio was calculated by method. *elovl5a* was higher in liver plus group, *elovl2* and *d6dA* were higher in intestine minus group. Except *elovl5a, elovl2* and *d6dA*, other genes (include *d6dB*) were with no significant differences between genetic groups in all tissues.

The present study has shown EPA and DHA synthesis capacity in liver was improved by gene selective breeding, but EPA and DHA deposition in flesh was not improved by this capacity when salmon fed marine diet. The muscle fatty acid composition is more likely depended on diet composition. Thus, further studies can focus on dietary effects on EPA and DHA synthesis and deposition capacity of fish selected by high and low EPA and DHA synthesis capacity.

Key words

Atlantic salmon, genetic selection, gene expression, fatty acid composition, total fat content, EPA, DHA, HUFA synthesis pathway.

Aim of study

The main objective is to study capacity of produce omega-3 fatty acids in families of Atlantic salmon selected for high and low capacity.

To achieve the main objective, there are some specific objectives as follows:

1. To study differences in EPA+DHA percentage and total fat content of the third generation of Atlantic salmon selected for high and low n-3 HUFA synthesis capacity (according to expression of d6dB in the liver) in tissues (tissues include liver, intestine and muscle).
2. To study gene expression difference of n-3 HUFA synthesis related genes (desaturase genes: *d5d, d6dA, d6dB* and *d6dC*; elongase genes: *elovl2, elovl5a* and *elovl5b*; β-desaturase gene: *ACO*) in tissues (liver, intestine and muscle).

1. Introduction

1.1 World market of salmon

Fish oil is the main source of highly unsaturated long-chain fatty acids, especially eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, in aquaculture feeds. Recently, the demand of fish oil and fish meal increases exponentially. Especially, in China fishmeal import grew 44%, in Vietnam and Thailand both almost doubled their imports (FAO, 2017). The aquaculture production increased around 20% between 1985 and 2014, while capture fisheries did not change or even decreased in this period (FAO, 2016). Moreover according to the report of FAO (2016), fish oil market faced pressures that unpredictable environmental changes, such as El Niño, caused decreasing of catches. Thus, captured fishes cannot satisfy the demands from a rapid developing aquaculture industry which highly depends on captured marine ingredients.

Modern human diet contain excessive n-6 PUFA, especially 18:2n-6, According to the research of Simopoulos (2002, 2009), the ratio of n-6 to n-3 fatty acids were 15:1-16.7:1 in modern western diet which is far more than the ratio of around 1:1 in early human diet. The high ratio of n-6 to n-3 fatty acid had been shown to relate several health problems, such as cardiovascular, inflammatory and neurological problems (Calder, 2006). The ability of conversion 18:3n-3 to HUFA was low in human, thus to increase n-3 PUFA level, seafood became the major source of n-3 HUFA, and in Western countries, minimum consumption levels of fish have been recommended by various health advisory bodies (WHO, 2002; FAO, 2010).

According to sustainable development principle (WCED 1987), fishing industry cannot fulfill the requirement of aquaculture industry and human needs any more. Two strategies were already considered under this situation. First was using vegetable oil to partially replace fish oil, and second was selective breeding fish with high EPA and DHA production capacity. Vegetable oils are mainly rich in n-6 and n-9 fatty acids, such as linoleic fatty acid (LA, 18:2n-6) and oleic acid (OA, 18:1n-9) (Orsavova et al., 2015). The commonly used vegetable oil in salmon feed is rapeseed oil which is particularly rich in 18:1n-9. Vegetable oils do not contain EPA and DHA, although some study showed a fish diet with mixed fish oil and vegetable oil had a positive effect on salmon growth (Ruyter et al., 2000a; Torstensen et al., 2005). However, the EPA and DHA deposit in fillet of salmon fed with vegetable oil became lower than salmon only fed with fish oil (Sargent et al., 2002; Bell et al., 2010). Selective breeding involved fish with high EPA and DHA synthesis capacity that were accomplished by combining with genetic engineering. The fish with high expression of EPA and DHA synthesis related genes were selected as parents and used to breed offspring (Yitbarak, 2013; Kolditz et al., 2008) .Combining these two strategies may be a feasible solution to improve DHA and EPA level in salmon fillet and reach the DHA and EPA requirements of human diet (Morais et al., 2011).

1.2 Lipids and fatty acids

Lipids include triaclyglycerols, phospholipids, wax esters, sphingolipids and sterols. Triacylglycerols (TGs) are the main energy storage form of fat in fish which consist of three molecules of fatty acids esterified to the sn-1, sn-2 and sn-3 postions of L-glycerol (Tocher, 2003). Phospholipids consist of two molecules of fatty acids esterified to L-glycerol 3-phosphate and are the main compounds of cell membrane bilayers (Sargent et al., 2002).

Fatty acid is one of the main constituents of lipids which is divided into three main groups— saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) according to their degree of unsaturation (the number of double bonds). Saturated fatty acids do not have double bonds, monounsaturated fatty acids have only one double bond, whereas, polyunsaturated fatty acids have two or more double bonds. Precisely, PUFA has over three double bonds and carbon chain length of over C20, is termed highly unsaturated fatty acids (HUFA) (Tocher, 2003). Moreover, one PUFA nomenclature is well known for specifying the

position of first double bond which counts the first carbon from methyl group. In that nomenclature, n-3 PUFA means the first double bond is in the third carbon atom, while n-6 PUFA means the first double is in the sixth carbon atom from the methyl terminus.

1.2.1 Function of lipids

The main function of lipids is providing metabolic energy through β-oxidation for growth and reproduction in fish. Atlantic salmon have poor ability in regulating blood glucose in the situation of over taking carbohydrates, therefore, protein and lipids became the major feed components of Atlantic salmon. (Hemre et al., 1996). Lipids have good protein-sparing effect (Wilson, 1989; Sargent et al., 1989) which can make most protein used for muscle growth instead of using for producing energy. Apart from providing energy, lipids also supply essential fatty acids (EFA). EFAs are the fatty acids which are critical to physiology, but cannot be synthesized in vivo, thus should be taken from diet. The EFAs of Atlantic salmon are mainly 18:3n-3, 18:2n-6, EPA and DHA. Although salmon can biosynthesis n-3 HUFA themselves, n-3 HUFA supplement in diet is still essential for Atlantic salmon growth and EPA/DHA deposition in fillet (Bou et al., 2017a; Bou et al., 017b). In the researches of Bou et al., (2017a), Ruyter et al., (2000b) and Dimitriou (2014), Atlantic salmon in early development stage fed with n-3 HUFA showed higher growth than fed with 18:3n-3 and 18:2n-6. Ruyter et al. (2000a) also found that feeding Atlantic salmon fry with n-3 PUFA can improve growth and survival rate, but n-6 PUFA had no benefit, and the growth-improving effect of EPA and DHA was better than 18:3n-3. This may due to higher nutritional efficiency of n-3 HUFA than 18:3n-3.

In human nutrition, EPA and DHA have many health benefits in the aspects of fetal development, cardiovascular disease, Alzheimer’s disease and so on (Swanson et al., 2012). Apart from EPA and DHA, 20:4n-6 is another important fatty acid which is the precursor of eicosanoids and has a wide range of physiological activities, for example, in the aspects of reproduction, cardiovascular tone, blood clotting and immune response. Over consumption of 18:2n-6 from vegetable source leads to synthesis of excessive level 20:4n-6. Thromboxane A2 (TxA2) which is the derivative of 20:4n-6 is adverse for heart, because it can cause vasospasm, thus excess level of 20:4n-6 is related to cardiovascular diseases (Anonymous 1992; Okuyama et al., 1996). 20:5n-3 can competitively interfere eicosanoid production from 20:4n-6 and 20:5n-3 itself converts to less

biological active derivates, therefore 20:5n-3 is beneficial for excessive 18:2n-6 (Sargent et al., 2002). In this point of view, increasing n-3 HUFA level in human diet is necessary.

1.2.2 Biosynthesis of fatty acids

Fatty acid composition in fish is not only influenced by diet fatty acid composition, but also the metabolism ability of tissues and organs, include desaturation and elongation (Holman 1986). In Salmon, liver is the main organ for regulating fatty acid catabolism and anabolsim (Sargent et al., 2002). Synthesis of the DHA and EPA is a complex process which using C18:3n-3 as a substrate. The elongation and desaturation processes happen alternately, and are finished in peroxisomes by β-oxidation. Going through one β-oxidation can shorten 2 carbon molecules.

The process of synthesizing DHA and EPA in fish is shown in figure 1. There are two alternative pathways for the synthesis from ALA to EPA and DHA (Monroig et al., 2013). One is starting from Δ6 desaturation, and followed by elongation and Δ5 desaturation, another is beginning with Δ8 desaturation, and followed by Δ5 desaturation. The next step of synthesizing DHA from EPA can be achieved by two pathways, one is EPA going through two elongations, one Δ6 desaturation and one β-oxidation to synthesize DHA (Sprecher, 2000), another is directly finished by Δ4 desaturase(Rosenthal, 1987). However, Δ4 desaturation is not yet shown to be functional in Atlantic salmon.

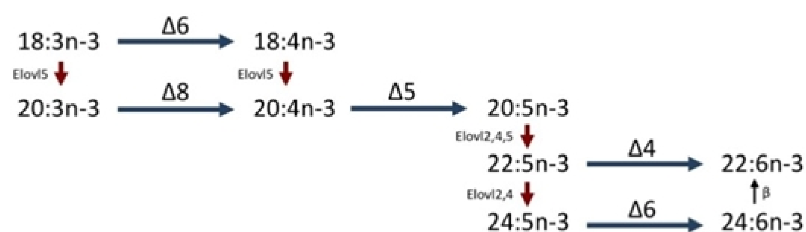

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Fig. 1 General pathway of biosynthesis of EPA and DHA in fish (Monroig, et al., 2013). Elovl2, 4, 5, elongase2, 4, 5 respectively; Δ4, Δ5, Δ6 Δ8, Δ4, Δ5, Δ6 Δ8 desaturase respectively; β, β-oxidation.

1.2.2.1 Elongation

Elongases of very long chain fatty acids (ELOVL) are the enzymes catalyzing carbon chain elongation of PUFA. ELOVL can elongate short chain fatty acids to long chain fatty acids by

introducing 2 carbon atoms each time. In mammals, ELOVL2, ELOVL4 and ELOVL5 were found that participate the HUFA synthesis, whereas in fish, only ELOVL2 and ELOVL5 were found that participate in the synthesis of HUFA. The elongase ELOVL5 was function characterized and cloned in many species, such as Atlantic cod (Agaba et al., 2005), rainbow trout (Meyer et al., 2004), Atlantic Salmon (Hastings et al., 2004; Morais et al., 2009), etc. The genes related to ELOVL2 and ELOVL5 are called *elong2* and *elovl5* respectively. ELOVL5 mainly is responsible for C18 elongation and ELOVL2 is responsible for C20 and C22 elongation (Morais et al., 2009; Agaba et al., 2005; Guillou et al., 2010). Two forms of ELOVL5 which are ELOVL5a and ELOVL5b, and ELOVL2 was found in Atlantic salmon (Morais et al., 2009). All the salmon ELOVL5 have the ability to elongate C18 and C20 PUFA, but had low ability to lengthen C22 (Hastings et al. 2005), whereas salmon ELOVL2 showed low ability in elongation of C18, but high ability in elongation of C20 and C22 (Morais et al., 2009).

1.2.2.2 Desaturation

Fatty acyl desaturase (Fad) is another kind of important enzyme which is responsible for dehydrogenation in synthesis of HUFA. When Fads have Δ4/Δ5/Δ6/Δ8 activities, it means they can dehydrogenate in Δ4/Δ5/Δ6/Δ8 position, and thus they are called Δ4/Δ5/Δ6/Δ8 desaturase/fads. Δ4/Δ5/Δ6/Δ8 position counts from the carboxyl terminus of a fatty acid. According to the "Sprecher pathway" (Sprecher, 2000), Δ6fad involves in synthesis 18:4n-3 and 24:6n-3 from 18:3n-3 and 24:5n-3 respectively. Δ5fad has the ability of introducing double bonds in Δ5 position of 20:4n-3 to form 20:5n-3. Marine fish have no capacity of synthesis LC-PUFAs which attributed to the lack of Δ5 activity (Sargent et al., 2002; Tocher & Chioni, 1999). The gene of Δ6 desaturase is termed as *d6d* which include *d6fad_a, d6fad_b* and *d6fad_c* (Zheng et al., 2009a; Monroig et al., 2010). *d6fad_b* and *d6fad_c* have activity towards Δ6 substrates 18:3n-3 and 18:2n-6, but no Δ5 and Δ4 desaturase activity, and *d6fad_b* protein was more active than *d6fad_c* (Monroig et al., 2010). Δ5 and Δ6 fads now have been isolated from Atlantic salmon (Hastings et al., 2005; Monroig et al., 2010), both Δ5 and Δ6 fad gene are highly expressed in liver and intestine, and Δ5 and Δ6 fad have relative specific desaturation activity on Δ5 and Δ6 positions (Zheng et al., 2005a). In the research of Monroig Ó, et al. (2010), salmon *d6fad_b* and *d6fad_c* gene were more active in catalyzing n-3 fatty acids than n-6 series. Moreover, Zheng et

al. (2009b) showed in Atlantic salmon and Atlantic cod that high level of dietary PUFA depressed Δ6 fad gene expression by binding to transcription factors (SREBP, LXR, etc.).

1.2.2.3 β-oxidation

β-oxidation is the last step in biosynthesis of LC-PUFA which can remove two carbon atoms at β-carbon position. After β-oxidation, an acetyl-CoA and a two-carbon-shortened fatty acyl-CoA are produced. β-oxidation is divided into mitochondrial and peroxisomal β-oxidation, that appears respectively in mitochondria and peroxisomes in heart, liver and red muscle of fish (Tocher, 2003). However, LC-PUFA β-oxidation can only happen in peroxiomes, because EPA and DHA are hard to be oxidized in mitochondria (Mannaerts and Veldhoven, 1993). Before β-oxidation is initiated, PUFA should be converted to its active form—fatty acyl-CoA which catalyzed by acyl CoA synthetase. After activation, activated PUFA will be transferred into peroxisomes. In peroxisomal matrix, β-oxidation goes through 4 steps which are dehydrogenation, hydration, dehydrogentation and tholysis, shown in figure2 (Scharader et al, 2015).

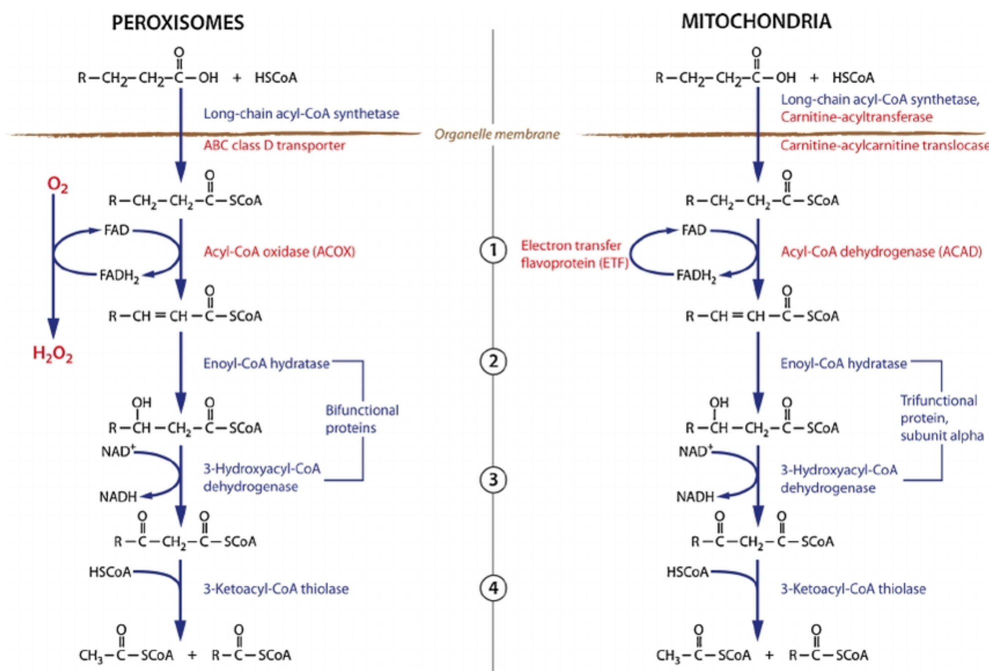


Fig.2 Overview of β-oxidation in peroxisomes and mitochondria (Scharader et al., 2015).

(1) dehydrogenation; (2)hydration; (3)dehydrogentation; (4)tholysis

In the first step, acyl-CoA is dehydrogenized to 2-trans-enoyl-CoA by acyl-CoA oxidase (ACO). ACO transfers two hydrogen atoms from the substrate to its FAD cofactor and then to O_2 which is reduced to H_2O_2. This is the main difference between mitochondrial and peroxisomal β-oxidation. Moreover, ACO is rate-limiting enzyme in peroxisomal β-oxidation because it determines the

substrate selectivity and oxidation capacity (Crockett et al., 1993). After that, 2-trans enoyl-CoA hydrated to 3-hydroxylacyl-CoA catalyzed by multifunctional enzyme. The step followed is a NAD^+-dependent dehydrogenation that 3-hydroxylacyl-CoA is transferred to 3-ketoacyl-CoA and is also catalyzed by multifunctional enzymes. The final step is 3-ketoacyl-CoA thiolized to an acetyl-CoA and an acyl-CoA with two carbon atoms shortened which catalyzed by 3-ketoacyl-CoA thiolase.

1.2.3 Fatty acid synthesis regulation

Fatty acid synthesis can be regulated by dietary fatty acids, hormone and gene transcription.

In the aspect of dietary fatty acids, there are already some evidences showing that replacement of some fish oil with vegetable oil actually have a up-regulated effect on gene expression of *fad* and *elovl* genes, thus affects EPA and DHA synthesis, e.g. the expression of *d6fad* genes in liver and intestine were significantly higher in fish fed diets containing vegetable oil compared to fish fed fish oil (Monroig et al., 2010); The transcription of *elovl5b* and *elovl2* genes were significantly increased in liver of salmon fed vegetable oils (Morais et al., 2009). In addition, dietary supplement of EPA and DHA inhibited desaturase and elongase activities in Atlantic salmon (Betancor et al., 2016).

In gene transcription level, the expression of genes related to LC-PUFA biosynthesis enzymes were regulated by cis-acting element of upstream primer and transcription factor (Zheng et al., 2009b; Geay et al., 2012). The transcription factors include peroxisome proliferator-activated receptors (PPARs), sterol regulatory element binding proteins (SREBPs), carbohydrate response element binding proteins (ChREBPs), liver X receptor (LXR) and retinoid X receptor (RXR). SREBPs include three subtypes which are SREBP-1a, -1c and -2. SREBP-1c mainly regulates the transcription of *d6d* gene in Atlantic salmon (Zheng et al., 2009b). The regulation from LXR can be completed by direct and indirect ways. The indirect way is through affecting activity of SREBP-1c to regulate gene expression of *fad* and *elovl5*, The direct way is straight regulating gene transcription of *d6fad* in Atlantic salmon (Carmona-Antonanzas, et al., 2014). The regulation from SREBPs and LXRs can be inhibited by LC-PUFA (Leaver et al., 2008; Vallim et al., 2010).

In hormone level, Insulin can induce SREBP-1c (Zheng, et al., 2009b) and also LXR (Sul and Smith, 2002). Leptin affects lipogenesis through SREBP-1 pathway (Kersten, 2001).

1.3 Genetic selection as a tool to improve the capacity for innate EPA and DHA production

Selective breeding is the traditional method for producing fish with expected traits, but only selecting phenotype is not accurate enough and takes a long period. Nowadays, genetic engineering is paying attention in identification or location of specific gene in order to improve the control of traits. This method can shorten the generation interval and increase breeding efficiency to the large extent.

Aims of breeding Atlantic salmon are to increase growth rate and quality (including filet color, body shape and total fat) and decrease the incidence of diseases (AquaGen, 2005; Gjedrem, 2010). However, the trait capacity for EPA and DHA synthesis is not included in the salmon production yet.

As mentioned above, for relieving the pressure of fishing industry and practicing sustainable development, vegetable oil was used to partially replace fish oil and fish meal, but this reduced n-3 LC-PUFA content in farmed fish flesh (Torstensen et al., 2005). Although Atlantic salmon fed vegetable oil showed an increased *Δ5 fad* and *Δ6 fad* gene expression level, the increase did not reach the expected n-3 LC-PUFA level after fed with fish oil (Leaver et al., 2008; Morais et al., 2011; Betancor et al., 2016). Therefore, gene selective breeding to find Atlantic salmon with higher n-3 LC-PUFA synthesis capacity is important to reach the expected n-3 LC-PUFA level in flesh as much as possible, since the level of n-3 LC-PUFA in muscle is a highly heritable trait (Leaver et al., 2010)

2. Material and methods

Table 1 Chemicals and equipments

Product	company	country
Benzene	VWR international, LLC	MA, USA
Chloroform	VWR international, LLC	MA, USA
Dimetoxypropan	Sigma Chemical Co., St. Louis	MO, USA
Ethnol absolute AnalaR NORMAPUR	VWR Interntional, LLC	MA, USA
Hexan	VWR International, LLC	PA, USA
Hewlett Packard 6890 gas chromatograph	Avondale	PA, USA
IKA® T25 digital ultra turrax	IKA®-Werke GmbH & Co. KG	Staufen, Germany
LightCycler® 480	Roche Diagnostcs	Basel, Switzerland
LightCycler® 480 Multiwell Plate 96, white	Roche Diagnostcs	Basel, Switzerland
LightCycler® 480 sealing Foil	Roche Diagnostcs	Basel, Switzerland
LightCycler® 480 SYBR Green I Mater	Roche Diagnostcs	Basel, Switzerland
Metanolic-HCL	Supelco Inc., Bellefonte	PA, USA
Methanol	VWR international, LLC	MA, USA
NanoDrop® ND-1000 Specrophotometer	Thermo Fisher Scientific Inc.	MA, USA
PureLink Pro96 RNA Purification Kit	Thermo Fisher Scientific Inc.	MA, USA
RNase-Free water	QIAGEN	Hilden,Germany
Sodium bicarbonate	VWR International, LLC	PA, USA
Sodium chloride	VWR international	PA, USA

TaqMan Reverse Transcription reagent	Applied Biosystems™ by Life Technologies	MA USA
Veriti 96-Well Thermal Cycler	Thermo Fisher Scientific Inc.	MA, USA
2,6-Di-t-butyl-p-cresol (BHT)	Sigma Chemical Co. St. Louis	MO, USA

Table 2 List of primers

Transcript	Accession no.	Primer name	Primer sequence (5'-3')
Elong2	TC91192	Ssa elong 2F1	CGGGTACAAAATGTGCTGGT
		Ssa elong 2R1	TCTGTTTGCCGATAGCCATT
ACO	DQ364432	Ssa ACO F1	CCTTCATTGTACCTCTCCGCA
		Ssa ACO R1	CATTTCAACCTCATCAAAGCCAA
D5d	AF478472	Ssa d5d F2	GCTTGAGCCCGATGGAGG
		Ssa d5d R2	CAAGATGGAATGCGGAAAATG
D6dC	GU207401	Ssa d6d C R2	CACAAACGTCTAGGAAATGTCC
		Ssa d6d C F2	TGAAGAAAGGCATCATTGATGTTG
D6dB	GU207400	Ssa d6d B F3	TGACCATGTGGAGAGTGAGGG
		Ssa d6d B R3	AACTTTTGTAGTACGTGATTCCAGC T
D6dA	AY458652	Ssa d6d A F3	TCCCCAGACGTTTGTGTCAGATGC
		Ssa d6d A R3	GCTTTGGATCCCCCATTAGTTCCTG
Elovl5b	NM_001136552	Ssa Elovl5b F2	GCAACCTTGACCCAAACAGG
		Ssa Elovl5b R2	CCTTGTCTCTACGCAAGGGA
Elovl5a	NM_001123567	Ssa Elovl5a F3	ACAGTAACCCCAGAGACCCA
		Ssa Elovl5a R3	TTGTCCCCACCACACTGAAG
RPL2	CA049789	Ssa RPL2 F	TAACGCCTGCCTCTTCACGTTGA
		Ssa RPL2 R	ATGAGGGACCTTGTAGCCAGCAA
Etif3	DW542195	Ssa etif3 F1	CAGGATGTTGTTGCTGGATGGG
		Ssa Etif3 R1	ACCCAACTGGGCAGGTCAAGA

2.1 Background of fish and tissue samples

Atlantic salmon (SalmoBreed) used in this trial was previously selected for high and low capacities for production of EPA and DHA from α-linolenic acid (ALA) in two generations. Briefly, Atlantic salmon were divided in the two genetic groups according to the expression of *d6dB* gene in liver, termed as plus group which has higher *d6dB* expression level, and minus group with lower *d6dB* expression level in the first parent generation. Offspring's (second generation) from the parent generations were produced from 60 fish with the highest and 60 fish

with the lowest gene expression of *d6dB*, respectively. When salmon from generation 2 reached brood stock size, eggs of female fish from families in the minus groups was fertilized with sperm from other male fish families in the minus groups, and eggs from female fish in the plus families were fertilized with male sperm from other fish families in the plus groups. The fish used in our trial was the offspring's from the second generation of fish selected for high and low capacities for EPA and DHA production. From start feeding of the fish larvae until slaughter when we sampled our fish at approximately 100 gram size, the fish from both genetic groups had been fed the same commercial Skretting feed.

Before slaughter, the 20 fish from each group were anesthetized in (MS 222) to mortality. The abdomen was cut open and the liver, muscle (Norwegian quality cut) and mid intestine were sampled.

For tissue gene expression profile, tissues (intestine, liver and muscle tissue) were dissected from Atlantic salmon and collected in 2ml microtubes, immediately frozen at -70℃ pending RNA extraction. For tissue fat content and fatty acid composition profile, muscle tissue was dissected from Atlantic salmon and cut in small pieces, immediately frozen at -70°C, whereas intestine and liver tissues were the same of tissue gene expression.

2.2 Tissue RNA extraction

Total RNA was extracted by organic solvent, according to manufacturer's instruction (PureLinkTM), and RNA quantity was assessed by Micro UV-Visible Spectrophotometer (NanoDrop 1000). The extraction of RNA followed steps of binding, washing and elution. Approximately 20mg tissue samples from liver, muscle and intestine respectively was added into 600µL lysis buffer with 4-5 4mm beads. Next step was homogenizing (Precyllis24) 2 times at 5500rpm for 2min and centrifuging at 12,000g for 30sec. There after, 600µL supernatant was mixed with 600µL 70% EtOH to eliminate protein and impurities, and the mixture was transferred to the filter column. The filter columns with the samples were centrifuged at 12,000g for 30sec. After centrifuging, RNA was bound in the filter column. The flow-through was removed, and then 300µL wash buffer I was added into filter column and centrifuged at 12,000g for 30sec to make sure that the contaminant from binding step was removed. To remove genomic DNA, 80µL DNase solution was added and incubated for 15min at room temperature (On-column PureLink

DNase I). The DNase solution was made by mixing 8μL 10xDNase Ⅰ buffer and 9,9μL DNase I with 62μL RNase-free water. After the DNase treatment, the samples were washed with 300μL wash buffer I and centrifuged at 12,000g for 30sec. After that, samples were washed again with 750μL wash buffer II and centrifuged at 12,000g for 30sec. Purpose of washing was to eliminate the contaminant which could be detected by Micro UV-Visible Spectrophotometer under 280nm, 260nm and 230nm. To ensure getting rid of the wash buffer residues, all the filter columns were centrifuged at 12,000g for 1 min. For the case of RNA elution, 50μL RNase free water was added to the filters and centrifuged at 12,000 for 2minutes. One drop of each sample was tested in Nano Drop 1000, according to the absorbance value in UV light, the RNA quantity of each sample was obtained.

2.3 qPCR

Polymerase chain reaction (PCR) is a method of DNA amplification in vitro. Through heating, DNA is degenerated and split into two single chains. There after, forward and reverse primers bind to target sequence and extend. After 2^n cycles of extension, DHA was amplified in large amount. Real time PCR (qPCR) is a kind of quantitative PCR. qPCR uses fluorescent dye which can bind to dsDNA. The dye fluorescence intensity has a linear relationship with DNA amount. Finally, DNA amplification curve is obtained in qRCR.

2.3.1 cDNA synthesis

qPCR reaction uses DNA polymerase which only works on cDNA, therefore cDNA corresbonding to mRNA should be synthesized first. 1000ng of total RNA per sample was reverse-transcribed into cDNA using TaqMan® reverse Transcription reagent, following manufacturer's instruction. And cDNA synthesis reaction was incubated in Veriti 96 well Thermal Cycler.

For 20μL cDNA synthesis reaction, 9.6μL RNA+H2O, 9.4μL mixture of 2μL RT buffer, 1,4μL MgCl2, 4μL dNTP, 1μL RNase inhibitor and 1μL Reverse trascriptase and 1μL Random hexamer were mixed together. Random hexamer was used to prime the reaction. 9.6μL RNA+H2O include 1000ng tissue RNA (in volume) and RNase-free water, the volume of RNase-free water varied according to tissue RNA.

The order of adding those reagents to each well is H2O, 9.4μL mixture, tissue RNA and then random hexamer. The plate was then centrifuged for 2 min with 2000 rpm. Finally, cDNA was

synthesized in a three reaction condition stages in Veriti 96 well Thermal Cycler which are 25℃ for 10min, 37℃ for 30min and 95℃ for 5 min. Finally, the plate were kept at 4℃ until stored at -20℃.

2.3.2 cDNA dilution

For diluting all samples to 1:10, 180μL of distilled water was added to each well and mixed well. After mixing, 100μL sample were transferred to each parallel well. There after, 4μL sample from each well was moved to another white LC plate.

2.3.3 qPCR analysis

cDNA was amplified by PCR, SYBR green was used as fluorescence signal for quantitative cDNA. The amount of cDNA in tissues was determined as Ct value.

For qPCR analysis, a mixture of 5μL SYBR green master mix (Roche Applied Science, Mannheim, Germany), 0.5μL forward primer and 0.5μL reverse primer (10M, Thermo Fisher, Waltham, Massachusetts, USA), was added to each well which contained 4μL cDNA (1:10 dilution) in specific LC480 plates (Roche Applied Science, Mannheim, Germany). SYBR green is a dye which has green excitation wavelength and bind to dsDNA. After mixing, the plate was sealed with a LC480 cover and centrifuged at 1500g for 2 min. The qPCR reaction was run on a LightCycler480 (Roche Diagnostics Gmbh, Germany) under the following conditions: Pre-incubation at 95°C for 5 minutes, amplification with 45 cycles at 95°C for 15 seconds and 60°C for 1 minutes, melting curve at 95°C for 5 seconds and 65°C for 1 minutes, cooling at 40°C for 10 seconds. All samples were analyzed in parallels, and a non-template control with water substituted for cDNA was run for each primer pair. A melting curve analysis was performed to confirm amplification of only one PCR product.

After the reaction of qPCR, the LC480 qPCR software was used to automatically analyze the Ct values of genes (Ct value means the numbers of experienced cycles before the fluorescence signal reaching the threshold). Each template's Ct value has the negative linear relationship with the logarithm of its initial copy number. The larger the initial copy number is, the smaller the Ct value will be. The $2^{-\Delta\Delta Ct}$ method is used to compare differences of mRNA among salmon tissues.

$$\Delta Ct_0 = Ct_t - Ct_r \quad (1)$$

ΔCt_0 means the difference between test gene and reference gene. Ct_t is the Ct value of test gene, Ct_r is the Ct value of reference gene.

Therefore the relative mRNA content (R) of different genes in different tissues is calculated as below:

$$R = 2^{-\Delta\Delta Ct} = 2^{-(\Delta Ct_0 - \Delta Ct_0 \text{average})} \quad (2)$$

In addition, in this experiment, two reference genes were tested which are RPF2 and etif3. After qPCR analysis, RefFinder (http://fulxie.0fees.us/?type=reference&ckattempt=1) was used to automatically analyze the stability of these two reference gene according to Ct value. And the more stable reference gene would be chose for $2^{-\Delta\Delta Ct}$ calculation.

2.4 Fat content and fatty acid composition

The muscle samples were homogenized with dry ice, dry ice is used for helping homogenized of muscle small pieces and will evaporated as CO_2 in the -18℃ freezer afterwards which has no contaminants to samples, whereas the liver and intestine samples were used directly without homogenization because the weights were lower than 2g.

Fat content

Fat extraction was done by Folch method (Folch et al., 1957). The Folch extraction solution is a mixture of polar (water and methanol) and non-polar solvents (organic chloroform). Fat is easily solved in non-polar solvent due to the compatibility principle that the polarity of fat is similar to non-polar solvent. Therefore, in the Folch method, fat was extracted by organic solvent and kept in organic phase, after evaporation of this organic phase, the mass difference before and after evaporation was the amount of fat. First, the whole liver and intestine samples and around 2g homogenized muscle sample were transferred to a 100mL Erlenmeyer flask. 6mL 0.9% NaCl and 50mL chloroform : methanol (2:1) mixture with 0.7mg/L BHT were added into the flask. The mixture was homogenized with a homogenizator (IKA T25 digital ultra turrax) at 17,000 rpm for 60s. After that, 6mL 0.9% NaCl was added and continued homogenizing for 5s. Then, the mix was separated into two phases, the lower phase was chloroform : methanol : water in the ratio of 86:14:1 which contained almost all lipids, and the upper phase was chloroform : methanol : water

in the ratio of 3:48:47 which contained mostly water soluble components. The homogenate was filtered through a cotton filter inside a cylinder. The cotton was for filtering the protein. When all the homogenate had passed the filter, the cylinder was capped and kept in the freezer to the next day.

In the second day, the upper phase was removed with a water-vacuum pump-pipette. 20mL lower phase was pipetted into a silver paper cup for measuring fat content. The rest lower phase was transferred to a test tube for preparing gas chromatograph (GC) to analyze fatty acid composition. Silver paper cup was chose due to its low weight which is more suitable for observing small weight changes. Before use, the cup was weighted. For fat content measurement, the silver cup was put on a heating plate to evaporate the solvent until dryness and then transferred into the dryer at 102℃ for 20min to dry the rest of water. The cup was weighted again after dryness. The formula for calculating fat content is as shown below.

By the use of 100mL chloroform/methanol:

$$\%\text{fat} = \frac{gfat * 100}{\frac{I * U}{37.5}}$$

g fat = weight change in silver paper cup in g

100=%

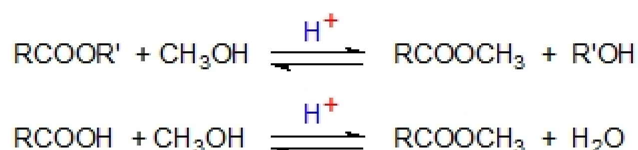
I=weight of the sample in g

U=pippetted chloroform phase (20mL)

37.5=total volume of solvent (chloroform in extract solution=50*2/3=33.3mL; after separation, 86 parts in choloroform phase and 3 parts in water phase=89, there was some methanol and water in choloroform phase, so the total volume is not 33.3mL, but 33.3*100/89=37.5mL. chloroform and methanol makes in total 100 parts, when water evaporated)

Fatty acid composition

Before fatty acid composition analysis in GC, fatty acids were methylated. The methylation reaction are showed as follow (Christie, 1993) by acid catalysis:



After reaction, free fatty acids are esterified and lipids, such as TGs are transesterified. The

amount of fatty acids was determined by GC that different retention time indicates different fatty acid and peak area means the amount of this fatty acid. 3mL chloroform extract after Folch extration from muscle samples and 5mL chloroform extract from liver and intestine samples were transferred into new test tubes, respectively, and evaporated at 60°C with nitrogen overflow until dryness. Nitrogen overflow was used for keeping oxygen away from fatty acids, due to its heavier density compared to oxygen. 2mL benzene, 2mL metanolic-HCl and 0.2mL dimetoxypropan were added into the tube for methylation and mixed well. 10μL and 20μL 0.6176g/50mL internal standard were added into tubes from muscle samples and tubes from liver and intestine samples, respectively. The internal standard is for quantifying relative amount of fatty acids, because internal standard is a fatty acid that is not found in the testing sample and its concentration is already known. The mix was incubated at room temperature until next day. Thereafter, all fatty acids were methylated.

In the next day, 2mL hexan and 2mL 6% $NaHCO_3$ were added. The $NaHCO_3$ was for neutralization. After that, the mix was separated into two phases, the upper phase was hexan and benzene with fatty acids and the lower phase was salt solution and water. The upper phase was removed to a new test tube and evaporated at 60°C with nitrogen overflow until dryness. Nitrogen overflow was again used for keeping oxygen away from fatty acids. 5 drops of hexan were added into the tube and transferred to a GC sample bottle. This step was repeated until the volume was enough for GC analysis. Hexan, GLC85 and GLC463 were used as standard samples in this GC analysis.

2.5 Statistic analysis

For the gene expression profiles, results were expressed as mean relative expression ratio (± standard error mean, SE). Differences in the expression of each *fad* and *elovl* gene between different genetic groups and among tissues were analyzed by one-way analysis of variance (ANOVA) and ranked using Duncan's multiple comparison test at a significance level of $P \leq 0.05$.

For the fat content and fatty acid composition profiles, results were presented as mean percentage in tissue (±SE). Differences of fat content and fatty acid composition between genetic groups and among tissues were analyzed by one-way analysis of variance (ANOVA) and ranked using Duncan's multiple comparison test at a significance level of $P \leq 0.05$.

3. Results

3.1 Expression of genes coding for proteins in the of omega-3 HUFA metabolic pathway

Gene expression analysis was done to know how the genes related to n-3 HUFA synthesis are expressed in different tissues from different genetic groups of Atlantic salmon. According to Ct value from qPCR, using $2^{-\Delta\Delta Ct}$ method, the relative mRNA content was calculated and represented as relative gene expression ratio.

In $2^{-\Delta\Delta Ct}$ method, the target-gene expression is normalized by one or more non-regulated reference gene (REF) expression, e.g. derived from classical and frequently described reference genes (Bustin, 2000; Pfaffl *et al*., 2005). In this experiment, normal reference genes--etif3 and RPF2 were tested. According to the RefFinder program which compared stability by Ct value, the gene with smaller change of Ct value in different samples is considered as more stable gene. Finally the RPF2 gene was chose as the more stable gene for further calculation in this experiment.

For comparing relative expression level between tissue groups, the relative expression ratio in the minus group of intestine was used as a control group. This control group was randomly selected, which can be anyone of the six groups.

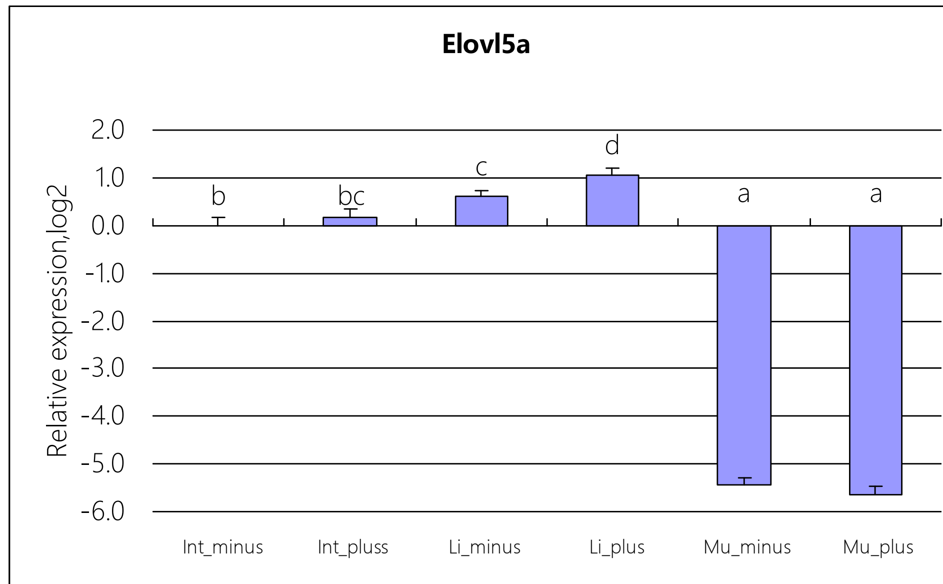


Fig.3 Tissue expression of elovl5a in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences (P≤0.05) between plus and minus groups and among different tissues The results show mean value of relative expression ratio in log2 between target gene and reference gene which presented in means and ± Standard error of means (SE). (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 individual fish samples respectively, while In_plus was got from 10 individual fish samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus genetic groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus genetic groups respectively.

According to fig.3, comparing within tissue groups, in the intestine, the expression of elovl5a between minus and plus groups showed no significant difference, in muscle, the expression between two genetic groups were also with no significant difference, whereas in liver, its plus group was significantly higher than minus group.

Comparing between tissue groups, in general, there was significantly higher expression in liver than in intestine and muscle, however, the intestine plus group was with no significant difference with liver minus group. In addition, there was also significant difference between intestine and muscle with a significant low expression in muscle.

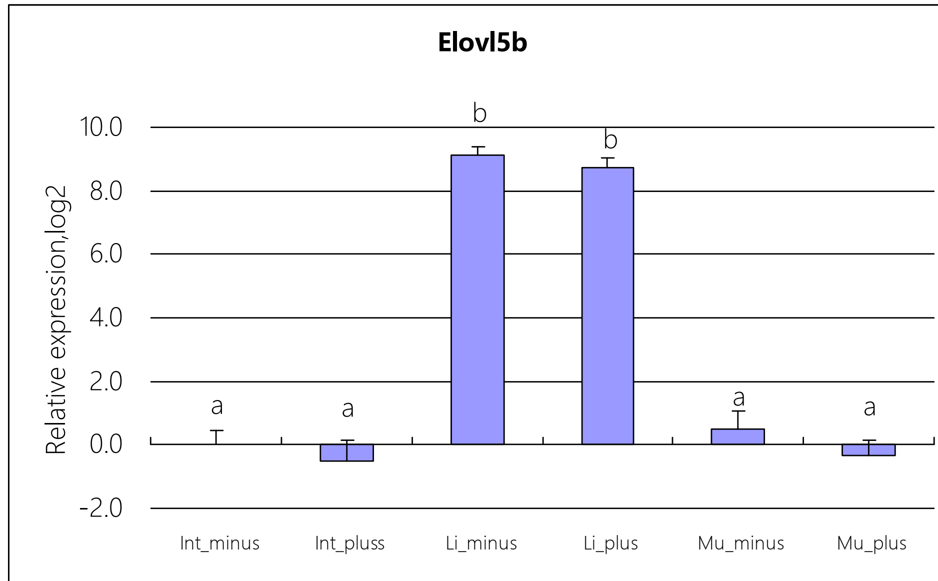


Fig.4 Tissue expression of elovl5b in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences (P≤0.05) between plus and minus groups and among different tissues. The results show mean value of relative expression ratio in log2 between target gene and reference gene which presented in means and ± Standard error of means (SE). (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 samples respectively, while In_plus was got from 10 samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

For elovl5b, comparing within tissue groups, there was no significant difference between genetic groups in each tissue group.

Comparing between tissue groups, the expression in liver was the highest with the significant difference compared to intestine and muscle. However, the expression between intestine and muscle was with no significant difference.

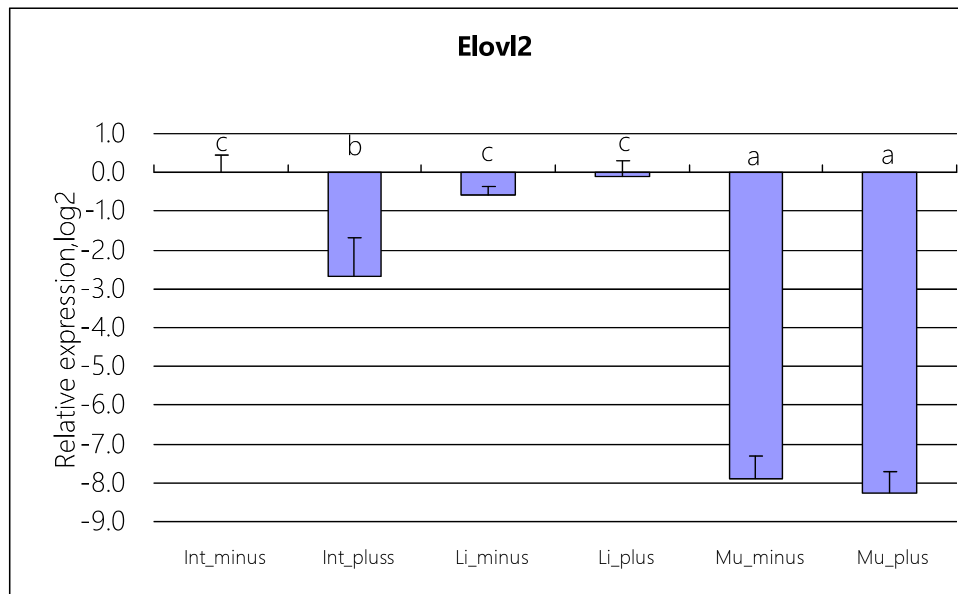


Fig.5 Tissue expression of elovl2 in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences (P≤0.05) between plus and minus groups and among different tissues. The results show mean value of relative expression ratio in log2 between target gene and reference gene which presented in means and ± Standard error of means SE. (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 samples respectively, while In_plus was got from 10 samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

For elovl2, comparing within tissue groups, in the intestine, the expression of plus group was significantly lower than minus group, whereas in liver and muscle, there were no significant differences between genetic groups.

Comparing between tissue groups, in general the expression in liver was significant higher than muscle and intestine. However, the intestine minus group was with no significant with liver minus and plus groups. Also, there was significant difference between intestine and muscle with a significant low expression in muscle.

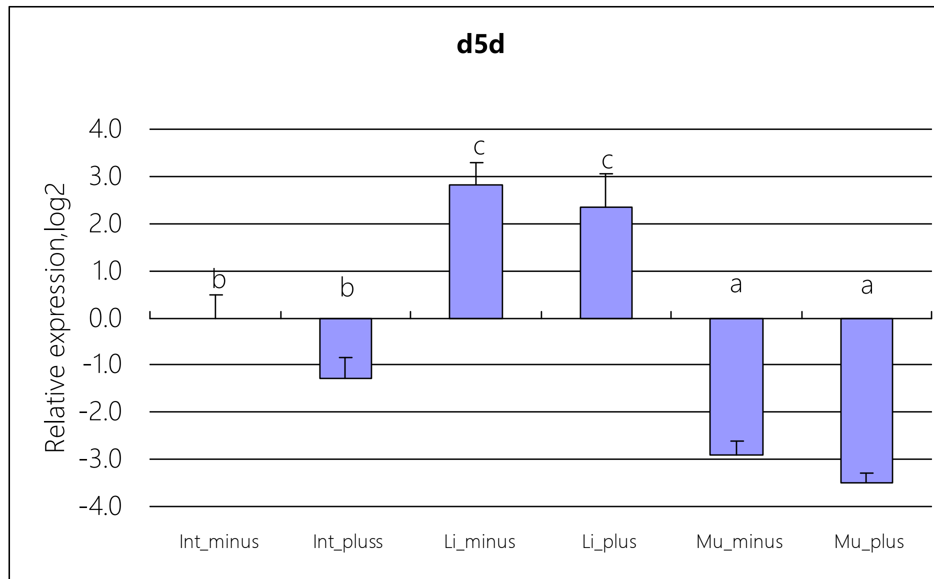


Fig.6 Tissue expression of d5d in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences (P≤0.05) between plus and minus groups and among different tissues. The results show mean value of relative expression ratio in log2 between target gene and reference gene which presented in means and ± Standard error of means (SE). (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 samples respectively, while In_plus was got from 10 samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

For d5d, comparing within tissue groups, there was no significant difference between genetic groups in each tissue.

Comparing between tissues, there was significant higher expression in liver than muscle and intestine in general. And there is also significant difference between intestine and muscle with a significant low expression in muscle.

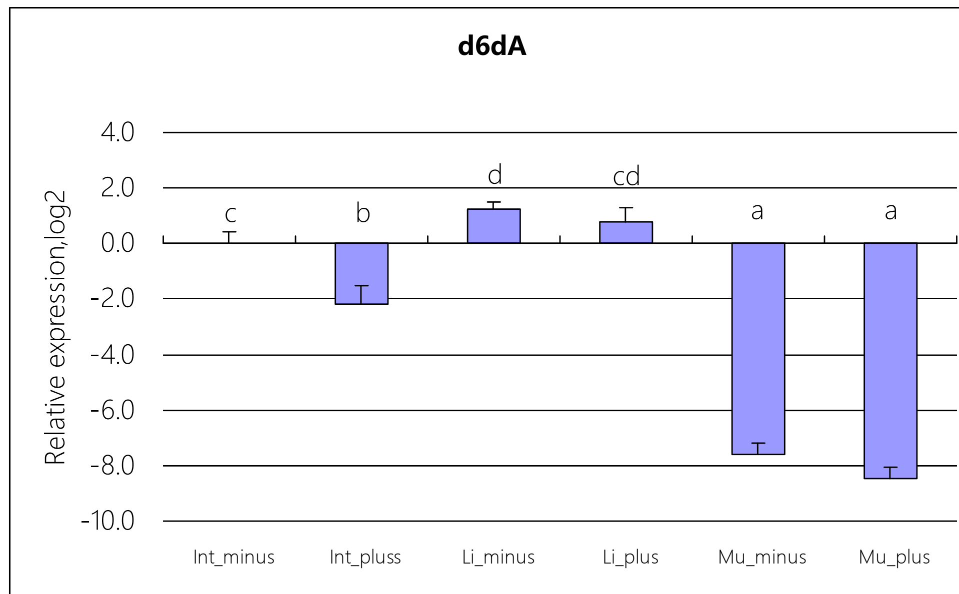


Fig.7 Tissue expression of d6dA in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences ($P \leq 0.05$) between plus and minus groups and among different tissues. The results show mean value of relative expression ratio in log2 between target gene and reference gene which presented in means and ±Standard error of means (SE). (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 samples respectively, while In_plus was got from 10 samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

For d6dA, comparing within tissue groups, in the intestine, plus group was significant lower than minus group, whereas, in liver and muscle, there was no significant difference between genetic groups.

Compared between tissues, the gene expressions of liver was significant higher than other tissues, however, there was no significant difference between intestine minus group and liver plus group. In addition, the expression between intestine and muscle was also with significant difference that expression in muscle was significantly lower than in the intestine.

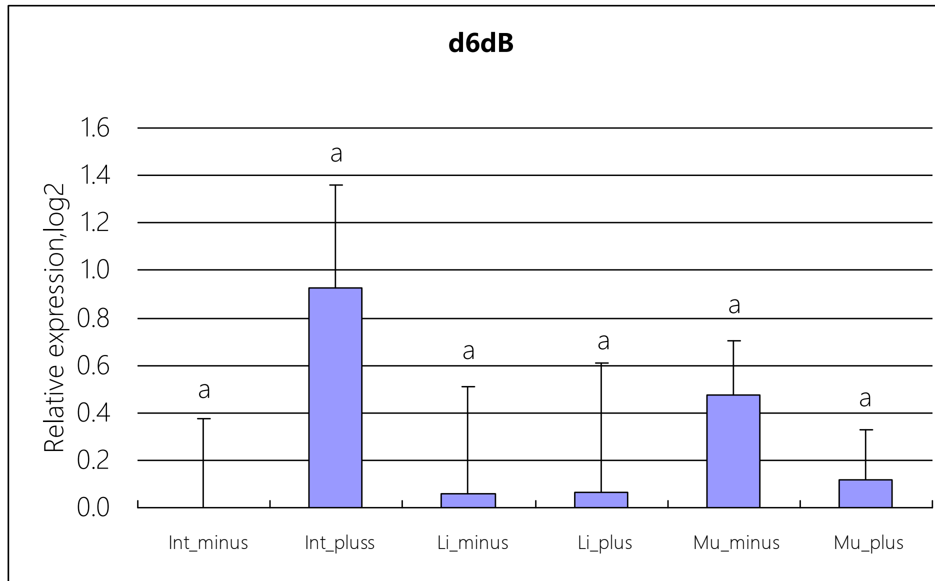


Fig.8 Tissue expression of d6dB in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences ($P \leq 0.05$) between plus and minus groups and among different tissues. The results show mean value of relative expression ratio in log2 between target gene and reference gene which presented in means and ±Standard error of means (SE). (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 samples respectively, while In_plus was got from 10 samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

For d6dB, comparing within tissue groups, the expression was with no significant difference between each two genetic groups.

Comparing between tissue groups, there was also no significant difference which means the expression in the intestine, liver and muscle was similar.

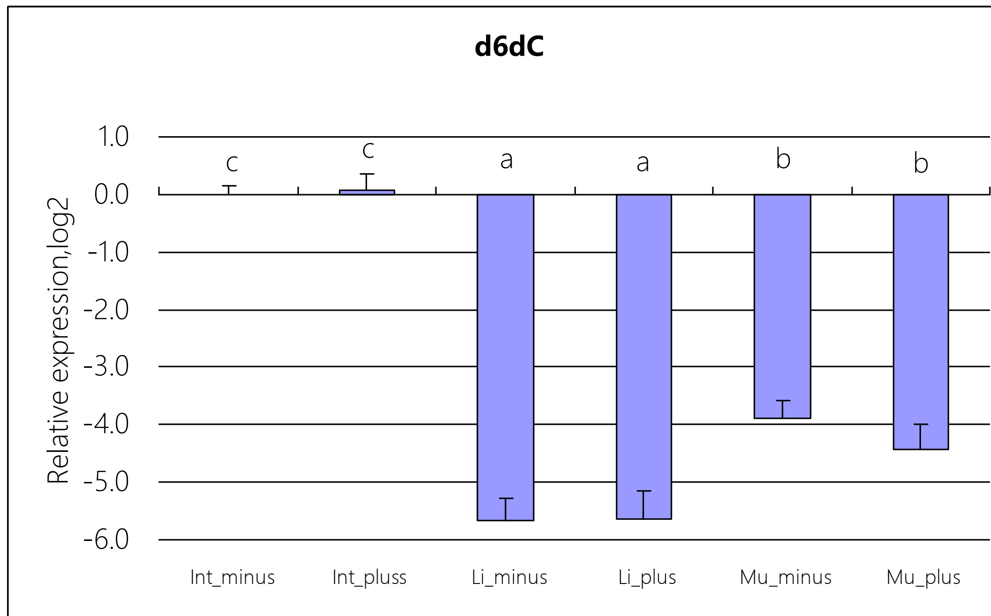


Fig.9 Tissue expression of d6dC in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences ($P \leq 0.05$) between plus and minus groups and among different tissues. The results show relative expression ratio log2 between target gene and reference gene mean and each point represents mean ± Standard error of means (SE). (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 samples respectively, while In_plus was got from 10 samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

For d6dC, comparing within tissue groups, there were no significant differences between minus and plus groups in each tissue group.

Comparing between tissues, liver showed significant lower level than muscle and intestine. And there was also significant difference between intestine and muscle that the expression in the intestine was significantly higher than in muscle.

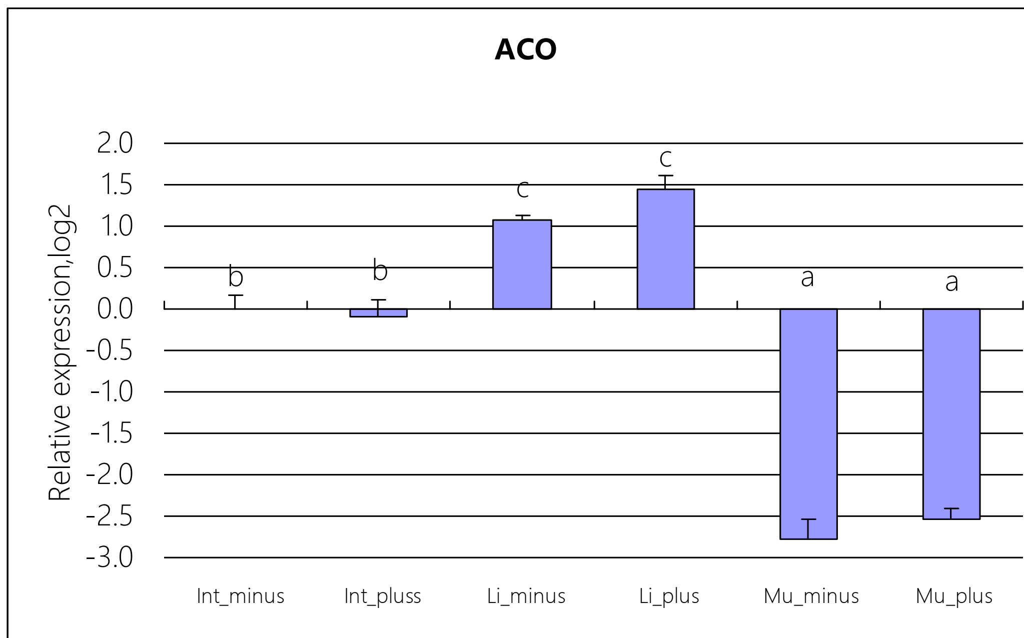


Fig.10 Tissue expression of ACO in Atlantic salmon, determined by RT-qPCR. Different letters indicate significant differences (P≤0.05) between plus and minus groups and among different tissues. The results show relative expression ratio log2 between target gene and reference gene mean and each point represents mean ± Standard error of means (SE). (Int_minus, Li_minus, Li_plus, Mu_minus and Mu_plus were got from 20 samples respectively, while In_plus was got from 10 samples). Int_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; Int_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

For ACO, comparing within tissue groups, there were no significant differences between plus and minus groups in each tissue groups.

Comparing between tissue groups, the expression in liver was the highest with significant differences from other two tissues. Moreover, there was also significant difference between intestine and muscle that the expression in muscle was significantly lower than in the intestine.

3.2 Total fat content and fatty acid composition in tissues from two genetic groups

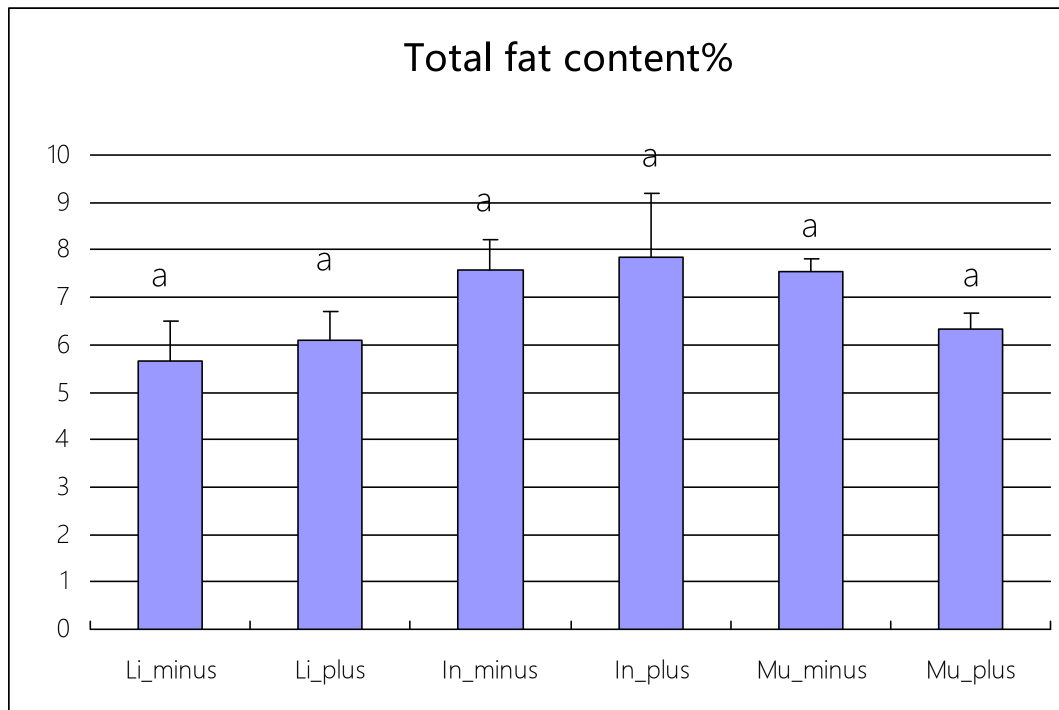


Fig.11 Total fat content (%) in different tissues from two genetic groups (in average). The results show mean fat content in percentage of each group and each point represents mean ±SE. (all groups were got from mean value of 6 samples). In_minus, Li_minus, Mu_minus, intestine, liver and muscle samples from minus groups respectively; In_pluss, Li_plus, Mu_plus, intestine, liver and muscle samples from plus groups respectively.

There were no significant difference in fat content between different tissues and genetic groups.

Table 3 Fatty acid composition of different tissue from two genetic groups (% in average)

	Li_plus	Li_minus	In_plus	In_minus	Mu_plus	Mu_minus
C 14:0	1.8±0.1[c][2]	1.9±0.2[c]	3.1±1.0[b]	3.3±0.8[b]	4.1±0.1[a]	4.5±0.1[a]
C 16:0	16.6±1.6[ab]	14.1±1.4[c]	18.2±3.3[a]	16.9±1.0[a]	14.2±0.1[c]	14.5±0.1[bc]
C 18:0	5.2±0.6[ab]	5.7±0.6[ab]	5.9±1.0[a]	5.1±0.4[b]	3.6±0.1[c]	3.5±0.1[c]
Sum SAT[1]	24.7±2.0[bc]	23.2±1.4[c]	29.7±5.2[a]	27.1±1.1[ab]	23.0±0.2[c]	23.7±0.2[c]
C 16:1 n-7	2.0±0.2[c]	2.7±0.5[c]	3.6±1.0[b]	4.1±0.8[b]	5.2±0.2[a]	5.8±0.1[a]
C 18:1 n-9	15.9±3.8[d]	20.8±3.5[bc]	19.5±3.3[c]	18.1±1.9[cd]	24.5±0.6[a]	23.2±0.2[ab]
C 18:1 n-7	2.5±0.7[b]	3.4±0.6[a]	3.0±0.1[a]	3.1±0.2[a]	3.3±0.0[a]	3.4±0.0[a]
C 20:1 n-11	0.1±0.1[c]	0.2±0.0[c]	0.7±0.2[b]	0.8±0.2[b]	1.1±0.0[a]	1.1±0.0[a]
C 20:1 n-9	2.3±0.8[b]	3.1±0.6[a]	3.0±0.5[a]	2.8±0.3[ab]	3.2±0.0[a]	3.0±0.1[a]
C 22:1 n-11	0.4±0.2[c]	0.6±0.1[c]	1.9±0.5[b]	1.9±0.3[b]	2.6±0.0[a]	2.6±0.1[a]
Sum MUFA[1]	25.6±5.3[c]	33.6±5.3[b]	34.2±5.7[b]	33.4±3.7[b]	42.5±0.5[a]	41.7±0.2[a]
C 18:2 n-6	4.1±1.0[c]	4.9±0.5[c]	6.0±1.2[b]	6.7±0.4[b]	7.8±0.2[a]	7.6±0.0[a]
C 20:2 n-6	1.1±0.3[a]	1.3±0.2[a]	1.2±0.4[a]	1.0±0.2[a]	0.7±0.0[b]	0.6±0.0[b]
C 20:4 n-6	3.2±0.5[a]	2.6±0.4[b]	1.2±0.4[c]	1.4±0.3[c]	0.7±0.0[d]	0.7±0.0[d]
Sum n-6[1]	9.2±0.9[b]	9.6±0.4[ab]	9.1±1.3[b]	10.3±0.4[a]	10.2±0.2[a]	10.1±0.0[ab]
C 18:3 n-3	1.0±0.3[c]	1.2±0.1[c]	1.7±0.5[b]	1.7±0.3[b]	2.6±0.1[a]	2.5±0.0[a]
C 20:5 n-3	5.3±1.6[a]	3.6±0.5[b]	3.4±0.8[b]	3.6±0.3[b]	4.0±0.2[b]	4.1±0.1[b]
C 22:5 n-3	1.3±0.4[c]	1.0±0.1[d]	1.3±0.3[c]	1.5±0.2[bc]	1.7±0.1[ab]	1.8±0.0[a]
C 22:6 n-3	30.9±3.4[a]	24.9±4.2[b]	17.7±5.2[c]	19.7±3.2[c]	13.0±0.4[d]	12.8±0.2[d]
Sum n-3[1]	38.9±4.7[a]	31.2±4.5[b]	24.5±5.7[c]	26.8±2.8[bc]	21.7±0.4[c]	21.7±0.3[c]
Sum EPA/DHA[1]	36.2±4.8[a]	28.5±4.6[b]	21.1±5.8[cd]	23.3±3.3[c]	17.0±0.4[d]	16.9±0.3[d]

1 Fatty acids lower than 1% in all tissues are not include in this table. Sum SAT (saturated fatty acids) also includes C15:0, C17:0, C20:0, C22:0 and C24:0; Sum MUFA (monounsaturated fatty acids) also includes C14:1n-5, C15:1, C16:1n-9 C16:1n-5, C17:1n-7, C18:1n-11, C22:1n-11, C24:1n-9 and C22:1n-9; Sum n-6 (n-6 series polyunsaturated fatty acids) also includes C16:2n-6, C18:3n-6, C20:3n-6; Sum n-3 (n-3 series poly unsaturated fatty acids) also includes C20:4n-3 and C20:3n-3.

2 Different letters means significant differences between those six groups

Each group was got from mean value of 6 samples.

The fatty acid composition in liver, intestine and muscle of different genetic groups is shown in table 3. Comparing within tissue groups, (1) in liver, plus group showed significantly higher percentages of n-3 PUFA and EPA/DHA, while minus group showed significantly higher percentage of MUFA. However, the percentages of SFA and n-6 PUFA were with no significant difference between two genetic groups; (2) in the intestine, minus group showed significantly higher percentage of n-6 PUFA. But, the percentages of SFA, MUFA, n-3 PUFA and EPA/DHA

were with no significant difference; (3) in muscle, the percentage of SFA, MUFA, n-6 PUFA, n-3 PUFA and EPA/DHA were all with no significant difference between two genetic groups.

Comparing between tissue groups, (1) the SFA in percentage of total fatty acids, was highest in the intestine with significant difference from other two tissues in general, however, the SFA percentage of liver plus group was with no significant with intestine minus group. Moreover, SFA percentage in liver and muscle was with no significant difference; (2) the percentage of MUFA was the highest in muscle, and was with significance from other tissues. But the MUFA percentage in liver and intestine was with no significant difference; (3) the percentage of n-6 PUFA between all tissue groups was with no significant difference in general. However, the n-6 PUFA percentage in muscle plus group was with significant difference compared to liver and intestine plus group that muscle plus group was the significantly highest; (4) the percentage of n-3 PUFA was significantly the highest in liver compared to other two tissues. Moreover, the n-3 PUFA percentage in intestine and muscle was with no significant difference; (5) and DHA/EPA percentage was the highest in liver with significant differences compared to other tissues. And there was no significant difference among intestine plus group and muscle minus and plus groups.

4. Discussion

4.1 Total fat content in liver

Liver is the main metabolic organ for lipid catabolism and anabolism (Sargent et al., 1989). Lipid is easily accumulated in liver when dietary fat content is excess or malnutrition (Badaloo, 2005). It is often shown in Atlantic salmon that when relative high level of plant oil introduced in diet, fatty liver occurs. (Torstensen, 2013). In the present study, there was no significant difference in total fat content of liver between the two genetic groups. This result was not in agreement with what was previously found in the parent generation, where it was observed that fish selected for high liver capacities for EPA and DHA production had significantly lower fat level in the liver than fish with low capacity when fed a diet rich in plant oil (Berge et al., 2015). The reason why no difference was found in fat percentage of liver between the genetic groups in my study may be that the fish were fed a diet rich in marine ingredients. Although not part of my thesis work, the fish of 100 grams from both genetic groups were after the end of my trial followed further and fed one of two diets, either a diet rich in plant oil or a diet rich in fish oil (FO) until they reached 200 g. Atlantic salmon in the low desaturase group had then significantly higher fat content in the liver than the high desaturase group, when both groups were fed a diet rich in plant oil (personal communication Bente Ruyter, unpublished data).

Therefore, fat content in liver may strongly relate to the diet--vegetable oil could increase hepatic fat deposition, whereas marine oil (contains EPA and DHA) has an effect of decreasing fatty liver risk. The mechanisms by which marine oil with EPA and DHA affects fat content in the liver can be through EPA and DHA decrease de novo lipogenesis by reducing expression of SREBP-1c, increase fatty acid oxidation by inducing PPAR-α (Shang et al., 2017; Dossi et al., 2014; Videla et al., 2012). This was however not investigated in my study. Selecting high EPA and DHA synthesis capacity only become important in preventing occurrence of fatty liver when fish fed a diet low in EPA and DHA, not in my study since the diet was rich in marine ingredients and EPA+DHA.

4.2 EPA+DHA percentage in liver

In the present study, EPA+DHA percentage was significantly higher in liver from high capacity group than low capacity group. The Atlantic salmon families used in this trial was selected by gene expression of *d6dB* in liver in the first generation as high and low capacity of HUFA

synthesis. *d6dB* is the gene of Δ6 desaturase, which is catalyzing the first and last desaturation step of HUFA synthesis pathway and is regard as rate-limiting enzyme (Sprecher, 2000; Cook, 2004). In Sun's study (2012) that parents fish selected for high *d6dB* gene expression in liver, transfer a higher capacity for EPA and DHA production to their offspring. This explain the high EPA+DHA content in liver from the high capacity group of the third generation, since the third generation inherited the trait of high HUFA synthesis capacity.

4.3 EPA+DHA percentage in muscle

Fillet is the main product of salmon and muscle EPA+DHA level is a critical trait in salmon production. In this study, EPA+DHA percentage in muscle showed no significant difference between two genetic groups. This may because diet given to Atlantic salmon in this trial was from marine source. Fish fillet fatty acid composition is highly influenced by diet fatty acid composition (Alhazzaa, 2012; Torstensen et al., 2000). In Yitbarak's study (2013) with Atlantic salmon (selected by high low d6dB gene expression in liver), two families were feed by FO diet and rapeseed oil (RO) replaced diet (25%, 50%, 70% and 100% replacement). This study showed no significant difference of EPA+DHA content in fish body between two families, except families fed by 25% RO diet in which high desaturase family showed significant higher EPA+DHA content than low desaturase family. This indicates that moderate level of dietary vegetable oil influences the capacity of producing EPA and DHA. Bell et al. (2010) found that fillet EPA+DHA percentage of Atlantic salmon (selected by high and low flesh adiposity) fed FO was higher than fed vegetable oil in both families, the EPA+DHA level was also affected by genotype but was with no significant difference. This may because vegetable oils are mainly rich in n-6 and n-9 fatty acids but lack of 18:3n-3 which is the precursor in EPA and DHA synthesis. In the study of Torstensen et al. (2005) with Atlantic salmon, largely replacing FO by 75% VO and 100% VO showed decrease of EPA and DHA percentage in fillet, and the reduced amount in 100% VO group was higher than 75% VO group. Thus, to reach the expected EPA and DHA content in fillet (close to the level of only fed FO), increasing EPA and DHA synthesis capacity by gene selective breeding is necessary when vegetable oil introduced into the diet, and the amount of vegetable oil added is also important.

Miller et al. (2008) compared flesh EPA+DHA content from Atlantic salmon after being fed canola oil (CO) diet, Echium oil (EO) diet and FO diet respectively. In this study, flesh

EPA+DHA content from fish fed CO and EO diet was significantly lower than fish fed FO diet, although the expression of *d5d* and *d6d* gene was significantly increased by CO and EO diet. CO is rich in 18:3n-3, EO is rich in 18:4n-3 and both are lack of EPA and DHA. Thus, diet rich in precursor of n-3 HUFA synthesis pathway still does not lead to high fillet EPA+DHA content as fish fed only FO diet. Bou et al. (2017a)'s study also confirmed that decreasing EPA and DHA level in diet led to dramatically reducing of EPA and moderately decreasing of DHA in Atlantic salmon muscle. This may come to the reason of fatty acid accumulation, since vegetable oil does not contain any EPA and DHA.

4.4 Total fat content in muscle

In present study, the total lipid content in muscle showed no significant difference between two genetic groups. However, in the research of Quillet et al. (2005) with rainbow trout (selected by flesh adiposity), the fat fish showed higher muscle fat content than lean fish fed by same diet. In study of Koldits et al. (2008) with rainbow trout (selected by low or high muscle fat content with a non-invasive measurement on live fish), the fat fish showed higher percentage of fillet lipid than lean fish when fed same diet. Quillet et al.'s (2007) study with rainbow trout (selected by high and low fillet adiposity) also showed an overall increase in fillet fat of fat fish fed the same diet. The results in these two studies were consists with the selection.

Moreover, in Koldit's (2008) study, both families showed higher muscle lipid content fed high-energy/lipid diet (23% lipid of DM) (close to commercial diet for rainbow trout) than low-energy/lipid diet (10% lipid of DM). Similar result showed in Quillet's study (2007) which compared between 27% lipid diet and 8% lipid diet. These results in agreement with that high energy diet leads to more fat deposition in fish body (Rasmussen et al., 2000).

The total fat content of the diet given to salmon in this study was around 30% of DM, which is close to the diet fat content in Kolditz's and Quillet's studies. Thus, the reasons for no significant difference of muscle fat content between two genetic groups may be muscle adiposity was not the selected trait in the present experiment. Nevertheless, Kause et al. (2002) also found a correlation between muscle fat and visceral fat in rainbow trout, that the correlation is negative which means high visceral fat content is partly independent of muscle fat deposition. Therefore, fat deposition in muscle of high capacity group may be not strongly related to its high HUFA synthesis capacity in liver.

4.5 Gene expression

In present study, the relative gene expression level of elongase genes (*elovl5a, elovl5b, elovl2*), desaturase genes (*d5d, d6dA*) and β-oxidation gene (*ACO*) was significantly higher in liver, whereas another desaturase gene (*d6dC*) were the significant highest in the intestine. This result consists with other studies that Δ6 desaturase, Δ5 desaturase and elongase genes are highly expressed in liver and intestine of Atlantic salmon (Monroig et al., 2010, Morais et al., 2009). Comparing between genetic groups, in liver, the expression of *elovl5a* was significantly higher in high capacity group; in the intestine, the expression of *elovl2* and *d6dA* was significantly higher in low capacity group, the expression of other genes was with no significant difference. Particularly, the expression of *d6dB* showed no significant difference between two genetic groups, which is incompatible to gene selection of the parents.

Many studies showed vegetable oil affect expression of genes related to n-3 HUFA synthesis pathway. Kjær et al., (2016) found that oleic acid up-regulates the expression of Δ6 desaturation gene (*d6dA, d6dB and d6dC*) and elongation gene (*elovl2*), whereas DHA down-regulates Δ5 desaturase gene (*d5d*), *d6dA, d6dB* and elongation gene (*elovl5b*), EPA also showed down-regulation effect to the expression of *d5d* and *d6dB*. Similar result was showed in the study of Zheng et al. (2004) that the expression of Δ5 desaturase gene and one elongase gene was increased by increasing dietary linseed oil and decreasing dietary FO. Monroig et al. (2010) also found that *d6fad* genes in liver and intestine were significantly higher in fish fed diets containing vegetable oil compared to fish fed FO.

The effect of vegetable oil in gene expression comes from competitive inhibition in HUFA synthesis pathway. Introducing vegetable oils equals to increasing level of n-6 fatty acids, and as we mentioned above, LC-PUFA in diet down-regulating LC-PUFA synthesis, thus a high vegetable oil diet leads to inhibition of n-6 LC-PUFA synthesis. Moreover, there is a competitive desaturation and elongation between n-3 and n-6 fatty acids in LC-PUFA synthesis pathway, since the desaturases and elongases for n-3 and n-6 substrate are the same (Lee et al., 2016). Therefore, inhibited activity of n-6 LC-PUFA synthesis leads to induced n-3 LC-PUFA synthesis.

Since the diet used in this study mainly from marine ingredients, n-6 LC-PUFA competitive regulation does not became a good reason for no significant difference in *d6dB* expression. Thus,

the main reason for this raises from feedback inhibition by high level of dietary EPA and DHA, since DHA is the end production in n-3 HUFA synthesis pathway (Nakamura and Nara, 2004). Moreover, *Δ6fad* gene in Atlantic salmon can be regulated by SREBP-1c and LXR and both of these two factors are inhibited by LC-PUFA (Carmona-Antonanzas et al., 2014).

5. Conclusion

In conclusion, gene selection can help breeding Atlantic salmon with high EPA and DHA synthesis capacity. Fish fillet is the main product of Atlantic salmon, gene selection in liver cannot make the EPA and DHA content in fillet significantly increasing, when fed Atlantic salmon with marine diet. This because dietary fat can strongly affect fat content, fatty acid composition in fillet and even HUFA synthesis related gene expression in liver. Therefore, gene selection is more necessary when vegetable oils partially replace fish oil, due to replacing fish oil by vegetable oil in fish diet decreasing EPA and DHA level in fillet and the reduced amount is increased when amount of vegetable oil added increases. Therefore, the amount of vegetable oil added into fish diet is important--for producing Atlantic salmon with EPA and DHA level as we expected (close to the level of feeding only fish oil), and n-6 to n-3 PUFA ratio is also crucial--for fish health.

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