Norwegian University of Life Sciences
Faculty of Biosciences
Department of Animal and Aquacultural Sciences

Philosophiae Doctor (PhD)
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The genetics of omega-3 fatty acids in fillets of Atlantic salmon (*Salmo salar* L.)

Genetisk karakterisering av omega-3 fettsyrer i filet av atlantisk laks (*Salmo salar* L.)

Siri Storteig Horn

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Ås, July 2019

Siri Storteig Horn

Contents

List of abbreviations

ALA: alpha linolenic acid

ARA: arachidonic acid

DHA: docosahexaenoic acid

DPA: docosepentaenoic acid

EPA: eicosapentaenoic acid

FA: fatty acid

FAS: fatty acid synthase

FO: fish oil

GWAS: genome-wide association studies

LC n-3 PUFA: long-chain omega-3 polyunsaturated fatty acid

LOA: linoleic acid

NIR: near infrared

PL: phospholipids

SNP: single-nucleotide polymorphism

TAG: triacylglycerides

Summary

Decreasing contents of the omega-3 fatty acids EPA and DHA in farmed Atlantic salmon reduces the nutritional value for consumers and influences physiological processes in the fish. Most studies of physiological omega-3 effects in Atlantic salmon have focused on responses to changed dietary levels. However, there are inherent differences in contents of EPA and DHA between Atlantic salmon fed the same diet. Revealing the metabolic differences between these individuals may contribute towards the goal of producing healthy fish of high nutritional value. Previous studies have showed the potential of selective breeding to increase the total n-3 LC-PUFA levels in salmon tissues, but knowledge on the genetic parameters for individual muscle fatty acids and their relationships with other traits were lacking.

The overall aim of this thesis was to identify the genetic basis and underlying biological mechanisms associated with omega-3 content in Atlantic salmon fillet. For this purpose, the Atlantic salmon from a SalmoBreed AS broodstock population studied were reared together under the same conditions and fed the same diet throughout their life, in order to reduce environmental factors to a minimum.

The EPA and DHA content of skeletal muscle of Atlantic salmon fed the same diet displayed large variation, and were both heritable traits (0.09 and 0.26, respectively). Muscle EPA and DHA traits had a polygenic architecture. However, a strong signal for a QTL was located at chromosome 21. Thus, changing muscle FA composition by selective breeding is possible. New technology that measures FA composition easier and faster may provide larger datasets for research and facilitate implementation in breeding programs.

Inherent differences in EPA and DHA in muscle were linked to fat deposition in liver, viscera and muscle. EPA had more favorable associations with the lipid deposition traits than DHA. This implies that EPA and DHA affect body fat deposition, also when the dietary requirements of these FAs are met.

Muscle EPA and DHA content was also associated with several metabolic pathways including carbohydrate metabolism, insulin signaling, muscle tissue development and lipid metabolism. There were no indications of omega-3 bioconversion being important for determining the content of these fatty acids in muscle of Atlantic salmon. The results

suggest that the reason why some individuals have a higher level of EPA in muscle is that this fatty acid is deposited rather than oxidized for energy.

Correlations between liver and muscle were very low regarding both EPA and DHA content, which implies that future studies of omega-3 in Atlantic salmon fillets should focus more on muscle tissue.

By applying an open approach using genomics and transcriptomics, this thesis has significantly contributed towards identifying the genetic basis and underlying biological mechanisms associated with inherent differences in EPA and DHA content in Atlantic salmon fillet.

Sammendrag

Minkende innhold av omega-3-fettsyrene EPA og DHA i oppdrettslaks reduserer næringsverdien for forbrukerne og påvirker fysiologiske prosesser i fisken. De fleste studier av fysiologiske effekter av omega-3 i atlantisk laks har fokusert på respons på forskjellige nivåer av omega-3 i fôret. Det er imidlertid individuelle forskjeller i innholdet av EPA og DHA mellom atlantisk laks gitt samme fôr. Å avdekke de metabolske forskjellene mellom disse individene kan bidra til målet om å produsere sunn fisk med høy næringsverdi. Tidligere studier har vist et potensial for selektiv avl for økt totalnivå av omega-3 fettsyrer i laksevev, men kunnskap om de genetiske parameterne for individuelle fettsyrer og deres sammenheng med andre egenskaper manglet.

Det overordnede målet med denne avhandlingen var å identifisere det genetiske grunnlaget og underliggende biologiske mekanismer assosiert med omega-3 innhold i filet av atlantisk laks. Derfor studerte vi oppdrettslaks, fra en SalmoBreed avlspopulasjon, som levde under de samme forholdene og fôret med samme diett gjennom hele livet, slik at miljøfaktorer ble redusert til et minimum.

Det var stor individuell variasjon i EPA- og DHA-innholdet i fiskenes muskelvev, og både EPA og DHA var arvelige egenskaper, med arvbarhet på henholdsvis 0,09 og 0,26. Dermed er det mulig å endre fettsyresammensetningen i laksemuskel ved selektiv avl. EPA- og DHA-egenskapene hadde en polygen arkitektur med en potensiell QTL lokalisert på kromosom 21. Ny teknologi som måler fettsyresammensetning enklere og raskere, kan gi større datasett for forskning og legge til rette for implementering i avlsprogrammer.

Iboende forskjeller i EPA og DHA i muskel var knyttet til fettdeponering i lever, innvoller og muskel. EPA hadde mere gunstige genetiske korrelasjoner med fettdeponeringsegenskaper enn DHA. Dette antyder at EPA og DHA påvirker fettavleiring i laksens kropp, også når de basale ernæringskravene er oppfylt for disse fettsyrene.

EPA- og DHA-innholdet i muskel var også forbundet med flere metabolske prosesser, inkludert karbohydratmetabolisme, insulinsignalisering, muskelutvikling og lipidmetabolisme. Det var ingen indikasjoner på at reaksjonsveien for omdanning av omega-3 (omega-3 bioconversion) var viktig for å bestemme innholdet av disse

fettsyrene i muskel av atlantisk laks. Resultatene tyder på at individer som har et høyere nivå av EPA i muskel, deponerer denne fettsyren i stedet for å oksidere den for energi.

Korrelasjonene mellom lever og muskel var svært lave med hensyn til innhold av både EPA og DHA, noe som indikerer at fremtidige studier av omega-3 i atlantisk laks bør fokusere mer på muskelvev.

Ved å anvende en åpen tilnærming ved hjelp av genomikk og transkriptomikk har denne avhandlingen bidratt betydelig til å identifisere genetisk bakgrunn og underliggende biologiske mekanismer knyttet til iboende forskjeller i EPA og DHA innhold i filet av atlantisk laks.

List of papers

This thesis is based on the papers listed below, which will be referred to by their roman numbers throughout the thesis.

I. Horn, S. S., Ruyter, B., Meuwissen, T. H. E., Hillestad, B. & Sonesson, A. K. (2018). **Genetic effects of fatty acid composition in muscle of Atlantic salmon.** Genetics Selection Evolution, 50 (1): 23.

II. Horn, S. S., Sonesson, A. K., Krasnov, A., Moghadam, H., Hillestad, B., Meuwissen, T. H. E. & Ruyter, B. (2019). **Individual differences in EPA and DHA content of Atlantic salmon are associated with gene expression of key metabolic processes.** Scientific Reports, 9 (1): 3889.

III. Horn, S. S., Ruyter, B., Meuwissen, T. H. E., Moghadam, H., Hillestad, B. & Sonesson, A. K. **Accuracy of selection for omega-3 fatty acid content in Atlantic salmon fillets.**

Manuscript submitted for publication in Aquaculture

IV. Horn, S. S., Ruyter, B., Meuwissen, T. H. E., Moghadam, H., Hillestad, B. & Sonesson, A. K. **GWAS identifies genetic variants associated with omega-3 fatty acid composition of Atlantic salmon fillets.**

Manuscript submitted for publication in Aquaculture

1. General introduction

Fish is the main source of the long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFAs) eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in human nutrition. EPA and DHA are known for having many beneficial health effects, especially in preventing and attenuating a range of inflammatory disorders, including cardiovascular disease, immune dysfunction, neurological conditions and obesity (Calder, 2015; Eilander et al., 2007; Rogero & Calder, 2018; Ruxton et al., 2007; Thota et al., 2018; Todorcevic & Hodson, 2015). The aquaculture industry is becoming increasingly more important for providing fish for human consumption, and has since 2014 provided more fish for human consumption than wild fisheries (FAO, 2016). Fatty fish, like Atlantic salmon (*Salmo salar* L.), are especially rich in LC n-3 PUFAs, and are therefore major sources of these important nutrients in the human diet (Henriques et al., 2014; Williams & Burdge, 2006).

Farmed salmon feed has traditionally contained high levels of LC n-3 PUFAs from fish oil (FO) and fishmeal, but while the production of these ingredients has been rather constant since the 1970's, the demand for marine oils containing omega-3 fatty acids has been increasing globally (Sprague et al., 2016; Sprague et al., 2017; Ytrestoyl et al., 2015). This has led to the need to replace a large proportion of the marine ingredients with more sustainable plant-based ingredients in aquaculture feed. While 90% of traditional Norwegian Atlantic salmon diets were composed of marine ingredients in 1990, current diets only contain approximately 30% marine ingredients (Ytrestoyl et al., 2015; Ytrestøyl et al., 2014). Although farmed Atlantic salmon is still considered a major source of LC n-3 PUFAs in the human diet (Henriques et al., 2014; Jensen et al., 2012; Sprague et al., 2016), the reduction in marine ingredients in fish feed has resulted in a substantial decline in the content of EPA and DHA in farmed salmon fillets (Nichols et al., 2014; Sprague et al., 2016; Ytrestoyl et al., 2015). Absolute content of EPA and DHA in Scottish farmed Atlantic salmon fillets have decreased from an average of 2.7 g in 2006 to 1.4 g per 100 g fillet in 2015 (Sprague et al., 2016), and in Norway the content was reported to be from 1.1 to 1.6 grams in 2017 (IMR, 2019; Lundebye et al., 2017). As further reductions of marine ingredients in the feed will not only reduce the nutritional value of the fish to consumers, but also fish health (Bou et al., 2017c), the limited supply

of omega-3 ingredients is a critical factor preventing increased production in aquaculture (Craze, 2018).

1.1 Omega-3 fatty acids

Fatty acids (FAs) are hydrocarbon chains with a carboxyl group at one end of the chain and a methyl group at the other. They vary in chain length, number and position of carbon–carbon double bonds, and have different properties and roles in the body based on these characteristics. FAs containing double bonds are referred to as unsaturated FAs, which have lower melting points and are more chemically reactive than saturated FAs. FAs with two or more double bonds are referred to as polyunsaturated fatty acids (PUFA) (McDonald et al., 2011). Omega-3 fatty acids are PUFAs with a double bond located three carbons from the methyl end of the hydrocarbon chain.

As the primary component of lipids, FAs play important roles in the body, including energy supply, structure and functions of cellular membranes (Sargent, 2002). In addition, certain FAs, and their derivatives, function as important regulators of metabolism (Miyazaki & Ntambi, 2008; Tocher, 2003; Tocher, 2010).

Omega-3 and omega-6 PUFAs are essential FAs for all vertebrate species, including fish, meaning that they, or their precursors, must be obtained from the diet (Bou et al., 2017a; Das, 2006; Ruyter et al., 2000; Ruyter & Thomassen, 1999). This is because vertebrates lack Δ12 and Δ15 desaturases needed to create double bonds at the omega-3 and omega-6 position of the FAs hydrocarbon chain (Tocher et al., 1998). Observed long-term consequences for Atlantic salmon fed omega-3 levels below the dietary requirements include reduction in growth, increased mortality in sea cages, inflammation in mid-intestine, and dysregulation of body fat deposition (Bou et al., 2017a; Bou et al., 2017c).

1.2 Atlantic salmon muscle fatty acid composition

The salmon fillet, i.e. muscle, consists of several tissues; mainly white skeletal muscle, connective and adipose tissues. Because it is the Atlantic salmon's main site of energy storage, the muscle has a high content of fat, in contrast to e.g. the cod, which stores most of its lipid-reserves in the liver (Aursand et al., 1994; Polvi & Ackman, 1992; Zhou et al., 1996). In Atlantic salmon, muscle fat is mainly stored in adipocyte clusters along the connective tissue sheets (Nanton et al., 2007; Polvi & Ackman, 1992; Zhou et al.,

1995). The muscle fat content of salmonid increases with dietary fat content, size, growth and development of the fish (Bell et al., 2004; Gjerde & Schaeffer, 1989; Rye & Gjerde, 1996). Several studies have reported seasonal variations in growth and lipid deposition of Atlantic salmon: increased feed intake, growth and lipid deposition is often observed during summer and autumn and correlates with high water temperatures and declining day length (Dessen et al., 2017; Mørkøre & Rørvik, 2001; Nordgarden et al., 2003; Oppedal et al., 2006).

The largest portion of FAs in muscle is found in triacylglycerides (TAG) (Nanton et al., 2007; Polvi & Ackman, 1992; Zhou et al., 1995), but FAs are also found in the cell membranes, as components of phospholipids (PL). The muscle fat content will influence the FA composition of muscle, because it affects the ratio of TAG to PL. The FA composition of TAG will reflect that of the feed, while the FA composition of the PL bilayer of cell membranes will remain relatively stable with a high level of LC n-3 PUFA (Ruyter et al., 2006; Tocher, 2003). This is why, typically, a lean salmon fillet has a higher proportional content (% of total muscle FAs) of EPA and DHA compared to a fatty fillet (Sargent, 2002).

There are several examples showing that the feed is the main factor determining FA composition of the muscle of Atlantic salmon (Bell et al., 2001; Bell et al., 2010; Torstensen et al., 2005). However, dietary fatty acids are not directly deposited into the fish muscle and other tissues, but are subject to different metabolic processes, such as energy production (β-oxidation), chain elongation and desaturation (bioconversion), and fatty acid synthesis (lipogenesis) (Tocher, 2003; Turchini et al., 2011). One of the processes that has received a lot of attention in omega-3 research in Atlantic salmon is bioconversion.

1.3 Omega-3 Bioconversion

The shorter omega-3 fatty acid alpha-linolenic acid (ALA; 18:3n-3), commonly found in some plant oils, can be converted into the LC n-3 PUFAs EPA and DHA through the omega-3 bioconversion pathway (Figure 1). This metabolic pathway was first described for mammals (Sprecher, 1981), and later for salmonids (Buzzi et al., 1996; Buzzi et al., 1997; Monroig et al., 2013), and consists of a series of desaturation and elongation reactions (presented in Figure 1). The first step is Δ6 desaturation of 18:3n-3 to produce

18:4n-3, which is then elongated to 20:4n-3 (Miyazaki & Ntambi, 2008). An alternative route to form 20:4*n*-3 is by elongation of 18:3n-3 to 20:3n-3 followed by a Δ8 desaturation. 20:4n-3 is desaturated by Δ5 desaturase to form EPA (Monroig et al., 2011; Park et al., 2009). In salmon, DHA is formed from EPA by two further elongation steps, a second Δ6 desaturation, and a chain-shortening step by peroxisomal β-oxidation (Sprecher, 2000). Some species have Δ4 desaturase activity, allowing a more direct formation of DHA from EPA by one elongation step, followed by Δ4 desaturation (Fonseca-Madrigal et al., 2014; Kuah et al., 2015; Morais et al., 2015). This has not been found in Atlantic salmon. The same enzymes also act on omega-6 FAs, converting linoleic acid (LOA; 18:2n-6) into arachidonic acid (ARA; 20:4n-6) (Miyazaki & Ntambi, 2008).

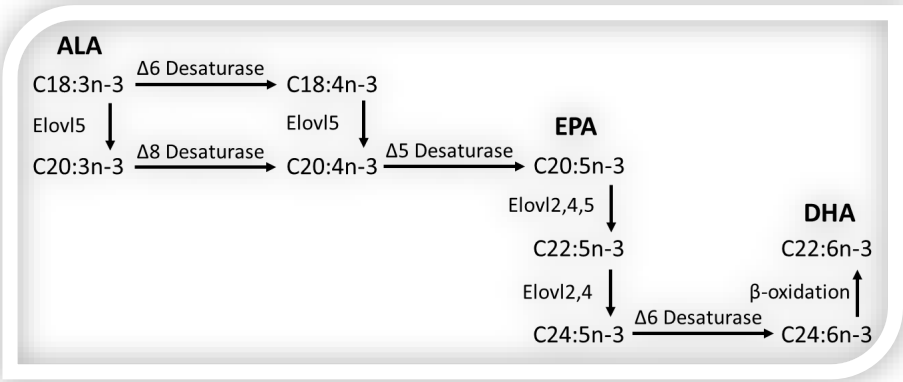

Figure 1. Omega-3 bioconversion pathway in Atlantic salmon.

In Atlantic salmon, three genes encode Δ6 desaturases: *Δ6fad_a*, *Δ6fad_b*, and *Δ6fad_c* (Monroig et al., 2010; Zheng et al., 2005a), and one gene encodes a Δ5 desaturase (Hastings et al., 2004). Four elongase genes have been cloned and characterized, encoding for elongases with different chain-length specificities: *elovl5a* and *elovl5b* (Agaba et al., 2005; Morais et al., 2009), *elovl2* (Morais et al., 2009), and *elovl4* (Carmona-Antonanzas et al., 2011). Delta 5 and Δ6 desaturases are positioned on chromosome 23, *elovl5a* on chromosome 1, *elovl5b* on chromosome 28, *elovl2* on chromosome 19 and *elovl4* on chromosome 6 (https://salmobase.org/). The presence and activity of the desaturase and elongase enzymes of the pathway determines the conversion capacity. Several factors influence the activities of these enzymes, including

feed, life-stage, sea temperature, and genetic factors (Berge et al., 2015; Kjær et al., 2008; Zheng et al., 2005b).

Through this bioconversion, Atlantic salmon has the potential to be a net producer of DHA, and to a certain degree EPA (Bou et al., 2017b; Rosenlund et al., 2016; Sanden et al., 2011; Turchini et al., 2011), but only when fed low dietary levels of EPA and DHA, as the activity of the enzymes in the pathway is inhibited by dietary EPA and DHA (Rosenlund et al., 2016; Sissener et al., 2016). The omega-3 bioconversion capacity is therefore limited and cannot result in higher levels of EPA and DHA tissue levels than addition of these FAs in the diet can (Betancor et al., 2015; Moya-Falcon et al., 2005; Tocher, 2015; Turchini & Francis, 2009; Turchini et al., 2011). The liver and intestine are considered the major bioconversion sites in Atlantic salmon, but omega-3 bioconversion genes are expressed also in muscle (Codabaccus et al., 2011). There is limited knowledge on its importance of omega-3 bioconversion in determining the FA composition of muscle.

In addition to omega-3 bioconversion, other metabolic processes that have been shown to affect muscle FA composition in Atlantic salmon include selective uptake, β-oxidation and deposition of FAs. Studies have observed selective deposition of DHA in muscle (Bell et al., 2004; Torstensen et al., 2004), which is primarily due to it being a relatively poor substrate for β-oxidation, likely because it is an important component of cellular membranes (Sargent, 2002). EPA, on the other hand, seems to be to a larger extent metabolized in muscle (Torstensen et al., 2004). This is probably because EPA is a more bioactive fatty acid that is involved in eicosanoid synthesis, is β-oxidized for energy and is used as substrate to produce DHA (Tocher, 2015). Vegusdal et al. (2004) found results indicating that Atlantic salmon muscle cells take up EPA more rapidly than 18:1n-9, which is in agreement with studies on human skeletal muscle cells (Aas et al., 2006; Lovsletten et al., 2018; Wensaas et al., 2009). Most of the studies on Atlantic salmon are feeding trials, making it difficult to determine what changes in metabolic pathways are the effect of feed and what is caused by genetic differences.

1.4 Physiological roles of EPA and DHA

Evidence from mammalian studies indicates that tissue content of EPA and DHA play important roles in the regulation of nutrient metabolism. Increased content of EPA and

DHA are reported to improve glucose uptake (Aas et al., 2006; Jeromson et al., 2018), have favorable effects on glucose utilization (Hessvik et al., 2010), and thereby have a preventive effect on development of metabolic syndrome (Lanza et al., 2013; Stephens et al., 2014). EPA and DHA may also improve skeletal muscle capability of switching between use of lipids, protein and glucose for production of energy (Jeromson et al., 2015). High levels of EPA may result in downregulated lipogenesis and upregulated mitochondrial β-oxidation (De Tonnac et al., 2016). It is also suggested by several studies that EPA and DHA have anabolic or anti-catabolic properties in skeletal muscle (reviewed in Jeromson et al. (2015)). There is limited knowledge on these roles of EPA and DHA in fish. With the shift in salmon diets causing major alterations in EPA and DHA content of skeletal muscle, it is especially important to gain knowledge on how this will influence nutrient metabolic pathways.

1.5 The genetic basis of muscle fatty acid composition

Atlantic salmon fed the same diet have variation in content of LC n-3 PUFA in muscle (Schlechtriem et al., 2007), but the mechanisms and genes causing these individual differences were not known prior to the work in this thesis. However, it was known that individual variation in omega-3 content is partly due to genetics, indicating a potential for selective breeding to increase the content of these FAs. One study estimated heritability of the total LC n-3 PUFA in muscle to 0.77, but this was based on pooled family samples of very small fish (Leaver et al., 2011). The heritability of omega-3 in slaughter-sized Atlantic salmon was unknown, as was basic genetic parameters of individual omega-3 FAs.

Selective breeding for increased omega-3 bioconversion has been tested recently: Selection based on liver expression of the Δ6 desaturase gene in a large breeding population resulted in increased bioconversion activity and EPA and DHA levels in liver tissue, but surprisingly the same response was not seen in muscle (Berge et al., 2015). This shows that we cannot assume that the knowledge gained on liver omega-3 FA metabolism can be applied also to muscle.

In order to increase the knowledge on what characterizes fish with high omega-3 in muscle at genetic and molecular level and find the potential for including EPA and DHA

in breeding programs, a broad approach is required. Several different genetic methods may be applied for this purpose:

1. Quantitative genetic analyses combines phenotypic recordings with pedigree information in order to estimate heritability and genetic correlations between the trait of interest and other important production and health traits. This is important knowledge both regarding the consequences of selecting for the new trait, and increasing the knowledge on the biology of the trait (Lynch & Walsh, 1998).

2. Gene expression studies: RNA sequencing allows a snapshot of the whole transcriptome, enabling identification of genes affecting a trait of interest without any prior assumptions. Revealing differences in gene expression between individuals with high and low levels of EPA and DHA may thus identify metabolic pathways associated with differences in these traits.

3. Genome-wide association studies (GWAS) combines quantitative phenotypic information with genotypic information, in the form of single-nucleotide polymorphisms (SNPs), and can identify genetic polymorphism affecting phenotypic traits by testing for association of SNPs with the phenotypic trait (Dekkers, 2012). Identification of the loci/markers affecting phenotypic traits makes it possible to apply marker-assisted selection, which could facilitate more effective breeding and implementation of new traits in breeding schemes (Meuwissen et al., 2013).

2. Aims of the thesis

The overall aim of this thesis was to identify the genetic basis and underlying biological mechanisms associated with omega-3 content in Atlantic salmon fillet. The main objective can be subdivided into the following specific aims related to each of the studies in this thesis:

- Estimate genetic parameters of individual fatty acids in the muscle of farmed Atlantic salmon to evaluate the potential for selection for increased LC n-3 PUFA levels and provide insight into the muscle's fatty acid metabolism (Paper I)
- Investigate how individual differences in EPA and DHA content in skeletal muscle of Atlantic salmon are associated with expression of genes involved in key physiological processes, particularly nutrient metabolism (Paper II)
- Compare the accuracy of selection for muscle content of EPA, DHA and fat in Atlantic salmon, by varying the sources of genetic information used in the estimation of breeding values (Paper III)
- Identify genetic variants associated with long-chain omega-3 fatty acid content in Atlantic salmon muscle, by performing a genome-wide association study (Paper IV)

3. Brief summary of papers

3.1 Paper I

Previous studies have showed the potential of selective breeding to increase LC n-3 PUFA levels in Atlantic salmon tissues, but knowledge on the genetic parameters for individual muscle fatty acids and their relationships with other traits is still lacking. Thus, we estimated genetic parameters for muscle content of individual FAs, and their relationships with lipid deposition traits, muscle pigmentation, sea lice and pancreas disease in slaughter-sized Atlantic salmon. Our aim was to evaluate the selection potential for increased LC n-3 PUFA content and provide insight into FA metabolism in Atlantic salmon muscle.

Among the omega-3 PUFAs, proportional content of ALA and DHA had the highest heritability (both 0.26) and EPA the lowest (0.09). Genetic correlations of EPA and DHA proportions with muscle fat differed considerably, 0.60 and 0.01, respectively. The genetic correlation of DHA proportion with visceral fat was positive and high (0.61), whereas that of EPA proportion with lice density was negative. FAs that are in close proximity along the omega-3 bioconversion pathway showed positive correlations with each other, whereas the start (ALA) and end-point (DHA) of the pathway were negatively correlated (-0.28), indicating active bioconversion of ALA to DHA in the muscle of fish fed high FO-diet.

Since content of individual FAs in Atlantic salmon muscle show additive genetic variation, changing FA composition by selective breeding is possible. Taken together, our results show that the heritabilities of individual LC n-3 PUFAs and their genetic correlations with other traits vary, which indicates that they play different roles in muscle lipid metabolism, and that proportional muscle content of EPA and DHA are linked to body fat deposition. Thus, different selection strategies can be applied in order to increase the content of healthy omega-3 FAs in the salmon muscle. We recommended selection for the proportion of EPA+DHA in the muscle because they are both essential FAs and because such selection had no clear detrimental effects on the other traits analyzed in this dataset.

3.2 Paper II

Decreasing content of the omega-3 fatty acids EPA and DHA in farmed Atlantic salmon reduces the nutritional value for consumers and influences physiological processes in the fish. Most studies of physiological omega-3 effects in Atlantic salmon have focused on responses to changed dietary levels. However, there are inherent differences in content of EPA and DHA between Atlantic salmon fed the same diet. Revealing the metabolic differences between these individuals may contribute towards the goal of producing healthy fish of high nutritional value. The main aim of this study was therefore to explore how individual differences in EPA and DHA content in muscle of Atlantic salmon fed the same diet, are associated with expression of genes involved in key physiological processes.

Muscle and liver transcriptome of 59 slaughter-sized Atlantic salmon were sequenced. Linear model association tests were performed between gene expression and the quantitative traits EPA, DHA and EPA+DHA (percentage of total fatty acids in skeletal muscle) to detect trait-associated genes. Higher DHA content was concurrent with increased expression of genes of the glycolytic pathway and the production of pyruvate and lactate, whereas EPA was associated with increased expression of pentose phosphate pathway and glycogen breakdown genes. Furthermore, EPA, but not DHA, was associated with expression of genes involved in insulin signaling. Expression of genes specific for skeletal muscle function were positively associated with both EPA and DHA. EPA and DHA were also associated with expression of genes related to eicosanoid and resolvin production. EPA was negatively associated with expression of genes involved in lipid catabolism. Thus, a possible reason why some individuals have a higher level of EPA in the muscle is that they deposit - rather than oxidize - EPA for energy.

3.3 Paper III

The main aim of this paper was to compare the accuracy of selection for muscle content of EPA, DHA and fat in Atlantic salmon, by varying the sources of genetic information used in the estimation of breeding values. The following genetic information sources were compared: pedigree, SNP-chip markers, imputed markers and allele-specific expression markers.

The results showed that differences between information sources were in general small, and different genetic information performed best for different traits. SNP-chip performed best for DHA, and pedigree performed best for EPA and muscle fat. Imputed markers did not improve selection accuracy for any of the traits studied, probably due to imputation errors caused by the large number of imputed SNPs combined with the small dataset. Markers based on allele-specific expression were able to capture a lot of genetic variation for DHA, but did not give higher accuracies when combined with SNP-chip or pedigree information.

The cross-validation accuracies for selection for DHA and EPA were moderate and offer possibilities for selection for these traits, especially if one extends the reference data set to a much bigger population.

3.4 Paper IV

The objective of this paper was to identify genetic variants associated with long-chain omega-3 fatty acid content in Atlantic salmon muscle, in order to identify genes underlying the genetic variation in these traits, by performing a GWAS.

Fatty acid composition, including EPA, DHA content and the ratios DHA/DPA (docosapentaenoic acid; 22:5n-3) and DHA/ALA of skeletal muscle was determined in 642 Atlantic salmon. Further, a 57K SNP array was used to genotype the 642 individuals to search for SNPs associated with skeletal muscle content of individual omega-3 fatty acids as well as ratios between fatty acids, using a mixed model approach. We identified markers showing a significant association with the ratio of DHA to DPA located on chromosome 19, close to the candidate gene *elovl2*, which is directly involved in the conversion of DPA to DHA. The results further suggested that genetic variation affecting fillet EPA and DHA content is present on Atlantic salmon chromosome 21, as the GWAS analysis of EPA, DHA and DHA/ALA ratio all pointed to this chromosome. No known genes of direct relevance to the omega-3 bioconversion pathway were found here. We discuss the relevance of other interesting genes related to lipid metabolism and health located in this region.

4. General Discussion

This thesis is a study of the genetics of omega-3 fatty acids in Atlantic salmon fillets, and contains four papers. An overview of the work in this thesis is presented in Figure 2. The results from Paper I revealed great variation in the content of EPA and DHA. This variation confirmed that there are inherent differences of these traits even among fish reared under the same conditions, thereby endorsing the continued studies of the genetics of these highly relevant traits in today's aquaculture. In Paper I, genetic parameters for muscle content of individual FAs were estimated, and the relationships between FAs and lipid deposition traits, muscle pigmentation, sea lice and pancreas disease were investigated. In Paper II, metabolic differences between animals inherently varying in muscle EPA and DHA content were explored by a gene expression association study. In Paper III, the accuracy of selection for muscle content of EPA, DHA and fat was compared between different sources of genetic information: pedigree, SNP-chip markers, imputed markers and allele-specific expression markers. In Paper IV, a GWAS was performed in order to identify genetic variants associated with LC n-3 PUFA content in muscle.

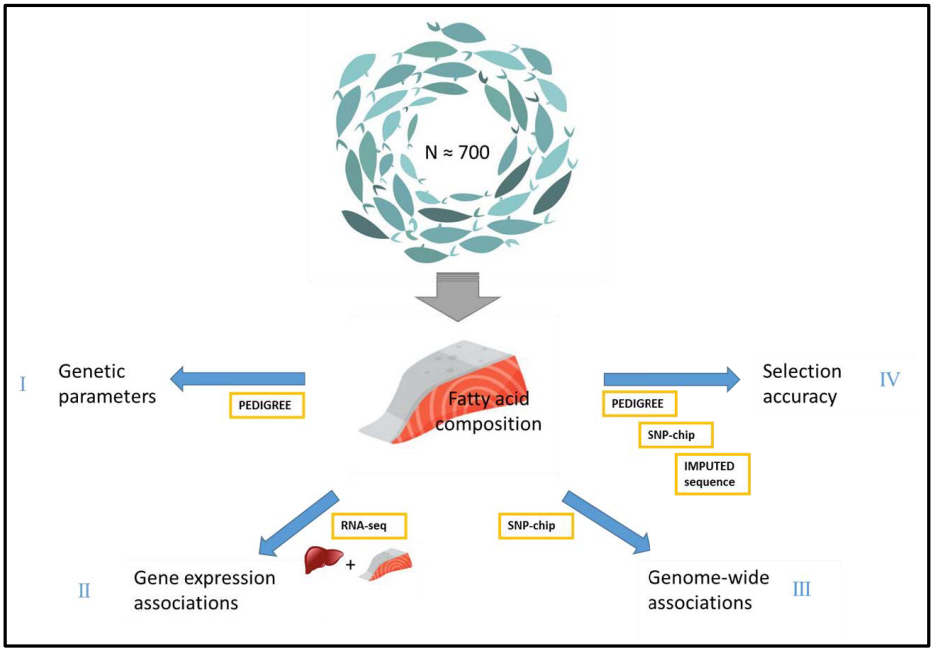

Figure 2. Methodological overview of how muscle fatty acid composition data were utilized in combination with different genetic information in the four papers of this thesis.

To achieve the aims of the thesis, the salmon studied were reared together under the same conditions and were fed the same diet throughout their life in order to reduce environmental factors to a minimum. The fish originated from an Atlantic salmon broodstock test-population of SalmoBreed AS that was reared to slaughter size. The fish were fed a commercial broodstock high-FO diet and fasted approximately 14 days prior to slaughter. From this population, 668 fish from 194 full-sib families were sampled for muscle FA composition, which laid the foundation of the studies performed (Paper I-IV). The FA traits analyzed were presented in percentage of total FAs.

In the following, consequences of the four studies performed (Papers I to IV) are put into the perspective of the literature and a more general perspective.

4.1 Muscle omega-3 content and metabolism is different from liver

Most studies of salmonid FA metabolism focus on liver, as liver is the more metabolically active organ (Tocher, 2003). Several previous studies have focused on omega-3 bioconversion genes in relation to LC n-3 PUFA content in Atlantic salmon (e.g. Giri et al., 2016; Kjaer et al., 2016; Monroig et al., 2010; Morais et al., 2009; Zheng et al., 2005b). It has even been suggested that Atlantic salmon can be a net producer of LC n-3 PUFA because if its omega-3 bioconversion ability (Sanden et al., 2011). Knowledge about the muscle omega-3 FA metabolism is scarce. The few transcriptome studies of Atlantic salmon skeletal muscle have showed that this tissue expresses FA desaturase and elongase genes (Codabaccus et al., 2011; Zheng et al., 2005a), as well as transcription factors known to regulate lipid metabolism genes (Vegusdal et al., 2004).

There seems to be a general assumption that the muscle FA composition is influenced by hepatic omega-3 bioconversion (in addition to diet). However, to the best of my knowledge, the correlation between liver and muscle LC n-3 PUFA content has not been reported previously. Feeding trials have indicated positive correlations between liver and muscle FA content, as EPA and DHA increases in both tissues with increased levels in the diet (Bell et al., 2001; Bou et al., 2017a; Brodtkorb et al., 1997; Torstensen et al., 2000). However, differences between liver and muscle deposition of EPA and DHA have been shown when dietary levels are very low (Bou et al., 2017a). In this thesis, comparing fish fed the same feed provided an opportunity to study the biological aspects of omega-3 FAs from a new angle, by eliminating the effect of feed and not

comparing groups. This enabled us to study the inherent differences between individual fish.

One important finding from Paper II was that omega-3 content in muscle and liver had a low correlation: the phenotypic correlation between liver and muscle regarding both EPA and DHA content was not significantly different from zero (n=59) (Figure 3). This finding is in agreement with a study by our group showing that selection for increased Δ6-desaturase expression in liver increased LC n-3 PUFA content in liver, but not in muscle (Berge et al., 2015).

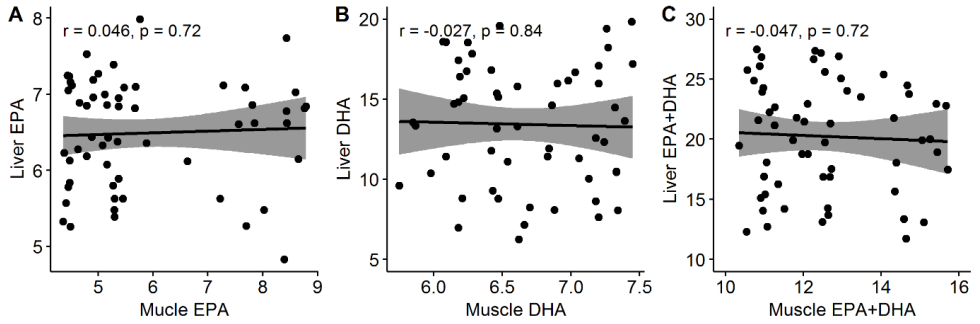

Figure 3. Correlation between liver and muscle content of A) EPA, B) DHA and C) EPA+DHA (% of total FAs).

The low correlation between liver and muscle omega-3 content demonstrates that it cannot be assumed that what happens in the liver determines muscle content, and that muscle must be studied separately in order to reveal what determines individual variation in levels of LC n-3 PUFA in Atlantic salmon fillet.

Further insight into the difference between liver and muscle can be gained by comparing the correlations between the main start- and end-points of the omega-3 and omega-6 bioconversion pathways (Figure 4), as correlations between start and end-point of the pathways are used as indicators of bioconversion activity. Whereas DHA (22:6n-3) is the main end product of desaturation and elongation of ALA (18:3n-3), ARA (20:4n-6) is the main end product of desaturation and elongation of LOA (18:2n-6) (Tocher, 2003). The same enzymes function in both bioconversion pathways (Miyazaki & Ntambi, 2008).

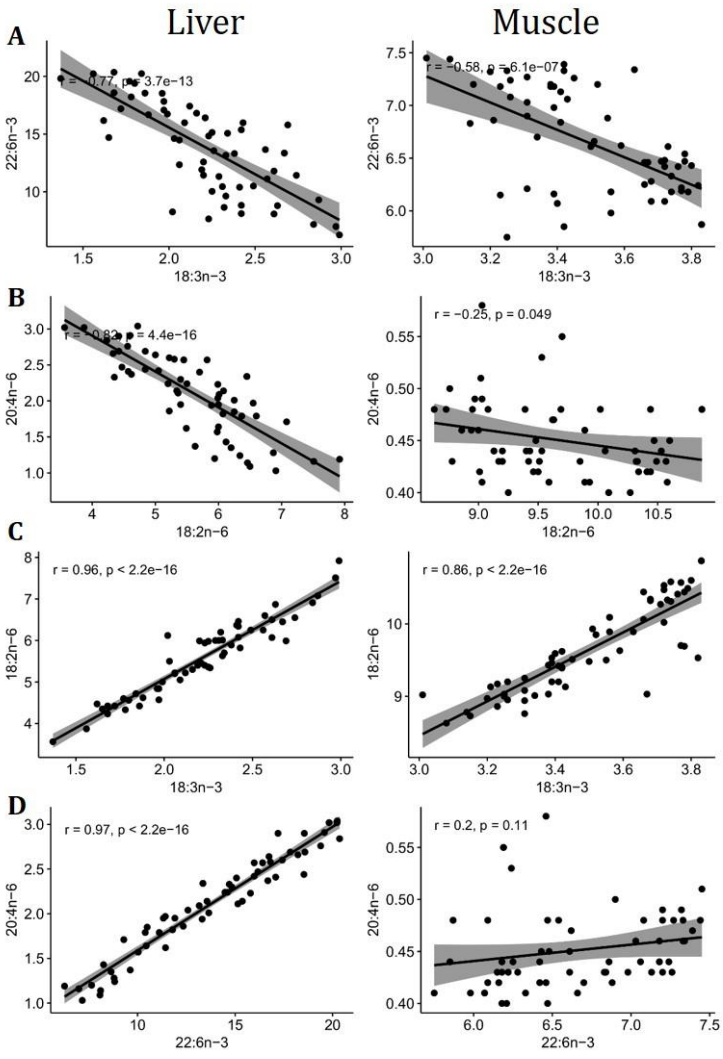

Figure 4. Correlations between proportional content of fatty acids within liver and muscle tissue. A) ALA vs DHA B) LOA vs ARA C) ALA vs LOA D) ARA vs DHA. Data from 59 fish of similar weight and muscle fat percentage (Paper II).

The correlation between ALA and DHA, which could indicate omega-3 bioconversion, was stronger in liver than muscle (-0.77 vs -0.58) (Figure 4A). The correlation between the start and end of omega-6 bioconversion showed even bigger differences between liver and muscle, again stronger in liver (-0.82) than muscle (-0.25) (Figure 4B). Further, there was a high correlation between the omega-3 and omega-6 start-substrate in both liver (0.96) and muscle (0.86) (Figure 4C), but between the two end-substrates (DHA

and ARA) there were clear differences between liver and muscle: in muscle the correlation was low (0.2), while in liver it was very high (0.97) (Figure 4D). These results show that generally, relationships are stronger in liver, whereas in muscle the correlations are weaker, indicating that bioconversion is less important in determining FA composition in muscle compared to liver. This can (partly) explain why there is a low correlation between liver and muscle EPA and DHA content.

The role of bioconversion in omega-3 FA composition in muscle discussed above was supported by the results from the gene expression association study (Paper II) and the GWAS (Paper IV): The gene expression analysis showed no positive association between muscle EPA and DHA content and expression of bioconversion-related genes in muscle (Paper II). The hepatic expression of the fatty acid desaturase genes *fadsd5* and *fadsd6* was negatively associated with muscle level of EPA and DHA (Paper II). Thus, it did not seem like hepatic omega-3 bioconversion caused increased muscular levels of EPA and DHA either. However, based on this we could not state with certainty that bioconversion is not important in determining individual differences in omega-3 FA content in muscle. This is because the gene expression results presents only a snapshot of the transcriptome at the time of sampling. Differences in expression of genes earlier in life may have caused the differences in omega-3 levels in muscle, and the fish with the highest levels may have a stronger inhibition of expression of bioconversion genes. In addition, the individual variation in omega-3 content may not be related to differences in gene expression, but rather differences in genetic variants. However, in the GWAS, no markers near genes related to the omega-3 bioconversion pathway were associated with EPA, DHA or DHA/ALA traits in muscle (Paper IV). What was found related to genes in omega-3 bioconversion was that the GWAS of DHA/DPA ratio identified a genome-wide significant peak located close to *elovl2*. This indicates that *elovl2* determines the ratio of DHA to DPA in muscle, but was not strongly associated with the content of EPA or DHA found in muscle. Hence, whilst this gene is involved in bioconversion, it (and thus bioconversion) was not important for EPA and DHA content in muscle.

The high dietary levels of FO may have repressed the omega-3 bioconversion activity and thereby affected the results (Bou et al., 2017b; Ruyter & Thomassen, 1999; Tocher et al., 2003; Zheng et al., 2004). On the other hand, in a study using a low FO diet, no differences in mRNA levels of hepatic desaturases and elongases were observed

between families with high and low muscle LC n-3 PUFA (Leaver et al., 2011). In addition, unpublished results from our group show that there was no re-ranking of groups of individuals regarding their omega-3 bioconversion capacity when the amount of FO in the feed was reduced. It is therefore expected that the fish that has the highest capacity when fed high levels of FO also have the highest capacity when fed lower levels of FO.

Overall, the results presented here indicate that omega-3 bioconversion is not the main factor determining EPA and DHA content in muscle of Atlantic salmon fed a high FO diet. Thus, research should focus on other mechanisms and physiological processes rather than omega-3 bioconversion, such as uptake, depositions and β-oxidation of FAs. Although cause and effect could not be determined by the association study in Paper II, the results suggested that β-oxidation might determine the content of EPA in fillet, as individuals with the highest EPA (and EPA + DHA) content had a lower expression of lipid catabolism genes in both liver and muscle. Since muscle is the main site of β-oxidation in Atlantic salmon (Froyland et al., 2000), and EPA is a particularly good substrate for β-oxidation (Bell et al., 2004; Kjær et al., 2008), it is plausible that differences in β-oxidation rate will affect EPA content in muscle. This may also explain why the same association was not found for DHA, since DHA is a relatively poor substrate for β-oxidation (Sargent, 2002).

4.2 Physiological roles of EPA and DHA in Atlantic salmon

It is known that dietary levels of EPA and DHA affect fat deposition in Atlantic salmon, where insufficient levels results in increased lipid deposition in liver and intestine, and decreased deposition in muscle (Bou et al., 2017c; Liland et al., 2015; Ruyter et al., 2006; Todorcevic et al., 2009). In mammals, fatty liver is associated with metabolic imbalance (Engin, 2017; Milic et al., 2014; Stefan et al., 2008), but there is no definition of fatty liver in fish. Results from both Papers I and II in this thesis indicated that inherent differences in EPA and DHA in muscle is likely to influence body fat deposits.

The results from Paper I and Paper II combined, gave a clearer picture for EPA than for DHA related to lipid deposition. EPA had a negative (-0.31) genetic correlation with liver fat, while DHA had a positive genetic correlation (0.32) (Paper I). The gene expression results from Paper II indicated that increasing skeletal muscle content of both EPA and

DHA reduces fat accumulation in liver, as these FAs were associated with reduced expression of genes involved in lipogenesis in liver. Expression of some genes had stronger associations with the sum of EPA and DHA, for example *fatty acid synthase* (*FAS*) and *1-acylglycerol-3-phosphate acyltransferase gamma-like* (*GPAT1*), a key enzyme for hepatic triglyceride synthesis. However, more genes were associated with EPA, and the associations with DHA were in general low. Hence, the gene expression associations for EPA were in agreement with the genetic correlation between EPA and liver fat.

The link between liver fat and muscle EPA and DHA content could not be seen in the phenotypic correlations, which were very low (<0.11) (Paper I). The fish in the current thesis were fed a high FO diet, and the mean liver fat scores were similar to what has previously been reported for Atlantic salmon fed a similar diet (Mørkøre et al. 2012). Excessive fat deposition in the liver has been consistently observed in Atlantic salmon fed insufficient levels of EPA and DHA (Bou et al., 2017c; Jordal et al., 2007; Torstensen et al., 2011). In the present thesis, salmon were fed a high FO diet with relatively high levels of these fatty acids. Therefore, it is possible that the associations between liver fat content and muscle EPA and DHA suggested here would be more apparent when fish are fed low levels of FO.

It should be mentioned that liver fat was recorded through a subjective scoring system, which proved not very accurate. Although this is a rapid and simple method of recording liver fat, which is optimal when dealing with large amounts of fish, its correlation with chemical analysis of liver fat was relatively low ($r = 0.45$, $p < 0.001$) (data from the 59 fish in Paper II). This highlights the need to improve the liver fat scoring system and that more documentation is needed before conclusions can be drawn on the effects of EPA and DHA on liver fat.

The results on the effects of EPA on fat deposition in muscle showed that EPA had positive genetic and phenotypic correlations with muscle fat (0.60 and 0.21, respectively)(Paper I), which is in agreement with feeding trials showing more lipid or TAG in muscle of Atlantic salmon fed FO diet vs VO diet (Beheshti Foroutani et al., 2018; Nanton et al., 2007; Vegusdal et al., 2004). Gene expression results indicated that EPA has a possible inhibitory effect on both lipogenesis and lipid catabolism in muscle. Expression of key genes involved in lipogenesis in muscle were negatively associated with EPA; for instance *2-acylglycerol O-acyltransferase 1*, which catalyzes the formation

of diacylglycerol, and *acetyl CoA carboxylase*, which is involved in regulation of fatty acid synthesis (Paper II). EPA was also negatively associated with expression of three genes coding for β-oxidation enzymes, as well as *PPAR β/δ*, a central regulator of energy metabolism that stimulates genes of β-oxidation (Dressel et al., 2003; Leaver et al., 2007) (Paper II). The negative associations between EPA and lipid metabolic genes are not in agreement with Lovsletten et al. (2018), who recently reported that processes of FA turnover and oxidation increased in human skeletal muscle cells exposed to EPA, although they also observed more muscle fat with EPA. This reflects the complexity of lipid metabolism in muscle. DHA had weak or no associations with genes involved in lipid metabolism in muscle (Paper II).

For visceral fat, the genetic correlation with DHA was very high (0.61), but with EPA it was low (-0.17). There is no more data on this trait in this thesis.

Overall, these results indicate that inherent differences in muscle EPA and DHA content are linked with body fat deposition in Atlantic salmon. This implies that EPA and DHA affect body fat deposition also when fish are fed sufficient amounts of EPA and DHA, i.e. not only as a dietary deficiency symptom.

The gene expression association analysis further showed that EPA and DHA in muscle are associated with many other physiological processes, such as muscle tissue development, insulin signaling and carbohydrate metabolism (Paper II). Especially interesting was that many genes involved in carbohydrate metabolism and insulin signaling were associated with differences in proportional content of EPA and DHA in muscle. It may be speculated that this is also related to metabolic balance (along with lipid deposition), as the main function of insulin is to shift the cellular metabolic state from catabolic to anabolic. The role of EPA and DHA in insulin signaling and carbohydrate metabolism has previously been documented in humans (Aas et al., 2006; Hessvik et al., 2010; Stephens et al., 2014), but not in Atlantic salmon, possibly indicating that salmon muscle is more similar to that of mammalians than previously assumed. Other positive effects of EPA and DHA supplementation reported in humans have been related to increased sensitivity to anabolic stimuli, muscle function, protein synthesis and muscle strength (Smith et al., 2011; Smith et al., 2015). In Paper II, positive associations with both EPA and DHA were found with expression of genes involved in muscle tissue development and function. Therefore, it may be speculated that selecting

for fish with higher levels of EPA and DHA may lead to increased muscle growth and fillet yield, which would be highly relevant to the Atlantic salmon industry. A recent feeding trial from our group showed a non-significant trend of improved fillet yield with increasing dietary level of EPA and DHA (FHF, 2019, unpublished results).

4.3 Methodology

Analyses of fatty acid composition of sampled fish resulted in the largest dataset on individual FAs in Atlantic salmon tissues currently available. However, in the context of genetic/genomic analyses, this dataset is not very large, and this was reflected in the results: the estimates of genetic parameters had high standard errors (Paper I), genomic prediction resulted in moderate to low prediction accuracies (Paper III), and GWAS resulted in low significance levels of SNPs (Paper IV). The size of the dataset was limited by the chosen method for phenotypic recording of FA composition. We used gas-chromatography, which is highly accurate for measuring individual FAs, but very time-consuming and costly.

The fish studied in this thesis were fasted for 14 days prior to slaughter. This may have had implications for the results presented in this thesis. Fasting responses in fish include reduced metabolism, reduced oxidative pressure and sparing of nutrients (O'Connor et al., 2000; Waagbø et al., 2017). This will in particular affect the gene expression results in Paper II, as it has been shown that enzymes involved in lipid breakdown and protein turnover will generally be up-regulated during starvation, and lipid anabolic enzymes will be down-regulated (Costas et al., 2011; Jagoe et al., 2002; Polakof et al., 2006; Salem et al., 2007; Suzuki et al., 2002). In the current thesis, the specific effects of fasting on gene expression could not be determined because all fish were fasted, and there was no control group as fasting effects were not the focus of these studies. Fasting may also influence the FA composition of muscle. Any individual differences in β-oxidation of specific FAs will likely be enhanced when the fish is fasted and FAs are used for energy to a larger degree, because muscle is the main site of β-oxidation in Atlantic salmon (Frøyland et al., 2000).

A 14-day fasting period is unusual for research trials, but is not uncommon for commercial Atlantic salmon production. Withdrawal of feed prior to stressful events such as transport, lice treatment and slaughter ensures that the gut is emptied and slows

down the metabolism of the fish, thereby reducing the negative impacts of stress. According to the Norwegian food authority, farmed salmon and trout are fasted between one to thirty days prior to slaughter, with an average of nine days (Kristiansen & Samuelsen, 2006).

The FA traits analyzed were presented as percentage of total FAs (proportional content) instead of g/100 g fillet (absolute content), as the latter reflects the amount of fat in the muscle because the absolute content of all FAs increases with increasing total muscle fat content. In Paper I, the estimated heritability for absolute content are also included. These are higher compared to proportional content, but reflect the high heritability of muscle fat. The proportional content of FAs enables us to study the relationship between FAs, and will more likely reveal links between FA composition and metabolic and physiological pathways.

In this thesis, the fish studied displayed a large variation in body weight (1.2 – 6.4 kg) and muscle fat content (5.5 – 27.7 %). The relationships between body weight, muscle fat, EPA and DHA are presented in Table 1. Muscle fat and body weight were highly correlated (0.71), which is expected, as fat deposits increase with the growth and development of the fish (Rye & Gjerde, 1996). We show that size (body weight) and muscle fat content influence FA composition in muscle. The proportional content of EPA and DHA decreases with increased body weight and muscle fat (Figure 5). This can be explained by that these FAs are mainly found as part of PL in cell membranes, which do not change much when fish gets fatter (Ruyter et al., 2006; Tocher, 2003). In this thesis, it was decided to correct for the effect of body weight. In the genetic models in Paper I, III and IV, body weight was fitted as a covariate. In the gene expression analysis in Paper II, the effect of body weight was removed by choosing fish of similar weight and muscle fat content (3.3 - 3.9 kg). Alternatively, muscle fat could have been fitted instead of body weight, but body weight had the strongest correlation with the FA traits (Table 1), and the objective was to study the relationships between omega-3 FAs and the body fat deposits, including muscle fat.

Table 1. Genetic and phenotypic correlations between EPA, DHA, muscle fat (MFAT) and body weight (BW). Genetic correlations in lower triangle. Phenotypic correlations in upper triangle. FAs in % of total FAs

	DHA	EPA	MFAT	BW
DHA		0.28	-0.40	-0.53
EPA	0.68		0.05	-0.15
MFAT	-0.57	-0.30		0.65
BW	-0.78	-0.84	0.71	

When correcting for body weight, some of the genetic variance of the FAs is removed because of the high genetic correlation with, and high heritability of, body weight. If we did not correct for body weight, all genetic parameter estimates would reflect those of body weight.

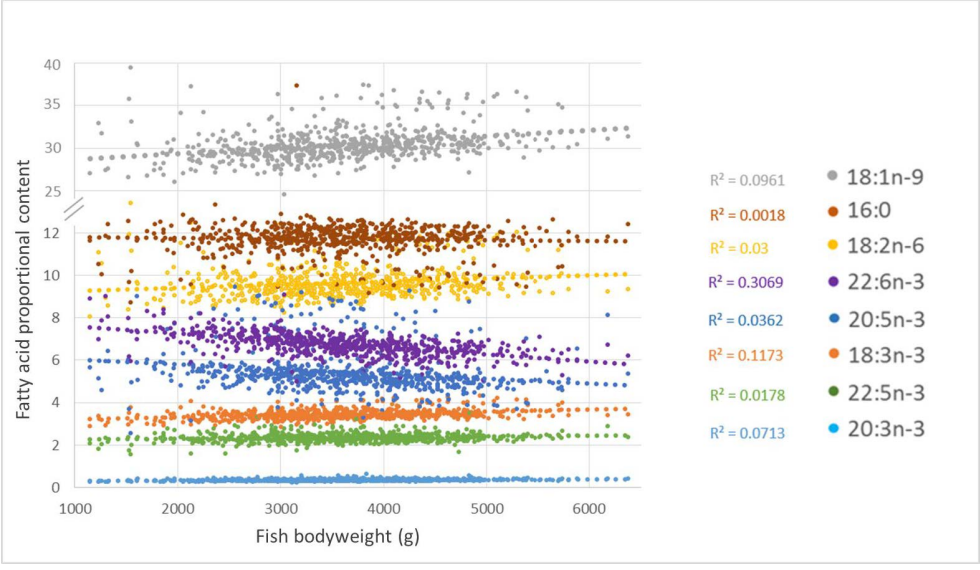

Figure 5. Relationship between fish body weight and proportional content of fatty acids in the muscle.

Genomic predictions using genotype data is generally considered to be more accurate than pedigree-based predictions (Correa et al., 2017; Tsai et al., 2016), and using imputed data has been shown comparable to using true genotype data in Atlantic salmon (Tsai et al., 2016; Tsai et al., 2017). However, in this thesis, the difference between pedigree and genomic (SNP chip) data was small, and pedigree yielded higher

accuracies than SNP-chip for EPA and muscle fat (Paper III). In addition, imputed data did not perform well; it did not yield higher selection accuracies compared to SNP-chip genotypes (Paper III), and when tested in GWAS, it did not give any additional information and the results looked noisier compared to using SNP-chip genotypes. The poor performance of genomic information in this thesis may be explained by that our dataset was not optimal for using genomic information, which was discussed in Paper III. We also had good pedigree information; six generations compared to two generations in Tsai et al. (2016) and Tsai et al. (2017). Imputation errors were likely to occur in this dataset where there were 5 million SNPs, only 563 genotyped fish, and 96 sequenced fish. Moreover, the imputation step from a 57K SNP-chip to 5 million markers may have been too large (Paper III), and is considerably larger compared to Tsai et al. (2017); Imputed from 6 K to 50 K, Yoshida et al. (2018); imputation from 0.5 K and 3 K to 50 K, and Yoshida et al. (2017); imputed from 50K to 200K.

The present thesis has proven the usefulness of genomics and transcriptomics for studying a trait where limited knowledge about underlying determinants is available. The open approach applied has greatly improved knowledge about molecular associations with inherent differences in muscle content of EPA and DHA. Although the associations with gene expression are strengthened by the large number of genes studied, additional studies of quantities and functions of proteins could potentially provide answers to which functional molecules are associated with variations in EPA and DHA content.

The gene expression study identified trait-associated genes through testing for association between continuous traits and mRNA expression by linear regression analysis. One limitation with this type of study is that it can only show associations, and not determine cause and effect relationships. Because EPA is such a highly metabolically active FA, it likely affects many of the metabolic pathways and genes identified as associated with EPA, but genes may also have an effect on the EPA muscle content.

4.4 Selection for increased EPA and DHA content in fillets of Atlantic salmon

The LC n-3 PUFA content in farmed Atlantic salmon feed and fillets is decreasing due to limited availability of FO, while the production of farmed fish is increasing (Ytrestøyl et al., 2015). In order to maintain fish health as well as the nutritional value of the products, this issue should receive attention from several research areas, including selective breeding, which may prove an important long-term contribution to aid the industry to produce Atlantic salmon with sufficient levels of EPA and DHA in the fillet, both for fish and human health.

Paper I revealed that EPA and DHA content of Atlantic salmon muscle are heritable traits, where DHA had the highest heritability (0.26) and EPA the lowest (0.09). Thus, one of the main findings of this thesis was that LC n-3 PUFA content of farmed Atlantic salmon fillets can be increased by selective breeding. The cross-validation accuracies for selection for DHA and EPA were moderate and offer possibilities for selection for these traits, especially if one extends the reference data set to a much bigger population (Paper III). Muscle EPA and DHA traits had a polygenic architecture with a potential QTL located at chromosome 21 (Paper IV). The GWAS analysis of EPA, DHA and DHA/ALA ratio all pointed to this chromosome, which indicates possible pleiotropic effects of this QTL.

Furthermore, this thesis has shown that selection for increased EPA and DHA content in Atlantic salmon fillet will likely affect several metabolic processes. These include lipid deposition and metabolism, carbohydrate metabolism, insulin signaling and eicosanoid production, as discussed above. The consequences of selection for altered muscle FA composition on these processes should ideally be further documented before implementation in breeding programs. For example, the results indicated that selection for increased DHA would increase liver and visceral fat (genetic correlations were 0.32 and 0.61, respectively), which is not desired and should therefore be monitored. In addition, the association of EPA and DHA with several metabolic processes may indicate that selection for altered muscle FA composition could change the nutrient metabolism in muscle, which could potentially change the nutritional requirements of the fish, such that the feed composition would require revision. Furthermore, if LC n-3 PUFA content in muscle were to be increased by selective breeding, the consequences for liver LC n-3

PUFA content are unknown as the genetic correlations between these traits have not been estimated. Since liver content of EPA and DHA is important for fish health (Glencross et al., 2015; Ruyter et al., 2006), caution should be taken not to reduce liver omega-3 in the process.

Overall, EPA had the most favorable correlations with lipid deposition traits, compared to DHA. The two FAs were differently associated with some genes and metabolic pathways (e.g. carbohydrate metabolism and insulin signaling), and seemed to have additive effects for others, such as muscle tissue development. From a human health perspective, selective breeding should ideally result in increases of the content of both health-promoting FAs EPA and DHA in Atlantic salmon fillets. The estimated genetic correlations for the sum of EPA and DHA showed no clear detrimental effects on lipid deposition traits (discussed further in Paper I).

Atlantic salmon breeding companies are not selecting for omega-3 FAs today, partly because there is no economic incentive for including this trait in breeding programs. The main obstacle for implementing selection for EPA and DHA in Atlantic salmon is that individual omega-3 FA composition is very expensive to record. In this thesis, in order to get accurate measures, this was done by gas-chromatography, which is very time consuming and laborsome – thus expensive. Spectroscopy methods are being explored as alternative ways of recording omega-3 FA composition, and technology development may even enable measurement of muscle omega-3 composition through the skin of the fish by near infrared spectroscopy ("SalmoNIR", Cargill, 2019). Raman spectroscopy is gaining interest as a rapid and non-destructive method for FA analysis. It has been shown that this method can be used to estimate FA unsaturation directly in Atlantic salmon muscle (Afseth et al., 2006), and can estimate EPA and DHA concentrations in fish oil supplements with high coefficients of determination and low estimation errors (Bekhit et al., 2014). Recently, Nofima has shown that Raman spectroscopy can be used to estimate single FAs like EPA and DHA in individual homogenized Atlantic salmon muscle with coefficients of determination in the region 0.7 – 0.8 (personal communication, Nils Kristian Afseth, 2019). This shows that Raman spectroscopy is a highly potent tool for FA phenotyping for an Atlantic salmon breeding purpose.

Implementing muscle omega-3 content in breeding programs could be done in different ways, through: i) sib-tests of breeding candidates, using slaughter-test data, ii)

individual measurements of FA composition directly on the candidates themselves, assuming technology development allows for non-invasive recording; e.g. SalmoNIR (Cargill, 2019). This would increase selection accuracy compared to the sib-tests. iii) Measurements of omega-3 content on pooled family muscle samples rather than individuals. In this way, a higher number of families may be recorded at lower costs compared to individual measurements, but with reduced selection accuracy because only family means of phenotypes will be obtained (Falconer & Mackay, 1996).

5. Concluding remarks and future perspectives

This thesis has significantly contributed towards identifying the genetic basis and underlying biological mechanisms associated with EPA and DHA content in Atlantic salmon fillet.

The EPA and DHA content of skeletal muscle of Atlantic salmon fed the same diet displayed large variation, and were both heritable traits, with heritabilities of 0.09 and 0.26, respectively. Thus, changing muscle FA composition by selective breeding is possible. Cross-validation accuracies for selection for EPA and DHA were moderate and offer possibilities for selection for these traits, especially if one extends the reference data set to a much bigger population. Muscle EPA and DHA traits had a polygenic architecture with a potential QTL located at chromosome 21. In order to identify causal mutations, future studies require larger datasets and improved annotation of the Atlantic salmon genome. New technology that measures FA composition easier and faster may provide larger datasets for research and facilitate implementation in breeding programs.

Inherent differences in EPA and DHA in muscle were linked to fat deposition in liver, viscera and muscle. EPA had more favorable associations with the lipid deposition traits than DHA. This implies that EPA and DHA affect body fat deposition, also when the dietary requirements of these FAs are met.

Muscle EPA and DHA content was also associated with several metabolic pathways, including carbohydrate metabolism, insulin signaling and lipid metabolism, but there were no indications of omega-3 bioconversion being important for determining the content of these FAs in muscle of Atlantic salmon. Results indicated that the reason why some individuals have a higher level of EPA in muscle is that this FA is deposited rather than oxidized for energy.

Correlations between liver and skeletal muscle were very low regarding both EPA and DHA content, which is valuable knowledge for future studies of omega-3 in Atlantic salmon. It would also be of value to discover the genetic and phenotypic correlation of LC n-3 PUFA levels at different life stages of the fish. Many studies are carried out using small fish because it is more practical and reduces costs. However, if the correlation

between small and slaughter-sized fish is low, it might not be relevant in all cases to study small fish.

One of the results from this thesis that deserve further exploration is the negative genetic correlation between muscle EPA content and sea lice density (Paper I), indicating that selecting for fish with higher levels of EPA and DPA in muscle would result in lower sea lice density. This deserves further attention as it could potentially have a huge impact on the welfare of the fish, as well as the cost of production.

Further research is needed on whether the results of this thesis are valid also for fish fed a low FO diet. This is valuable knowledge for breeding companies, as broodstock fish, where selection takes place, and production fish are fed different diets. One way of answering this question, that would increase our knowledge considerably, would be to study two large groups of fish from the same population, one group on a high FO diet, the other on a low FO diet. Results on associations, variations and genetic parameters from within each group would then be compared between the groups. In order to gain more insight into the physiological effects of differences in EPA and DHA levels, this study should include health and quality parameters.

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Paper I

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GSE Genetics Selection Evolution

RESEARCH ARTICLE Open Access

Genetic effects of fatty acid composition in muscle of Atlantic salmon

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Abstract

Background: The replacement of fish oil (FO) and fishmeal with plant ingredients in the diet of farmed Atlantic salmon has resulted in reduced levels of the health-promoting long-chain polyunsaturated omega-3 fatty acids (n-3 LC-PUFA) eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in their filets. Previous studies showed the potential of selective breeding to increase n-3 LC-PUFA levels in salmon tissues, but knowledge on the genetic parameters for individual muscle fatty acids (FA) and their relationships with other traits is still lacking. Thus, we estimated genetic parameters for muscle content of individual FA, and their relationships with lipid deposition traits, muscle pigmentation, sea lice and pancreas disease in slaughter-sized Atlantic salmon. Our aim was to evaluate the selection potential for increased n-3 LC-PUFA content and provide insight into FA metabolism in Atlantic salmon muscle.

Results: Among the n-3 PUFA, proportional contents of alpha-linolenic acid (ALA; 18:3n-3) and DHA had the highest heritability (0.26) and EPA the lowest (0.09). Genetic correlations of EPA and DHA proportions with muscle fat differed considerably, 0.60 and 0.01, respectively. The genetic correlation of DHA proportion with visceral fat was positive and high (0.61), whereas that of EPA proportion with lice density was negative. FA that are in close proximity along the bioconversion pathway showed positive correlations with each other, whereas the start (ALA) and end-point (DHA) of the pathway were negatively correlated (− 0.28), indicating active bioconversion of ALA to DHA in the muscle of fish fed high FO-diet.

Conclusions: Since contents of individual FA in salmon muscle show additive genetic variation, changing FA composition by selective breeding is possible. Taken together, our results show that the heritabilities of individual n-3 LC-PUFA and their genetic correlations with other traits vary, which indicates that they play different roles in muscle lipid metabolism, and that proportional muscle contents of EPA and DHA are linked to body fat deposition. Thus, different selection strategies can be applied in order to increase the content of healthy omega-3 FAin the salmon muscle. We recommend selection for the proportion of EPA + DHA in the muscle because they are both essential FA and because such selection has no clear detrimental effects on other traits.

Background

Traditionally, farmed Atlantic salmon (*Salmo salar* L.) were fed diets rich in fish oil (FO) and fishmeal. Limited and decreasing availability of raw materials from wild fisheries have led to the replacement of a large portion of the marine ingredients with more sustainable plant-based ingredients in aquaculture feed [1]. Well-documented consequences of this change are decreased levels of the health-promoting omega-3 long-chain polyunsaturated fatty acids (n-3 LC-PUFA) eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acids (DHA; 22:6n-3) in salmon filets, because these fatty acids (FA) are not present in plant oils [2, 3].

Atlantic salmon store most of their energy in the form of lipids in their muscle. The largest portion of such lipids is in the adipocytes along connective tissue sheets known as myocepta [4, 5]. Lipids present in the myocepta have a high proportion of triacylglycerol (TAG), with a FA composition that is strongly influenced by the diet. Thus,

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muscle lipids have high levels of 18-carbon unsaturated FA 18:1n-9, 18:2n-6 and 18:3n-3 and saturated FA palmitic acid (PA; 16:0) [6]. Another important lipid group in muscle is phospholipids (PL), which are located mainly in cell membranes [7]. Compared to TAG, PL have high levels of n-3 LC-PUFA, and their FA composition is more strictly regulated in order to maintain cell membrane functionality [8, 9]. Fat percentage in the muscle increases with growth and development of the fish [10, 11]. A higher fat content results in an increased ratio of TAG versus PL and, thus, influences FA composition.

Muscle FA composition is strongly influenced by the diet, although not in an entirely linear way [12]. It is a function of several processes, each regulated on a cellular, tissue and whole-body level for maintaining lipid homeostasis [8]. Several studies have reported selective oxidation and deposition of individual FA in salmonids [13–16] and Vegusdal et al. [17] provided evidence of preferential uptake of specific FA into the muscle. Another process that influences the FA composition is bioconversion. Salmonids can convert the shorter-chained FA alpha-linolenic acid (ALA; 18:3n-3), which is commonly found in some plant oils, into longer chained EPA and DHA [18, 19]. This metabolic pathway was first described in mammals by Sprecher [20] and consists of a series of desaturation and elongation reactions. The first step is Δ6 desaturation of 18:3n-3 to produce 18:4n-3 that is elongated to 20:4n-3. Alternatively, 18:3n-3 is elongated to 20:3n-3 followed by a Δ8 desaturation. 20:4n-3 is desaturated by Δ5 desaturase to form EPA [21, 22]. DHA synthesis from EPA requires two more elongation steps, a second Δ6 desaturation, and a chain-shortening step by peroxisomal β-oxidation [23]. Activity of the desaturase and elongase enzymes determines the relative amounts of EPA and DHA formed. Several factors influence the activities of these enzymes: nutrition, environment, hormones and genetics. In salmonids, liver, intestinal and muscle cells can convert ALA to DHA [24, 25]. Thus, this bioconversion is expected to influence the muscle FA composition, although less than the diet.

There is evidence that genetics affects muscle FA composition in Atlantic salmon [26]; Schlechtriem et al. [27] reported individual variation in the content of n-3 PUFA in the flesh of Atlantic salmon fed the same feed, which indicates a strong genetic influence on this trait. Similarly, Leaver et al. [28] found that the content of total n-3 LC-PUFA in salmon muscle differed between families and was highly heritable. Selection for increased liver expression of genes encoding enzymes in the bioconversion pathway has led to increased levels of DHA in the liver of salmon [29]. Hence, there is potential in using selective breeding as a tool to increase levels of n-3 LC-PUFA in Atlantic salmon muscle by increasing the salmon's natural capacity to convert the shorter-chained 18:3n-3 FA from plant oils into n-3 LC-PUFA and to efficiently deposit these FA in the muscle. However, knowledge on the genetic parameters of individual muscle FA and their relationships with lipid deposition traits and other breeding traits (e.g. carcass, quality and disease) is lacking, but is key to predicting the consequences of selection for higher n-3 LC-PUFA levels. Thus, the objective of this study was to estimate genetic parameters of individual FA in the muscle of farmed Atlantic salmon to evaluate the potential for selection for increased n-3 LC-PUFA levels and provide insight into the muscle's FA metabolism.

Methods

Fish populations and trait recording

Slaughter test

The data represented one year-class of the Atlantic salmon breeding population of SalmoBreed AS. The fish were transferred to net pens in the sea at a mean weight of 113.1 g and harvested at a mean slaughter weight of 3605 g. In total, 668 fish that were reared under the same conditions were included in this study. They were fed a commercial broodstock feed with a high FO content (see Additional file 1) and were fasted 13 to 14 days prior to slaughter. These fish came from 194 full-sib families that originated from 92 sires and 194 dams. All sires had four or more offspring from more than one dam.

The following traits were recorded at slaughter: body weight (g); length (cm); sex, determined visually by inspection of the gonads; muscle pigment (mg/kg), the carotenoid astaxanthin was measured immediately after slaughter using a commercial NIR imaging scanner (Tomra Sorting Solutions, Leuven, Belgium) [30, 31]; liver fat, an indicator of the degree of fatty liver, determined visually on a scale of 1 (darkest color i.e. healthy) to 5 (lightest color i.e. fatty liver), which is reversed compared to the scale presented in [32] to facilitate interpretation of the results; visceral fat, determined visually on a scale of 1 (lowest amount of fat) to 5 (highest) [32].

Muscle samples from the Norwegian Quality Cut (NQC) of the fillet were collected at harvest from each fish, frozen, and stored at − 20 °C.

Sea lice and pancreas disease challenge tests

Data from sea lice and pancreas disease (PD) challenge tests of siblings of the analyzed fish were obtained from SalmoBreed AS, as part of their larger challenge tests of the 2014 year class.

In the sea lice challenge test, 2207 post-smolts (body weight ~ 60 g) were infected by mixing sea lice copepodites in a water basin (12 °C) with an infection rate of about 30 copepodites per fish. The sea lice strain was LS

Gulen from 2006. The fish were sedated and the number of lice per fish was counted after sea lice reached the motile stage (~14 days) (personal communication Borghild Hillestad). Sea lice density was calculated as lice count/body weight$^{2/3}$, following [33].

In the PD challenge test, 2426 post-smolts (body weight ~ 65 g) were infected with the SAV3 virus by direct intraperitoneal injection, following the VESO Vikan protocol that was approved by the Norwegian Animal Research Authority (personal communication Borghild Hillestad). The total PD survival rate at the end of the challenge was 60%.

Lipid and fatty acid analyses

Total lipids were extracted from homogenized NQC muscle samples of individual fish sampled at slaughter, according to the method described by Folch et al. [34]. Using 1 mL of the chloroform–methanol phase, FA composition of the total lipids was analyzed according to the method described by Mason and Waller [35]. The extract was dried quickly under nitrogen gas and the residual lipid extract was trans-methylated overnight with 2,2-dimethoxypropane, methanolic-HCl, and benzene at room temperature. The methyl esters formed were separated in a gas chromatograph with a split injector, using an SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm, and film thickness 0.25 µm; SGE Analytical Science, Milton Keynes, UK) and a flame ionization detector. The results were analyzed using the HP Chem Station software (Hewlett Packard 6890; HP, Wilmington, DE, USA). The carrier gas was helium, and both injector and detector temperatures were 270 °C. The oven temperature was raised from 50 to 170 °C at a rate of 4 °C/min and then raised to 200 °C at a rate of 0.5 °C/min and finally to 240 °C at a rate of 10 °C/min. Individual FA methyl esters were identified by reference to well-characterized standards. The proportional content of each FA was expressed as a percentage of the total amount of FA in the analyzed sample. Absolute content of each FA was calculated as described in Folch et al. [34]: FA in g per 100 g muscle = (% FA of total FA/00) × (Muscle fat % × 0.9).

Presentation of the results will focus on the most abundant FA in the fillet (16:0, 18:1n-9, 18:2n-6) and the following FA that are part of the bioconversion pathway: 18:3n-3, 20:3n-3, 20:4n-3, 20:5n-3, 22:5n-3 and 22:6n-3.

Statistical analyses

Variance and covariance components were estimated by residual maximum likelihood procedures using the ASReml Package [36]. Bivariate analyses were performed to estimate genetic correlations between traits, using the following bivariate animal model [37]:

$$\mathbf{Y} = \mathbf{XB} + \mathbf{U} + \mathbf{E},$$

where $\mathbf{Y}$ is a matrix of phenotypic records for individuals $i = 1, 2, \ldots, n$ and traits $j = 1, 2$, $\mathbf{X}$ is a matrix of the fixed effects of animal i on trait j, $\mathbf{B}$ is a matrix of fixed effect solutions. $\mathbf{U}$ is a matrix containing the random effects of animal i on trait j, with variance $\mathbf{G} \otimes \mathbf{A}$, where $\mathbf{A}$ is the relationship matrix between individuals and $\mathbf{G}$ is a genetic variance–covariance matrix among traits. $\mathbf{E}$ is a matrix of residual effects, that is assumed to have a variance of $\mathbf{R} = \begin{bmatrix} \sigma^2_{e1} & \sigma_{e1,e2} \\ \sigma_{e1,e2} & \sigma^2_{e2} \end{bmatrix}$, where 1 and 2 indicate traits. Univariate analyses were performed to estimate heritabilities for all traits. For the univariate analyses, matrices $\mathbf{Y}$, $\mathbf{B}$, $\mathbf{U}$ and $\mathbf{E}$ were reduced to vectors and matrices $\mathbf{G}$ and $\mathbf{R}$ were reduced to scalars. Heritability estimates (h^2) were calculated as the ratio of additive genetic (σ^2_A) to total phenotypic (σ^2_p) variance ($h^2 = \sigma^2_A/\sigma^2_p$). Correlations between two traits (r) were calculated as the covariance divided by the square root of the product of two variances i.e. $r = \mathrm{cov}_{1,2}/\sqrt{\sigma^2_1 \times \sigma^2_2}$.

Body weight was included as a covariate and sex was included as a fixed effect for FA, pigment, visceral fat, liver fat and muscle fat. For PD survival, cage was included as random effect. For lice density, the person performing the counting and the day of recording were included as fixed effects and cage was included as a random effect. Four generations of pedigree information on direct ancestors of the fish in the tests were available (n = 11,801). Estimates were considered as significantly different from zero if they deviated more than two times their standard error from zero ($p < 0.05$).

Results

Data description

The coefficients of variation (CV) showed that traits varied extensively among the sampled fish (Table 1), especially body weight, visceral fat and liver fat, which each had a CV above 20%. The average fat content of NQC muscle samples was 19% (Table 1), which is high for fish of this size but not extreme. The average liver fat score was within the normal range (2.13).

The major muscle FA were 18:1n-9, 18:2n-6 and the saturated FA 16:0 (Table 2). The mean muscle contents of EPA and DHA were approximately twice as high as in commercially-produced salmon, which reflects the high level of these FA in the feed (see Additional file 1). The absolute content of individual muscle FA varied greatly, as shown by their CV, which ranged from 15 to 33%, with EPA showing the largest variation. The muscle content of each individual FA was approximately normally distributed. The distributions of proportional contents of

Table 1 Descriptive statistics for body size, fat deposition, fillet quality and disease traits

Trait category	Trait	N	Mean	SD	Min	Max	CV (%)
Body size	Body weight (g)	668	3605	861	1150	6380	24
	Length (cm)	668	66.5	5.1	50	78	8
Fat deposition	Muscle fat (%)	668	19.07	3.16	5.47	27.65	17
	Visceral fat (1–5)	668	3.40	0.83	1	5	24
	Liver fat (1–5)	668	2.13	0.76	1	5	26
Fillet quality	Pigment (mg/kg muscle)	668	7.7	0.9	3.9	10.7	11
Disease	Sea lice density[a]	2207	64.25	26.25	0	202.98	41
	PD survival[b]	2426	0.62	–	0	1	–

N number of observations, *SD* standard deviation, *Min* minimum value, *Max* maximum value, *CV* coefficient of variation (SD/mean × 100)

[a] Sea lice density = lice count/body weight$^{2/3}$

[b] Scored 0 or 1, where 0 is dead and 1 is alive after the challenge test

Table 2 Descriptive statistics of proportional and absolute content of selected fatty acids in muscle

Fatty acid		N	Proportional content (% of total FA)			Absolute content (g FA/100 g muscle)		
			Mean	SD	CV (%)	Mean	SD	CV (%)
16:0	Palmitic acid (PA)	668	11.69	0.71	6	2.01	0.38	19
18:1n-9	Oleic acid (OA)	668	30.45	1.91	6	5.22	0.90	17
18:2n-6	Linoleic acid (LA)	668	9.65	0.73	8	1.65	0.27	16
18:3n-3	Alpha-linolenic acid (ALA)	668	3.46	0.23	7	0.59	0.10	17
20:3n-3	Eicosatrienoic acid	668	0.36	0.05	13	0.06	0.01	23
20:4n-3	Eicosatetraenoic acid	634	0.54	0.16	30	0.09	0.03	33
20:5n-3	Eicosapentaenoic acid (EPA)	668	5.42	1.01	19	0.93	0.24	25
22:5n-3	Docosapentaenoic acid (DPA)	668	2.36	0.21	9	0.41	0.08	19
22:6n-3	Docosahexaenoic acid (DHA)	668	6.75	0.51	8	1.15	0.18	15
	Sum EPA + DHA	668	12.17	1.26	10	2.08	0.38	18

N number of observations, *SD* standard deviation, *CV* coefficient of variation (SD/Mean × 100)

individual FA in the muscle across fish are in Additional file 2. The FA that are not shown in Table 2 amounted to approximately 30% of total FA.

The proportional contents of muscle FA changed with increasing body weight (Fig. 1). The content of PA (16:0) remained stable, whereas that of 18-carbon FA increased and those of EPA and DHA were the only ones that decreased with increasing body weight. In the remaining results, all estimates were corrected for body weight.

Metabolism

The heritability of the absolute content of the n-3 PUFA, in g per 100 g muscle, was generally moderate to high, which reflects the high heritability of muscle fat (0.46) (see Additional file 3). DHA had the highest heritability (0.46) (Table 3). The absolute content of EPA + DHA had a heritability of 0.35 (Table 3), whereas proportional content had a heritability of 0.09 (SE 0.06). For proportional contents of FA, the start and end-point of the bioconversion pathway (ALA and DHA) had the highest heritabilities, whereas EPA had the lowest heritability (0.09, Table 4).

Estimates of genetic and phenotypic correlations both showed the same pattern: contents of FA that were in close proximity in the pathway had positive correlations with each other, whereas correlations between contents of the start- (ALA) and end-points (DHA) of the pathway were negative (Table 4). The genetic correlation between the contents of 20:4n-3 and DHA was high.

Correlations between fatty acid contents and fat deposition traits

Muscle fat had positive phenotypic correlations with proportional contents of PA and EPA (Table 5), but a negative correlation with the proportional contents of

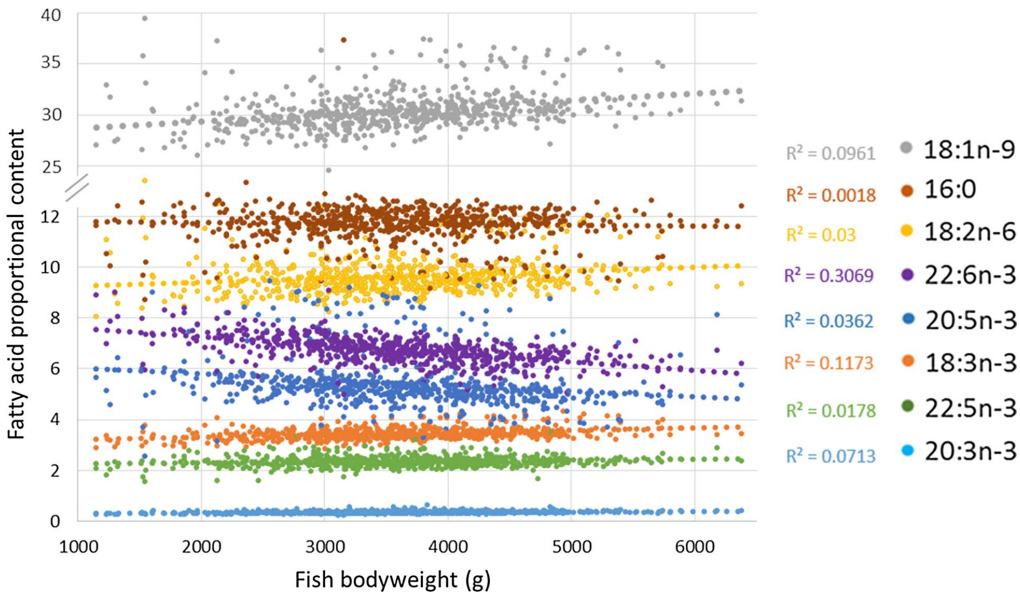

Fig. 1 Relationship between fish body weight and proportional content of fatty acids in the muscle

Table 3 Estimates of heritability for absolute content of selected fatty acids

FA (g)	Heritability (SE)
18:3n-3	0.34 (0.09)
20:3n-3	0.23 (0.08)
20:4n-3	0.17 (0.08)
20:5n-3	0.25 (0.07)
22:5n-3	0.37 (0.09)
22:6n-3	0.46 (0.09)
EPA + DHA	0.35 (0.09)
Ratio DHA:ALA	0.20 (0.08)

FA (g): muscle fatty acid content in grams per 100 grams of muscle

Standard errors in brackets

the 18-carbon FA. Phenotypic correlations of proportional contents of DPA (22:5 n-3) and DHA with muscle fat were close to zero. Estimates of genetic correlations between muscle fat and proportional contents of FA showed the same pattern, but were higher than the phenotypic correlations, except for the proportional contents of the very long-chain (VLC) ($\geq C_{22}$) PUFA DPA and DHA, which had genetic correlations of nearly zero with muscle fat when correcting for body weight. As expected, genetic correlations between absolute amounts of all FA and muscle fat were highly positive, because an increase in muscle fat increases the absolute amount of all FA (see Additional file 4), and because muscle fat percentage was used to calculate the absolute amount of FA.

Estimates of genetic and phenotypic correlations of visceral fat correlations with FA reflected those with muscle fat for all FA except for EPA, 20:4n-3, and DHA (Table 5). Proportional contents of both EPA and PA had positive genetic correlations with muscle fat; PA also had a positive genetic correlation with visceral fat (0.66) but EPA had a negative correlation with visceral fat (− 0.17). DHA had a high positive genetic correlation with visceral fat (0.61).

Correlations of FA with liver fat were weaker than those with visceral fat but showed the same pattern (Table 5). The genetic correlations for liver fat were similar to the phenotypic correlations, except for EPA, which had a positive phenotypic but a negative genetic correlation with liver fat.

Overall, Table 5 shows that, for fish at the same age and weight, a fatter muscle had a higher absolute content but lower proportion of the 18-carbon FA and higher absolute and proportional contents of EPA and PA, than a leaner muscle. The proportional content of DHA did not

Table 4 Estimates of heritability and of genetic and phenotypic correlations for proportional content of selected fatty acids

FA (%)	18:3n-3	20:3n-3	20:4n-3	20:5n-3	22:5n-3	22:6n-3
18:3n-3	*0.26 (0.08)*	0.21 (0.04)	−0.21 (0.04)	[a]	−0.15 (0.04)	−0.56 (0.03)
20:3n-3	−0.03 (0.28)	*0.18 (0.08)*	−0.20(0.04)	0.40 (0.03)	0.43 (0.03)	−0.06 (0.04)
20:4n-3	0.03 (0.31)	0.07 (0.33)	*0.14 (0.07)*	−0.33 (0.04)	−0.14 (0.04)	0.25 (0.04)
20:5n-3	[a]	0.01 (0.42)	−0.21 (0.40)	*0.09 (0.06)*	0.69 (0.02)	0.23 (0.04)
22:5n-3	−0.44 (0.23)	0.30 (0.25)	0.19 (0.32)	0.42 (0.26)	*0.22 (0.07)*	0.32 (0.04)
22:6n-3	−0.28 (0.22)	0.33 (0.28)	0.64 (0.25)	0.16 (0.34)	0.41 (0.21)	*0.26 (0.08)*

FA (%): muscle fatty acid content in percentage of total muscle fatty acids. Heritability on the diagonal. Phenotypic correlations on the upper triangle. Genetic correlations on the lower triangle. Standard errors in brackets

[a] Parameters not converged

Table 5 Estimates of phenotypic and genetic correlations of the proportional content of selected muscle fatty acids with muscle, visceral and liver fat

FA (%)	Muscle fat		Visceral fat		Liver fat	
	r_P	r_G	r_P	r_G	r_P	r_G
16:0	0.44 (0.03)	0.86 (0.10)	0.43 (0.03)	0.66 (0.24)	0.12 (0.04)	0.20 (0.27)
18:1n-9	−0.38 (0.05)	−0.67 (0.15)	−0.41 (0.03)	−0.67 (0.27)	−0.14 (0.04)	−0.17 (0.28)
18:2n-6	−0.46 (0.03)	−0.78 (0.11)	−0.47 (0.03)	−0.63 (0.24)	−0.14 (0.04)	−0.23 (0.26)
18:3n-3	−0.35 (0.04)	−0.72 (0.15)	−0.41 (0.03)	−0.67 (0.26)	−0.11 (0.04)	−0.23 (0.28)
20:3n-3	−0.01 (0.04)	−0.28 (0.22)	−0.02 (0.04)	0.25 (0.46)	0.10 (0.03)	0.02 (0.33)
20:4n-3	0.01 (0.04)	−0.12 (0.25)	0.11 (0.04)	0.82 (0.40)	0.06 (0.04)	0.79 (0.31)
20:5n-3	0.21 (0.04)	0.60 (0.28)	0.12 (0.04)	−0.17 (0.51)	0.10 (0.04)	−0.31 (0.41)
22:5n-3	0.05 (0.04)	0.02 (0.21)	0.01 (0.04)	0.20 (0.38)	0.11 (0.04)	0.19 (0.28)
22:6n-3	−0.06 (0.04)	0.01 (0.21)	0.24 (0.04)	0.61 (0.37)	0.10 (0.04)	0.32 (0.27)
EPA + DHA	0.14 (0.04)	0.58 (0.32)	0.19 (0.04)	0.36 (0.52)	0.11 (0.04)	−0.04 (0.42)
Ratio DHA:ALA	0.11 (0.04)	0.38 (0.22)	0.34 (0.04)	0.98 (0.35)	0.11 (0.04)	0.35 (0.29)

FA (%): muscle fatty acid content in percentage of total muscle fatty acids

Standard errors in brackets

r_P phenotypic correlations, r_G genetic correlations

increase with increasing muscle fat at a constant body weight.

Correlations of fatty acid contents and fat deposition traits with other traits

PD survival had a negative genetic correlation with muscle fat (−0.29) but, surprisingly, a positive genetic correlation with liver fat (0.28, Table 6). The correlations between PD survival and the proportional contents of FA were generally weak. The estimate of heritability for sea lice density was 0.21. Proportional contents of EPA and DPA had negative genetic correlations with sea lice density. The proportional content of all other FA had close to zero genetic correlations with sea lice density. Pigment was positively correlated with all three fat deposit traits. Only the proportional content of the saturated FA PA had a positive correlation with pigment, while the proportional contents of all unsaturated FA had negative correlations with pigment. These correlations with pigment reflected the correlations of muscle fat with the proportional contents of FA in Table 5, except for EPA and DHA.

Discussion

Genetic parameters of individual muscle FA and their correlations with lipid deposition traits and other traits (carcass, quality, and disease traits) were estimated for 668 slaughter-sized (3.6 kg) Atlantic salmon that were fed a high FO-diet. Our aim was to evaluate the selection potential for increased long-chain omega-3 polyunsaturated FA and provide insight into FA metabolism in salmon muscle.

Heritability estimates

The results showed presence of additive genetic variation in the individual FA contents of salmon muscle,

Table 6 Estimates of genetic correlations of quality and disease traits with fat deposit traits and muscle fatty acids

Trait	PD survival	Sea lice density	Pigment
Heritability	0.28 (0.05)	0.21 (0.04)	0.29 (0.08)
	r_G	r_G	r_G
Muscle fat	−0.29 (0.15)	−0.22 (0.16)	0.47 (0.17)
Visceral fat	0.16 (0.20)	0.10 (0.21)	0.44 (0.34)
Liver fat	0.28 (0.20)	0.21 (0.20)	0.50 (0.27)
16:0 (%)	−0.17 (0.18)	0.07 (0.18)	0.36 (0.21)
18:1n-9 (%)	0.12 (0.19)	0.06 (0.20)	−0.14 (0.23)
18:2n-6 (%)	0.18 (0.17)	0.06 (0.19)	−0.27 (0.21)
18:3n-3 (%)	0.08 (0.18)	0.10 (0.20)	−0.19 (0.22)
20:3n-3 (%)	0.30 (0.21)	−0.33 (0.22)	−0.18 (0.29)
20:4n-3 (%)	−0.06 (0.23)	0.26 (0.23)	0.02 (0.29)
20:5n-3 (%)	−0.13 (0.28)	−0.47 (0.28)	−0.09 (0.33)
22:5n-3 (%)	0.05 (0.18)	−0.40 (0.19)	−0.35 (0.23)
22:6n-3 (%)	−0.02 (0.18)	0.09 (0.19)	−0.29 (0.23)
EPA + DHA	−0.11 (0.27)	−0.39 (0.29)	−0.26 (0.34)
Ratio DHA:ALA	−0.07 (0.20)	0.00 (0.22)	−0.13 (0.26)

r_G genetic correlations

Standard errors in brackets. Fatty acids in proportion of total muscle fatty acids

thus changing FA composition by selective breeding is possible. For several FA, both absolute and proportional contents had a high heritability (Tables 3 and 4). The absolute and proportional contents of DHA had heritabilities of 0.46 and 0.26, respectively. The start- and end-points of the n-3 bioconversion pathway (ALA and DHA) had higher heritabilities than the intermediate FA (20:3n-3, 20:4n-3, 20:5n-3 and 22:5n-3). A possible explanation for this is that the levels of intermediate FA may not be stable since they are shuttled through the pathway. In addition, since contents of these FA are very low, errors in their measurement increase. The proportional content of EPA was high (5.42%) but had the lowest heritability of all FA (0.09) (Tables 2 and 4), which may be explained by its many metabolic roles in the body, i.e. it is converted to DHA, used for eicosanoid synthesis, or directed to produce energy [38]. Thus, the "pool" of EPA is highly variable over time [39], which was reflected by the high CV of EPA content, compared to that of the other FA (Table 2).

Overall, the results of this study show that additive genetic variation can be exploited to change the muscle FA composition by selective breeding. This agrees with studies in both pigs [40] and Atlantic salmon [28] that showed that n-3 LC-PUFA content in muscle has a heritable component. Our estimates of heritability are not as high as those reported by Leaver and Taggart [28], probably because they used pooled family records instead of individual records, and because of differences in the age and diet of the fish studied.

Effect of body weight

Our statistical model included body weight as a fixed effect, which decreased the heritability estimates, because of the high heritability of body weight. We corrected for the effect of body weight because the fish studied displayed a large variation in body weight, and body weight had a significant effect on most traits. When correcting for this trait, we removed the effect seen in Fig. 1, i.e. that the proportional contents of some FA increased or decreased with increasing body weight, which renders the relationships between these FA predictable.

Metabolism

The additive genetic variation observed in muscle FA composition could be due to differences in the transport and deposition of these FA from the feed, or due to differences in endogenous production of these FA. When Atlantic salmon are fed diets with high levels of DHA, their endogenous capacity for bioconversion from ALA to DHA is expected to decrease because DHA is known to down-regulate the activity of the Δ6-desaturase enzyme [41]. It has also been reported that FA desaturation activity decreases with the age of the fish [18]. However, although the bioconversion capacity is relatively low, our data indicates that active bioconversion of 18:3n-3 (ALA) to 22:6n-3 (DHA) in muscle takes place in slaughter-sized salmon that are fed a high FO diet. One indicator for this active bioconversion is the negative phenotypic correlation (−0.56) between proportional contents of ALA and DHA, the start and end-points of the pathway (Table 4). Another indicator is the positive correlations between adjacent FA in the pathway. This assumption is supported by recent studies that indicated that, although the Δ6-desaturase conversion of 18:3n-3 to 18:4n-3 is greatly inhibited in fish fed a high FO diet, the activity of the last Δ6-desaturase step of the bioconversion pathway (converting 24:5n-3 to 24:6n-3) is not lower than in fish fed a low FO diet [42, 43]. Therefore, DHA production from ALA is, to some extent, maintained [42, 43], which has been suggested to be due to an increase in the direct elongation of 18:3n-3 to 20:3n-3, bypassing the first Δ6 step (18:3n-3 to 18:4n-3). The FA 20:3n-3 is then converted to 20:4n-3, which is shuttled into the main pathway and then converted to EPA and DHA. Our detection of 20:3n-3 in the muscle further supports this assumption because 20:3n-3 is a FA that is not commonly present in significant amounts in Atlantic salmon feed. The genetic correlation of 20:3n-3 was negative with ALA and positive with DHA (Table 4), which also agrees with

the above results. Although our study used large fish on a FO-rich feed, there were still indications of bioconversion influencing muscle FA composition.

Because levels of EPA and DHA in the feed are proven to affect bioconversion activity, it would be interesting to perform the same analysis on fish fed a low FO diet. Unpublished results from our group have shown that the fish with the highest conversion capacity on a high FO diet also retained this capacity when the amount of FO in the feed was reduced (personal communication Bente Ruyter). In addition, n-3 bioconversion is not expected to be the only factor that determines the FA composition of the muscle. Thus, the results of our study are relevant also for salmon in commercial production in which diets with low contents of marine ingredients are used.

Correlations of fatty acid contents with fat deposition traits

Although our dataset of 668 fish is larger than data used in previous studies on the genetic variation of FA composition in fish (416 fish in [28] and 514 fish in [44]), only some of the genetic correlations were significant and all had large standard errors. Thus, the interpretation of the results is based on the patterns of correlations estimates across traits, rather than on individual estimates.

Individual FA appear to play different roles in the lipid metabolism, since their proportional contents differed in heritability and in correlations with fat deposition traits. In general, we found that proportional contents of all 18-carbon FA had similar correlations with the fat deposition traits, and that they followed a pattern; correlations of these FA with visceral fat and liver fat reflected those with muscle fat, and the genetic and phenotypic correlations were similar. The correlations of the saturated FA PA (16:0) with the fat deposition traits displayed opposite signs compared to the 18-carbon FA, but followed a similar pattern. However, correlations of proportional contents of EPA, DPA and DHA did not follow this pattern; their correlations with visceral fat and liver fat did not reflect their correlations with muscle fat, and the genetic correlations often differed from the phenotypic correlations.

The proportional content of EPA had favorable genetic correlations with different body fat deposits; a higher proportion of EPA in the muscle was associated with a higher amount of fat in the muscle but with less fat in liver and viscera (Table 5). The proportional content of DHA had a positive genetic correlation with visceral fat (0.61), indicating a possible genetic link between high deposition of DHA in the muscle and a high level of visceral fat (Table 5). The DHA:ALA ratio had an even higher genetic correlation with visceral fat. The basis for these correlations is unknown. Genetic correlations between proportional contents of EPA and DHA in muscle and different fat deposits for salmon fed a traditional high FO-diet have never been reported. However, feeding trials have shown that very high levels of EPA and DHA in the diet reduce the amount of visceral adipose tissue in Atlantic salmon [45], whereas very low dietary amounts of EPA and DHA are linked to fatty liver [9, 46]. Since there were no health-risk levels of liver fat in the fish of this study, the correlations of liver fat with other traits found here are not comparable to those found in feeding trials or other studies that analyzed fish with a high occurrence of health-impairing fatty liver. It should be noted that the accuracy of the visual scoring method used for liver and visceral fat is low compared to that of chemical analyses, especially of liver fat in big fish.

Correlations of fatty acid contents with other phenotypic traits

The metabolic role of EPA may explain the correlation found between its proportional contents and sea lice density (-0.47 (0.28), Table 6), which indicated that a high proportion of EPA in muscle is associated with increased resistance to sea lice. Since the EPA content in muscle is reflected in the skin [15], this correlation may be due to the increased levels of EPA in the skin having anti-inflammatory and immunological effects [47]. The effect of EPA may be similar to that of other anti-inflammatory factors that protect Atlantic salmon against sea lice infection [48]. To date, there is no evidence that supports a possible genetic effect of EPA on sea lice resistance, but it could be of great practical importance to the industry. However, because of the large standard error of the genetic correlation estimated here, this finding requires further investigations and should be verified in salmon fed a low FO diet.

The limitations of the method used to determine liver fat must also be taken into account when considering the observed positive correlation between liver fat and PD survival, as we do not have a biological explanation for this correlation. We found a negative genetic correlation between PD survival and muscle fat, which suggests that increased levels of fat in the muscle reduce the PD survival rate. Pancreas disease is caused by a virus that triggers inflammation in pancreas, heart and skeletal muscle tissues [49]. High levels of lipids increase the amount of inflammatory factors [50], which may explain why resistance to the PD virus infection decreases.

Muscle fat had a positive genetic correlation with pigment (Table 6). This may result from the hydrophobic nature of carotenoids, which results in their transport and absorption being closely linked to the transport of FA [51, 52]. Estimates of genetic correlations between proportional content of FA with pigment reflected those of the proportional content of FA with muscle fat, except

for EPA, DPA and DHA. Since the proportional content of EPA had a positive genetic correlation with muscle fat, it was expected to have also a positive correlation with pigment, but a negative correlation of -0.09 was found. Proportional contents of DPA and DHA also had negative correlations with pigment. Previous studies reported that n-3 LC-PUFA have positive effects on the pigment content of salmon fed low levels of FO [53]. The reason why we observed the opposite may be that the fish in this study were fed high levels of these FA. Highly unsaturated FA are more susceptible to oxidation [54], which can lead to degradation of carotenoids [55].

Possible selection strategies

From a breeder's perspective, the goal is to increase the content of healthy omega-3 FA in salmon fillets. This can be achieved in two ways: by increasing the absolute content (grams per 100 g muscle), or by increasing the proportional content (percentage of total muscle FA). The absolute content is an important trait from the perspective of human health. The heritability for absolute content was quite high for all FA, which is linked to the high heritability of muscle fat (0.46) since estimates of genetic correlations between the absolute content of FA and muscle fat were close to 1. Thus, increased absolute contents of, e.g., EPA and DHA could be achieved by selecting for muscle fat [which had positive genetic correlations with both visceral and liver fat (see Additional file 2)]. However, the salmon breeding industry considers increased levels of muscle fat as undesirable (personal communication Håvard Bakke). Since this applies for the absolute content of all FA, the following discussion is based on proportional content only.

Based on the overall analysis of the results, among all FA, the proportional content of EPA showed the most beneficial genetic correlations with other traits, since an increased proportion of EPA in the muscle was concurrent with less liver fat and visceral fat, as well as increased resistance to sea lice. Thus, increasing the proportion of this FA could be a desirable objective, but unfortunately, its heritability is relatively low (0.09).

DHA was the most abundant n-3 LC-PUFA in the muscle and had the highest heritability (0.26). Selecting for fish with a high proportion of DHA in the muscle would lead to small and lean fish, because the proportional content of DHA decreases with increasing body weight as the TAG:PL ratio increases (Fig. 1). However, our results show that there is variation in the proportional content of DHA that is independent of body weight. The proportion of DHA corrected for body weight was not genetically correlated with muscle fat content but was positively correlated with visceral fat content. Selection for higher proportional content of DHA in the muscle may lead to improved bioconversion capacity, but it may also affect other physiological mechanisms related to FA, e.g. transport, oxidation, and deposition.

Another trait relevant for selection is the DHA:ALA ratio, since it can be an indirect measure of the bioconversion of ALA to DHA. We found that it had moderate heritability (0.20) and its correlations with other traits were similar to those of proportional content of DHA, but its correlation with visceral fat was even higher (although with a high standard error). The viscera represent a natural and healthy place for the salmon to store excess energy as fat, and thus, biologically it is meaningful. However, for Atlantic salmon breeding and production companies, visceral fat represents production loss. Visceral fat content is part of the existing breeding programs and can be dealt with as for other negatively correlated traits by using selection index theory [56]. In addition, optimizing the protein-to-lipid ratio of the feed can significantly reduce visceral lipid deposition in Atlantic salmon [57, 58]. Hence, combining selection and feeding management could prevent increased amounts of visceral fat while selecting for DHA or DHA:ALA ratio. Increasing the ratio of DHA:ALA could increase the bioconversion capacity but would not necessarily increase the absolute amount of DHA in the fillet. Furthermore, beyond bioconversion capacity, several other factors and processes probably play a role in determining the FA composition of the fillet.

From a human health perspective, selective breeding should ideally result in increases of the content of both essential FAs EPA and DHA in salmon muscle. Since EPA and DHA have different physiological functions and correlations with other traits, it may be relevant to include both FA in the selection strategy for achieving a healthy end-product. The sum of EPA and DHA (EPA + DHA) showed similar estimated genetic correlations with PD survival, lice density and pigment as the intermediate of the genetic correlations of EPA and DHA with these traits. The estimate of heritability of EPA + DHA was similar to that of EPA (0.09). In spite of this relatively low heritability, our recommendation is to genetically improve the proportion of EPA + DHA in Atlantic salmon muscle, mainly because of its aforementioned human health benefits and because it has no clear detrimental effects on other traits.

Implementing selection for changes in FA composition requires costly and time-consuming chemical analyses, which make large-scale data recording challenging. New methods for predicting FA composition may provide rapid, cost-effective measurements of muscle FA composition, and thus practical implementation of selection for this trait may become more realistic in the future.

Conclusions

We conclude that there is additive genetic variation in the content of individual FA in salmon muscle, thus changing its FA composition by selective breeding is possible. Combined, our results indicate that individual n-3 LC-PUFA play different roles in the lipid metabolism of muscle since their heritabilities and phenotypic and genetic correlations with other traits varied. Proportional contents of EPA and DHA in the muscle were linked to body fat deposition in different ways. Several observations indicated that an active bioconversion of 18:3n-3 (ALA) to 22:6n-3 (DHA) in muscle takes place in slaughter-sized salmon fed a high FO diet. Different selection strategies could be applied to increase the content of the healthy omega-3 FA in salmon muscle. We recommend selection for proportion of EPA + DHA in the muscle since both are essential FA and because such selection has no clear detrimental effects on the other traits.

Additional files

Additional file 1. Mean gross fatty acid composition of the feed provided during the final seawater stage. The values are calculated on the basis of analyses of the raw materials included in the prescription of the feed (Skretting Norway).

Additional file 2. Distribution of proportional content of individual fatty acids in muscle of all fish in the study. The figure shows how the muscle content of individual fatty acids (in % of total FA) is approximately normally distributed for the fish studied.

Additional file 3. Heritability, genetic and phenotypic correlations for fat deposition traits. Genetic parameters for muscle fat, visceral fat and liver fat. Heritability on the diagonal. Phenotypic correlations in the upper triangle and genetic correlations in the lower triangle. Standard errors in brackets.

Additional file 4. Phenotypic and genetic correlations between absolute content of selected muscle fatty acids and muscle fat. FA g = fatty acid content in g per 100 g of muscle. r_P = phenotypic correlations. r_G = genetic correlations. Standard errors in brackets.

Authors' contributions

All authors planned and designed the experiment. SSH performed fatty acid analysis and was responsible for data analysis and writing the manuscript. AKS, BR, SSH and THEM participated in the interpretation of results and the writing of the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The datasets generated and analyzed during the current study are not publicly available since they contain commercially sensitive data.

Consent for publication

Not applicable.

Ethics approval and consent to participate

The study was based on *post-mortem* sampling of material from fish harvested from a commercial breeding program for other purposes. The experimental plan was evaluated according to the Norwegian regulation for use of animals in experiments (FOR-2015-06-18-761, Forskrift om bruk av dyr i forsøk). The regulation states that activities related to non-experimental aquaculture activities are exempt from the regulation, and that it is legal to sample from animals *post-mortem* without a specific license. Thus, the samples used in this study were collected in accordance with the Norwegian legislation for animal experiment.

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Paper II

SCIENTIFIC REPORTS

OPEN

Individual differences in EPA and DHA content of Atlantic salmon are associated with gene expression of key metabolic processes

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The aim of this study was to explore how individual differences in content of the omega-3 fatty acids EPA and DHA in skeletal muscle of slaughter-sized Atlantic salmon, are associated with expression of genes involved in key metabolic processes. All experimental fish were fed the same diet throughout life and fasted for 14 days prior to slaughter. Still, there were relatively large individual variations in EPA and DHA content of skeletal muscle. Higher DHA content was concurrent with increased expression of genes of the glycolytic pathway and the production of pyruvate and lactate, whereas EPA was associated with increased expression of pentose phosphate pathway and glycogen breakdown genes. Furthermore, EPA, but not DHA, was associated with expression of genes involved in insulin signaling. Expression of genes specific for skeletal muscle function were positively associated with both EPA and DHA. EPA and DHA were also associated with expression of genes related to eicosanoid and resolvin production. EPA was negatively associated with expression of genes involved in lipid catabolism. Thus, a possible reason why some individuals have a higher level of EPA in the skeletal muscle is that they deposit - rather than oxidize - EPA for energy.

Farmed Atlantic salmon has traditionally been very rich in the healthy long-chain omega-3 polyunsaturated fatty acids (LC n-3 PUFA) eicosapentaenoic- (EPA) and docosahexaenoic (DHA) acid, and thereby they are a valuable source of these fatty acids in the human diet. Limited availability of fishmeal and fish oil on the world market, in conjunction with a growing aquaculture industry, has led to a substantial substitution of marine ingredients with plant ingredients in feed for farmed salmon in the last few decades[1]. Consequently, decreased levels of EPA and DHA in farmed salmon tissues and organs are reported in Norwegian, Scottish and Tasmanian Atlantic salmon[1–3]. This is not only reducing the nutritional value of Atlantic salmon fillet to consumers, but may also influence fish health and quality[4].

The LC n-3 PUFA content of fish tissues is a complex trait influenced by feed, life stage, and metabolic processes including digestibility and uptake of fatty acids (FA) in the gut, as well as selective transport, deposition and β-oxidation in organs and tissues[4–8]. In addition, endogenous omega-3 bioconversion enables salmonids to convert the shorter chain alpha-linolenic acid (ALA) to the longer chain PUFAs EPA and DHA through a series of desaturation and elongation steps, followed by peroxisomal β-oxidation[9,10]. In Atlantic salmon, the rate of bioconversion is inversely related to the amounts of fish oil in the diet[9,11–13]. The omega-3 bioconversion is also influenced by water temperature, the genetic background, and life stage of the fish[14,15].

Both EPA and DHA fulfill numerous biological functions in the animal body. EPA and DHA are to some extent metabolically interchangeable making it in some cases difficult to pinpoint functions specific to each of them. However, it is known that DHA is more critical than EPA as a structural component of cell membranes including lipid rafts, while EPA is believed to play a more central role in regulation of several processes related to immunity and inflammation[16,17]. Atlantic salmon fed only EPA but no DHA developed abnormal intestinal morphology, while the fish fed only DHA but no EPA developed a normal intestine[4], pointing to the importantce

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of DHA for functional intestinal cell membranes. Other studies have shown that EPA content of heart and head kidney tissues of Atlantic salmon is associated with reduced severity of the inflammatory response to the viral disease Heart and skeletal muscle inflammation[18]. Specific functions of EPA and DHA in production of eicosanoids and their role in inflammatory responses has been described both in fish[19] and in mammals[20]. Further, different pro-resolving lipid mediators, resolvins and protectins, can also be generated. Resolvins are derived from both EPA and DHA, categorized as either E-series (from EPA) or D-series (from DHA), while protectins are derived from DHA[21,22]. Although little is known for fish, the different resolvins and protectins are shown to have potent specific immunoregulatory actions in mammals[21,22].

Evidence from mammalian studies further indicates that tissue contents of EPA and DHA play important roles in the regulation of carbohydrate metabolism. Increased contents of EPA and DHA are reported to improve glucose uptake[23,24], have favorable effects on glucose utilization[25], and thereby have a preventive effect on development of metabolic syndrome[26,27]. EPA and DHA may also improve skeletal muscle capability of switching between use of lipids, protein and glucose for production of energy[28]. High levels of EPA may result in downregulated lipogenesis and upregulated mitochondrial β-oxidation[29]. The systems that regulate lipogenesis and LC n-3 PUFA biosynthesis in mammals are largely conserved in Atlantic salmon[30], and dietary LC n-3 PUFA levels have been shown to influence lipid metabolism also in Atlantic salmon[31,32]. With the shift in salmon diets causing major alterations in EPA and DHA content of skeletal muscle, it is especially important to gain knowledge on how these alterations influence lipid and carbohydrate metabolic pathways.

Most gene expression studies of omega-3 effects in Atlantic salmon have focused on the liver transcriptome and its responses to changed dietary levels of LC n-3 PUFAs[13,33–36]. Skeletal muscle is highly relevant as it constitutes the largest part of the Atlantic salmon body, and expresses desaturase and elongase genes[37,38], as well as transcription factors known to regulate lipid metabolism genes[8]. Further, there are inherent differences in the muscle content of EPA and DHA between Atlantic salmon fed the same diet[39], but the metabolic differences between individuals with high and low levels remain unknown.

The main aim of this study is to investigate how individual differences in EPA and DHA content in skeletal muscle of Atlantic salmon are associated with expression of genes involved in key physiological processes, particularly nutrient metabolism. We limit this study to the contents of EPA and DHA in the skeletal muscle and transcriptome of skeletal muscle and liver.

Methods

Fish populations and recordings. The fish studied originated from families and documentation groups from the entire 2014 year-class of the Atlantic salmon breeding population of SalmoBreed AS. The fish were transferred to sea at a mean weight of 0.1 kg, and slaughtered approximately 12 months later, at a mean weight of 3.6 kg. The fish were fed a commercial broodstock feed from Skretting (https://www.skretting.com/en/products/atlantic-salmon/?lifephase=474980) with a high fish oil content, and were fasted 13–14 days prior to slaughter. All the fish were reared under the same conditions.

Skeletal muscle and liver tissue samples for RNA-sequencing were taken from each individual fish at harvest, immediately frozen in liquid nitrogen, and subsequently stored at −70 °C. Skeletal muscle samples for lipid and FA analysis were taken from Norwegian Quality Cut collected at harvest, frozen and stored at −20 °C. In total, 668 fish were analyzed for skeletal muscle FA composition. The ranges in bodyweight and fat content of skeletal muscle were 1.2–6.4 kg and 5.5–27.7%, respectively. In order to minimize the effects of size and lipid deposition, 59 fish were selected for RNA-sequencing based on bodyweight (3.3 to 3.9 kg) and skeletal muscle fat level (16 to 25%). The selected individuals came from 48 full sib families, originating from 39 sires and 48 dams.

Lipid and fatty acid analysis. Total lipids were extracted from homogenized liver and skeletal muscle samples of individual fish, according to the Folch method[40]. Using one milliliter from the chloroform-methanol phase, FA composition of total lipids was analyzed following the method described by Mason and Waller[41]. The extract was dried briefly under nitrogen gas and residual lipid extract was trans-methylated overnight with 2′,2′-dimethoxypropane, methanolic-HCl, and benzene at room temperature. The methyl esters formed were separated in a gas chromatograph (Hewlett Packard 6890; HP, Wilmington, DE, USA) with a split injector, using an SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm, and film thickness 0.25 μm; SGE Analytical Science, Milton Keynes, UK) and a flame ionization detector. The results were analyzed using HP Chem Station software. The carrier gas was helium, and the injector and detector temperatures were both 270 °C. The oven temperature was raised from 50 to 170 °C at the rate of 4 °C/min, and then raised to 200 °C at a rate of 0.5 °C/min and finally to 240 °C at 10 °C/min. Individual FA methyl esters were identified by reference to well-characterized standards. The content of each FA was expressed as a percentage of the total amount of FAs in the analyzed sample. Presentation of the results will focus on the most abundant LC n-3 PUFAs of the fillet: 20:5n-3 (EPA) and 22:6n-3 (DHA), and the sum of EPA and DHA (EPA + DHA).

Gene expression analysis. Total RNA was extracted from liver and skeletal muscle tissues of each individual fish using the PureLink Pro 96 RNA Purification Kit (Invitrogen), according to the manufacturer's instruction. RNA was treated with PureLink On-Column DNase Digestion (Invitrogen) to remove any contaminating DNA. Samples were shipped to The Norwegian High-Throughput Sequencing Centre, where the mRNA library preparation and sequencing of transcripts were performed using standard protocols (www.illumina.com). Samples were sequenced on an Illumina HiSeq platform as paired-end 151 bp reads. After final quality control, results from 48 liver- and 59 skeletal muscle samples remained for analyses.

Processing of reads, alignment and annotation was performed according to Moghadam *et al.*[42]. Expression data were normalized via the median of the geometric means of fragment counts across all sample[43]. Cufflinks and Cuffdiff were used to estimate the expression abundances of the assembled genes and transcripts[44].

Trait	N	Mean	SD	Min	Max
Bodyweight (g)	59	3634	161	3330	3890
Muscle fat (%)	59	19.7	1.99	16.2	25.3
EPA (%)*	59	5.81	1.46	4.36	8.79
DHA (%)*	59	6.63	0.49	5.75	7.45
EPA + DHA (%)*	59	12.45	1.60	10.35	15.71

Table 1. Descriptive statistics of the fish material. N: Number of observations, SD: Standard deviation, Min: Minimum value. Max: Maximum value. *% of total FAs in skeletal muscle.

Statistical analysis. Gene expression data were normalized by calculating the aligned fragments per kilobase per million mapped fragments (FPKM). Normalized gene expression data were log2 transformed prior to the statistical analysis.

Trait-associated genes were defined by using linear regression analysis, testing for an association between continuous traits and mRNA expression. The FA content was considered the response variable and each individual gene expression an explanatory variable in the model. As suggested by Seo *et al.*[45], univariate analyses were carried out for each FA trait. The following general linear mixed model was fitted for each trait:

$$Y_{jk} = \mu + sex_j + fat + gene_k + family + e$$

where Y is the skeletal muscle FA content (EPA, DHA or EPA + DHA) of one individual, μ the overall mean, sex_j the fixed effect of sex (male or female), *fat* the percentage of fat in the tissue of the gene expression as a covariate (muscle or liver), $gene_k$ gene expression level of gene k, *family* random effect of family (1–48), and e random residual variation.

Genes were considered significantly associated with traits when the p-value of the regression coefficient was < 0.05. Genes of interest are presented with their regression coefficients. All significant genes are presented in Supplementary Table S1.

Enrichment analysis. A search for enriched GO classes and KEGG pathways in the list of trait-associated genes was performed by counting of genes among the trait-associated genes and all genes that passed quality control. Enrichment was assessed with Yates' corrected chi square test ($p < 0.05$). Terms with less than five genes were not taken into consideration.

The enrichment analysis was used for an initial screening to outline the functional groups and pathways of interest. Next, we focused on individual genes, taking into account the regression coefficients as metrics of expression differences between the phenotypes.

Ethics statement. The study was based on *post mortem* sampling of material from fish harvested from a commercial breeding program for other purposes. The experimental plan was evaluated towards the Norwegian regulation for use of animals in experiments (FOR-2015-06-18-761, Forskrift om bruk av dyr i forsøk). The regulation states that activities related to non-experimental aquaculture activities are exempt from the regulation, and that it is legal to sample from animals *post mortem* without a specific license. Thus, the samples used in this study were collected in accordance with the Norwegian legislation for animal experiment.

Results and Discussion

Description of phenotypes of experimental fish. In this study, we investigated how individual differences in EPA and DHA content (% of total FAs) in skeletal muscle of Atlantic salmon were associated with muscular and hepatic expression of genes involved in key physiological processes, particularly nutrient metabolism. The fish originated from the same strain of a breeding population, were reared under the same conditions and were all fed the same diet (as described previously in Horn *et al.*[37]), and fasted for two weeks prior to slaughter.

The EPA and DHA content of skeletal muscle changes with fish size and fat content, therefore fish of approximately the same size (3.6 kg) were selected for the gene expression analyses, as shown in Table 1. The mean skeletal muscle fat content was 19.7%. The mean contents (in percentage of total FAs) of EPA and DHA were 5.8 and 6.6%, respectively (Table 1). This is approximately twice as high as the EPA and DHA content found in today's commercially produced Atlantic salmon in Norway, which reflects the high levels of these FA in the feed used in the current study[4,46]. Standard diets for Atlantic salmon breeding populations are based on high levels of marine ingredients, usually a ratio of 70/30 between marine and plant ingredients, and the diets are therefore rich in EPA and DHA. Commercial diets, on the other hand, are based on 70% plant ingredients and are therefore low in EPA and DHA[1].

Relatively large variations in EPA and DHA contents were found in the skeletal muscle of the 59 experimental fish, even though the fish were of approximately the same size, reared under the same experimental conditions and fed the same diet throughout their life. The contents of EPA varied between 4.4 to 8.8% of total FAs, while DHA varied from 5.8 to 7.5% (Table 1). This indicates that the genetic variation between individuals may cause metabolic differences in one or several of the processes; dietary uptake, deposition, utilization or bioconversion of dietary fatty acids, and thereby result in individual differences in EPA and DHA contents of muscle despite the fact that the fish has been fed the same diet. Genetic variation was expected in the current study considering the family structure (48 full-sib families and 39 half-sib families). It has previously been shown that both EPA and DHA content of skeletal muscle are heritable traits[34,37].

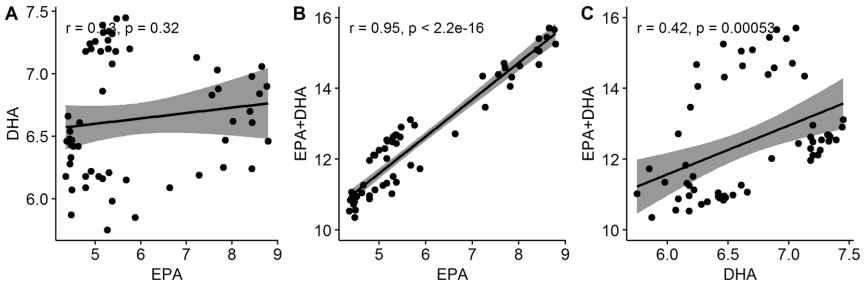

Figure 1. Correlation between contents of fatty acids in skeletal muscle. (**A**) Correlation between EPA and DHA (% of total FAs). (**B**) Correlation between EPA and EPA + DHA. (**C**) Correlation between DHA and EPA + DHA.

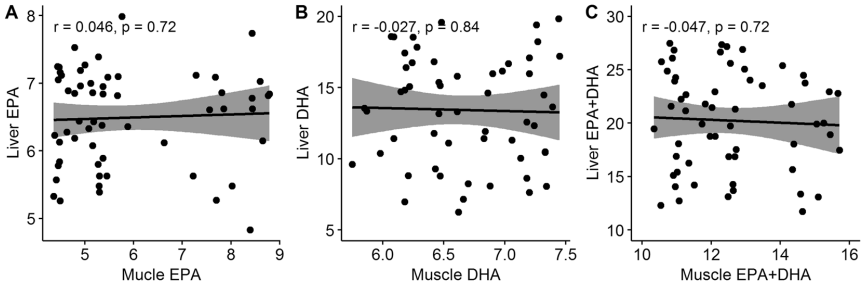

Figure 2. Correlation between liver and skeletal muscle content of fatty acids. Correlation between liver and skeletal muscle content of (**A**) EPA, (**B**) DHA, and (**C**) EPA + DHA (% of total FAs).

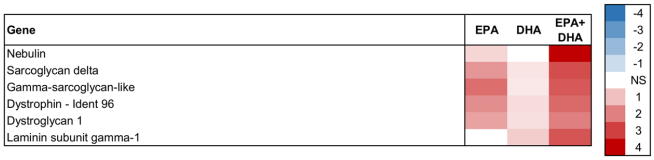

Figure 3. Skeletal muscle tissue development. Association between EPA, DHA or EPA + DHA content in muscle and expression of genes in skeletal muscle. Color scale shows linear regression coefficients. NS = no significant association.

There was a low correlation between EPA and DHA percentage in skeletal muscle (Fig. 1A). A similar correlation was found in an analysis of a larger dataset of the same population ($r = 0.23$, $n = 668$)[37]. The fish with the highest DHA percentage had a low EPA percentage (Fig. 1A), indicating that some individuals had higher capacities for the conversion from EPA to DHA, or higher β-oxidation rate of one of the two FAs. EPA + DHA was more strongly correlated to EPA than DHA (Fig. 1B,C), because there was greater variation in EPA content (Table 1).

Correlations between liver and skeletal muscle EPA and DHA content were very low (Fig. 2). This is most likely due to the differences between liver and skeletal muscle lipid metabolism. Feed trials with Atlantic salmon have shown that the tissues respond differently to increased dietary levels of EPA and DHA and that the skeletal muscle FA composition is to a much higher extent than the liver influenced by dietary FA composition[4].

Gene expression differences between EPA and DHA. The number of trait-associated genes identified by the linear model association tests was more than 50% higher for the EPA trait than for the DHA trait in both skeletal muscle and liver. In skeletal muscle, the number of trait-associated genes were 3487, 1915 and 3169 for EPA, DHA and EPA + DHA, respectively. The association tests of skeletal muscle FAs with liver gene expression identified 4005 EPA-, 2519 DHA- and 4285 EPA + DHA-associated genes. EPA was not only associated with a higher number of genes, but also had stronger associations compared to DHA (Figs 3–8). This could be related to that EPA is a more bioactive FA compared to DHA, as it is a precursor for many bioactive molecules influencing for instance immunity and inflammatory responses[17]. The transcriptome pattern of skeletal muscle for

Muscle Gene Expression			Liver Gene Expression		
EPA Muscle	**Vocabulary**	**Genes***	**EPA Muscle**	**Vocabulary**	**Genes***
Citrate cycle (TCA cycle)	KEGG	15/89	Fatty acid beta-oxidation	GO	14/53
Fatty acid metabolism	KEGG	18/109	Lipid metabolic process	GO	44/343
Mitochondrion	GO	266/2005	Mitochondrion	GO	270/2005
Pentose phosphate pathway	KEGG	13/81	Peroxisome	GO	24/140
Insulin signaling pathway	KEGG	54/442	Triglyceride catabolic process	GO	8/34
PPAR signaling pathway	KEGG	21/164	Insulin signaling pathway	KEGG	59/442
Fat cell differentiation	GO	17/129	PPAR signaling pathway	KEGG	36/164
Skeletal muscle tissue development	GO	23/190			
DHA Muscle	**Vocabulary**	**Genes***	**DHA Muscle**	**Vocabulary**	**Genes***
Citrate cycle (TCA cycle)	KEGG	11/89	Biosynthesis of unsaturated fatty acids	KEGG	8/40
Fatty acid metabolism	KEGG	10/109	Fatty acid elongation in mitochondria	KEGG	6/30
Gluconeogenesis	GO	10/90	Lipid metabolic process	GO	37/343
Glycolysis/Gluconeogenesis	KEGG	15/198	Lipid particle	GO	15/88
Mitochondrion	GO	176/2005	Mitochondrion	GO	207/2005
Pyruvate metabolism	KEGG	13/119	PPAR signaling pathway	KEGG	16/164
Skeletal muscle tissue development	GO	17/190			
Striated muscle cell development	GO	6/39			

Table 2. Enriched functional groups. *Number of genes corresponding to term in the list of trait associated genes/total number of genes of GO/KEGG term.

EPA + DHA was relatively similar to the pattern found for EPA, while DHA was different, most likely because EPA + DHA was more strongly correlated with EPA than DHA (Fig. 1).

Searches for enriched functional classes of GO and KEGG pathways were performed in order to find thematic associations among the trait-associated genes. These identified in total 55 groups enriched for the trait EPA in skeletal muscle and 47 for DHA. In the liver, 62 groups were enriched for skeletal muscle content of EPA and 45 for DHA (Supplementary Table S2). From these, the functional groups considered relevant for the scope of the study were selected (Table 2).

The enrichment analyses identified several functional groups related to nutrient metabolism as associated with skeletal muscle content of EPA and DHA. The following functional groups were enriched for both FAs; TCA cycle, FA metabolism, mitochondrion and skeletal muscle development (in muscle), lipid metabolic process, peroxisome proliferator activated receptor (PPAR) signalling and mitochondrion (in liver) (Table 2). Functional groups enriched for EPA only included pentose phosphate pathway, insulin and PPAR signalling in muscle, and insulin signalling and lipid catabolic processes in liver. Functional groups enriched for DHA only included glycolysis and gluconeogenesis in muscle, and biosynthesis of unsaturated FAs and FA elongation in liver. No associations were found with protein metabolism. In the following sections, trait-associated genes from these functional groups will be discussed.

It should be noted that the fish in this study was fed a diet rich in EPA and DHA and further fasted for two weeks prior to slaughter, both being factors known to influence nutrient metabolism. Whether the suggested associations of EPA and DHA in Atlantic salmon skeletal muscle with the different metabolic pathways applies to fish fed a commercial diet and with a shorter fasting period prior to slaughter, remains to be elucidated.

Muscle content of EPA and DHA was associated with skeletal muscle tissue development and function. Several studies on mammals suggest that EPA and DHA can influence the response of skeletal muscle to exercise and nutrient utilization, and improve muscle anabolic processes, although the underlying mechanisms are unclear (reviewed in Jeromson *et al.*[28]).

The enrichment analysis identified skeletal muscle tissue development as an overrepresented group for both EPA and DHA (Table 2). Positive associations with both EPA and DHA were found with expression of the genes *sarcoglycan*, *dystrophin*, *dystroglycan* and *laminin* - all involved in the Dystrophin-Associated Protein Complex (DAPC) in skeletal and cardiac muscle (Fig. 3). The DAPC plays a structural role in the muscle by stabilizing the sarcolemma during contraction and relaxation, and transmits force generated in the muscle sarcomeres to the extracellular matrix[47].

There was a clear additive effect of EPA and DHA, as shown by the higher regression coefficients of EPA + DHA (Fig. 3). EPA + DHA had an especially strong positive association with expression of *nebulin*. Nebulin is a structural protein that functions in numerous cellular processes including regulation of muscle contraction, improving muscle force efficiency, Z-disc formation, and myofibril assembly[48].

In conclusion, these results indicate that EPA and DHA are associated with skeletal muscle function and/or development in Atlantic salmon.

EPA and DHA were associated with carbohydrate metabolism in skeletal muscle. Carnivorous fish have a low capacity to utilize dietary digestible carbohydrates, and have slow blood glucose clearance,

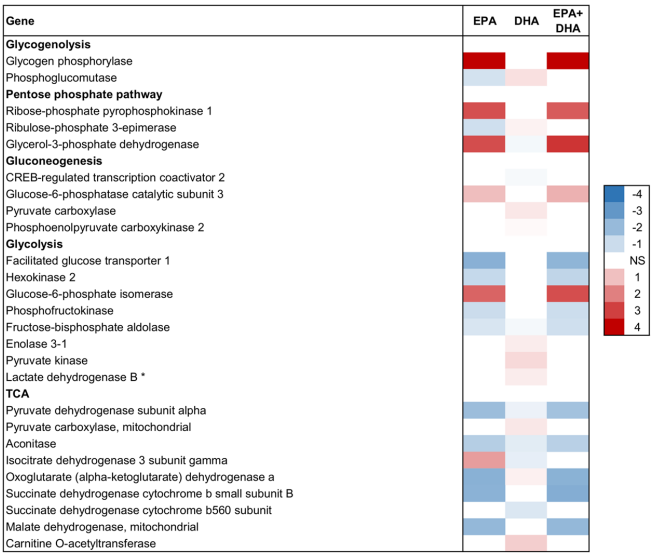

Figure 4. Carbohydrate metabolism in skeletal muscle. Association between EPA, DHA or EPA + DHA content in muscle and expression of genes in skeletal muscle. Color scale shows linear regression coefficients. NS = no significant association. *Anaerob glycolysis.

however, they do have an active glucose homeostatic system with the presence of almost all the essential biological elements (reviewed in[49] and[50]).

EPA and DHA were differently associated with the expression of genes involved in carbohydrate metabolism in the skeletal muscle (Fig. 4). While increasing DHA content was concurrent with increased expression of genes in the glycolytic pathway and the production of pyruvate and lactate (anaerobic glycolysis), EPA was associated with increased expression of genes involved in the pentose phosphate pathway and nucleotide synthesis, in addition to increased glycogen breakdown (Fig. 9).

Carbohydrates are stored as glycogen in the muscle, which can be broken down to glucose units. Gene expression of the catalyzer of the rate-limiting step of glycogen degradation to glucose, *glycogen phosphorylase*, was positively associated with EPA and EPA + DHA (Fig. 4). In addition, EPA was positively associated with expression of the gluconeogenesis-specific gene *glucose-6-phosphatase* that catalyzes the hydrolysis of glucose-6-phosphate to glucose, which can then be released into the blood for use in other tissues. The glucose-6-phosphate derived from the breakdown of glycogen can also be utilized as a substrate for glycolysis, or enter the pentose phosphate pathway to yield ribose derivatives and NADP, which is used in multiple anabolic pathways (Fig. 9).

The results from this study indicate that increased EPA content in skeletal muscle is concurrent with reduced glycolytic activity. Glycolysis and gluconeogenesis are opposing processes, and share several enzymes, acting in reversible reactions. There are three critical stages separating the two pathways. EPA was negatively associated with expression of two of the three glycolysis-specific genes (*hexokinase* and *phosphofructokinase*), and positively associated with expression of the gluconeogenesis-specific gene *glucose-6-phosphatase* that catalyzes the opposite reaction of *hexokinase* (Fig. 4). DHA, on the other hand, was positively associated with gene expression of the glycolysis-specific enzyme *pyruvate kinase* (the last step of glycolysis, forming pyruvate). However, DHA was also positively associated with *pyruvate carboxylase* and *phosphoenolpyruvate carboxykinase*, two gluconeogenesis-specific enzymes that catalyzes the opposite reaction of *pyruvate kinase*. Further, DHA was positively associated with *lactate dehydrogenase*, which converts pyruvate to lactate, and negatively associated with *pyruvate dehydrogenase*, which converts pyruvate to acetyl-coA. Hence, increased DHA content was concurrent with increased anaerobic glycolytic potential, where the pyruvate produced by glycolysis is shuttled towards lactate to a larger degree than to the TCA cycle (Fig. 9).

EPA, however, had a clear negative association with TCA cycle gene expression in muscle, possibly related to the reduced glycolytic activity to form pyruvate; expression of pyruvate dehydrogenase, plus four of the enzymes catalyzing TCA cycle, were negatively associated with EPA. The only TCA-cycle gene whose expression was positively associated with EPA content was α-*ketoglutarate dehydrogenase* (Fig. 4).

The enrichment analysis suggested an association between the pentose phosphate pathway (PPP) and EPA (Table 2). The regression coefficients showed that *ribose-phosphate pyrophosphokinase 1* (*PRPS1*) expression was positively associated with EPA content in the muscle (Fig. 4). *PRPS1* catalyzes the synthesis of phosphoribosylpyrophosphate (PRPP) that is essential for nucleotide synthesis. This may indicate that EPA stimulates nucleotide synthesis in muscle, although the cause-effect relationship is unknown. Nucleotides participate in nearly all biochemical processes in the body as for instance precursors for DNA and RNA, or energy, metabolic regulators,

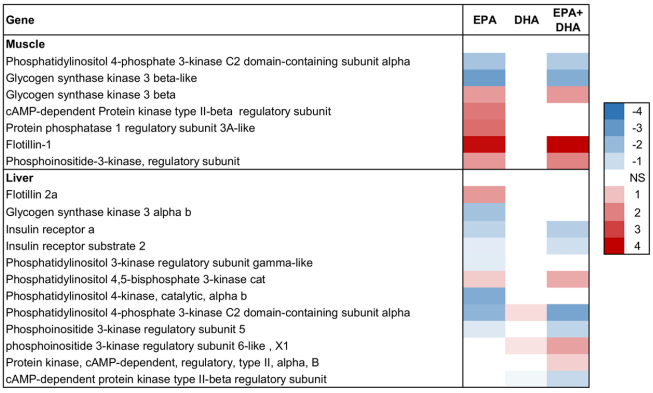

Figure 5. Insulin signaling in muscle and liver. Association between EPA, DHA or EPA + DHA content in muscle and expression of genes in skeletal muscle and liver. Color scale shows linear regression coefficients. NS = no significant association.

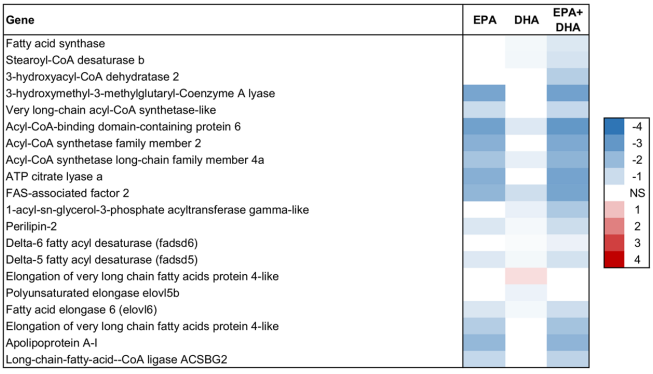

Figure 6. Lipogenesis and fatty acid bioconversion in liver. Association between EPA, DHA or EPA + DHA content in muscle and expression of genes in liver. Color scale shows linear regression coefficients. NS = no significant association.

coenzymes and activated intermediates in biosynthesis[51]. The results therefore suggest that in a skeletal muscle rich in EPA, rather than entering glycolysis, the glucose-6-phosphate from breakdown of glycogen seems to be released as glucose, or shuttled into the PPP.

In conclusion, this study indicates that muscle EPA and DHA content has apparent links with carbohydrate metabolism in Atlantic salmon, as has previously been demonstrated in mammals.

Insulin regulation in Atlantic salmon and its association with EPA and DHA. Skeletal muscle is a major site of insulin action, and muscle LC n-3 PUFA effects on insulin sensitivity has been observed in mice[52], pigs[53,54] and human[23,24]. Carnivorous fish possess an insulin-signaling system similar to mammals, although its role is not completely clarified[49,50]. In Atlantic salmon adipose tissue, insulin improves the differentiation capacity of preadipocytes towards mature adipocytes, similar to what happens in human adipose tissue[55].

The enrichment analysis identified the insulin-signaling pathway as an enriched group for EPA, but not DHA, in both skeletal muscle and liver (Table 2), and expression of several genes related to insulin were associated with EPA content in skeletal muscle. In skeletal muscle, expression of the two genes *phosphatidylinositol 4-phosphate 3-kinase C2 domain-containing subunit alpha-like*, and *phosphatidylinositol 3-kinase regulatory subunit beta-like*, in the phosphoinositide 3-kinase (PI3K) family were positively associated with EPA content (Fig. 5). PI3-kinases are known to play key roles in many signaling pathways in mammals[56,57], including glucose uptake and insulin signaling[58,59]. Further, expression of *cAMP-dependent protein kinase type II-beta regulatory subunit-like*, which belongs to the protein kinase A (PKA) family of enzymes, was also positively associated with EPA. Studies in mammals have shown that a correct regulation of cAMP/PKA activity is crucial for glucose homeostasis and skeletal muscle insulin sensitivity[60]. The gene expression of *flotillins*, which are considered as markers of lipid rafts

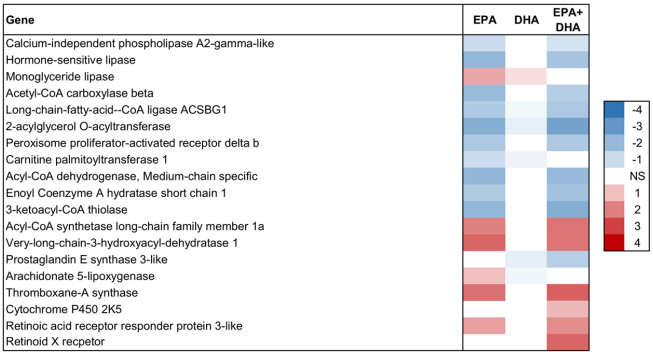

Figure 7. Lipid metabolism in skeletal muscle. Association between EPA, DHA or EPA + DHA content in muscle and expression of genes in skeletal muscle. Color scale shows linear regression coefficients. NS = no significant association.

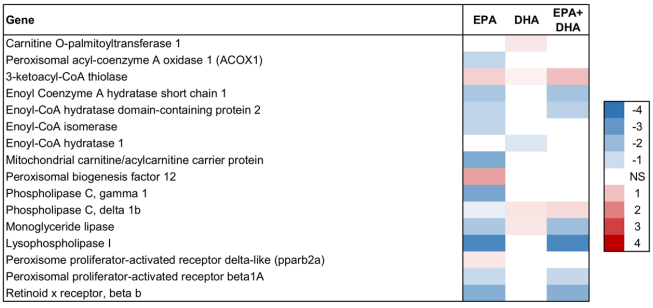

Figure 8. Lipid catabolism in liver. Association between EPA, DHA or EPA + DHA content in muscle and expression of genes in liver. Color scale shows linear regression coefficients. NS = no significant association.

and implicated in numerous signaling events, including insulin signaling[61], increased in both skeletal muscle and liver with increasing content of EPA in muscle (Fig. 5).

These results indicate that EPA is involved in insulin regulation in Atlantic salmon. This is in agreement with what was previously demonstrated in mammals[25,52,62].

High skeletal muscle content of EPA and DHA was negatively associated with hepatic lipogenesis. Lipogenesis is the formation of lipids, and includes formation of triglycerides for storage as well as *de novo* lipogenesis of FAs. The crucial carbon source for *de novo* lipogenesis is acetyl-CoA formed in mitochondria from the oxidative decarboxylation of pyruvate or the oxidative degradation of some amino acids.

The analysis of the liver transcriptome showed that both EPA and DHA were associated with reduced expression of genes involved in lipogenesis (Fig. 6). For example, several *acyl-CoA synthethases*, *fatty acid synthase (FAS)*, *FAS-associated factor 2*, as well as *1-acylglycerol-3-phosphate acyltransferase gamma-like* (*GPAT1*), a key enzyme for hepatic triglyceride synthesis. Several studies have shown reduced hepatic triglyceride synthesis with increasing dietary levels of EPA and/or DHA, both in mammals[63,64] and salmonid fish including rainbow trout[65] and Atlantic salmon[66]. Our study found an association between reduced lipogenesis and increased EPA in the skeletal muscle tissue of fish that received the same feed.

Although the cause-effect relationship cannot be determined, these results may indicate that increasing the skeletal muscle EPA and DHA content lowers the risk of developing fatty liver, which may promote health as fatty liver is recognized as a health problem in farmed Atlantic salmon[67]. However, this effect was not seen on the phenotype of the fish; the correlation between liver fat percentage and skeletal muscle EPA and DHA content was close to zero (Supplementary Fig. S1).

In skeletal muscle, expression of the key genes involved in lipogenesis were negatively associated with EPA, for instance *2-acylglycerol O-acyltransferase 1* that catalyzes the formation of diacylglycerol, and *acetyl CoA carboxylase* (ACC) that is involved in regulation of fatty acid synthesis (Fig. 7). These results indicate a possible inhibitory effect of EPA on lipogenesis in Atlantic salmon skeletal muscle, which has not been reported before.

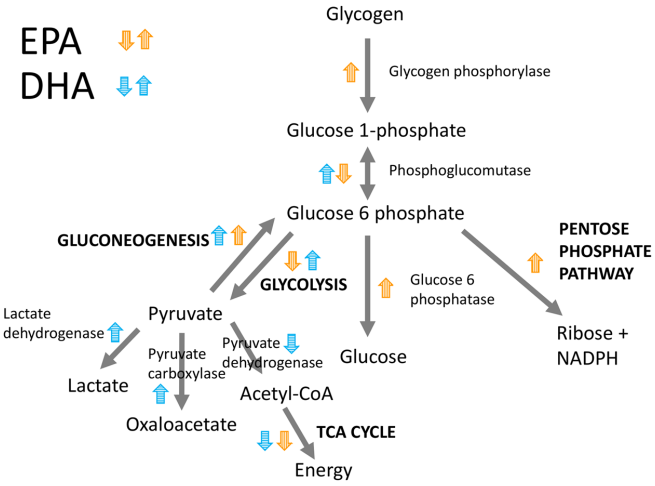

Figure 9. Overview of main results regarding carbohydrate metabolism in skeletal muscle. Colored arrow up and down indicate positive and negative associations, respectively.

Lipid catabolism decreased with increasing EPA in skeletal muscle. Fatty acid catabolism is the major source of energy in salmonid fish. The process is termed β-oxidation, and occurs in the mitochondria and peroxisomes[68]. In Atlantic salmon, β-oxidation is an important source of energy in several tissues[5,69,70]. Increased EPA content in skeletal muscle was negatively associated with expression of lipid catabolic genes in both liver and skeletal muscle (Figs 7 and 8). DHA had weak associations compared to EPA.

In the liver, we observed that EPA was associated with reduced expression of the lipid catabolic genes *monoglyceride lipase* and *phospholipase C* (Fig. 8), as well as e*noyl-CoA hydratase* and *Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase*, which are both involved in β-oxidation. Expression of the mitochondrial β-oxidation biomarker *carnitine palmitoyltransferase 1 (CPT1)*, was also negatively associated with EPA.

In skeletal muscle, EPA and EPA + DHA were negatively associated with expression of three genes coding for β-oxidation enzymes: *3-ketoacyl-CoA thiolase*, *medium-chain specific acyl-CoA dehydrogenase* and *enoyl Coenzyme A hydratase* (Fig. 7). In addition, expression of *PPAR β/δ*, a central regulator of energy metabolism that stimulates genes of β-oxidation[71,72], and *CPT1*, had negative associations with EPA.

Mitochondrial β-oxidation capacity is determined by many factors, for instance mitochondrial biogenesis and function in general. Expression of a number of mitochondrial genes were associated with EPA and DHA content in skeletal muscle, both positively and negatively, thus, the overall effect was difficult to determine, although the majority of the associations were negative (Table 3).

Increased EPA in the diet generally increases the content of EPA in both liver and skeletal muscle, and has been reported to result in increased FA oxidation in liver tissue in mammals[73–76]. In Atlantic salmon, however, there are varying results from feeding trials regarding the effect of high EPA and DHA on hepatic β-oxidation capacity[77–79]. Regarding skeletal muscle, Aas *et al.*[23] reported that preincubation of human skeletal muscle cells with EPA decreased FA oxidation, which concurred with increased incorporation of EPA into complex cellular lipids. Thus, the variation in β-oxidation capacity observed in this study may reflect on the ratio between utilization of EPA for energy production and its deposition in skeletal muscle.

Overall, expression of genes involved in lipid catabolism decreased in both liver and skeletal muscle with increasing content of EPA and EPA + DHA in skeletal muscle.

Eicosanoids, resolvins and protectins. In the muscle, genes related to conversion of long chain FAS were positively associated with EPA and EPA + DHA. These included *very-long-chain-3-hydroxyacyl-dehydratase 1*, which catalyzes the third of the four reactions of the long-chain fatty acids elongation cycle, as well as *long-chain Acyl-CoA synthetase* (Fig. 7). This indicates further bio-conversion of EPA and DHA to very long chain n-3 PUFAs and possibly formation of resolvins and protectins. It has been suggested that resolvins and protectins are mediators of the beneficial effects of EPA on insulin signaling[80,81]. Expression of a few eicosanoid-related genes, such as *Arachidonate 5-lipoxygenase (ALOX)* and *Thromboxane-A synthase*, were positively associated with EPA (Fig. 7). Further, *Cytochrome P450* was positively associated with EPA + DHA. ALOX metabolizes EPA to 5-hydroperoxy-EPA which is then converted to 5-series of Leukotriene products[82]. ALOX is also known to cooperate with other lipoxygenase and cyclooxygenase cytochrome P450 enzymes in metabolic pathways that metabolize EPA to resolvins of the E series[82]. These results indicate that EPA and DHA contents of muscle are associated with skeletal muscle production of eicosanoids and resolvins.

Trait	Total no. of associated genes	Negatively associated	Positively associated	Mean regression coefficient
Muscle				
EPA	268	154	114	−0.06
DHA	176	128	48	−0.19
EPA + DHA	192	116	76	−0.11
Liver				
EPA	265	215	50	−1
DHA	206	165	41	−0.28
EPA + DHA	259	208	51	−1.21

Table 3. Genes classified in the GO functional group *Mitochondrion* that were significantly associated with the fatty acid traits.

What determines EPA and DHA content in skeletal muscle? Individual differences in EPA and DHA content were observed in fish of similar size fed an identical diet. This individual variation may result from many factors, for instance individual differences in FA deposition, β-oxidation of FAs, omega-3 bioconversion in skeletal muscle, or uptake of these FAs from blood, which again can be influenced by omega-3 bioconversion in the liver and intestine, which are the major sites of omega-3 bioconversion in Atlantic salmon[5].

In the current study, the hepatic expression of the fatty acid desaturase genes *fadsd5* and *fadsd6* was negatively associated with skeletal muscle level of EPA and DHA (Fig. 6). In the skeletal muscle, no desaturases or elongases involved in the omega-3 bioconversion pathway showed any association with either EPA or DHA (although *fadsd5*, *fadsd6* and *elovl5* were expressed in skeletal muscle tissue). This may point to factors other than omega-3 bioconversion which are more important for determining the EPA and DHA content in skeletal muscle. Albeit we cannot determine cause and effect based on the association analyses, our results point to reduced β-oxidation as a possible determining factor for EPA and the sum of EPA + DHA, since the individuals with the highest EPA and EPA + DHA content had a lower expression of β-oxidation genes. Studies in Atlantic salmon have shown that LC n-3 PUFAs given in surplus are oxidized for the production of energy[83]. Thus, our results suggest that the reason why some individuals have a higher level of EPA and DHA in the skeletal muscle is that these FAs are deposited rather than oxidized for energy.

Conclusions

The current study shows that there are big individual variations in EPA and DHA content of skeletal muscle between Atlantic salmon fed the same diet, and that these variations are associated with differences in expression of genes involved in nutrient metabolism. The main results are:

- Correlations between liver and skeletal muscle were very low regarding both EPA and DHA content.
- The correlation between EPA and DHA percentage in skeletal muscle was low, and over 50% more genes were associated with EPA than DHA. Also, the two FAs were associated with different gene expression profiles. This information is relevant for consideration of future feed production strategies, since several of new alternative feed ingredients contain either EPA or DHA, not both.
- Skeletal muscle content of EPA and DHA was positively associated with muscle tissue development and function.
- Skeletal muscle content of EPA and DHA was associated with carbohydrate metabolism. While increasing DHA content was concurrent with increased expression of genes in the glycolytic pathway and the production of pyruvate and lactate, EPA was associated with increased activity of the pentose phosphate pathway and nucleotide synthesis in addition to increased glycogen breakdown. Furthermore, skeletal muscle content of EPA, but not DHA, was associated with expression of genes involved in insulin signaling in both skeletal muscle and liver tissues.
- EPA and DHA were associated with expression of genes involved in eicosanoid and resolvin production.
- EPA was negatively associated with expression of genes involved in lipid catabolism, in both liver and muscle. Thus, a possible reason why some individuals have a higher level of EPA in the skeletal muscle is that they deposit - rather than oxidize - EPA for energy.

Data Availability

The datasets generated and analyzed during the current study are not publicly available since it contains commercially sensitive data, but are available from the corresponding author on reasonable request.

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Author Contributions
A.K.S., B.R., T.H.E.M. conceived and designed the study. S.S.H., B.R., A.K.S., B.H. collected the data. S.S.H., A.K., H.M. analyzed the data. S.S.H., B.R., A.K.S., A.K. wrote the first draft of the manuscript, and all authors contributed to editing and revising the manuscript.

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Paper III

Accuracy of selection for omega-3 fatty acid content in Atlantic salmon fillets

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Abstract

The main aim of the current study was to compare the accuracy of selection for muscle content of EPA, DHA and fat in Atlantic salmon, by varying the sources of genetic information used in the estimation of breeding values. The following genetic information sources were compared: pedigree, SNP-chip markers, imputed markers and allele-specific expression markers.

The results showed that differences between information sources were in general small, and different genetic information performed best for different traits. SNP-chip performed best for DHA, and pedigree performed best for EPA and muscle fat. Imputed markers did not improve selection accuracy for any of the traits studied, probably due to imputation errors caused by the large number of imputed SNPs combined with the small dataset. Markers based on allele-specific expression were able to capture a lot of genetic variation for DHA, but did not give higher accuracies when combined with SNP-chip or pedigree information.

The cross-validation accuracies for selection for DHA and EPA were moderate and offer possibilities for selection for these traits, especially if one extends the reference data set to a much bigger population.

Keywords: Selection accuracy; Omega-3; Atlantic salmon; Genomic prediction.

1. Background

Atlantic salmon (*Salmo salar* L.) is an important farmed fish species, known for its high content of the health-promoting long-chain polyunsaturated omega-3 fatty acids (n-3 LC-PUFA) eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3). The replacement of fish oil and fishmeal with plant ingredients in the diet of farmed Atlantic salmon has resulted in reduced levels of EPA and DHA in the fish fillet (Sprague et al. 2016; Ytrestoyl et al. 2015). Quantitative genetic analyses have demonstrated the potential of selective breeding to increase n-3 LC-PUFA levels in salmon tissues (Horn et al. 2018; Leaver et al. 2011). Muscle content of EPA and DHA appear to be genetically different traits, with heritability of 0.09 and 0.26, respectively (Horn et al. 2018).

Selective breeding programs have historically been based on best linear unbiased prediction (BLUP) of individual breeding values that use pedigree-based relationship matrices. Genomic

selection (GS) is a method that uses DNA marker-based relationship matrices as the genetic information to predict the breeding value of all genotyped individuals (Meuwissen et al. 2001). The GS methodology is of particular relevance for traits that cannot be measured directly on selection candidates, such as muscle EPA and DHA content, because it allows prediction of individual breeding values for non-phenotyped individuals. GS has been shown to increase the accuracy of breeding values for several traits in salmonids compared to conventional selection based on pedigree data (Bangera et al. 2017; Tsai et al. 2016; Yoshida et al. 2018).

GS may be performed using several different sources of genetic information, where dense SNP-chip is the most common. Another source of genetic information is imputed genotypes, where genotypes are inferred for individuals genotyped with a low-density panel using a reference population sequenced or genotyped with a high-density marker panel (Habier et al. 2009). It has been shown in Atlantic salmon that the performance of imputed genotype data (from 7.8 K to 78 K density) in genomic prediction was comparable to using true genotype data (Tsai et al. 2017). However, improvements due to the use of imputed whole-genome sequence, with millions of markers, are unclear.

An alternative source of genetic information is markers of variation in gene expression. Variation in gene expression has the potential to contribute significantly to phenotypic variation (Pritchard et al. 2006; Wray et al. 2003). One technique to identify this variation is to screen for allele-specific expression (ASE) – unequal expression of the two alleles of a gene, caused by cis-regulatory elements (Yan et al. 2002). ASE markers are more closely linked to causative loci affecting traits than random markers, and can potentially be applied in selection programs. ASE is widespread within a large number of species. ASE genes have been found related to complex, economically important traits in chicken, pigs and cattle (Cheng et al. 2015; Muráni et al. 2009; Olbromski et al. 2013). Selection using ASE SNPs reduced disease incidence after one generation of selection in chicken (Cheng et al. 2015).

The main aim of the current study was to compare the accuracy of selection for muscle content of EPA, DHA and fat in Atlantic salmon, by varying the sources of genetic information used in the estimation of breeding values.

2. Materials and Methods

2.1 Fish population and recordings

The 563 fish studied consisted of 174 full-sib families from 92 sires and 174 dams. All fish originated from year-class 2014 of the Atlantic salmon breeding population of SalmoBreed AS. Four generations of pedigree information on direct ancestors of the fish were available. The fish were transferred to sea at a mean weight of 0.1 kg, and slaughtered approximately 12 months later, at a mean weight of 3.6 kg. The fish were fed a commercial broodstock feed from Skretting (https://www.skretting.com/en/products/atlantic-salmon/?lifephase=474980) with a high fish oil content, and were fasted 13-14 days prior to slaughter. All fish were reared under the same conditions.

At slaughter, sex was determined visually by inspection of the gonads, body weight was recorded, and skeletal muscle samples for lipid and fatty acid analysis taken from Norwegian Quality Cut were collected, frozen and stored at −20 °C.

2.2 Muscle fat and fatty acid analysis

The traits analyzed in this study were muscle EPA and DHA content; expressed as a percentage of the total amount of fatty acids in the analyzed sample, and, because it is a trait highly interconnected with muscle fatty acid composition (Horn et al. 2018), muscle fat (MFAT); expressed as total lipid percentage in the sampled muscle tissue.

Muscle fat content was measured by extracting total lipids from homogenized skeletal muscle samples of individual fish, according to the Folch method (Folch et al. 1957). Using one milliliter from the chloroform-methanol phase, fatty acid composition of total lipids was analyzed following the method described by Mason and Waller (1964). The extract was dried briefly under nitrogen gas and residual lipid extract was trans-methylated overnight with 2′,2′-dimethoxypropane, methanolic-HCl, and benzene at room temperature. The methyl esters formed were separated in a gas chromatograph (Hewlett Packard 6890; HP, Wilmington, DE, USA) with a split injector, using an SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm, and film thickness 0.25 μm; SGE Analytical Science, Milton Keynes, UK) and a flame ionization detector. The results were analyzed using HP Chem Station software. The carrier gas was helium, and the injector and detector temperatures were both 270 °C. The oven temperature was raised from 50 to 170 °C at the rate of 4 °C / min, and then raised to 200 °C at a rate of 0.5 °C / min and finally to 240 °C at 10 °C / min. Individual fatty acid methyl esters were identified by reference to well-characterized standards.

2.3 Genotyping and sequencing

The fish were genotyped using a customized 57K axiom Affymetrix SNP Genotyping Array (NOFSAL02). 78 sires and 18 dams of the population were sequenced on Illumina HiSeq X platform (Illumina, CA, USA) according to the manufacturer's protocol, at The Norwegian Sequencing Centre in Oslo, Norway. These genome sequences were used in the imputation of the 563 fish, as part of a bigger dataset where the genotypes of 1168 non-sequenced offspring were imputed based on the 78 sequenced sires and 18 dams using the Beagle4.1 software (faculty.washington.edu/browning/beagle/). 1168 offspring were SNP-chip genotyped resulting in 44438 SNP genotypes per individual which served as anchor points for the imputation to the whole genome sequence. The imputation to whole genome sequence resulted in 5,360,704 genotypes available. The error rate of the genotype imputation was estimated at 10%.

The SNP-chip genotypes and the imputation genotypes were used to construct the genomic relationship matrices $G_{SNPCHIP}$ and $G_{IMPUTED}$, respectively (see section Genomic relationship matrices).

2.4 Allele specific expression

An observation of allele specific expression in RNA-seq data indicates the presence of one or more variants that act in cis to affect the expression level of the gene. Genes exhibiting allele specific expression were identified by first selecting all genes whose expression was identified in Horn et al. (2019) as associated with EPA and/or DHA in muscle and/or liver. Here, we detected 6194 trait-associated (TA) genes at a nominal P-value of 0.10. Second, within each RNA-seq sample derived from individual fish, that were called heterozygote by the Freebayes software (https://github.com/ekg/freebayes) for a SNP in the TA genes, we compared the relative expression levels of the reference and the alternative allele. Lastly, we counted in how many of the heterozygous individuals the alternative allele was more often expressed than the reference allele. This number was compared to the Binomial distribution with p=0.5 as a null-hypothesis distribution to determine statistical significance in a one-sided-test for overexpression of the alternative allele at a P-value of 0.001. A one-sided statistical test was performed because we observed a general tendency for the reference allele to be over-expressed (possibly because the reads with the reference allele are more likely to align with the reference genome than reads with alternative alleles). This statistical test resulted in a total of 537 significant genes across the tissues liver and muscle. Within the 57K

SNP-chip we found 395 SNPs within these 537 genes, which were used to construct a genomic relationship matrix (G-matrix); G_{ASE}.

In order to compare this method to a random set of markers, 400 random markers were sampled from the 57K SNP-chip markers, and a G-matrix was constructed based on these; G_{RANDOM}.

2.5 Statistical analyses

The different models compared in the current study differed solely with respect to their specification of the relationship matrix that was fitted:

- PED: Classical pedigree-based analysis with a numerator relationship matrix (A).
- SNPCHIP: G-matrix based on 57K genome-wide SNP markers ($G_{SNPCHIP}$).
- IMPUTED: G-matrix based on imputed whole genome sequence data ($G_{IMPUTED}$).
- ASE: G-matrix based on 395 markers identified in the allele specific expression analysis (G_{ASE}).
- RANDOM: G-matrix based on 400 randomly selected markers (G_{RANDOM}).
- ASE+PED: Using both A and the G_{ASE}
- ASE+SNPCHIP: using the two G-matrices G_{ASE} and $G_{SNPCHIP}$.

2.5.1 Genomic relationship matrices

The G-matrices were constructed using the GCTA software, following the method by Yang et al. (2011), using the following equation to estimate the genetic relationship between individuals j and k:

$$G_{jk} = \frac{1}{N}\sum_{i=1}^{N} \frac{(x_{ij} - 2p_i)(x_{ik} - 2p_i)}{2p_i(1 - p_i)}$$

In order to stabilize the relationship matrix (make it easier invertable), a small value (0.05) was added to the diagonal, in accordance with Forni et al. (2011).

The following G-matrices were constructed: $G_{SNPCHIP}$, $G_{IMPUTED}$, G_{ASE} and G_{RANDOM}.

2.5.2 Breeding value estimation

The following linear mixed model was applied for the estimation of breeding values:

$$y = \mu + X_1 b_1 + X_2 b_2 + Z u_1 + (Z u_2 +) e \quad (1)$$

where y is a vector of EPA, DHA or MFAT content, μ is the overall mean, b_1 is the fixed effect of body weight as a covariable, b_2 is a vector of fixed effect of sex (Horn et al. 2018), X_1 and X_2 are incidence matrices for the effects contained in b_1 and b_2, u_1 is a vector of additive genetic effects distributed as $u_1 \sim N(0, G\sigma^2_{u_1})$ (or $u_1 \sim N(0, A\sigma^2_{u_1})$ in case of PED), where $\sigma^2_{u_1}$ is the additive genetic variance, G and A are genomic and pedigree relationship matrices, respectively; Z is the corresponding incidence matrix to additive genetic effects, and e is the vector of random residual effects with $e \sim N(0, I\sigma^2_e)$. In models where two relationship matrices are fitted, the additive genetic effect of the second relationship matrix is included ($Z u_2$) with distribution $u_2 \sim N(0, G_2\sigma^2_{u_2})$, where G_2 is the second genomic relationship matrix, and $\sigma^2_{u_2}$is its associated variance component. Variance components and breeding values were estimated by ASReml 4.0 (Gilmour et al. 2015). Heritability (narrow sense) was estimated as the ratio of additive genetic variance to total phenotypic variance. Estimated Breeding Values (EBV) were obtained as $EBV = \hat{u_1}(+ \hat{u_2})$, where ^ denotes estimates of the effects.

2.5.3 Selection accuracy

Selection accuracies of the different models were assessed through predictive ability, using a cross-validation scheme by randomly masking the phenotype of one of the siblings in every full-sib family consisting of more than three full-sibs, resulting in 87 validation and 476 training individuals.

The mean selection accuracy (Acc) of 100 replicates was computed as:

$Acc = Corr(EBV_v * \hat{y}) / \sqrt{h^2}$,

where EBV_v represents breeding values for validation individuals estimated using the reference data, $\hat{y}$ is y adjusted for fixed effects by calculating $\hat{y} = EBV_a$ + residual, with EBV_a and h^2 denoting the pedigree based estimates of breeding values and heritability using all data.

To test if the accuracy-estimates for the different methods were significantly different from each other, paired two-sided t-tests were applied in R 3.4.3 (R Core Team 2017). For each of the 100 replicates, a data subset was created. The same 100 data subsets were used for

estimating accuracy for all methods. Thus, we obtained accuracy-estimate 1 to 100 for each method (for data subset 1 to 100), which were used for the paired t-test.

3. Results and discussion

The variance components showed that the genetic variance (and thus heritability) estimates with genomic information were lower than the estimates using pedigree, except for DHA where PED, SNPCHIP and IMPUTED were very similar (Table 1). Several authors have previously reported a reduction in heritability estimate with marker information (Boison et al. 2019; Erbe et al. 2013; Robledo et al. 2018). This can be explained by factors such as lack of markers that are in linkage disequilibrium with the causative mutations, and large numbers of markers that are in linkage equilibrium with the causative mutations (de Los Campos et al. 2015). It should also be noted that the standard errors of the estimates of the variance components were high (Table 1).

Table 1. Estimates of variance components for EPA, DHA and muscle fat (MFAT) using different genetic information.

		DHA	**EPA**	**MFAT**
PED				
	h^2	0.21 (0.09)	0.06 (0.05)	0.36 (0.10)
	V_a	0.038 (0.017)	0.061 (0.057)	2.216 (0.665)
	V_e	0.144 (0.016)	0.988 (0.078)	3.877 (0.542)
SNPCHIP				
	h^2	0.20 (0.07)	0.04 (0.04)	0.25 (0.07)
	V_a	0.036 (0.013)	0.044 (0.043)	1.505 (0.486)
	V_e	0.144 (0.014)	1.001 (0.072)	4.501 (0.455)
IMPUTED				
	h^2	0.21 (0.08)	0.04 (0.04)	0.28 (0.08)
	V_a	0.038 (0.015)	0.046 (0.047)	1.687 (0.548)
	V_e	0.141 (0.015)	1.00 (0.075)	4.297 (0.504)
ASE				
	h^2	0.14 (0.05)	0.03 (0.03)	0.16 (0.05)
	V_a	0.026 (0.010)	0.033 (0.031)	0.960 (0.316)
	V_e	0.154 (0.012)	1.013 (0.067)	5.012 (0.372)
RANDOM				
	h^2	0.02 (0.04)	0.02 (0.03)	0.15 (0.05)

	V_a	0.003 (0.007)	0.026 (0.030)	0.872 (0.304)
	V_e	0.177 (0.120)	1.021 (0.068)	5.106 (0.382)
ASE+PED				
	h^2	0.21 (0.08)	0.05 (0.05)	0.36 (0.10)
G_{ASE}	V_a	0.020 (0.011)	0.020 (0.041)	0.324 (0.321)
A	V_a	0.018 (0.017)	0.031 (0.073)	1.861 (0.710)
	V_e	0.143 (0.015)	0.996 (0.079)	3.877 (0.541)
ASE+SNPCHIP				
	h^2	0.20 (0.07)	0.04 (0.04)	0.24 (0.07)
G_{ASE}	V_a	0.022 (0.016)	0.025 (0.073)	0.970 (0.599)
$G_{SNPCHIP}$	V_a	0.015 (0.012)	0.016 (0.056)	0.463 (0.399)
	V_e	0.143 (0.014)	1.004 (0.073)	4.553 (0.456)

V_a : Additive genetic variance. V_e: Residual variance. h^2: Estimated heritability. Standard errors in brackets.

The accuracies were generally low (0.27-0.61; Table 2), which is in agreement with a study on fatty acid traits in cattle (N=1366), where the accuracy of genomic prediction was <0.40 for the majority of the fatty acids (Chen et al. 2015). The trait with the highest heritability (MFAT) had highest accuracy. Higher heritability may be expected to result in increased accuracy of genomic prediction (e.g., Sonesson and Meuwissen 2009). Moreover, several studies have shown that low heritability can contribute to low prediction accuracy (Nirea et al. 2012; Vela-Avitúa et al. 2015).

Inaccurate estimates of variance components may have reduced cross-validation prediction accuracies in Table 2. In fact, for EPA none of the heritability estimates were significantly different from zero, which may explain the low cross validation accuracies. In a quantitative genetic analysis using a bigger dataset (668 fish), the heritability estimate was higher (0.09 ± 0.06)(Horn et al. 2018), suggesting that a larger dataset is needed for EPA. EPA muscle content is highly variable over time because EPA serves many metabolic roles in the body, such as energy production and conversion to bioactive components (Glencross et al. 2014; Sanden et al. 2011). DHA content, on the other hand is more stable as it is mainly incorporated into phospholipids in cell membranes (Ruiz-Lopez et al. 2015). This is reflected in the higher heritability of DHA compared to EPA (Table 1).

Table 2. Estimated accuracies by cross-validation for EPA, DHA and muscle fat (MFAT) for six different genetic information sources.

Trait	PED	SNPCHIP	IMPUTED	ASE	ASE+PED	ASE+SNPCHIP
DHA	0.328 (0.018)	0.413 (0.019)	0.397 (0.019)	0.359 (0.02)	0.342 (0.019)	0.369 (0.019)
EPA	0.374 (0.046)	0.321 (0.042)	0.311 (0.042)	0.331 (0.039)	0.268 (0.041)	0.267 (0.04)

MFAT	0.603 (0.013)	0.565 (0.014)	0.576 (0.014)	0.564 (0.013)	0.606 (0.013)	0.566 (0.014)

Standard errors in brackets.

Based on the results of this study, it cannot be concluded which genetic information source is best because different genetic information performed best for different traits (Table 2). Differences between information sources were in general small, except for DHA, where SNPCHIP (and IMPUTED) performed considerably better than the other information sources. The use of SNPCHIP genotypes resulted in 15.5% (on average) higher selection accuracy than all other information sources for DHA (Tables 2 & 3). For EPA, PED gave significantly higher accuracy than the other sources, except for ASE, which was similar to PED (Tables 2 & 4). Thus, in this study GS did not result in higher accuracy than pedigree for a low heritability trait (EPA, 0.06). This is contrary to a previous study by Calus et al. (2008), and may be due to the low heritability of EPA combined with the small dataset.

For MFAT, PED and ASE+PED resulted in similar accuracies, and both were significantly higher than those of the other information sources (6.5% on average; Tables 2 & 5). The high heritability of MFAT may explain why PED performed best. Moreover, the performance of SNPCHIP is dependent on the presence of markers in genes influencing the trait. The NOFSAL02 SNP-chip does not contain markers in the genes of fatty acid synthase (*LOC106610271*), peroxisome proliferator-activated receptors *pparb2b* and *pparb2a,* carnitine palmitoyltransferases *cpt1b* and *cpt2*, hormone-sensitive lipase, and acetyl-CoA carboxylase (*LOC106603271*), which are all known to influence lipid metabolism in mammals and/or fish (Sul & Smith 2008; Tocher 2003; Varga et al. 2011).

Table 3. P-values from paired t-test indicating significant differences in accuracies between sources of genetic information for DHA.

DHA	PED	SNPCHIP	IMPUTED	ASE	ASE+PED	ASE+SNPCHIP
PED	-	**	**	ns	ns	**
SNPCHIP		-	**	**	**	**
IMPUTED			-	**	**	*
ASE				-	*	ns
ASE+PED					-	**
ASE+SNPCHIP						-

* = $p < 0.05$, ** = $p < 0.01$, ns= not significant ($p>0.05$)

Table 4. P-values from paired t-test indicating significant differences in accuracies between sources of genetic information for EPA.

EPA	PED	SNPCHIP	IMPUTED	ASE	ASE+PED	ASE+SNPCHIP
PED	-	**	**	ns	**	**
SNPCHIP		-	ns	ns	**	**
IMPUTED			-	ns	**	**
ASE				-	**	**
ASE+PED					-	ns
ASE+SNPCHIP						-

* = p < 0.05, ** = p < 0.01, ns= not significant (p>0.05)

Table 5. P-values from paired t-test indicating significant differences in accuracies between sources of genetic information for muscle fat (MFAT).

MFAT	PED	SNPCHIP	IMPUTED	ASE	ASE+PED	ASE+SNPCHIP
PED	-	**	**	**	ns	**
SNPCHIP		-	**	ns	**	ns
IMPUTED			-	ns	**	**
ASE				-	**	ns
ASE+PED					-	**
ASE+SNPCHIP						-

* = p < 0.05, ** = p < 0.01, ns= not significant (p>0.05)

The G-matrix based on ASE markers seemed able to capture a relatively high portion of genetic variation for DHA (although standard errors of the components were high), indicating that markers selected through the ASE method were relevant for this trait. This was further supported by testing the G_{RANDOM} matrix, which for DHA resulted in a very low estimate of genetic variance (Table 1). This confirms that the 395 markers selected based on ASE explain substantially more variance for DHA than 400 random markers. ASE alone did not give significantly lower selection accuracies than PED for DHA (Tables 2 & 3). The ASE markers did not capture a higher portion of genetic variance than RANDOM markers for EPA, probably because of the very low, non-significant heritability of EPA in this dataset. For MFAT, ASE was not better than 400 random markers, which can be explained by that the ASE markers were not selected for this trait.

When combining ASE with either PED or SNPCHIP, ASE surprisingly explained the greatest portion of the genetic variation (Table 1). However, ASE did not improve prediction accuracies when combined with SNP-chip or pedigree information (Table 2). ASE+SNPCHIP surprisingly resulted in lower accuracy than SNPCHIP alone (11% lower) for DHA. Moreover, ASE+PED did not give significantly higher accuracy than PED alone. This is possibly because in the combined models, there are twice as many effects to estimate ($\hat{u}_1$ and $\hat{u}_2$), but the size of the reference data is too small to accurately estimate that many effects. In

addition, the variance due to ASE may be overestimated due to sampling, which may explain why the accuracy is not improved.

The imputed genetic information did not perform well in this study. For EPA and MFAT there was no significant difference between IMPUTED and ASE. For DHA, IMPUTED gave significantly lower selection accuracy than SNPCHIP (Table 2). The reason why IMPUTED was not better may be explained by imputation errors, which were estimated to be 10 %. Imputation errors were likely to occur in this dataset where there were 5 million SNPs, only 563 genotyped fish, and 96 sequenced fish. Moreover, the step from a 57K SNP-chip to 5 million SNPs may have been too large. I.e. with 5 million SNPs, the imputation software identifies even in relatively small regions many haplotypes, but the 57K SNP panel has insufficient SNPs in these regions to identify the correct haplotype. In addition, the Atlantic salmon genome is rather complex, which further increases likelihood of imputation errors. Pérez-Enciso et al. (2015) found that there is a law of diminishing returns with increasing SNP density. The little that is to be gained from increasing marker density may easily be lost due to imputation errors. In addition, increasing the marker density of commercial chips has not increased accuracy of genomic predictions (VanRaden et al. 2013). As a result, use of whole-genome sequence data may not result in a highly increased selection response over high-density genotyping.

The cross-validation method is unfavorable for this dataset, because we are masking one individual from each family whilst we have small family sizes. The latter hampers the accuracy of GS. Yet, the choice of this validation scenario is realistic because for most traits in aquaculture (and especially for carcass quality traits), selection of breeding candidates is based on full- and half-sib phenotypic information. However, to obtain high accuracies of GS we want high numbers of validation animals per family (Odegård et al. 2014).

The accuracies of selection for DHA and EPA were moderate and offer possibilities for selection for these traits, especially if one extends the reference data set to a much bigger population. The latter requires the cost-effective recording of DHA and EPA in large reference populations, which is currently unavailable. When using the 395 ASE markers alone, the reference population does not need to be so large since there are not as many marker effects to estimate. In the current case we cannot recommend using ASE markers, but the results show that knowledge from gene expression analysis of a few individuals (59 in this case) can be utilized to select a small panel of markers that perform relatively well.

4. Conclusions

The results of this study show that different genetic information performed best for different traits. SNPCHIP performed best for DHA, and PED performed best for EPA and MFAT. The relatively poor performance of GS may be due to the small reference population size and the small number of animals per family.

Differences between information sources were in general small, except for DHA, where SNPCHIP (and IMPUTED) performed considerably better than the other information sources.

IMPUTED did not improve selection accuracy for any of the traits studied, probably due to imputation errors and the large step from a 57K SNP-chip to 5 million markers, combined with a small dataset.

Markers based on allele-specific expression were able to capture a lot of genetic variation for DHA, but did not give higher accuracies when combined with SNP-chip or pedigree in this dataset.

The cross-validation accuracies for selection for DHA and EPA were moderate and offer possibilities for selection for these traits, especially if one extends the reference data set to a much bigger population.

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Paper IV

GWAS identifies genetic variants associated with omega-3 fatty acid composition of Atlantic salmon fillets

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Abstract

The objective of this study was to identify genetic variants associated with long-chain omega-3 fatty acid content in Atlantic salmon muscle, in order to identify genes underlying the genetic variation in these traits, by performing a genome-wide association study.

Fatty acid composition, including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) content and the ratios DHA/DPA (docosepentaenoic acid) and DHA/ALA (α-linolenic acid) of skeletal muscle was determined in 642 Atlantic salmon individuals from the Salmobreed broodstock program. Further, a 57K single-nucleotide polymorphism (SNP) array was used to genotype the 642 individuals to search for SNPs associated with skeletal muscle content of individual omega-3 fatty acids as well as ratios between fatty acids, using a mixed model approach. We identified markers showing a significant association with the ratio of DHA to DPA located on chromosome 19, close to the candidate gene *elovl2*, which is directly involved in the conversion of DPA to DHA. The results further suggested that genetic variation affecting fillet EPA and DHA content is present on Atlantic salmon chromosome 21, as the GWAS analysis of EPA, DHA and DHA/ALA ratio all pointed to this chromosome. No known genes of direct relevance to the omega-3 bioconversion pathway were found here. We discuss the relevance of other interesting genes related to lipid metabolism and health located in this region.

1. Background

Atlantic salmon (*Salmo salar* L.) is an important farmed fish species, known for its high content of the health-promoting omega-3 long-chain polyunsaturated fatty acids (n-3 LC PUFA) eicosapentaenoic (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) in muscle. Levels of n-3 LC PUFA have decreased significantly the last few decades because of the substitution of a large portion of marine ingredients with more sustainable plant ingredients in the diet of farmed Atlantic salmon (FAO 2016; Sprague et al. 2016; Ytrestoyl et al. 2015). Quantitative genetic analyses have estimated genetic parameters of Atlantic salmon omega-3 fatty acids (FA) and showed the potential of selective breeding to increase n-3 LC PUFA levels in salmon fillets, as they are heritable traits (Horn et al. 2018; Leaver et al. 2011).

The omega-3 bioconversion pathway enables salmonids to convert the shorter-chained fatty acid alpha-linolenic acid (ALA; 18:3n-3), into EPA and DHA (Bou et al. 2017; Monroig et al. 2013; Tocher et al. 2003). Several of the genes of this pathway have been the focus of research in salmonids. One Δ5 fatty acid desaturase (fad) gene and three Δ6fad genes, in addition to three elongase (elovl) genes (elovl2, elovl4 and elovl5), have been cloned and functionally characterized in Atlantic salmon (Hastings et al. 2004; Kjaer et al. 2016; Monroig et al. 2010; Zheng et al. 2005). The fad and elovl enzymes of this pathway are known to influence n-3 LC PUFA content in liver (Berge et al. 2015; Bou et al. 2017; Castro et al. 2016). The genes of the omega-3 bioconversion pathway are expressed in skeletal muscle tissue of Atlantic salmon (Codabaccus et al. 2011; Horn et al. 2019). However, a recent gene expression study of Atlantic salmon indicated that the omega-3 bioconversion pathway is not an important factor in determining n-3 LC PUFA content in muscle (Horn et al. 2019). The study found that EPA was negatively associated with expression of genes involved in lipid catabolism and hypothesized that individual differences in EPA content is caused by selective oxidation of FAs.

Genome-wide association studies (GWAS) are a powerful tool that can identify Single Nucleotide Polymorphisms (SNPs) linked to genes associated with phenotypic traits. GWAS of muscle FA composition have been performed in cattle (Chen et al. 2015; Saatchi et al. 2013; Zhu et al. 2017) and pigs (Ramayo-Caldas et al. 2012), where several significantly associated regions and loci were identified. In cattle, FA composition is influenced by a few host genes with major effects and many genes of smaller effects (Chen et al. 2015). In fish, one study in Asian Seabass identified multiple quantitative trait loci (QTL) for omega-3 FA in muscle and different QTL were associated with different omega-3 FA (Xia et al. 2014), showing the complexity of the genetic architecture of FAs in fish. To increase the understanding of the genetics of muscle n-3 LC PUFA content in Atlantic salmon, GWAS is a relevant approach that has not yet been explored in Atlantic salmon.

The aim of the current study was to identify genetic variants associated with long-chain omega-3 fatty acid content in Atlantic salmon muscle, by performing a genome-wide association study.

2. Methods

2.1 Fish population and recordings

A total of 642 fish from 92 sires and 194 dams were included in the analysis. All sires had four or more offspring from more than one dam. The fish originated from one year-class of the Atlantic salmon breeding population of SalmoBreed AS. The fish were transferred to sea at a mean weight of 0.1 kg, and slaughtered approximately 12 months later, at a mean weight of 3.6 kg (ranging from 1.2 to 6.4 kg). The fish were fed a commercial broodstock feed from Skretting (https://www.skretting.com/en/products/atlantic-salmon/?lifephase=474980) with a high fish oil content, and were fasted 13-14 days prior to slaughter. All the fish were reared under the same conditions.

At slaughter, sex was determined visually by inspection of the gonads, body weight (g) was recorded, and skeletal muscle samples for lipid and FA analysis were taken from Norwegian Quality Cut were collected, frozen and stored at −20 °C.

2.2 Muscle fat and fatty acid analysis

Muscle fat content was measured by extracting total lipids from homogenized skeletal muscle samples of individual fish, according to the Folch method [36]. Using one milliliter from the chloroform-methanol phase, FA composition of total lipids was analyzed following the method described by Mason and Waller [37] The extract was dried briefly under nitrogen gas and residual lipid extract was trans-methylated overnight with 2′,2′-dimethoxypropane, methanolic-HCl, and benzene at room temperature. The methyl esters formed were separated in a gas chromatograph (Hewlett Packard 6890; HP, Wilmington, DE, USA) with a split injector, using an SGE BPX70 capillary column (length 60 m, internal diameter 0.25 mm, and film thickness 0.25 μm; SGE Analytical Science, Milton Keynes, UK) and a flame ionization detector. The results were analyzed using HP Chem Station software. The carrier gas was helium, and the injector and detector temperatures were both 270 °C. The oven temperature was raised from 50 to 170 °C at the rate of 4 °C / min, and then raised to 200 °C at a rate of 0.5 °C / min and finally to 240 °C at 10 °C / min. Individual FA methyl esters were identified by reference to well-characterized standards. The content of each FA was expressed as a percentage of the total amount of FAs in the analyzed sample.

In total, 12 traits were analyzed: six for muscle FA content and six for ratios of FA as indices of desaturase and elongase enzyme (omega-3 bioconversion) activity. This includes the following omega-3 FAs in the bioconversion pathway; 18:3n-3 (ALA), 20:3n-3, 20:4n-3,

20:5n-3 (EPA), 22:5n-3 (DPA) and 22:6n-3 (DHA). In addition, the following ratios between FAs in the omega-3 bioconversion pathway was run: 20:3n-3/18:3n-3, 20:5n-3/20:3n-3, 20:5n-3/18:3n-3, 22:5n-3/20:5n-3, 22:6n-3/22:5n-3 and 22:6n-3/18:3n-3.

2.3 Genotyping

The fish were genotyped using a customized 57k axiom Affymetrix SNP Genotyping Array (NOFSAL02).

From the initial 57k SNPs on the NOFSAL02 SNP chip, we retained those with call rate greater than 0.8, minor allele frequencies greater than 0.05, and Hardy-Weinberg equilibrium correlation p-value > 0.001. A total of 49 726 SNPs passed quality control filtering and were used in the GWAS.

2.4 Statistical analyses

The GWAS was carried out with the SNP and Variation Suite v8.x (SVS; Golden Helix, Inc., Bozeman, MT, www.goldenhelix.com).

A single-locus mixed linear model (EMMAX; Kang et al., 2010) was used with body weight and sex as covariates (Horn et al. 2018), and a genomic relationship matrix constructed in SVS:

$$y = \mu + X_1 b_1 + X_2 b_2 + S_i a_i + Zu + e$$

where y is a vector of FA trait records, μ is the overall mean, b_1 is a fixed regression coefficient of body weight as a covariable, b_2 is a (2x1) vector of fixed effects of sex (Horn et al. 2018), X_1 and X_2 are incidence matrices for the effects contained in b_1 and b_2, a_i is the regression coefficient of the genotype of SNP i; S_i is a vector of genotypes of SNP i (coded as 0, 1, or 2 for the homozygote, heterozygote, and alternative homozygote genotype, respectively); u is a vector of additive genetic effects distributed as $u \sim N(0, G\,\sigma^2_u)$, where σ^2_u is the additive genetic variance, G is the genomic relationship matrix; Z is the corresponding incidence matrix to additive genetic effects, and e is the vector of random residual effects with $e \sim N(0, I\,\sigma^2_e)$.

Genomic narrow-sense heritability was estimated using the equation, $h^2 = \sigma^2_g / (\sigma^2_g + \sigma^2_e)$, where σ^2_g is the genetic variance and σ^2_e is the environmental variance (Kang et al., 2010).

SNPs were considered genome-wide significant when they exceed the Bonferroni threshold for multiple testing (alpha = 0.05) of $0.05/tg$, where tg = 49 726 (total number of genome-

wide SNPs). The genome-wide significant threshold used in this study was $P \leq 1.0 \times 10^{-6}$, which is equivalent to $-\log10(P) > 6$.

Manhattan plots were created to visualize the association analyses.

Quantile–quantile (Q–Q) plots were used to analyze the extent to which the observed distribution of the test statistic followed the expected (null) distribution in order to assess potential systematic bias due to population structure or to the analytical approach.

3. Results and discussion

In this study, GWAS was performed on 642 slaughter sized Atlantic salmon displaying variation in EPA and DHA content. The traits that were analyzed included the following FAs in the omega-3 bioconversion pathway; 18:3n-3 (ALA), 20:3n-3, 20:4n-3, 20:5n-3 (EPA), 22:5n-3 (DPA) and 22:6n-3 (DHA), in addition to the following ratios between FAs: 20:3n-3/18:3n-3, 20:5n-3/20:3n-3, 20:5n-3/18:3n-3, 22:5n-3/20:5n-3, 22:6n-3/22:5n-3 and 22:6n-3/18:3n-3. Most traits did not show genome-wide significant p-values. The exceptions were DHA/ALA ratio and DHA/DPA ratio. Therefore, the focus of this paper is on these two traits, in addition to the major n-3 LC PUFAs: DHA and EPA. Table 1 shows descriptive statistics for these traits. The mean content of EPA and DHA was 5.42 and 6.75 % of total muscle FA, respectively. EPA content displayed much greater variation and lower heritability compared to DHA. The genomic heritability of EPA and DHA estimated here was lower than the pedigree-based heritability estimates from Horn et al. (2018). The ratio-traits had slightly higher genomic heritability-estimates than DHA, with 0.21 for DHA/DPA and 0.25 for DHA/ALA (Table 1).

Table 1. Descriptive statistics for fatty acid traits.

TRAIT	Mean	SD	Min	Max	CV	h^2
Fatty acids*						
EPA 20:5n-3	5.42	1.02	2.57	9.45	18.72	0.03
DHA 22:6n-3	6.75	0.51	5.01	9.10	7.57	0.20
Ratios						
Ratio DHA/ALA	1.97	0.25	1.27	3.10	12.54	0.25
Ratio DHA/DPA	2.88	0.30	1.94	4.25	10.24	0.21

*: percentage of total FA in muscle. N: number of observations; SD: standard deviation; Min: minimum value; Max: maximum value; CV: coefficient of variation (SD/mean * 100); h^2: genomic heritability.

GWAS resulted in few significant peaks in general. However, considering that the Bonferroni test (0.05/numbers of SNPs) criterion is extremely strict (Yang et al., 2011; Wang et al., 2012), the top ten SNPs are presented in Table 2 regardless of p-value. Quantile-quantile plots presenting the distribution of observed vs expected p-values are presented in Fig 1. These showed no indication of generally inflated p-values of the GWAS results.

The GWAS results for the ratio of DHA to DPA showed a clear signal with a genome-wide significant peak on chromosome 19 (Fig 2). DHA/DPA ratio can be an indicator of conversion of DPA to DHA. There are several processes involved in this conversion. Firstly, 22:5n-3 is elongated to 24:5n-3 by elolv2 or elovl4 (Morais et al. 2009), then Δ6fad forms 24:6n-3, and lastly peroxisomal β-oxidation forms DHA (Sprecher et al. 1995). The strongest association for DHA/DPA ratio mapped to AX-87328720 on chromosome 19 (p-value: $4.28 \times e^{-8}$) (Table 2). Six of the top 10 SNPs were located in the region ~70-74,000 K. One of the genes in this interval is *fatty acid elongase 2* (*elovl2)* (located at ~73,300 K), which is directly involved in the conversion of DPA to DHA by elongating DPA to 24:5n-3, and is therefore a strong candidate to explain this association.

The third most significant SNP for DHA/DPA ratio (although it did not surpass the Bonferroni significance threshold), AX-87250961, was located on chromosome 19 at ~ 57,323 K, and had a p-value of 2.06e-6 (Table 2). Located less than 600 K base pairs (bp) from this SNP is *long-chain acyl-CoA synthetase family member 5 (acsl5*). The different long chain acyl-CoA synthases are important in channelling FAs to either β-oxidation or storage (Digel et al. 2009), and may in this way affect the DHA/DPA ratio. A recent study with primary human muscle cells concluded that *acsl5* in human skeletal muscle functions to increase mitochondrial β-oxidation (Kwak et al. 2019).

Table 2. Top 10 significant markers for the traits DHA, EPA, ratio DHA/ALA and ratio DHA/DPA.

SNP ID	Chromosome	Position	P-Value	Regression β	β SE	FDR	MAF
DHA							
AX-87919346	ssa21	49151954	1.15E-06	-0.131	0.027	0.057	0.459
AX-87124183	Unknown	0	3.09E-05	0.116	0.028	0.768	0.357
AX-87389789	ssa21	50479456	3.21E-05	-0.112	0.027	0.532	0.354
AX-98320019	ssa21	30018523	3.23E-05	0.198	0.047	0.402	0.087

AX-98318665	ssa21	14757981	4.05E-05	0.199	0.048	0.403	0.081
AX-87378441	ssa21	22867458	4.10E-05	0.142	0.034	0.340	0.183
AX-86905307	ssa21	22867671	4.10E-05	0.142	0.034	0.291	0.183
AX-88216662	ssa21	22948217	4.10E-05	0.142	0.034	0.255	0.183
AX-87919493	ssa21	22911827	4.62E-05	0.141	0.034	0.255	0.184
AX-87357378	ssa21	23255715	4.72E-05	0.209	0.051	0.235	0.075
EPA							
AX-87786769	ssa21	19598596	7.10E-06	0.252	0.056	0.353	0.384
AX-87661754	ssa21	19597227	1.36E-05	0.243	0.055	0.337	0.386
AX-87382389	ssa18	6097605	3.82E-05	0.258	0.062	0.632	0.298
AX-96229572	ssa04	52366314	4.36E-05	0.247	0.060	0.542	0.347
AX-87191103	ssa13	9497671	6.27E-05	0.323	0.080	0.624	0.136
AX-87910325	ssa07	31838925	6.64E-05	0.313	0.078	0.550	0.142
AX-96289868	ssa05	13946696	6.98E-05	0.225	0.056	0.496	0.465
AX-96203291	ssa05	13952012	7.21E-05	0.225	0.056	0.448	0.466
AX-96169902	ssa05	14065480	8.43E-05	-0.225	0.057	0.466	0.351
AX-97890796	ssa05	14064912	8.58E-05	-0.224	0.057	0.426	0.349
Ratio DHA/ALA							
AX-88119767	ssa21	26322077	8.00E-07	0.095	0.019	0.040	0.113
AX-87378441	ssa21	22867458	9.79E-07	0.080	0.016	0.024	0.183
AX-86905307	ssa21	22867671	9.79E-07	0.080	0.016	0.016	0.183
AX-88216662	ssa21	22948217	9.79E-07	0.080	0.016	0.012	0.183
AX-87919493	ssa21	22911827	1.11E-06	0.080	0.016	0.011	0.184
AX-87357378	ssa21	23255715	3.13E-06	0.114	0.024	0.026	0.075
AX-87919346	ssa21	49151954	5.07E-06	-0.058	0.013	0.036	0.459
AX-87224882	ssa21	23643657	7.29E-06	0.087	0.019	0.045	0.116
AX-87996038	ssa21	50488762	1.16E-05	0.062	0.014	0.064	0.271
AX-97891041	ssa21	24382679	1.57E-05	0.076	0.017	0.078	0.138
Ratio DHA/DPA							
AX-87328720	ssa19	73821250	4.28E-08	0.087	0.016	0.002	0.307
AX-88283832	ssa19	73714400	1.39E-06	0.073	0.015	0.035	0.391
AX-87250961	ssa19	57323866	2.06E-06	-0.070	0.015	0.034	0.489
AX-88124297	ssa19	73347846	2.25E-06	-0.075	0.016	0.028	0.416
AX-88213817	ssa19	70394436	2.79E-06	0.069	0.015	0.028	0.431
AX-88114105	ssa19	67322609	3.85E-06	-0.080	0.017	0.032	0.262
AX-96481712	ssa19	52010916	5.36E-06	0.073	0.016	0.038	0.339
AX-88299830	ssa19	66731046	6.41E-06	0.068	0.015	0.040	0.484
AX-87910497	ssa19	69919222	6.93E-06	0.074	0.016	0.038	0.309
AX-96150449	ssa19	73734851	7.23E-06	0.068	0.015	0.036	0.495

194 Regression β: Indicates the direction of the effect. β SE: The standard error corresponding to the β
195 value. FDR: False discovery rate. MAF: Minor allele frequency.

For DHA, the GWAS resulted in a peak on chromosome 21 with one SNP close to surpassing the Bonferroni-corrected genome-wide significance threshold (Fig 3, Table 2). The second most significant marker had unknown location. Four of the top 10 SNPs were located in a similar region on chromosome 21, less than 400 K bp apart, with p-values ranging from $4.1e^{-5}$ to $4.7e^{-5}$ (Table 2). For EPA, no SNPs surpassed the significance threshold and the pattern looked more noisy compared to DHA, but the two most significant SNPs were located on the same chromosome as the DHA peak (Fig 3), although in a different region (Table 2). EPA had a very low genomic heritability (0.03; Table 1), considerably lower compared to the pedigree-based heritability (0.09; Horn, 2018).

The GWAS results for DHA to ALA ratio further strengthened the significance of chromosome 21 in relation to n-3 LC PUFA content in Atlantic salmon muscle. The analysis of this trait resulted in a clear signal on chromosome 21, with four SNPs surpassing significance threshold (Fig 3, Table 2). These SNPs were located at ~22,900 – 26,300 K. Six of the top 10 markers for DHA/ALA ratio were also among the top 10 markers for DHA.

To identify candidate genes, we searched annotated genes with functional relevance to FA or lipid metabolism. No known genes of direct relevance to omega-3 FA metabolism were found in the region of interest mentioned above. However, a few potential candidate genes linked to EPA and DHA in a less direct way were annotated to this region. Three of these genes are related to T-cell signaling; *T-cell surface glycoprotein CD3 zeta chain-like (LOC106581970), Toll-like receptor 7 (LOC106581875)* and *Toll-like receptor 8 (LOC106581917)*. Toll like receptors (TLR) are a family of transmembrane proteins that plays an essential role in the innate immune system through controlling inflammatory and immunological responses (Moresco et al. 2011; Rogero & Calder 2018). TLRs controls T-cell responses both directly and indirectly (Rahman et al. 2009). N-3 LC PUFAs, such as EPA and DHA are shown to exert anti-inflammatory actions through the TLR signaling pathways, and modulation of T-cell activation (Denys et al. 2005; Lee et al. 2003; Rogero & Calder 2018). In Atlantic salmon, it has been shown that EPA and DHA affect TLR activation (Arnemo et al. 2017). Although the effect of EPA and DHA on expression of these genes have been documented, it is unknown whether the genes can influence EPA and DHA content in muscle.

A few other genes that could be related to EPA and DHA were also found in the region of the most significant SNPs on chromosome 21; such as *renin receptor (renr)*, which was located close to the top significant SNP for ratio DHA/ALA (~150 K bp apart). Renin receptor has in

mammalian studies been shown to be involved in the renin–angiotensin system, which regulates blood pressure (Fyhrquist & Saijonmaa 2008). It is documented that EPA and DHA reduce blood pressure in mammals (Guo et al. 2019; Miller et al. 2014), and it has been suggested that this effect is via the renin-angiotensin system (Francois & Coffman 2004; Jayasooriya et al. 2008). However, it is not known if EPA and DHA have a cardiovascular effect through renin–angiotensin system in Atlantic salmon. Another role of *renin receptor* is that it may reduce excessive autophagy in skeletal muscle during starvation (Mizuguchi et al. 2018). Because the fish in this study were starved for 14 days prior to slaughter, it can be hypothesized that this role of renin receptor may influence muscle FA composition in the fish of this trial.

The positional candidate genes discussed above are only indirectly linked to EPA and DHA, and have no documented direct effect on FA metabolism or content. Therefore, they are not considered strong candidate genes. There may be several reasons why we do not find any strong candidate genes for the n-3 LC PUFA traits on chromosome 21:

- The SNP-chip does not contain markers in several of the genes known to influence lipid metabolism in mammals and/or fish, such as fatty acid synthase, peroxisome proliferator-activated receptors *pparb2b* and *pparb2a,* carnitine palmitoyltransferases *cpt1b* and *cpt2*, hormone-sensitive lipase, and acetyl-CoA carboxylase. Including SNPs in these genes could potentially identify QTLs not in linkage disequilibrium with the SNPs in this study.
- It is also possible that the GWAS could not directly identify the causal mutations in our study since causal mutations are unlikely to be included in the 57K SNP-chip.
- The region of the most significant SNPs on chromosome 21 contained several uncharacterized genes. Some of these might prove to be involved in omega-3 FA metabolism in the future. Additional studies including fine mapping and functional validation are needed to identify the causal variants for further elucidation of the variants underlying the FA composition traits
- The identified regions may be regulatory hotspots. Genetic variation does not lead directly to phenotype, but instead alters intermediate molecular pathways that in turn affects the traits. Transcriptional regulatory sequences are as important for gene function as the coding sequences that determine the linear array of amino acids in a protein (Wray et al. 2003).

- The power of GWAS to detect genetic variation affecting Atlantic salmon FA traits is influenced by the number of samples, sampling methods and the procedure chosen for phenotypic recording. In this study, phenotypic recordings for FA traits were available for only 642 Atlantic salmon. However, they were all reared and slaughtered under the same conditions, fed the same feed and slaughtered at the same age, reducing the influences of environmental factors to a minimum. FA composition was determined by gas chromatography, which is highly accurate. The latter may explain why we found some genome-wide significant results, despite the relatively small number of records.

Based on the results of this study, we cannot pinpoint which genes are determining EPA and DHA content in skeletal muscle of Atlantic salmon. DHA/ALA ratio can act as an indicator of the rate of omega-3 bioconversion activity, thus, the genes of this pathway should have been identified in this analysis if this pathway really was important for determining EPA and DHA content in muscle of Atlantic salmon. For DHA/DPA ratio, we found candidate genes directly involved in the conversion, but for DHA/ALA ratio, we did not find a signal in or near genes known to be involved in the omega-3 bioconversion of ALA to DHA. Because we do not find any peaks near the genes related to this pathway, this study suggests that the genes of the omega-3 bioconversion pathway are not clearly associated with EPA and DHA content in Atlantic salmon muscle. This is in line with the results from a gene expression study of the same fish material, where the genes in the omega-3 bioconversion pathway did not appear to play a major role for EPA and DHA content in muscle, although they are important for omega-3 bioconversion in liver (Bou et al. 2017). This may be explained by that n-3 LC PUFA content in liver and muscle are two different traits with a very low correlation, as was shown in a recent paper (Horn et al. 2019).

4. Conclusions

Our results suggest that skeletal muscle n-3 LC PUFA content traits have a polygenic architecture with potentially a few QTL explaining moderate levels of the genetic variation in Atlantic salmon.

Markers showing a significant association with the DHA/DPA ratio were located on chromosome 19, close to the candidate gene *elovl2*, which is directly involved in the conversion of DPA to DHA.

The results suggest that genetic variation affecting fillet EPA and DHA content is present on Atlantic salmon chromosome 21.

A larger dataset is needed to validate these QTLs. Including SNPs in genes known to be involved in fatty acid metabolism could potentially identify additional QTLs not in linkage disequilibrium with the SNPs in this study.

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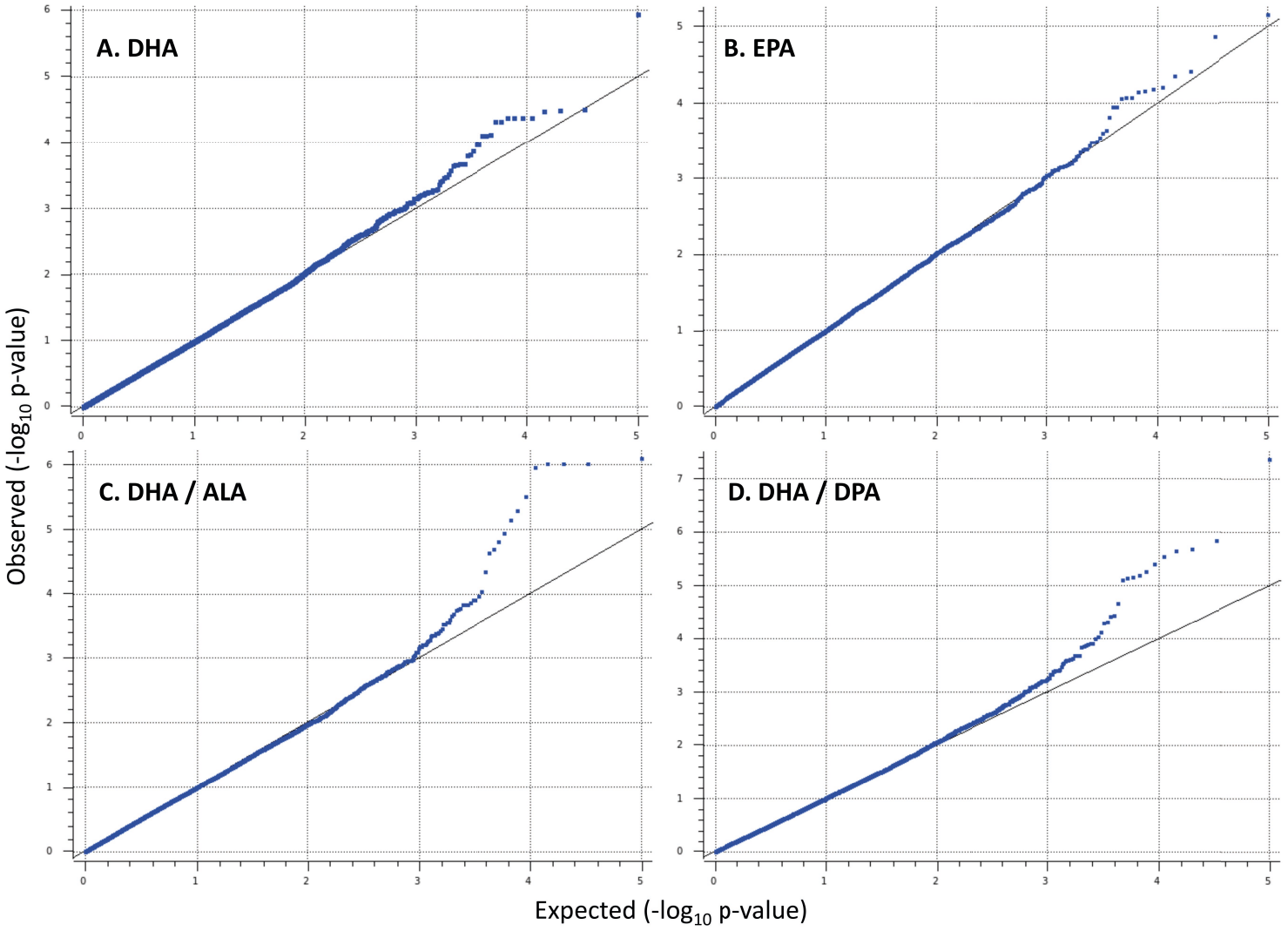
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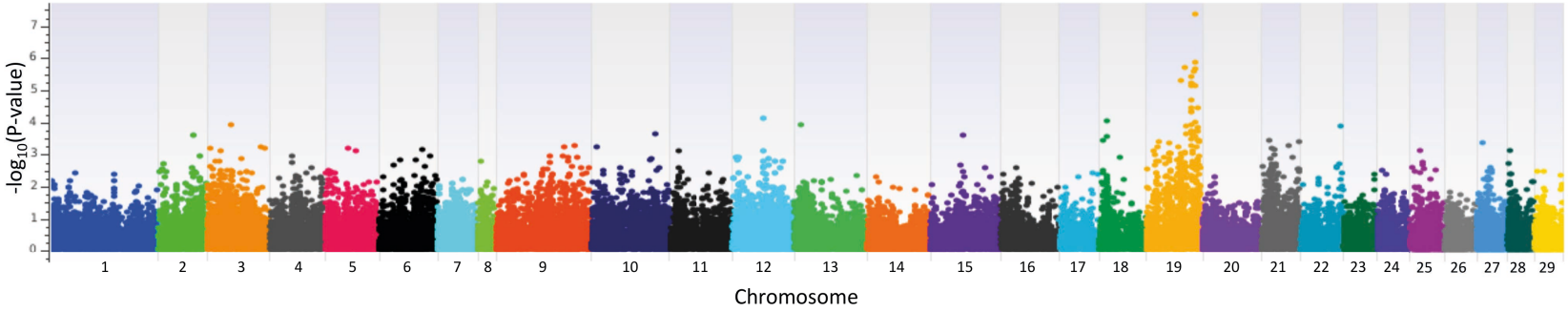
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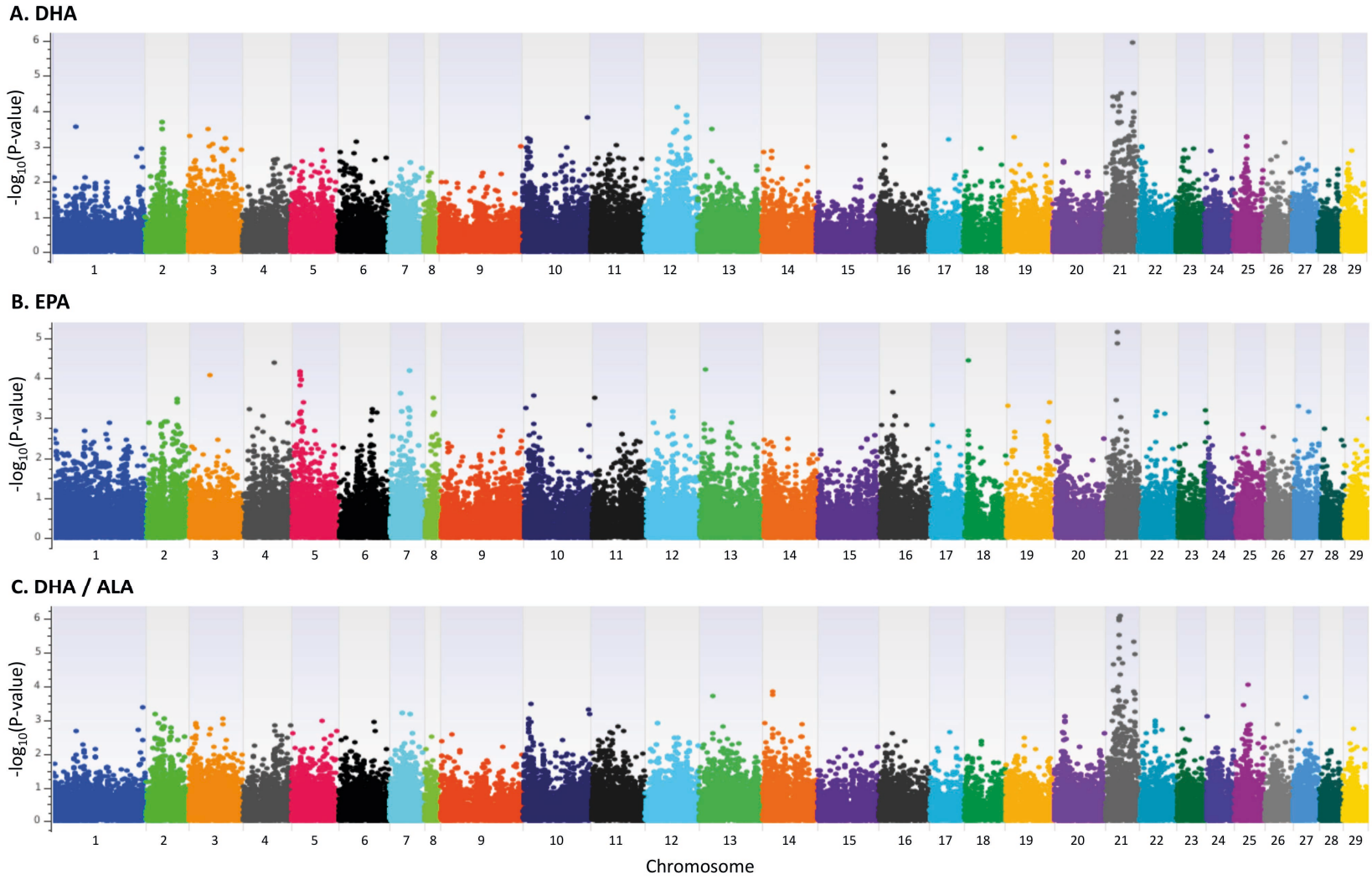
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