Norwegian University of Life Sciences
Faculty of Biosciences

Philosophiae Doctor (PhD)
Thesis 2019:40

Towards a genome wide understanding of salmon lipid metabolism gene regulation across tissues and life stages

Regulering av gener involvert i lipidmetabolisme i ulike vev og livsstadier i laks

Thomas Nelson Harvey

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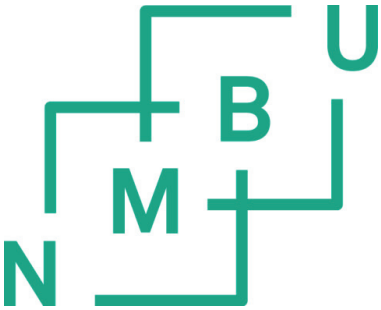
Ås (2019)



Thesis number 2019:40
ISSN 1894-6402
ISBN 978-82-575-1599-7

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Acknowledgements

The work presented in this thesis has been carried out at the Department of Biosciences (BIOVIT) at the Norwegian University of Life Sciences (NMBU). It was funded by NMBU and the research council of Norway (project 244164; Integrating genomics and system biology to improve the capacity for synthesis, transport, and filet deposition of EPA/DHA in salmon).

Moving to Norway to pursue a PhD certainly has been an adventure; one I could not have completed successfully without the ongoing support of colleagues, friends, and family.

First, I would like to thank my main supervisor, Simen Rød Sandve, for being the best supervisor a PhD student could ask for. Thanks for always being available to discuss results, work through figures, or just kick back and get a beer. Another big thanks to my co-supervisors Jacob Torgersen, Jon Olav Vik, and Torgeir Hvidsten. I don't think I would have managed without your incredible support, interest, and enthusiasm.

Thanks to all my colleagues and friends at NMBU. Especially Gareth Gillard for being patient while explaining the complexities of RNAseq normalization over and over and Yang Jin for being the best fish dissection specialist I've seen. Thanks to Matilde Holen and Line Røsæg for always making our shared office life exciting. Thanks to Thales Costa and the ones that got away, Róbert Hafþórsson and Kiira Vuoristo, for making life outside of work interesting in oh so many ways. Thanks to everyone else at CIGENE and NMBU that I don't have space to mention by name – you all make this one of the best places I've had to privilege to work and live.

I would like to give a huge thanks to my family and friends. Thanks to my parents Dave and Vicky for allowing me to move to Norway without putting up too much of a fight, my brother Dan for providing much needed distraction during his visits, and all my friends back home for still talking to me even though I've been absent for the past three years – you know who you are. Lastly, thanks to my girlfriend, Line Hansen (and our pets – Dali, Suzie, and Nougat), for maintaining my mental health and putting up with me during all the ups and downs of my PhD adventures. I couldn't have done it without you.

Ås, March 2019

Thomas Harvey

List of papers

Paper 1

Thomas N. Harvey, Simen R. Sandve, Yang Jin, Jon Olav Vik, Jacob S. Torgersen (2019) **Liver slice culture as a model for lipid metabolism in fish.** Manuscript.

Paper 2

Gareth Gillard*, **Thomas N. Harvey***, Arne Gjuvsland, Yang Jin, Magny Thomassen, Sigbjørn Lien, Michael Leaver, Jacob S. Torgersen, Torgeir R. Hvidsten, Jon Olav Vik, Simen R. Sandve (2018) **Life-stage associated remodeling of lipid metabolism regulation in Atlantic salmon. Molecular Ecology.** 27 (5): 1200-1213. DOI: 10.1111/mec.14533

* Co-first authors

Paper 3

Thomas N. Harvey, Jon Olav Vik, Torgeir R. Hvidsten, Simen R. Sandve (2019) **Mapping the transcriptomic landscape of Atlantic salmon during smoltification.** Manuscript.

Summary

Atlantic salmon is an important source of essential omega-3 fatty acids in human diets; however, omega-3 content in farmed salmon has been decreasing over the past decade due to the replacement of fish oil in aquafeeds with more sustainable and less expensive plant oil. To alleviate this, knowledge based breeding and feeding strategies need to be employed to produce nutritious salmon high in omega-3 in a sustainable and commercially viable way. These strategies require a genome wide understanding of lipid metabolism regulation. The work presented here brings us one-step closer to this goal through the development of an advanced cell culture systems (paper I), functional annotation of lipid metabolism genes (paper II), and interrogation of lipid metabolism regulation across life stages and tissues using transcriptomics in combination with two salmon trials (papers II and III).

In paper I, we aim to establish precision cut liver slice culture (PCLS) as a platform for studying lipid metabolism in Atlantic salmon. To accomplish this, we carried out three main experiments: (i) a time course to identify culturing associated effects, (ii) a fatty acid and insulin supplementation experiment to test the liver slice response, and (iii) a primary cell culture experiment to benchmark PCLS culture against traditional cell culture and whole liver. Overall, liver slices acclimatize to the cell culture environment after three days, they actively respond to insulin and fatty acids in the media in a liver-like way, and liver slices are closer to liver *in vivo* than primary cell culture in terms of expression of lipid metabolic pathway genes and liver marker genes.

In papers II and III, we aim to characterize changes in lipid metabolism-related gene expression across tissues and life stages in Atlantic salmon. Paper II involves the functional annotation of lipid metabolism genes and identification of genome wide trends in lipid metabolism regulation in liver and gut across diets and life stages. Metabolic plasticity in liver decreases after transition to seawater and lipid metabolism-related gene expression shifts from 'liver centric' biosynthesis in freshwater to 'gut centric' digestion and absorption in seawater. Paper III focuses more on how lipid metabolism changes during smoltification and subsequent seawater transfer. Lipid metabolism related gene expression generally decreases in smolts before transition to seawater. Additionally, shortened photoperiod likely does not influence smoltification-associated changes in lipid metabolism gene expression.

Sammendrag

Atlantisk laks er en viktig kilde til essensielle omega-3-fettsyrer for mennesker, men omega-3-innholdet i oppdrettslaks har gradvis sunket de siste 10 årene. Denne nedgangen i sunne omega-3-fettsyrer skyldes at fiskeolje i fôret har blitt erstattet med mer bærekraftige planteoljer. To mulige mottiltak er å avle frem laks som er bedre til å lagre eller lage omega-3 fettsyrer, eller finne nye fôrsammensetninger som sikrer høyt innhold av omega-3. Dette krever dybdeforståelse av hvordan laksen regulerer lipidmetabolismen, som er temaet for dette doktorgradsarbeidet. Avhandlingen beskriver et nyskapende system for dyrking av tynne skiver av vev (artikkel I), funksjonell annotering av gener involvert i lipidmetabolismen (artikkel II), og et dypdykk i genregulering av lipidmetabolismegener på tvers av livsstadier og vev (artikkel II og III).

Artikkel I etablerer leversnittkultur som laboratoriemetode for å interaktivt studere lipidmetabolisme i laks. Arbidet inkluderer (i) et molekylært tidsserie-studium av i hvilken grad leversnittet bevarer sin karakter over tid, (ii) *in vitro* tilførsel av fettsyrer og insulin for å gjenskape aspekter ved normal leverfunksjon, og (iii) sammenlignende analyse av leversnitt og primærcellekultur. Resultatene viser at leversnitt stabiliserte seg i *in vitro*-miljøet etter 3 dager, responderte realistisk på endringer i insulin og fettsyretilførsel, og oppfører seg mer likt intakt lever enn primærcellekultur gjør, betraktet gjennom genuttrykk i lipidmetabolske reaksjonsveier.

Artikkel II og III karakteriserer endringer i genuttrykk for lipidmetabolisme over ulike livsstadier og vev i laks. Artikkel II annoterer funksjonen til lipidgener og beskriver genregulering i lever og tarm hos laks på plante- vs fiskeoljediett i ferskvann, under smoltifisering (den fysiologiske omstillingen til saltvann) og i sjø. Effekten av diettforskjeller på stoffskiftet i lever ble svakere etter overgang til sjøvann, mens transkripsjon av gener for lipidopptak i tarm øker i sjøvann relativt til ferskvann. Artikkel III fokuserer på hvordan lipidmetabolisme-genuttrykk endres gjennom smoltifiseringsprosessen. Her fant vi at det relative uttrykket til gener involvert i lipidmetabolisme gradvis synker i smolt før de overføres til sjøvann. Simulert årstidsvariasjon i daglengde påvirket ikke genuttrykket i lever.

1 Introduction

The current human population is approximately 7.7 billion and by 2050 it is projected to approach 10 billion [1]. Meeting the nutritional demands of so many people in a sustainable way will be one of the great challenges of our generation and will require the concerted effort of the global community. In line with this, Norwegian aquaculture production is projected to expand in the coming decades, from 1.3 million tonnes in 2016 to 5 million tonnes in 2050 [2]. This is necessary, since Atlantic salmon is an important source of the essential omega-3 long chain polyunsaturated fatty acids (LC-PUFA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). However, traditional salmon aquaculture relies heavily on capture fisheries for fish oil high in LC-PUFA and demand has already far surpassed stagnating supply. This has led to the substitution of fish oil for terrestrial ingredients like vegetable and seed oils. While plant oils are cheaper and more sustainable than fish oil, they are also devoid of LC-PUFA, translating to diminished EPA and DHA content of commercial salmon filets and reduced health benefits to the human consumer [3].

There is hope, however. Plant oils typically contain high levels of the shorter chain PUFA alpha-linolenic acid (ALA) and Atlantic salmon possess all of the enzymes to biosynthesize EPA and DHA from ALA. A decade of research on the effects of plant oil in salmon feed has revealed that salmon increase endogenous LC-PUFA production in response to dietary availability, but not enough to maintain LC-PUFA at levels resembling salmon fed fish oil [4–6]. To solve this problem there are two options: 1) design next-generation feeds to fit the fish, or 2) design next-generation fish to fit the feed. The latter approach involves selection for genetic variation in broodstock associated with increased total fat or proportion LC-PUFA, or if GMO legislation changes in the future, use gene editing to produce high LC-PUFA fish.

On our way to implementation of both ‘designer feeds’ and ‘designer fish’ approaches, a detailed understanding of Atlantic salmon lipid metabolism is important. This thesis takes an important step in this direction through the development of an advanced cell culture system that maintains the three-dimensional liver structure, an improved annotation of genes involved in lipid metabolism, and the use of transcriptomics to analyze gene expression regulation under different dietary levels of LC-PUFA and life stages.

Before venturing into details of the main results from my PhD project, I will provide an overview of important aspects regarding lipid biochemistry, lipid metabolism, and approaches to study this, both *in vitro* and *in vivo*.

1.1 The Lipids

Lipids, amino acids, carbohydrates, and nucleic acids are the four biomolecular roots anchoring the tree of life. They cooperate in vast interconnected structural and functional networks to form every species of plant, animal, fungus, bacteria, and archaea – all life as we know it. In this thesis, I will focus on the lipid domain, but will touch on other aspects including amino acid and carbohydrate metabolism.

Lipids are formally defined as any biomolecule that is soluble in a nonpolar solvent [7]. This includes over 43,000 distinct species belonging to eight categories including fatty acids (FA), sterols, glycerolipids, and glycerophospholipids to name a few [8]. Structurally, lipids in the form of glycerophospholipids and sterols form the cell membrane separating the exterior and interior of the cell and provide intracellular membrane surfaces for many important biochemical reactions. Functionally, lipids in the form of fatty acids and glycerolipids act to store energy in times of plenty and release it in times of scarcity. Lipid metabolism as a whole is regulated by metabolites and hormones in response to environmental, physiological, dietary, and developmental factors.

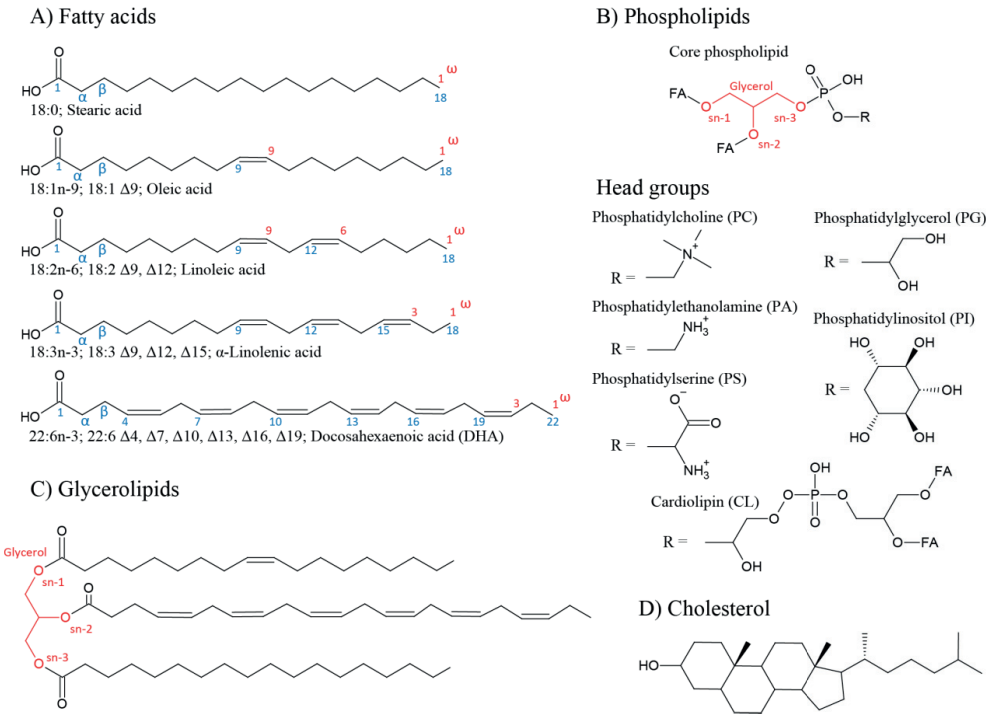

Figure 1: Lipid structure and nomenclature. A) Structure of stearic acid, oleic acid, linoleic acid, α-linolenic acid, and docosahexaenoic acid. Important carbons are numbered starting from the carboxyl end (blue) and the methyl end (red). B) Structure of the core phospholipid and six head groups. The glycerol backbone and substitution number (sn) positions are shown in red. FA stands for fatty acid. C) Structure of a typical glycerolipid. The glycerol backbone and SN positions are shown in red. In this glycerolipid, the SN-1 position is occupied by oleic acid, the SN-2 position by DHA, and the SN-3 position by stearic acid. D) Structure of cholesterol.

1.1.1 Fatty acids

All fatty acids consist of a carbon chain with a carboxyl group at one end. They are key components of glycerolipids and are named according to the length and number of double bonds in their carbon chain. A fatty acid with 18 carbons and no double bonds is denoted as 18:0, while a fatty acid with 18 carbons and 3 double bonds is 18:3 (figure 1a). Furthermore, the first carbon from the hydroxyl end is considered the alpha carbon, the second the beta carbon, and the last the omega carbon. The distance of the first double bond from the omega carbon is denoted as n-#, so if a double bond of 18:3 is three carbons from the omega end, then it is denoted as 18:3n-3 (figure 1a).

This nomenclature is of critical importance because the distance of the first double bond from the omega end is a key difference between categories of polyunsaturated fatty acid (PUFA). Those with the first double bond three or six carbons from the omega end are broadly referred to as “omega-3s” or “omega-6s”, respectively. Many popular dietary trends consider omega-6s to be bad fats and omega-3s to be good fats. Reality is more complicated, as both are essential dietary nutrients with very distinct, sometimes opposing, biological roles. For example, arachidonic acid (ARA, 20:4n-6) and its omega-3 counterpart eicosapentaenoic acid (EPA, 20:5n-3) are precursors to the important short range signaling molecules called eicosanoids. ARA derived eicosanoids are generally considered to be pro-inflammatory, playing an important role in immune response, while those derived from EPA are considered neutral or even anti-inflammatory, counteracting the effects of n-6 eicosanoids [9]. The primary health issue lies in the old cliché “you are what you eat”, and that modern western diets are primarily composed of terrestrial ingredients high in omega-6s, so many people do not consume sufficient amounts of omega-3s.

1.1.2 Sterols

Sterols contain four interconnected carbon rings with a hydroxyl group on one end (figure 1d). The most common sterol in animal cells is cholesterol, which is an essential component of cell membranes, and plays a number of other roles as a signaling molecule and precursor to other bioactive compounds such as steroids, vitamin D, and bile acids [10]. The polar hydroxyl group of cholesterol interacts with water and the head group of membrane phospholipids, while the non-polar body interacts with the phospholipid tails, so cholesterol is imbedded in cell membranes with the hydroxyl pointing outward. Cholesterol can be obtained from the diet or synthesized *de novo*, which will be discussed later. Transport of cholesterol around the body requires the hydroxyl group to be esterified with a fatty acid, forming non-polar cholesteryl ester, which migrates to the inner hydrophobic region of circulating lipoproteins.

1.1.3 Glycerolipids

Glycerolipids are composed a glycerol molecule with one (monoacylglycerol, MAG), two (diacylglycerol, DAG), or three (triacylglycerol, TAG) fatty acids attached with an ester

bond. The three positions on the glycerol molecule are referred to as substitution number (sn) 1, 2, or 3 (figure 1c). Any fatty acid can occupy any of these sn positions, but in animals the sn-1 and sn-3 position are typically occupied by polyunsaturated fatty acid (PUFA), while the sn-2 position usually contains a saturated fatty acid (SFA) or monounsaturated fatty acid (MUFA). In plants it is typically the opposite, with PUFA or MUFA occupying the sn-2 positions and SFA occupying the sn-1 and sn-3 positions [11]. TAGs act as an energy reserve, actively being synthesized and stored in adipose tissue when resources are abundant and broken down when resources are scarce.

1.1.4 Phospholipids

Phospholipids are structurally similar to glycerolipids, but with the sn-3 position of glycerol occupied by a phosphate containing head group (figure 1b). They are amphiphilic by nature with a polar head group and two non-polar fatty acid tails. The specific proportion of SFA, MUFA, and PUFA in the phospholipids is what gives cell membranes many of their structural characteristics. For example, a membrane with a high proportion of SFA to PUFA will have low fluidity and be more sensitive to low temperatures than a membrane with high levels of PUFA. Additionally, PUFAs in the sn-2 position of membrane phospholipids are the source for biologically active eicosanoids and docosanoids. The PL head group determines the PL type and many of the functional properties. The simplest form is phosphatic acid, which consists of a hydroxyl group attached to a phosphate group. More complex forms include phosphatidylcholine, phosphatidylenthanolamine, phosphatidylserine, phosphatidylglcerol, phosphatidylinositol, and cardiolipin [12]. See figure 1 for their molecular structures.

1.1.5 Lipids in aquaculture

Salmon aquafeeds have two main components, protein and oil. Traditionally, both of these came from fishmeal and fish oil from the capture fishery industry and was relatively cheap and abundant. However, around the turn of the century aquaculture production was continuing to grow, while fisheries production stagnated due to overfishing that triggered governmental regulations imposing catch limits. In turn, demand for fishmeal and fish oil continued to increase while supply remained the same, leading to skyrocketing prices [13]. For this reason, many aquaculture producers have substituted fishmeal and fish oil

with plant based ingredients like soybean oil, linseed oil, and rapeseed oil that are cheaper and more sustainable.

Plant based ingredients have a very different composition than fish based ingredients. For example, plant oils are completely lacking EPA and DHA found in fish oil, but have higher levels of shorter chain ALA and linoleic acid (LA). DHA and EPA are essential fatty acids for salmon, so aquafeeds usually contain some fish oil so the fish remain healthy, but levels are not high enough to maintain high tissue LC-PUFA levels [3]. Plant oils are also typically higher in omega-6 fatty acids, leading to higher levels of omega-6 in the salmon filet and decreased endogenous EPA and DHA production since omega-6s compete with omega-3s for elongation and desaturation enzymes. Additionally, cholesterol is only synthesized by animals, so plant based feeds have a very low cholesterol content meaning that the deficit must be made endogenously by the fish. Plants do, however, make phytosterols that are structurally different from cholesterol and could have an impact on lipid metabolism [14]. Overall, substitution of plant oil for marine ingredients presents new challenges, but may open other avenues for sustainable growth of the aquaculture industry.

1.2 Lipid metabolism

This thesis discusses various aspects of lipid metabolism regulation in salmon, but especially emphasizes fatty acid metabolism and cholesterol metabolism. In order to understand the effects of changes in lipid-related gene expression, some background on the underlying biochemical processes is required. For this reason, I will discuss fatty acid metabolism, cholesterol metabolism, and their regulation in detail in this section. All animals share the basic steps in these processes, so I will describe them generally first, followed by salmon specific features.

1.2.1 Fatty acid metabolism

Fatty acid metabolism involves all of the biochemical reactions involved in the storage of energy through synthesis of fatty acids (anabolism), the release of energy through the breakdown of fatty acids (catabolism), and everything in between. Fatty acids in salmon can be synthesized in one of two ways, the *de novo* biosynthesis pathway mediated by fatty acid synthase (FAS) and the elongation/desaturation pathway mediated by dedicated elongase and desaturase genes. Shorter chain saturated fatty acids are

produced by the former whereas long chain polyunsaturated fatty acids (LC-PUFA) are produced by the latter.

1.2.1.1 De novo fatty acid biosynthesis

The basic building block of all fatty acids is essentially vinegar (minus the water), two-carbon acetic acid. In its native state acetic acid is inactive, so it must first be activated by acetyl coenzyme A synthetase (ACS) to combine with coenzyme A (CoA) forming bioactive acetyl-CoA [15]. At this point, a carboxyl group from bicarbonate is added to the methyl group of acetyl-CoA via acetyl-CoA carboxylase (ACC) to form one molecule of malonyl-CoA [16]. Next, the acyltransferase domain of the homodimeric super-enzyme FAS loads one molecule of acetyl-CoA onto an acyl-carrier protein (ACP) domain on one monomeric FAS arm and one molecule of malonyl-CoA onto the ACP domain of the other. From there, the attached acetyl-CoA is translocated to four catalytic domains within the FAS complex that attach two carbon from malonyl-CoA, extending the attached acetyl-CoA by two carbons. Another malonyl-CoA is transferred to the now vacant ACP domain and the entire process repeats, iteratively growing the chain two carbons at a time [17]. Once the nascent fatty acid has reached a certain length (14 to 18 carbons) it is released as a free fatty acid (FFA) by a thioesterase domain [18]. Chain length is determined based on complementary substrate specificities between the elongation domain (KS) and the thioesterase domain. The KS has decreased activity above 14 carbons, while the TE has decreased activity below 16 carbons. Thus, 16:0 is the primary product of FAS with lower levels of 14:0 and 18:0 [19].

Once FAs are released from the FAS complex, they can be further processed by stearoyl-CoA desaturase (SCD). This will introduce a double bond 9 carbons from the hydroxyl end in 14:0, 16:0, or 18:0 to produce the MUFA 14:1n-5, 16:1n-7, or 18:1n-9, respectively (figure 2) [20]. Alternatively, SFAs or MUFAs can be further elongated to longer chain FAs by Elovl6, which has substrate specificity to 12-16 carbon FAs [21]. SFAs and MUFAs produced this way can have a number of fates. They can be converted to phospholipids where they become integral parts of the plasma membrane, converted to glycerolipids as an energy reserve, or used in a variety of post-translational modifications.

Atlantic salmon possess two copies of *fas*, both of which are highly expressed in liver (table 1). There are two *Scd* genes in animals, *Scd* and *Scdb*, of which salmon possess two

copies and four copies, respectively. Only one of these (LOC106577210) is highly expressed in liver. For *elovl6*, salmon have two copies, both of which are expressed in liver (table 1).

1.2.1.2 Polyunsaturated fatty acid biosynthesis

Long chain polyunsaturated fatty acids (LC-PUFA) cannot be synthesized *de novo* in any animal. Instead, they must be directly obtained from the diet or biosynthesized from shorter chain PUFA (ALA or LA) through sequential elongation and desaturation steps. The exact enzymes used to accomplish this vary between species, but the basic process is the same. All of the enzymes involved have substrate specificity to the alpha end of the fatty acid, so omega-3s and omega-6s compete for the same active sites. I will describe the process beginning from 18:3n-3, but it is the same when beginning from 18:2n-6 (figure 2). Additionally, all enzymes in the pathway only act on FAs that have been activated through the addition of CoA by long-chain acyl-CoA synthetases (ACSL), so from now on when discussing FAs in a biosynthetic pathway I am referring to the acyl-CoA form.

First, a double bond is added to 18:3n-3 six carbons from the alpha end by a Δ6 desaturase (Δ6FAD) to produce 18:4n-3. Then, two carbons are added to the alpha end by elongase 5 (ELOVL5) to produce 20:4n-3. The four step elongation reaction is as follows: rate limiting condensation of acyl-CoA (in this case 18:4n-3) and malonyl-CoA by ELOVL5 to form ketoacyl-CoA; reduction by 3-keto acyl-CoA reductase to form 3-hydroxyacyl-CoA; dehydration by β-hydroxyacyl-CoA dehydrase to form trans-2,3-enoyl-CoA; and reduction of enoyl-CoA by trans-2,3-enoyl-CoA reductase to form the final product 20:4n-3 [20]. Next, a double bond is added five carbons from the alpha end by a Δ5 desaturase (Δ5FAD) to form 20:5n-3 (EPA) followed by two more elongation steps involving elovl5, elongase 2 (ELOVL2), and elongase 4 (ELOVL4) to form 22:5n-3 and then 24:5n-3 [20]. ELOVL5 has higher affinity for 18 carbon FAs, ELOVL2 for 20 carbon FAs, and ELOVL4 for 22 and longer FAs [22]. Another desaturation step by Δ6FAD transforms 24:5n-3 to 24:6n-3, then a series of peroxisomal chain shortening steps occur to produce 22:6n-3 (DHA). The elongation step involving 24:5n-3 and subsequent chain shortening is collectively referred to as the Sprecher shunt [23].

Atlantic salmon possess a full complement of enzymes to synthesize EPA and DHA from shorter chain ALA (18:3n-3). Notably, *Δ6fada* is the most highly expressed of the three

copies in salmon and while for *elovl5a* and *elovl5b* are both highly expressed. See figure 2 for the number of copies of each gene and table 1 for their tissue expression profiles.

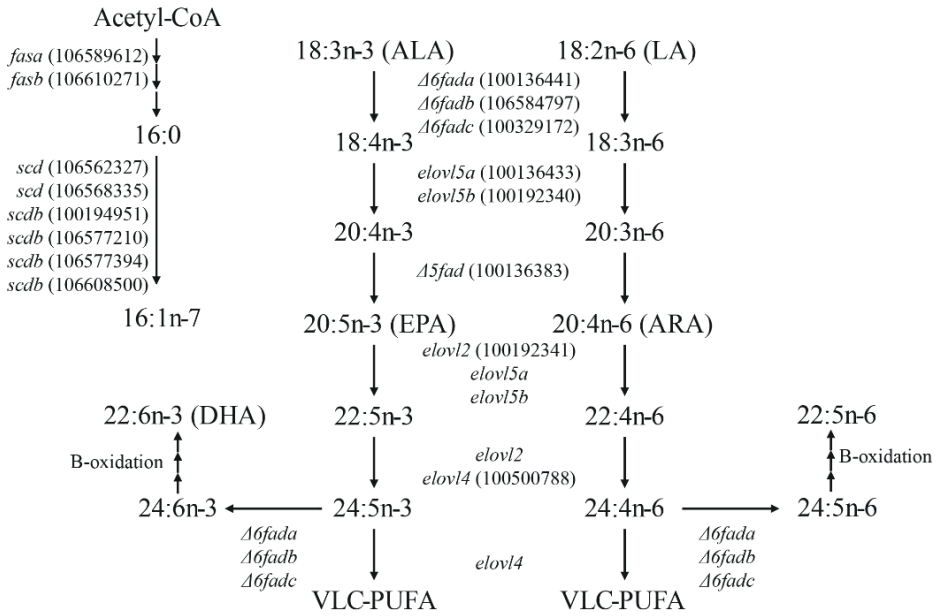

Figure 2: FA and PUFA biosynthesis pathways in Atlantic salmon. Fatty acid biosynthesis from acetyl-CoA to 16:1n-7 is in the top left. Omega-3 and omega-6 PUFA biosynthesis is in the middle. Gene names are in italics followed by NCBI identifiers in parenthesis.

1.2.1.3 Beta-oxidation

Mitochondrial fatty acid oxidation is a key energy producing process that allows animals to transform stored fatty acids into energy in the form of ATP. The first step, like in FA biosynthesis, is activation of FAs by ACSLs on the outer mitochondrial membrane to form acyl-CoAs [24]. These cannot cross the inner mitochondrial membrane, so the CoA moiety must be replaced by a carnitine moiety, catalyzed by carnitine palmitoyltransferase 1 (CPT1) located on the outer mitochondrial membrane. This step is rate limiting, so the expression of *cpt1* can be an indicator of β-oxidation activity [25]. Acyl-carnitine is transported into the mitochondrial matrix by carnitine-acylcartnitine translocase where it is transformed back to acyl-CoA by carnitine palmitoyltransferase 2 (CPT2) located on the inner mitochondrial membrane [24]. Transformation of acyl-CoA into energy then proceeds via the β-oxidation spiral.

The four catalytic domains required for mitochondrial β-oxidation in order of reaction sequence are acyl-CoA dehydrogenase (AD), enoyl-CoA hydratase (EH), 3-hydroxyacyl-CoA dehydrogenase (HD), and 3-ketoacyl-CoA thiolase (KT) [24]. The first step is performed by a set of AD enzymes with substrate specificity to varying length carbon chains, ensuring that acyl-CoAs of any length are efficiently oxidized. The last three steps are performed by mitochondrial trifunctional protein (MTP) attached to the inner mitochondrial membrane. MTP is a hetero-octomer composed of four alpha (HADHA) and four beta subunits (HADHB) [26]. In fish, the HADHA has two additional subunits, HADHAA and HADHAB. The electron that will eventually be fed into the electron transport chain to produce energy in the form of ATP is provided in the first step when AD abstracts a hydrogen and hydride from acyl-CoA [24]. The β-oxidation spiral proceeds iteratively, shortening the fatty acyl chain two carbons at a time until the entire FA is consumed.

Atlantic salmon has two copies of *cpt1* and one copy of *cpt2*, which are all expressed ubiquitously. For MTP, we identified two copies of *hadhab* in salmon and one copy of *hadhb*, but could not identify *hadhaa* using our orthogroup pipeline. All identified copies of the MTP complex are expressed ubiquitously, but are more highly expressed in energy intensive tissues like muscle and heart (table 1).

1.2.2 Cholesterol metabolism

All animals maintain cholesterol homeostasis by strictly balancing dietary intake and *de novo* biosynthesis. If dietary cholesterol levels are too high, excess cholesterol is transported from peripheral tissues back to the liver via reverse cholesterol transport and converted to bile acids, which are secreted into the gut to assist in digestion. Conversely, if dietary cholesterol is too low, it is synthesized *de novo* from acetyl-CoA in a complex 24-step process [10]. Nearly all genes in the cholesterol biosynthetic pathway respond to low dietary cholesterol by upregulating their expression. This is mediated by the master cholesterol regulator, sterol regulatory element binding protein 2 (SREBP2) [27] which is discussed in a later section. The third step in cholesterol biosynthesis, mediated by 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), is considered to be rate limiting [28]. This is the target of statins, a family of common cholesterol reducing drugs, and has been the subject of extensive research [29]. Salmon have two gene encoding the HMGCR protein, *hmgcr* and *hmdh*, the first of which is highly expressed in liver (table 1).

1.2.3 Regulation of lipid metabolism

Regulation of lipid metabolism is complex, involving allosteric, posttranslational, and transcriptional mechanisms. I will focus exclusively on transcriptional regulation since other types of regulation are out of scope for this thesis, but they can all be reviewed here [18]. The three main transcription factors governing expression of most lipid-related genes in salmon are sterol regulatory element binding protein (SREBP), liver X receptor (LXR), and peroxisome proliferator-activated receptor (PPAR) [20]. Since lipid metabolic networks are highly conserved across vertebrates [30], I will discuss lipid metabolism regulation in general terms. The main difference in salmon is the presence of duplicate genes resulting from the salmonid specific whole genome duplication (WGD) event.

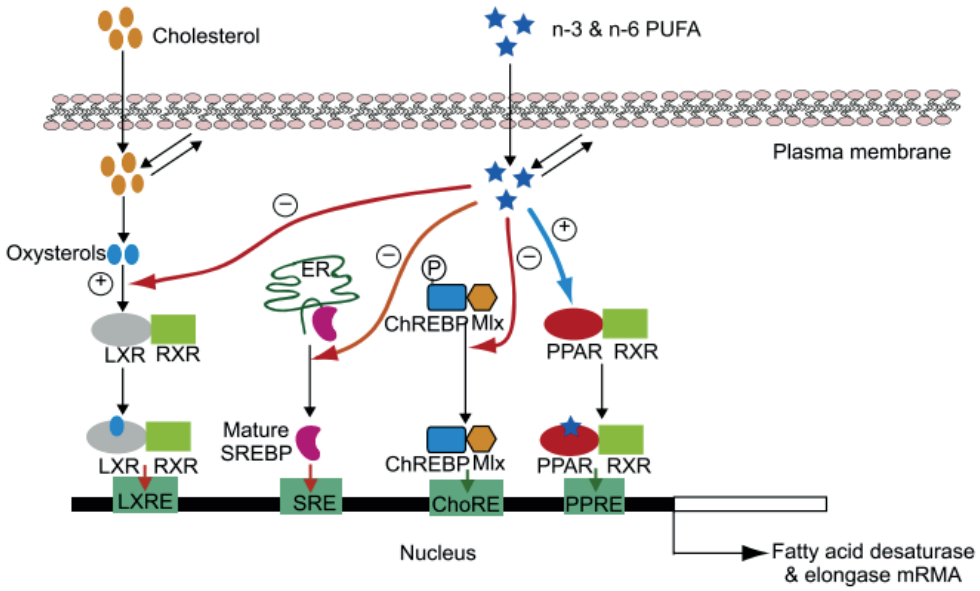

Figure 3: Transcriptional regulation of lipid metabolism. Lipid metabolism in salmon is mainly regulated by LXR, SREBP, and PPAR. Carbohydrate response element binding protein (ChREBP) does not play a major role in salmon and will not be discussed. This figure was obtained from Bond et. al. 2016 [20] under license number 4534220331354.

1.2.3.1 Sterol regulatory element binding protein

In mammals there are two sterol regulatory element binding protein (SREBP) genes encoding three isoforms – SREBP1a, SREBP1c, and SREBP2. Of the first two, SREBP1-c is primarily expressed in lipogenic tissue such as liver and both are generally involved in

regulating fatty acid biosynthesis. SREBP2 primarily regulates cholesterol biosynthesis and is expressed ubiquitously [18]. Activation of SREBP requires transport of endoplasmic reticulum (ER) bound preSREBP to the golgi followed by proteolytic cleavage to release the mature SREBP protein. This translocates to the nucleus where it binds sterol regulatory elements (SRE) in the promoter region of certain genes and activates transcription. Cleavage of preSREBP is suppressed by LC-PUFA and cholesterol, so in this way SREBP is a sensor of intracellular LC-PUFA and cholesterol, activating biosynthesis of fatty acids and cholesterol when levels are low (figure 3) [20].

At least one SRE site can be found in the promoters of nearly all cholesterol biosynthetic genes and many lipogenic genes including *acc*, *fas*, *scd*, *Δ6fad*, *Δ5fad*, and *elovl5* [30,31]. Additionally, a SRE site is present in the *srebp* promoter itself, so SREBP activation promotes its own expression in a positive feedback loop [32].

1.2.3.2 Liver X receptor

Liver X receptor (LXR) is a transcription factor that acts as a sensor of cellular cholesterol; it activates gene expression upon binding of oxysterols (figure 3) [20]. Two genes, *Lxrα* and *Lxrβ*, exist in vertebrates and have distinctly different tissue expression profiles. *Lxrα* is highly expressed in lipogenic tissue and plays a larger role in regulating lipid metabolism [18]. LXR binds to LXR response elements (LXRE) in the promoter region of some genes as an obligate heterodimer with retinoid x receptor α (RXRα). Upon oxysterol binding, LXR/RXR releases a bound co-repressor complex and recruits a co-activator complex including the histone acetylase P300 [33].

The main function of LXRα in liver is to maintain cholesterol homeostasis through regulation of genes responsible for cholesterol elimination. This includes excretion of cholesterol through reverse cholesterol transport and the direct upregulation of CYP7A1, responsible for the rate limiting step in bile acid synthesis [34]. LXR also plays a role in activating genes involved in fatty acid biosynthesis. This is mainly mediated through the upregulation of SREBP1c, which contains several LXREs in its promoter region, however LXR can also directly activate some lipogenic genes that have LXREs in their promoters [35]. Atlantic salmon have two copies of LXRα, both of which are highly expressed in liver (table 1).

1.2.3.3 Peroxisome proliferator-activated receptor

PPARs bind fatty acids and regulate a wide variety of cellular tasks including lipid metabolism, development, growth, apoptosis, inflammation, and immune response [36]. Like LXR, PPARs form an obligate heterodimer with RXR and remain bound to DNA response elements even in the absence of ligand (figure 3) [20]. Upon fatty acid binding, they undergo a conformational change, which releases the bound co-repressor complex and recruits a co-activator complex [36]. There are three PPAR genes – *Pparα* which is mostly expressed in tissues with high oxidative capacity such as liver and muscle, *Pparβ* which his ubiquitously expressed, and *Pparγ* which is mainly expressed in adipocytes [37]. The main function of PPAR in liver is activation of β-oxidation when excess fatty acid is detected, such as when fatty acid influx increases during fasting [38]. There are two copies of *pparα* in Atlantic salmon and both are highly expressed in liver (table 1).

1.2.3.4 Regulation if lipid metabolism by insulin

Insulin is a well-known anabolic hormone secreted by the pancreas during feeding. Its main function is to shift the cellular metabolic state from catabolic to anabolic since it would be a profound waste of energy to break down storage molecules like TAG and glycogen while building them up at the same time. Insulin binding to insulin receptors (INSR) located on the cell surface triggers a complex signaling cascade beginning with the phosphorylation of INSR and insulin receptor substrates and ending in a systemic anabolic shift mediated by several signaling pathways including the MAP kinase and PI3K/Akt/mTOR pathways [39]. A main result of activation of the latter signaling pathway is upregulation of SREBP1c which in turn upregulates *de novo* and PUFA biosynthesis pathway genes [40]. This is coordinated with downregulation of glycolysis and β-oxidation genes, completely transforming the cellular metabolic state.

Table 1: Tissue distribution of key lipid metabolism genes in Atlantic salmon. Genes mentioned above in salmon with NCBI identifiers. Gene expression is measured relative to all other expressed genes and denoted as transcripts per million mapped reads (TPM). ++++ = TPM >500, +++ = TPM >100, ++ = TPM >10, + = TPM >1. L = liver, PC = pyloric caeca, G = gut, M = muscle, H = heart, B = brain.

			Tissue expression					
Common name	Salmon name	NCBI ID	L	PC	G	M	H	B
De novo fatty acid biosynthesis								
Accα	*accαa*	106568086	+				+	+
	LOC106603271	106603271	+	+	+		+	++
Accβ	*accβa*	106580110	+	++	+	+	++	+
	LOC106585658	106585658		++	+	+	+	
Fas	*fasa*	106589612	+		+	+	+	++
	fasb	106610271				+	+	++
Scd	LOC106562327	106562327						+
	LOC106568335	106568335						
Scdb	*acod*	100194951			+			++
	LOC106577210	106577210	+++	+	++	+	+++	+++
	LOC106577394	106577394						++
	LOC106608500	106608500	+		+	+	+	++
Elovl6	*elovl6*	106611586	++				+	+
	LOC106604515	106604515	+				+	++
PUFA biosynthesis								
Δ5fad	*Δ5fad*	100136383	++++	+++	++	+	+++	++
Δ6fad	*Δ6fada*	100136441	++++	++++	++		++++	++
	Δ6fadb	106584797	++	++	+		++	++
	Δ6fadc	100329172	++	+	+		+	++
Elovl5	*elovl5a*	100136433	++	++	+++	+	++	++
	elovl5b	100192340	+++				+++	++
Elovl2	*elovl2*	100192341	+++	+++	++		++	+
Elovl4	*elovl4*	100500788						+
	LOC106571493	106571493		+	+	+		++
B-oxidation								
Cpt1	*cpt1b*	106587782	+	+	+	+	+	+
	LOC106560707	106560707	+	++	++	++	++	+
Cpt2	*cpt2*	106560431	++	++	++	++	++	++
Hadhab	LOC106603767	106603767	+	++	++	++	+	++
	LOC106611334	106611334	++	++	++	++	++	++
Hadhb	*hadhb*	106608385	++	++	++	++	++	++
Cholesterol biosynthesis								
Hmgcr	*hmgcr*	106570829	+		+		+	++
	hmdh	100380797	+	+	+	+	+	+
Regulation								
Srebp1	*srebp1*	100502556	+	++	+	+	+	++
	LOC106606621	106606621	+	+	+	+	+	++
	LOC106606632	106606632	+	+	+	+	+	++
Srebp2	*srebp2*	100502557	++	+	++	+	++	++
	LOC106607132	106607132	++	+	++	+	+	++
	LOC106607120	106607120	++	+	++	+	++	++
LXRα	*lxrα*	100270809	++	+	++	+	++	++
	LOC106561932	106561932	+	+	+	+	+	+
PPARα	LOC100136415	100136415	++	++	++	+	++	+
	LOC106584489	106584489	++	+	+	+	++	+

1.3 Lipid metabolism and life stage transitions

Atlantic salmon have a complex life history. They begin life in freshwater rivers as alevin and thrive on egg sac nutrients until they become fry and transition to a primarily insect-based diet (figure 4). Salmon continue to grow in freshwater, eventually becoming parr and developing a golden color with vertical black stripes, which help them to blend in to a rocky riverine habitat (figure 4). Next, typically after short photoperiods associated with winter, salmon begin a process called smoltification where they transform physiology, behavior, and appearance in preparation for life at sea [41]. They become schooling open water swimmers and develop a silvery appearance allowing them to blend into deep oceans environments (figure 4) [42]. Eventually, after continuing to grow and mature at sea, salmon will migrate back upstream to their birthplace to spawn and the cycle begins anew.

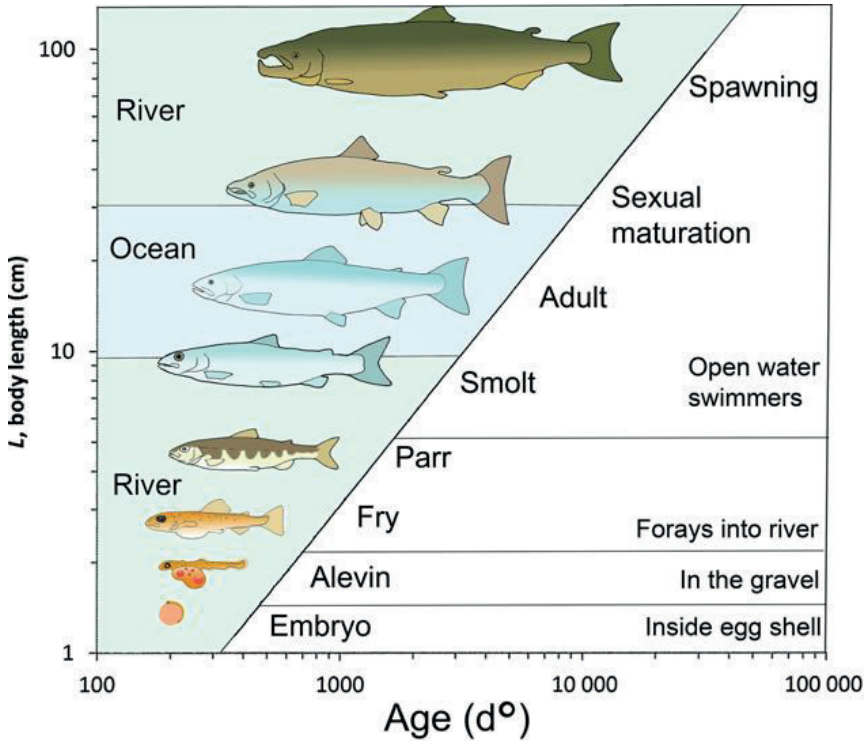

Figure 4: Life stages of Atlantic salmon. Salmon begin life in freshwater before undergoing smoltification and migrating to sea. This figure was adapted from Kryvi et. al. (2017) [43] according to the terms and conditions of the creative commons license 4.0 (https://creativecommons.org/licenses/by/4.0/).

Just as life stage transitions are associated with changes in appearance and behavior, they are also associated with changes in how salmon metabolize lipids. Recently, Yang et.al. (2019) [44] demonstrated that phospholipid and lipoprotein synthesis gene expression increase when salmon transition from egg sac to exogenous feeding. This was associated with increases in free fatty acids and decreases in triacylglycerol, demonstrating that remodeling of gene expression across life stages leads to meaningful changes in lipid composition. Lipid metabolism transforms again during the parr-smolt transition. Overall, smoltification triggers increased lipid breakdown and decreased biosynthesis [45]. This results in transformation of their tissue fatty acid composition from freshwater-like, low in LC-PUFA to seawater-like high in LC-PUFA [46] and interestingly is independent of diet [47,48]. This convergence of tissue fatty acid composition regardless of dietary intake suggests a genetically pre-determined LC-PUFA requirement for seaward migration. Prior to this thesis, the extent of metabolic transformation was unknown, however we demonstrate that it is a genome wide trend and is specific to lipid metabolism.

1.4 The Atlantic salmon genome

Transcriptomic studies in salmon have until recently been based on microarray technology. Although, these microarray-based studies revolutionized the salmon functional genomics field, the microarray technology did not allow us to distinguish signals from highly similar genes and was limited to the genes that were 'printed' on to the microarray chip. The recent publication of a good reference genome for Atlantic salmon [49] have made it possible to use RNAseq and thereby accurately measure the expression of all genes at once, and even distinguishing expression of all genes belonging to larger gene families. The Atlantic salmon genome assembly totals 2.97 gigabases (Gb) of sequence information, 2.24 Gb of which were assembled into 29 chromosomes. Annotation based on transcriptomic and comparative genomic data identified 37,206 high-confidence protein coding gene loci [49]. Much of the rest of the salmon genome is repetitive sequence (58-60%) which is higher than zebrafish (52.2%), the previous record holder among vertebrates [50]. Short segments of DNA with the ability to insert themselves into the genome called transposable elements [51], contribute much of the repeat-DNA, with the most prevalent class being Tc1-*mariner* at 12.89% of total genome sequence [49].

1.4.1 Ss4R genome duplication

An ancestor of Atlantic salmon underwent a whole genome duplication (WGD) roughly 80 million years ago (figure 5a). This sudden increase in genetic redundancy let to a period of genomic instability with a high rate of chromosomal rearrangements thought to be due to increased TE activity causing errors in homeologous chromosomal pairing during meiotic division [52]. These rearrangements are evident by the presence of large blocks of homeologous sequences between chromosomes (figure 5b) [53]. This is relevant for this thesis for two reasons. First, this salmonid WGD event has resulted in an increase in gene copies, and in Atlantic salmon, approximately half (55%) of all genes in have a retained functional duplicate [49]. This must be taken into consideration when analyzing gene expression data. Second, many of these salmonid specific gene copies have evolved novel regulation, which also is relevant when interpreting transcriptomics data under the assumption that similar genes in different species perform the same job. Gene regulatory divergence after WGD may take several forms. Some duplicates have retained the ancestral regulation, potentially increasing gene dosage, while others display novel regulatory phenotypes [54].

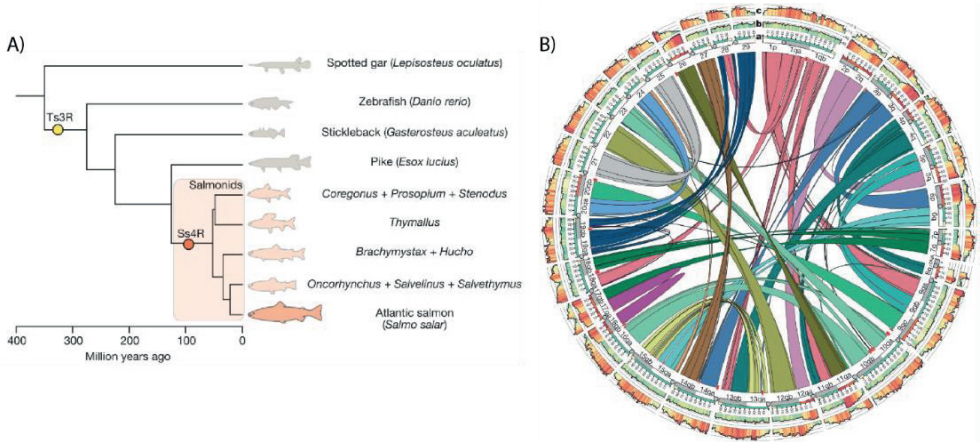

Figure 5: Duplication of the Atlantic salmon genome. A) Phylogeny of a subset of salmonids and non-salmonid outgroup species. The salmonid specific WGD (Ss4R) is shown in orange and the teleost specific WGD (Ts3R) is in yellow. B) Circos plots of the Atlantic salmon genome. Colored blocks connect homeologous regions between chromosomes. The three outer tracks represent a) sequence similarity between homeologous regions grouped as high (red), elevated (orange), low (green) similiarity, or telomeric (yellow); b) sequence similarity between homeolog blocks in 1Mb windows; and c) frequency of the Tc1-mariner transposon in the genome. This figure was adapted from Lien et. al. (2016) [49] according

1.4.2 Functional annotation of genes

Predicting gene function based on DNA sequence requires comparison to other model organisms where protein function has been previously determined. To do this, genes that are functionally equivalent between species (orthologs) are identified by constructing gene trees (orthogroups) based on protein sequence using a number of closely and distantly related species [55]. For example, in paper II we generated orthogroups from four salmonid species, four non-salmonid species (including zebrafish), and two mammalian species as outgroups. This allowed us to assign a zebrafish gene with a known function to a salmon gene with a previously unknown function.

Once function has been assigned, annotation of lipid metabolism genes only involved selecting a set of zebrafish genes considered lipid-related. This is not a straightforward task since many proteins have promiscuous activities or functions that crossover to several different aspects of metabolism. Therefore, designating a group as "lipid metabolism genes" must rely heavily on domain knowledge. In our work, we therefore created a manually curated lipid gene list by selecting genes within 19 lipid-related zebrafish KEGG pathways plus a few well-known genes missing from those pathways.

Having a high confidence curated list of lipid metabolism genes enables genome scale interrogation of lipid metabolism in ways that were not previously possible. For example, lists of differentially expressed genes could be filtered to remove irrelevant genes and reveal patterns that could not be previously detected. Additionally, statistical enrichment tests could reveal if a list of genes contains a higher than random number of lipid genes. In papers II and III, we use our lipid annotation to provide unprecedented genome scale insights into lipid metabolism gene regulation across life stages and tissues.

1.5 Methods to study lipid metabolism

There are numerous ways of studying lipid metabolism in animals. In this thesis, we leverage both live animal trials and cell culture approaches to study lipid-related gene expression changes across diets, tissues, and life stages. We mostly take an indirect approach to measuring lipid metabolism by focusing on tracing shifts in relative

abundance of different mRNA. Since mRNA is translated to proteins that perform the majority of biochemical tasks in the cell, changes in transcript abundance can provide insight into the cellular metabolic state under different conditions. In the following sections, I will describe and discuss the experimental approaches and main methods we used.

1.5.1 Live animal trials

Feeding trials are the gold standard to study metabolism in animals. A feed containing a novel ingredient or composition is fed to animals for a period, then they are slaughtered and organs harvested for analysis. This strategy is straightforward with many advantages since all measurements are taken in a relevant biological context including all organs and circulating hormones.

They also have many drawbacks. Since animals are complex systems there are a plethora of confounding factors like age, feed status, size, health, genetics, and behavior. Unless working with clonal animals, each individual will have a slightly different genetic background that can influence gene expression in unpredictable ways. In addition, many animals including salmon have complex behavioral hierarchies that can further complicate experimental designs. There are also practical limitations, becoming very expensive and time consuming depending on the size of the experiment and the life history characteristics of the animal of interest. Salmon, for example, require at least 6 months in freshwater before they are large enough to smoltify and move to seawater.

1.5.2 *In vitro* systems

1.5.2.1 Monocyte culture

Some studies would require too many animals to be sacrifice or are simply are not suited to live animals, so cell culture is typically used. There are two broad categories of monocyte culture, primary cell culture and immortalized cell lines. The former uses cells that come directly from an animal and cannot be cultured indefinitely. There is a hard-coded limit to the number of times somatic cells can divide, known as the hayflick limit [56], so primary cells must be obtained from a new individual each time. The latter have typically been genetically modified to overcome the hayflick limit and so can be cultured

continuously. This improves reproducibility by eliminating genetic variability. An immortalized Atlantic salmon liver cell line does not yet exist, so we use primary hepatocytes as a benchmark for liver slice culture.

The method to culture primary hepatocytes is relatively straightforward. The liver is dissected from an animal, washed by transfusion, and treated with collagenase solutions to disassociate cells. They are then filtered through a fine mesh to remove cell clumps before centrifugation and washing. Finally, cells are grown as a monolayer in a sterile petri dish where they anchor themselves to the bottom of the plate. Cell culture allows for a much higher degree of control over extraneous factors since many different variables can be tested under the exact same conditions on a single individual.

Unfortunately, this reduction in complexity compared to live fish can mean that results obtained from cell culture are not fully applicable to living animals. For this reason, it is essential that *in vitro* results can be followed up with *in vivo* experiments.

1.5.2.2 Precision cut liver slice culture

A promising alternative to traditional primary cell culture is precision cut liver slice (PCLS) culture. Liver slices are fast and easy to produce and more closely resemble whole liver by maintaining the complex three-dimensional structure with all interacting cell types. To generate liver slices, liver is first removed from an animal and cut into thin strips. One piece is then superglued to a plastic piston and encased in low melting agarose to give support to the tissue while cutting. The piston containing tissue is inserted into a vibratome and automatically sliced into 300µm thick sections in an ice-cold saline solution. Slices are carefully removed from the saline and placed into a sterile petri dish containing fresh culture media (figure 6). Dozens liver slices can be prepared from a single animal, so PCLS culture is useful for complex experimental designs where many conditions need to be tested in parallel.

PCLS culture was first introduced in 1923 and quickly fell out of practice due to low reproducibility since slices had to be created by hand [57]. The invention of the Krumdieck automated tissue slicer in the 1980s [58] solved this problem and spurred renewed interest in PCLS culture as a viable cell culturing method. Since then, PCLS culture has mainly been used in toxicological and immunological studies [59,60], however a few studies have used PCLS culture to investigate metabolism. For example, Janssen et.

al. [61] demonstrated the effects of PPARα on desaturation and elongation in human PCLS and Fortin et. al. [62] recently used PCLS from dairy cows to investigate the relationship between PUFAs and oxidative stress. Prior to this thesis there have been no metabolic PCLS studies in fish, so protocol development and proper validation was required to take full advantage of this technique in salmon.

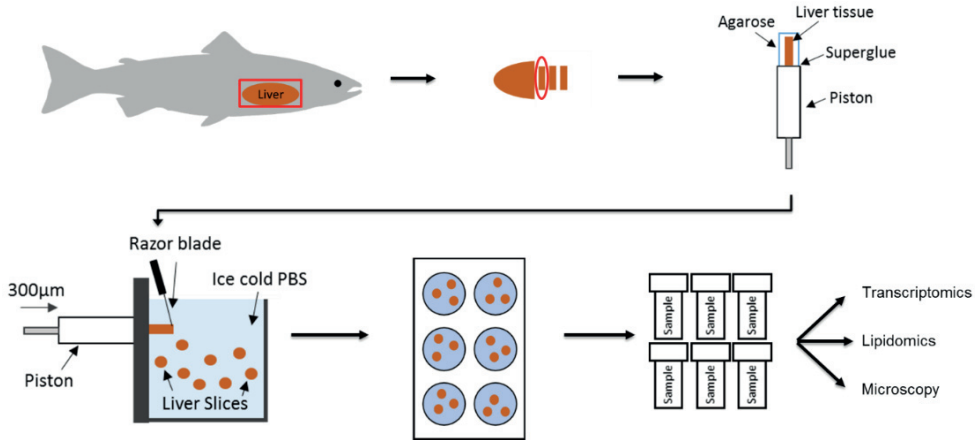

Figure 6: Precision cut liver slice culture. Schematic of the liver slice culturing process. Liver is first removed from the fish and cut into strip. One strip is superglued to a plastic piston and encased in agarose. The piston is placed into a vibratome and tissue is sliced into 300µm thick sections. Slices are placed in culture media and sampled according to the experimental design.

1.5.3 Omics

There are three primary levels of sequential information in a cell. First, DNA is the cellular blueprint containing many thousand discrete pieces of information called genes, collectively referred to as the genome. Second, RNA is the mediator of information from gene to protein in the form of transcripts, collectively referred to as the transcriptome. Finally, proteins are the molecular machines that perform the majority of cellular tasks, collectively referred to as the proteome. Modern techniques allow us to take a snapshot of each of these domains at a given point in time. Genomics, transcriptomics, and proteomics measure the entirety of cellular DNA, RNA, or protein, respectively. The majority of this thesis relies on the use of transcriptomics to measure changes in gene expression across feeds, life stages, and liver slice conditions, so I will discuss this technique exclusively.

1.5.3.1 RNA sequencing

In the past, biologists could only measure the expression of a handful of genes at a time, however with the advent of massively parallel sequencing the expression of all genes could be measured simultaneously. This miraculous task is accomplished as follows: First, total RNA is extracted from a tissue of interest and used to make RNA sequencing libraries. This involves enriching for mRNA using poly-T primers that bind to the poly-A mRNA tail, fragmenting the mRNA to a desired size, reverse transcribing to cDNA, and ligating unique indexes to both ends of the cDNA fragments (figure 7). Once indexed, libraries from different samples can be pooled and separated later bioinformatically since the index sequence is unique for each sample. Next, if using the illumine short read sequencing technology, the libraries are loaded onto a flowcell and replicated using bridge PCR to generate discrete clusters of identical sequences. Fluorescently labelled nucleotide terminators (A, C, T, or G modified so that only one attaches at a time) are then added which bind to the complimentary base on the library fragments and are covalently linked by DNA polymerase. A laser excites the newly added labeled nucleotides and a camera takes a picture of the flowcell. A new batch of labeled nucleotides is then added and the process is repeated a number of times depending on the desired read length (typically 100-200 times). By reading the color of the dots in the flowcell images sequentially, a sequence for each cluster can be deduced (figure 7). An Illumina Hiseq flowcell has eight lanes that can produce about 300 million reads each, so a single sequencing run can produce roughly 2.4 billion mRNA 'reads' at a time.

Once the raw sequences are generated, individual samples are separated based on index sequences and aligned to a reference genome sequence. The number of reads corresponding to each gene are counted to determine the relative expression level of each gene (figure 7). Data from each sample can be represented in a counts table, where each row is a gene and each column is a sample, allowing easy manipulation of the data and statistical tests between groups. Before the data can be analyzed, it must be normalized since the total amount of RNA sequenced can vary between samples. We use trimmed mean of M-values (TMM) normalization, which calculates a scaling factor for each sample based on the assumption that less than half of the gene are differentially expressed between samples [63]. The total library size is multiplied by the scaling factor to give an

effective library size that is used to calculate counts per million (ignoring gene length, CPM) or transcripts per million (accounting for gene length, TPM).

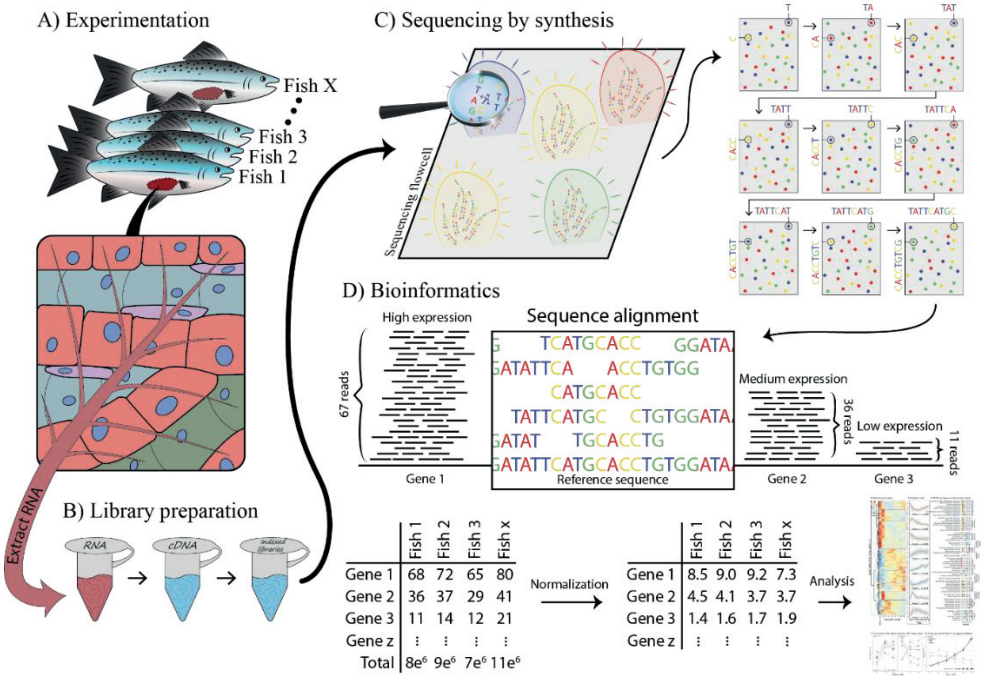

Figure 7: Transcriptomics experiment from start to finish. A) Experiments are designed using at least three biological replicates and the tissue of interest is dissected from the fish for RNA extraction. B) Libraries are made where mRNA is purified and converted to cDNA. Unique indexes are added and samples pooled for sequencing. C) Sequencing by synthesis where libraries are amplified on a sequencing flowcell to generate clusters. Then labeled nucleotides are added that fluoresce when bound to a complimentary DNA strand. The flowcell is photographed and sequence deduced by sequentially reading of cluster signal. D) Sequences are aligned to a reference genome and number of reads per gene are counted. Read counts are placed in a data matrix and normalized to account for differences in library size. Transcriptomic data is then ready for analysis.

1.5.3.2 Analytical methods

Generating a massive amount of sequencing data is relatively straightforward; figuring out what it all means is much more complicated. A good starting point for this type of data is differential expression analysis (DEA), which compares gene expression between groups of replicate samples to find genes that change in abundance given a significance

threshold (typically false discovery rate (FDR)<0.05) [64]. This can be done pairwise or using an ANOVA-like test that finds all differentially expressed genes (DEGs) over time or across a gradient. When the experimental design involves a set of serial conditions, DEG lists can be broken down further using hierarchical clustering to generate groups of genes with similar expression patterns.

Today, in the post-genomic era a fundamental goal of any omics study is to move from statistics on single genes to a system wide understanding of cellular processes. Once a list of genes that change in expression across different samples is established, we can use enrichment analysis to link these genes to biological processes. One way of doing this is to use KEGG annotations. KEGG stands for Kyoto Encyclopedia of Genes and Genomes and is a manually curated database with known functions for enzymes and signaling molecules, arranged into biochemical pathways. In transcriptomic studies like ours, where the focus is genes involved in well-described biochemical pathways such as lipid biosynthesis, we can take advantage of this a priori pathway knowledge to link single gene measurements to system wide pathway-centric statistics.

1.5.3.3 Limitations

A key assumption when using transcriptomic data is that transcript abundance correlates to protein abundance, and that protein abundance correlates to pathway flux. This is of course not always true since net protein abundance is determined by the rate of synthesis minus the rate of degradation and neither of these values are static [65]. Additionally, metabolic flux is the product of protein abundance, protein activity, and the relative concentration of substrates and products. For this reason, we must be careful about the conclusions we draw from transcript level data that is not supplemented by other measurements. Differential expression of a single gene in a pathway should not be extrapolated to control pathway flux unless the protein is well known to be rate limiting and correlated to gene expression (HMGCR in cholesterol biosynthesis for instance). Instead, gene set analysis can reveal overall trends in the expression data to identify large-scale changes in cellular state. Expression of individual genes, however, can still be useful in hypothesis generation, but follow-up by protein and metabolite measurements is critical for validation.

There is also the question of biological significance. DEA may yield many genes that are statistically significant, but changes in expression of these genes may not actually have an impact on the cell. On the other hand, small changes in the expression of some genes (like the beginning of signaling cascades) may have a large effect on the cell, but are unlikely to be detected due to low abundance or low fold change. Many studies use a log fold change cutoff as a proxy for biological significance, but this is almost certainly an oversimplification. In reality, biological significance is likely different for each gene, so interpretation of statistical tests must always be advised by domain knowledge when analyzing transcriptomic datasets.

2 Aim of thesis

In this thesis, we approach a genome wide understanding of lipid metabolism regulation by leveraging large scale feeding trials, advanced cell culture techniques, and transcriptomics. Specifically, our aims are to:

i) validate and benchmark PCLS culture for studying lipid metabolism *ex vivo* (paper I)

ii) describe tissue and life stage specific transcriptome-wide shifts in lipid metabolism regulation, with a particular focus on liver (papers II and III)

iii) test the hypothesis that lipid-metabolism regulation is contingent on the physiological transformation occurring when Atlantic salmon undergo smoltification (paper III).

3 Results in brief

3.1 Paper I

In paper I, we developed a method for *ex vivo* experiments on lipid metabolism using precision cut liver slice culture. Liver slices have never been used to study metabolism in fish, so we designed a series of experiments to test 1) the effect of culturing on liver slices, 2) if liver slices respond to their environment in a liver-like way, and 3) how liver slices compare to liver *in vivo* and primary cell culture in terms of gene expression similarity. Our results provide a foundation for future experiments interrogating various aspects of lipid metabolism *ex vivo*.

3.1.1 Liver slice performance over time

We carried out a nine-day time course experiment where we took liver slice samples every day (except day 2) for viability testing, microscopy, and transcriptomic analysis. We found that liver slices remained highly viable through nine days in culture and that thickness and general morphology remained constant (paper I, figure 1). Differential expression analysis yielded 16,267 genes that significantly (FDR<0.01) changed in expression between any of the time points. Grouping these differentially expressed genes (DEGs) by expression similarity over time revealed a striking shift in gene expression relating to signal transduction (up) and genetic information processing (down) one day after slicing (paper I, figure 2c). Fortunately, pathways relating to lipid, amino acid, carbohydrate, and vitamin metabolism experienced an initial decrease in relative expression in the first three days after slicing, but remained stable after this through day nine (paper I, figure 2c).

3.1.2 Liver slice response to ALA and insulin

In order for liver slices to be a useful in modeling lipid metabolism, we need to be sure that they have the ability to interpret nutritional and hormonal signals from their environment in a biologically meaningful way. To test this we designed two experiments 1) a fatty acid gradient experiment where liver slices were cultured in media containing increasing concentration of ALA from 0 to 140μM and 2) an insulin gradient experiment where liver slices were cultured with increasing concentrations of insulin in the presence

and absence of ALA. We found a positive correlation between lipid metabolism-related gene expression and media ALA concentration up to 70µM (paper I, figure S1) and a binary effect of insulin on gene expression, where major anabolic pathways were turned on while other catabolic pathways were turned off (paper I, figure S2).

We also observed an interaction between ALA and insulin within the PUFA biosynthesis pathway. ALA alone strongly upregulated key desaturase genes *Δ5fad* and *Δ6fada* while insulin alone strongly upregulated *elovl5a*, *elovl5b*, and *Δ6fada* (paper I, figure 4b and c). Interestingly, when the two were combined insulin had the opposite effect on *Δ6fada* and strongly decreased expression of *Δ5fad* while activation of *elovl5a* and *elovl5b* was maintained (paper I, figure 4c).

Finally, we obtained strong evidence that ALA is taken up by liver slices and enters the biosynthetic pathway. We fed slices increasing concentrations of ALA and measured a proportional increase in both ALA and its elongation product, 20:3n-3 (paper I, figure 4d). We were however not able to detect a shift in EPA and DHA with increasing ALA concentration with the approach we used, most likely because any small changes in abundance would have been masked by the large intracellular EPA and DHA pools.

3.1.3 Comparison of PCLS to primary hepatocyte culture and *in vivo* liver

In order to evaluate how liver slice culture performs relative to more traditional cell culture we cultured primary hepatocytes and compared the RNA sequencing results to liver slice culture and *in vivo* liver from a large feeding trial (from paper II). We found that in nearly every case gene expression in liver slice culture was more similar to *in vivo* liver on both the pathway level and gene level (paper I, figure 5). In particular, gene expression in the pathways *"fatty acid biosynthesis"*, *"glycerolipid metabolism"*, *"biosynthesis of unsaturated fatty acids"*, and *"steroid biosynthesis"* was nearly identical to whole liver in slices, but lower in primary cells. Additionally, expression of several liver marker genes was maintained at levels resembling *in vivo* liver in PCLS culture while highly downregulated in primary hepatocytes.

3.2 Paper II

In paper II, we used comparative genomics to annotate lipid metabolism genes in Atlantic salmon. We then used this annotation in combination with a feeding trial to determine

how lipid metabolism in gut and liver changes across different feeds and life stages. We also identified signatures of selection pressure on salmon lipid metabolism gene duplicates by comparing lipid-related gene expression in salmon to their closest sister lineage, the pike.

3.2.1 Annotation of lipid metabolism genes

In order to enable genome wide identification of lipid metabolism gene expression trends, we curated a list of lipid metabolism genes based on 19 zebrafish KEGG pathways and other known regulators. Assignment of gene function using orthogroup prediction yielded 1421 salmon lipid metabolism genes, 23% of which had a 2:1 relationship with the zebrafish counterpart, corresponding to duplicates retained after the Ss4R WGD. We validated this novel annotation across 15 tissue expression profiles and found that our lipid metabolism genes were indeed expressed at an overall higher level in tissues known to have high lipid metabolic activity like liver, gut, heart, and brain.

3.2.2 Dietary effect across life stages

We reared Atlantic salmon on diets based on vegetable oil (VO) or fish oil (FO), then took samples of liver and gut for RNA sequencing in freshwater and again after transition to saltwater. Differential expression tests between diets revealed a higher proportion of DEGs in liver from freshwater fish (479 DEGs) than saltwater fish (405 DEGs) (paper II, figure 3a). This trend was more striking when subset by genes related to lipid metabolism, with 66 DEGs in freshwater and 31 DEGs in saltwater (paper II, figure 3b). Specifically, we found that cholesterol and PUFA biosynthesis were much more responsive (upregulated) to diet in freshwater than saltwater (paper II, figure 3c). In freshwater, *elovl5a*, *elovl4*, *Δ6fada*, *Δ5fad*, and nearly all cholesterol biosynthesis genes were upregulated by VO diet, whereas in saltwater only the desaturases and three cholesterol genes were significantly upregulated (paper II, figures 4 and 5).

3.2.3 Shifts in lipid metabolism between tissues and life stages

To further investigate life stage associated changes in lipid metabolism, we performed differential expression tests between freshwater and saltwater salmon fed the same diet. For lipid metabolism genes, we found most were downregulated in saltwater compared

to freshwater regardless of diet. In gut however, the opposite was seen, with more active expression in saltwater samples (paper II, figure 6b). Partitioning these genes into KEGG pathways revealed that in liver genes in pathways "*Fatty acid biosynthesis*", "*Glycerolipid metabolism*", "*Biosynthesis of unsaturated fatty acids*" were downregulated in saltwater, while in gut "*Steroid biosynthesis*" and "*Fat digestion and absorption*" were upregulated in saltwater (paper II, figure 6c and d).

3.2.4 Evolution of gene duplicates

We assessed the effect of duplicate retention on lipid metabolism by testing whether gene duplicates were retained in lipid pathways at a higher or lower rate than the average across all lipid pathways. We found that duplicates within the pathways "*Glycerophospholipid metabolism*", *"Bile secretion*", "*Glycerolipid metabolism*", and "*Fat digestion and absorption*" were retained at a high than average rates, while pathways "*Biosynthesis of unsaturated fatty acids*" and "*Steroid hormone biosynthesis*" were retained at lower rates (paper II, figure 7a). We also tested for overrepresentation of duplicate pairs with similar regulation across life stages and found several cases of higher or lower than average coregulation, but found no relationship between duplicate retention and duplicate coregulation. Finally, we identified potential markers for increased gene dosage after the Ss4R WGD by comparing gene expression of duplicates to the corresponding singleton in northern pike. We identified three lipid metabolism duplicates with increased gene dosage: *hadhab*, *elovl6*, and *elovl5*.

3.3 Paper III

In paper III, we took a deeper look at how lipid metabolism is regulated across life stages. Smoltification and seawater transfer were confounded in paper II, so it was not possible to tell if the observed shifts in lipid metabolism related to transition from freshwater to saltwater occurs prior to seawater transfer or is a response to seawater itself. To address this shortcoming, we designed a smoltification trial where salmon reared on diets high in EPA and DHA were induced to smoltify by exposure to shortened photoperiod and subsequently transferred to seawater, termed the "experimental" group. In this study we also included the effect of short photoperiod history (i.e. winter) as a factor (see paper III, figure 1 for full experimental design). We sequenced RNA from liver samples before

winter (week 1), immediately after winter (week 10), after smoltification in freshwater (week 19), and after transfer to seawater (week 25). Additionally, at each of these time points we prepared liver slices and fed them C14 labeled ALA to directly assess life stage associated changes in elongation and desaturation of PUFA. Unfortunately, the downstream products of ALA could not be detected using our LC-MS/MS method, so these results could not be included in this thesis.

3.3.1 Timing of lipid metabolism remodeling

ANOVA-like differential expression analysis yielded 6,054 genes differentially expressed at some point during the smoltification trial. Assigning these to coexpression clusters and then KEGG pathways revealed that nearly all lipid metabolism pathways decreased in expression after smoltification, but before transition to seawater. This was distinct from amino acid and carbohydrate metabolism, which decreased only after transition to seawater. Specifically, all key elongase and desaturase genes within the PUFA biosynthesis pathway, six *acsl* genes responsible for activation of FAs, and three *dgat* genes responsible for TAG synthesis were associated with smoltification, decreasing in expression between week 10 and week 19.

3.3.2 Photoperiod history and lipid-related gene expression

To determine the effect of having experienced 'winter' on lipid metabolism, we performed differential expression tests between winter control and experimental groups on weeks 10 and 19. On week 10, we identified 292 DEGs of which only 16 were lipid related. Included in these were phosphocarboxykinase 1 (*pck1*), the rate-limiting step in gluconeogenesis (-7.2 logFC); hormone sensitive lipase (*lipe*), responsible for releasing FAs from TAG (3.8 logFC); and apolipoprotein B-100 (*apob*), responsible for lipoprotein mediated export of TAG and cholesterol from the liver (1.14 logFC). By week 19, all salmon appeared to be smolts based on their silvery appearance. While this is not surprising since other studies have demonstrated that salmon can still smoltify without winter [66], we were surprised to find zero DEGs between experimental and control groups (figure 8).

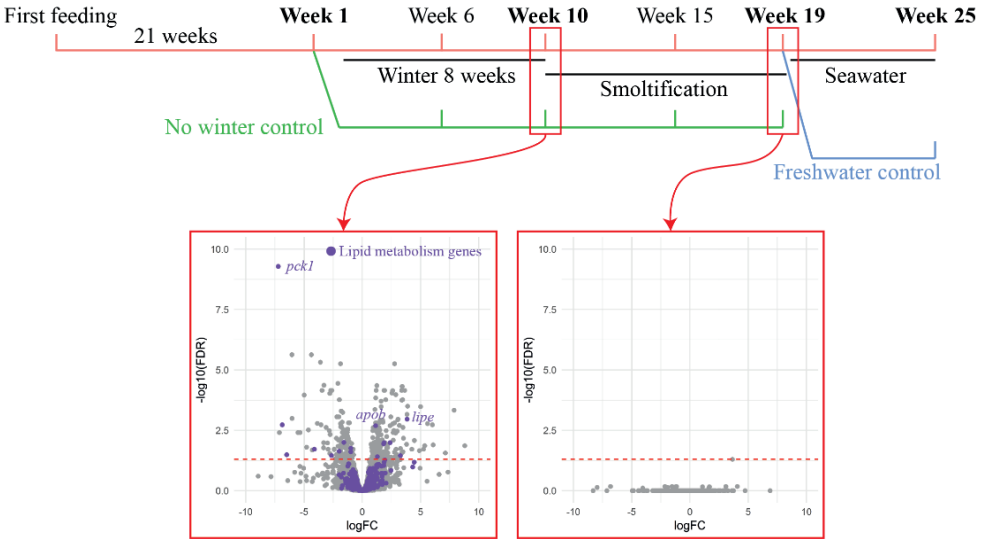

Figure 8: Differential expression between experimental and control groups in smoltification trial. Volcano plots from differential expression tests between experimental and control groups on weeks 10 and 19. For week 10, all lipid metabolism genes are colored purple. Points above the red dotted line have an FDR < 0.05.

3.3.3 Expression of epigenetic remodeling genes across life stages

We found that winter influenced the expression of several known epigenetic remodeling genes. Specifically, two *de novo* methyltransferases were significantly downregulated during winter then upregulated after smoltification. Additionally, two histone modifying genes, *p300* and *ezh1*, were significantly upregulated during winter. On the other hand, seawater transfer altered the expression of a different suite of epigenetic remodeling genes, including downregulation of the histone deacetylase *hdac1b* and upregulation of the histone demethylase *kmd6a*.

4 Discussion

4.1 Are liver slices useful for studying lipid metabolism?

In paper I, we carried out a series of experiments intended to provide much needed validation to the PCLS method for metabolic studies in fish. This included a time course experiment, a fatty acid and insulin gradient experiment, and an experiment directly comparing PCLS culture to primary hepatocyte culture. In general, we conclude that PCLS culture is suitable and for some purposes preferable to primary cell culture for studying lipid metabolism in Atlantic salmon. I outline the reasons for this conclusion below.

4.1.1 Culturing associated effects

The time course experiment revealed several direct effects of the culturing process, especially in the first three days after slicing. The observed early response (days 0-3) is likely due to the harsh slicing process and adaptation to the foreign culture environment. Studies in mice demonstrate that the first stages of liver regeneration involve upregulation of a variety of signaling pathways similar to what we see on day one in our experiment [67].

Some liver slice studies have shown slices to continue to grow in culture over time, increasing in thickness due to high cell proliferation [68]. Slices in our study, however remained approximately the same thickness through nine days. We interpret the combination of stabilization of metabolic gene expression and high viability as liver slices adapting to the culture environment after three days. For this reason, we recommend a three-day recovery period after slicing to ensure that the observed effects are the result of the experimental treatment rather than the culturing process itself.

4.1.2 Responsiveness of liver slices

Experiments supplementing media with ALA and insulin yielded a clear response. Measurements of 20:3n-3 increasing proportionally with ALA directly demonstrate that fatty acids from the media are taken up by the cells and enter the PUFA biosynthetic pathway. Furthermore, ALA led to up-regulation of PUFA biosynthesis gene expression in

a dose dependent manner in agreement with results from paper II and a decade's worth of research on the effect of vegetable oil based diets high in ALA [6,69].

Insulin is a hormone that is secreted by the pancreas during feeding that switches the metabolism from catabolic to anabolic so that energy storage molecules are not broken down while at the same time being built up [70]. Indeed, in liver slices insulin led to an anabolic shift in gene expression even at the lowest concentration. Pathways relating to fatty acid and PUFA biosynthesis were largely upregulated while a suite of other catabolic genes was downregulated as expected. Taken together, these observations are in line with known Atlantic salmon lipid biology, providing convincing evidence that liver slices retain their liver-like properties even after five days in culture, making liver slice culture a valid proxy for *in vivo* lipid metabolism.

We took this a step further and identified an unreported interaction between insulin and ALA where several desaturases were upregulated by both ALA and insulin and downregulated when the two were combined. Additionally, *elovl5* genes were only upregulated by insulin and not by ALA. Taken together, these two observations suggest a conflict between nutritional and hormonal regulatory networks, with *Δ5fad* and *Δ6fada* regulated by both ALA and insulin and *elovl5a* and *elovl5b* exclusively regulated by insulin. This demonstrates the utility of liver slice culture in unraveling the complexity of signaling networks governing lipid metabolism in liver.

4.1.3 Performance relative to whole liver and primary cell culture

We show that gene expression in liver slice culture is consistently more similar to *in vivo* liver than primary hepatocyte culture across a number of pathways and genes. This is probably because liver slices are a step up from hepatocyte culture in terms of complexity since they maintain the three-dimensional structure of the liver will multiple interacting cell types. This complexity is important since hepatocytes primarily house the biochemical conversion properties of liver, but other processes like tumor suppression, filtering, and matrix formation are partitioned to non-parenchymal cells [71]. Additionally, eicosanoids influence gene expression by transmitting signals between different cell types in the liver and are exclusively produced in non-parenchymal cells [72]. These effects are completely lost in primary hepatocytes and likely contribute to

transcriptomic drift away from an *in vivo* expression phenotype. These results provide a strong case for the use of liver slice culture to study lipid metabolism *ex vivo*.

4.2 Shifts in lipid metabolism across environments

In paper II, we observe a decrease in the number of lipid metabolism DEGs between fish fed VO and FO diets after salmon transition to sea. This agrees with results from Bell et. al. (1997) [47] who measured desaturation rates of ALA before and after seawater transfer and found that desaturation was more active in VO versus FO fed fish in freshwater than in saltwater. Together, this suggests a diminished capacity of Atlantic salmon to regulate lipid metabolism in response to diet after seawater transition. We also observed a life stage associated shift in lipid metabolism-related gene expression in liver and gut that was independent of diet. Lipid biosynthesis related gene expression decreased in liver while digestion and absorption related gene expression increased in gut after seawater transfer. In nature, freshwater salmon subsist mostly on flies and other insects that are low in EPA and DHA and higher in shorter chain ALA [41,73], so the ability to regulate PUFA biosynthesis is important. At sea, however, salmon are flooded with consistent high levels of EPA and DHA from a diet of marine fish and crustaceans, so PUFA absorption in gut becomes more critical. We thus hypothesize that anadromous salmonids have evolved this regulatory dichotomy of lipid metabolism between fresh- and saltwater environments as an adaptation to increase fitness in freshwater and minimize energy expenditure in saltwater environments.

4.3 Lipid metabolism and smoltification

4.3.1 Genome wide remodeling of lipid metabolism

Atlantic salmon are known to transform their fatty acid composition before transfer to seawater [45,48], but the extent of this lipid metabolism transformation is unknown. Paper II demonstrates that remodeling of lipid metabolism-related gene expression between fresh- and saltwater is a genome wide trend, but since we did not sample smolts in freshwater, we could not say anything about the timing of this transformation. Therefore, in paper III we were sure to take samples before smoltification, after smoltification in freshwater, and after seawater transfer. We show that genome wide lipid

metabolism-related gene expression decreases while the fish are still in freshwater, and this trend was distinct from other aspects of metabolism like carbohydrate and amino acid metabolism (figure 9). This means that the observed changes in lipid metabolism are the result of coordination between hundreds of genes after smoltification. Since seawater environments are low in shorter chain ALA and high in DHA and EPA, this supports the idea that lipid metabolism is linked to smoltification and remodeled in preparation for life at sea.

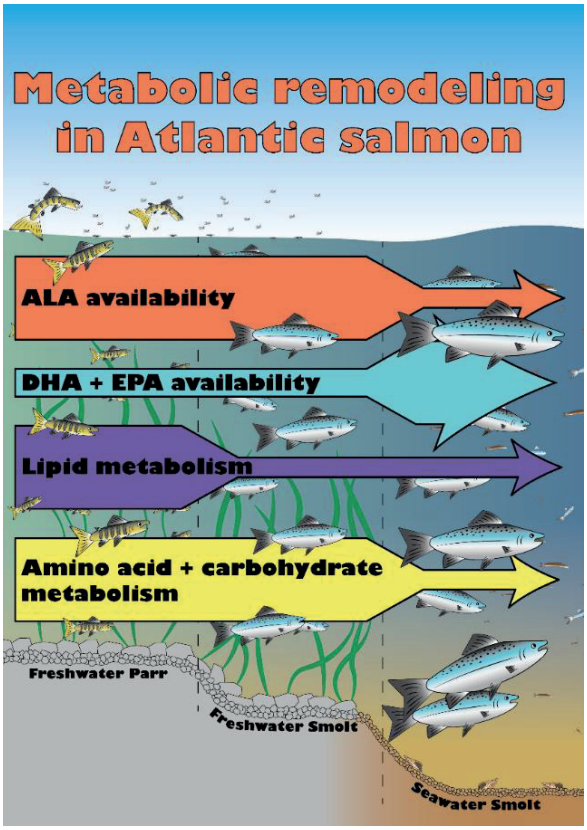

Figure 9: Metabolic remodeling in Atlantic salmon. Lipid metabolism is remodeled in liver of salmon after smoltification and before migration to sea. Lipid related gene expression decreases, especially in anabolic pathways like FA and PUFA biosynthesis. Other metabolic pathways like amino acid and carbohydrate metabolism are remodeled only after seaward migration. Diet also changes from primarily insect based high in ALA in rivers to fish and crustacean based high in EPA and DHA at sea, so lipid metabolism remodeling is likely a preparatory physiological adaptation to life at sea.

4.3.2 The effect of photoperiod on lipid metabolism

Having experienced a period of shortened photoperiod (i.e. similar to a winter) is well documented to improve salinity tolerance of Atlantic salmon entering seawater [74,75], acting as an external cue to coordinate the smoltification process [76]. Since we demonstrate in paper III that lipid metabolism in liver is also transformed during smoltification, we naturally wondered whether winter was modulating this effect as well.

We found a few hundred DEGs between fish experiencing short or long photoperiods eight weeks into winter (before smoltification), but just nine weeks after the short photoperiod exposure (after smoltification) there was no clear difference between the liver transcriptomes. This convergence in gene expression could mean one of two things: 1) the short photoperiod exposure does change the liver lipid metabolism but we cannot measure this at the transcript level or 2) photoperiod history is not influencing liver lipid metabolism of later life stages.

4.3.3 Possible molecular mechanisms

Much research has been done on the physiological and environmental drivers of smoltification, but the underlying molecular mechanisms driving changes in gene expression are still unknown. Other studies have associated epigenetic changes with migratory phenotypes [77] and seawater acclimation [78] in salmonids, leading us to look for epigenetic remodeling genes differentially expressed in our study. We found several epigenetic remodelers, including several DNA methyltransferases and histone acetyl- and methyl- transferases, associated with shortened photoperiod. These epigenetic genes could be modulating the expression of other genes differentially expressed during short photoperiod, but likely do not contribute to the liver metabolism smoltification phenotype since there were no DEGs between these groups on week 19. We also found several epigenetic remodelers differentially expressed after seawater transfer. Specifically, the genes we found are responsible for removal of epigenetic marks and could provide evidence that gene expression changes after seawater transfer are modulated to some extent by removal of epigenetic marks added to histones earlier in the life cycle. Since these observations are only based on a few genes at the transcript level, they are far from proof that epigenetic remodeling is important in driving the smoltification process at the molecular level, but they do open interesting avenues for future research surrounding epigenetics, photoperiod, and seawater transfer.

4.4 Evolution of lipid metabolism following WGD

Our findings that some pathways had higher than average duplicate coregulation could have implications into the ability of ancestral salmonids to adapt to freshwater environments low in dietary LC-PUFA. Specifically, increased transcript dosage of *elovl5*

and generally higher coregulation of gene duplicates in PUFA biosynthesis and cholesterol biosynthesis pathways compared to random gene duplicates could mean that there has been selective pressure to maintain two functioning copies in these pathways. It is plausible that this has resulted in a fitness advantage due to the salmon to having improved ability of endogenous production of DHA and EPA from shorter chain PUFA in freshwater ecosystems low in LC-PUFA.

5 Future perspectives

By combining transcriptomics with large scale feeding trials and advanced cell culture techniques we have contributed novel resources and insights for future lipid metabolism research in salmon. PCLS culture shows much promise as a high throughput platform for interrogating metabolic pathways in a more biologically relevant environment compared to traditional techniques. One promising application for PCLS culture is in computational biology. The digital salmon project here at NMBU aims to generate an explanatory metabolic model to allow *in silico* testing of different feeding conditions. High quality models require iterative testing under a wide variety of experimental conditions, a task that is prohibitively expensive, time consuming, and would require sacrificing a large number of fish. This could instead be accomplished cheaply and quickly by feeding liver slices experimental diets containing novel feed ingredients and monitoring the impact on metabolism.

Our work on transcriptome wide changes across life stages and tissues is an important step towards a genome wide understanding of lipid metabolism in salmon. Yet, many important aspects of the molecular regulation of salmon lipid metabolism remain unknown or poorly understood. Lipid metabolism is regulated through a variety of allosteric and post-translational mechanisms as well [18], so both protein and metabolite measurements are required to illuminate these aspects of regulation. For example, an experiment where liver slices are fed radiolabeled ALA to measure metabolic flux at different points in the smoltification gradient could uncover life stage associated differential elongation and desaturation activities. Based on findings in papers II and III, we would expect EPA and DHA production to be high in freshwater parr and then decrease in freshwater smolts. This in combination with protein measurements using antibodies to Δ6FADa, Δ5FAD, and ELOVL5, would add another important layer to the smoltification associated metabolic remodeling story.

For a truly genome wide understanding of how lipid metabolism is regulated, epigenetic measurements at different life stages are required. In paper III, we provide evidence that expression of epigenetic remodeling genes are modulated by short photoperiod and seawater transfer. An experiment measuring the state of the epigenome at these points

would provide insight into the regulatory mechanisms driving gene expression changes during winter and seawater transfer.

This work brings us one-step closer to a systems level understanding of lipid metabolism transcriptional regulation in Atlantic salmon. If we are to provide sustainable nutritious salmon high in essential omega-3s to 10 billion people, we need to continue down this path. Future researchers will harness the knowledge we have accumulated to design data driven feeding and breeding strategies that improve the omega-3 content of farmed salmon fed sustainable aquafeeds.

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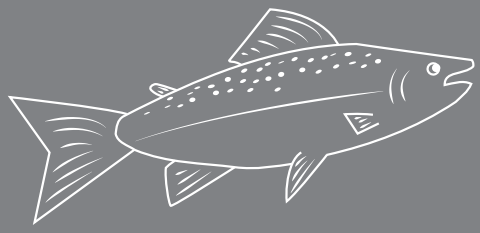
Papers I-III

Paper I

Thomas N. Harvey, Simen R. Sandve, Yang Jin, Jon Olav Vik, Jacob S. Torgersen (2019) **Liver slice culture as a model for lipid metabolism in fish.** Manuscript.



Liver slice culture as a model for lipid metabolism in fish

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Abstract

Hepatic lipid metabolism is traditionally investigated *in vitro* using hepatocyte monocultures lacking the complex three-dimensional structure and interacting cell types essential liver function. Precision cut liver slice (PCLS) culture represents an alternative *in vitro* system, which benefits from retention of tissue architecture. Here we present the first comprehensive evaluation of the PCLS method in fish (Atlantic salmon) and validate it in the context of lipid metabolism using feeding trials, extensive transcriptomic data, and fatty acid measurements. We observe an initial period of post-slicing global transcriptome adjustment, which plateaued after three days in major metabolic pathways and stabilized through nine days. PCLS fed alpha-linolenic acid (ALA) and insulin responded in a liver-like manner, increasing lipid biosynthesis gene expression. We identify interactions between insulin and ALA, where two PUFA biosynthesis genes that were induced by insulin or ALA alone, were highly down-regulated when insulin and ALA were combined. We also find that transcriptomic profiles of liver slices are exceedingly more similar to whole liver than hepatocyte monocultures, both for lipid metabolism and liver marker genes. PCLS culture opens new avenues for high throughput experimentation on the effect of "novel feed composition" and represent a promising new strategy for studying genotype-specific molecular features of metabolism.

1 Introduction

Liver is the metabolic transformation hub. It is responsible for receiving nutrients absorbed in the gut through the portal vein, processing these nutrients for storage or energy production, and subsequent transportation of metabolic products throughout the body. Essential to proper function, the complex three-dimensional structure of liver consists of intrahepatic microcirculatory units (lobules) of tightly associated cells that communicate through paracrine and autocrine effects [1]. The liver is also the main organ for detoxification, so *in vitro* methods are commonly applied for toxicological studies to reduce use of *in vivo* experiments. Hepatocyte cultures were established in the 1970s [2], and rapidly became the preferred model system for toxicology. Liver slice culture was first introduced in 1923 [3], but seldom used due to a lack of reproducibility since slices needed to be cut by hand. The development of automated tissue slicers in the 1980s [4] solved this problem, so liver slices became a viable option. One of the main advantages of liver slices is the retention of normal cell composition and 3D structure. In addition, the preparation is fast, reproducible, without enzymatic cell dissociation, and no need for coating the growth surface. Together with established protocols, this has heralded the return of liver slices for *in vitro* studies.

Precision cut liver slice (PCLS) cultures have been applied in a number of toxicology studies and most recently also immunology [5], however the use of PCLS to study central liver metabolism is sparse, with few PCLS studies investigating aspects of lipid metabolism, all of which are in mammals [6–9]. We provide a critical evaluation of PCLS as a metabolic model system in fish by characterizing whole transcriptome changes in the context of lipid metabolism. We chose Atlantic salmon for its economic importance; and because development of feeding and breeding strategies that optimize omega-3 production require a better understanding lipid metabolism. Additionally, the effect of altered feed fatty acid profile on liver gene expression is well documented [10–12] making this an ideal system for assessing the effects of altering media fatty acid composition and comparing to expected *in vivo* gene expression.

Here we integrate transcriptomics data with domain knowledge to describe a method for using PCLS as a model system to study lipid metabolism. We aim to 1) characterize transcriptome wide changes in liver slice culture over time, 2) demonstrate the utility of

using liver slice culture to study lipid metabolism, and 3) compare gene expression patterns between liver slice culture, 2D hepatocyte culture, and whole liver *in vivo*.

2 Results

2.1 Viability and morphology

Liver slices were highly viable in all experiments with a mean viability of 90.3±2.7% (figure 1B). We did not observe any viability effects of ALA, insulin nor methyl-β-cyclodextrin (BCD) used as a lipid carrier in the experiments. More generally, across all liver slice experiments performed to date (data not shown) we find that viability at the end of an experiment is similar to the viability at the beginning of an experiment. This implies that preparation of the slices is most critical to viability, as opposed to culture time. Morphological analysis of liver slice cross-sections did not reveal any increase in the thickness or decrease in cell density of slices over a five day period (figure 1C).

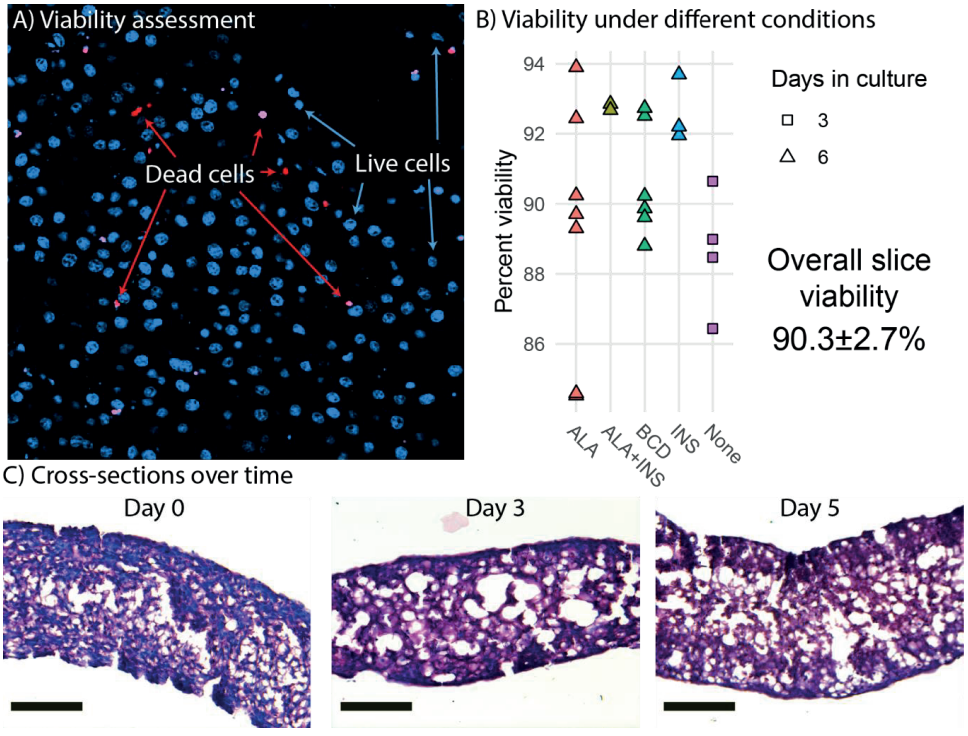

Figure 1: Viability and morphology of liver slices. (A) Confocal scanning laser microscope (CLSM) image of a liver slice. Cells are stained with Hoechst (blue) and dead cells with propidium iodide (red). (B) Cell viability when incubated in media (none) and media supplemented with alpha-linolenic acid (ALA), insulin

(INS), empty methyl-β-cyclodextrin (BCD), or a combination as measured by CLSM live/dead counts. (C) Cross sections of paraffin embedded liver slice sampled at day zero (immediately after slicing), day three, and day five. The sections were stained with hematoxylin and eosin and photographed using light microscopy at 20x magnification. Scale bars are 100μM in length.

2.2 Time Course experiments

In order to study how the liver slices change in culture over time, we sequenced RNA from three experiments lasting for nine days. In time course one, media was changed every three days and samples were taken before slicing (day 0) and 1, 3, 4, 5, 6, 7, 8, and 9 days after slicing. On day three, slices were fed a control diet consisting of empty BCD. We use BCD to deliver the FAs to the cells, so in this case empty BCD was used as a control for FA supplementation conditions. The second and third time course experiments differed from the first in terms of media change frequency (daily or every third day) and inclusion of insulin in the media (20nM).

To characterize the behavior of liver slices over time under control conditions, we performed ANOVA-like differential expression analysis testing for differentially expressed genes between any of the time points in time course one. This yielded 16,267 differentially expressed genes (DEG) with a false discovery rate (FDR) < 0.01 and a log2 fold change (log2FC) >1 (figure 2A). We used hierarchical clustering to group genes with a similar expression trend into eight gene clusters (figure 2B), then searched for enriched pathways from the Kyoto encyclopedia of genes and genomes (KEGG) in each of these clusters ($p < 0.0001$) to characterize the overall trend of various physiological and metabolic processes (figure 2C). Interestingly, almost all pathways related to protein, lipid, carbohydrate and vitamin metabolism belong to clusters two and three, which decreased between day zero (before slicing) and day three, followed by an overall stabilization in expression through day nine. Pathways related to signal transduction were mostly enriched in clusters seven and eight, which increased expression greatly between day zero and day one (before and 24 hours after slicing), then decreased to original levels by day 9. Pathways related to cell growth and death were mostly enriched in clusters four, five, and six, which in general increased during 9 days of liver slice culture.

Since cell culture aims to mimic the conditions and behavior of tissue *in vivo*, we compared gene expression patterns between whole liver and liver slices for all three

experiments. To assess the similarity in expression patterns over time we calculated Spearman co-expression correlations between mean whole liver gene expression and gene expression from each day in three time course experiments for all genes and genes within seven relevant lipid metabolism pathways (figure 3). For time course one, correlation between liver slices and whole liver decreased gradually over time from 0.90 on day 1 to 0.83 on day 4, then stabilized around 0.8 through day 9. A similar effect was observed in time course two and time course three with co-expression correlation to whole liver stabilizing around 0.82 through day 8 then decreasing to 0.78 and 0.79 respectively on day 9 (figure 3). The greatest difference between whole liver and liver slices was in the pathway "*Steroid biosynthesis*" with co-expression correlations hovering between 0.48 and 0.28 during days three through nine for all three experiments. Co-expression correlation was slightly more stable over time when media was refreshed daily, especially "*Steroid biosynthesis*"; however, overall expression similarity to whole liver was high for nearly all pathways and time points examined.

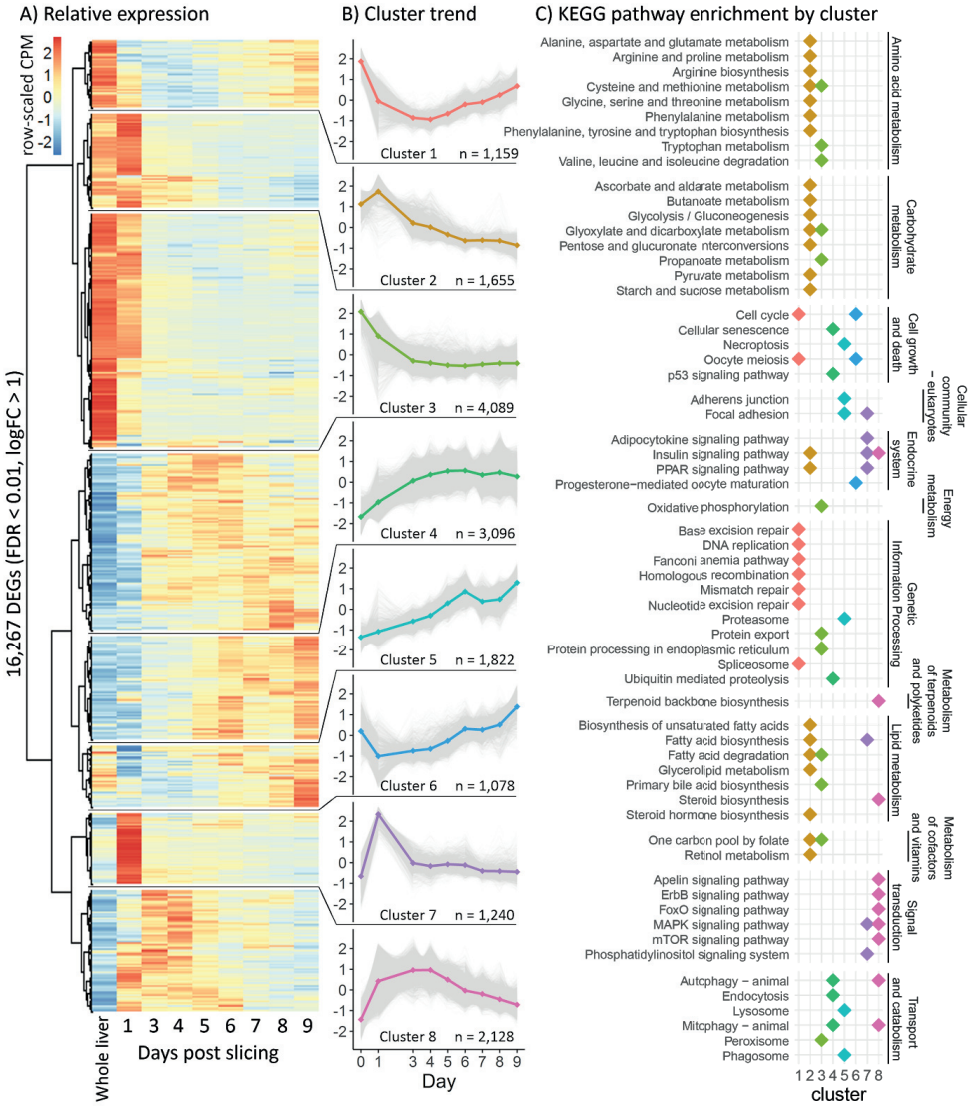

Figure 2: Global gene expression patterns over time. A) Heatmap showing changes in the liver slice transcriptome over time. Heatmap includes 16,267 genes significantly differentially expressed (FDR <0.01, log2FC >1) over the course of 9 days. Transcript abundance is expressed in counts per million and were individually scaled across days to highlight changes in gene expression. B) Genes behaving similarly over time were clustered using Ward's method and broken into eight groups. Trend lines are based on mean scaled values in each cluster. C) KEGG pathway enrichment analysis was run on each cluster to determine how the liver slices are changing over time. Each point represents a significantly enriched pathway (p <0.001).

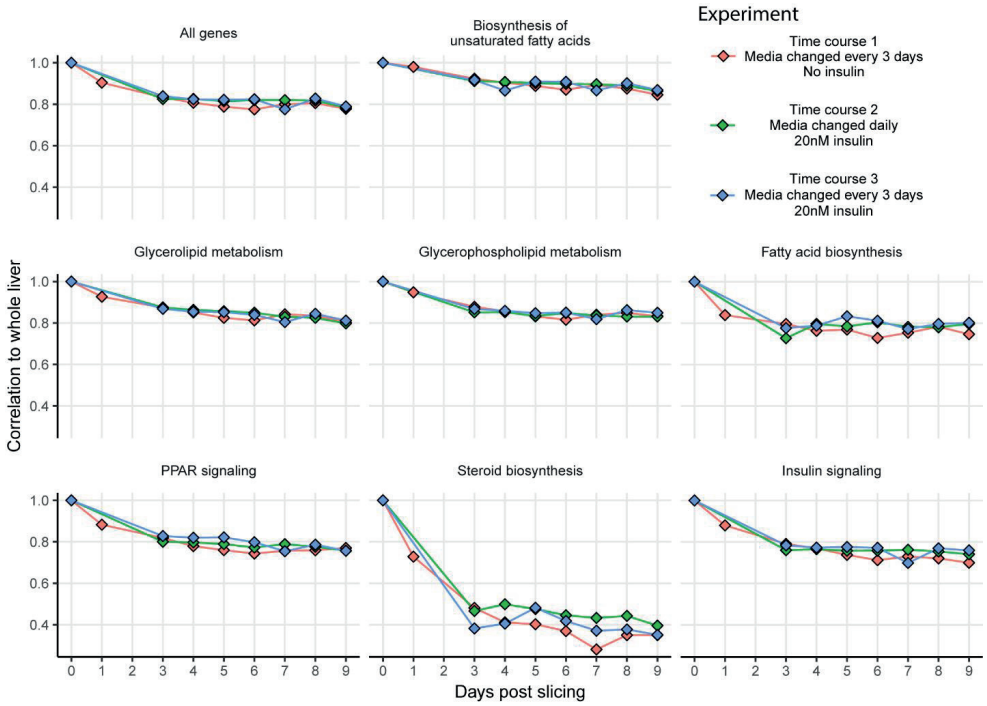

Figure 3: Co-expression correlation of liver slices over time for select lipid metabolism pathways. Co-expression correlations (Spearman) between mean whole liver expression and gene expression from different days in time course one (red), time course two (green), and time course three (blue). Experiments were divided by insulin and media change regime. Time course two and three contained insulin, time course one and three had media changed every three days, and time course two had media changed daily. Correlations were calculated for all expressed genes (top left) and genes from seven lipid metabolism pathways.

2.3 Fatty acid and insulin gradient experiments

In order to evaluate fatty acid uptake and transcriptomic response in liver slices, we added alpha-linolenic acid (ALA) to the media in increasing concentrations from 20μM up to 100μM. We expect this to trigger upregulation of lipid metabolism-related gene expression as observed in liver of fish fed vegetable oil diets high in ALA [10]. ANOVA-like differential expression analysis testing for differences between any of the ALA concentrations yielded 8,282 DEGs (FDR < 0.01, figure S1A). We then broke these DEGs into four expression clusters as previously explained (figure S1B). KEGG enrichment analysis on these four clusters yielded 37 total pathways significantly enriched ($p < 0.001$) in one or more cluster (figure S1C). We found that all enriched pathways relating

to lipid metabolism belonged to the same cluster which increased with increasing ALA concentration, especially between 40 and 70μM. Specifically, the pathways *"biosynthesis of unsaturated fatty acids"*, *"fatty acid degradation"*, *"glycerolipid metabolism"*, *"steroid biosynthesis"*, and *"PPAR signaling pathway"* were all enriched in this cluster (figure S1B and C).

In order to better characterize the effect of ALA supplementation on PUFA biosynthesis, we analyzed individual gene expression of key genes in the PUFA biosynthesis pathway (figure 4). The five key genes involved in PUFA biosynthesis that are differentially expressed at some point in the ALA concentration gradient include delta-5 desaturase (*Δ5fad*), delta-6 desaturase a (*Δ6fada*), fatty acid elongase 2 (*elovl2*), fatty acid elongase 5a (*elovl5a*), and fatty acid elongase 5b (*elovl5b*). All five genes displayed an overall positive correlation with ALA concentration (figure 4B) with *Δ5fad*, *Δ6fada*, and *elovl2* responding strongly to ALA between 40μM and 70μM and both *elovl5* genes less influenced, slightly increasing with increasing ALA concentration. Counterintuitively, at low ALA concentration (20μM) all genes except *elovl2* and *elovl5a* significantly decreased ($q<0.05$) in expression compared to control samples (no fatty acid). Between 70μM and 100μM ALA, expression of *Δ5fad*, *Δ6fada*, and *elovl2* did not significantly change (figure 4B).

To assess the impact of ALA supplementation (up to 140μM) on the fatty acid profile of liver slices we conducted a second ALA concentration gradient experiment. As expected, percent ALA increased with increasing media ALA concentration from 0.87% with no ALA supplementation to 6.5% with 140μM ALA supplementation (figure 4D). In addition, the elongation product of ALA, 20:3n-3, increased with increasing media ALA concentration from 0.35% with no ALA supplementation to 2.7% with 140μM ALA supplementation (figure 4D). EPA and DHA levels do not significantly ($q<0.05$) change at any point in the ALA gradient (figure 4D). There was a large difference in proportions of 18:0, EPA, and DHA between fresh liver and liver slices after four days of incubation (table S1). 18:0 doubled, increasing from 5.8% in fresh liver to 12.3% in liver slices. Both EPA and DHA decreased in liver slices, from 6.4% to 3.5% and 29.6% to 25.1%, respectively (table S1).

The effect of insulin supplementation on liver slices was assessed by incubating slices with two different concentrations of insulin, 10nM and 100nM. To test for an interaction between insulin and fatty acid supplementation, we also tested these insulin levels with

and without supplementation of 70μM ALA. Differential expression analysis testing for changes in expression between any of the conditions (without ALA supplementation) yielded 11,898 DEGs (FDR <0.01, figure 5A). Approximately half of these genes were upregulated (5,889 DEGs) and half were downregulated (6,012 DEGs) regardless of insulin concentration (figure S2B). Only 13 genes were differentially expressed between the two insulin concentrations. KEGG pathway enrichment on these gene clusters revealed that most metabolism related pathways were upregulated with the addition of insulin. Specifically relating to lipid metabolism, "*biosynthesis of unsaturated fatty acids*", "*fatty acid biosynthesis*", and "*PPAR signaling pathway*" were significantly enriched in the upregulated gene set (figure S2C). Pathways related to metabolism enriched in the downregulated gene set included "*glycerophospholipid metabolism*", "*inositol phosphate metabolism*", and interestingly "*insulin signaling pathway*" (figure S2C).

Insulin supplementation alone tended to increase expression of key PUFA biosynthesis genes except for *Δ5fad*, which did not significantly (q<0.05) change with increasing insulin concentration (figure 4C). Increasing insulin concentration from 10nM to 100nM did not significantly change the expression of any of the five genes. Addition of 70μM ALA had a large effect on the expression of *Δ5fad* and *Δ6fada*, which were expressed most in the absence of insulin, then downregulated upon insulin supplementation. ALA supplementation did not appear to have a large effect on the expression of *elovl5a* and *elovl5b*, which agrees with findings from the ALA gradient experiment.

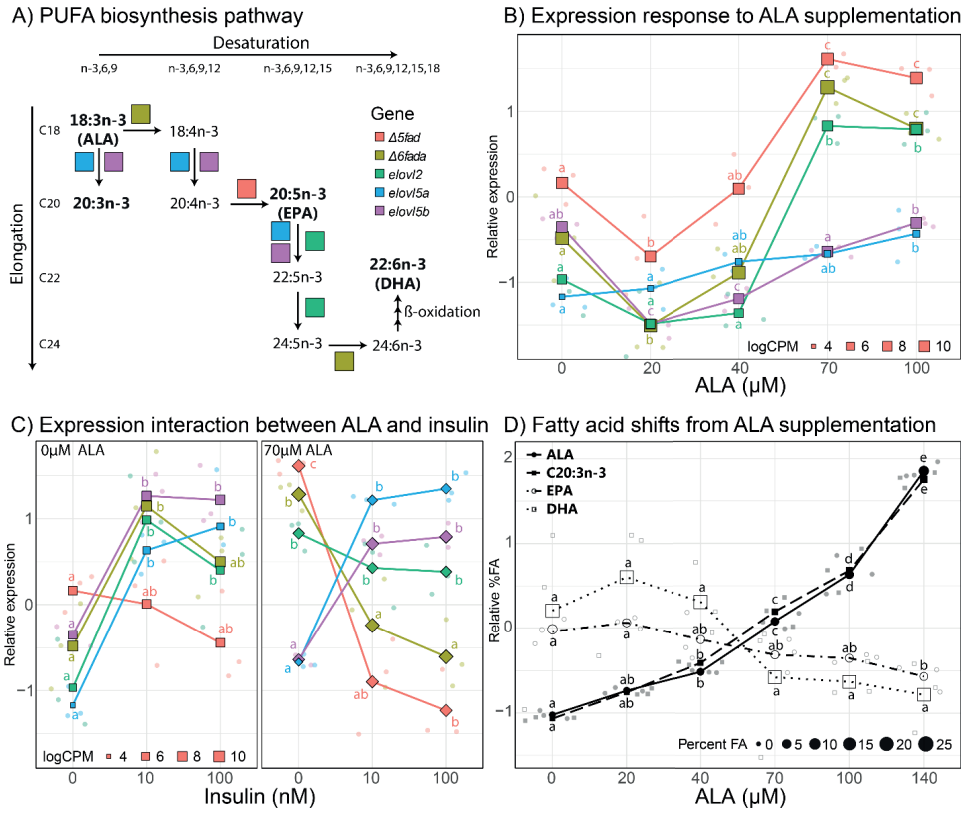

Figure 4: Effect of ALA and insulin on PUFA biosynthesis in liver slices. A) Schematic diagram of the PUFA biosynthesis pathway. B) Gene-scaled log counts per million (CPM) of PUFA biosynthesis genes with increasing ALA concentration. C) Gene-scaled logCPM of PUFA biosynthesis genes with increasing insulin concentration with and without ALA supplementation. D) Relative abundance of ALA, 20:3n3, EPA, and DHA with increasing ALA concentration. For all plots, large square, diamond, or circle points show mean scaled values (logCPM or percent FA) while small points show scaled values of individual replicates. Point size corresponds to unscaled values (logCPM or percent FA) of the mean. Letters indicate significant ($q<0.05$) differences between groups (ALA or insulin concentration) for corresponding genes or FAs.

2.4 Liver slice culture versus primary cell culture

To assess how liver slice culture compares to widely used hepatocyte culture and liver *in vivo*, we compared RNA sequencing data from hepatocyte culture (n = 16), liver slice culture (n = 89), and whole liver (n = 210). Hepatocytes were sampled after 5, 6, and 8 days in culture, so only liver slices incubated at least 5 days were used for comparison. Data on whole liver was obtained from a feeding trial where salmon were fed either a fish oil or plant oil based diet [10]. All data from each source was pooled to give a range of possible expression patterns from that source.

We find that overall, liver slice culture more closely resembles whole liver than hepatocyte culture. Gene expression in the lipid related KEGG pathways *"fatty acid biosynthesis"*, *"glycerolipid metabolism"*, *"biosynthesis of unsaturated fatty acids"*, and *"steroid biosynthesis"* was much lower in hepatocyte culture relative to both liver slice culture and whole liver (figure 5A). This was reflected in the expression of all underlying key PUFA biosynthesis genes except *elovl5a* (figure 5B). Expression of genetic marker genes characteristic of functional liver was generally lower in hepatocyte culture, while liver slice culture was closer, but not identical to whole liver expression patterns (figure 5C). Specifically, albumin genes *alb1*, *alb2-1*, and *alb2-2* and glucose-6-phosphatase genes *g6pcl-3* and *g6pcl-4* had lowest expression in hepatocyte culture followed by liver slice culture and highest expression in whole liver (figure 5C).

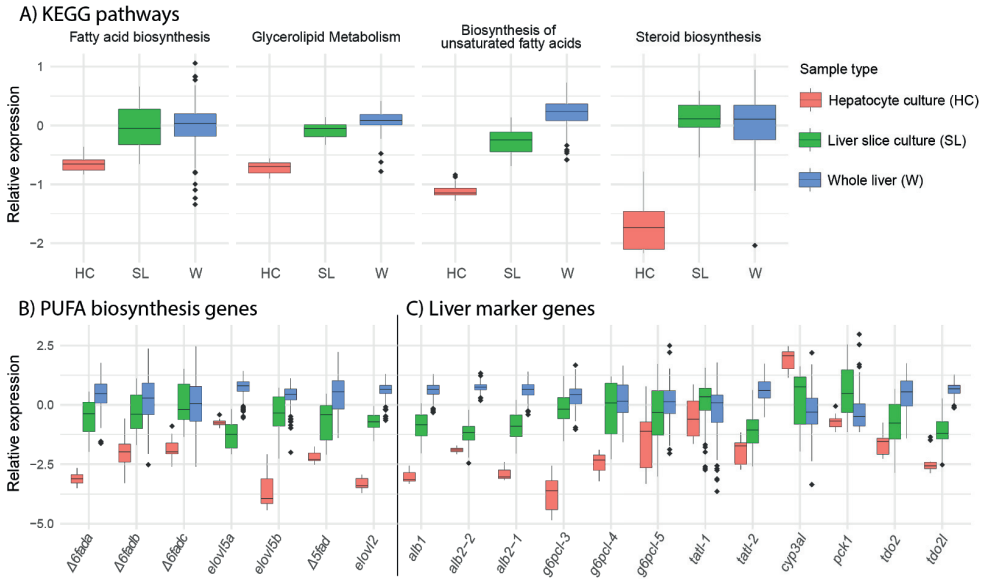

Figure 5: Expression of select pathways and genes in hepatocyte culture, liver slice culture, and whole liver. A) Relative expression profiles for five selected lipid metabolism pathways. Values are expressed mean scaled log2 CPM of all genes within a pathway for each sample with a minimum CPM of 1. B) Relative expression of key genes in the PUFA biosynthesis pathway with a minimum CPM of 10. Abbreviations: *Δ6fad* = delta-6 fatty acid desaturase, *elovl5* = fatty acid elongase 5, *Δ5fad* = delta-5 fatty acid desaturase, *elovl2* = fatty acid elongase 2. C) Relative expression of select liver marker genes with a minimum CPM of 10. Abbreviations: *alb* = albumin, *g6pcl* = glucose-6-phosphatase-like, *tatl* = tyrosine aminotransferase-like, *cyp3al* = cytochrome P450 3A27-like, *pck1* = phosphoenolpyruvate carboxykinase 1, *tdo2* = tryptophan 2,3-dioxygenase, *tod2l* = tryptophan 2,3-dioxygenase-like.

3 Discussion

3.1 Liver slice metabolism stabilizes after three days and remains liver-like through nine days in culture

We find that time, up to 9 days tested, does not have a large effect on the viability or thickness of the slices. This is encouraging, since morphometric analysis of Atlantic cod liver slices showed an increase in the proportion of dead cells at 72 hours in culture [13] and studies on rat liver slices have shown that changes in viability and slice thickness over time is highly dependent on the culture media used [14].

We do, however, observe a time dependent drift in gene expression patterns. Slices most resemble whole liver 24 hours after slicing with a correlation coefficient of 0.90 and gradually decrease in similarity over time (figure 3). A similar effect has been observed in rat liver slices [15], however, the rate that slices diverged from whole liver was much lower in our experiments. High correlation (>0.8) to whole liver was maintained through day four in time course one and through day six in time courses two and three. Genes that are highly upregulated 24 hours after slicing were mostly enriched in signaling pathways (figure 2), likely related to repair and inflammatory response processes known to be triggered by physical liver damage that is unavoidable during the slicing process [16]. Since metabolic gene expression stabilizes after three days (figure 2, clusters 2 and 3), we used a three day recovery period for future metabolic studies so that changes in gene expression are more likely to be the result of the treatment rather than time. The gradual downward trend in co-expression correlation over time represents a slow drift in the global gene expression phenotype as opposed to a rapid gene expression change upon hepatocyte culturing. This is a known problem with hepatocyte cultures resulting from a combination of factors, especially the lack of circulating hormones produced elsewhere in the body causing time-dependent de-differentiation of hepatocytes [17]. Expression of liver marker genes was markedly higher in our liver slice culture than 2D hepatocyte culture (figure 6C) representing an improvement in long-term hepatocyte stability.

3.2 Exogenous ALA is taken up and triggers a liver-like response

ALA complexed with BCD was efficiently delivered to cells in a dose dependent manner. We observed a proportional increase in ALA and 20:3n-3 with increasing ALA concentration. While the ALA increase could be due to residual ALA sticking to the cells

from the media, the proportional increase in 20:3n-3 with media ALA concentration supports active uptake and elongation of exogenous FAs (figure 4D). We did not observe any adverse effect of BCD on liver slices in any of our experiments, establishing the suitability of this delivery system for use in PCLS studies. There was no significant change in EPA or DHA after ALA supplementation. This does not mean that EPA and DHA are not being produced, but rather that the amount of ALA in the media is too low to cause a measurable increase in the already abundant pool of EPA and DHA in the cells. Indeed, both ALA and 20:3n-3 are low (0.88% and 0.35% respectively) in control slices, so a small increase in abundance could be detected.

ALA fed to slices has two fates within the PUFA biosynthesis pathway. The first and most common is the canonical pathway, where ALA is first desaturated by a Δ6 desaturase to 18:4n-3, then elongated and desaturated to EPA and DHA via Sprecher's shunt [18]. The second occurs when ALA is first elongated to 20:3n-3, presumably by ELOVL5. In this case, a Δ8 desaturase is required to form 20:4n-3, which can then continue to EPA and DHA via the canonical pathway. This does however not happen efficiently in Atlantic salmon because of the low Δ8 desaturase activity of Δ6FADb [19] in combination with low expression in liver (0.5-3.2 counts per million). Rather, 20:3n-3 accumulates in the cells or is catabolized for energy [20], which is consistent with observations in feeding trials where fish fed vegetable oil based diets high in ALA contained higher tissue levels of 20:3n-3 [12,21]. This can explain why we measure increased levels of 20:3n-3, but not other PUFA intermediates.

We also observe that saturated fatty acids 16:0 and 18:0 increase between whole liver and liver slices. This agrees with the transcriptomic data where both fatty acid synthase genes, *fasa* and *fasb,* increased in expression one day after slicing and the pathways "*fatty acid biosynthesis*" and "*glycerolipid metabolism*" were enriched in clusters that spike one day after slicing (clusters two and seven, figure 2C). Immediately after slice preparation, the tissue must recover by cellular repair and proliferation, which means there is a need for cell membranes that are composed of phospholipids rich in 16:0 and 18:0. These are synthesized *de novo* from fatty acid synthase and then incorporated into phospholipids through the glycerolipid and glycerophospholipid metabolism pathways [22].

Overall, ALA concentration was positively correlated to lipid metabolism related gene expression, especially in PUFA biosynthesis with expression of all key pathway genes

increasing with ALA. The same effect is known to occur in Atlantic salmon livers where fish fed vegetable oil-based diets high in ALA have higher PUFA biosynthesis gene expression relative to salmon fed fish oil-based diets low in ALA and high in EPA/DHA [10]. Additionally, this has been observed *in vitro* using Atlantic salmon primary hepatocytes [23] and *in vivo* on rat liver [24]. At very low concentration (20μM), expression of *Δ5fad*, *Δ6fada*, and *elovl5b* actually decreased relative to control slices with no ALA supplementation. In this experiment samples were taken two days after exposure to ALA, so it is possible that in 48 hours all of the ALA in the media was depleted, presumably taken up by the cells and anabolized to longer chain FA products that have an inhibitory effect on expression. Additionally, the "*PPAR signaling pathway*", which includes PPARs and target genes, was significantly enriched in cluster three which increases with increasing ALA concentration (Figure S1). PPARs are well known transcriptional factors that bind FAs and in turn activate genes involved in a wide range of cellular functions, most notably lipid metabolism [25]. Taken together our results demonstrate the ability of our PCLS model to accurately mimic expected shifts in lipid metabolism genes, highlighting its quality as an *in vitro* system.

3.3 Insulin triggers an anabolic response

Lipid metabolism, like other metabolic processes, is highly influenced by the feed status of the fish with insulin production triggered by feeding [26]. In order to ensure that the liver slices behaved similarly to liver in fed fish, we assessed the inclusion of insulin in the media. A main function of insulin is to shift the metabolic state from catabolic to anabolic, since it would be counterproductive for cells to actively produce energy by breaking down organic macromolecules while at the same time storing energy by building them up [27]. In line with this we observe a binary response with several thousand genes either upregulated or downregulated in the presence of insulin, regardless of concentration (figure S2). Major anabolic pathways including "*biosynthesis of unsaturated fatty acids*" and "*fatty acid biosynthesis*" are upregulated in the presence of insulin in agreement with an anabolic response. Physiological range for circulating insulin is 0.2-5nM [28], so it is plausible that raising insulin concentrations to 100nM has little effect because all of the insulin receptors are bound at 10nM.

3.4 Insulin and ALA interact to regulate PUFA biosynthesis gene expression

Insulin and ALA displayed complex interaction effects on expression of genes related to PUFA biosynthesis in liver slices. Unaffected by ALA concentration, *elovl5a* and *elovl5b* were highly upregulated in the presence of insulin. On the other hand, genes that were upregulated in response to ALA tended to be upregulated in the presence of insulin alone, but then downregulated in the presence of insulin when combined with ALA (figure 4C). An important regulator of lipid metabolism in liver, sterol regulatory element binding protein 1 (*srebp-1*), is known to be upregulated by insulin through the PI3K/Akt/mTOR signaling pathway [29], and indeed *srebp-1* is upregulated in response to insulin in our experiments. Both *elovl5a* and *elovl5b* contain sterol regulatory elements in their promoter regions [30], and along with *Δ6fada* have been shown to increase in expression when co-transfected with *srebp-1* [31]. On the other hand, activation of PPARα by ALA could work in opposition to insulin-mediated effects by stimulating beta-oxidation and ketogenesis. There is evidence in rats that Δ5 desaturase (D5D) and Δ6 desaturase (D6D) are under dual regulation by both SREBP-1 and PPARα [32], and given that regulation of lipid metabolism is highly conserved across species [31] it is likely a similar effect is present in salmon. The contrasting effect of insulin and ALA supplementation highlights the complex interplay between signaling networks balancing hormonal and nutritional input to optimize regulation of PUFA metabolism in Atlantic salmon.

3.5 Liver slice culture outperforms 2D hepatocyte culture in terms of gene expression similarity to whole liver

We find that gene expression in liver slice culture more closely resembles whole liver than hepatocyte culture. This is intuitive, as liver slices themselves more closely resemble the complex three-dimensional organization of whole liver with all interacting cell types. While hepatocytes are generally responsible for the metabolic activities associated with liver, regulation of these functions is controlled in concert with nonparenchymal cells through complex endocrine and autocrine signaling networks [33]. Eicosanoid signaling is a key component of these networks, which represents a layer of information that is completely lost in 2D hepatocyte cultures since eicosanoids are only produced in nonparenchymal cells [34]. In mammals, glucose metabolism has been demonstrated to

be influenced by nonparenchymal produced eicosanoids [35] and there is evidence that regulation of lipogenesis and PUFA metabolism is influenced by eicosanoid-mediated effects [36]. In addition to eicosanoid production, interactions between hepatocytes and nonparenchymal cells are known to play a role in cell proliferation and differentiation [33] which could explain the higher liver slice culture expression of liver marker genes. This, along with many other factors likely contribute to the observed differences between liver slice culture and 2D hepatocyte culture. Many of the metabolic processes in the liver are also regulated by circulating hormones produced in other parts of the body, so while liver slice culture is not identical to whole liver, we assert that liver slice culture is superior to 2D hepatocyte culture for metabolic studies.

4 Conclusion

Taken together, our results demonstrate the utility and effectiveness of precision cut liver slices as a tool for studying lipid metabolism in Atlantic salmon. We found that when studying metabolism in liver slices, it is best to allow the slices to recover for three days before adding fatty acids, since gene expression in pathways relating to metabolism remains stable after three days in culture. Liver slices were highly responsive to both exogenous fatty acids and insulin in line with current understanding of lipid metabolism of Atlantic salmon. Supplementation with ALA induced expression of lipid metabolism genes and pathways while supplementation with insulin shifted gene expression to an anabolic state as expected. We also observed a different, sometimes opposing, regulatory effect of insulin and ALA on expression of genes involved in PUFA biosynthesis. Liver slices mimic the complex three dimensional structure of the liver and produce results that are more relatable to liver *in vivo* than 2D hepatocyte culture. For this reason, liver slices are an attractive alternative to 2D hepatocyte culture for interrogating metabolic pathways.

5 Materials and Methods

5.1 Liver slice culture

Atlantic salmon used in this study were treated according to the Norwegian Animal Research Authority (NARA); use of the experimental animals and in accordance with the Norwegian Animal Welfare Act of 19th of June 2009. The liver was removed immediately after euthanization and placed in ice cold Hank's balanced salt solution (HBSS, Thermofisher). Livers were cut into approximately 4 mm x 4 mm x 8 mm strips before being superglued to a plastic piston and encased in ultra-low melt agarose (Merck). Liver strips were sliced to a thickness of 300 µm using a compresstome VF-300 (Precisionary Instruments) and collected in ice cold HBSS before being transferred to 15°C Leibovitz 15 medium (L15, Thermofisher) containing 5% fetal bovine serum (FBS, Merck) and 1% penicillin - streptomycin (PS, Thermofisher) which will now be referred to as base media. Liver slices were incubated in sterile 6 (2mL media per well) or 12 (4mL media per well) well cell culture plates with netwell inserts (Corning, 500µm membrane size) for up to 9 days at 15°C.

5.2 Time course experiments

We performed two time course experiments, the first to test the effect of culturing time on the liver slices, and the second as a follow up to test the effect of media change frequency and inclusion of insulin over time. In both experiments, liver slices were generated immediately after euthanization and viability measurements were taken every day in the first experiment and on days three and six in the second experiment. All samples were stored in RNAlater at -20°C. In the first experiment we generated slices from a saltwater life-stage Atlantic salmon (~200g) reared on a marine oil based diet high in DHA and EPA. Immediately after euthanization, liver slices were generated as described above. Media was changed on days three and six using base media supplemented with 700µM randomly methylated beta cyclodextrin (BCD) and 0.7% ethanol. Samples were taken before slicing (whole liver) and 1, 3, 4, 5, 6, 7, 8, and 9 days after slicing. In the second time course experiment we used Atlantic salmon in the freshwater life-stage reared on a marine oil diet high in EPA and DHA. Human insulin (Sigma) was included in the media at 20nM and media was refreshed either every day or

every third day with fresh base media containing 20nM insulin. Samples were taken in triplicate before slicing (whole liver) and days 3, 4, 5, 6, 7, 8, and 9.

5.3 Fatty acid and insulin gradient experiments

We performed two concentration gradient experiments, the first was used for transcriptomic analysis, the second for fatty acid analysis. In the first experiment liver slices were prepared from two freshwater stage Atlantic salmon (~50g), one for use in the fatty acid gradient experiment and one for use in the insulin gradient experiment. We used randomly methylated beta-cyclodextrin (BCD) as our fatty acid delivery system since it has been demonstrated to efficiently deliver fatty acids across membranes in other *in vitro* systems [37]. Alpha-linolenic acid (ALA) was stored at 10mM in ethanol then mixed 1:1 with 100mM BCD in water for a final molar ratio of 1:10 fatty acid to BCD. From this stock ALA was added to the media at a concentration of 0 (empty BCD), 20, 40, 70, and 100μM, aliquoted into a new six well culture plate, and placed at 15°C to equilibrate for at least 30 minutes. For all ALA treated samples, liver slices were transferred to ALA supplemented base media after a three day recovery period. For the insulin containing samples, human insulin (Sigma) was diluted in base media to a final concentration of 10 or 100nM and incubated with liver slices from the beginning of the experiment. All liver slices were sampled on day five and stored in RNAlater at -20°C. In the second concentration gradient experiment, liver slices were prepared from freshwater stage fish (~500g) and supplemented with 0 (empty BCD), 20, 40, 70, 100, and 140 on day three as described except this time ethanol was evaporated under a stream of nitrogen before mixing with BCD. Samples were taken in triplicate on day four, washed in ice cold HBSS, flash frozen in an ethanol dry ice slurry, and stored at -80°C.

5.4 2D hepatocyte culture experiment

Primary cells were isolated from salmon liver as described [38], with some modifications. After euthanization, the liver was removed and rinsed in ice cold Mg2+/Ca2+ free HBSS, before ~100 ml of the same buffer was injected with a 50 ml syringe and 27G needle, at various places to wash out blood cells. Then, 30 ml of HBSS with 150U/ml Collagenase (Sigma) was injected, before the tissue was finely chopped. The tissue suspension was incubated for 1h at 10-12°C with agitation. Dissociated cells were collected by cell straining (70 μm) and centrifugation for 10 min at 100g. After three washes in HBSS, the pellet was dissolved in base media supplemented with 10 μM insulin (Merck) and grown

at 200k density at 15°C. Cells were supplemented with ALA on day five as previously described and collected in triplicate using a cell scraper on days five (before ALA), six, and eight by flash freezing and storing at -80°C.

5.5 Viability measurement

Slice viability was assessed by staining with Hoechst and propidium iodide to identify live and dead cells. Slices were transferred to L15 medium containing 10μg/mL Hoechst and 10μg/mL propidium iodide for 5 minutes at 15°C. Slices were then transferred to fresh L15 medium and placed on ice until being imaged with a scanning laser confocal microscope (CLSM, Leica). Live/dead ratios were determined using Icy (http://www.bioimageanalysis.org/). We compared the proportions of live and dead cells in several locations per slice to determine overall slice viability.

5.6 Microscopy

We made cross sections of liver slices at three different time points during culturing and observed morphological changes using light microscopy. All samples for microscopy were fixed using 4% formalin in phosphate buffered saline (PBS) for 1 hour then transferred to 70% ethanol stepwise (PBS-25%-50%-70%) for 5 minutes at each step and stored at -20°C until microscopic analysis was performed. Prior to paraffin embedding liver slices were transferred to 96% ethanol stepwise (70%-85%-96%-96%) for 5 minutes at each step then washed twice with histoclear (National diagnostics) for 5 minutes each. Next, liver slices were embedded in paraffin (Sigma) by incubating in paraffin at 61°C three times for 10 minutes each. Paraffin was allowed to solidify at room temperature. Liver slice cross-sections were prepared using a rotary microtome (Leica) at a thickness of 7μm, placed on the surface of a 43°C water bath, and floated onto a clean microscopy slide. Sections were deparaffinized by washing twice with histoclear for 5 minutes each and rehydrated by transferring to 70% ethanol stepwise (histoclear-96%-85%-70%-70%) for 5 minutes each followed by a brief wash in distilled water. Sections were stained with a 1% hematoxylin solution (Mayer's) for eight minutes, rinsed in running tap water for 10 minutes followed by 96% ethanol and counterstained with a 0.25% eosin-phloxine B solution for 30 seconds. Stained sections were washed twice with histoclear for five minutes each and mounted with DPX (Sigma). Micrographs of cross-sections were taken at 20x magnification on a light microscope (Leica).

5.7 RNA sequencing

Slices were stored in RNAlater (Sigma) at -20°C until RNA extraction using the RNeasy universal kit (QIAGEN). RNA concentration was determined on a Nanodrop 8000 and quality was determined on an Agilent 2100 bioanalyzer using Agilent RNA 6000 nano chips. All RNA samples had a RNA integrity number greater than 7. mRNA libraries were prepared using the Trueseq library preparation kit (Agilent). Concentration and mean length were determined by running cDNA libraries on a bioanalyzer 2100 using DNA 1000 chips (Agilent). RNA libraries were sequenced on an Illumina HiSeq 2500 with 100-bp single end reads.

5.8 RNAseq analysis

All RNA sequencing and demultiplexing was done at the Norwegian sequencing center (Oslo, Norway). Fastq files were trimmed and mapped the the salmon genome (ICSASG_v2) using STAR (v2.5.2a) [39]. Mapped reads for each gene were counted with HTSeq-count (v0.6.1p1) [40]. Differential expression analysis was performed in R (v3.2.5) using the edgeR package [41]. All counts were normalized to library size using TMM normalization within edgeR. For the time course and gradient experiments an ANOVA-like differential expression test was used to find difference between any of the conditions (see edgeR manual). This yielded log2 fold change to the reference level (day0 or ALA0) and false discovery rate (FDR) for each gene. For the time course experiments we considered genes with a FDR of <0.01 and log2FC >1 as differentially expressed while for the gradient experiments genes with a FDR of <0.01 were considered differentially expressed. Gene expression clusters were generated by applying wardD2 hierarchical clustering to gene-scaled mean counts per million. KEGG enrichment was performed on each gene cluster using edgeR. Pathways with a p-value <0.001 were considered significantly enriched. To compare gene expression between whole liver, liver slice, and hepatocyte culture, we pooled data from each source to give an overall expression phenotype. Data on whole liver was obtained from a previously published feeding trial [10] and whole liver samples taken before generating liver slices. Data on liver slice and hepatocyte culture was obtained from the previously described experiments.

5.9 Lipid analysis

Fatty acid methyl esters (FAME) were prepared from liver slices according to established protocols [42] with minor changes to account for the small size of liver slices. We used 13:0 as an internal standard in all samples and FAMEs were separated by gas chromatography on a Trace GC Ultra (Thermo Scientific) using a flame ionization detector. Relative fatty acid abundance was calculated from the resulting chromatograms.

5.10 Statistical analysis

All statistical analysis was performed in R (v3.2.5). Correlation analysis between whole liver and liver slice samples was calculated using the mean counts per million (CPM) of each gene across the three time course experiments (whole liver) and triplicate samples within each experiment (liver slice) for each day followed by Spearman's rank correlation test. Comparison of gene expression (CPM) between groups in the ALA and insulin gradient experiments was calculated using a one-way analysis of variance (ANOVA) test followed by a Tukey-HSD test. Differences with a p-value <0.05 were considered significant.

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7 Supplementary data

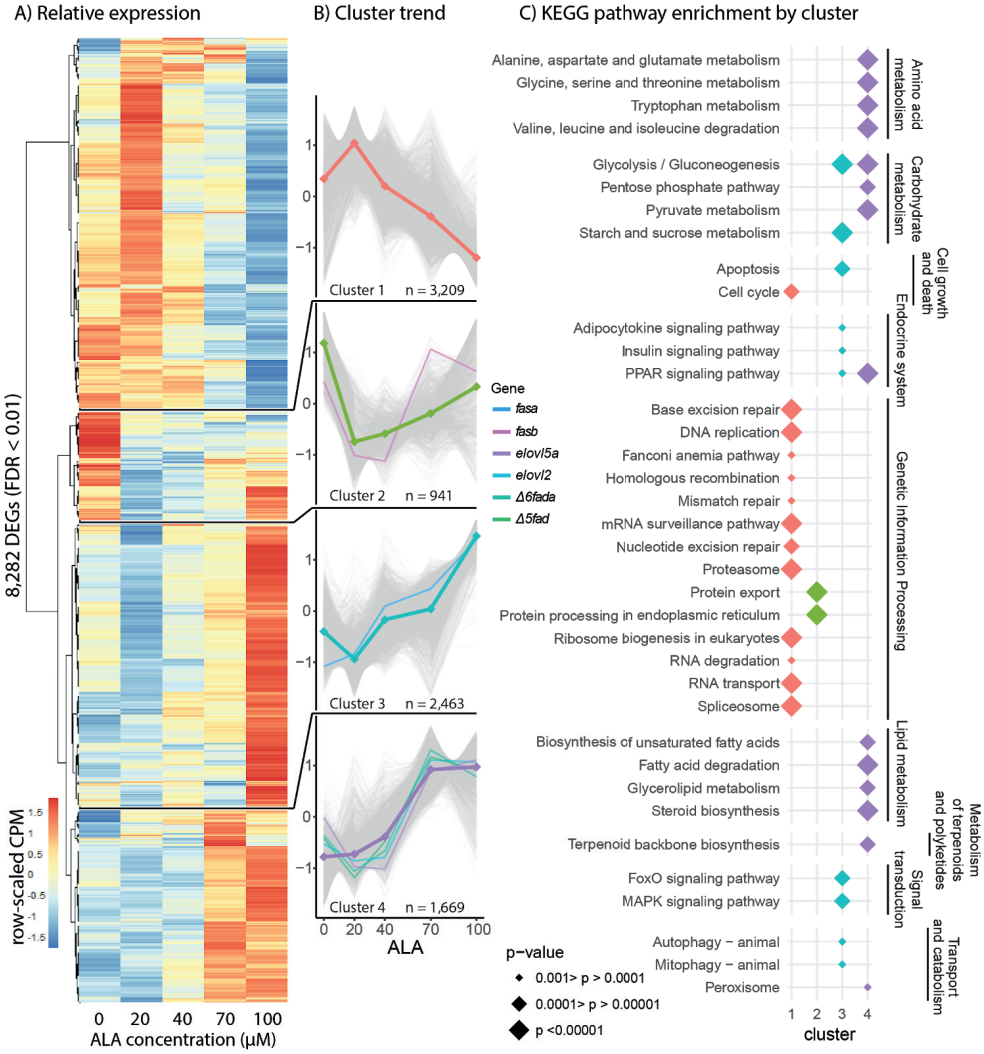

Figure S1: Global gene expression patterns with increasing ALA concentration. A) Heatmap showing changes in relative gene expression with increasing concentration of ALA. Heatmap contains 8,282 genes differentially expressed (FDR <0.01) at any point in the ALA gradient (ANOVA-like). Transcript abundance is expressed CPM and were row-scaled to highlight changes in individual gene expression. Genes were broken up into four clusters with similar expression patterns across the ALA gradient. B) Mean row-scaled CPM (thick lines with points) colored by cluster overlays row-scaled CPM of individual genes (gray). Expression of fatty acid synthase a (*fasa*), fatty acid synthase b (*fasb*), elongase 5a (*elovl5a*), elongase 2 (*elovl2*), Δ 6 desaturase a (*Δ6fada*), and Δ 5 desaturase (*Δ5fad*) are also plotted (thin lines colored by gene). C) Pathway enrichment analysis on each gene cluster. All pathways shown are significantly enriched (p <0.001) in the corresponding expression cluster with point size corresponding to p-value.

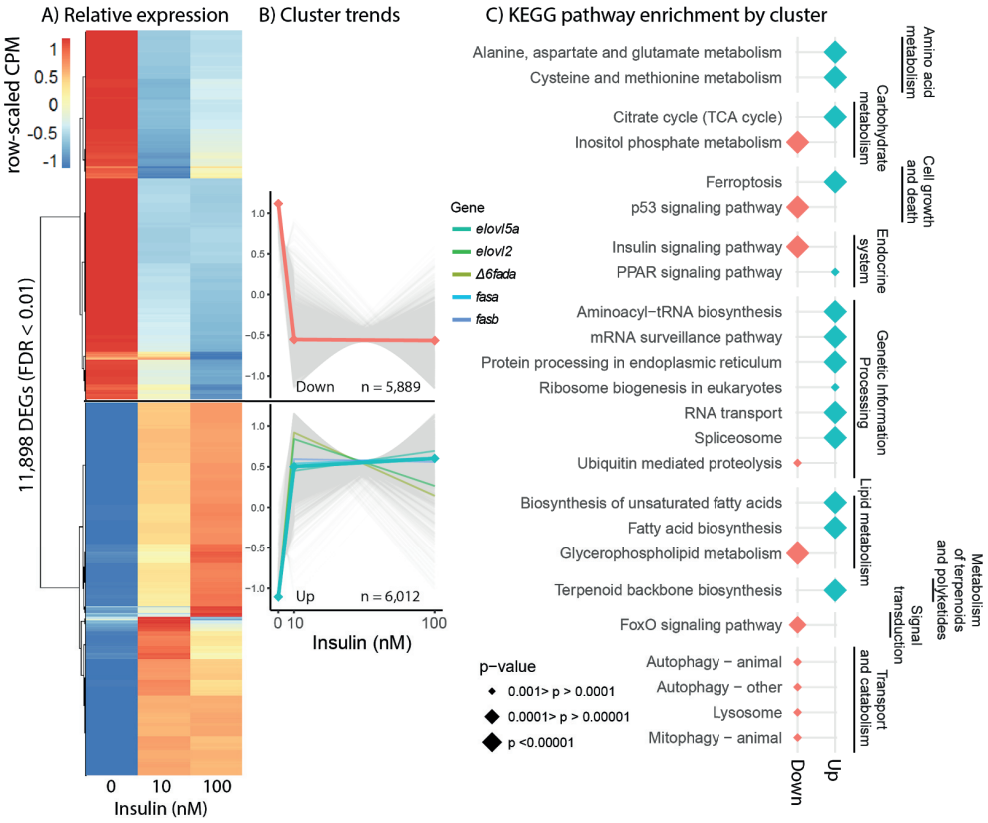

Figure S2: Global gene expression patterns with increasing insulin concentration. A) Heatmap showing changes in relative gene expression with increasing concentration of insulin. Heatmap contains 11,898 genes differentially expressed (FDR <0.01) at any point in the ALA gradient (ANOVA-like). Transcript abundance is expressed counts per million (CPM) and were row-scaled to highlight changes in individual gene expression. Genes were broken up into two clusters, either downregulated or upregulated. B) Mean row-scaled CPM (thick lines with points) overlays row-scaled CPM of individual genes (gray). Expression of fatty acid synthase a (*fasa*), fatty acid synthase b (*fasb*), elongase 5a (*elovl5a*), elongase 2 (*elovl2*), and Δ 6 desaturase a (*Δ6fada*) are also plotted (thin lines colored by gene). C) Pathway enrichment analysis on each gene cluster. All pathways shown are significantly enriched (p <0.001) in the corresponding expression cluster with point size corresponding to p-value.

Table S1: Fatty acid profile of liver slices incubated with increasing concentration of ALA. Fatty acid profile of whole liver and liver slices fed increasing amounts of ALA. All values are expressed as triplicate mean percent of total FAs.

		ALA concentration (μM)					
Fatty acid	Whole liver	0	20	40	70	100	140
14:0	1.18	1.03	0.98	1.01	1.08	1.09	0.98
16:0	16.21	15.01	14.58	14.89	14.05	14.14	13.65
18:0	5.8	12.29	12.08	11.66	10.40	10.33	10.56
20:0	0.12	0.37	0.35	0.34	0.30	0.29	0.30
22:0	0.04	0.07	0.05	0.05	0.05	0.05	0.05
Total Saturates	23.35	28.77	28.04	27.95	25.87	25.88	25.55
16:1n7	1.02	0.81	0.77	0.73	0.81	0.82	0.70
18:1n9	14.11	12.84	12.81	11.40	12.79	12.29	11.17
20:01	1.49	2.08	2.09	1.80	1.98	1.86	1.72
22:1n9	0.08	0.22	0.26	0.20	0.23	0.21	0.20
24:1n9	0.35	0.44	0.44	0.43	0.44	0.44	0.39
Total MUFA	17.04	16.40	16.37	14.55	16.24	15.62	14.18
18:2n6	4.68	3.52	3.61	3.32	3.69	3.61	3.29
20:2n6	1.05	1.43	1.49	1.41	1.37	1.33	1.33
20:3n6	0.87	0.79	0.82	0.78	0.74	0.72	0.73
20:4n6	3.59	2.96	3.06	3.01	2.86	2.81	2.80
22:02	0.73	0.67	0.73	0.70	0.78	0.79	0.79
Total n-6 PUFA	10.92	9.38	9.71	9.21	9.45	9.27	8.95
18:3n3	1.17	0.88	1.44	1.88	3.04	4.12	6.52
20:3n3	0.24	0.35	0.61	0.91	1.42	1.83	2.74
20:5n3	6.38	3.51	3.58	3.45	3.32	3.29	3.14
22:5n3	2.07	2.26	2.34	2.33	2.14	2.12	2.09
22:6n3	29.64	25.07	25.83	25.26	23.58	23.48	23.19
Total n-3 PUFA	39.5	32.07	33.79	33.83	33.49	34.84	37.68

Paper II

Gareth Gillard*, **Thomas N. Harvey***, Arne Gjuvsland, Yang Jin, Magny Thomassen, Sigbjørn Lien, Michael Leaver, Jacob S. Torgersen, Torgeir R. Hvidsten, Jon Olav Vik, Simen R. Sandve (2018) **Life-stage associated remodeling of lipid metabolism regulation in Atlantic salmon. Molecular Ecology.** 27 (5): 1200-1213. DOI: 10.1111/mec.14533

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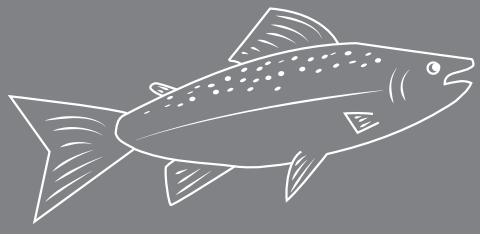

Received: 7 July 2017 | Revised: 7 December 2017 | Accepted: 13 December 2017

DOI: 10.1111/mec.14533

ORIGINAL ARTICLE

WILEY MOLECULAR ECOLOGY

Life-stage-associated remodelling of lipid metabolism regulation in Atlantic salmon

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Abstract

Atlantic salmon migrates from rivers to sea to feed, grow and develop gonads before returning to spawn in freshwater. The transition to marine habitats is associated with dramatic changes in the environment, including water salinity, exposure to pathogens and shift in dietary lipid availability. Many changes in physiology and metabolism occur across this life-stage transition, but little is known about the molecular nature of these changes. Here, we use a long-term feeding experiment to study transcriptional regulation of lipid metabolism in Atlantic salmon gut and liver in both fresh- and saltwater. We find that lipid metabolism becomes significantly less plastic to differences in dietary lipid composition when salmon transitions to saltwater and experiences increased dietary lipid availability. Expression of genes in liver relating to lipogenesis and lipid transport decreases overall and becomes less responsive to diet, while genes for lipid uptake in gut become more highly expressed. Finally, analyses of evolutionary consequences of the salmonid-specific whole-genome duplication on lipid metabolism reveal several pathways with significantly different ($p < .05$) duplicate retention or duplicate regulatory conservation. We also find a limited number of cases where the whole-genome duplication has resulted in an increased gene dosage. In conclusion, we find variable and pathway-specific effects of the salmonid genome duplication on lipid metabolism genes. A clear life-stage-associated shift in lipid metabolism regulation is evident, and we hypothesize this to be, at least partly, driven by nondietary factors such as the preparatory remodelling of gene regulation and physiology prior to sea migration.

KEYWORDS
adaptation, fish, life stage, metabolism, transcriptomics

1 | INTRODUCTION

Atlantic salmon lives a "double life." It starts its life in rivers, before transforming its physiology and behaviour and migrating to sea to grow and accumulate resources for reproduction. This shift in environment requires preparatory remodelling of physiology prior to sea migration (referred to as smoltification), which encompasses a suite of coordinately regulated processes involving hormonal changes and large-scale alteration of gene expression. The resulting adaptations to a marine environment include transformation of salt tolerance, coloration, behaviour, growth rate and metabolism (reviewed in Stefansson, Björnsson, Ebbesson, & McCormick, 2008).

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A key difference between freshwater and sea habitats is the dietary availability of essential long-chain polyunsaturated fatty acids. Salmon in rivers mostly eat invertebrates that are low in physiologically critical n-3 and n-6, 20 and 22 carbon long-chain polyunsaturated fatty acids (n-3LC-PUFA and n-6LC-PUFA), arachidonic acid (20:4n-6), eicosapentaenoic acid (20:5n-3) and docosahexaenoic (22:6n-3), while marine habitat food chains are high in available LC-PUFAs. Possibly, as an adaptation to this (Leaver, Bautista et al., 2008), salmon have evolved a high capacity for endogenous production of LC-PUFAs by elongation and desaturation of essential dietary 18 carbon precursor linoleic and linolenic acids (18:2n-6 and 18:3n-3; Figure 4) and the ability to increase or decrease this endogenous production as a response to the dietary availability (Kennedy et al., 2006; Leaver, Villeneuve et al., 2008; Morais et al., 2011; Ruyter, Røsjø, Måsøval, Einen, & Thomassen, 2000; Tocher, Bell, MacGlaughlin, McGhee, & Dick, 2001; Tocher et al., 2002; Zheng et al., 2005). During smoltification and after sea migration, Atlantic salmon have been shown to undergo transformation of lipid metabolism function, by decreasing lipid syntheses and increasing lipid breakdown (Sheridan, 1989). However, very little is known about the molecular nature of this life-stage-associated transformation physiological function.

The evolution of novel traits in salmonids, such as increased plasticity and the ability to migrate to sea, may have been facilitated by their ancestral whole-genome duplication (called Ss4R) some 80 Ma (Allendorf & Thorgaard, 1984; Lorgen et al., 2015; Macqueen & Johnston, 2014; Robertson et al., 2017). Gene duplication can give rise to new adaptive phenotypes in different ways: through evolution of novel functions or gene regulation, subdivision and/or specialization of function among duplicates, or via an adaptive increase in gene dosage. The Atlantic salmon genome contains ~10,000 pairs of Ss4R gene duplicates, of which ~50% have evolved some novel regulation (Lien et al., 2016; Robertson et al., 2017). Indeed, in the context of lipid metabolism, it has recently been shown that a Ss4R duplicate of elovl5, a key enzyme in LC-PUFA syntheses, has gained expression compared to its ancestral regulation with likely implications for the ability to synthesize LC-PUFAs (Carmona-Antoñanzas, Zheng, Tocher, & Leaver, 2016). This is believed to have facilitated evolution of novel traits, including flexible phenotypes necessary for an anadromous life history (Stefansson et al., 2008). However, no systematic genomewide study has yet been conducted to assess the importance of the Ss4R in evolution of salmon lipid metabolism.

In this study, we integrate comparative genomics with transcriptomic data from a feeding trial carried out across the freshwater to saltwater transition to build a functional annotation of lipid metabolism pathway genes in salmon. We use this annotation to elucidate (i) the nature of the transformation of lipid metabolism from freshwater to saltwater life stages and (ii) the impact of whole-genome duplication on evolution of the lipid gene repertoire and metabolic function. Our results indicate a striking shift in lipid metabolism after transition to sea water and show that lipid pathways differ with respect to selection pressure on gene duplicates from the salmonid whole-genome duplication.

2 | MATERIALS AND METHODS

2.1 | Orthogroup prediction

Protein sequences were obtained from seven teleost fish species: *Danio rerio* (zebrafish), *Gasterosteus aculeatus* (three-spined stickleback), *Oryzias latipes* (medaka), *Oncorhynchus mykiss* (rainbow trout), *Oncorhynchus kisutch* (coho salmon), *Salmo salar* (Atlantic salmon), *Thymallus thymallus* (grayling), *Esox lucius* (northern pike), and two mammalian outgroup species: *Homo sapiens* (human), *Mus musculus* (house mouse). Human, mouse, zebrafish, medaka and stickleback protein fasta data were obtained from ENSEMBL (release 83). Atlantic salmon (RefSeq assembly GCF_000233375.1, Annotation Release 100) and northern pike (RefSeq assembly GCF_000721915.2, Annotation Release 101) proteins were obtained from NCBI RefSeq. Rainbow trout proteins were obtained from an assembly and annotation of the genome (Berthelot et al., 2014). Grayling proteins were obtained from an assembly and annotation of the genome (Varadharajan et al., 2017). The coho salmon transcriptome (Kim, Leong, Koop, & Devlin, 2016) was obtained from NCBI (GDQG00000000.1). Where transcriptome data were used, protein sequences were translated using TRANSDECODER (v2.0.1, http://transdecoder.github.io/). Protein fasta files were filtered to retrieve only the longest protein isoform per gene. ORTHOFINDER (v0.2.8; Emms et al., 2015) assigned groups of orthologs based on protein sequence similarity. Proteins within an orthogroups were further aligned using MAFFT (v7.130; Katoh, Misawa, Kuma, & Miyata, 2002), and maximum-likelihood trees were estimated using FASTTREE (v2.1.8; Price et al., 2010).

2.2 | Annotation of salmon lipid metabolism genes

A list of zebrafish proteins obtained from 19 manually selected zebrafish KEGG pathways related to lipid metabolism (Appendix S1: Table S1) were used to search for Atlantic salmon orthologs. Orthogroups that contained a selected zebrafish protein were identified. Salmon proteins within those orthogroups were assigned as orthologs of the closest zebrafish protein based on the orthogroup tree distance. A lipid metabolism gene list was created including salmon orthologs to the selected zebrafish genes. Additional salmon genes related to lipid metabolism not included in KEGG pathways (e.g., regulators or transporters, SREBP, LXR, FABP) were manually searched for through NCBI and added to the list.

2.3 | Tissue expression

Atlantic salmon RNA-Seq samples from 15 different tissues (liver, gut, pyloric caeca, heart, kidney, muscle, gill, eye, skin, ovary, nose, testis, brain, head kidney and spleen) were obtained from NCBI SRA (PRJNA72713; Lien et al., 2016). Fastq files were adapter trimmed

before alignment to the Atlantic salmon genome (RefSeq assembly GCF_000233375.1; Lien et al., 2016) using STAR (v2.5.2a; Dobin et al., 2013). HTSeq-count (v0.6.1p1; Anders, Pyl, & Huber, 2015) counted the sum of uniquely aligned reads in exon regions of each gene in the annotation (RefSeq Annotation Release 100). Gene FPKM values were calculated based on the gene count over the samples effective library size (see TMM method from EDGER (Robinson, McCarthy, & Smyth, 2010) user manual) and the mean gene transcript isoform length.

2.4 | Feed trial

Atlantic salmon fry were obtained from AquaGen Breeding Centre, Kyrksæterøra, Norway, and reared in the Norwegian Institute for Water Research (NIVA), Solbergstranda, Norway, in four partitioned 1,000-L tanks on vegetable oil (VO)- or fish oil (FO)-based diets continuously from first feeding (fry weight <0.2 g). Daily feed amount was calculated based on total biomass in each tank and decreased as the fish grew, from 3% at first feeding to 1.2% by the end of the trial. Fish were euthanized periodically throughout the experiment to maintain appropriate levels of dissolved oxygen. VO-based feeds contained a combination of linseed oil and palm oil at a ratio of 1.8:1, and FO-based feeds contained only North Atlantic fish oil. Percentage of protein in feed decreased with fish size from 56% at first feeding to 41% at the end of the trial. This corresponded to an increase in percentage of lipid from 16% at first feeding to 31% at the end of the trial. At the time of sampling, the proportion of lipid in the feed was 22% in freshwater and 31% in saltwater (Appendix S1: Table S2). Increasing lipid proportion in feed with fish size is standard practice in the aquaculture industry as this maintains optimal growing conditions by decreasing the digestible protein to digestible energy ratio (Storebakken, 2002). All feeds were formulated and produced by EWOS innovation (Supplementary File 3). Local groundwater was UV-sterilized for use in the freshwater life stage, and water from the Oslofjord taken from 60 metres below sea surface (~3%–3.5% salinity) was UV-sterilized for use in the saltwater life stage. Fish were raised under constant light and water temperature (~12°C) for 26 weeks. Then, 40 presmolt salmon (~50 g) from each control tank (~240 fish per control tank) were switched to the contrasting diet (VO to FO and vice versa) by physically moving them to the empty partition of the tank receiving the appropriate feed (Figure 8a). Five fish from each of the control tanks (2 VO tanks and 2 FO tanks) were sampled before switching feeds (D0), and then, fish from both control and feed switch conditions were similarly sampled 1, 2, 5, 9, 16 and 20 days after switching feeds (5 fish × 2 replicate tanks × 4 conditions = 40 fish per time point, Figure 8b). Two weeks after freshwater sampling (31 weeks after first feeding), smoltification was triggered by 5 weeks of winter-like conditions with decreased light (12 hr/day) and water temperature (~8°C), immediately followed by 5 weeks of spring-like conditions, returning to normal light (24 hours per day) and water temperature (~12°C). All salmon from the control groups (VO or FO) were then switched to saltwater and allowed to acclimate for 3 weeks. The feed switch was repeated in saltwater by transferring half (~40 fish) of the postsmolt salmon (~200 g) from each control tank to the contrasting feed condition. Again, preswitch control samples were taken (D0) followed by sampling 1, 2, 6, 9, 16 and 20 days postdiet switch (Figure 8b). For both freshwater and saltwater samplings, feeding was stopped in the mornings of each of the sampling days. All fish were euthanized by a blow to the head and samples of liver and midgut (gut section between pyloric caeca and hindgut) were flash-frozen in liquid nitrogen and stored under −80 °C. A subset of the samples taken were used for further RNA-Seq analysis (see Figure 8c for details).

2.5 | RNA sequencing

Total RNA was extracted from selected feed trial samples (see Figure 8c for details) using the RNeasy Plus Universal Kit (QIAGEN). Quality was determined on a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent). Concentration was determined using a Nanodrop 8000 spectrophotometer (Thermo Scientific). cDNA libraries were prepared using the TruSeq Stranded mRNA HT Sample Prep Kit (Illumina). Library mean length was determined by running on a 2100 Bioanalyzer using the DNA 1000 Kit (Agilent) and library concentration was determined with the Qbit BR Kit (Thermo Scientific). Single-end sequencing of sample libraries was completed on an Illumina HiSeq 2500 with 100-bp reads.

2.6 | Differential expression analysis between feed conditions and life stages

To analyse gene expression differences between feed conditions and life stages, samples from the feed trial were selected for RNA-Seq. Liver and gut tissue RNA were sequenced from fish fed each of the feeds (FO, VO) at day 0 of the diet switch, both before (freshwater) and after (saltwater) smoltification (see Figure 8c for the number of RNA-Seq replicates and sampling details). Fastq files were processed to produce gene count and FPKM data using the same protocol described under the Section 2.3. For the feed comparison, changes in gene expression were tested between FO and VO feed conditions for both freshwater and saltwater samples, and liver and gut tissues. For the life-stage comparison, changes in gene expression were tested between freshwater and saltwater stages for both FO and VO feed conditions, and liver and gut tissues. Using RNA-Seq gene count data, lowly expressed genes were filtered prior to testing, retaining genes with a minimum of one read count per million (CPM) in two or more samples. Differential expression analysis was carried out using a standard EDGER (Robinson et al., 2010) protocol. Effective library sizes were calculated using the EDGER TMM normalization procedure allowing effective comparison of expression data between different sample types (see EDGER user manual). An exact test between expression levels of a pair of conditions gave the log2-fold change, *p*-value and false discovery rate (FDR) for each gene. Genes with FDR <0.05 were considered differentially expressed genes (DEGs).

2.7 | Identification of Ss4R duplicates

To identify putative gene duplicates stemming from the Ss4R, we used the same approach as in Lien et al. (2016). All-vs-all protein blast was run with e-value cut-off of 1e−10 and pident (percentage of identical matches) ≥80 and blast hit coverage of ≥50% of protein length. Only the best protein hits between the 98 defined synteny blocks (see Lien et al., 2016) were considered as putative Ss4R duplicates. Blast result ranking was carried out using the product of pident times bitscore to avoid spurious "best blast matches" with low pident (<85), but high bitscore.

2.8 | Duplicate analysis

Genes from the lipid metabolism gene list were paired together with their putative Ss4R duplicates identified above. The retention of gene duplicates (i.e., whether both genes in a pair were retained, or just one) was compared between all identified duplicates in the salmon genome annotation and the lipid metabolism gene list. Pathway-level retention was explored by comparing the number of genes in each of the 19 selected KEGG pathways (Appendix S1: Table S1) in a duplicate pairing to that of the total list of lipid genes, to find pathways with significantly less or more duplicate retention (Fisher's exact test, p-value <.05). Regulatory conservation of lipid gene duplicates was explored by correlation of gene expression changes between duplicates over the course of the feed trial described above. RNA-Seq data were generated from liver samples of salmon from 38 sampling time points (19 in freshwater and 19 in saltwater). Fastq files were processed to produce gene count and FPKM data using the same protocol described under the Section 2.3. For each duplicate pair, mean FPKM values were retrieved for each time point and used to calculate a freshwater and saltwater correlation value. Duplicates with Pearson correlation ≥0.6 were considered correlated (p-value <.003 from 19 sample points). The number of duplicates with correlated expression profiles was counted for each pathway and compared to all lipid genes to find pathways with significantly less or more correlated duplicates (Fisher's exact test, p-value <.05). The effect of gene duplication on gene dosage was estimated by calculating a dosage ratio between the FPKM value of a salmon ortholog (sum of gene expression in duplicate pairs) over the FPKM value of the nonduplicated ortholog from northern pike. For salmon, the RNA-Seq data from the freshwater and saltwater FO feed trial was used (samples used in Section 2.6). For pike, RNA-Seq from livers of four individuals were aligned (see Section 2.3 for protocol) to their respective genomes (see genomes in Section 2.1). RSEM (v1.2.31; Li & Dewey, 2011) was used to generate FPKM values for genes so that nonuniquely mapped reads between salmon duplicate genes were not ignored but instead assigned proportionately to each gene to match the proportions of uniquely mapped reads between the genes. Gene dosage levels for duplicate pairs with correlated expression (see above), noncorrelated expression and single genes were compared for all lipid metabolism genes and for each pathway.

3 | RESULTS AND DISCUSSION

3.1 | Annotation of lipid metabolism genes

To identify genes involved in lipid metabolism in Atlantic salmon, we initially assembled groups of orthologous genes (orthogroups) using protein sequence similarity. We included proteins from four salmonid species sharing the Ss4R genome duplication, in addition to four nonsalmonid fish genomes and two model mammalian outgroup species (Figure 1a) to aid in distinguishing Ss4R copies from other gene duplicates. Next, we aligned orthogroup proteins and constructed

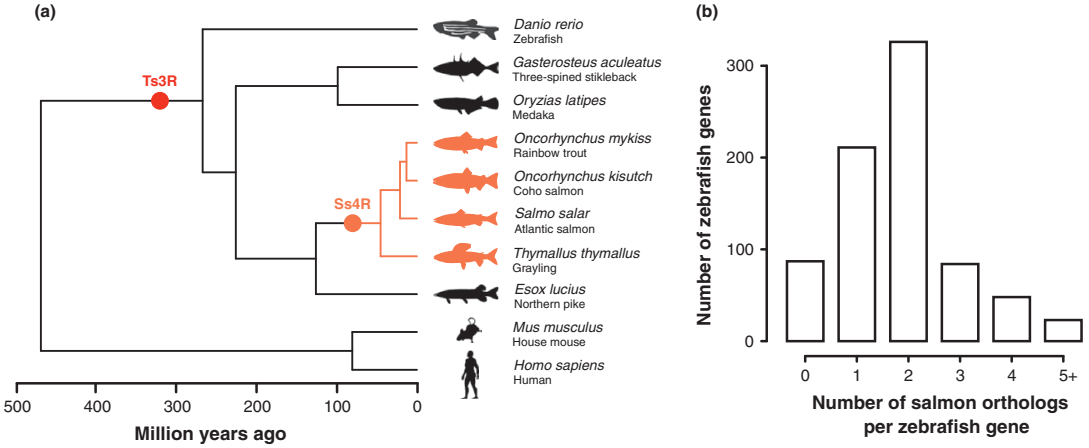


FIGURE 1 Ortholog annotation. (a) Species used to construct ortholog groups and their evolutionary distance. Points in the phylogenetic tree show the time of the teleost-specific (Ts3R) and salmonid-specific (Ss4R) whole-genome duplications. (b) The number of salmon orthologs found (1,421 genes in total) per zebrafish gene in 19 selected KEGG pathways involved in lipid metabolism [Colour figure can be viewed at wileyonlinelibrary.com]

maximum-likelihood gene trees. The majority (82%–98%) of proteins from each species were represented in 23,782 ortholog gene trees. The salmonid species had significantly higher number of proteins included in ortholog gene trees compared to nonsalmonid fish (Appendix S1: Figure S1), reflecting the salmonid-specific whole-genome duplication. We then used the evolutionary distances in gene trees to infer the most likely salmon sequence orthologs of zebrafish genes selected from 19 KEGG pathways involved in lipid metabolism (File S1). This resulted in the annotation of 1421 (File S2) salmon lipid metabolism genes, of which 326 (23%) showed a 2:1 ortholog ratio between salmon and zebrafish (Figure 1b). Only 87 (6%) of the zebrafish genes could not be assigned a salmon ortholog.

To validate our ortholog annotation pipeline used to identify lipid metabolism genes, we analysed the tissue specificity of these genes using gene expression data from 15 tissues (File S3) of Atlantic salmon (Lien et al., 2016). Genes in certain fatty acid metabolism-related pathways ("fatty acid metabolism," "PPAR signalling pathway," "fat digestion and absorption") had higher overall expression in tissues known to have high lipid metabolism activity (i.e., pyloric caeca, liver, heart and brain; Glatz, Luiken, & Bonen, 2010; Rimoldi, Benedito-Palos, Terova, & Pérez-Sánchez, 2016; Tocher, 2003; Figure 2). Examples include the following: (i) liver was the site of highest expression for all genes in the LC-PUFA biosynthesis pathway (the desaturases Δ6FAD and Δ5FAD, and the elongases elovl5, elovl2 and elovl4). (ii) Bile acids are essential for fat digestion in the gut, but are synthesized in liver. As expected, the rate-limiting step for bile syntheses, cytochrome P450 7A1 (CYP7A1), has the highest expression in the liver. (iii) Cholesterol, an essential component of cell membranes and precursor to bile acids, is known to be synthesized in all tissues, but primarily in liver, intestine and brain (Brown & Sharpe, 2016). This is reflected in our annotation by high expression of the key cholesterol biosynthesis genes 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), isopentenyl-diphosphase Δisomerase (IDI1), squalene epoxidase (SM) and lanosterol synthase (LS) in these tissues. (iv) Several known regulators of lipid metabolism show high expression in liver, heart, brain and pyloric caeca, as expected, including liver X receptor (LXR), peroxisome proliferator-activated receptor-alpha (PPARα), sterol regulatory element-binding protein 1 (SREBP1), and sterol regulatory element-binding protein 2 (SREBP2). Taken together, the tissue distribution of lipid metabolism gene expression is in line with knowledge about vertebrate physiology in general, and supports the validity of our annotation of lipid metabolism genes in salmon. To make all data underlying our annotation easily available, and to facilitate further refinement through manual community curation, we have created an interactive Web server available online (goo.gl/8Ap89a).

3.2 | Life-stage-associated remodelling of lipid metabolism

We conducted a feeding trial to study how salmon adjusts its lipid metabolism to different levels of LC-PUFA in freshwater and saltwater (see Figure 8 for experimental details). Groups of salmon were fed contrasting diets from hatching until after transition to sea water. One feed was based on vegetable oil (VO) and hence low in LC-PUFA, similar to river ecosystem diets, whereas the other was based on fish oil (FO) and high in LC-PUFA as expected in a marine-type diet (see Appendix S1: Tables S2 and S3 for details on feed composition). VO-based diets are also low in cholesterol (Ciftci, Przybylski, & Rudzińska, 2012; Verleyen et al., 2002). The proportion of fat in feed also increased between FW and SW (Appendix S1: Table S2), as is standard practice in the aquaculture industry to maintain optimal growth conditions (Storebakken, 2002). Moreover, total lipid availability is also expected to increase between natural

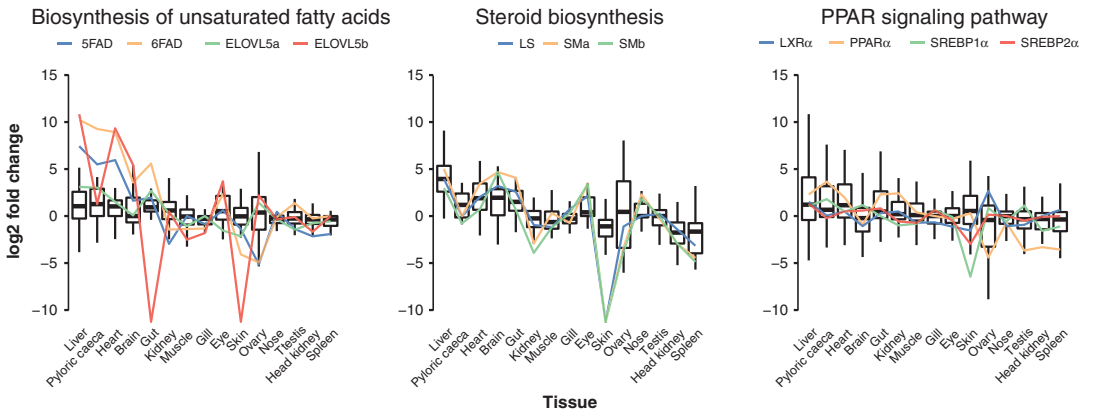


FIGURE 2 Tissue expression profiles of salmon genes in lipid metabolism pathways. Tissue expression profiles of our annotated lipid metabolism genes were consistent with expectations. Gene expression levels are shown as the log2-fold change difference between the FPKM value of each tissue and the median FPKM across all tissues. Expression profiles for selected genes in each pathway are shown (see Figures S2 and S3 for all pathways and gene details) [Colour figure can be viewed at wileyonlinelibrary.com]

riverine and marine ecosystem diets. The contrasting levels of EPA/DHA between FO and VO diets remained constant across life stages. In total, 32 and 23 fish were sampled for RNA-Seq of liver and gut, respectively, including up to eight biological replicates from each diet and life stage (freshwater and saltwater, see Figure 8c for details). Fish in the different dietary groups were given FO and VO feed from first feeding (<0.2 g body weight) until sampling.

In general, global gene expression levels were more affected by dietary composition in liver than in gut (which was largely unresponsive), and the effect was more pronounced in freshwater than in saltwater (Figure 3a). VO diets, compared to FO diets, increased lipid metabolism-related gene expression in liver. In freshwater, 66 genes were differentially expressed with 57 (86%) of these upregulated, while in saltwater, 31 genes were differentially expressed with 23 (74%) of these upregulated (Figure 3b). The increased activity of liver lipid metabolism under VO diets confirms the well-known ability of salmon to regulate endogenous synthesis of LC-PUFA and cholesterol in response to VO diets (Kortner, Björkhem, Krasnov, Timmerhaus, & Krogdahl, 2014; Leaver, Villeneuve et al., 2008; Zheng et al., 2005).

Fish sampled in freshwater and saltwater shared a relatively small number of differentially expressed genes (DEGs) for each pathway (Appendix S1: Table S4). We found that most pathways had more DEGs in freshwater ("fatty acid biosynthesis," "steroid biosynthesis" and its precursor "terpenoid backbone biosynthesis"), whereas few had more DEGs in saltwater ("fat digestion and absorption" and "steroid hormone biosynthesis"; Figure 3c). Of 87 lipid metabolism DEGs in the dietary contrast, 56 (64%) were freshwater-specific, 21 (24%) were saltwater-specific, and 10 (11%) shared dietary response. For example, only two genes in the FA and LC-PUFA biosynthesis pathways (Δ6FADa and Δ5FAD) shared response to diet in freshwater and saltwater (Figure 4). Similarly, in the pathways responsible for cholesterol biosynthesis, there were more DEGs between diets in FW (21 DEGs in FW, 4 shared and no SW-specific; Figure 5). The few genes that showed diet effects specific to saltwater included bile salt-activated lipase, responsible for the hydrolysis of free fatty acids from TAG obtained from the diet (Tocher, 2003). Two of these genes, carboxyl ester lipase, tandem duplicate 2a (CEL2a) and b (CEL2b), are highly upregulated in saltwater in response to VO diet. Taken together, our results show higher metabolic plasticity in parr-stage salmon, suggesting a life-stage-associated remodelling of lipid metabolism in liver. This corroborates the idea of a postsmoltification phenotype adapted to an environment with a surplus of n-3LC-PUFA.

To further investigate the life-stage-associated changes in lipid metabolism, we tested for differential expression between salmon in freshwater and saltwater fed diets with identical n-3LC-PUFA profiles (Figure 6). Liver and gut showed contrasting effects of saltwater on lipid gene expression with extensive downregulation in liver and upregulation in gut (Figure 6b). The number of DEGs in each tissue was similar for the environment comparison (Figure 6a), unlike for the diet comparison (Figure 3).

Further examination of key lipid metabolism genes revealed that after life-stage transition, the systemwide lipid metabolism

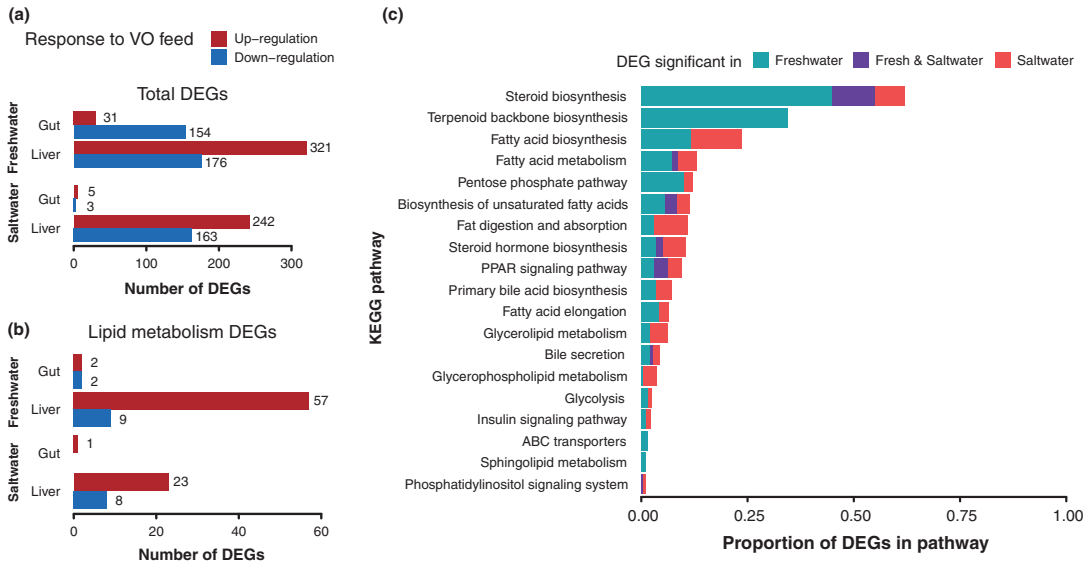


FIGURE 3 Gene regulation in response to feed type. (a) Total number of significant (FDR <0.05) differentially expressed genes (DEGs) between fish oil (FO)- and vegetable oil (VO)-fed salmon in the liver and gut tissues of freshwater and saltwater stage Atlantic salmon (see Files S4 (liver) and S5 (gut) for underlying data). (b) As above, but for lipid-associated genes only. (c) Proportions of genes in each KEGG pathway that had significantly different liver expression between the two feed types only in freshwater, only in saltwater or in both stages [Colour figure can be viewed at wileyonlinelibrary.com]

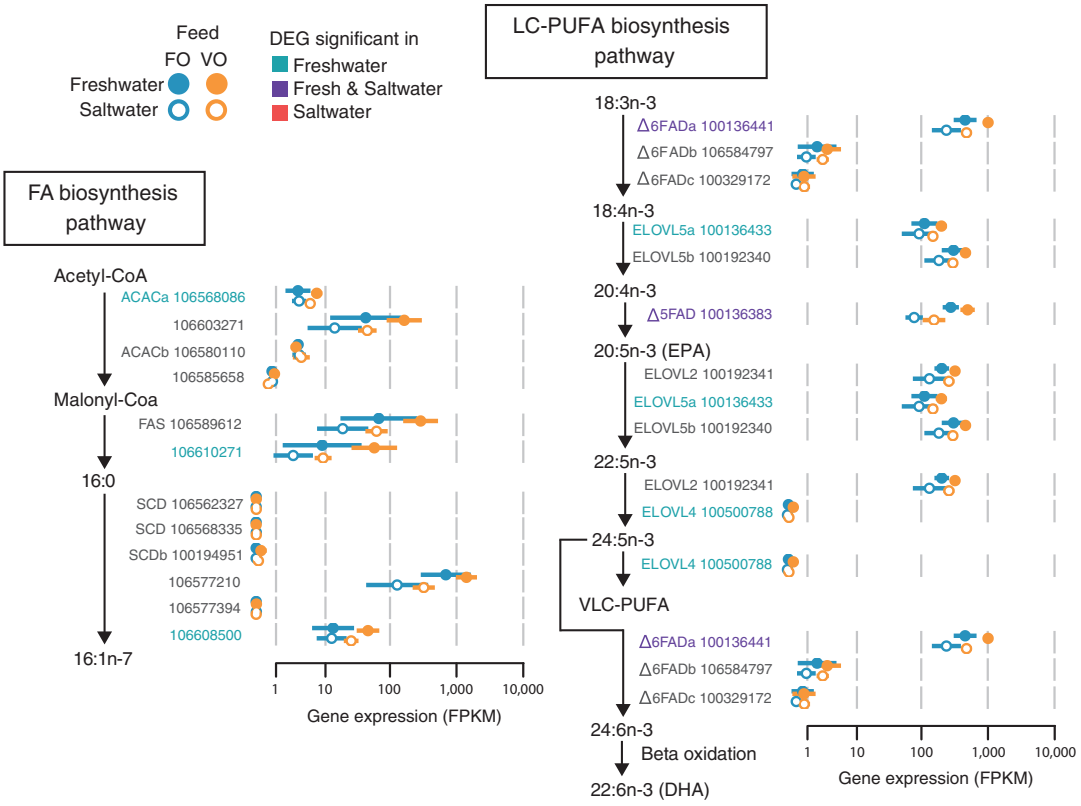


FIGURE 4 Diet and life-stage effects on FA and LC-PUFA biosynthesis in salmon liver. Core fatty acid (FA) biosynthesis and biosynthesis of unsaturated fatty acids pathways with Atlantic salmon genes annotated to each catalytic step (enzyme names followed by NCBI gene numbers). Gene expression levels are shown as mean (point) and standard deviation (line) of expression in eight samples (measured in log (FPKM + 1)) from each diet (FO, VO feeds) and life-stage (freshwater, saltwater) combination. Genes significantly (FDR < 0.05) differentially expressed (DEG) between diets in a life stage are highlighted [Colour figure can be viewed at wileyonlinelibrary.com]

remodelling represented a concerted shift in the metabolic role of liver and gut. After the salmon entered the marine stage, lipogenic gene expression in the liver was significantly decreased, as evident by the markedly lower expression (2.2- to 3.3-fold) of the master regulator of lipid metabolism SREBP1, a fivefold decrease in expression of fatty acid synthase and a two- to threefold decrease in rate-limiting enzymes in LC-PUFA synthesis (i.e., Δ5FAD, Δ6FADa; Figure 4). Liver and gut gene expression also indicated increased catabolic activity in saltwater, with upregulation of the carnitine palmitoyltransferase 1 and 2 genes, responsible for uptake of fatty acids into mitochondria for β-oxidation (Lehner & Quiroga, 2016). Finally, expression of lipid transport genes shifted from liver to gut with the transition to seawater (apolipoproteins, pathway "Fat digestion and absorption" in Figure 6). Four apolipoproteins (of 11 annotated) were differentially regulated in liver between different life stages, with a 2.4- to 5-fold decrease in saltwater compared to freshwater. In stark contrast, nine of the diet-regulated apolipoproteins in gut increased their expression in saltwater between 1.8- and 9.7-fold. The results point to an adaptive shift in lipid metabolism, with increased ability to take up lipids in the gut after Atlantic salmon migrates to sea where lipid availability is higher. Remodelling of lipid metabolism across life stages is likely the result of a combination of factors, including the direct regulatory effect of dietary fat itself, effect of salinity and smoltification-induced physiological changes influencing gene regulation. Although the relative importance of these factors is undetermined in our study, the fact that DEGs in the VO versus FO feed contrast were mostly life-stage-specific (Figure 3) supports that factors other than the diet itself contribute significantly to the freshwater and seawater metabolic phenotypes.

Interestingly, diet had a strong influence on the number and direction of gene expression changes between freshwater and saltwater (Figure 6). In gut, about twice as many DEGs (with respect to the fresh- to saltwater transition) were observed in salmon when fed

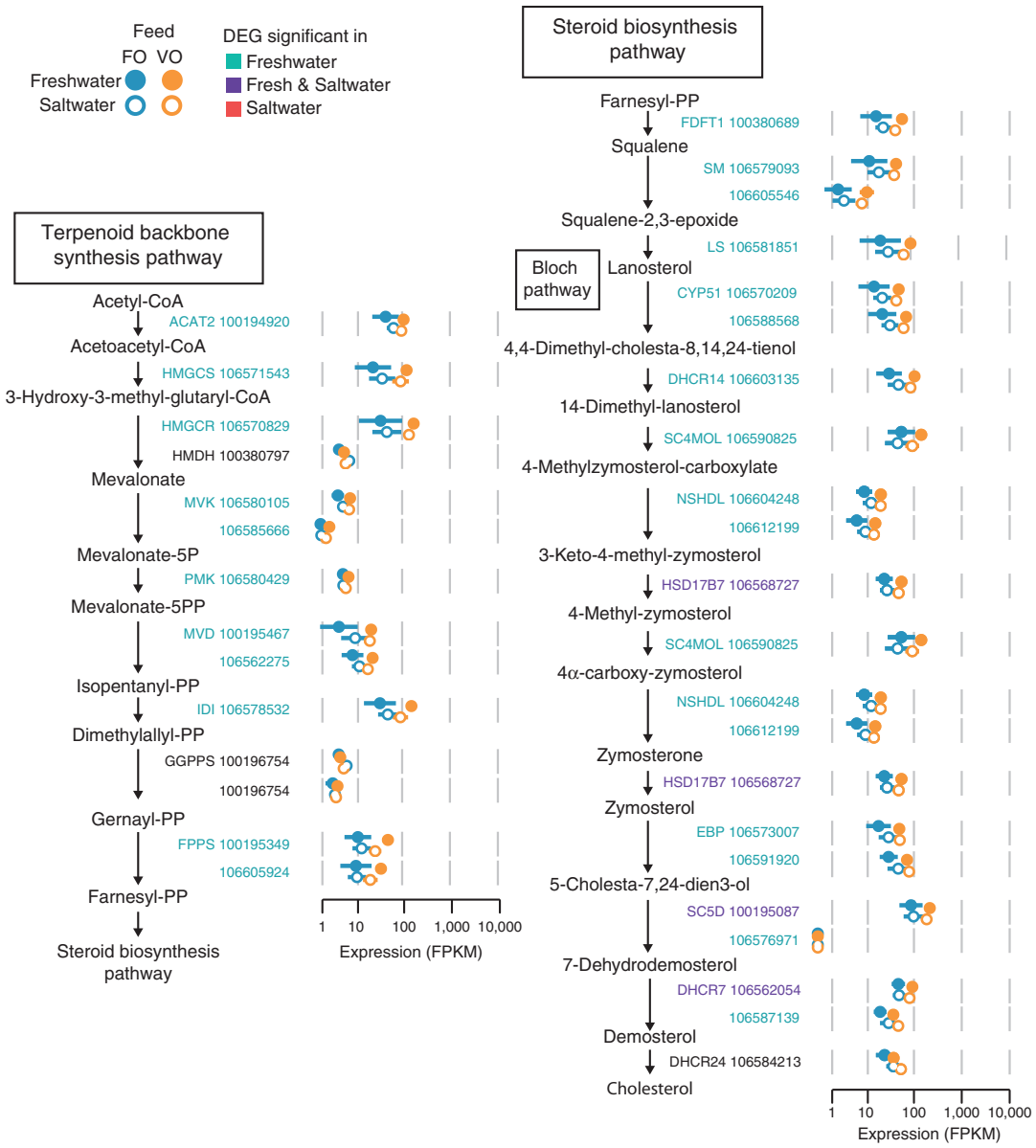


FIGURE 5 Diet and life-stage effects on cholesterol biosynthesis in salmon liver. Terpenoid backbone synthesis and steroid biosynthesis pathways with Atlantic salmon genes annotated to each catalytic step (enzyme names followed by NCBI gene numbers). Gene expression levels are shown as mean (point) and standard deviation (line) of expression in eight samples (measured in log(FPKM + 1)) from each diet (FO, VO feeds) and life-stage (freshwater, saltwater) combination. Genes significantly (FDR < 0.05) differentially expressed (DEG) between diets in a life stage are highlighted [Colour figure can be viewed at wileyonlinelibrary.com]

FO diet than VO diet (Figure 6a). In liver, the diet effect was less pronounced, with the FO group containing 46% more DEGs than the VO group (Figure 6a). This diet effect pattern was reflected in the lipid metabolism genes with 89% and 16% more DEGs in the FO group for gut and liver, respectively (Figure 6b). As this diet and life-stage interaction is a genomewide trend, and more pronounced in

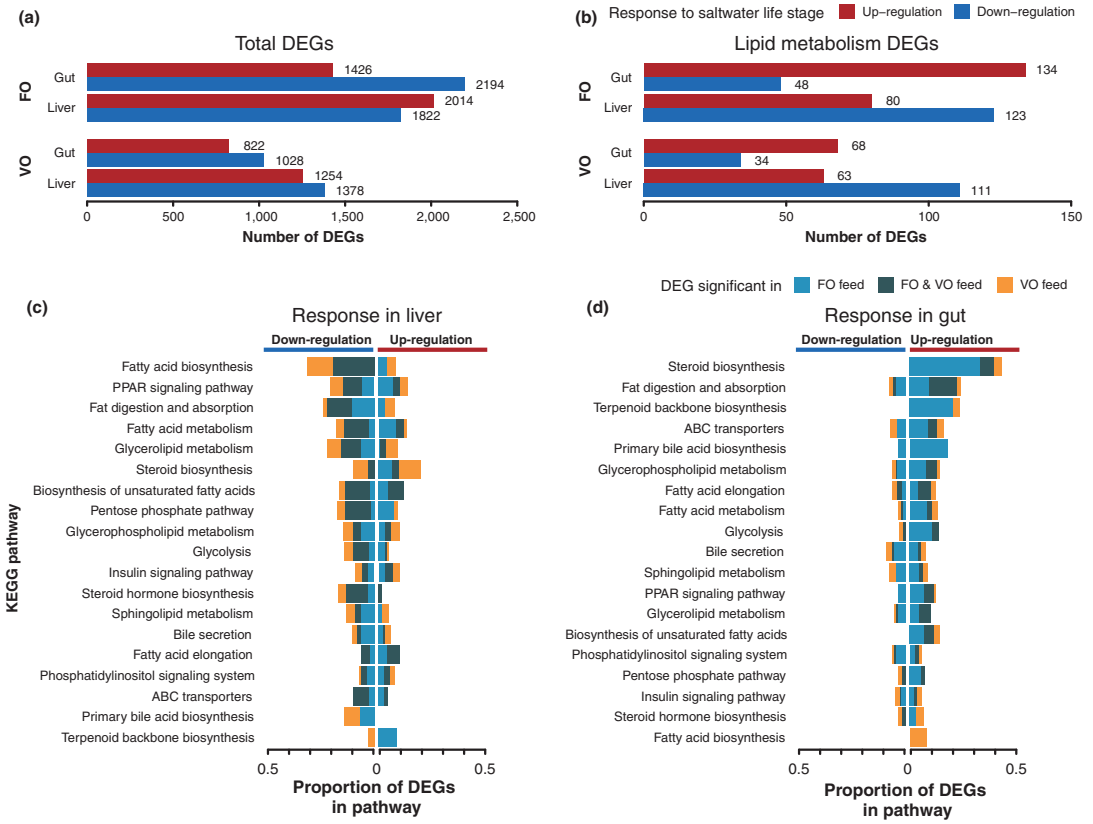


FIGURE 6 Gene regulation in response to life stage. (a) Total number of significant (FDR < 0.05) differentially expressed genes (DEGs) between freshwater and saltwater life stages in the liver and gut tissues of Atlantic salmon fed fish oil (FO) or vegetable oil (VO) diets (see Files S6 and S7 for underlying data). (b) As above, but for lipid metabolism DEGs. (c) Proportion of genes in each KEGG pathway that are DEGs in liver and (d) gut, coloured by DEG significance in only FO, only VO or both diets, and separated into up- or downregulation in saltwater samples [Colour figure can be viewed at wileyonlinelibrary.com]

gut tissue than in liver, this pattern could be related to differences in osmoregulation and adaptation to saltwater. Two studies have suggested that Atlantic salmon raised on VO-based feeds more closely resembling riverine diets adapt to saltwater sooner and better than salmon raised on FO-based diets (Bell et al., 1997; Tocher et al., 2000). Conversely, there has been evidence that VO-based diets can reduce markers for stress response upon saltwater challenge, resulting in reduced osmoregulatory capacity (Oxley et al., 2010). Regardless of the effect, it is clear that diet can modulate the smoltification process and could explain the discrepancy between diets in number of life-stage-related DEGs. Another possibility is that the different levels of fatty acids in the diets, for example DHA, affect DNA methylation and thus trigger genomewide divergence in gene regulation (Kulkarni et al., 2011).

Our results clearly demonstrate very different baseline lipid metabolic functions in pre- and postsmolt salmon, as well as life-stage-associated changes in the plasticity of lipid metabolism, for example the ability to regulate endogenous LC-PUFA synthesis as a response to changes in diet (i.e., fatty acid composition). As opportunistic carnivores, salmon tend to eat whatever the local environment provides. Thus, in freshwater, insects and amphipods provide variable, mostly low amounts of essential LC-PUFA and total fat (Jonsson & Jonsson, 2011; Sushchik, Gladyshev, Moskvichova, Makhutova, & Kalachova, 2003), favouring a metabolic function that can efficiently regulate endogenous lipid synthesis based on dietary availability (Carmona-Antonanzas, Tocher, Martinez-Rubio, & Leaver, 2014). Conversely, in marine environments, amphipods and smaller fish provide a higher, more stable source of n-3LC-PUFA and total fat (Baeza-Rojano, Hachero-Cruzado, & Guerra-García, 2014; Jonsson & Jonsson, 2011), promoting a metabolic function that allocates less energy to endogenous synthesis of essential lipids.

3.3 | Selection on gene duplicates after whole-genome duplication

Carmona-Antonanzas et al. (2014), Carmona-Antoñanzas et al. (2016) proposed that the salmonid whole-genome duplication may have adaptively increased the potential for endogenous lipid synthesis. We pursued this hypothesis by searching for distinct signatures of selection pressure on lipid metabolism genes in salmon. Specifically, we compared pathways in terms of their tendency to retain both duplicates of gene pairs, in terms of whether duplicates showed similar regulation (expression patterns across diets and environments) and in terms of total gene dosage (for the one or two genes retained of a pair) in salmon compared to pike, its closest unduplicated sister lineage.

To assess the level of Ss4R duplicate retention, we first defined 10,752 Ss4R duplicate pairs (21,504 genes) in the NCBI RefSeq annotation using the same approach as Lien et al. (2016). Of the 1,421 annotated lipid metabolism genes, 867 (61%) were retained as duplicated genes after Ss4R (Figure 7a; in contrast to 47% of the 45,127 salmon genes assigned to ortholog groups). Moreover, our results showed large variation in the proportion of retained duplicates in each lipid metabolism pathway (Figure 7), with the most extreme case being "fat digestion and absorption" with 80% retained duplicates and "steroid hormone biosynthesis" with only 27% retained Ss4R duplicates.

The regulatory conservation of the duplicates was then estimated by calculating co-expression correlation between Ss4R duplicates from RNA-Seq data representing a time-course of dynamic changes in gene expression and lipid metabolism function in liver. Fish in the same feeding trial were switched from VO to FO feed and vice versa, in both fresh- and saltwater conditions (see Figure 8 for details). In total, 38 sampling time points (20 in freshwater and 18 in saltwater) from the feed switch experiment were used. Pathway-level analyses showed that regulatory conservation was not associated with duplicate retention (Figure 7). For example, the "*biosynthesis of unsaturated fatty acids*" pathway had significantly fewer duplicates retained than expected by chance (*p*-value <.0234), but a significant overrepresentation of duplicate pairs that display highly similar regulation (*p*-value <.0142 and <.0361 in freshwater and saltwater, respectively). Interestingly, the "insulin signalling pathway" also showed higher-than-expected duplicate coregulation. This pathway has been shown to be

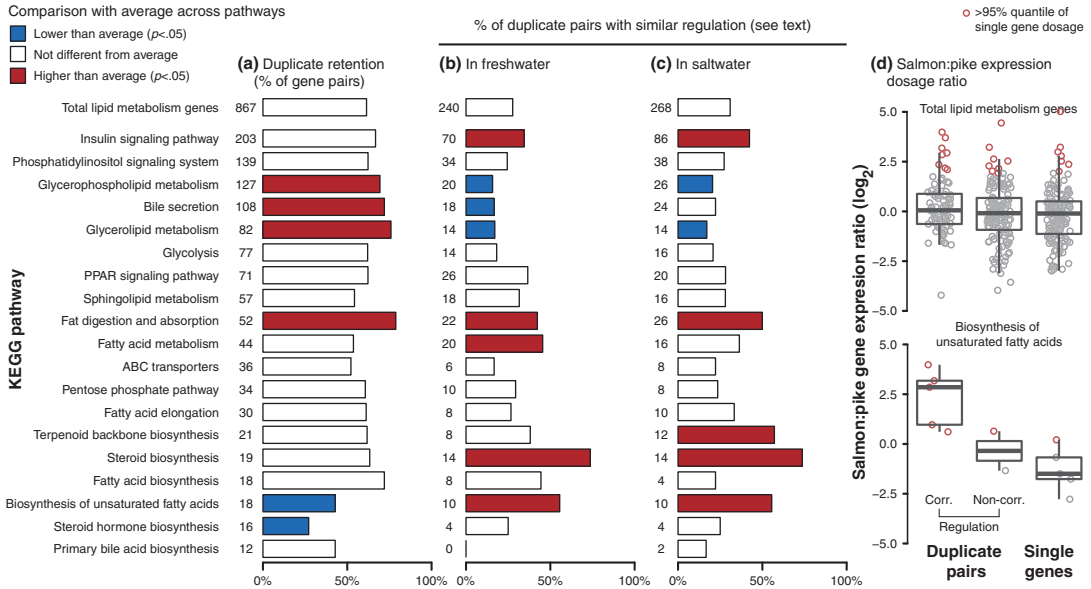
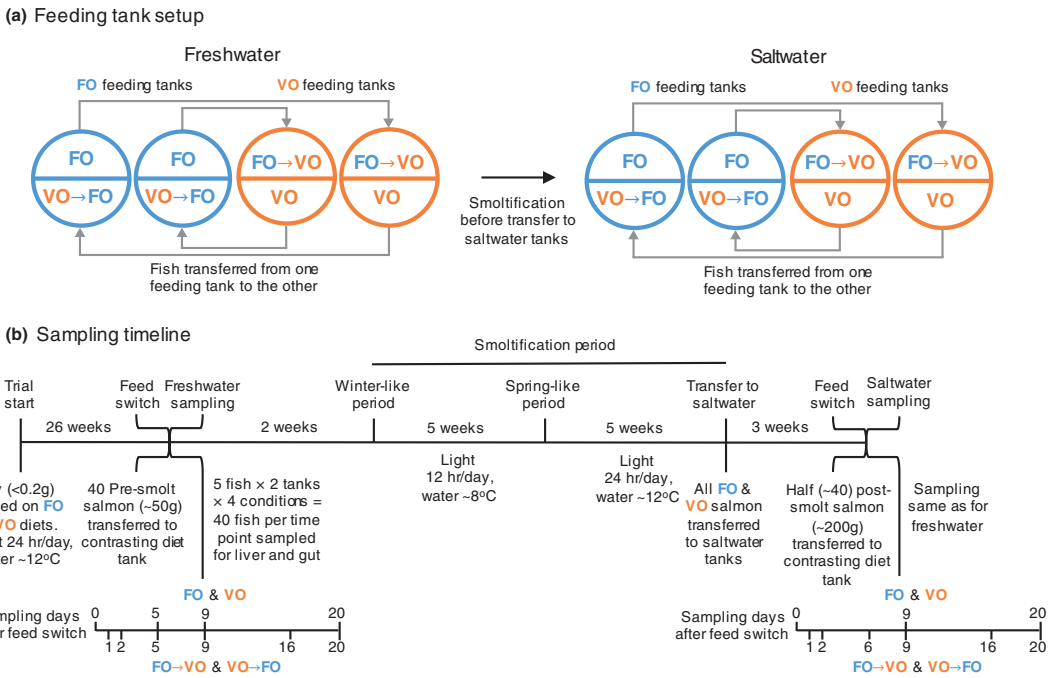


FIGURE 7 Gene duplication in lipid metabolism pathways. For the total list of lipid metabolism genes in Atlantic salmon, and sets of genes belonging to different KEGG pathways: (a) number and percentage of genes with a duplicate homolog from the Ss4R duplication. (b) Number and percentage of duplicate genes with correlated liver expression response to feed in freshwater and (c) saltwater (correlation ≥.6, *p*-value <3.306e−3, using 19 time points from feed trial for each water condition). Fisher's exact test was used to detect pathways with significant enrichment compared to all gene (*p*-value <.05) (d) Log2 gene dosage ratios (salmon/pike) in liver from fish in freshwater, where the ratio is computed between expression in the salmon duplicates (FPKM, sum of the two duplicates) and the expression of the corresponding pike ortholog. Ratios were computed for all lipid metabolism genes and genes in the pathway "biosynthesis of unsaturated fatty acids." For comparison, ratios were also computed for genes without retained duplicates, that is with a 1:1 orthology between salmon and pike. Duplicates were grouped into correlated (corr.) or noncorrelated (noncorr.) based on saltwater correlation result in (c). Dosage ratios (points) greater than the 95% quantile of single gene dosages are marked in red [Colour figure can be viewed at wileyonlinelibrary.com]

important in regulating uptake and transport of FAs in adipose tissue, liver and muscle of Atlantic salmon (Sánchez-Gurmaches et al., 2011). Other pathways showing signatures of increased duplicate coregulation were "terpenoid backbone biosynthesis," "steroid biosynthesis," "fat digestion and absorption" and "fatty acid metabolism" (Figure 7b,c). Overall, the distinct differences in duplicate retention and conservation of regulatory mechanisms across the lipid metabolism pathways suggest differences in selective pressures shaping duplicate evolution following Ss4R. Moreover, the pathways with highly conserved duplicate coregulation were also those that were most responsive to dietary differences in fatty acid composition (Figure 3).



(c) Samples sequenced for total RNA

		Freshwater sampling days							Saltwater sampling days						
Feed	Tissue	0	1	2	5	9	16	20	0	1	2	6	9	16	20
FO	Liver	8			8	8		8	8				8		8
	Gut	4				4		4	8				8		8
VO	Liver	8			8	8		8	8				8		8
	Gut	4				4		4	7				8		8
VO→FO	Liver		4	4	4	4	4	4		4	4	4	4	4	4
	Gut		4	4	4	4	4	4		4	4	4	4	4	4
FO→VO	Liver		4	4	4	4	4	4		4	4	4	4	4	4
	Gut		4	4	4	4	4	4		4	4	4	4	4	4

FIGURE 8 Overview of feed trial experiment. (a) Atlantic salmon fry were reared in four feeding tanks containing freshwater; two continuously fed fish oil (FO) and two vegetable oil (VO). A feed switch involved the transfer of fish from one tank to an empty partition of another tank fed the opposite diet. After smoltification, fish from FO and VO tanks were transferred to four new feeding tanks containing saltwater and the feed switch was repeated. (b) Timeline of feed trial showing fish sampling and smoltification periods. Fish were sampled before (D0) and up to 20 days after the fresh- or saltwater feed switch. (c) Total RNA was sequenced from select fish tissue samples. The number of RNA-Seq replicates is shown for each tissue, condition and time point [Colour figure can be viewed at wileyonlinelibrary.com]

Finally, to link duplicate retention and coregulation to signals of increased gene dosage following Ss4R, we used RNA-Seq data from the northern pike (*Esox lucius*), a species that belongs to the unduplicated sister lineage (see Section 2 for details). For each duplicate pair, we computed the ratio between the sum of Ss4R duplicate expression and its nonduplicated ortholog in pike and compared these ratios to those observed for salmon genes that had not retained two Ss4R duplicates. In total, 69 duplicate pairs from 18 different lipid metabolism-related pathways displayed a combined dosage increase relative to single-copy genes, of which 26 had highly conserved regulation (i.e., correlated expression; File S8). We saw no systematic effect of gene dosage when comparing the total gene expression of duplicate pairs with that of single-copy genes, nor did coregulation of duplicates associate with increased gene dosage (Figure 7d). This pattern was also true for most individual lipid pathways (Appendix S1: Figures S4, S5), except for "*biosynthesis of unsaturated fatty acids*," "*fatty acid metabolism*" and "*fatty acid elongation.*" These three pathways showed a link between coregulation of duplicated genes and higher total gene dosage (Figure 7d; Appendix S1: Figures S4, S5). Underlying this link were three genes with coregulated dosage effects shared between all three pathways; trifunctional enzyme alpha subunit b (hadhab), elovl6 and the previously identified elovl5 (Carmona-Antonanzas et al., 2014, 2016). Only elovl5 is known to be directly involved in core PUFA biosynthesis. Hadhab is involved in mitochondrial β-oxidation/elongation, and elovl6 is involved in elongation of saturated and monounsaturated fatty acids (Bond, Miyazaki, O'Neill, Ding, & Ntambi, 2016). Although we do not see a general trend of increased gene dosage effects on lipid metabolism genes after whole-genome duplication, it is likely that an increased dosage of elovl5 and the 68 other duplicate pairs has affected the function of lipid metabolism in salmon.

4 | CONCLUSION

Atlantic salmon needs great plasticity of physiology and behaviour to adapt for migration between freshwater and sea. By analysing transcriptomic changes through the transition from fresh- to saltwater and the associated increase in dietary lipids, we identified an overall remodelling of lipid metabolism, with liver reflecting higher lipid metabolic plasticity and higher capacity of endogenous synthesis of LC-PUFAs in freshwater, while gut lipid uptake genes become more active in saltwater. These results indicate adaptive optimization of the Atlantic salmon lipid metabolism to account for life-stage-specific dietary availability. Moreover, we found signatures of pathway-specific selection pressure on gene duplicates, including a gene dosage increase in three genes involved in fatty acid metabolism. This illustrates possible adaptive consequences of the salmonid whole-genome duplication for the evolution of lipid metabolism. Future studies should attempt to decipher how the life-stage-related metabolic reprogramming is controlled (e.g., through epigenetic mechanisms). Understanding this will have important implications for understanding evolution of genome regulatory processes in anadromous salmonids and potentially have economically important implications for Atlantic salmon aquaculture.

ACKNOWLEDGEMENTS

This work was financed by the projects DigiSal NFR 248792 and GenoSysFat NFR 244164. We also thank anonymous reviewers for comments on the earlier version of the manuscript as well as Dominic Nanton and Cargill Innovation Center Dirdal for producing the fish trial feeds.

DATA ACCESSIBILITY

- Supplementary files have been deposited to datadryad.org under the accession: https://doi.org/10.5061/dryad.j4h65.
- All gene expression results can be accessed through the interactive shiny Web server: https://goo.gl/8Ap89a.
- Lipid metabolism gene annotation can be accessed from https://goo.gl/VVUVWr.
- Raw RNA-Seq data have been deposited into European Nucleotide Archive (ENA) under the project Accession no. PRJEB24480.

AUTHOR CONTRIBUTIONS

S.R.S., J.O.V., A.G., and J.S.T. conceived of the study and designed the feeding trial experiment. S.R.S., J.O.V., and A.G. carried out orthogroup prediction and lipid metabolism annotation. T.N.H., Y.J., J.S.T., and J.O.V. carried out the feeding trial. T.N.H. and Y.J. prepared the samples for RNA sequencing. M.T., S.L., and M.L. provided input on the experimental design and helped interpreting the results from the transcriptomic and gene duplicate analysis. G.G. and T.R.H. performed the bioinformatic analyses. T.N.H., G.G., S.R.S., and T.R.H. wrote the manuscript. All authors reviewed the final manuscript draft.

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SUPPORTING INFORMATION

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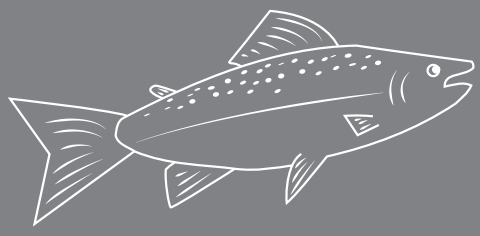
How to cite this article: Gillard G, Harvey TN, Gjuvsland A, et al. Life-stage-associated remodelling of lipid metabolism regulation in Atlantic salmon. *Mol Ecol*. 2018;27:1200–1213. https://doi.org/10.1111/mec.14533

Paper III

Thomas N. Harvey, Jon Olav Vik, Torgeir R. Hvidsten, Simen R. Sandve (2019) **Mapping the transcriptomic landscape of Atlantic salmon during smoltification.** Manuscript.



Mapping the transcriptomic landscape of Atlantic salmon during smoltification

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Abstract

Atlantic salmon undergo a metabolic transformation during smoltification and seawater transfer. Lipid biosynthesis in liver generally decreases and fatty acid composition changes from freshwater-like to seawater-like. Recent studies have demonstrated that this shift is a genome wide trend; however, whether it is associated with smoltification or seawater transfer is not clear. Other aspects of smoltification are known to be coordinated by shortened photoperiod, but whether this is true for lipid metabolism in liver is unknown. In this study, we aim to elucidate the timing of lipid metabolism transformation and determine whether it is influenced by photoperiod history by mapping the transcriptomic landscape in liver during smoltification and seawater transfer. We observe an overall decrease in lipid metabolism related gene expression in smolts before transition to seawater, followed by decreased gene expression of other metabolic pathways after seawater transfer. Photoperiod history does not appear to play a role in modulating changes in lipid metabolism gene expression during smoltification. Additionally, several known epigenetic remodeling genes were differentially expressed during shortened photoperiod and seawater transfer and could be evidence that epigenetics plays a role in regulating gene expression across life stages.

1 Introduction

Atlantic salmon are an anadromous species. They begin life in freshwater riverine habitats, then migrate to sea to grow and mature before returning to freshwater to spawn. Seawater habitats are drastically different in many ways such as salinity, predation, and dietary availability, so migration is preceded by morphological, behavioral, physiological transformation collectively referred to as smoltification. This transformation is associated with changes in a number of processes and tissues including ionoregulation in gill chloride cells [1], visual pigmentation in the retina [2], and importantly for this study, lipid metabolism in the liver and gut. Prior to seawater transfer salmonids transition their fatty acid (FA) composition from freshwater-like low in LC-PUFA, to seawater-like high in LC-PUFA [3]. Two separate studies have demonstrated that lipid composition of tissues in Atlantic salmon reared on diets with drastically different PUFA levels converge just before seawater transfer, suggesting a specific LC-PUFA compositional requirement for migration to sea [4,5]. More generally, smoltification triggers increased lipid breakdown and decreased lipid biosynthesis [6]. Recently, the first transcriptome wide study of lipid metabolism across fresh and seawater life-stages revealed that decreased lipid metabolism in Atlantic salmon smolts is a genome wide trend and that decreased biosynthesis in liver was compensated by increased absorption in the gut [7]. Unfortunately, in this study smoltification and seawater transfer were confounded (i.e. smolts in freshwater were not sampled), so whether or not the observed change in lipid metabolism was associated with smoltification or seawater transfer could not be determined. We aim to address this knowledge gap in the current study.

Smoltification is an inherently disjointed process with the various aspects (growth rate, coloration, body shape, etc.) under independent circannual control. Photoperiod does not act as a trigger for smoltification, but rather an external cue to synchronize the various smoltification processes [8]. Information on photoperiod is transmitted through a light-brain-pituitary axis where changes in day length influence levels of circulating hormones (melatonin, growth hormone, insulin-like growth factor) and cause physical changes to brain structure [9]. Salmon exposed to constant light will still become smolts, but will suffer from reduced fitness at sea compared to salmon that have experienced a shortened photoperiod. The effect of photoperiod on observed changes in lipid metabolism is wholly

unknown, so we address this by comparing the liver transcriptome of salmon with and without shortened photoperiod during smoltification.

Although we recognize the environmental and physiological mediators of smoltification and seaward migration, the underlying molecular mechanisms governing these processes are still poorly understood. Several studies have begun to elucidate these and have implicated epigenetics in the form of DNA methylation as a driving force. For example, Morán et. al. (2013) [10] found changes in DNA methylation patterns were associated with salt-induced acclimation to seawater environments in Brown trout and Baerwald et. al. (2016) [11] identified differential methylation patterns between resident and migratory Rainbow trout populations. These observations suggest that pre-adaptive smolt phenotypes are linked to changes in the epigenome, however studies on Atlantic salmon and on other aspects of epigenetic change such as histone modification and chromatin dynamics are completely lacking.

In this study, we aim to map the transcriptomic landscape of Atlantic salmon liver across the smoltification gradient and subsequent seawater transition for the first time. We identify processes modulated by winter, smoltification, and seawater transfer using RNAseq data from a large smoltification trial controlling for changes in photoperiod and salinity. Finally, we provide evidence that epigenetic remodeling could contribute to the observed smoltification and seawater associated metabolic remodeling.

2 Results and discussion

2.1 Fish performance

Our goal in this study was to determine effects of smoltification on metabolism, deconvolute smoltification-related effects from seawater-related effects, and determine whether there was an effect of shortened photoperiod on the liver transcriptome. To accomplish this, we reared salmon for 21 weeks on commercial diets high in DHA and EPA as they would be in an aquaculture environment. Salmon were divided into three cohorts: 1) “experimental” which were exposed to shortened photoperiod, allowed to smoltify, and subsequently transferred to seawater; 2) “winter control” which were split off from the experimental group at week 1 and did not experience winter; and 3) “freshwater control” which were split off from the experimental group on week 19 and remained in freshwater (figure 1). We induced smoltification in experimental fish by

exposure to an artificial winter with 8 hours of light per day. Fish that were given an artificial winter grew more slowly than light control fish in the five weeks following winter with specific growth rates of 1.48 and 2.37 respectively (figure 1). This is normal and attributed to lower levels circulating plasma growth hormone in fish during winter [12]. Similarly, fish that were transferred to seawater after smoltification grew more slowly than fish that remained in freshwater with specific growth rates of 0.99 and 1.78, respectively (figure 1). There was no mortality throughout the freshwater portion of the trial, however when fish were transferred to seawater eight fish in one of the two tanks died after one day. We later realized that this was due to improper oxygenation of the water in one of the two replicate tanks. Liver samples were taken from randomly sampled fish at six timepoints representing different stages in the smoltification process and four of these time points were selected for transcriptomic analysis (figure 1).

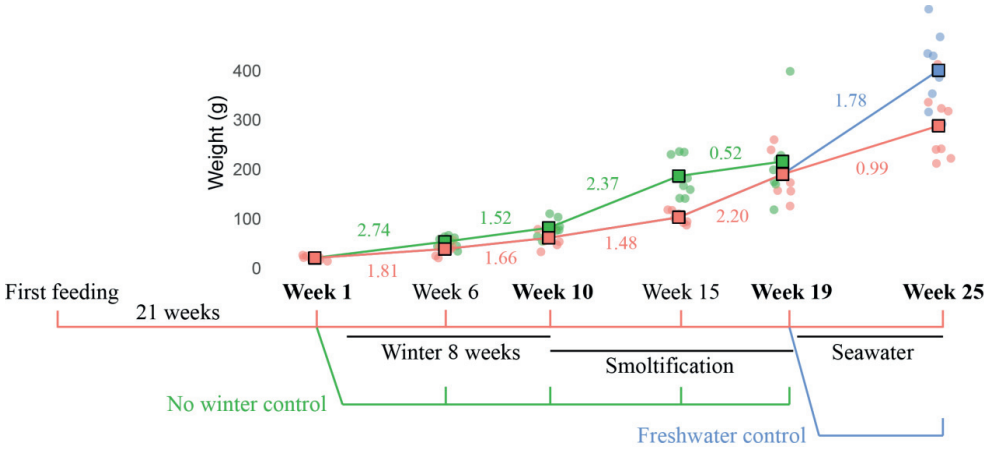

Figure 1: Salmon growth over time – Schematic diagram of the experimental design and growth rates of salmon over time. Experimental fish (red) were raised for 21 weeks after first feeding, then sampled six times over a 25-week period. Fish were split into replicate control tanks after the first sampling (green) and again before seawater transfer (blue). Large squares represent mean weight (n=8) and small circles indicate weight of individual fish. Numbers above and below the lines represent specific growth rate between the two corresponding sampling points. Weeks selected for transcriptomic analysis are in bold.

2.2 Transcriptome changes associated with smoltification and seawater transfer

We characterized global transcriptome response to winter, smoltification, and seawater by sequencing RNA from liver at four key time points: before winter (week 1);

immediately after winter, but before smoltification (week10); immediately before seawater transfer, but after smoltification (week 19); and after seawater transfer (week 25). We then identified any genes that change in expression between any of the time points by performing an ANOVA-like differential expression test. This yielded 6,054 differentially expressed genes (DEGs, FDR <0.05) which we assigned to five co-expression clusters using hierarchical clustering (figure 2A). Each of these clusters displayed a unique expression trend; genes within clusters one and three changed the most after seawater transfer whereas genes within clusters two and five had the largest expression shifts on week 19, after smoltification, but before seawater transfer (figure 2B). Cluster four was most responsive to winter, with gene expression increasing on week 10, immediately after eight weeks of winter-like conditions (figure 2B). We then performed KEGG enrichment analysis on each of these clusters, yielding 51 significantly ($p < 0.01$) enriched pathways (figure 2C).

Strikingly, all lipid metabolism pathways were enriched in the smoltification-associated cluster two, which strongly decreased in expression four weeks after winter (figure 2C). Additionally, the lipid metabolism-related signaling pathways *"Adipocytokine signaling pathway"* and *"PPAR signaling pathway"* were enriched in this cluster. Alternatively, almost all other metabolism related pathways (amino acid and carbohydrate) were enriched in the seawater-related cluster one, which greatly decreases in expression after seawater transfer (figure 2C). This coincided with a decrease in specific growth rate (figure 1) and downregulation of pathways related to cell division and growth (i.e. 11 pathways under "*Genetic information processing*" and the pathway "*Cell cycle*").

Since many KEGG pathways contain different enzymes with reciprocal activities, deeper examination of key genes within these pathways is required to reveal exactly what metabolic processes are driving enrichment trends. We observed a distinct bias towards genes relating to long-chain fatty acids downregulated in freshwater smolts. For example, we find six long-chain-fatty-acyl-CoA ligase (*acsl*) genes (three *acsl3* and three *acsl4*) and three acetyl-CoA synthetase genes (*acs2l-1*, *acs2l-1*, and *acs2l-1*) downregulated in freshwater smolts (figure 3B). Synthesis of acetyl-CoA by ACSs and activation of long-chain fatty acids by ACSLs is the first obligatory step for entry into beta-oxidation or biosynthesis pathways, so a decrease in these gene products means that metabolism of their substrates (acetate and C12 to 20 FAs) also decreases [13]. We also found that key

genes related to PUFA biosynthesis *5fad*, *6fada*, *6fadb*, *elovl5b*, and *elovl2* all significantly decrease (FDR <0.05) in freshwater smolts (figure 3B). Finally, three copies of the key gene diacylglycerol acetyltransferase (two *dgat1* and one *dgat2*) which catalyzes the last committed step in triacylglycerol biosynthesis [14] decreased in freshwater smolts (figure 3B). Collectively, co-downregulation of these important genes is a strong indicator of decreased utilization and processing of FAs, especially LC-PUFA, in smolts preparing to enter seawater environments.

We identified genes directly influenced by seawater transfer by comparing gene expression between the experimental group and freshwater control group on week 25, yielding 1,296 DEGs (FDR <0.05). Most of these (713 DEGs) were shared with genes belonging to clusters one and three above. Regarding lipid metabolism, processes related to *de novo* fatty acid synthesis decrease in seawater relative to control. Both copies of fatty acid synthase (*fas1* and *fas2*) and one acetyl-CoA carboxylase (*acc1*) decreased expression in seawater (figure 3C), all of which catalyze key steps in de novo fatty acid synthesis [15]. Additionally, two other ACSL genes (*acsbg2* and *acsl1*) known to be involved in saturated and monounsaturated FA activation were downregulated in seawater [16]. This coincided with an increase in five thioesterase genes, two *acot1* genes and three *acot5* genes (figure 4B), responsible for de-activation of FAs through the hydrolysis of acyl-CoAs [17]. It is unlikely that de-smoltification occurred in the freshwater control smolts because expression of these genes remains stable between week 19 and 25 in the control fish. This combination of decreased expression of key de novo biosynthesis genes and increased FA de-activation through greatly increased thioesterase expression suggests a diminished metabolic state in liver of fish after transition to sea, in line with previous findings [7].

While lipid metabolism-related gene expression is known to decrease in liver of seawater stage Atlantic salmon [7], this is the first report that systemic downregulation of lipid metabolism gene expression actually occurs before transition to sea. Furthermore, this trend is specific to lipid metabolism, since other metabolic pathways (amino acid, carbohydrate, and vitamin metabolism) are unaffected by smoltification (figure 2C). Given that availability of PUFA in seawater environments is higher than freshwater [18] and that the body lipid composition changes to match this in freshwater smolts [19], it is

likely that the observed decrease in lipid metabolism is a genetically programmed preadaptation to life at sea.

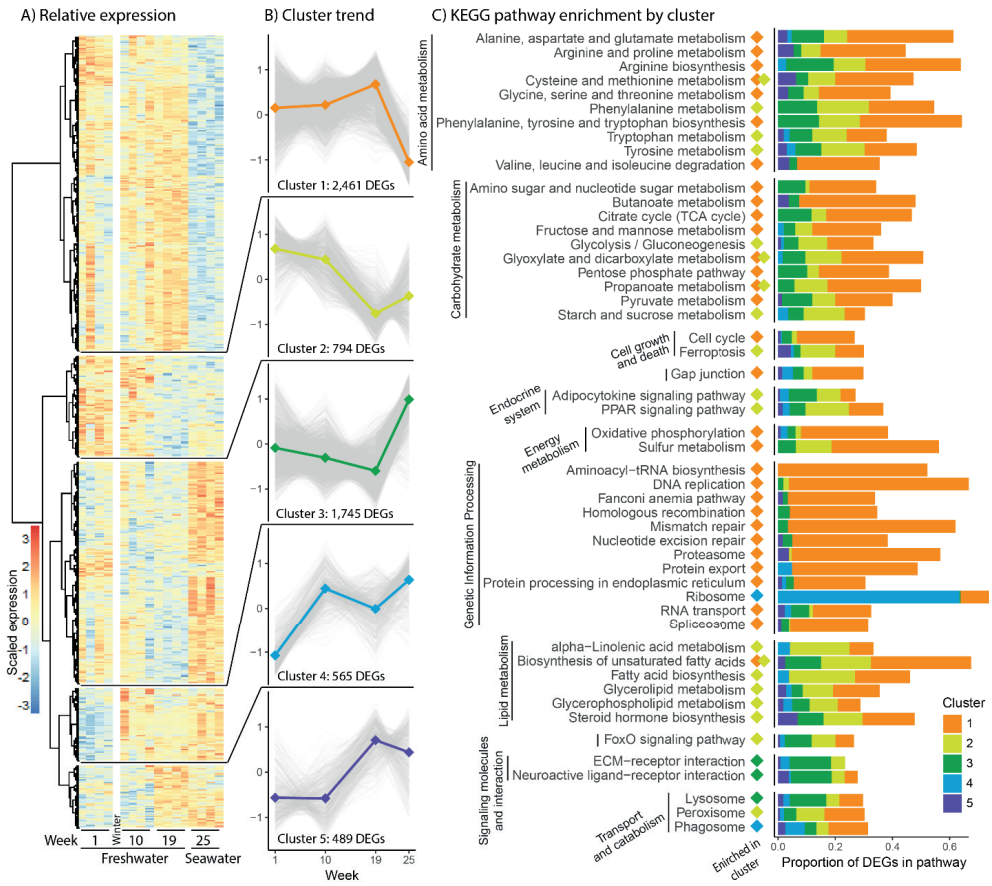

Figure 2: Global gene expression across life-stage – A) Relative expression of 6,054 genes differentially expressed between any time point in the experimental fish cohort (FDR <0.05). Scaled expression is denoted as gene-scaled counts per million. Genes were partitioned into five co-expression clusters by hierarchical clustering. B) Gene expression trends over time by cluster. Colored line indicates mean relative expression while gray lines are relative expression of individual genes. C) KEGG pathway enrichment by cluster. All pathways are significantly enriched (p <0.01) in the indicated expression cluster. Bars indicate the proportion of DEGs belonging to each cluster within a given pathway.

2.3 Effect of photoperiod history on smolt transcriptome

We also identified photoperiod-associated genes by performing differential expression analysis between the experimental group and the winter control group. On week 10, we identified 292 DEGs (FDR <0.05). The single most strongly downregulated gene was

phosphocarboxykinase 1 (*pck1*) (figure 3A), the rate limiting step in gluconeogenesis. Additionally, vitamin D hydroxylase (*cyp2r1*) (figure 3A), the first step in the formation of biologically active vitamin D [20], was strongly downregulated in winter. This is likely due to decreased UV mediated vitamin D synthesis in the skin resulting from shortened photoperiod. Winter had the opposite effect on retinoic acid signaling, strongly inducing expression of both copies of retinol dehydrogenase 7 (*rdh7*) (figure 3A). This converts vitamin A (retinol) to retinal, the precursor to retinoic acid (RA), a potent transcriptional regulator that binds to the transcription factor retinoic acid receptor and is involved in developmental regulation [21]. Additionally RA binds to and activates, retinoid X receptor (RXR), which forms obligate heterodimers with liver X receptor (LXR) and peroxisome proliferator-activated receptor (PPAR), two well-known lipid metabolism regulators [22]. It is possible that increased RA signaling and decreased vitamin D signaling during winter is a yet undescribed mediator of winter-associated effects in Atlantic salmon.

Surprisingly, on week 19 we found zero DEGs (FDR <0.05). This, in combination with the silvery coloration of the winter control salmon, likely means that the control salmon had smoltified anyway by week 19. Since the transcriptome profiles of fish converged regardless of photoperiod history, it is likely that either winter associated effects could not be detected at the transcript level or photoperiod history does not play a role in mediating the observed gene expression changes in the liver after smoltification.

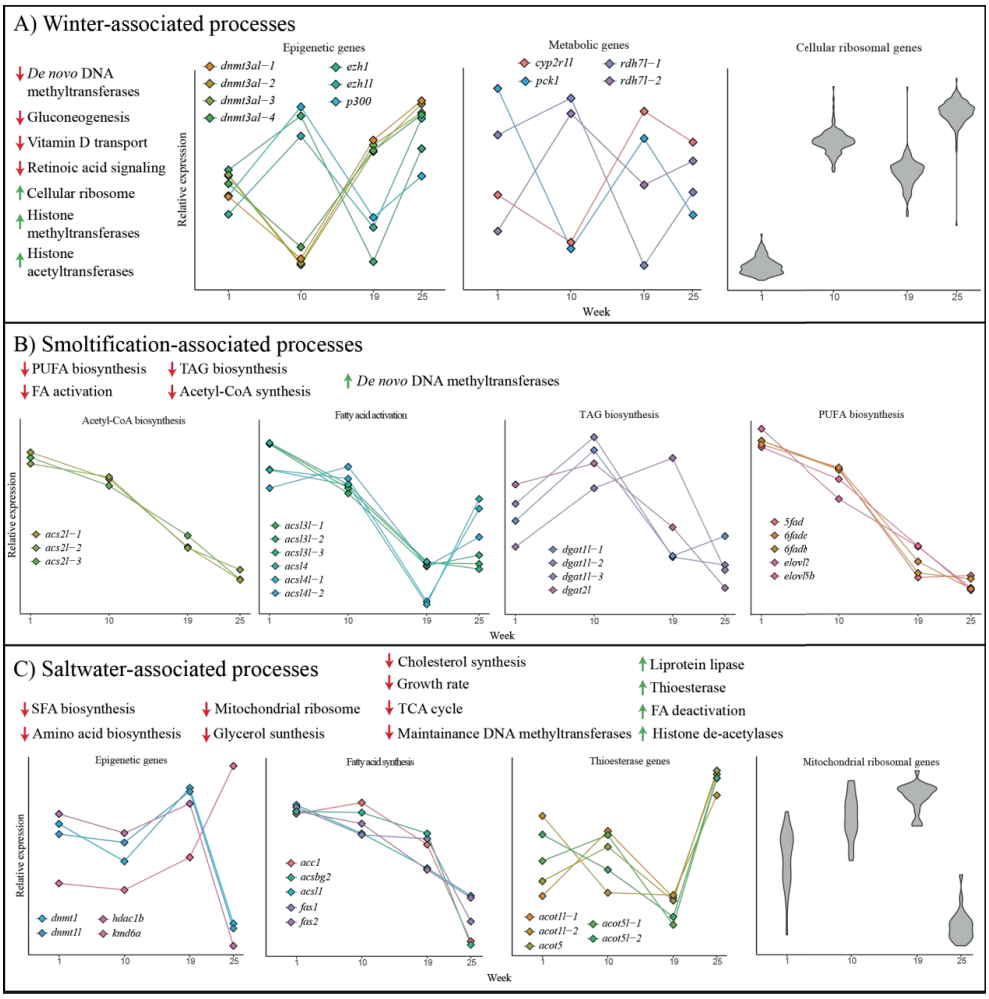

Figure 3: Processes influenced by winter, smoltification, and seawater – All gene expression levels are represented as gene-scaled counts per million. Points indicate mean relative expression across four replicate samples. Relative expression of ribosomal genes are represented as a distribution of 158 cellular ribosomal genes (A) and 23 mitochondrial ribosomal genes (C) at each of the four time points. A) Processes associated with winter. Genes were manually curated based upon results from the winter-no winter differential expression test and genes belonging to cluster four in figure 2. B) Processes associated with smoltification. Genes were selected based upon membership in clusters two and five in figure 2. C) Processes associated with seawater. Genes were selected based upon results from the seawater-freshwater differential expression test and membership in clusters one and three in figure 2.

2.4 Expression of cellular and mitochondrial ribosomal genes across life-stages

We observed a distinct expression pattern in the ribosomal protein genes. Cellular ribosomal protein genes behaved strikingly different from mitochondrial ribosomal protein genes across the smoltification gradient, the former being upregulated after winter and the latter upregulated in freshwater smolts then strongly downregulated in seawater. We found that 158 cellular ribosomal genes significantly increased in expression after winter (figure 3A) while 23 ribosomal genes significantly decreased in expression after seawater transfer (figure 3C). This is in line with previous findings that the number of mitochondria increase in freshwater smolts [24]. Unfortunately, seawater mitochondrial levels have not been studied, but our data suggests that after seawater transfer mitochondrial counts would greatly decrease. All cellular and mitochondrial ribosomal protein genes shared respective expression trends across the experiment, indicating tight co-regulation since the stoichiometry is very important to proper ribosomal assembly and could play a role in translational regulation [25]. Since mitochondria are the energy powerhouses of the cell, the strong decrease in mitochondrial ribosomal protein expression in seawater is indicative of a drastically reduced energy state in the liver of seawater life-stage salmon. Additionally, it has been proposed that ribosomes play a more active role in metabolic regulation through differential translation or changes in translational efficiency [26], so our observation of strong differential expression trends of ribosomal protein genes at various life-stages could be evidence for an additional layer of metabolic regulation at the ribosomal level in salmon.

2.5 Expression of epigenetic remodelers across life-stages

We also identified potential markers for winter and seawater induced epigenetic remodeling. Two *de novo* DNA methyltransferase 3A (*dnmt3a*) genes are significantly downregulated by winter while two genes known to modify histones, histone acetyltransferase P-300 (*p300*) and histone methyltransferase *ezh1*, are significantly upregulated by winter relative to winter control fish (figures 3A and 4A, FDR<0.05). P300 is responsible for acetylation of all four core histones, generally associated with gene activation [27,28]. EZH1 cooperates with polycomb repressive complex 2 to mono, di, and tri methylate histone 3 (H3K27me1,2,3) generally resulting in gene silencing [28,29].

Both of these genes have been implicated in a wide variety of cellular tasks including development, growth, pluripotency, and oncogenesis [30], so it is conceivable that they also play a role in the developmental task of smoltification in preparation for life at sea.

Seawater had a distinctly different effect on the epigenetic remodeling gene repertoire. Two DNA methyltransferase 1 genes (*dnmt1*) were downregulated in seawater relative to freshwater control fish (figures 3C and 4B). These have a high affinity for hemimethylated DNA and are therefore traditionally thought of as "maintenance methylases" which mediate the heritability of methylation patterns during cell division [31], so it is likely that this is related to the observed decrease in specific growth rate. Simultaneously, *hdac1b* involved in histone deacetylation and *kmd6a* involved in histone demethylation were significantly down and up regulated, respectively (figures 3C and 4B). The substrate activity for HDAC1 remains ambiguous, but there have been reports that it can deacetylate all four core histones at all acetylation marks [32]. KMD6A works in opposition to the previously mentioned EZH histone methylases, selectively removing methyl groups from H3K27me3 and activating gene expression [33]. The differential expression of these two genes with histone remodeling activities could indicate epigenetic remodeling specific to the seawater environment.

Our observations of changes in known epigenetic remodelers coordinated with changes in gene expression during shortened photoperiod and seawater transfer could be evidence for epigenetic remodeling during these life-stages. Specifically, our results suggest that winter is associated with gene activation by histone acetylation and gene silencing by EZH mediated trimethylation of H3K27. Moreover, they suggest that seawater is associated with depletion of H3K27me3 epigenetic marks and maintenance of histone acetylation patterns. Since all measurements are at the transcript level, it is not possible to say whether epigenetic remodeling is contributing to observed expression patterns, but it does provoke interesting questions concerning the molecular nature of gene regulation during winter, smoltification, and seawater transfer. Future studies should focus on experimentally defining the extent of epigenetic remodeling across life-stages by identifying genomic regions differentially methylated or differentially accessible and link this to gene expression.

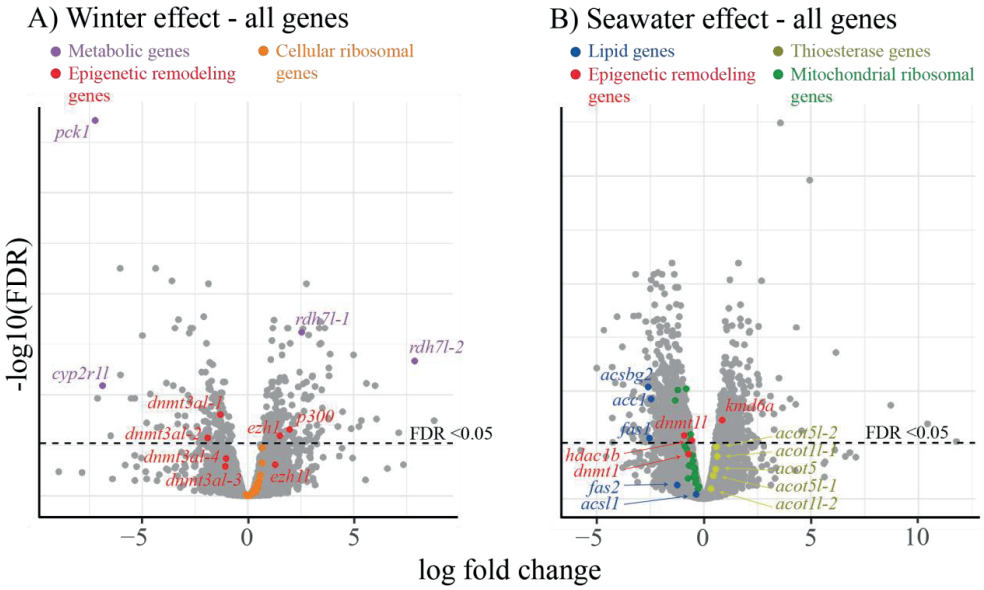

Figure 4: Genes influenced by winter and seawater relative to control – A) Volcano plot of all genes from the week 10 winter-no winter control differential expression test. Genes of interest are colored and labeled. Genes above the black dashed line are considered significant with a FDR <0.05. B) Same as in A, but for the week 25 seawater-freshwater control differential expression test.

3 Conclusion

We confirm previous findings that lipid metabolism is reduced after seawater transfer and provide the first evidence that this is a genome wide trend that occurs after smoltification while salmon are still in freshwater. Furthermore, other aspects of metabolism such as amino acid and carbohydrate metabolism follow the global downregulation patterns observed in lipid metabolism after transfer to sea. This in combination with drastic downregulation of mitochondrial ribosomal genes indicates a diminished energy state in salmon at sea. Our finding that photoperiod history does not induce any clear differences in the transcriptomic profiles of freshwater smolts likely means that winter does not play a role in smoltification associated remodeling of lipid metabolism. Both shortened photoperiod and seawater transfer were associated with altered expression of epigenome remodeling genes, suggesting that epigenetic remodeling could be occurring at both of these stages. As a first step in understanding the underlying molecular mechanisms of metabolic remodeling, this work provides the

foundation and direction for future studies identifying specific epigenetic marks differentially occurring across the smoltification gradient in Atlantic salmon.

4 Materials and methods

4.1 Smoltification trial

All animals used in this study were handled in accordance with the Norwegian Animal Welfare Act of 19th June, 2009. Atlantic salmon eggs were provided by AquaGen Breeding Centre Kyrksæterøra, Norway. Eggs were sterilized at the Norwegian University of Life Sciences (NMBU) fish lab and incubated at 350 to 372 day-degrees until hatching. First feeding of salmon fry took place 5 weeks after hatching when the egg sac had been depleted. Fry were then randomly divided into two replicate tanks and reared on a standard commercial diet high in EPA and DHA for the remainder of the trial. Fish occasionally needed to be euthanized as they grew to maintain dissolved oxygen levels in the tanks. Sampling began 21 weeks after first feeding (week 1) and again 6, 10, 15, 19, and 25 weeks after that. At each sampling point fish were euthanized by a blow to the head and samples of liver from eight fish were cut into ~5mm cubes and placed in RNAlater. Samples were incubated for at least 30 minutes at room temperature before long-term storage at -20°C. One week after the first sampling 30 fish from each tank were transferred to replicate light control tanks and sampled in the same manner as the experimental fish. At the same time, the experimental fish were switched to "winter-like" lighting conditions with 8 hours of light per day for 8 weeks to trigger smoltification, before returning to "spring-like" conditions with 24 hours of light per day. Immediately after the week 19 sampling, 20 fish from each tank were transferred to seawater conditions at the Norwegian Institute for Water Research (NIVA), Solbergstranda, Norway. UV-sterilized seawater used in this life-stage had a salinity of 3%-3.5% and was obtained from the Oslofjord. Fish were sedated before transport and allowed to acclimatize for several hours before being slowly introduced to the new water conditions. The fish that were not transferred to seawater were sampled as a freshwater control at the same time as the experimental fish.

4.2 RNA sequencing

We extracted total RNA from liver samples taken on weeks 1, 10, 19, and 25 with the RNeasy Plus Universal Kit (QIAGEN). Concentration was determined with a nanodrop

8000 spectrophotometer (Thermo Scientific) and quality was assessed by running on a 2100 bioanalyzer using the RNA 6000 Nano Kit (Agilent). Extracted RNA with an RNA integrity number (RIN) of at least eight was used to make RNAseq libraries using the TruSeq Stranded mRNA HT Sample Prep Kit (Illumina). Mean length and library concentration was determined by running libraries on a 2100 bioanalyzer using a DNA 1000 Kit (Agilent). RNAseq libraries were sequenced by the Norwegian Sequencing Center (Oslo, Norway) on an Illumina HiSeq 4000 using 100-bp single end reads.

4.3 RNAseq analysis

After sequencing, samples were demultiplexed by the Norwegian Sequencing Center (Oslo, Norway) before being trimmed, mapped, and counted using all using bcbio-nextgen (https://github.com/bcbio/bcbio-nextgen). STAR [35] was used to align the reads agains the Atlantic salmon reference genome [34] and featureCounts [36] was used to count reads aligned to genes. QC metrics were obtained using FastQC (Andrews S. (2010). FastQC: a quality control tool for high throughput sequence data. Available online at:http://www.bioinformatics.babraham.ac.uk/projects/fastqc) and reporting was done using MultiQC [37]. All statistical analysis was performed in R (v3.5.1). Differential expression analysis was done in edgeR [38] using TMM normalization. Global expression trends across all samples in the smoltification gradient were analyzed using an ANOVA-like differential expression test (see edgeR manual) and a false discovery rate (FDR) cutoff of <0.05. Subsequent hierarchical clustering of DEGs was completed using ward's D2 method on gene-scaled counts per million (cpm). KEGG pathway enrichment was performed on gene expression clusters within edgeR using a p-value cutoff of <0.01. Differential expression analysis between experimental and control fish on weeks 10 and 25 was performed using an exact test and a FDR cutoff of <0.05.

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