The impact of micro-mineral sources and their availability on hepatic lipid metabolism in Atlantic salmon *(Salmo salar)*

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Abstract

The increased use of plant-based ingredients in aquafeeds for Atlantic salmon has led to an increase in phytate, an antinutrient binding micro minerals and reducing their bioavailability. It has been suggested that the chemical form of the minerals (organic/inorganic) can alter their bioavailability, especially in feeds with high phytate content. The functional role of minerals in hepatic intermediary metabolism is poorly understood in fish, though studies have shown that dietary mineral levels can affect hepatic lipid metabolism. However, this effect has not been examined in the nutritionally relevant context of dietary mineral availability in plant ingredient based diets. The aim of this study was to investigate whether availability and chemical form of zinc, selenium and manganese affected liver lipid metabolism of Atlantic salmon.

A feeding trial involving five different diets was performed. The two control diets contained inorganic Zn, Se and Mn with different phytate contents. Unfortunately, the difference in phytate turned out to be too small to have any effect on the mineral digestibility. The three other diets all had the higher phytate content and in each diet one of the inorganic minerals Zn, Se and Mn were exchanged with chelate of Zn, selenium methionine or chelate of Mn, respectively.

The mineral content of the liver was investigated to see if there had been any changes to the mineral status. No significant differences were found. Genes involved in β-oxidation (PPARα, CPT1), lipogenesis (LXR, SREBP1, FAS), bioconversion into LC-PUFA (Δ5Fad, Δ6Fad) and transport out of the liver (ApoB100) were examined to see if there were any effects on hepatic lipid metabolism. There were no significant effects on LXR, FAS, PPARα, CPT1, Δ5Fad or Δ6Fad. ApoB100 and SREBP1 were significantly reduced in the higher phytate control group compared to the lower phytate control. However, these two groups had the same chemical form of all the minerals, no impact of phytate on mineral digestibility was detected and there was similar hepatic content of all three examined minerals. Thus, these effects are probably random effects rather than induced by the diets. As the minerals and phytate likely were not the cause of differences between the two control diets, the organic Mn is probably not the cause of the difference between the higher phytate control diet and the diet containing organic Mn either. Finally, a lipid class distribution analysis was also performed, but no significant effects of minerals on the hepatic lipid composition was discovered. Thus it was concluded that the chemical form of the micro-minerals Zn, Se and Mn probably have no effect on the hepatic lipid metabolism of Atlantic salmon.

List of abbreviations

ACC	Acetyl-CoA carboxylase
ALA	Alpha-linolenic acid
ApoB100	Apolipoprotein B 100
Aq	Aqueous
BHT	Butylhydroksytoluen
ChREBP	Carbohydrate responsive element-binding protein
CPT1	Carnitine palmitoyltransferase 1
CPT2	Carnitine palmitoyltransferase 2
CF	Condition factor
Δ5Fad	Delta 5 fatty acid desaturase
Δ6Fad	Delta 6 fatty acid desaturase
DAG	Diacylglycerol
DHA	Docosahexaenoic acid
DPA	Docosapentaenoic acid
EPA	Eicosapentaenoic acid
FxR	Farnesoid receptor x
FA	Fatty acid
FAS	Fatty acid synthase
FO	Fish oil
FM	Fishmeal
FFA	Free fatty acid
HSI	Hepatosomatic index
HDL	High density lipoprotein
LA	Linoleic acid
LXR	Liver x receptor
LC-PUFA	Long chain polyunsaturated fatty acid
LDL	Low density lipoprotein
MAG	Monoacylglycerol
PPAR	Peroxisome proliferator-activated receptor
PUFA	Polyunsaturated fatty acid
RXR	Retinod x receptor
Se-Met	Selenium methionine
Se-Cys	Selenocysteine
SREBP	Sterol regulatory element-binding protein
TAG	Triacylglycerol
VO	Vegetable oil
VLDL	Very low density lipoprotein

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1 Introduction

1.1 General introduction

Atlantic salmon *(Salmo salar)* has traditionally been fed a diet based on fishmeal (FM) and fish oil (FO). Its production is based on designated wild capture fisheries, using mainly small pelagic fish (Alder et al., 2008). FM and FO has long been considered the gold standard ingredients in fish feed as they have a near ideal nutrient composition (Sørensen et al., 2011). Historically they also represented cost-effective feed ingredients (Tacon and Metian, 2008), and represented a beneficial supply of the n-3 long-chain fatty acid (Sargent et al., 2002). Owing to the ideal composition, the FM use more than doubled from 1995 – 2005, and FO use nearly doubled in the same period (Tacon and Metian, 2008). This increased use of and reliance on FM and FO from the capture fisheries is an unsustainable practice, simply because there soon will not be enough wild fish to capture. The FAO (2016) reported that approximately 90 % of the world's wild fish stocks are either fully or overexploited. That leaves mere 10 % underexploited, and does not leave much room for growth in the capture fisheries. Indeed, the capture fisheries have remained stable over the past three decades with an annual production at 93.4 million tonnes per 2014, with a significant, although declining, fraction being used for FM and FO production (FAO, 2016). Concurrently, the aquaculture industry reported an annual growth of 5.8 % from 2005 – 2014 (FAO, 2016). While the current use of FM and FO arguably could be considered sustainable assuming responsible management, the expected growth in aquaculture, and hence aquaculture feed production, will soon exceed the supply of FM and FO. Thus the increased demand for FM and FO combined with a stagnating supply has led to increased prices, reducing the viability of their use in fish feeds (Tacon and Metian, 2008)

The feed production industry has long since acknowledged the viability of using plant origin feed stuffs in replacing FM and FO (Gatlin et al., 2007).This is evident from the use of marine ingredients in feeds for Atlantic salmon in Norway, which has been reduced from around 90% in 1990 to less than 30% in 2013 (Ytrestøyl et al., 2015). Even so, plant ingredients all have some characteristics that puts them at a disadvantage compared to marine ingredients (Gatlin et al., 2007). Generally, all plant protein sources have an imbalanced amino acid profile for fish. Soy for instance contains too little methionine, lysine and threonine (Gatlin et al., 2007). Many plant-based ingredients also contain excessive amounts of carbohydrate of which fish only have limited metabolic capabilities, particularly carnivores like salmonids (Hemre et al., 2002). Vegetable oils (VO) differs from FO in that they are generally lacking in the long chain

polyunsaturated fatty acids (LC-PUFA), such as eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), even though they often provide the 18 carbon precursor α-linolenic acid (ALA, 18:3n-3). Generally, partial replacement of FO with VO have little or no effect on growth in salmonids (Petterson et al., 2009, Torstensen et al., 2005). However, it leads to changed lipid composition of the tissues with reduced levels of EPA and DHA (Bell et al., 2003, Bransden et al., 2003, Bou et al., 2017). The lack of EPA and DHA in VO has been linked to several possible health effects, such as accumulation of visceral fat (Torstensen et al., 2011, Todorčević et al., 2008), changes in the stress response (Holt, 2011) and increased hepatic lipid content, especially at low temperatures (Sissener et al., 2016, Ruyter et al., 2006).

It could be argued that fish do not really need FM and FO, but the nutrients they contain. This was illustrated by Espe et al. (2006), who reported that salmon fed diets with only 5% FM with the rest made from plant meal and crystalline amino acids utilise these diets satisfactory as long as the amino acid profile mimics the composition of fishmeal. Provided we tailor the feed to contain all essential nutrients at required levels the fish will have normal growth regardless of the feed ingredient source.

1.2 Organic and inorganic forms of supplemented micro-minerals

Most plant ingredients used in fish feed are known to contain undesirable components called antinutrients (Francis et al., 2001). They are defined as substances either natural or synthetic that by themselves or through their metabolic products inhibit or decrease the digestion and absorption of other nutrients (Francis et al., 2001). One common antinutrient in plant ingredients is phytic acid, which is the main storage form of phosphorous in seeds (Morales et al., 2016). Phytic acid is a strong chelating agent that binds to divalent cations and renders them unavailable to the animal, whereupon they are lost to the environment through faeces (Morales et al., 2016, Katya et al., 2016). There have been numerous reports of the addition of phytase improving the availability of minerals, indicating that they had previously been bound to phytic acid (Morales et al., 2016, Gatlin et al., 2007, Cheng and Hardy, 2003, Sugiura et al., 2001). Organic forms of micro-minerals are expected to have a higher bioavailability than the inorganic forms (Mantovani et al., 2010), and particularly in feeds with a high phytic acid content as the organic forms can protect the micro-minerals from forming insoluble complexes. Micro-minerals have traditionally been provided as inorganic salts. Now their respective organically bound forms are being considered as an alternative, particularly in the presence of

antinutrients. Several studies report better micro-mineral bioavailability when they are provided as organic bound forms, such as for rainbow trout (*Oncorhynchus mykiss*) (Apines-Amar et al., 2004), pacific white shrimp (*Litopenaeus vannamei)* (Bharadwaj et al., 2014, Lin et al., 2013, Katya et al., 2016) and European sea bass (*Dicentrarchus labrax*) (Fountoulaki et al., 2010). However, studies in some species have reported lower availability with organic minerals, such as Nile tilapia (*Oreochromis niloticus*) (Do Carmo e Sá et al., 2005). For gilthead seabream (*Sparus aurata*), a reduced bioavailability for Mn and Zn was found, whereas increased bioavailability for Se was observed with organic minerals (Domínguez et al., 2017). Selenium is reported to be the only element with a clear advantage of using organic forms over inorganic ones for all fish (Prabhu et al., 2014).

1.2.1 Metabolic fate of different chemical forms

Theoretically, the main advantage of using organic micro-minerals over inorganic, is to reduce the inhibitory interactions with antinutrients in the feed. This means that the mineral can be transported to the site of absorption uninterrupted, hence increasing the bioavailability. However, this increased absorption is dependent on the organic form remaining table through the digestive system to the site of absorption. Any direct metabolic effects of organic minerals in target tissues is dependent on the chemical form remaining different also after absorption. If the organic mineral complex is broken up after absorption, then any metabolic effects seen by using organic minerals could be ascribed to increased mineral absorption, rather than a direct function of the organic micro-mineral complexes themselves. Although there is not much information on the metabolic fate of the micro-minerals after absorption, some research has been done.

In mammals, selenium methionine (Se-Met) can either be used directly for protein synthesis or be metabolised to Se-Cys and then serine and selenide. This selenide can then be used for the production of selenoproteins or be excreted (Schrauzer, 2000). Selenite can also be used for the production of Se-Cys, but not for the production of Se-Met as fish cannot synthesise Met (Wilson, 2002). This is also seen in Atlantic salmon, where supplementation of Se-yeast (which contains mainly Se-Met) led to higher retention of Se as Se-Met in muscle than selenite (Sele et al., 2018). A similar result was found in rainbow trout fry, where Se-yeast led to higher accumulation of total Se in whole body than selenite (Godin et al., 2015). Se-Met and selenite achieved similar amounts of Se-Cys in rainbow trout fry (Godin et al., 2015), indicating that

they have similar ability of being metabolised into Se-Cys. Selenite inclusion in the diet gave no increase in Se-Met in neither Atlantic salmon (Sele et al., 2018) nor rainbow trout fry (Godin et al., 2015). As protein synthesis does not distinguish between Se-Met and Met (Suzuki, 2005), this means that adding Se-Met in the feed rather than selenite can lead to an increased retention of Se through storage in muscle.

Regarding Zn and Mn there is very little information. However, there are some indications that different chemical forms of Zn are metabolised differently. Rider et al. (2010) found no significant differences in digestibility of Zn-proteinate and Zn-sulphate in rainbow trout, but dissimilar retention in different tissues. Zn-sulphate led to a higher increase in Zn retention in all tissues that responded to Zn-supplementation, with the exception of the liver. This showed that Zn-sulphate has a higher retention than Zn-proteinate, at least when provided at above requirements. If the organic minerals still are distinct chemical species post-absorption, with differing metabolic routes compared to the inorganic forms, then it is possible that they could affect lipid metabolism in dissimilar ways.

1.3 Requirements

The bioavailability of minerals in aquafeeds is of primary importance for their nutritional value to the fish. Increasing the efficiency of mineral uptake would decrease the required dietary inclusion, in addition to reducing the environmental impact. Most aquaculture fish and shrimp live in open sea cages, leaving feed spill and faeces with high mineral content to sink to the seabed under the net pen, or alternatively float with the current. This mineral and organic enrichment can lead to a build-up of anoxic sediments under the net pens, alter the seabed fauna and flora and lead to eutrophic waters (Lall and Milley, 2008)

Increasing environmental concerns also put pressure on the authorities to implement stricter regulations regarding feed additives. The upper limit for Zn, for instance, is being lowered from 200 mg/kg to 180 mg/kg for salmonids in the time period from 2016 to 2018 (EU regulation 2016/1095) The requirements of Atlantic salmon, upper limit values and current content of Zn, Se an Mn in Norwegian commercial feeds can be found in Table 1.

In addition to reduced upper limits, marine ingredients are being replaced with vegetable sources. Not only do they contain antinutrients hindering uptake, they also contain lower amounts of minerals such as Se and Zn. However, Cu and Mn are actually present in higher amounts in plant feed ingredients (Sanden et al., 2017). Consequently, discovering whether

adding the minerals in other chemical forms increases the bioavailability is pivotal; it would allow for the addition of less mineral without affecting fish health, or perhaps with positive health effects at the same inclusion levels.

Table 1: Mineral requirements in Atlantic salmon, upper limit values and content in Norwegian commercial fish feeds

Trace mineral	Requirement Atlantic salmon	Upper limit (EFSA)[1]	Content in Norwegian feeds with min/max [3]
Mn	10 mg/kg[4]	100 mg/kg	42 mg/kg (20-110)
Se	Not tested (0.15 mg/kg in Rainbow trout)[4]	0.5 mg/kg	1.1 mg/kg (0.3-17.0)
Zn	37 mg/kg[4]	200 mg/kg [2]	158 mg/kg (100-280)

[1] European food safety authorities. The upper limit applies to the sum of naturally occuring and added minerals, but only if the mineral has been added to the feed
[2] EU regulation 2016/1095 to reduce max Zn to 180 mg/kg for salmonids is being implemented
[3] Sanden et al. (2017)
[4] NRC (2011)

1.4 Lipid metabolism and its regulation

Lipids are a diverse group of organic molecules of biological origin with the common feature of being soluble in organic solvents, but insoluble in water (Lehninger et al., 2013d, Leaver et al., 2008). They have various important functions in fish; they are structural components of membranes, precursors to hormones, energy storage molecules and they are used in β-oxidation for energy production, to mention a few (Torstensen et al., 2001, Leaver et al., 2008). The pathways and regulatory processes of lipid metabolism are well defined (see Fig. 1), and the lipid homeostasis is a result of the balance between the dietary absorbed fat, biosynthesis of lipids (lipogenesis), catabolism via β-oxidation (lipolysis), transport and storage (Lehninger et al., 2013a, Lehninger et al., 2013c). These processes are tightly regulated through transcriptional factors and hormones in response to feedback and feed forward signals in order to maintain optimal homeostasis (Lehninger et al., 2013a, Lehninger et al., 2013c). A fat accumulation or depletion will be the result of an imbalance between these processes.

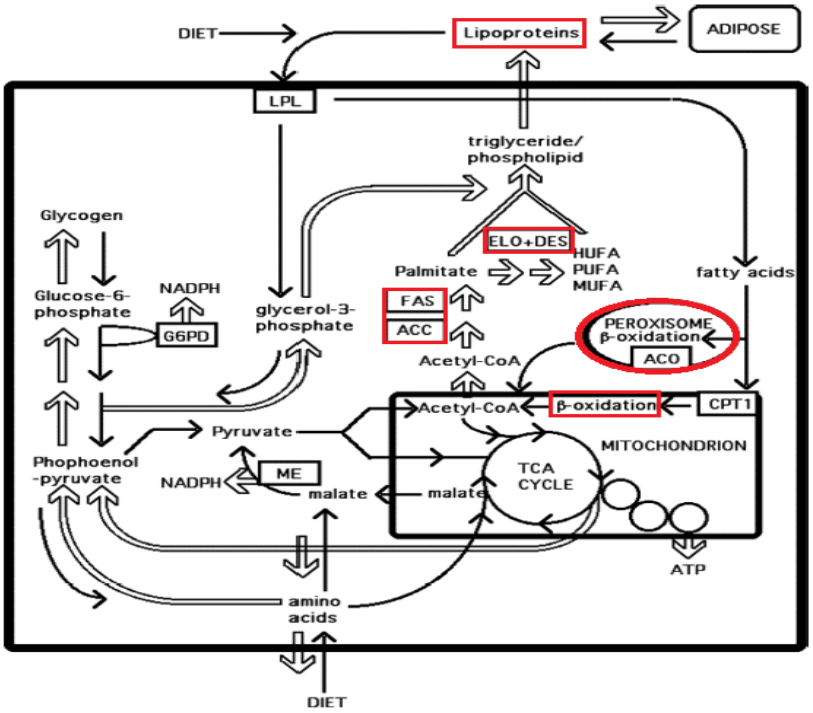

Fig. 1: Schematic overview of the interconnection of the main metabolic pathways of the liver. The processes marked in red are the main processes for lipid metabolism. Normal arrows indicate catabolic routes, open arrows anabolic. Adapted from Leaver et al. (2008).

1.4.1 Fatty acid synthesis

The process of synthesising endogenous fatty acids (FA) in the liver is called *de novo* lipogenesis. This process occurs in the cytoplasm and the first, committed step is catalysed by acetyl-CoA carboxylase (ACC) where acetyl-CoA is turned into malonyl-CoA. The enzyme fatty acid synthase (FAS) then catalyses the assembly of C16:0 (palmitic acid) and C18:0 (stearic acid) from malonyl-CoA and acetyl-CoA (Berlanga et al., 2014, Lehninger et al., 2013c). This is a process that requires substantial amount of reducing power in the form of NADPH (Leaver et al., 2008). Thus the main two limiting substrates for the FA synthesis is malonyl-CoA and NAPDH (Leaver et al., 2008).

To avoid a futile cycle of simultaneous synthesis and degradation of FAs, they are separated into different compartments of the cell. Additionally, the rate-limiting step of β-oxidation is inhibited by malonyl-CoA, the first intermediate in the FA synthesis (Lehninger et al., 2013a).

Fish have biosynthetic capabilities of the saturated FAs 16:0 (palmitate) and 18:0 (stearic acid) and the reactions are catalysed by FAS, as in all other living organisms (Sargent et al., 2002,

Leaver et al., 2008). Many fish have an appreciable proportion of lipids in their diets, and as a result, they have a reduced need for biosynthesis. In some cases it could even be repressed.

1.4.2 β-oxidation

The oxidation of FAs occur within the mitochondria, peroxisomes and the endoplasmic reticulum (Berlanga et al., 2014). In general, the short-, medium- and long-chain FA are oxidised in the mitochondria (β-oxidation). However, FA longer than 20 carbons cannot cross the mitochondrial membrane and must first be oxidised in the peroxisomes to shorten the chain for transport across the mitochondrial membrane. The peroxisomes do not have the electron transport chain, so much of the energy is released as heat (Torstensen et al., 2001, Leaver et al., 2008). FAs longer than 12 carbons cannot diffuse directly through the mitochondrial membrane. They have to form an acyl-carnitine to facilitate transport, a reaction catalysed by carnitine palmitoyltransferase I (CPT1). The acyl-carnitine is then transported over the membrane via a translocase and acyl-CoA is reformed with carnitine palmitoyltransferase 2 (CPT 2). The transport of FAs is thought to be the rate-limiting step of β-oxidation. As mentioned, malonyl-CoA inhibits β-oxidation. It does this by inhibiting CPT1, hence CPT1 is considered the main regulatory enzyme of β-oxidation (Lehninger et al., 2013b). The mitochondrial β-oxidation is a four step reaction resulting in the successive removal of two-carbon units in the form of acetyl-CoA. The acetyl-CoA is then used in the citric acid cycle to produce NADH and $FADH_2$ which will enter the electron transfer chain to produce ATP (Lehninger et al., 2013a, Lehninger et al., 2013b).

1.4.3 Bioconversion

As mentioned, the saturated FAs 16:0 and 18:0 can be synthesized *de novo* by fish, but the unsaturated FAs linoleic acid (LA, 18:2n-6) and ALA cannot be synthesized and are thus termed essential (Sargent, 1995, Miller et al., 2008). As a consequence, fish are dependent on dietary sources to get enough of these FAs. Whereas most freshwater fish have the ability to elongate and desaturate ALA into EPA and DHA, most marine species seem to have lost this capability (Tocher and Ghioni, 1999). As their diet is naturally rich in EPA and DHA, the need for synthesis is attenuated, hence also the desaturase activity (Tocher, 2010, Sargent, 1995).

The synthesis of EPA from ALA starts with a desaturation with a Δ6 desaturase (Δ6Fad) to 18:4n-3, which is then elongated to 20:4n-3 and a final desaturation with Δ5 desaturase (Δ5Fad)

into EPA. The synthesis of LA into ARA is essentially the same, and they compete for the same enzymes (Sprecher, 2000, Leaver et al., 2008). To produce DHA, EPA is first elongated to docosapentaenoic acid (DPA, 22:5n-3), but then there is not a direct Δ4-desaturation of DPA to DHA, rather it goes through another elongation step to 24:5n-3 before a Δ6Fad desaturation to get 24:6n-3. The final step to produce DHA is a peroxisomal chain-shortening step (Sprecher, 2000, Leaver et al., 2008). The sequence of enzymes acting on ALA and LA into their respective LC-PUFA is as shown in Fig. 2.

The bioconversion of ALA into LC-PUFA has been shown to increase with decreased fish oil content of the feed (Zheng et al., 2005b, Kjaer et al., 2016) , but also environmental cues such as temperature have been shown to modulate this synthesis (Zheng et al., 2005b, Ruyter et al., 2003). Atlantic salmon has the capability for the synthesis of EPA and DHA from ALA to some degree (Sargent, 1995, Tocher, 2010, Bou et al., 2017, Rosenlund et al., 2016). Decreasing EPA+DHA in the feed results in increased elongation and desaturation, and especially when fed diets containing less than 1% EPA+DHA of the total FAs (Bou et al., 2017). Hence it has been suggested that the elongation and desaturation products can have a negative feedback inhibition on these enzymes (Tocher, 2003).

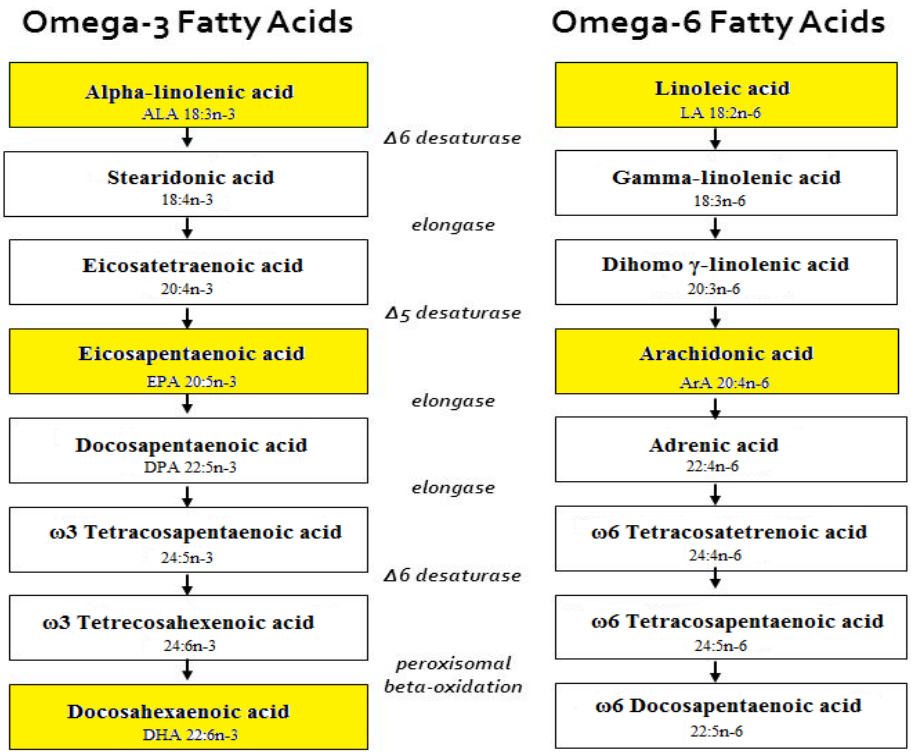

Fig. 2: Sequence of enzymes and intermediates in the synthesis of LC-PUFA

The first Δ6 step has long been considered the rate-limiting step of this biosynthesis (Gregory et al., 2011), however increasing the availability of 18:4n-3 to bypass this step did not result in improved bioconversion into EPA and DHA (Alhazza et al., 2011). Rather than having one singular rate-limiting step, studies now report that there are a combination of regulations at each step that together regulate the synthesis of LC-PUFA (Alhazzaa et al., 2013, Thanuthong et al., 2011). It has also been suggested that since the products of this bioconversion, namely EPA and DHA, are inhibitors of these enzymes, that the EPA produced from ALA could slow down further elongation and desaturation reactions (Alhazzaa et al., 2013).

1.4.4 Uptake, packaging and transport of fatty acids

Due to their hydrophobic properties, lipids need to be emulsified for uptake into the enterocytes. The formation of micelles greatly increases the accessibility of the lipids to the actions of water-soluble lipases, which breaks triacylglycerol (TAG) into monoacylglycerol (MAG), diacylglycerol (DAG) and free FAs (FFA) (Lehninger et al., 2013a). Although, some data indicate that TAG is completely broken down to glycerol and free fatty acids (FFA) in salmon as the main hydrolysis products (Bogevik et al., 2008). This enables them to diffuse into the intestinal mucosa where they can be re-esterified and packaged together with apolipoproteins to form chylomicrons (Lehninger et al., 2013a). Chylomicrons are first transported to the tissues to deliver the majority of their lipids. They then become chylomicron remnants, which are mainly absorbed by the liver (Tocher, 2003). Together with FAs synthesised by the liver they can be packaged into very-low-density lipoprotein (VLDL), which requires apolipoprotein B100 (ApoB100). VLDL's function is to transport lipids from the liver to peripheral tissues. After delivering lipids to the tissues it becomes low-density lipoprotein (LDL). LDL provides cholesterol to peripheral tissues. High-density lipoprotein (HDL) is also produced in the liver and its task is to return lipids and cholesterol to the liver from peripheral tissues (Tocher, 2003).

1.4.5 Transcriptional regulation of lipid metabolism

A transcription factor is a nuclear protein which binds to the promoter sequence of a gene, thereby either activating or repressing its transcription (Lehninger et al., 2013e). The rate of lipogenesis is mainly regulated at the transcriptional level, and include several transcription factors such as liver X receptor (LXR), sterol regulatory element-binding protein (SREBP),

carbohydrate responsive element-binding protein (ChREBP), farnesoid receptor x (FxR) and enzymes such as FAS and ACC (see Fig. 3) (Berlanga et al., 2014).

SREBP is a transcription factor that acts on genes involved in FA synthesis and biosynthesis of LC-PUFA (Dong et al., 2017, Minghetti et al., 2011). Reduced cellular concentration of cholesterol activates SREBP and induces expression of genes involved in cholesterol and fat synthesis (Horton et al., 2002). There are three different types of SREBP in mammals, SREBP1c, SREBP1a and SREBP2. SREBP1c preferentially regulates lipogenic genes, SREBP2 primarily targets those of cholesterol synthesis while SREBP1a activates both of these (Horton et al., 2002, Jeon and Osborne, 2012). Only two isoforms, the SREBP1 and SREBP2, have been identified in salmon (Minghetti et al., 2011).

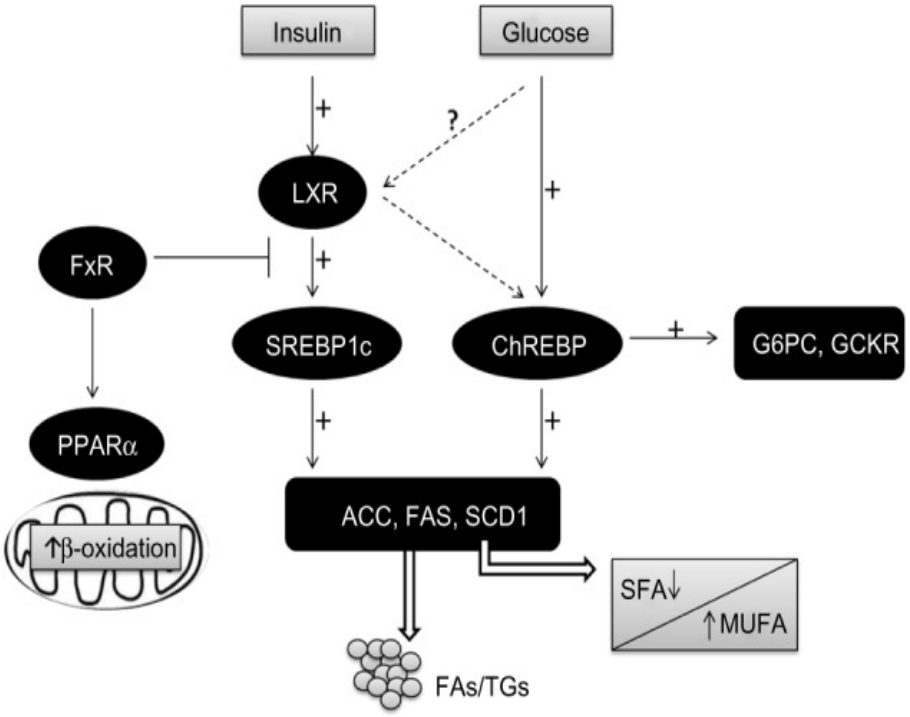

Fig. 3: Transcriptional control of lipogenesis and glycolysis. Image from Berlanga et al. (2014). Abbreviations: ACC: Acetyl-CoA carboxylase, ChREBP: carbohydrate-responsive element binding protein, FA: fatty acid, FAS: Fatty acid synthase, FxR: Farnesoid X receptor, G6PC: glucose 6-phosphatase, GCKR: glucokinase regulatory protein, LXR: Liver-X receptor, MUFA: monounsaturated fatty acid, PPARα: peroxisome proliferator-activated receptor alpha, SCD1: steroyl CoA desaturase 1, SFA: saturated fatty acid, SREBP1: sterol regulatory element-binding protein 1c, TG triglyceride

SREBP can also be induced through another transcription factor called LXR. LXR forms a heterodimer with retinoid x receptor (RXR) and then binds to the DNA consensus sequence of the target gene, the LXR response elements (Zhao and Dahlman-Wright, 2010, Lehninger et al., 2013c). It is activated by oxysterols, the catabolic products of cholesterol (Zhao and Dahlman-Wright, 2010). High cholesterol levels in the cell will result in more oxysterols, leading to activation of LXR (Zhao and Dahlman-Wright, 2010, Lehmann et al., 1997). LXR can activate the synthesis of several proteins involved in lipogenesis (Lehninger et al., 2013c). It induces the production of FAS directly by binding the FAS promoter, and also indirectly through SREBP1 (Joseph et al., 2002, Carmona-Antoñanzas et al., 2014). LXR also modulates the catabolism of cholesterol through cholesterol 7α-hydroxylase (Chiang, 2009). An increased cholesterol level in salmon led to higher expression of LXR in salmon SHK-1 cells (Minghetti et al., 2011), and an upregulation of LXR resulted in an increased expression of FAS, SREBP1 and SREBP2 in the same cell type (Carmona-Antoñanzas et al., 2014).

Furthermore, SREBP1 has a regulatory function in the biosynthesis of LC-PUFA in fish. An SREBP binding site has been identified in the promoter region of the salmon Δ6Fad (Zheng et al., 2009). Only moderate expression of SREBP1 was found in Japanese seabass with inclusion of n-3 FA (Dong et al., 2015), corresponding with a previous study where they found low expression of Fad with high inclusion of n-3 LC-PUFA (Xu et al., 2014). Dong et al. (2017) and Carmona-Antoñanzas et al. (2014) both found that an increased inclusion of vegetable oils, which are devoid of LC-PUFA, gave a higher expression of SREBP1. This again gave rise to higher expression of Δ6Fad genes. Conversely, high levels of LC-PUFA repress the expression of SREBP1 (Desvergne et al., 2006, Betancor et al., 2014), hence also the Δ6Fad genes.

The peroxisome proliferator-activated receptors (PPAR) are a group of transcription factors that alter the expression of genes involved in lipid and carbohydrate metabolism. They dimerize with RXR and bind to peroxisome proliferator response elements (PPREs), their DNA consensus sequence. They are responsive to dietary lipids, their ligands being FAs (mainly PUFAs) and their derivatives (Lehninger et al., 2013b, Tocher, 2003). There are three genes for PPARs in mammals, PPARα, –δ /β and -γ (Tocher, 2003, Desvergne et al., 2006). They are all part of the nuclear receptor family, but they have dissimilar tissue distributions and have functionally opposite effects (see Fig. 4) (Evans et al., 2004).

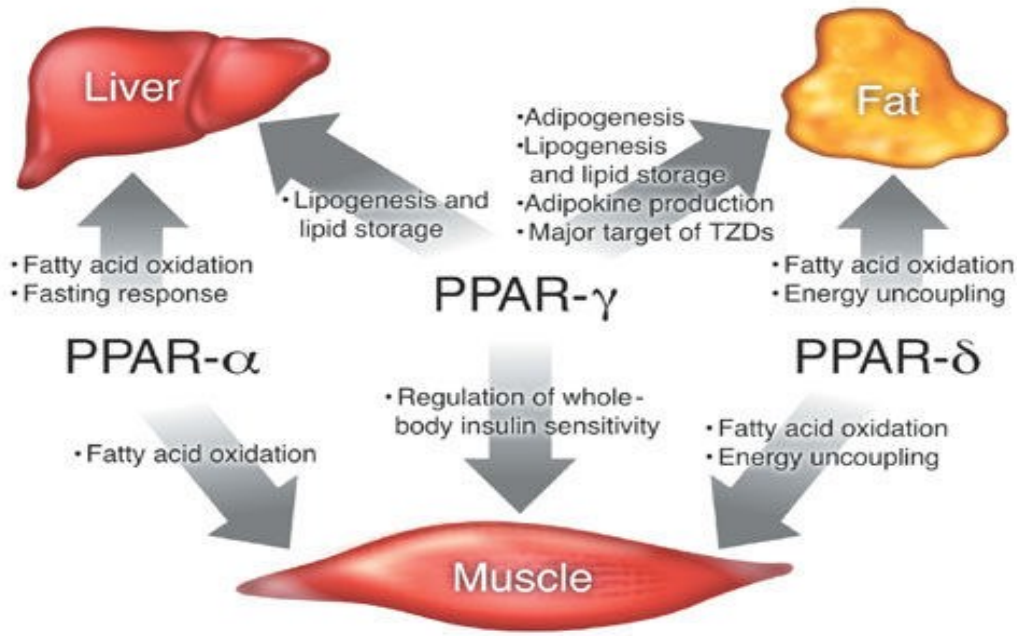

Fig. 4: The three isoforms of PPAR regulate lipid and carbohydrate homeostasis through their concerted effects on gene expression in different tissues. PPARα and δ /β regulate lipid utilisation, whereas PPARγ regulated lipid storage. Image from Evans et al. (2004).

PPARα is expressed in tissues with high β-oxidation activity, reflecting its role in the regulation of FA oxidation. In mammals, the highest expression is found in liver and brown adipose tissue, then heart, kidneys, enterocytes and muscles (Desvergne et al., 2006). PPARα also plays a role in activating genes involved in transport and uptake of FAs (Desvergne et al., 2006, Tocher, 2003). The importance of PPARα is particularly highlighted during fasting, when stored FAs are used as an energy source (Desvergne et al., 2006). PPARδ /β is expressed amply throughout the body, but in lower levels in the liver. It activates genes involved in FA catabolism and thermogenesis (mitochondrial uncoupling; resulting in heat production rather than ATP) (Evans et al., 2004). PPARγ is mainly present in adipose tissue and promotes lipid uptake and adipogenesis, which is the development of the adipocyte phenotype (Tocher, 2003, Desvergne et al., 2006). All three PPAR isoforms have been identified in salmon (Ruyter et al., 1997, Leaver et al., 2006). They are homologues of the mammalian PPARs and are expected to have relatively conserved functions (Tocher, 2003).

1.5 Minerals in fat metabolism

The chemical definition of a mineral is an element in its pure form or its simple, chemical compound. There are approximately 20 elements that are considered essential in the diet of fish. An essential element is required for the maintenance of life, and a deficient intake will lead to impairment of normal physiological function. Recovery of physiological levels of the element will prevent such impairment or alleviate deficiency (Lall, 2002) Minerals can roughly be divided into two groups called macro- and micro-minerals, dependent on how much is needed in the diet (Lall, 2002). Macro-minerals are required at quite high concentrations (in grams) and have functions in for instance bone structures and hard tissues, osmoregulation and acid-base regulation (NRC, 2011, Lall, 2002). The macro-minerals include sodium, magnesium, chlorine, potassium, phosphorous and calcium (NRC, 2011). Micro-minerals are required at much lower concentrations than the macro-minerals (in milli- or micrograms) and participate in a variety of functions as components of hormones and enzymes and as cofactor and coactivators of enzymes (NRC, 2011, Lall, 2002). The most commonly recognised micro-minerals according to the NRC (2011) include zinc, iron, selenium, chromium, manganese, iodine and copper.

1.5.1 Zinc

Zinc is a micro-mineral that is an integral part of around 20 metalloenzymes (Chanda et al., 2015) and also functions as a cofactor and activator for many Zn-dependent enzymes (NRC, 2011, Lall, 2002). Zinc is important in the metabolism of proteins, carbohydrates and lipids (Lall, 2002). Fish can both show reduced growth performance during Zn deficiency (Luo et al., 2011) or no significant changes (Zheng et al., 2015, Giri et al., 2016). Zheng et al. (2015) found that deficiency of Zn tends to increase hepatic lipid content in yellow catfish, whereas excess tends to reduce it. The Zn deficiency reduced the activity of CPT1, which is thought to be the main regulatory enzyme of β-oxidation. Reduced CPT1 activity would mean a reduced lipid utilisation. This is in accordance with results from Luo et al. (2011) who found that increased dietary levels of Zn significantly reduced whole body lipid content in yellow catfish. They also found increased lipoprotein lipase and hepatic lipase activities, thought to indicate increased lipolysis. Giri et al. (2016) and Lewis et al. (2013) also found increased β-oxidation with increased fortification of a mixture of cofactors and coenzymes which included zinc.

A study investigating the effect of Zn, Fe, Mg, niacin, riboflavin, pyridoxine and biotin on the n-3 LC-PUFA biosynthesis in rainbow trout showed significantly higher activity of Δ5Fad on 20:3n-3. This effect was only significant at double and triple levels of the requirement, with no additional benefits of higher inclusion levels. Elongase activity on 18:4n-3 was also increased at double requirement level compared to the requirement. Increased transcription of elongase 5 was found at double levels, with no further increase with increasing supplementation (Lewis et al., 2013*).* The authors suggested this hinted at an optimal level of supplementation, and that co-factor fortification can promote *in vivo* n-3 LC-PUFA biosynthesis in rainbow trout fed VO-based diets. Another study on Atlantic salmon using the same co-enzymes and co-factor (excluding pyridoxine) in the feed showed significantly improved levels of EPA and ARA in the fillet with supplementation, though DHA was not affected. A numerical trend showed an increase in the activity of the enzymes involved in bioconversion, though there was no significant increase (Giri et al., 2016).

1.5.2 Selenium

Selenium is an essential trace element, which functions as the active part of selenoproteins. It is widely recognised for its antioxidant function as a part of the enzyme glutathione peroxidase (Lall, 2002, Bell et al., 1987), but it is also linked to the thyroid hormone production (Lorentzen et al., 2001). Se is also known to interact with vitamin E and they show sparing effects for one another, delaying the onset of deficiency symptoms (Lin and Shiau, 2009). Salmonid tissues are rich in PUFAs, which are highly susceptible to lipid peroxidation. It follows that Se plays a vital role in the protection against oxidative damage (Bell et al., 1987).

Infante (1986) suggested early on that Se, together with vitamin E, play an important role in the desaturation of n-3 and n-6 FAs. Se-deficiency in rats resulted in reduced concentration of the Δ6-desaturase (Δ6Fad) product 22:6n-3, which could signify an inhibition of this enzyme (Schafer et al., 2004). Silva-Brito et al. (2016) found that Meagre *(Argyrosomus regius)* fed a diet of FO with adequate Se content exhibited reduced expression of Δ6Fad genes in the liver, but not when fed VO. When fed FO diets deficient of Se, they found increased expression of genes of PUFA biosynthesis. Sufficient supply of Se when fed FO was thought to protect PUFA from peroxidation, and when deficient of Se they had to express genes of PUFA biosynthesis to counteract the damage. During feeding of VO diets, Se stimulated PUFA biosynthesis. This could indicate that the effect of Se on desaturation is dependent on the FA composition.

In Se-adequate rats increased levels of TAG were found compared to Se-deficient rats (Schafer et al., 2004), indicating that Se have an impact on TAG metabolism in the liver. In rainbow trout higher levels of Se led to decreased liver TAG content (Knight et al., 2016), whereas in Atlantic salmon no significant change in TAG was uncovered, but a reduction of DAG and MAG in high selenite diets and reduced MAG in high Se-Met diets (Berntssen et al., 2017). In Atlantic salmon, it was suggested of being due to increased β-oxidation as they also found a reduction of coenzyme A (Berntssen et al., 2017).

1.5.3 Manganese

Manganese is a cofactor for various metal-enzyme complexes and is an integral part of metalloenzymes, and there is a greater concentration inside the mitochondria than in other organelles or the cytoplasm (Watanabe et al., 1997). As its chemistry is very similar to that of magnesium, Mg and Mn can often be used interchangeably by many enzymes (Lall, 2002). Some enzymes are specific for manganese, most notably the manganese superoxide dismutase (MnSOD). MnSOD is responsible for dismutation of reactive oxygen species (ROS) and is thus an essential part of the antioxidant defence of the cell (Holley et al., 2011).

Some earlier work indicated that manganese could have a lipotropic effect, as manganese deficiencies were shown to cause enlarged abdominal fat deposits and fatty liver in mice and increased body fat content in pigs (Keen et al., 1984). Correspondingly, Tan et al. (2012) fed yellow catfish *(Pelteobagrus fulvidraco)* diets with increasing Mn content and found that the highest amounts of Mn led to the lowest whole body lipid content.

Manganese acts as a cofactor for mevalonate kinase and farnesyl pyrophosphate synthetase, both of which are enzymes in this biosynthetic pathway of cholesterol (Johnson, 1986). However, experiments performed on manganese deficient Wistar and RICO rats showed no significant effects on the cholesterol metabolism (Klimis-Tavantzis et al., 1983). They did however find slightly reduced levels of LDL which they proposed could be caused by Mn's role in apolipoprotein glycosylation, lipoprotein structure and membrane integrity. This would affect VLDL structure and catabolism, consequently affecting LDL the same way.

1.6 Research questions

The functional role of minerals in the hepatic intermediary metabolism in fish is not very well understood. Some recent research has indicated that dietary minerals can have an effect on hepatic lipid metabolism. This has however, usually been performed with varying levels of the minerals and not in the context of plant-based ingredients with different chemically bound mineral sources. Phytic acid is expected to reduce the availability of the inorganic minerals, while changing from inorganic to organic forms may improve availability again, which in turn may affect liver lipid metabolism.

This study aims to examine whether different digestibility and the chemical form of the micro-minerals Zn, Se and Mn affect the liver lipid metabolism, and whether it could be of interest to conduct further investigation to attain a better understanding of the role of micro-minerals in the hepatic intermediary metabolism.

2 Materials and methods

This master is part of the project "Apparent availability and requirements of micro-minerals in salmon", which is funded by the Research Council of Norway (grant no. 244490). Vegetable ingredients contain fibre and phytic acid, and can thus decrease the mineral availability. The experiment was designed to elucidate the apparent digestibility of feed additives (minerals) in different chemical forms (organic or inorganic), to see how it is influenced by the presence of other feed additives and how phytic acid will affect mineral availability.

In this master project, changes in the lipid metabolism of Atlantic salmon liver as affected by organic Zn, Se or Mn in high phytic acid diets was studied.

2.1 Composition of diets

The diets were produced at the pilot-scale feed mill facility for research diets at Skretting ARC, Stavanger. Five different diets were examined in this study. They were chosen to examine the effect organic Zn, Se and Mn in high phytic acid diets on the liver lipid metabolism of Atlantic salmon. In diet LPI, all three minerals were added in inorganic form in combination with low phytate level, in diet HPI the inorganic minerals were combined with high phytate, while in the three final diets (HPOZn, HPOSe and HPOMn) one of the three inorganic minerals were replaced by an organic form (Table 2). Table 3 shows the formulation of the low phytate and high phytate diets, respectively.

Table 2: Overview of the different diets used. LPI: low-phytate inorganic, HPI: high-phytate inorganic, HPOZn: high-phytate organic Zn, HPOSe: high-phytate organic Se and HPOMn: high-phytate organic manganese

Diet	Zn source	Se source	Mn source	Phytic acid level
LPI	Zinc sulphate	Selenite	Manganous sulphate, monohydrate	Low
HPI	Zinc sulphate	Selenite	Manganous sulphate, monohydrate	High
HPOZn	Chelate of glycine	Selenite	Manganous sulphate, monohydrate	High
HPOSe	Zinc sulphate	Selenium methionine	Manganous sulphate, monohydrate	High
HPOMn	Zinc sulphate	Selenite	Chelate of glycine	High

Table 3: Formulation of basal diets (g/100g)

Diet	Low phytic acid	High phytic acid
Wheat	8.29	8.15
Corn gluten	14.97	15.00
Hi-pro soya	14.39	10.00
Wheat gluten	20.00	14.35
Soy protein concentrate	10.00	20.00
FM North-Atlantic	5.00	5.00
Rapeseed oil	12.27	12.56
FO North-Atlantic	9.86	10.09
Monoamoniumphosphate	1.85	1.81
Astaxanthin	0.05	0.05
Histidine HCl	0.50	0.48
Internal premixes	3.27	3.12
Yttrium	0.10	0.10
Premix vitamins (at requirement)	0.10	0.10
Premix minerals (at requirement, excluding Zn, Se, Mn)	0.10	0.10
Correction for moisture	-0.77	-0.91

2.2 Fish and fish experiment

The feeding trial was carried out at Skretting ARC Research station at Lerang, Norway, and was conducted according to the guidelines of the Norwegian State Commission for Laboratory animals. Atlantic salmon *(Salmo salar)* with an initial mean body weight of about 250 g were distributed in 15 tanks with seawater, with 33 or 32 individuals in each tank. The fish were acclimatised for 20 days in their respective tanks, and feeding of the experimental diets started when feed intake was as expected (0.8% of body weight per day). The tanks were supplied with flow through seawater at 12˚C and were exposed to a 24-hour light regime. Each experimental diet was distributed to triplicate tanks and the fish were fed to apparent satiation three times per day, with a minimum of 10% overfeeding. Surplus feed was collected to determine feed intake. Acclimatisation started the 07.09.17, with sampling being performed the 9th, 10th and 11th of October. Fish sampled on the respective days started their experimental feeds on the 29th Sep, 30th Sep and 1st Oct to ensure all fish were fed the experimental diets the same amount of time. The fish were euthanized using a stock solution of Tricain (MS222) containing 40 g/L. Of this solution 5 mL/L was used.

2.3 Sampling procedures

The phase-feeding procedure prior to sampling was performed so that all the groups were sampled at the same definite time post last meal. The sampled fish were anesthetised and then killed with an overdose of anaesthetic. The weight and length of all fish were measured and liver samples were taken from six individual fish per tank. Thus, a total of 18 fish were sampled from each diet. Livers were weighed and deviating colour or appearance were noted. Samples for different analyses were taken from the same part of the liver each time (Fig. 5). The samples were flash frozen in liquid nitrogen, transported on dry ice and then stored at -80°C until analysis.

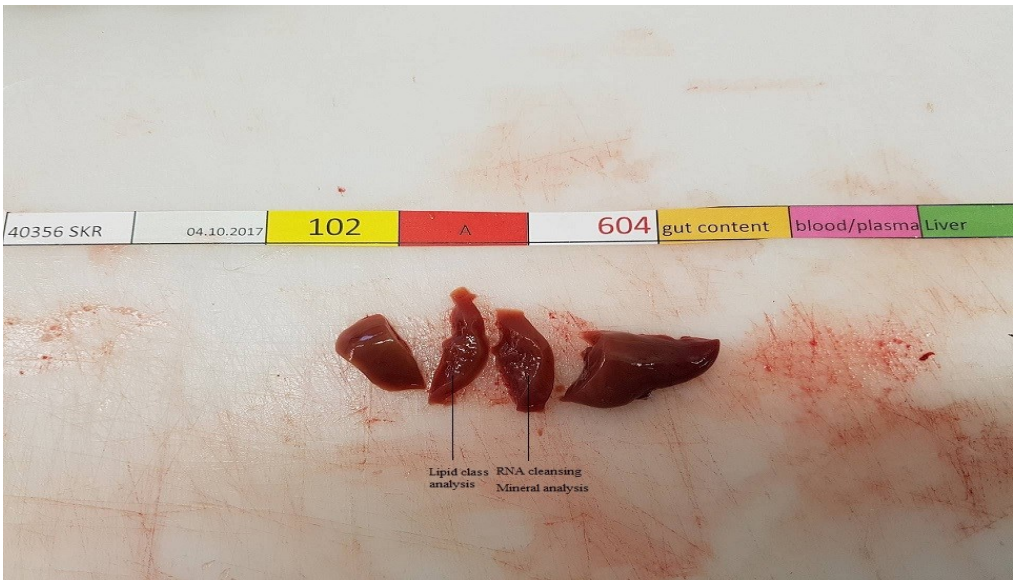

Fig. 5: Liver cut up for different analyses. The same part of the liver was used for the same type of analysis.

2.4 Analytical methods

2.4.1 Fatty acid analysis

The fat was extracted from the feed sample by adding 20x weighed in sample of chloroform:methanol (2:1. v/v). The FA 19:0 methyl-ester was added as an internal standard in a concentration of 10%<>30% of the total fat content. The samples were then left overnight in -20°C for extraction. After extraction, the samples were filtered and evaporated in RapidVap (Labconco, Kansas City, MO, USA) to remove all chloroform:methanol. Then 1 mL of 0.5 M NaOH was added and the samples were boiled at 100°C for 15 minutes before saponification and methylation with 2 ml of BF_3 and boiling at 100°C for 5 minutes. Then 2 mL of hexane

was added to extract the fats before the sample was centrifuged and the hexane phase was pipetted out. After a second extraction with hexane, the samples were diluted to an appropriate concentration for analysis using gas-liquid chromatography (Autosystem XL, Perkin Elmer Inc, Waltham, MA, USA). A flame-ionization detector was used for the determination. The FAs were identified using a standard mixture of FAs and quantified using the internal standard. Chromeleon version 7.2.6 was used to integrate the chromatogram.

2.4.2 Mineral analysis

Due to little sample material, three liver samples were pooled to have enough for parallel runs. Thus, two pooled samples from each tank were used for this analysis.

Digestion of sample

The samples were weighed and placed in Teflon bombs 0.5 mL 30% H_2O_2 and 2 mL concentrated HNO_3 for digestion in Milestone Microwave digestion system MLS-1200 MEGA for 20 minutes for complete digestion of organic matter. After water cooling, the content of the bombs was placed into 25 mL volumetric flasks and diluted using milli-Q water. The solution was clear after this process.

Multielement determination with inductively coupled plasma mass spectrometer (ICPMS)

The solution is nebulised and carried into argon plasma (~7000˚C), were it is vaporised and the elements are ionised, mainly creating singly charged atoms. This is important as the mass filter separates the ions based on their mass/charge (m/z) ratio. Doubly charged ions would have different m/z ratio, and would thus be forced away from the detector. However, this also means that the MS is not able to separate between different substances with the same m/z ratio. The ions are then carried to the detector, which is an electron multiplier which releases an electron cascade each time an ion hits, leading to an electrical signal that can be read as hits per second. Quantification of the elements is determined with an external standard curve and an internal standard is also used.

The method is accredited for Zn (0.5-1400 mg/kg dw) and Se (0.01-8 mg/kg dw). It is not accredited for Mn, but can be measured in the range 0.03-19 mg/kg dw.

2.4.3 Gene expression analysis

RNA isolation

All equipment and work areas were cleaned using RNase Zap (Sigma-Aldrich) to avoid contamination of RNase (ribonuclease), an RNA degrading enzyme. All samples were stored on dry ice prior to homogenisation to avoid degradation of RNA. EZ1 RNA universal tissue kit (Qiagen, Crawley, UK) was used to extract RNA from liver. Approximately 50 mg liver was used and homogenised with 750 µL Qiazol lysis reagent (Qiagen) and precellys beads in the Precellys 24 (Bertin Instruments, Montigny-le-Bretonneux, France) at 6000 rpm for 10 seconds 3 times. After 5 minutes, the RNA was then separated from protein and DNA using 150 µL chloroform. Then it was hand shaken for approximately 30 seconds, before it was incubated at room temperature for 3 minutes. The sample was then centrifuged at 4°C at 12000 rpm for 15 minutes before collection of the supernatant. The Biorobot EZ1 (Qiagen) was used to purify the RNA according to the producer's instruction, with the inclusion of 10 µL DNase. The samples were frozen at -80°C until further analysis.

Quality control of RNA

The Nanodrop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used to measure the concentration and to give a measure of the purity of the RNA. The quality of twelve randomly selected samples was checked using the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies). If all 12 samples showed adequate integrity, the same was expected to be true for the remaining samples. Integrity is measured in RIN (RNA integrity number), where 1 means completely degraded RNA and 10 means intact RNA (Schroeder et al., 2006). The RIN of all selected samples was above 8.6 in this study.

Reverse transcription reaction

A pooled sample was used to make a standard curve with concentrations from 31 ng/µL to 1000 ng/µL RNAm, which was set up in triplicate on a 96 well cDNA plate. The samples were set up in duplicate with two negative controls; the non-amplification control (nac) and non-template control (ntc) to ensure that only one PCR product was amplified, and that stock solutions were not contaminated. The nac and ntc were without enzymes and RNA,

respectively.

10 µL sample diluted to 50 ng/µL (±5%) and 40 µL TaqMan® reverse transcriptase kit with oligo (dT) primers (Applied Biosystems, AB) were added in duplicate, and the reverse transcription reaction to make cDNA was performed in the GeneAmp PCR 9700 (Applied Biosystems) with the thermal program given in Table 4. The cDNA plate was then stored at -20°C.

Table 4: Temperature program for the reverse transcription reaction

Step	Incubation	Reverse transcription activation	Reverse transcription inactivation	End
Temperature °C	95	48	95	4
Time (minutes)	10	60	5	∞

Real-time quantitative PCR (qPCR)

The cDNA was thawed on ice before vortexing on MixMate at 1500 rpm for 5 minutes. A mixture of 575 µL SYBR Green Mastermix (Roche applied science, Basel, Switzerland). Then a mixture of 322 µL MiliQ water, 11.5 µL forward primer and 11.5 µL reverse primer was prepared. The primer sequence of the target genes are given in Table 5. B-actin (Bact), phosphoprotein P0 (ARP) and elongation factor lab (EF1A) were used as reference genes. A BioMek® 3000 pipetting robot (Beckmann Coulter. Fullerton. USA) was used to transfer 8 µL of the mixture and 2 µL cDNA into a 384 well qPCR plate. The plate was spun down at 1500 rpm for 2 minutes before the real time PCR in LightCycler 480 Real-time PCR system (Roche Applied science). The thermal program was as given in Table 6.

Table 5: Forward and reverse primers for target genes

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Source of primer	Accession number
CPT1	CTTTGGGAAGGGCCTGATC	CATGGACGCCTCGTACGTTA	Nøstebakken et al. (2012)	AM230810
PPAR-α	TCTCCAGCCTGGACCTGAAC	GCCTCGTAGACGCCGTACTT	Søfteland et al. (2016)	NM001123560
FAS	GTGCCCACTGAATACCATCC	ATGAACCATTAGGCGGACAG	Morais et al. (2011)	CK876943
SREBP1	GCCATGCGCAGGTTGTTTCTTCA	TCTGGCCAGGACGCATCTCACACT	Minghetti et al. (2011)	TC148424
LXR	TGCAGCAGCCGTATGTGGA	GCGGCGGGAGCTTCTTGTC	Cruz-Garcia et al. (2011)	NM001159338
Δ5Fad	GGAACCACAAACTGCACAAGT	GTGCTGGAAGTGACGATGGT	Torstensen et al. (2005)	AF478472.1
Δ6Fad	GGGATTTAATCCATCGCATATTAACT	CGTCACAACAAAATACAGCATCTG	Zheng et al. (2005a)	AY458652
ApoB100	TTGCAGAGACCTTTAAGTTCATTCA	TGTGCAGTGGTTGCCTTGAC	Torstensen et al. (2011)	gi:854619

Table 6: Temperature program for the qPCR reaction

Step	Preincubation	45 cycles of amplification with 3 steps			Melting point analysis			Cooling
Temperature (°C)	95	95	60	72	95	65	97	40
Time	5 min	10 sec	10 sec	10 sec	5 sec	1 min		10 sec

2.4.4 Lipid class analysis

Liver samples were weighed and fat extracted by adding 20x weighed in sample of chloroform:methanol (2:1. v/v), with butylhydroksytoluen (BHT) to avoid oxidation of the FAs. The samples were left overnight at -20 °C for extraction. After extraction, the samples were filtered and evaporated in RapidVap (Labconco) to remove all chloroform:methanol. The sample was then diluted with chloroform with added BHT until a concentration of approximately 5 mg/mL. Analysis was performed using high performance thin layer chromatography in a HPTLC system (Camag, Berlin, Germany). The HPTLC plate was washed in a polar solution (KCl, methanol, chloroform, isopropanol, methylacetate), and then evaporated for 10 minutes before activation at 110°C for 30 minutes. It was then cooled down in an exicator. Application of 1.0 µL sample to the prewashed plate was performed using an automatic sample applicator (ATS4, Camag). Elution was performed using an automatic system (AMD2, Camag). Elution up to 48 mm of the plate with the polar solution was performed first, and then a second elution with a neutral solution (isohexane, diethyl ether, acetic acid) to 88 mm. The plate was developed using a CuAc(aq) solution, and then it was quantified in Densitometer-Camag TLC-scanner with a deuterium lamp at 350 nm. The data was integrated using WinCats version 1.3.3 (Camag). Quantification of the lipid classes in mg lipid class/g tissue was carried out with established external standard curves and a standard sample per run.

2.5 Data analysis

The stability of the reference genes was calculated using GeNorm version 3.5, as the M-value. Bact and ARP were the most stable and were thus used to determine the normalisation factors. Ct values for the target genes were then used to calculate normalised gene expression with their respective normalisation values.

All statistical analyses were performed using the free software environment R version 3.4.3 (R Development Core Team, 2011). Best-fit regression lines were found using the linear model (lm) function. The data were analysed using Levene's test to check for homogeneity of variance

and Shapiro Wilk's test was used to check for normality, as well as being evaluated graphically using quantile-quantile (QQ) plots. Data were modelled using one way ANOVA, and any significant differences among treatments given by the ANOVA model were evaluated using Tukey's honestly significant (HSD) test, using the package multcomp. SREBP1 and ApoB100 did not meet the assumptions of normality or homogeneity, and a Kruskal-Wallis nonparametric test was used. When evaluating correlations, Spearman's rank correlation coefficient was used. The package ggpubr was used for this.

Significance was set at $p < 0.05$ for all statistical tests.

3 Results

3.1 Feeds

The analysed values for proximate composition, mineral content (Zn, Se and Mn) and fatty acid composition of the feeds are given in Table 7, Table 8 and Table 9, respectively. The analysis of proximate composition (Table 7) and the mineral content (Table 8) were performed by the project, but were not a part of this thesis. Thus the method descriptions are not detailed, but the results are given due to their importance in explaining the results of this thesis. The dry weight, the content of fat, protein and ash was fairly consistent among the diets (Table 7). There was only a slight difference in planned phytate levels, as it was attempted to only use regular feed ingredients and not add extra phytate. The fatty acid composition (Table 9) was very similar across all five diets.

The amount of Zn, Se and Mn added to the feeds (Table 8) were at levels several times the requirement (NRC, 2011), at relevant levels to what is used in commercial feeds (Sanden et al., 2017), and can be assumed to satisfy at least the minimal needs for Atlantic salmon.

Table 7: Analysed proximate composition of the diets. Dry matter, protein, fat and ash of the diets given in g/100g.

Diet	LPI	HPI	HPOZn	HPOSe	HPOMn
Dry matter	91	93	92	93	93
Protein	46.7	44.7	46.7	48	46.3
Fat	22	22	22	22	22
Ash	3.9	4.2	4.2	4.3	4.3
Phytate[1]	6.4	7.2	7.2	7.2	7.2

[1] Theoretical calculated values, as analysis by the project was not complete prior to deadline

Table 8: Amount of Zn, Se and Mn in the experimental diets. Given as mg/kg wet weight

Diet	Zn	Se	Mn
LPI	130	0.56	26
HPI	140	0.55	26
HPOZn	140	0.63	26
HPOSe	150	0.58	27
HPOMn	140	0.55	24

Table 9: Fatty acid composition of the diets, given in both % of total fatty acids and in mg fatty acid per g feed

	LPI		HPI		HPOZn		HPOSe		HPOMn	
	%	mg/g	%	mg/g	%	mg/g	%	mg/g	%	mg/g
14:0	2.3	4.79	2.3	4.85	2.3	4.80	2.3	4.70	2.3	4.74
15:0	0.2	0.48	0.2	0.49	0.2	0.47	<0.1	<0.01	0.2	0.48
16:0	10.5	22.15	10.4	22.04	10.5	21.98	10.3	21.34	10.3	21.61
16:1n-9	0.2	0.45	0.2	0.46	0.2	0.45	<0.1	<0.01	0.2	0.46
16:1n-7	2.2	4.55	2.2	4.65	2.2	4.61	2.2	4.53	2.2	4.62
17:0	0.3	0.68	0.3	0.63	0.3	0.70	0.3	0.67	0.3	0.71
16:2n-4	0.3	0.60	0.3	0.62	0.3	0.59	0.3	0.56	0.3	0.59
18:0	2.3	4.92	2.3	4.98	2.3	4.92	2.3	4.84	2.3	4.91
18:1n-9	38.6	81.05	38.8	82.35	38.4	80.81	38.7	80.27	38.1	80.03
18:1n-7	2.7	5.57	2.7	5.63	2.6	5.55	2.6	5.49	2.7	5.58
18:2n-6 (LA)	15.7	32.97	15.2	32.15	15.4	32.34	15.2	31.47	15.2	31.88
20:0	0.4	0.94	0.4	0.82	0.4	0.80	0.4	0.88	0.4	0.85
18:3n-3 (ALA)	5.6	11.78	5.6	11.96	5.6	11.74	5.6	11.65	5.6	11.83
20:1n-11	0.2	0.49	0.2	0.49	0.2	0.51	0.2	0.48	0.2	0.49
20:1n-9	2.6	5.51	2.6	5.59	2.6	5.54	2.6	5.39	2.6	5.55
18:4n-3	1.0	2.04	1.0	2.10	1.0	2.10	1.0	2.04	1.0	2.09
20:2n-6	0.2	0.34	0.2	0.37	0.2	0.41	0.2	0.39	0.2	0.38
20:4n-6 (ARA)	0.8	1.78	0.9	1.85	0.9	1.80	0.9	1.89	0.8	1.76
22:1n-11	2.8	5.90	2.9	6.10	2.9	6.15	2.9	5.98	2.9	6.17
22:1n-9	0.5	0.98	0.5	1.03	0.5	1.13	0.5	1.14	0.5	1.10
20:4n-3	0.2	0.46	0.2	0.49	0.2	0.49	0.2	0.48	0.2	0.48
20:5n-3 (EPA)	3.2	6.77	3.3	6.96	3.3	6.91	3.3	6.81	3.3	6.93
21:5n-3	<0.1	<0.01	<0.1	<0.01	<0.1	<0.01	<0.1	<0.01	0.1	0.22
24:1n-9	0.4	0.78	0.4	0.83	0.4	0.84	0.4	0.81	0.4	0.81
22:5n-6	0.1	0.29	0.1	0.26	0.1	0.27	0.1	0.25	0.1	0.25
22:5n-3 (DPA)	0.4	0.79	0.4	0.79	0.4	0.77	0.4	0.74	0.4	0.76
22:6n-3 (DHA)	4.9	10.24	5.0	10.55	5.0	10.51	5.0	10.38	5.0	10.56
Sum saturated	16.2	34.0	15.9	33.8	16.0	33.7	15.8	32.7	15.8	33.3
Sum monounsaturated	50.1	105	50.5	107	50.2	106	50.4	104	49.9	105
Sum EPA + DHA	8.1	17.0	8.3	17.5	8.3	17.4	8.3	17.2	8.3	17.5
Sum n-3	15.3	32.1	15.5	32.8	15.5	32.6	15.5	32.1	15.6	32.9
Sum n-6	16.8	35.4	16.3	34.6	16.6	34.8	16.4	34.0	16.3	34.3
Sum polyunsaturated	32.4	68.1	32.1	68.1	32.4	68.0	32.2	66.7	32.2	67.7
n-3/n-6	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	1.0	1.0
n-6/n-3	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.0	1.0
Sum unidentified	1.0	2.20	1.1	2.43	1.1	2.33	1.4	2.84	1.9	3.93
Sum identified	99.0	208	98.9	210	98.9	208	98.6	204	98.1	206
Sum FA	100.0	210	100.0	212	100.0	210	100.0	207	100.0	210

3.2 Growth

The fish grew from a mean weight of approximately 253 g to a mean final body weight of 296 ± 64 g, and the final weight of the fish per diet group is reported in Table 10. No significant differences in final weight were found between the different diet groups. There were also no significant differences in the condition factor (CF). For hepatosomatic index (HSI), it was significantly higher in diet groups HPOZn ($p = 0.0041$) and HPOMn ($p = 0.0143$) when compared to control diet LPI. Otherwise no significant differences occurred. The CF and HIS are given in Table 10 for each diet group.

Table 10: Final weight, condition factor (CF) and hepatosomatic index (HSI) of Atlantic salmon fed the five different experimental diets. Final weight and CF were recorded for all fish (33/32 per tank). HSI and liver lipid was measured for six fish per tank. Numbers are mean and standard deviation (SD). Statistical difference is denoted by different letters.

Diet	LPI	HPI	HPOZn	HPOSe	HPOMn
Final weight, g	284 ± 54	306 ± 64	295 ± 63	292 ± 69	306 ± 59
CF	1.34 ± 0.10	1.38 ± 0.11	1.39 ± 0.12	1.37 ± 0.11	1.39 ± 0.11
HSI	0.96 ± 0.15 [a]	1.00 ± 0.11 [ab]	1.12 ± 0.15 [b]	1.01 ± 0.12 [ab]	1.11 ± 0.14 [b]

3.3 Mineral content

The hepatic mineral content is given in Table 11. There were no significant differences in mineral content between any of the diet groups. However, the chelated Zn tended to give a lower liver Zn content than the inorganic form ($p = 0.065$).

Table 11: Mineral content of Zn, Se and Mn in the liver of the different diet groups given in mg/kg ww.

Diet	LPI	HPI	HPOZn	HPOSe	HPOMn
Zn (mg/kg ww)	21.5 ± 1.6	21.5 ± 1.2	19.7 ± 0.8	21.5 ± 0.5	21.0 ± 1.1
Se (mg/kg ww)	1.64 ± 0.33	1.58 ± 0.17	1.45 ± 0.08	1.47 ± 0.10	1.33 ± 0.15
Mn (mg/kg ww)	1.48 ± 0.08	1.48 ± 0.10	1.40 ± 0.06	1.45 ± 0.08	1.42 ± 0.11

3.4 Gene expression

A series of genes involved in lipogenesis, β-oxidation, FA transport and bioconversion were selected to perform a qPCR analysis on RNA extracted from the liver of Atlantic salmon fed the different experimental feeds. The dietary mineral source only had minor effects on the liver gene expression, with no significant effects on LXR, FAS, PPARα, CPT1, Δ5Fad or Δ6Fad (Fig. 6, Fig. 7). However, the expression of ApoB100 was significantly reduced ($p = 0.0243$) in diet group HPI compared to diet group LPI (Fig. 6). The expression of ApoB100 in diet group HPOMn was similar to that of LPI, and significantly higher than HPI ($p = 0.0407$). SREBP1 exhibited a similar expression pattern as ApoB100. There was a significantly lower

expression of SREBP1 (p = 0.0464) in diet group HPI compared to LPI, and although not significant (p = 0.145) there was also a trend towards higher expression in diet group HPOMn (Fig. 6).

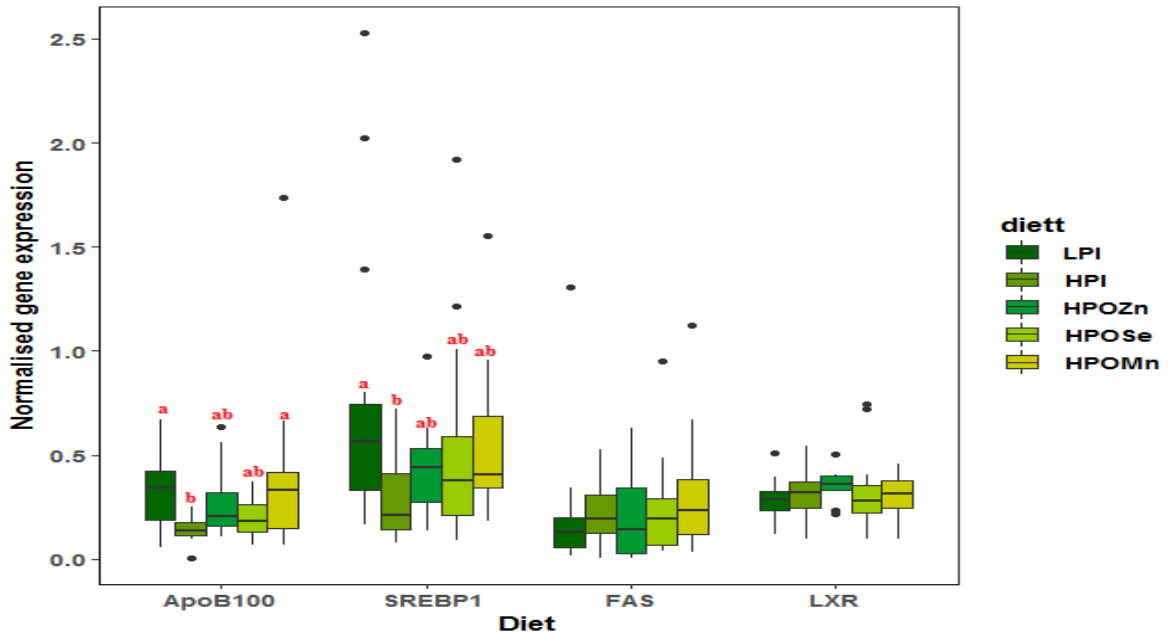

Fig. 6: Normalised expression of genes involved in lipid metabolism in liver of Atlantic salmon fed different diets. Statistical difference is denoted by different letters. Boxes provide 25th to 75th percentile, with the line giving the median. Error bars show minimum and maximum values, except for outliers which are provided as circles. Outliers are defined as 1.5 times the length of the box away from the box.

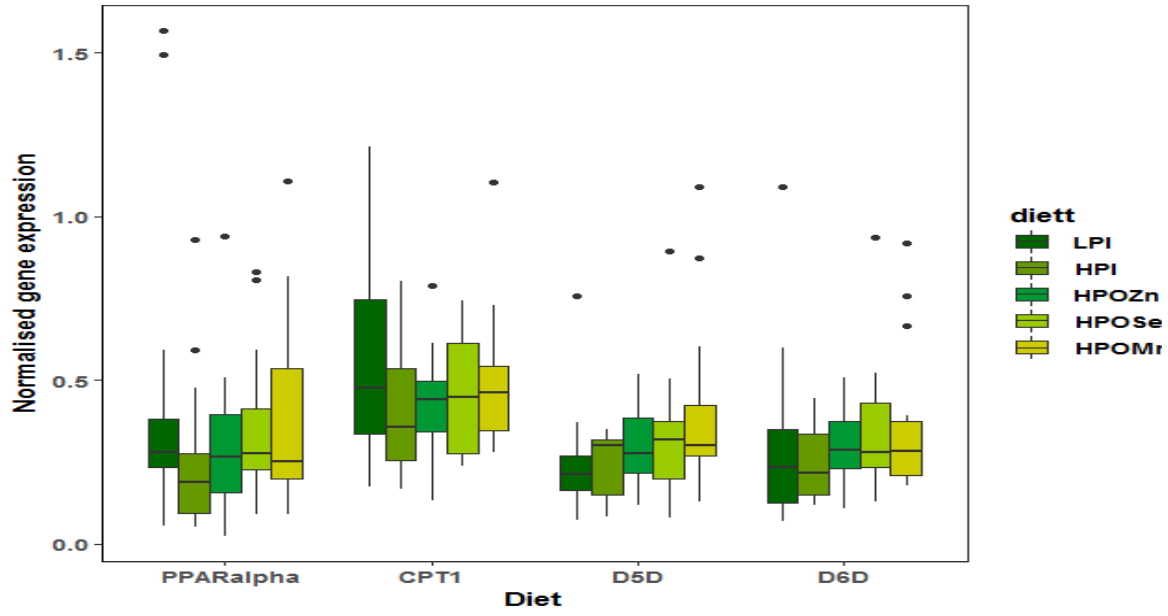

Fig. 7: Normalised expression of genes involved in lipid metabolism in liver of Atlantic salmon fed different diets. Boxes provide 25th to 75th percentile, with the line giving the median. Error bars show minimum and maximum values, except for outliers which are provided as circles. Outliers are defined as 1.5 times the length of the box away

3.5 Liver lipids

The liver lipid class distribution was very similar in all diet groups (Table 12). Except for a significantly lower amount of FFA in diet group HPOMn compared to diet group HPI ($p = 0.008$), no diet-induced differences were revealed by this analysis.

Table 12: Lipid class composition in liver. Results are presented as mean ± standard deviation in mg/g. Six fish were analysed per tank, thus a total of 18 fish per diet. One tank in diet D only had four fish analysed for lipid class composition.

	LPI	HPI	HPOZn	HPOSe	HPOMn
Total lipids	44.9 ± 15	52.6 ± 6.4	51.5 ± 10	56.7 ± 17	56.6 ± 8.4
Total NL	7.9 ± 3.8	10.6 ± 3.2	9.86 ± 3.3	10.1 ± 4.2	11.5 ± 4.2
Total PL	37.1 ± 12	42.0 ± 4.6	41.7 ± 8.3	46.6 ± 14	45.1 ± 8.0
TAG	3.01 ± 2.3	4.59 ± 3.0	4.54 ± 3.0	4.13 ± 2.5	6.22 ± 4.3
Cholesterol	3.25 ± 0.91	3.26 ± 0.28	3.22 ± 0.62	3.5 ± 0.92	3.47 ± 0.47
FFA	1.05 ± 0.97 [ab]	2.09 ± 0.50 [a]	1.39 ± 0.81 [ab]	1.68 ± 0.98 [ab]	1.14 ± 0.59 [b]
DAG	0.55 ± 026	0.67 ± 0.13	0.69 ± 0.27	0.79 ± 0.31	0.67 ± 0.15
PE	6.39 ± 2.7	7.16 ± 1.3	7.29 ± 1.7	8.01 ± 3.5	8.36 ± 1.8
CL	0.48 ± 0.28	0.61 ± 0.08	0.49 ± 0.21	0.67 ± 0.19	0.49 ± 0.26
PI	2.29 ± 0.93	2.77 ± 1.2	2.34 ± 0.62	2.86 ± 0.75	2.86 ± 0.90
PS	2.07 ± 0.93	2.44 ± 0.25	2.04 ± 0.72	2.59 ± 0.77	2.02 ± 0.59
PC	23.2 ± 7.7	26.0 ± 3.4	27.0 ± 4.7	29.3 ± 8.7	28.7 ± 4.7
SM	2.25 ± 0.73	2.30 ± 0.19	2.01 ± 0.63	2.49 ± 0.63	2.24 ± 0.53

NL = neutral lipid; PL = polar lipid; TAG = triacylglycerol; FFA = free fatty acid; DAG = diacylglycerol; PE = phosphatidylethanolamine; CL = cardiolipin; PI = phosphatidylinositol; PS = phosphatidylserine; PC = phsophaticylcholine; SM = sphingomyelin

There was a positive correlation between the amount of TAG in the liver and the expression of FAS ($r = 0.41$, $p = 0.00038$, see Fig. 8). Additionally, the expression of FAS was positively correlated with the expression of Δ5Fad ($r = 0.48$, $p < 0.0001$) and Δ6Fad ($r = 0.52$, $p < 0.0001$) (See Fig. 9 and Fig. 10). The Δ5Fad and Δ6Fad also exhibited a positive correlation with liver TAG, with respectively $r = 0.29$, $p = 0.012$ and $r = 0.35$, $p = 0.0024$ (data not shown).

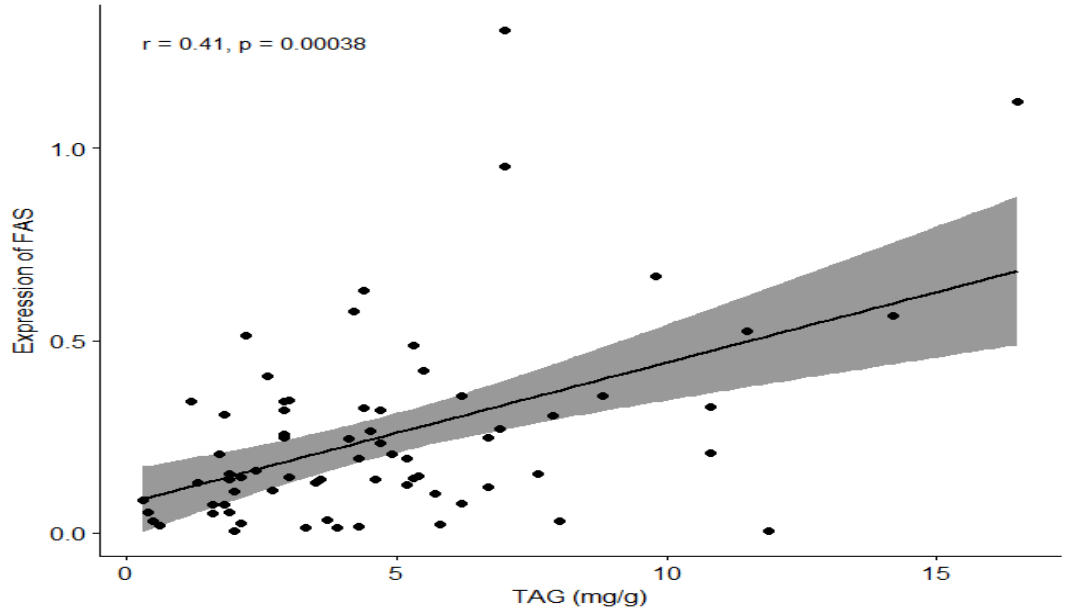

Fig. 8: Correlation between liver TAG and the normalised expression of FAS.

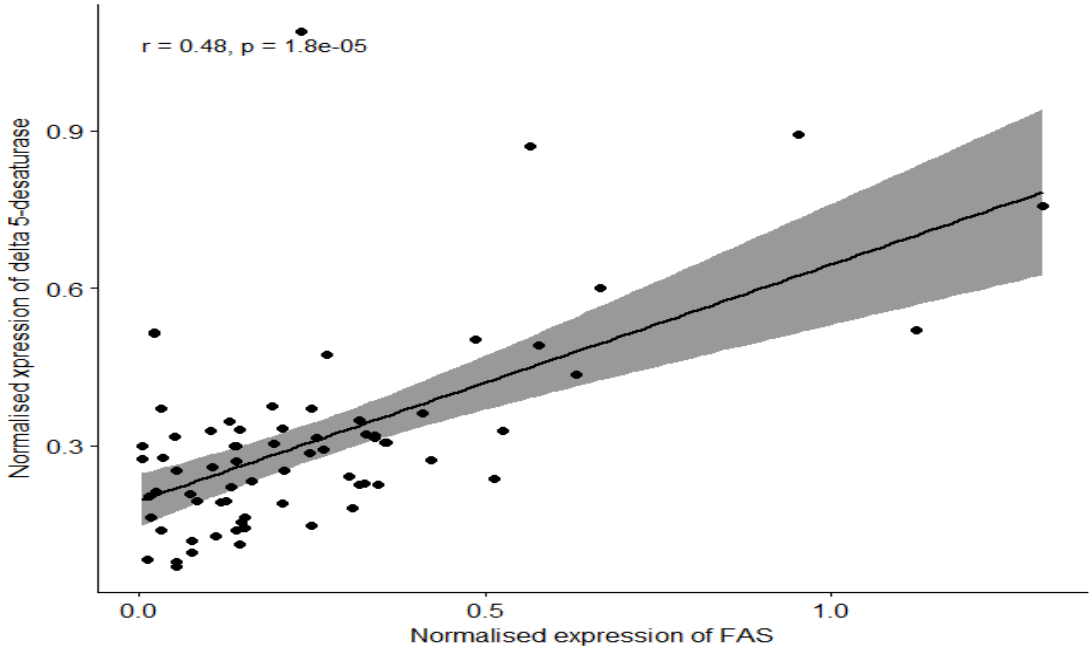

Fig. 9: Correlation between the normalised expression of FAS and Δ5 -desaturase

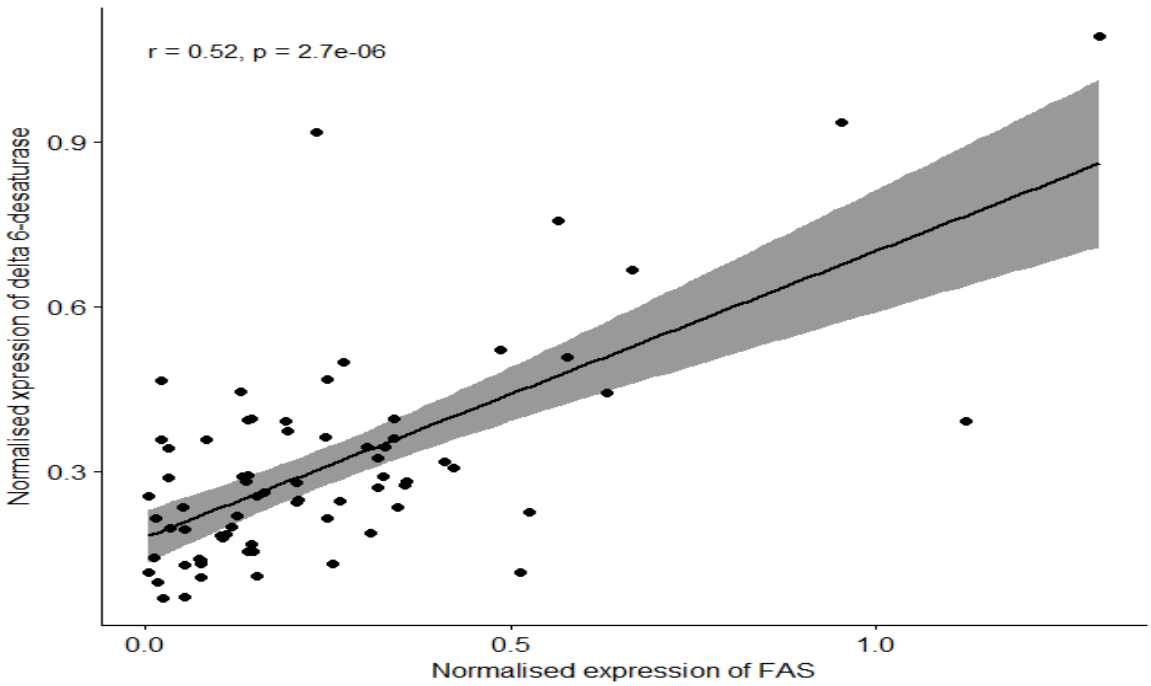

Fig. 10: Correlation between the normalised expression of FAS and Δ6-desaturase

4 Discussion

4.1 Experimental design and feeds

The concept was that the two control diets were both supplemented with inorganic micro-minerals, but in either a low or high phytate environment. Since it is well known that phytate can cause inhibition of the micro-mineral uptake (Gatlin et al., 2007), it was expected that the bioavailability of the minerals would be lower in the HPI diet group than in the LPI diet group. It was then to be investigated whether this lowered bioavailability would have an effect on selected lipid metabolism markers. A difference between diet group LPI and HPI would indicate an effect of phytate. Restoration of marker levels similar to diet group LPI in the HPO diet groups, could then indicate improved digestibility of the organic minerals. No differences between the control groups, but with different levels in the HPO groups could then indicate an effect of the chemical form.

However, there was a lack of impact of the phytate inclusion in the high and low phytate diets on the apparent digestibility (Prabhu, 2018), which seem to suggest that the difference in phytate might have been smaller than anticipated. This could be on account of wanting only to have phytate levels similar to what can be achieved in practical diets, and not add extra phytate to get artificially high levels. The analysis for accurate phytate content is not yet completed, but is performed as part of the project. The phytate levels used in the current study are not expected to have any effect on the digestibility of protein or lipid (Denstadli et al., 2006).

4.2 Mineral availability

The bioavailability of the different micro-minerals forms was, as mentioned, investigated as part of the larger project. Although not performed by the undersigned, a brief summary of the bioavailability in the current study is warranted. Yttrium was added to the feed as an inert marker, and the relationship between yttrium and the micro-minerals in the feed and faeces were used to calculate the apparent digestibility coefficient (ADC). This analysis showed that there was an improved uptake of organic selenium (Se-Met) compared to inorganic Se (selenite) (Prabhu, 2018), concurring with results in gilthead seabream (Domínguez et al., 2017), rainbow trout (Küçükbay et al., 2009) and the general consensus that organic Se has superior bioavailability compared to inorganic Se (Prabhu et al., 2014). There is some variability in the literature regarding the bioavailability of the other micro-minerals, and this could possibly be

species specific. In the current study, the inorganic Mn (manganous sulphate) achieved better ADC than organic Mn (chelate of glycine) (Prabhu, 2018). This is akin to results in gilthead seabream (Domínguez et al., 2017) but contrasting results in rainbow trout (Apines-Amar et al., 2004). Neither the chelated Zn nor the Zn-sulphate showed a clear superiority over the other (Prabhu, 2018). This corresponds rather well with the findings of Maage et al. (2001) which indicated that organic and inorganic (glycine chelate of Zn and Zn-sulphate) were equally efficient Zn supplements for Atlantic salmon.

Despite these differences in bioavailability, no significant difference in mineral content of the liver were induced. This means that the post-absorptive fate chemical form, i.e if still intact after absorption, is probably what would cause any changes on hepatic lipid metabolism markers.

4.3 Desaturase genes

No significant effects of the different chemical forms of micro-mineral supplementation on the expression of Δ5Fad and Δ6Fad were found in this study. This is despite that Zn has been linked to the desaturation process, owing to the similarities of Zn deficiency symptoms and EFA deficiency symptoms (Knez et al., 2017). Eder and Kirchgessner (1996), Cunnane et al. (1984) and Horrobin and Cunnane (1980) all discovered reduced desaturation capabilities in rats suffering from Zn deficiencies. Zn supplementation, together with other micro-nutrients, is needed for the biosynthesis of LC-PUFA in Atlantic salmon, but supplementation above the requirement did not result in any additional effect on LC-PUFA biosynthesis (Giri et al., 2016). Thus, the current results fit well with previous studies, as none of the groups in the current study were expected to suffer from Zn deficiency. If both mineral forms have the same chemical form after uptake, then it would be logical that no differences were observed when no differences in apparent digestibility were found. However, Rider et al 2010 found that Zn-sulphate and Zn-proteinate did not have the same retention in different tissues of rainbow trout when supplied above requirements, suggesting a different metabolism of the two forms. Even so, the data from the current study indicate that Zn chelate of glycine and Zn-sulphate do not affect the desaturation process differently, at least when the total Zn levels in the diet are above the recommended requirement levels.

Additionally, a relatively large proportion of the lipids in the present study were provided from FO, at around 44% of total lipids. FO contains a high percentage of the essential fatty acids

EPA and DHA, and they constituted 8% of the total lipids in the current diets. Hence, EPA and DHA were not limiting factors in the present experiment. It is well known that dietary EPA and DHA can affect the expression of fads genes in Atlantic salmon; Rosenlund et al. (2016), Betancor et al. (2014) and Bou et al. (2017) all found that increased dietary content of EFA led to reduced expression levels of Δ5Fad and Δ6Fad. Increased activity of desaturase and elongase enzymes with diets containing low EPA and DHA is also reported (Zheng et al., 2005b, Tocher et al., 2003). Furthermore, there exists evidence that low levels of EPA and DHA in the feed is necessary to see noticeable changes in the biosynthesis of LC-PUFA. Sanden et al. (2011) found that Atlantic salmon can have such high rates of elongation and desaturation as to be a net producer of DHA, but this effect was only noticeable with low levels of DHA in the feed. Turchini et al. (2011) found that Rainbow trout can also be a net producer of EFA when fed linseed oil, which is low in n-3 LC-PUFA. Bou et al. (2017) found that EPA+DHA below 1% in the feed resulted in high synthesis of LC-PUFA, but the effect was markedly reduced with levels above 1%. Consequently, as higher levels of EPA and DHA in the feed results in lower expression of desaturase and elongase genes, the expression in this study with 8% EPA+DHA would be relatively low. Hence, any eventual effects of the chemical form of the investigated micro-minerals on the expression of Δ5Fad and Δ6Fad might have been masked by the high FO content.

4.4 Expression of ApoB100 and SREBP1

The expression pattern of ApoB100 and SREBP1 in the current study seems to indicate a different effect of the chelated Mn than the Mn-sulphate. Despite the difference in digestibility between the two, the two chemical forms did not lead to significant differences in hepatic Mn content. This could indicate that it was the chemical form rather than the difference in bioavailability that caused the observed effects. The metabolism of Zn chelate and Zn-sulphate in rainbow trout appear to differ (Rider et al., 2010), so it is possible that the same may apply to Mn and thus lead to different responses.

ApoB100 is critical for the production of VLDL (Sundaram and Yao, 2010) and SREBP1 is known to affect VLDL secretion (Wang et al., 1997). SREBP1 can cause increased synthesis of lipids for TAG production, and there is a close relationship between hepatic TAG synthesis and ApoB100 secretion (Yao and McLeod, 1994). Mn plays a role in activating glycosyltransferases (Chanda et al., 2015), and mammalian ApoB is dependent on

glycosylation of the N-terminal end for normal stability and secretion (Vukmirica et al., 2002). However, ApoB100 mRNA and protein level do not necessarily correlate well, and ApoB mRNA levels can remain very stable under metabolic conditions that alter secretion dramatically (Yao and McLeod, 1994). Furthermore, ApoB100 and SREBP1 are the only two parameters showing any response. There were no significant differences for the expression of FAS, which is the major enzyme catalysing synthesis of fatty acids for TAG used in VLDL production. Nor was there any significant changes in the expression of LXR, which also affects the VLDL assembly in mammals (Sundaram and Yao, 2010). There were also no significant differences in the hepatic TAG content, suggesting no increased clearance of hepatic lipid content. Furthermore, the phytate levels made no significant impact on the digestibility (Prabhu, 2018), which is supported by the similar hepatic mineral content. As phytate did not impact mineral digestibility and diets LPI and HPI both had the same form of all the minerals, the reduced expression of ApoB100 and SREBP1 in diet group HPI was probably random rather than caused by the diets. Hence, it is also possible that the chelated Mn was not the cause of the differences in ApoB100 expression between the HPI and HPOMn diet groups, as the low expression in the HPI group seems to be a random occurrence.

4.5 β-oxidation genes

In mammalian studies, Zn has repeatedly demonstrated its anorexigenic properties. In mice, it ameliorated alcohol-mediated increased in hepatic TAG, cholesterol and FFA (Kang et al., 2010). A decrease in expression of genes related to β-oxidation and VLDL production and –secretion has also been shown (Kang et al., 2010). Simple Zn deficiency has also been shown to cause dysregulation in a large number of genes involved in lipid metabolism (Dieck et al., 2003), with a clear trend of decreasing hepatic lipolytic processes and increase in hepatic lipogenic activity (Dieck et al., 2005, Dieck et al., 2003). None of the lipolytic or lipogenic enzymes themselves are known to contain Zn as an essential element. However, PPARα, a key regulator of lipid degradation, contain at least 2 zinc finger domains (Kang et al., 2010, Dieck et al., 2005), and the DNA binding capabilities of PPARα is significantly reduced during Zn deprivation (Kang et al., 2010). Thus, Kang et al. (2010) suggested that the effect of Zn on lipid metabolism is due to dysfunctional PPARα. Similar effects of Zn are present in yellow catfish, in which Zn deficiencies tended to increase hepatic lipid content and excess reduced it (Zheng et al., 2015), and a 96 h chronic Zn exposure had the same effect (Zheng et al., 2013). In both cases the activity of CPT1 increased with higher Zn content, and Zheng et al. (2013) found a

positive relationship between the activity and expression of CPT1, indicating a pre-translational effect of Zn. This is in accordance with the possibility of PPARα being the mediator of the effects of Zn deficiencies and Zn excess on hepatic lipid content.

There were no clear increases in any of the genes related to FA oxidation investigated in this study, with no significant differences between the diet groups for neither PPARα nor CPT1. If organic (or inorganic) micro-mineral supplementation had had any effect on the FA oxidation, likely similar effects as described in Zn excess/deficiency would have been seen. The non-existent changes in gene expression corresponds well with the lipid class distribution in the liver, as an increase in FA oxidation should have been seen as a lowered lipid content. However, the experiment was probably too short for any noticeable changes in the lipid composition, to be further discussed below.

4.6 Lipid class composition

The lipid class composition analysis didn't show many significant differences between the different diets in this study. There was a significantly higher amount of FFA in the diet group HPI than HPOMn, but this is not necessarily caused by biological reasons. The TAG proportion of the samples is highly sensitive to lipid hydrolysis, during which it is broken down to its constituents. This process is temperature sensitive, with higher temperatures leading to higher degree of hydrolysis, especially in samples with a high FA content (Rudy et al., 2016). Thus, the difference in FFA found between the two diet groups is probably due to degradation of TAG, most likely during weighing in for analysis, rather than differences caused by the diets.

It is well known that salmonid tissues are affected by the dietary lipid composition (Glencross, 2009, Turchini et al., 2009). The dietary lipids of the present study had the same amount of total lipids and the same FA profile, hence the dietary lipid would not cause any differences. All the investigated minerals have some direct or indirect connection to the lipid metabolism, but the chemical form seems to have had no effect on the hepatic lipid composition. This was not an unexpected result as there were few differences in the gene expression. Possibly, the experiment was too short for any effects to be seen on liver lipid class composition. Experiments investigating the effects of micro-minerals on lipid content usually last several weeks (e.g. Zheng et al. (2015), Tan et al. (2012), Luo et al. (2011), Maage and Julshamn (1993)). It should be mentioned that the liver lipid content of Atlantic salmon can change quite rapidly (Sissener

et al., 2017). However, it is possible that there was not enough time for the diets to affect the hepatic mineral status sufficiently for this to translate into the lipid distribution.

4.7 Correlation between FAS and TAG, co-regulation with fads2 genes

The expression of FAS correlated positively with the amount of liver TAG. It was not a strong correlation, but there are many factors affecting liver TAG in addition to FA synthesis, such as absorbed dietary lipids and lipid hydrolysis. An increased amount of FAS will still lead to increased FA synthesis which can be used to build up TAG, which is where fats are stored. As such, this correlation makes sense. That there was a correlation between the Fads and TAG is less intuitive, but it is probably derived from a connection between the expression of Fads genes and FAS. There are several examples of concurring increased expression of FAS together with Δ5Fad and Δ6Fad (Carmona-Antoñanzas et al., 2014, Martinez-Rubio et al., 2013). This could be on the account of them being regulated by the same transcription factors; FAS is a direct target of LXR in Atlantic salmon (Carmona-Antoñanzas et al., 2014), and LXR also led to up-regulated expression of Fad and elovl genes in zebrafish (Pinto et al., 2016). FAS is a target of SREBP1 in Atlantic salmon (Minghetti et al., 2011), and so is Δ6Fad (Zheng et al., 2009, Carmona-Antoñanzas et al., 2014). Additionally, Minghetti et al. (2011) showed a clear co-expression of FAS with Δ5Fad and Δ6Fad after lipid depletion. This suggests a co-regulation between FAS and Δ5Fad + Δ6Fad, and that the correlation found between Δ5Fad and Δ6Fad with TAG is a result of this co-regulation.

5 Conclusion and future perspectives

The current results show no major overall effects of the chemical micro-mineral form on the selected hepatic metabolism markers, at least when supplied at above recommended requirement levels, as is often the case in commercial salmon feeds. Although some significant differences were seen in ApoB100 and SREBP1 expression it seems most probable that these were just random effects. The largest differences were seen between diets LPI and HPI, which both had the same mineral form and there was no effect of phytate on the mineral digestibility. Additionally, both diet groups had similar mineral content in the liver.

It is possible the experiment was too short for the diets to bring about changes in the hepatic mineral status. Therefore, it could be of interest to see if any effects would occur over time with

longer experiments. To see if the organic forms can prevent inhibitory interactions, perhaps using diets with larger differences in phytate could be worth considering. If effects on the synthesis of LC-PUFA from the bioavailability or the chemical form is to be studied, then diets containing lower levels of EPA and DHA should be used. Furthermore, it may be of interest to examine whether the chemical forms remain the same after uptake and see if they have different metabolic pathways. If they do, they could have an impact on lipid metabolism.

References

ALDER, J., CAMPBELL, B., KARPOUZI, V., KASCHNER, K. & PAULY, D. 2008. Forage Fish: From Ecosystems to Markets. *Annual Review of Environment and Resources,* 33**,** 153-166.

ALHAZZA, R., BRIDLE, A. R., NICHOLS, P. D. & GARTER, C. 2011. Replacing dietary fish oil with echium oil enriched barramundi with C18 PUFA rather than long-chain PUFA. *Aquaculture,* 312**,** 162-171.

ALHAZZAA, R., SINCLAIR, A. J. & TURCHINI, G. M. 2013. Bioconversion of alpha-Linolenic Acid into n-3 Long-Chain Polyunsaturated Fatty Acid in Hepatocytes and Ad Hoc Cell Culture Optimisation. *PLOS One,* 8.

APINES-AMAR, M. J. S., SATOH, S., CAIPANG, C. M. A., KIRON, V., WATANABE, T. & AOKI, T. 2004. Amino acid-chelate: a better source of Zn, Mn and Cu for rainbow trout, Oncorhynchus mykiss. *Aquaculture,* 240**,** 345-358.

BELL, J. G., COWEY, C. B., ADRON, J. W. & PIRIE, B. J. S. 1987. Some effects of selenium deficiency on enzyme activities and indices of tissue peroxidation in Atlantic salmon parr (Salmo salar). *Aquaculture,* 65**,** 43-54.

BELL, J. G., TOCHER, D. R., HENDERSON, R. J., DICK, J. R. & CRAMPTON, V. O. 2003. Altered fatty acid compositions in Atlantic salmon (Salmo salar) fed diets containing linseed and rapeseed oils can be partially restored by a subsequent fish oil finishing diet. *Journal of Nutrition,* 133**,** 2793-2801.

BERLANGA, A., GUIU-JURADO, E., PORRAS, J. A. & AUGUET, T. 2014. Molecular pathways in non-alcoholic fatty liver disease. *Clinical and Experimental Gastroenterology,* 7**,** 221-39.

BERNTSSEN, M. H. G., SUNDAL, T. K., OLSVIK, P. A., AMLUND, H., RASINGER, J. D., SELE, V., HAMRE, K., HILLESTAD, M., BUTTLE, L. & ØRNSRUD, R. 2017. Sensitivity and toxic mode of action of dietary organic and inorganic selenium in Atlantic salmon (Salmo salar). *Aquatic Toxicology,* 192**,** 116-126.

BETANCOR, M. B., HOWARTH, F. J. E., GLENCROSS, B. D. & TOCHER, D. R. 2014. Influence of dietary docosahexaenoic acid in combination with other long-chain polyunsaturated fatty acids on expression of biosynthesis genes and phospholipid fatty acid compositions in tissues of post-smolt Atlantic salmon (Salmo salar). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 172-173**,** 74-89.

BHARADWAJ, A. S., PATNAIK, S., BROWDY, C. L. & LAWRENCE, A. L. 2014. Comparative evaluation of an inorganic and a commercial chelated copper source in Pacific white shrimp Litopenaeus vannamei (Boone) fed diets containing phytic acid. *Aquaculture,* 422-423**,** 63-68.

BOGEVIK, A. S., OXLEY, A. & OLSEN, R. E. 2008. Hydrolysis of acyl-homogeneous and fish oil triacylglycerols using desalted midgut extract from Atlantic salmon, Salmo salar. *Lipids,* 43**,** 655-662.

BOU, M., BERGE, G. M., BAEVERFJORD, G., SIGHOLT, T., ØSTBYE, T.-K., ROMARHEIM, O. H., HATLEN, B., LEEUWIS, R., VENEGAS, C. & RUYTER, B. 2017. Requirements of n-3

very long-chain PUFA in Atlantic salmon (Salmo salar L): effects of different dietary levels of EPA and DHA on fish performance and tissue compositiion and integrity. *British Journal of nutrition,* 117**,** 30-47.

BRANSDEN, M. P., CARTER, C. G. & NICHOLS, P. D. 2003. Replacement of fish oil with sunflower oil in feeds for Atlantic salmon (Salmo salar L.): effect on growth performance, tissue fatty acid composition and disease resistance. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 135**,** 611-625.

CARMONA-ANTOÑANZAS, G., TOCHER, D. R., MARTINEZ-RUBIO, L. & LEAVER, M. J. 2014. Conservation of lipid metabolic gene transcriptional regulatory networks in fish and mammals. *Gene,* 534**,** 1-9.

CHANDA, S., PAUL, B. N., GHOSH, K. & GIRI, S. S. 2015. Dietary essentiality of trace minerals in aquaculture-A Review. *Agricultural Reviews,* 36.

CHENG, Z. J. & HARDY, R. W. 2003. Effects of extrusion and expelling processing, and microbial phytase supplementation on apparent digestibility coefficients of nutrients in full-fat soybeans for rainbow trout (Oncorhynchus mykiss). *Aquaculture,* 218**,** 501-514.

CHIANG, J. Y. 2009. Bile acids: regulation of synthesis. *Journal of Lipid Research,* 50**,** 1955-66.

CRUZ-GARCIA, L., SANCHEZ-GURMACHES, J., GUTIERREZ, J. & NAVARRO, I. 2011. Regulation of LXR by fatty acids, insulin, growth hormone and tumor necrosis factor-alpha in rainbow trout myocytes. *Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology,* 160**,** 125-36.

CUNNANE, S. C., HORROBIN, D. F. & MANKU, M. S. 1984. Essential fatty acids in tissue phospholipids and triglycerides of the zinc-deficient rat. *Proceedings of the Society for Experimental Biology and Medicine,* 177**,** 441-6.

DENSTADLI, V., SKREDE, A., KROGDAHL, Å., SAHLSTRØM, S. & STOREBAKKEN, T. 2006. Feed intake, growth, feed conversion, digestibility, enzyme activities and intestinal structure in Atlantic salmon (Salmo salar L.) fed graded levels of phytic acid. *Aquaculture,* 256**,** 365-376.

DESVERGNE, B., MICHALIK, L. & WAHLI, W. 2006. Transcriptional regulation of metabolism. *Physiological Reviews,* 86**,** 465-514.

DIECK, H. T., DÖRING, F., FUCHS, D., ROTH, H.-P. & DANIEL, H. 2005. Transcriptome and Proteome Analysis Identifies the Pathways that Increase Hepatic Lipid Accumulation in Zinc-Deficient Rats. *Journal of Nutrition,* 135**,** 199-205.

DIECK, H. T., DÖRING, F., ROTH, H.-P. & DANIEL, H. 2003. Changes in Rat Hepatic Gene Expression in Response to Zinc deficiency as Assessed by DNA Arrays. *Journal of nutrition,* 133**,** 1004-1010.

DO CARMO E SÁ, M. V., BARROS, L. E. P. M. M. & PADILHA, P. M. 2005. Apparent absorption of zinc from supplemental inorganic and organic sources to Nile tilapia *Oreochromis niloticus* juveniles. *Journal of the world aquaculture society,* 36**,** 375-383.

DOMÍNGUEZ, D., RIMOLDI, S., ROBAINA, L. E., TORRECILLAS, S., TEROVA, G., ZAMORANO, M. J., KARALAZOS, V., HAMRE, K. & IZQUIERDO, M. 2017. Inorganic, organic, and encapsulated minerals in vegetable meal based diets for Sparus aurata (Linnaeus, 1758). *PeerJ,* 5**,** e3710.

DONG, X., TAN, P., CAI, Z., XU, H., LI, J., REN, W., XU, H., ZUO, R., ZHOU, J., MAI, K. & AI, Q. 2017. Regulation of FADS2 transcription by SREBP-1 and PPAR-alpha influences LC-PUFA biosynthesis in fish. *Scientific Reports,* 7**,** 40024.

DONG, X., XU, H., MAI, K., XU, W., ZHANG, Y. & AI, Q. 2015. Cloning and characterization of SREBP-1 and PPAR-α in Japanese seabass Lateolabrax japonicus, and their gene expressions in response to different dietary fatty acid profiles. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 180**,** 48-56.

EDER, K. & KIRCHGESSNER, M. 1996. Zinc deficiency and the desaturation of linoleic acid in rats force-fed fat-free diets. *Biological Trace Element Research,* 54**,** 173-83.

ESPE, M., LEMME, A., PETRI, A. & EL-MOWAFI, A. 2006. Can Atlantic salmon (Salmo salar) grow on diets devoid of fish meal? *Aquaculture,* 255**,** 255-262.

EVANS, R. M., BARISH, G. D. & WANG, Y. X. 2004. PPARs and the complex journey to obesity. *Nature Medicine,* 10**,** 355-61.

FAO 2016. The state of world fisheries and aquaculture 2016. Contributing to food security and nutrition for all. Rome.

FOUNTOULAKI, E., MORGANE, H., RIGOS, G., ANTIGONI, V., MENTE, E., SWEETMAN, J. & NENGAS, I. 2010. Evaluation of zinc supplementation in European sea bass (Dicentrarchus labrax) juvenile diets. *Aquaculture Research,* 41**,** e208-e216.

FRANCIS, G., MAKKAR, H. P. S. & BECKER, K. 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture,* 199**,** 197-227.

GATLIN, D. M., BARROWS, F. T., BROWN, P., DABROWSKI, K., GAYLORD, T. G., HARDY, R. W., HERMAN, E., HU, G., KROGDAHL, Å., NELSON, R., OVERTURF, K., RUST, M., SEALEY, W., SKONBERG, D., J SOUZA, E., STONE, D., WILSON, R. & WURTELE, E. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. *Aquaculture Research,* 38**,** 551-579.

GIRI, S. S., GRAHAM, J., HAMID, N. K. A., DONALD, J. A. & TURCHINI, G. M. 2016. Dietary micronutrients and in vivo n − 3 LC-PUFA biosynthesis in Atlantic salmon. *Aquaculture,* 452**,** 416-425.

GLENCROSS, B. D. 2009. Exploring the nutritional demand for essential fatty acids by aquaculture species. *Reviews in Aquaculture,* 1**,** 71-124.

GODIN, S., FONTAGNE-DICHARRY, S., BUENO, M., TACON, P., PRABHU, P. A., KAUSHIK, S., MEDALE, F. & BOUYSSIERE, B. 2015. Influence of Dietary Selenium Species on Selenoamino Acid Levels in Rainbow Trout. *Journal of Agricultural and Food Chemistry,* 63**,** 6484-92.

GREGORY, M. K., GIBSON, R. A., COOK-JOHNSON, R. J., CLELAND, L. G. & JAMES, M. J. 2011. Elongase reactions as control points in long-chain polyunsaturated fatty acid synthesis. *PLOS One,* 6**,** e29662.

HEMRE, G. I., MOMMSEN, T. P. & KROGDAHL, A. 2002. Carbohydrates in fish nutrition: effects on growth, glucose metabolism and hepatic enzymes. *Aquaculture Nutrition,* 8**,** 175-194.

HOLLEY, A. K., BAKTHAVATCHALU, V., VELEZ-ROMAN, J. M. & ST CLAIR, D. K. 2011. Manganese superoxide dismutase: guardian of the powerhouse. *Int J Mol Sci,* 12**,** 7114-62.

HOLT, G. J. 2011. *Larval fish nutrition,* Chichester, UK, Wiley-Blackwell.

HORROBIN, C. F. & CUNNANE, S. C. 1980. Interactions between zinc, essential fatty acids and prostaglandins: relevance to acrodermatitis enteropathica, total parental nutrition, the glucagonoma syndrome, diabetes, anorexia nervosa and sickle cell anemia. *Medical hypotheses,* 6**,** 277-296.

HORTON, J. D., GOLDSTEIN, J. L. & BROWN, M. S. 2002. SREBPs: activators of the complete program of cholesterol and fatty acid synthesis in the liver. *Journal of Clinical Investigation,* 109**,** 1125-1131.

INFANTE, J. P. 1986. Vitamin-E and Selenium Participation in Fatty-Acid Desaturation - a Proposal for an Enzymatic Function of These Nutrients. *Molecular and Cellular Biochemistry,* 69**,** 93-108.

JEON, T. I. & OSBORNE, T. F. 2012. SREBPs: metabolic integrators in physiology and metabolism. *Trends in Endocrinology & Metabolism,* 23**,** 65-72.

JOHNSON, J. M. 1986. *Manganese and lipid metabolism as affected by dietary manganese and fat.* Ph.D., The unisversity of Nebraska - Lincoln.

JOSEPH, S. B., LAFFITTE, B. A., PATEL, P. H., WATSON, M. A., MATSUKUMA, K. E., WALCZAK, R., COLLINS, J. L., OSBORNE, T. F. & TONTONOZ, P. 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. *Journal of Biological Chemistry,* 277**,** 11019-25.

KANG, X., ZHONG, W., LIU, J., SONG, Z., MCCLAIN, C. J., KANG, Y. J. & ZHOU, Z. 2010. Zinc supplementation reverses alcohol-induced steatosis in mice through reactivating hepatocyte nuclear factor-4α and peroxisome proliferators activated receptor-α. *Hepatology,* 50**,** 1241-1250.

KATYA, K., LEE, S., YUN, H., DAGOBERTO, S., BROWDY, C. L., VAZQUEZ-ANON, M. & BAI, S. C. 2016. Efficacy of inorganic and chelated trace minerals (Cu, Zn and Mn) premix sources in Pacific white shrimp, Litopenaeus vannamei (Boone) fed plant protein based diets. *Aquaculture,* 459**,** 117-123.

KEEN, C. L., LÕNNERDAL, B. & HURLEY, L. S. 1984. Manganese. *In:* FRIEDEN, E. (ed.) *Biochemistry of the essential ultratrace elements.* Boston: Springer-Verlag US.

KJAER, M. A., RUYTER, B., BERGE, G. M., SUN, Y. & OSTBYE, T. K. 2016. Regulation of the Omega-3 Fatty Acid Biosynthetic Pathway in Atlantic Salmon Hepatocytes. *PLOS One,* 11**,** e0168230.

KLIMIS-TAVANTZIS, D. J., LEACH, R. M., JR. & KRIS-ETHERTON, P. M. 1983. The effect of dietary manganese deficiency on cholesterol and lipid metabolism in the Wistar rat and in the genetically hypercholesterolemic RICO rat. *Journal of Nutrition,* 113**,** 328-36.

KNEZ, M., STANGOULIS, J. C. R., GLIBETIC, M. & TAKO, E. 2017. The Linoleic Acid: Dihomo-gamma-Linolenic Acid Ratio (LA:DGLA)-An Emerging Biomarker of Zn Status. *Nutrients,* 9.

KNIGHT, R., MARLATT, V. L., BAKER, J. A., LO, B. P., DEBRUYN, A. M. H., ELPHICK, J. R. & MARTYNIUK, C. J. 2016. Dietary selenium disrupts hepatic triglyceride stores and transcriptional networks associated with growth and Notch signaling in juvenile rainbow trout. *Aquatic Toxicology,* 180**,** 103-114.

KÜÇÜKBAY, F. Z., YAZLAK, H., KARACA, I., SAHIN, N., TUZCU, M., CAKMAK, M. N. & SAHIN, K. 2009. The effects of dietary organic or inorganic selenium in rainbow trout (Oncorhynchus mykiss) under crowding conditions. *Aquaculture Nutrition,* 15**,** 569-576.

LALL, S. P. 2002. The minerals. *In:* HALVER, J. E. & HARDY, R. W. (eds.) *Fish nutrition.* Third ed.: Academic press.

LALL, S. P. & MILLEY, J. E. 2008. Impact of aquaculture on aquatic environment: trace minerals discharge. *In:* SCHLEGEL, P., DUROSOY, S. & JONGBLOED, A. W. (eds.) *Trace elements in animal production systems.* The Netherlands: Wageningen Academic publishers.

LEAVER, M. J., BAUTISTA, J. M., BJÖRNSSON, B. T., JÖNSSON, E., KREY, G., TOCHER, D. R. & TORSTENSEN, B. E. 2008. Towards Fish Lipid Nutrigenomics: Current State and Prospects for Fin-Fish Aquaculture. *Reviews in Fisheries Science,* 16**,** 73-94.

LEAVER, M. J., TOCHER, D. R., OBACH, A., JENSEN, L., HENDERSON, R. J., PORTER, A. R. & KREY, G. 2006. Effect of dietary conjugated linoleic acid (CLA) on lipid composition, metabolism and gene expression in Atlantic salmon (Salmo salar) tissues. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* 145**,** 258-267.

LEHMANN, J. M., KLIEWER, S. A., MOORE, L. B., SMITH-OLIVER, T. A., OLIVER, B. B., SU, J. L., SUNDSETH, S. S., WINEGAR, D. A., BLANCHARD, D. E., SPENCER, T. A. & WILLSON, T. M. 1997. Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *Journal of Biological Chemistry,* 272**,** 3137-40.

LEHNINGER, A., NELSON, D. L. & COX, M. M. 2013a. Fatty acid catabolism. *In:* NELSON, D. L. & COX, M. M. (eds.) *The principles of biochemistry (sixth edition).* New York: W.H. Freeman and company.

LEHNINGER, A., NELSON, D. L. & COX, M. M. 2013b. Hormonal regulation of and integration of mammalian metabolism. *In:* NELSON, D. L. & COX, M. M. (eds.) *The principles of biochemistry (sixth edition).* New York: W. H. Freeman & company.

LEHNINGER, A., NELSON, D. L. & COX, M. M. 2013c. Lipid biosynthesis. *In:* NELSON, D. L. & COX, M. M. (eds.) *The principles of biochemistry (sixth edition).* New York: W.H. Freeman and company.

LEHNINGER, A., NELSON, D. L. & COX, M. M. 2013d. Lipids. *In:* NELSON, D. L. & COX, M. M. (eds.) *The principles of biochemistry (sixth edition).* New York: W. H. Freeman & company.

LEHNINGER, A., NELSON, D. L. & COX, M. M. 2013e. Regulation of metabolic pathways. *In:* NELSON, D. L. & COX, M. M. (eds.) *The principles of biochemistry (sixth edition).* New York: W. H. Freeman & company.

LEWIS, M. J., HAMID, N. K. A., ALHAZZAA, R., HERMON, K., DONALD, J. A., SINCLAIR, A. J. & TURCHINI, G. M. 2013. Targeted dietary micronutrient fortification modulates n−3 LC-

PUFA pathway activity in rainbow trout (Oncorhynchus mykiss). *Aquaculture,* 412-413**,** 215-222.
LIN, S., LIN, X., YANG, Y., LI, F. & LUO, L. 2013. Comparison of chelated zinc and zinc sulfate as zinc sources for growth and immune response of shrimp (Litopenaeus vannamei). *Aquaculture,* 406-407**,** 79-84.
LIN, Y.-H. & SHIAU, S.-Y. 2009. Mutual sparing of dietary requirements for alpha-tocopherol and selenium in grouper, Epinephelus malabaricus. *Aquaculture,* 294**,** 242-245.
LORENTZEN, M., BERNTSSEN, M. H. G. & MAAGE, A. 2001. Mineraler og sporelementer. *In:* WAAGBØ, R., ESPE, M., HAMRE, K. & LIE, Ø. (eds.) *Fiskeernæring.* Kystnæringen, Forlag & Bokklubb AS.
LUO, Z., TAN, X.-Y., ZHENG, J.-L., CHEN, Q.-L. & LIU, C.-X. 2011. Quantitative dietary zinc requirement of juvenile yellow catfish Pelteobagrus fulvidraco, and effects on hepatic intermediary metabolism and antioxidant responses. *Aquaculture,* 319**,** 150-155.
MAAGE, A. & JULSHAMN, K. 1993. Assessment of zinc status in juvenile Atlantic salmon *(Salmo salar)* by measurement of whole body and tissue levels of zinc. *Aquaculture,* 117**,** 179-191.
MAAGE, A., JULSHAMN, K. & BERGE, G. E. 2001. Zinc gluconate and zinc sulphate as dietary zinc sources for Atlantic salmo. *Aquaculture Nutrition,* 7.
MANTOVANI, A., FRAZZOLI, C. & CUBADDA, F. 2010. Organic forms of trace elements as feed additives: Assessment of risks and benefits for farm animals and consumers. *Pure and Applied Chemistry,* 82**,** 393-407.
MARTINEZ-RUBIO, L., WADSWORTH, S., GONZÁLEZ VECINO, J. L., BELL, J. G. & TOCHER, D. R. 2013. Effect of dietary digestible energy content on expression of genes of lipid metabolism and LC-PUFA biosynthesis in liver of Atlantic salmon (Salmo salar L.). *Aquaculture,* 384-387**,** 94-103.
MILLER, M. R., NICHOLS, P. D. & CARTER, C. G. 2008. n-3 Oil sources for use in aquaculture--alternatives to the unsustainable harvest of wild fish. *Nutrition Research Reviews,* 21**,** 85-96.
MINGHETTI, M., LEAVER, M. J. & TOCHER, D. R. 2011. Transcriptional control mechanisms of genes of lipid and fatty acid metabolism in the Atlantic salmon (Salmo salar L.) established cell line, SHK-1. *Biochimica et Biophysica Acta,* 1811**,** 194-202.
MORAIS, S., PRATOOMYOT, J., TAGGART, J. B., BRON, J. E., GUY, D. R., BELL, J. G. & TOCHER, D. R. 2011. Genotype-specific responses in Atlantic salmon (Salmo salar) subject to dietary fish oil replacement by vegetable oil: a liver transcriptomic analysis. *BMC Genomics,* 12**,** 255.
MORALES, G. A., DENSTADLI, V., COLLINS, S. A., MYDLAND, L. T., MOYANO, F. J. & ØVERLAND, M. 2016. Phytase and sodium diformate supplementation in a plant-based diet improves protein and mineral utilization in rainbow trout (Oncorhynchus mykiss). *Aquaculture Nutrition,* 22**,** 1301-1311.
NRC 2011. *Nutrient requirements of fish and shrimp,* Washington, D.C National Academic Press.
NØSTEBAKKEN, O. J., BREDAL, I. L., OLSVIK, P. A., HUANG, T. S. & TORSTENSEN, B. E. 2012. Effect of Marine Omega 3 Fatty Acids on Methylmercury-Induced Toxicity in Fish and Mammalian Cells In Vitro. *Journal of Biomedicine and Biotechnology*.
PETTERSON, A., JOHNSSON, L., BRÄNNÄS, E. & PICKOVA, J. 2009. Effects of rapeseed oil replacement in fish feed on lipid composition and self-selection by rainbow trout (Oncorhynchus mykiss). *Aquaculture nutrition,* 15**,** 577-586.
PINTO, C. L., KALASEKAR, S. M., MCCOLLUM, C. W., RUIU, A., JONSSON, P., LOPEZ, J., SWINDELL, E., BOUHLATOUF, A., BALAGUER, P., BONDESSON, M. & GUSTAFSSON, J.-Å. 2016. Lxr regulates lipid metabolic and visual perception pathways during zebrafish development. *Molecular and cellular endocrinology,* 5**,** 29-43.
PRABHU, A. J. 2018. Speciation and bio-availability of Zinc and Selenium in Atlantic salmon feeds. Oral presentation. *HAVBRUK2018. Oslo, Norway. 18-20 April, 2018.*
PRABHU, A. J., SCHRAMA, J. W. & KAUSHIK, S. J. 2014. Mineral requirements of fish: a systematic review. *Reviews in aquaculture*.

R DEVELOPMENT CORE TEAM 2011. R: A language and environment for statistical computing. *Foundation for Statistical Computing, Vienna. Vienna, Austria*.

RIDER, S. A., DAVIES, S. J., JHA, A. N., CLOUGH, R. & SWEETMAN, J. W. 2010. Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (Oncorhynchus mykiss) diet. *Journal of animal physiology and animal nutrition,* 94**,** 99-110.

ROSENLUND, G., TORSTENSEN, B. E., STUBHAUG, I., USMAN, N. & SISSENER, N. H. 2016. Atlantic salmon require long-chain n-3 fatty acids for optimal growth throughout the seawater period. *Journal of nutritional science,* 5**,** e19.

RUDY, M. D., KAINZ, M. J., GRAEVE, M., COLOMBO, S. M. & ARTS, M. T. 2016. Handling and Storage Procedures Have Variable Effects on Fatty Acid Content in Fishes with Different Lipid Quantities. *PLOS One,* 11**,** e0160497.

RUYTER, B., ANDERSEN, Ø., DEHLI, A., FARRANTS, A.-K., GJØEN, T. & THOMASSEN, M. S. 1997. Peroxisome proliferator activated receptors in Atlantic salmon *(Salmo salar):* effects on PPAR transcription and acyl-CoA oxidase activity in hepatocytes by peroxisome proliferators and fatty acids. *Biochimica et biophysica acta,* 1348**,** 331-338.

RUYTER, B., MOYA-FALCÓN, C., ROSENLUND, G. & VEGUSDAL, A. 2006. Fat content and morphology of liver and intestine of Atlantic salmon (Salmo salar): Effects of temperature and dietary soybean oil. *Aquaculture,* 252**,** 441-452.

RUYTER, B., RØSJØ, C., GRISDALE-HELLAND, B., ROSENLUND, G., OBACH, A. & THOMASSEN, M. S. 2003. Influence of temperature and high dietary linoleic acid content on esterification, elongation, and desaturation of PUFA in Atlantic salmon hepatocytes. *Lipids,* 38**,** 833-40.

SANDEN, M., HEMRE, G. I., MAAGE, A., LUNESTAD, B. T., ESPE, M., LIE, K. K., LUNDEBYE, A.-K., AMLUND, H., WAAGBØ, R. & ØRNSRUD, R. 2017. Rapport 2017. Program for overvåking av fiskefôr. Årsrapport for prøver innsamlet i 2016. Nasjonalt institutt for ernærings- og sjømatforskning (NIFES).

SANDEN, M., STUBHAUG, I., BERNTSSEN, M. H., LIE, O. & TORSTENSEN, B. E. 2011. Atlantic salmon (Salmo salar L.) as a net producer of long-chain marine omega-3 fatty acids. *Journal of agricultural and food chemistry,* 59**,** 12697-706.

SARGENT, J. R., BELL, J. G., BELL, M. V., HENDERSON, R. J. & TOCHER, D. R. 1995. Requirement for essential fatty acids. *Journal of applied ichthyology,* 11**,** 183-198.

SARGENT, J. R., TOCHER, D. R. & BELL, J. G. 2002. The lipids. *In:* HALVER, J. E. & HARDY, R. W. (eds.) *Fish nutrition (Third edition).* Academic press.

SCHAFER, K., KYRIAKOPOULOS, A., GESSNER, H., GRUNE, T. & BEHNE, D. 2004. Effects of selenium deficiency on fatty acid metabolism in rats fed fish oil-enriched diets. *Journal of trace elements in medicine and biology,* 18**,** 89-97.

SCHRAUZER, G. N. 2000. Selenomethionine: a review of its nutritional significance, metabolism and toxicity. *Journal of Nutrition,* 130**,** 1653-6.

SCHROEDER, A., MUELLER, O., STOCKER, S., SALOWSKY, R., LEIBER, M., GASSMANN, M., LIGHTFOOT, S., MENZEL, W., GRANZOW, M. & RAGG, T. 2006. The RIN: an RNA integrity number for assigning integrity values to RNA measurements. *BMC Molecular Biology,* 7**,** 3.

SELE, V., ØRNSRUD, R., SLOTH, J. J., BERNTSSEN, M. H. G. & AMLUND, H. 2018. Selenium and selenium species in feeds and muscle tissue of Atlantic salmon. *Journal of Trace Elements in Medicine and Biology,* 47**,** 124-133.

SILVA-BRITO, F., MAGNONI, L. J., FONSECA, S. B., PEIXOTO, M. J., CASTRO, L. F., CUNHA, I., DE ALMEIDA OZORIO, R. O., MAGALHAES, F. A. & GONCALVES, J. F. 2016. Dietary Oil Source and Selenium Supplementation Modulate Fads2 and Elovl5 Transcriptional Levels in Liver and Brain of Meagre (Argyrosomus regius). *Lipids,* 51**,** 729-41.

SISSENER, N. H., LILAND, N. S., HOLEN, E., STUBHAUG, I., TORSTENSEN, B. E. & ROSENLUND, G. 2017. Phytosterols are not involved in the development of fatty liver in plant

oil fed Atlantic salmon (Salmo salar) at high or low water temperature. *Aquaculture,* 480**,** 123-134.

SISSENER, N. H., TORSTENSEN, B. E., OWEN, M. A. G., LILAND, N. S., STUBHAUG, I. & ROSENLUND, G. 2016. Temperature modulates liver lipid accumulation in Atlantic salmon (Salmo salar L.) fed low dietary levels of long-chain n-3 fatty acids. *Aquaculture Nutrition,* 23**,** 865-878.

SPRECHER, H. 2000. Metabolism of highly unsaturated n-3 and n-6 fatty acids. *Biochimica et biophysica acta,* 1486**,** 219-31.

SUGIURA, S. H., GABAUDAN, J., DONG, F. M. & HARDY, R. W. 2001. Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout [*Oncorhynchus mykiss*(Walbaum)] fed soybean meal-based diets. *Aquaculture Research,* 32**,** 583-592.

SUNDARAM, M. & YAO, Z. 2010. Recen progress in understanding protein and lipid factors affecting hepatic VLDL assembly and secretion. *Nutrition & metabolism,* 7.

SUZUKI, K. T. 2005. Metabolomics os selenium: Se metabolites based on speciation studies. *Journal of health science,* 51**,** 107-114.

SØFTELAND, L., BERNTSSEN, M. H. G., KIRWAN, J. A., STØRSETH, T. R., VIANT, M. R., TORSTENSEN, B. E., WAAGBØ, R. & OLSVIK, P. A. 2016. Omega-3 and alpha-tocopherol provide more protection against contaminants in novel feeds for Atlantic salmon (Salmo salar L.) than omega-6 and gamma tocopherol. *Toxicology Reports,* 3**,** 211-224.

SØRENSEN, M., BERGE, G. M., THOMASSEN, M., RUYTER, B., HATLEN, B., YTRESTØYL, T., AAS, T. S. & ÅSGÅRD, T. 2011. Today's and tomorrows feed ingredients in Norwegian aquaculture. *Nofima*.

TACON, A. G. J. & METIAN, M. 2008. Global overview on the use of fish meal and fish oil in industrially compounded aquafeeds: Trends and future prospects. *Aquaculture,* 285**,** 146-158.

TAN, X. Y., XIE, P., LUO, Z., LIN, H.-Z., ZHAO, Y.-H. & XI, W.-Q. 2012. Dietary manganese requirement of juvenile yellow catfish Pelteobagrus fulvidraco, and effects on whole body mineral composition and hepatic intermediary metabolism. *Aquaculture,* 326-329**,** 68-73.

THANUTHONG, T., FRANCIS, D. S., SENADHEERA, S. P., JONES, P. L. & TURCHINI, G. M. 2011. LC-PUFA biosynthesis in rainbow trout is substrate limited: use of the whole body fatty acid balance method and different 18:3n-3/18:2n-6 ratios. *Lipids,* 46**,** 1111-27.

TOCHER, D. R. 2003. Metabolism and Functions of Lipids and Fatty Acids in Teleost Fish. *Reviews in Fisheries Science,* 11**,** 107-184.

TOCHER, D. R. 2010. Fatty acid requirements in ontogeny of marine and freshwater fish. *Aquaculture Research,* 41**,** 717-732.

TOCHER, D. R., BELL, J. G., DICK, J. R. & CRAMPTON, V. O. 2003. Effects of dietary vegetable oil on Atlantic salmon hepatocyte fatty acid desaturation and liver fatty acid compositions. *Lipids,* 38.

TOCHER, D. R. & GHIONI, C. 1999. Fatty acid metabolism on marine fish: Low activity of fatty acyl Δ5 desaturation in gilthead sea bream *(Sparua aurata)* cells. *Lipids,* 34**,** 433-440.

TODORČEVIĆ, M., VEGUSDAL, A., GJØEN, T., SUNDVOLD, H., TORSTENSEN, B. E., KJÆR, M. A. & RUYTER, B. 2008. Changes in fatty acids metabolism during differentiation of Atlantic salmon preadipocytes; Effects of n-3 and n-9 fatty acids. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids,* 1781**,** 326-335.

TORSTENSEN, B. E., BELL, J. G., ROSENLUND, G., HENDERSON, R. J., GRAFF, I. E., TOCHER, D. R., LIE, Ø. & SARGENT, J. R. 2005. Tailoring of Atlantic salmon (Salmo salar L.) flesh lipid composition and sensory quality by replacing fish oil with a vegetable oil blend. *Journal of agricultural and food chemistry,* 53.

TORSTENSEN, B. E., ESPE, M., STUBHAUG, I. & LIE, O. 2011. Dietary plant proteins and vegetable oil blends increase adiposity and plasma lipids in Atlantic salmon (Salmo salar L.). *British Journal of nutrition,* 106**,** 633-47.

TORSTENSEN, B. E., FRØYLAND, L. & LIE, Ø. 2001. Lipider. *In:* WAAGBØ, R., ESPE, M., HAMRE, K. & LIE, Ø. (eds.) *Fiskeernæring.* Kystnæringen, Forlag & Bokklubb AS.

TURCHINI, G. M., FRANCIS, D. S., KEAST, R. S. J. & SINCLAIR, A. J. 2011. Transforming salmonid aquaculture from a consumer to a producer of long chain omega-3 fatty acids. *Food Chemistry,* 124**,** 609-614.

TURCHINI, G. M., TORSTENSEN, B. E. & NG, W.-K. 2009. Fish oil replacement in finfish nutrition. *Reviews in Aquaculture,* 1**,** 10-57.

VUKMIRICA, J., NISHIMAKI-MOGAMI, T., TRAN, K., SHAN, J., MCLEOD, R. S., YUAN, J. & YAO, Z. 2002. The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL. *Journal of Lipid Research,* 43**,** 1496-1507.

WANG, S.-L., DU, E. Z., MARTIN, T. D. & DAVIS, R. A. 1997. Coordinate reuglation of lipogenesis, the assembly and secretion of apolipoprotein B-containing lipoproteins by sterol response element binding protein 1. *The journal of biological chemistry,* 272**,** 19351-19358.

WATANABE, T., KIRON, V. & SATOH, S. 1997. Trace minerals in fish nutrition. *Aquaculture,* 151**,** 185-207.

WILSON, R. P. 2002. Amino acids and proteins. *In:* HALVER, J. E. & HARDY, R. W. (eds.) *Fish nutrition.* Third ed.: Academic press.

XU, H., DONG, X., AI, Q., MAI, K., XU, W. Y., ZHANG, Y. & ZUO, R. 2014. Regulation of Tissue LC-PUFA Contents, Δ6 Fatty Acyl Desaturase (FADS2) Gene Expression and the Methylation of the Putative FADS2 Gene Promoter by Different Dietary Fatty Acid Profiles in Japanese Seabass (Lateolabrax japonicus). *PLOS One*.

YAO, Z. & MCLEOD, R. S. 1994. Synthesis and secretion of hepatic apolipoprotein B-containing lipoproteins. *Biochim Biophys Acta,* 1212**,** 152-66.

YTRESTØYL, T., AAS, T. S. & ÅSGÅRD, T. 2015. Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. *Aquaculture,* 448**,** 365-374.

ZHAO, C. & DAHLMAN-WRIGHT, K. 2010. Liver X receptor in cholesterol metabolism. *Journal of Endocrinology,* 204**,** 233-40.

ZHENG, J.-L., LUO, Z., HU, W., LIU, C.-X., CHEN, Q.-L., ZHU, Q.-L. & GONG, Y. 2015. Different effects of dietary Zn deficiency and excess on lipid metabolism in yellow catfish Pelteobagrus fulvidraco. *Aquaculture,* 435**,** 10-17.

ZHENG, J.-L., LUO, Z., LIU, C.-X., CHEN, Q.-L., TAN, X.-Y., ZHU, Q. L. & GONG, Y. 2013. Differential effects of acute and chronic zinc (Zn) exposure on hepatic lipid deposition and metabolism in yellow catfish Pelteobagrus fulvidraco. *Aquatic Toxicology,* 132-133**,** 173-181.

ZHENG, X., LEAVER, M. J. & TOCHER, D. R. 2009. Long-chain polyunsaturated fatty acid synthesis in fish: Comparative analysis of Atlantic salmon (Salmo salar L.) and Atlantic cod (Gadus morhua L.) Δ6 fatty acyl desaturase gene promoters. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology,* 154**,** 255-263.

ZHENG, X., TOCHER, D. R., DICKSON, C. A., BELL, J. G. & TEALE, A. J. 2005a. Highly unsaturated fatty acid synthesis in vertebrates: New insights with the cloning and characterization of a Δ6 desaturase of Atlantic salmon. *Lipids,* 40**,** 13-24.

ZHENG, X., TORSTENSEN, B. E., TOCHER, D. R., DICK, J. R., HENDERSON, R. J. & BELL, J. G. 2005b. Environmental and dietary influences on highly unsaturated fatty acid biosynthesis and expression of fatty acyl desaturase and elongase genes in liver of Atlantic salmon (Salmo salar). *Biochimica et biophysica acta,* 1734**,** 13-24.