Norwegian University of Life Sciences
Faculty of Veterinary Medicine
Department of Paraclinical Sciences

Philosophiae Doctor (PhD)
Thesis 2020:27

Choline is an essential nutrient for post-smolt Atlantic salmon (*Salmo salar* L)

Kolin er et essensielt næringsstoff for post-smolt Atlantisk laks (*Salmo salar* L)

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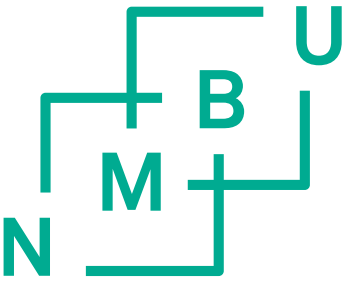
Adamstuen 2020



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Anne Kristine Grostøl Hansen

Trondheim, 2020

SUMMARY

The aquaculture feed industry continuously works to decrease the share of marine ingredients in the diets by searching for alternative feed ingredients. The increased use of such ingredients, on one hand expose the fish for new components, and on the other hand reduce the levels of components typical for diets rich in marine ingredients. Salmon diets have, over the last decades, also changed regarding lipid content. The level has increased, whereas the proportion of n-3 fatty acids has decreased. These changes may present challenges for the aquaculture industry. Fish farmers have, since 2000, reported symptoms of lipid malabsorption (LMS) in young as well as more mature salmon. The typically characteristics of LMS are swollen, pale and coarse appearance of the pyloric and mid intestine as a result of lipid accumulation in the tissue, seen histologically as large vacuoles. Previous studies have indicated that impaired lipoprotein formation, as a result of lack of phosphatidylcholine, could be the cause of the formation of excessive intestinal lipid vacuolation. Phosphatidylcholine is not considered as an essential nutrient for any fish species as it can be synthesized if sufficient choline is present. However, essentiality of choline has been established for many animal species, including some fish species in particularly for early stages, but not for any life stages of Atlantic salmon.

In this thesis, three separate experiments were conducted to increase knowledge on the effects of choline and other key components involved in lipid and sterol metabolism on the development of LMS in Atlantic salmon in seawater. The experiments included firstly investigating whether choline chloride could prevent LMS. Secondly, whether other key components in lipid and sterol metabolism may modify the development of symptoms of LMS. The goal of the last experiment was to define the dietary requirement of choline for Atlantic salmon in seawater.

The results from the first experiment showed that fish fed a diet with a choline level of 944 mg/kg developed clear signs of LMS whereas a diet supplemented to contain 4250 mg/kg choline, did not develop such signs. The level of triglycerides (TAG) in the pyloric caeca of

fish fed the high choline diet was reduced by 65%. This reduction was reflected in a parallel reduction in relative weight of the pyloric intestine. Choline altered the intestinal expression of genes related to phosphatidylcholine synthesis (*chk* and *pcyt1a*), cholesterol transport (*abcg5* and *npc1l1*), lipid metabolism and transport (*mgat2a* and *fabp2*), lipoprotein formation (*apoAI* and *apoAIV*) and the surface marker of intestinal lipid droplets (*plin2*). Furthermore, fish growth increased by 18% in the choline supplemented group. Taken together, these results demonstrated the importance of choline in lipid turnover in the intestine and its ability to prevent LMS.

The results in the second experiment confirmed those of the first, showing that fish fed a diet low in fish meal (LF), with a choline level of 1190 mg/kg, developed clear LMS-symptoms, whereas fish fed diets with high fish meal content and a choline level of 1860 mg/kg, did not show clear signs of LMS. Supplementation of LF with phosphatidylcholine or choline chloride, resulting in choline levels of 2870 and 2980, respectively, eliminated the LMS symptoms. Supplementing the low fishmeal basal diet, containing 1190 mg/kg choline, with either taurocholate at two levels (3.5 and 6.9 g/kg), cholesterol (2.0 g/kg), taurine (0.8 g/kg), cysteine (0.8 g/kg) or methionine (1.0 g/kg) did not reduce the LMS symptoms. No significant dietary effects on growth rate were observed in this experiment. The results from the intestinal gene expression evaluation showed that phosphatidylcholine altered the expression of *pcyt1a*, a gene involved in phosphatidylcholine synthesis and the cholesterol transporter *abcg5*. Both choline and phosphatidylcholine altered the intestinal gene expression for *apoAIV* related to lipoprotein formation and markedly suppressed *plin2*, the surface marker of intestinal lipid droplets. Supplementation with methionine, did not alter expression of genes involved in endogenous synthesis of choline. Cholesterol supplementation suppressed the expression of genes involved in sterol uptake and de novo cholesterol synthesis and induced genes involved in sterol efflux from the intestinal mucosa. Taurocholate and taurine induced expression of genes involved in their respective metabolic pathways. These results confirmed the importance of choline observed in the first experiment, which was supplied either as choline chloride, phosphatidylcholine or as a constituent in fishmeal, in lipid turnover in the intestine as well as its ability to prevent LMS.

The last experiment, a dose-response study testing nine levels of choline ranging from 1340 to 4020 mg/kg choline, demonstrated a clear inverse relationship between dietary choline level and the degree of vacuolation in the mucosa of the pyloric intestine of Atlantic salmon. Analyses of the lipid content in the tissue confirmed that this was mainly due to reduction in the level of TAG and diacylglycerol (DAG). Fish growth was not affected by dietary choline in this study. Other biomarkers, such as relative weight of the proximal intestine including the pyloric caeca and mid intestine, macroscopically observed whiteness and histologically observed vacuolation of the pyloric intestine, and expression of the genes *pcyt1a*, *apoAIV, apoAI* and *plin2* (biomarkers with a close functional association to lipid transport) also showed a clear dose-response relationship with dietary choline level. This make them suitable as biomarkers for the estimation of the choline requirement. Based on the results for these biomarkers an average choline requirement, covering 50% of the population, of 2936 mg/kg was estimated. To cover the needs of 95% of the population, a level as high as 3350 mg/kg choline would be required.

The results from all three experiments clearly showed that Atlantic salmon in seawater, fed a low fishmeal diet containing between 944-1340 mg/kg choline and 26-29% lipid, must be supplemented with a choline source in an amount which increases the level to 3350 mg/kg, to prevent developed LMS. Insufficient supply of choline impairs assembly of the intestinal lipoproteins engaged in lipid transport, resulting in impaired lipid transport capacity across the enterocytes and intracellular lipid accumulation. At higher feed intake and/or higher lipid levels in the diet than in the present studies, an even higher level may be required.

The work presented in this thesis provides important knowledge on choline's role in lipid transport in the intestine, and has, for the first time, established a basis for defining choline requirement in salmon kept in seawater. It will further help to improve the gut health of Atlantic salmon in Norway.

SAMMENDRAG (SUMMARY IN NORWEGIAN)

Fiskefôrindustrien jobber kontinuerlig for å redusere innholdet av marine ingredienser i fôrene ved å søke etter alternative ingredienser. Den økte bruken av alternative ingredienser utsetter imidlertid fisken for nye næringsstoffer, og reduserer nivåene av næringsstoffer som er typiske for de marine ingrediensene. I de siste tiårene har fôrene til oppdrettslaks endret seg betydelig, også når det gjelder innhold av lipid. Nivået av lipid har økt, mens andelen av n-3 fettsyrer på samme tid har gått ned. Disse endringene kan ha medført utfordringer for fisken. Oppdrettere har siden 2000 rapportert om symptomer på at opptaket av lipider hos oppdrettslaksen ikke er optimalt, og at det medfører såkalt lipid-malabsorpsjon (LMS). Dette gjelder fisk både i ferskvann og i sjø. Typiske symptomer på LMS er fortykket tarmvev og en hvitfarget og grov overflate i både fremre og midtre deler av tarmen, i motsetning til den normale, lyserosa fargen. Symptomene er et resultat av unormal akkumulering av lipid i vevet, som vises som store, intracellulære vakuoler i histologiske snitt. Tidligere studier indikerer at nedsatt dannelse av lipoproteiner på grunn av mangel på fosfatidylkolin, kan være årsaken til økningen av lipidvakuoler. Fosfatidylkolin er ikke regnet som et essensielt næringsstoff, ettersom fisken kan syntetiserer denne viktige komponenten når kolin er til stede. Kolin derimot, er definert som essensielt næringsstoff for mange dyr, oftest i tidlige livsstadier. For fisk er behovet for kolin definert for tidlige stadier for noen fiskearter, men ikke for noen livsstadier hos Atlantisk laks.

Denne avhandlingen omfatter resultater fra tre forsøk, alle med mål om å øke kunnskapen angående effekter av kolin og andre nøkkelkomponenter involvert i lipid og sterol metabolismen på utviklingen av LMS hos atlantisk laks i sjøvann. I det første forsøket ble det undersøkt om kolinklorid kunne forhindre utvikling av LMS hos atlantisk laks i sjøvann. Det påfølgende forsøket fulgte opp resultatene fra første forsøk og undersøkte om andre nøkkelkomponenter i lipid og sterol metabolismen kan påvirker utviklingen av LMS-symptomer. Målet med det siste forsøket var å definere kolinbehovet for laksens i sjø.

Resultater fra det første forsøket viste at fisk som fikk et fôr som inneholdt 944 mg/kg kolin, utviklet tydelige tegn på LMS mens et fôr med et kolininnhold på 4250 mg/kg, ikke utviklet slike tegn. Den relative vekten av fremre del av tarm og innholdet av triglyserider (TAG) i blindsekkene var redusert med henholdsvis 40% og 65%, hos fisk som fikk fôret med høyt kolininnhold. Høy kolintilførsel endret uttrykket av gener som er involvert i syntesen av fosfatidylkolin (*chk* and *pcyt1a*), transport av kolesterol (*abcg5* and *npc1l1*), metabolisme og transport av lipid (*mgat2a* and *fabp2*), dannelse av lipoproteiner (*apoA1* and *apoAIV*) og dannelse av lipidvakuoler i tarmen (*plin2*). Tilsetning av kolin ga dessuten en økning i tilvekst på 18%. Tilsammen viser disse resultatene kolins viktige rolle i lipidomsetningen i tarmen og dermed for å forhindre LMS.

Det andre forsøket bekreftet funnene fra det første forsøket og viste at fisk gitt et fôr med lavt fiskemel (LF) og med kolininnhold på 1190 mg/kg ga klare LMS-symptomer, mens fisk som ble gitt fôr med høyt fiskemelinnhold, med et kolininnhold på 1860 mg/kg, ikke hadde vesentlige tegn på LMS. Tilskudd av fosfatidylkolin eller kolinklorid til LF, som ga kolinnivåer i fôrene på hhv 2870 og 2980 mg/kg, eliminerte symptomene på LMS. I dette forsøket var det ingen signifikante effekter av kolinnivå i fôret på veksthastighet hos fisken. Tilskudd av andre komponenter som er viktige i omsetningen av lipider, dvs. to nivåer av taurokolat (3.5 and 6.9 g/kg), kolesterol (2.0 g/kg), taurin (0.8 g/kg), cystein (0.8 g/kg) eller metionin (1.0 g/kg) til LF, ga ingen reduksjon i LMS-symptomene. Evalueringen av effekter av tilskuddene på genuttrykk viste at fosfatidylkolin påvirket uttrykket av *pcyt1a*, som er involvert i syntesen av fosfatidylkolin, og kolesterol transportøren, *abcg5.* Et økt uttrykk av *apoAIV*, indikator for lipoproteindannelse, og en markert nedregulering av *plin2*, indikator for dannelse av lipidvakuoler, ble observert i både kolin- og fosfatidylkolin fôret fisk.

Tilsetning av metionin, en viktig metyldonor som er nødvendig for kolinsyntese, ga ikke økninger i uttrykk av gener i synteseveiene for kolin. Økt innhold av kolesterol i fôret medførte i en reduksjon i uttrykket for gener involvert i opptak av steroler og *de novo* syntese av kolesterol, mens uttrykket av gener involvert i transporten av steroler gjennom slimhinnene i tarmen økte. Tilsetning av taurokolat og taurin induserte uttrykk av gener i

deres respektive metabolske reaksjonsveier. Resultatene fra dette andre forsøket bekreftet den sentrale rollen kolin spiller i lipid omsetningen i tarmen og for å forhindre LMS.

Det siste forsøket, som hadde som mål å estimere behovet for kolin hos post-smolt laks, var et dose-respons forsøk med ni nivåer av kolin fra 1340 til 4020 mg/kg. Resultatene viste et klart, omvendt forhold mellom kolinnivået i fôret og graden av lipidvakuolisering i tarmslimhinnene. Analyser av lipid innholdet i vevet fra den proksimale delen av tarmen viste at årsaken til reduksjonen i vakuolisering hovedsakelig var en reduksjon i innholdet av TAG og diacylglycerol (DAG). Veksten hos fisken var i dette forsøket ikke påvirket av kolininnholdet i fôret. Andre biomarkører, dvs relativ vekt av fremre del av tarm inkludert blindsekker og midttarm, makroskopisk synlig hvitfarget overflaten av fremre del av tarm og midttarm, vakuolisering av enterocyttene i blindsekkene, og ekspresjon av genene *pcyt1a*, *apoAIV, apoAI* and *plin2* (biomarkører med en funksjonell tilknytning til lipidtransport) viste også et klart dose-responsforhold med kolinnivå i fôret. Dette gjør dem egnet som biomarkører for å estimere behovet for kolin. Basert på resultatene for disse biomarkørene ble det gjennomsnittlige behovet for kolin, det vil si nivået som dekker 50% av populasjonens behov, estimert til 2936 mg/kg. For å dekke behovet til 95 % av fiskene trengs 3350 mg/kg.

Resultatene fra de tre forsøkene viser klart at fôr med lavt innhold av fiskemel, med kolininnhold mellom 944-1340 mg/kg, og som inneholder 26-29% lipid, må tilsettes en kolinkilde, i en mengde som gir et nivå på 3350 mg/kg fôr, for å forhindre utviklingen av LMS. Utilstrekkelig tilgang på kolin forhindrer dannelsen av lipoproteiner i enterocyttene, og derved transporten av lipid gjennom cellene til blodet, med intracellulær opphoping av lipid i store vakuoler som resultat. Ved høyere fôrinntak og/eller høyere lipid nivå i fôrene enn det som var tilfelle i disse forsøkene, er behovet kanskje enda høyere, noe som bør studeres i framtidige forsøk.

Dette doktorgradsarbeidet har frembragt viktig informasjon om kolins rolle i lipidtransport i tarmen og har, for første gang, gitt grunnlag for å definere behov for kolin hos atlantisk laks i sjøvann. Resultatene vil bidra til forbedring av tarmhelsen hos norsk oppdrettslaks.

LIST OF ABBREVIATIONS

AD: apparent digestibility
CDP-choline: cytidine-diphosphocholine
CHT1: choline high-affinity transporter
CTL family: choline intermediate-affinity transporters
DAG: diacylglycerol
DEG: differentially expressed genes
DI: distal intestine
DI1: proximal half of distal intestine
DI2: distal half of distal intestine
FFA: free fatty acids
HDL: high-density lipoproteins
HF: 30% high fishmeal diet
IBW: initial body weight
LAP: leucine aminopeptidase activity
LDL: low-density lipoproteins
LF: 10% low fishmeal diet
LFC: choline supplemented low fishmeal diet
LI: liver
LMS: lipid malabsorption syndrome
MAG: monoacylglycerol
MI: mid intestine
OCT family: choline low-affinity organic cation transporters
OSI: organosomatic indices
PCR: polymerase chain reaction
PI: pyloric intestine
PI1: proximal half of pyloric intestine
PI2: distal half of pyloric intestine
PL: phospholipid
qPCR: quantitative real-time polymerase chain reaction
RNA: ribonucleic acid
SEM: standard error of the mean
SGR: specific growth rate
TAG: triacylglycerol

Notification: Responses referred to as significant different means a $p < 0.05$.

LIST OF ARTICLES

Paper I

Choline supplementation prevents diet induced gut mucosa lipid accumulation in post-smolt Atlantic salmon (*Salmo salar* L.)

Anne Kristine G. Hansen, Trond M. Kortner, Aleksei Krasnov, Ingemar Björkhem, Michael Penn, Åshild Krogdahl

Paper II

Choline and phosphatidylcholine, but not methionine, cysteine, taurine and taurocholate, eliminate excessive gut mucosal lipid accumulation in Atlantic salmon (*Salmo salar* L)

Åshild Krogdahl, Anne Kristine Grostøl Hansen, Trond M. Kortner, Ingemar Björkhem, Aleksei Krasnov, Gerd M. Berge and Vegard Denstadli

Paper III

Dose-response relationship between dietary choline and lipid accumulation in pyloric enterocytes of Atlantic salmon (*Salmo salar* L.) in seawater

Anne K. G. Hansen, Trond M. Kortner, Vegard Denstadli, Kjell Måsøval, Ingemar Björkhem, Hans J. Grav, Åshild Krogdahl

INTRODUCTION

The foreseen increase in world aquaculture production has put further pressure on marine ingredients and has stimulated the need for alternative ingredients. A blue-green shift has also been seen in recent years in the selection of raw materials used for commercial Atlantic salmon feed production. In salmon feed, the level of marine raw materials decreased from 90% in 1990 to around 30% in 2013 (Ytrestøyl et al., 2015) with reported levels as low as 25% in 2016 (Aas et al., 2019). The feed industry works continuously to decrease the amount of marine raw materials used by searching for alternative feed ingredients. Moreover, the feeds have become increasingly energy dense. A typical diet for salmon, from 500 g and above, contains roughly 10% of both fishmeal and marine oil with a total lipid content up to 32%. Two main concerns when replacing fishmeal with alternative plant ingredients are decreased growth performance and weakened fish health. Several studies have shown that refined plant ingredients are good alternatives to fishmeal when used within their recommended range in nutritionally balanced diets (Collins et al., 2013, 2012; Hartviksen et al., 2014). However, since the requirement for several nutrients have yet to be well defined, formulation of balanced diets can be challenging (NRC, 2011). Prevalence of various intestinal disturbances has increased with the decrease of fishmeal and the concomitant increase of plant meals in fish feed, indicating that diets with high levels of plant ingredients may be deficient or imbalanced in some essential nutrients. One of the intestinal disorders seen, is the lipid malabsorption syndrome (LMS). The first reports of LMS came in the summer 2002. The symptoms seemed to disappear the following winter but reoccurred in the spring 2003. Thereafter, no observations were reported until summer 2010 after which the symptoms have regularly been observed in cultivated Atlantic salmon. The first signs were observed by the farmers as large accumulations of white or yellow floating material accumulating on the water surface around the farm (Figure 1).

Figure 1: Floating faeces on the water surface at an aquaculture farm in Norway. Picture: Ragnhild Hanche-Olsen

This floating material turned out to be faeces from the fish, which attracted birds and predators to the sea cages causing stress for the fish and polluted the nearby seashores. This phenomenon was occurring in all regions in Norway. Among neighbouring farms, some suffered and others not. Even within a farm some sea cages were affected, while others were not. No clear patterns were observed which could explain the cause for the floating faeces. The only clear observed trend was that LMS peaked in periods with high water temperatures. The fish had been reported to have high feed intake prior to signs of LMS and fish suffering from LMS differed in behaviour, some exhibited reduced feed intake while others continued to eat well (Penn, 2011).

Figure 2 shows a typical picture of the macroscopic appearance of LMS in the pyloric intestine in Atlantic salmon. The typically characteristics of LMS is a swollen and coarse structure and pale appearance of the pyloric caeca and intestine as a result of lipid accumulation. The picture further illustrates that the pyloric caeca closer to the stomach are pale in colour, while those closer to the mid intestine have a “normal” darker appearance. In the most severe cases, a white “mayonnaise” like liquid could be seen “leaking” from the pyloric caeca (Figure 3). The pale, swollen and coarse appearance may, in the most severe cases, cover all the pyloric region as well as the mid intestine (Figure 4).

Figure 2: Macroscopic appearance of LMS of the pyloric caeca. Note both the swollen and pale caeca, a result of excessive lipid accumulation and the darker appearance indicating normal caeca.
Photo: Vegard Denstadli.

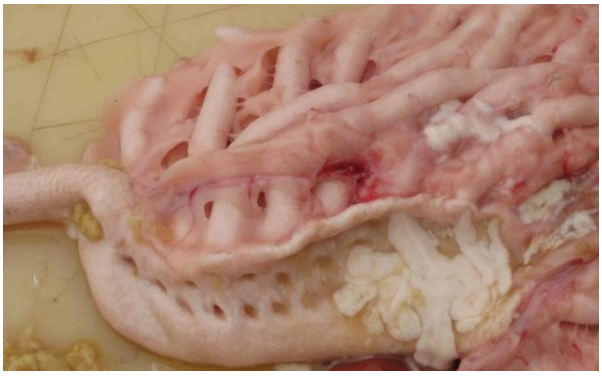

Figure 3: Example of white "mayonnaise" like liquid "leaking" from the pyloric caeca in a fish with LMS.
Photo: Arne Guttvik.

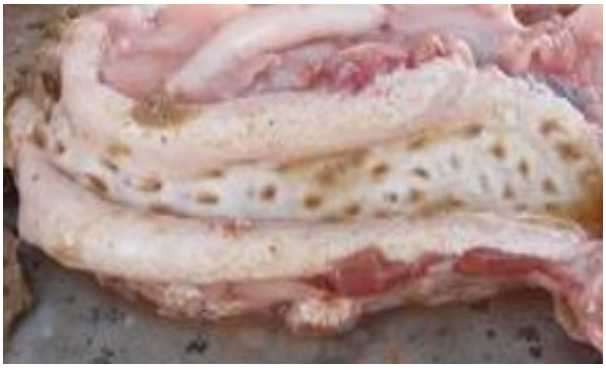

Figure 4: Pale, swollen and coarse appearance of the proximal intestine.
Photo: Arne Guttvik.

The present work addresses questions whether LMS is a result of a deficiency of one or more essential nutrients, present at high levels in fishmeal, which are insufficiently supplied by plant-based raw materials. The first on the list of possible nutrients was choline. Therefore, the ability for choline to prevent LMS was the focus of this PhD work. As present knowledge on intestinal lipid transport is limited, this work also strengthens the knowledge of intestinal lipid metabolism.

The following chapters present background information and knowledge of relevance for the present thesis.

The alimentary tract of Atlantic salmon

The main function of the alimentary tract is to digest and absorb nutrients from the ingested feed and to excrete undigested feed components. It is also the excretion route of several waste products processed by the liver. The alimentary tract in salmon is often divided into three main sections: the pre-gastric, gastric and post-gastric compartments. The pre-gastric segment comprises the mouth, pharynx and the esophagus (E), the latter forming a tube connecting the pre-gastric section to the gastric section, encompassing the stomach (S; Figure 5). The post-gastric compartment covers the intestine, which can be further divided into three regions: the pyloric intestine (PI), including the pyloric caeca (PC) and the mid (MI) and distal intestine (DI). The PI covers the section from the pyloric sphincter to the distal-most pyloric caecum; MI: from the distal end of PI to the increase in intestinal diameter defining the entrance to the DI, which stretches from the distal end of MI to the anus.

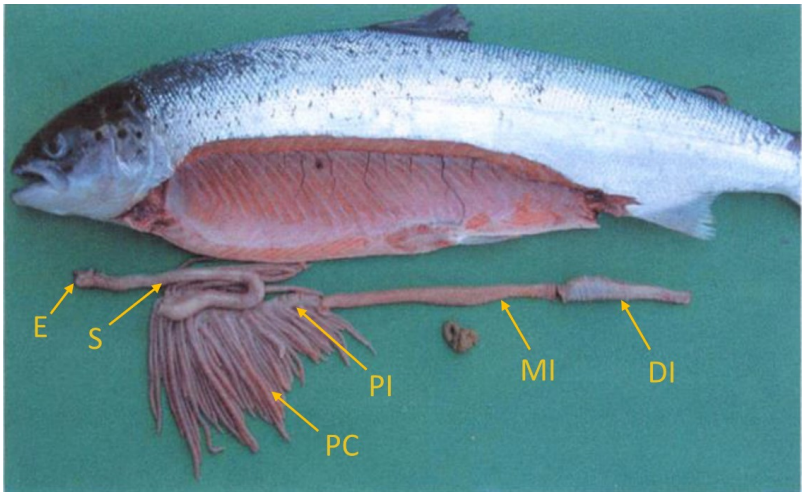

Figure 5: The alimentary tract of Atlantic salmon. Abbreviations: E, esophagus; S, stomach; PC, pyloric caeca; PI, pyloric intestine; MI, mid intestine; DI, distal intestine.
Photo: Åshild Krogdahl.

Intestinal lipid absorption and transport

Lipids are one of the major nutrients in feeds for salmon and the richest source of energy on a weight basis (Gunstone, 1999). They also play an essential role in cell structure and metabolic regulation (Sargent et al., 2003). Dietary lipids can be divided into TAG, wax esters, phosphoglycerides also referred to as phospholipids, sphingolipids and sterols. Triacylglycerols (TAG) provide fatty acids for energy production (Sargent et al., 2003). Wax esters also provide fatty acids for energy production, but they have lower digestibility than TAG. Phospholipids play a crucial role in intestinal lipid emulsification, digestion and absorption, and as a component in lipoproteins for lipid transport. Furthermore, they act as a substrate for the production of the neurotransmitter acetylcholine, as a methyl donor in a wide range of methylation processes and for cell structure and function (Li and Vance, 2008; Tocher et al., 2008). Sphingolipids have a structural function in cell membranes and an important role in signal transmission. The major sterol in animals tissues is cholesterol, which serves as an integral component of cell membranes and as a precursor for important

metabolites such as steroid hormones and bile acids (Kortner et al., 2014). Dietary lipids enter the intestinal lumen from the stomach as emulsified lipid droplets (Figure 6). The PI and the PC are the predominant segments for lipid absorption of the gastrointestinal tract of Atlantic salmon. However, a study with Atlantic salmon have shown that lipid absorption occurs along the whole pyloric segment and also in MI if absorbable lipid is still present (Denstadli et al., 2004).

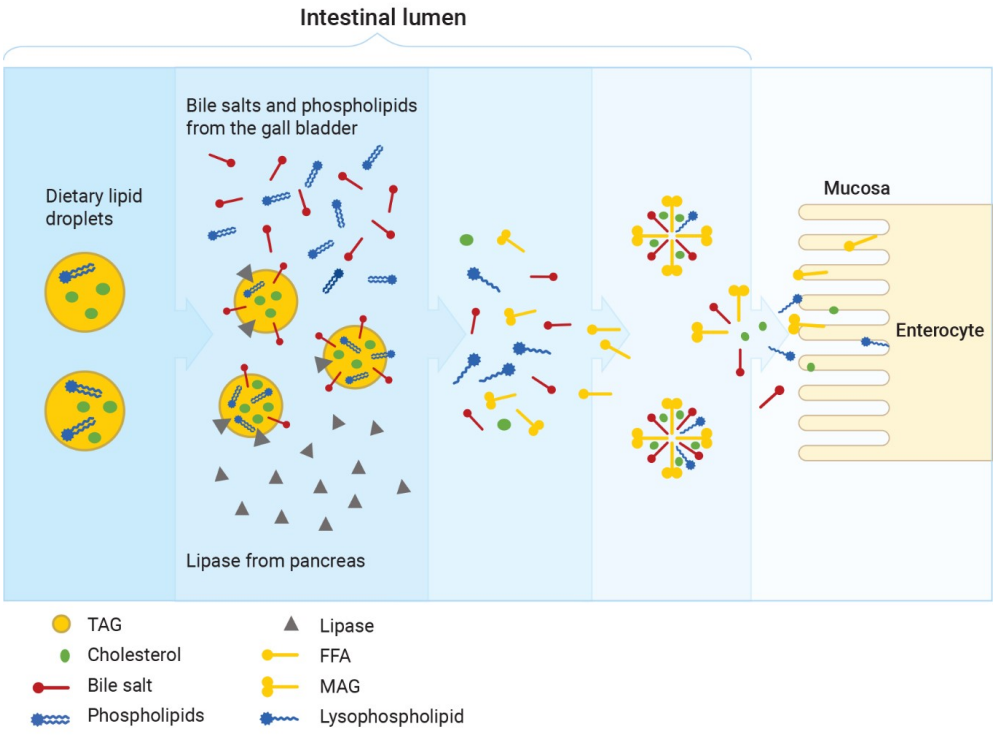

Figure 6: Lipid digestion and absorption. Dietary lipids (TAG, phospholipids and cholesterol esters) enter the intestinal lumen from the stomach as emulsified lipid droplets. These components are hydrolysed to 2-monoacylglycerols, lysophospholipids, cholesterol and free fatty acids (Gunstone, 1999) by various lipases before incorporation into micelles. The micelles dissolve when they come in contact with the mucosa and the lipid components are subsequently taken across the membrane and into the enterocyte by diffusion or bound to transporters.

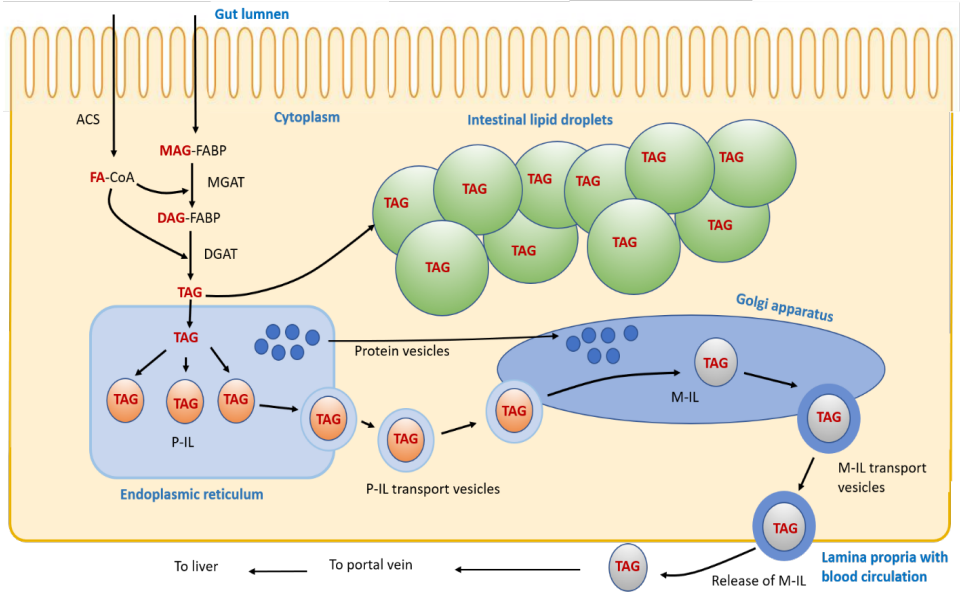

Figure 7: Lipid transport within the enterocyte. FFA are primarily activated by ASC to form FA-CoA, utilized for the two-step re-synthesis of TAG through the MGAT pathway. First step involves the formation of DAG from MAG and a FA-CoA by MGAT activity. The second step involves the synthesis of TAG from DAG and a FA-CoA by DGAT activity. The synthesized TAG could either be transported to the intestinal lipid droplets synthesis or to the endoplasmic reticulum for intestinal lipoprotein synthesis. Abbreviations: FFA, free fatty acids; ACS, acyl-CoA synthetase; FA, fatty acids; FA-CoA, fatty acyl-CoA; FABP, fatty acid binding protein; MGAT, monoacylglycerol acyltransferase; DGAT, diacylglycerol acyltransferase; P-IL, pre-intestinal lipoproteins; M-IL, mature intestinal lipoprotein. Figure modified from Åshild Krogdahl.

The mechanisms of lipid absorption and transport in fish are presumed to be quite similar to those in mammals (Sargent et al., 2003) and is suggested to involve two routes. The first is a fast route for short and medium chain fatty acids. These components are transferred across the mucosa of the intestinal epithelium by diffusion and carried into the cytoplasm by specific protein transporters which can then enter into the portal blood and further to the liver (Denstadli et al., 2004; Lie et al., 1993). The second, assumed to be slower, is

handling long chain free fatty acids (FFA) which are re-esterified and incorporated into TAG rich lipoproteins before being excreted into the lamina propria (Figure 7).

Lipoproteins are often divided into three classes: chylomicrons, very low-density lipoprotein (VLDL) and high-density lipoprotein (HDL) (Babin, 1987; Field and Mathur, 1995; Gunstone, 1999). Chylomicrons are the predominant lipoprotein released from the enterocytes of the pyloric intestine and in particular responsible for the intracellular transport of TAG (Field and Mathur, 1995). Teleost species appear to differ from mammal by lacking a lymphatic system for conveying lipid to the peripheral circulation and tissues (Sire et al., 1981). The intestinal lipoproteins are presumably drained into the portal vein system from the lamina propria (Thorarensen et al., 1991) and passed on to the liver (Lie et al., 1993). A secondary, intestinal circulatory system has been described in fish (Olson, 1996), but whether it plays any role in lipid transport is not known.

Accordingly, it may be discussed if the term chylomicrons are the correct term for this lipoprotein. In the following, the term intestinal lipoproteins, will be used. The core of the intestinal lipoproteins contains mainly triglycerides, esterified cholesterol as well as lipid soluble vitamins and other highly lipophilic compounds. The surface consisting of apolipoproteins (1-2%), phosphatidylcholine (6-12%) and free cholesterol (1-3%) (Field and Mathur, 1995) (Figure 8).

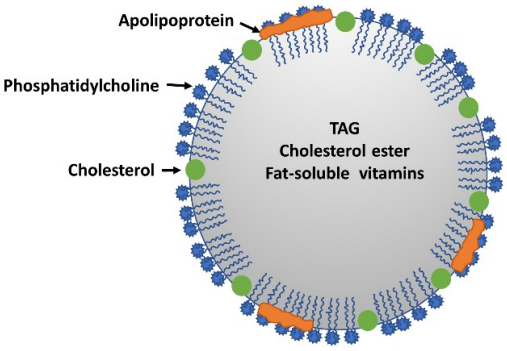

Figure 8: Mature intestinal lipoprotein, modified from Åshild Krogdahl.

The intestinal lipoproteins are taken up by the liver where they are dismantled and re-assembled into VLDL (van der Veen et al., 2017) for further transportation to peripheral tissues. After delivering TAG and cholesterol to peripheral tissues, VLDL is transformed into the VLDL remnant, low-density lipoprotein (LDL), which again can be absorbed by the liver and peripheral tissues. The surplus of cholesterol in LDL and peripheral tissues are absorbed by HDL and transported back to the liver for reuse (Lie et al., 1993).

Intestinal lipid droplets have been observed in the enterocytes of many fish species (Arai, S., Nose, T., 1972; Fontagne et al., 1998; Gu et al., 2014; Olsen et al., 2003, 2000; Oxley et al., 2005; Sire et al., 1981) (Figure 7). These lipid droplets are considered to be a temporary storage site for lipid when the rate of lipid absorption (lipid load) exceeds the rate of lipoprotein synthesis (Gunstone, 1999), with TAG being the primary lipid for storage in almost all plants and animals, including Atlantic salmon (Tocher et al., 2008).

Choline

Choline, $C_5H_{14}NO^+$, is defined as a vitamin-like compound. The main function of choline is as a substrate for the synthesis of phosphatidylcholine (Gibellini and Smith, 2010). Around 95% of dietary choline is rapidly incorporated into phosphatidylcholine upon entering the enterocyte (Gibellini and Smith, 2010; Li and Vance, 2008). Phosphatidylcholine functions as a methyl donor for the production of the neurotransmitter acetylcholine and in a wide range of methylation processes, is also essential for cell structure and functions in all biological membranes (da Silva et al., 2015; Li and Vance, 2008; Tocher et al., 2008; Yeh et al., 2015). Phosphatidylcholine is also an essential component for the assembly of intestinal lipoproteins and for the formation and secretion of VLDL from the hepatocytes, both playing important roles in lipid transport and absorption (Li and Vance, 2008). Phosphatidylcholine constitutes for over 80% of the total phospholipid in lipoproteins (Daum and Vance, 2002; Peter Wood and Kinsell, 1964).

Choline is found in a variety of foods and feeds mostly as phosphatidylcholine, but also as free choline. The levels vary between raw materials, however higher levels are often found

in marine versus plant ingredients. Atlantic and South American fishmeal contain roughly 3000 mg/kg of choline, whereas krill meal can have levels of about 15000 mg/kg. Soya protein concentrate (SPC), which is widely used as a plant alternative for fishmeal, contains roughly 600 mg/kg. Sunflower expeller and pea protein concentrate (50%) have, on the other hand, a choline content in the range 2200 to 2400 mg/kg. Choline may also be supplied as choline chloride. There are several suppliers marketing a broad range of products with varying choline concentrations. Choline chloride is regarded as an effective source of choline and is used in feed for animals both in agriculture and aquaculture. The usage is considered as safe for consumers and not harmful to the environment (EFSA, 2016). The European Food Safety Authority (EFSA) states that it is safe to use between 400-1500 mg/kg choline chloride in feed for fish (McDowell, 2000).

Choline absorption, transport and endogenous synthesis

Phosphatidylcholine supplied by the diet is hydrolysed to lysophosphatidylcholine in the intestine, absorbed and re-esterified to phosphatidylcholine in the enterocyte, whereas dietary free choline is transported as such through the brush border into the enterocyte. In mammals, the uptake of choline is mediated by three classes of choline transporters: the high-affinity transporter (CHT1), the intermediate-affinity transporters (CTL family) and the low-affinity organic cation transporters (OCT family) (van der Veen et al., 2017). When entering the enterocyte, choline is incorporated into phosphatidylcholine by the CDP-choline pathway, also called the Kennedy pathway (Carmona-Antoñanzas et al., 2015; Li and Vance, 2008) (Figure 9). In several animals, the liver has the capacity for de novo synthesis of phosphatidylcholine through the CDP-ethanolamine pathway, which is similar to the CDP-choline pathway except it starts from ethanolamine (Li and Vance, 2008). Plants and algae can synthesize ethanolamine from serine, whereas animals cannot, meaning that they must obtain ethanolamine from their diet. Phosphatidylcholine can also be produced from phosphatidylserine. Phosphatidylserine is decarboxylated into phosphatidylethanolamine and further by three methylation steps into phosphatidylcholine (Gibellini and Smith, 2010).

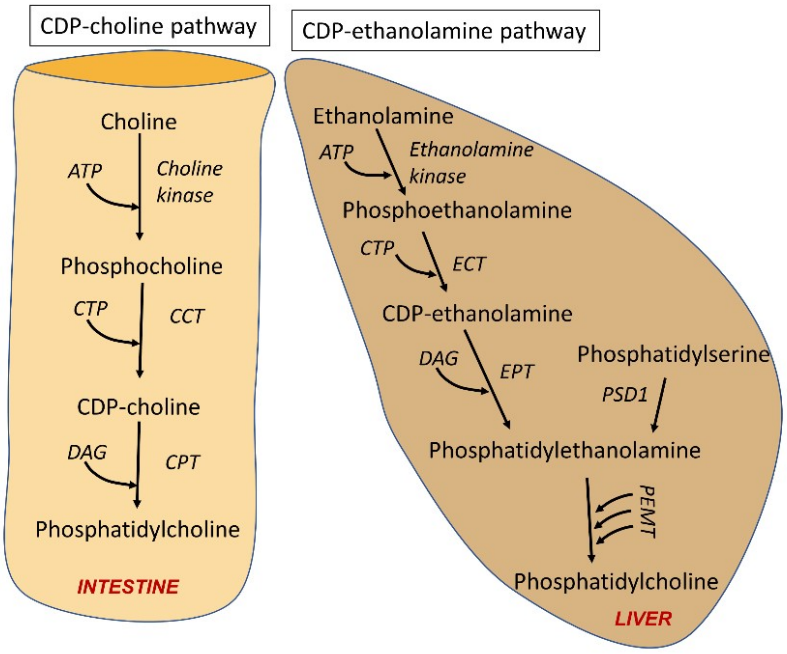

Figure 9: Pathways of phosphatidylcholine synthesis. **CDP-choline pathway**. In the first step of the pathway, choline is rapidly phosphorylated by ATP to phosphocholine mediated by the enzyme choline kinase encoded by two chk genes. In the second step, considered to be the rate-limiting step, phosphocholine is converted to cytidine-diphosphocholine (CDP-choline) by phosphocholine and cytidine-5'-triphosphate (CTP) mediated by the enzyme CTP:phosphocholine cytidylyltransferase (CCT), encoded by the *pcyt1a* gene. In the final step CDP-choline: sn-1,2-diacylglycerol cholinephosphotransferase (CPT), encoded by chpt1, produces phosphatidylcholine (PC). **CDP-ethanolamine pathway**. In the first step of the pathway, ethanolamine is rapidly phosphorylated by ATP to phosphoethanolamine mediated by the enzyme ethanolamine. In the second step, considered to be the rate-limiting step, phosphoethanolamine is converted to cytidine-diphosphoethanolamine (CDP-ethanolamine) by phosphoethanolamine and cytidine-5'-triphosphate (CTP) mediated by the enzyme CTP:phosphoethanolamine cytidylyltransferase (ECT). In the final step CDP-ethanolamine: sn-1,2-diacylglycerol ethanolaminephosphotransferase (EPT) produce phosphatidylethanolamine (PE). PC could also be produced from phosphatidylserine (PS) which a decarboxylation turns into phosphatidylethanolamine (PE), catalysed by phosphatidylserine decarboxylase (PSD1). PE is further transformed to PC by three methylation steps, catalysed by phosphatidylethanolamine N-methyltransferase (PEMT).

One of the goals in the present work was to gain more information of the effects on lipid transport of other compounds related to the supply of phosphatidylcholine. Phosphatidylcholine circulating in the bile is part of the phosphatidylcholine pool (Figure 10).

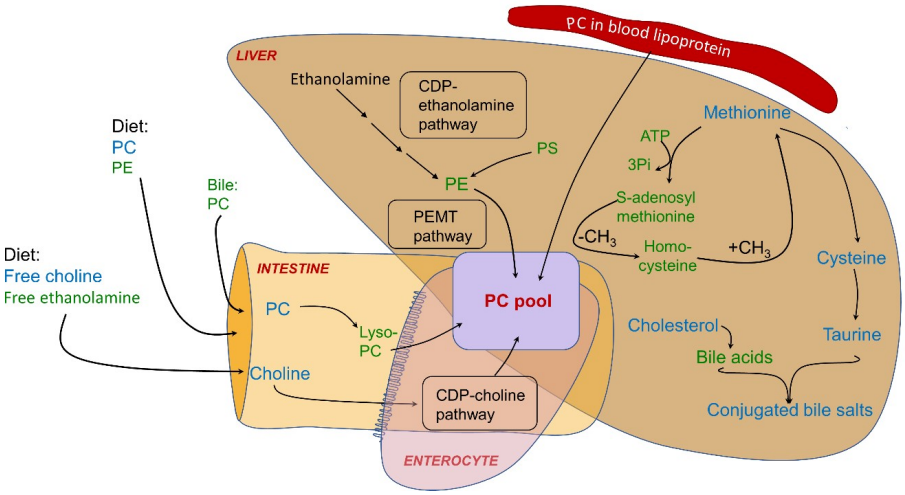

Figure 10: Main pathways of compounds important for the supply of phosphatidylcholine (PC). Blue coloured compounds indicate those investigated in the present study. Dietary free choline and phosphatidylcholine are contributing to the production of phosphatidylcholine. Phosphatidylethanolamine (PE) can also be supplied from the diet either as such or as free ethanolamine and follows the same pathways of absorption as indicated for dietary phosphatidylcholine and choline. Phosphatidylcholine circulating in the blood and bile is also part of the phosphatidylcholine pool. Endogenous synthesis from PE and phosphatidylserine (PS) contributes to the pool. Dietary supply of taurine may also be of importance for the size of the phosphatidylcholine pool, as taurine is needed for the conjugation of bile acids and is produced from methionine, via cysteine.

Another supply pf phosphatidylcholine is from the endogenous synthesis in the liver from phosphatidylethanolamine, involving methyl groups from methionine. Supplementing with

methionine could then potentially promote lipid transport in the gut mucosa. If so, would also the level of cysteine, produced from methionine, may play a role for production of choline and have an impact on LMS. Cysteine is also a key substrate/metabolite in the production of taurocholate (Schubert et al., 2003). Low supply of these components may reduce availability of methyl groups for the formation of choline.

Choline requirement and deficiency

Choline is defined as an essential nutrient for mammals (Gibellini and Smith, 2010). However in fish, choline has only been established as essential in early life stages for some species; with the estimated requirement differing between those species (Duan et al., 2012; Halver and Hardy, 2002a; Kennedy et al., 2007; NRC, 2011; Ogino et al., 1970; Qin et al., 2016; Shiau and Lo, 2000; Wilson and Poe, 1988). The US National Research Council (NRC), reports a choline requirement for Pacific salmon larvae, including rainbow trout, of 800 mg/kg (NRC, 2011). However, Halver and Hardy (Halver and Hardy, 2002a) reported requirements as high as 3000 mg/kg. Several studies on juvenile rainbow trout suggest an inverse relationship between body weight and choline requirement, which estimated dietary choline requirements for 0.12 g, 1.4 g, 3.2 g and 3.5 g fish to be 3000, 813, 714 and between 50-100 mg/kg, respectively (NRC, 2011; Poston, 1990; Rumsey, 1991). Currently, there are no estimates of choline requirements published in the scientific literature for any life stages of Atlantic salmon (NRC, 2011). The available studies, addressing the requirement for early life-stages of fish, show that a deficient supply of choline can cause poor growth, low feed efficiency, fatty liver, haemorrhagic kidney and intestine, high mortality and anorexia (Duan et al., 2012; Halver and Hardy, 2002a; Ketola, 1976; NRC, 2011; Poston, 1990; Rumsey, 1991). Lipid accumulation in the intestinal mucosa is seldom an observed endpoint in studies of choline deficiency and requirement but was observed in an early study of Japanese eel (Anguilla japonica) as “white-grey coloured intestines” (Arai, S., Nose, T., 1972). In studies with many species of fish larvae (Daprà et al., 2011; De Santis et al., 2015; Fontagne et al., 1998; Olsen et al., 2003) deficiency of phosphatidylcholine, and likely also choline, seemed to lead to an insufficient assembly of lipoproteins and transport

of lipids out of the intestines. The same has been seen in a previous study involving Atlantic salmon (initial weight of 442 g) in seawater (Gu et al., 2014).

Estimating the requirement of a specific nutrient

Detailed knowledge on a species' requirement for all essential nutrients is the foundation for animal health and the development of high performance and cost-effective feeds. The book, Nutrient requirement of fish and shrimp (NRC, 2011), provides guidance for the aquaculture feed industry on the methodology and data analysis for nutrient requirements studies, in addition to provide nutrient requirements for many farmed species. There are, however, no general agreements on how to choose biomarkers to get the most relevant requirement estimates. It is therefore up to the researcher to choose and design the experiment with the understanding of which physiological responses that will confidently and accurately provide an appropriate measure of the animal's response to the nutrient in focus (NRC, 2011). A condition, which is generally agreed upon, is that the biomarker should have a close functional relationship with the nutrient in question and show a clear dose-response relationship with an intake at levels below requirement. However, information on the estimated requirements is limited or even absent for many fish species, therefore caution should be taken when using available requirement estimates. Furthermore, requirements may also differ depending on fish culture system used, life-stage development, the response variables that are measured, environmental conditions, experimental design and the statistical methods used to estimate the requirement. Dietary nutrient requirements in fish has often been estimated using a basal diet deficient in the nutrient in question, with the addition of graded levels of the specific nutrient. The dose-response relationship is then examined, and the nutrient requirement are estimated from the level producing the maximum/minimum response for the chosen end sampling criteria. Experimental design and statistical methods used to define nutrient requirements in fish and other animals have been addressed in several studies (Baker, 1986; Cowey, 1992; Mercer, 1992, 1989, 1982; Mercer et al., 1978; Robbins et al., 1979; Shearer, 2000). These papers agree that a dose-response design should be used in studies addressing a nutrient

requirement level. According to the review of Shearer (2000), the broken-line method has been the most common statistical method used to estimate a nutrients requirement followed by the ANOVA and then the quadratic model (a second-order polynomial). The review further concluded that the quadratic model produced the most accurate estimate of a nutrient requirement level and that both the broken-line method and ANOVA have shown a tendency to underestimate the requirement (Pesti et al., 2009; Shearer, 2000). The broken-line method uses two straight lines to model the dose-response relationship. The ascending or descending line represents increases in response with increasing nutrient intake, while the horizontal line represents the nutrient abundance. The break point would then indicate the estimated nutrient requirement. ANOVA would treat the nutrient levels in a dose-response study as discrete rather than continuous and the requirement level will be estimated as the range between two input levels. Often, both the broken-line and the ANOVA methods are used in combination. For example, the data would first be analysed with ANOVA to determine the break point at the lowest nutrient level above which no significant difference among the studied levels could be found. The quadratic method uses the least square to estimate the requirement level and the method forms a symmetric parabola. The least square describes the variance in a prediction of the dependent variable (response criterion) as a function of the independent variable (nutrient level) and the deviations from the fitted curve. The statistical analyses will reveal the average requirement for the nutrient studied. An average requirement means that the estimated requirement of the nutrient covers 50% of the populations. To find the level which will cover the needs of 95% of the population, 2 * SEM should be added to the average requirement level.

Hypotheses

The following hypotheses were the basis for the thesis:

- Low fishmeal and high energy feeds to Atlantic salmon in seawater can induce LMS.

- Insufficient dietary levels of key components in lipid metabolism, in particular choline, can result in inefficient intestinal lipid uptake and transport.

- Atlantic salmon in seawater fed low fishmeal and high energy feeds requires dietary supply of choline.

AIMS OF THE STUDY

The overall goal of the present study was to elucidate the role and the requirement of choline in lipid transport across the intestinal mucosa and to be able to formulate diets securing optimal lipid absorption and healthy mucosa in post-smolt Atlantic salmon.

The strategies chosen for achieving the overall goal had the following aims:

- To investigate the development of LMS in post-smolt Atlantic salmon given a low fishmeal and high lipid diet (**Papers I, II and III**).

- To investigate the effect of choline on lipid accumulation in the intestine of Atlantic salmon in seawater (**Papers I, II and III**).

- To study the effects of choline on expression of genes involved in phospholipid and lipid synthesis and transport pathways in the pyloric intestine and liver of Atlantic salmon in seawater (**Paper I**)

- To study the effect of supplementation with other key components in lipid and sterol metabolism on lipid accumulation and on expression of genes involved in phospholipid and lipid synthesis and transport pathways in the pyloric intestine of post-smolt Atlantic salmon (**Paper II**).

- To define the dietary required level of choline supplementation in feed to Atlantic salmon in seawater (**Paper III**).

SUMMARY OF RESULTS

Paper I provide an assessment of whether supplementation with choline chloride would prevent LMS, characterized by high OSIPI as well as hypervacuolation of the pyloric enterocytes due to lipid accumulation. Post-smolt Atlantic salmon, kept in flow-through tanks with seawater for 11 weeks, were fed two diets, a low fishmeal (LF; 10%) diet and another where the LF diet was supplemented with 4 g/kg choline chloride, equivalent to 3306 mg/kg of choline (LFC). Analysed total choline was 944 and 4250 mg/kg for the LF and LFC diets, respectively. Dietary lipid level was 29% (Table 1).

Choline supplementation increased fish growth by 18%. Furthermore, showed the choline fed fish a significant reduced level, by 65%, of TAG in the pyloric caeca tissue compared to the LF fed fish. This reduction was reflected in a parallel reduction in OSIPI by 40% and no histological signs of hypervacuolation of the pyloric enterocytes were observed in fish fed the LFC diet, whereas all fish fed the LF diet showed hypervacuolation. On the other hand, there was no significant difference in apparent lipid digestibility between the two experimental diets. Furthermore, the molecular analyses of the pyloric caeca revealed an alteration in the expression of genes related to pathways involved in lipid metabolism in the fish fed the LFC diet. The expression of the *pcyt1a* gene was significantly down-regulated and *chk* showed a similar trend; both involved in the CDP-choline pathway of phosphatidylcholine biosynthesis. No effect of choline supplementation was observed in the expression of *pemt*, coding for the methylation steps transforming phosphatidylethanolamine to phosphatidylcholine, called the PEMT pathway. On the other hand, an up-regulation, as a result of choline supplementation, was observed for genes playing important roles in lipoprotein formation (*apoA1* and *apoAIV*), DAG re-esterification and intracellular lipid transport (*mgat2a* and *fabp2*), cholesterol transport (*abcg5* and *npc1l1*). Another important finding was the observed down-regulation of the expression of *plin2*, the general marker for lipid accumulation in non-adipogenic cells, indicating a reduction of intracellular lipid storage. The alterations of these genes confirmed the importance of choline in lipid turnover in the intestine.

The analyses of lipoproteins in blood plasma revealed a decreased level of TAG, whereas an increased level of cholesterol was found in fish fed the LFC diet.

Choline supplementation did not affect the liver to the same magnitude as in the pyloric caeca. The choline fed fish (LFC) had significantly lower hepatosomatic index than the LF group. Calculation of the absolute amount of liver lipid in grams showed no significant differences between the two experimental diets nor did the histological degree of liver vacuolation. Furthermore, choline supplementation caused only minor effects to the hepatic transcriptome and the expression of one gene related to lipid metabolism, *plin2*, was down-regulated.

Paper II presents work assessing whether key components in lipid and sterol metabolism, other than choline, may modify hypervacuolation of the pyloric enterocytes and development of symptoms of LMS and alter the expression of genes related to pathways involved in lipid metabolism in the pyloric caeca in Atlantic salmon. Post-smolt fish, kept in flow-through tanks with seawater, were fed ten diets for 12 weeks. Two reference diets, one with 30% fishmeal (HF) and one with 10% fishmeal diet (LF) and eight experimental diets were made by supplementing the LF diet with one of the seven selected key components of lipid and sterol metabolism; taurocholate (TC1 and TC2, 6.9 and 3.5 g/kg respectively), cholesterol (CH, 2 g/kg), taurine (TA, 0.8 g/kg), phosphatidylcholine (PC, 15.3 g/kg), choline chloride (CL, 3.7 g/kg), cysteine (CY, 0.8 g/kg) and methionine (ME, 1.0 g/kg) (Table 2 and 3). Analysed choline was 1860 mg/kg in the HF diet, an average of 1200 mg/kg in LF and the diets supplemented with TC, CH, TA, CY and ME, 2870 mg/kg in the PC diet and 2980 mg/kg in the CL diet. Analysed lipid level was 29% in the HF diet and 27%, on average, for the supplemented diets. The feeding trial lasted for 12 weeks.

Supplementation with PC significantly increased lipid and lowered starch digestibility compared to LF. An increased protein digestibility was observed for both TC2 and TA compared to the LF group. No significant effect by any diets were seen on growth rate nor on feed efficiencies. The relative weight of the pyloric intestine (OSIPI) was significantly

reduced only in the fish fed the supplemented diets with PC and CL diets compared to LF fed fish. Fish fed the HF diet showed also a significantly lower OSIPI compared to fish fed the LF diet. Furthermore, showed the fish fed the HF diet a higher OSIPI compared to the PC and CL groups. The relative weight of the mid intestine (OSIMI) were significantly affected by the same diets as OSIPI. The HF fed fish and the LF diet supplemented with PC and CL eliminated the hypervacuolation of pyloric enterocytes and signs of LMS, however none of the other supplements had an effect. No significant differences were observed between the fish fed the PC and CL diets and the HF fed group.

Both microarray and qPCR were used to study the effects of the supplements on gene expression in pyloric caeca. The microarray analyses revealed few changes overall in the expression of genes involved in lipid metabolism except for a clear transcriptional suppression of cholesterol uptake and biosynthesis in the CH fed group compared to the LF fed group. The suppression was also verified by qPCR, showing down-regulation of the expression of the cholesterol influx transporter, *npc1l1*, whereas the apical efflux transporter, *abcg5*, was induced indicative of a reduction of cholesterol uptake from the gut in the CH group. The marked down-regulation of cholesterol biosynthesis was confirmed by reduced expression levels of *idi1* and *cyp51*.

The qPCR analyses revealed alterations in the expression of genes involved in both the phosphatidylcholine and sterol metabolic pathways for the PC and CL groups. Of the genes involved in the CDP-choline pathway, *pcyt1a* was significantly down-regulated only in the PC fed group whereas no effect of supplementations was observed for *chk* for any of the supplementations. Neither was the expression of *pemt*, altered by PC or CL supplementation. On the other hand, an up-regulation in the expression of *apoAIV* was observed in both the PC and CL group, whereas *apoB* was significantly induced only in the PC group. The apolipoproteins are playing important roles in lipoprotein formation and are essential for exporting TAG on the basolateral side of the intestinal mucosa. For genes involved in cholesterol transport, both PC and CL fed fish showed an induced expression of *npc1l1*, whereas an increased expression of *abcg5* was only observed in the PC fed fish. Another important finding was a down-regulation of *plin2*, indicating a reduction of

intracellular lipid storage in both the PC and CL groups. For the other supplemented diets, few significant changes in gene expression were seen.

The qPCR revealed significant differences between the two reference diets, HF and LF, for the expression of *apoAIV*, *plin2* and *abcg5*. The expression of *apoAIV* and *plin2* were significantly reduced in the HF group compared to LF group, whereas *abcg5* was significantly induced.

The analyses of lipoproteins in blood plasma revealed a significantly increased level in fish fed the CH diet.

Paper III presents results which founded the basis for the estimation of choline requirement for post-smolt Atlantic salmon. The post-smolt fish, kept in flow-through tanks with seawater for 13 weeks, were fed nine experimental diets. The first was a reference diet with 10% fishmeal (LF), while the additional eight diets were based on the LF diet but supplemented with either 0.75, 1.50, 2.25, 3.00, 3.75, 4.50, 5.25 or 6.00 g/kg of choline chloride (70%). Analysed choline level in the nine experimental diets was 1340, 1530, 1760, 2310, 2600, 2850, 3330, 3830 and 4020 mg/kg respectively and lipid level was 29% in all diets (Table 4 and 5).

The biomarkers for the estimation of choline requirement in the present study were those showing a significant correlation to dietary choline levels (with a response curve which made them potential indicators for choline requirement). The relative weight of PI and MI decreased significantly with increasing choline levels before levelling off at the higher inclusion levels, making them suitable biomarkers for the estimation of choline requirement. An average required level of 3593 ± 226 mg/kg was revealed for OSIPI and 3031 ± 195 mg/kg for OSIMI. Macroscopically observed whiteness of the pyloric intestine and histologically observed hypervacuolation of enterocytes, both signs of LMS, decreased with increasing choline levels and were also suitable as biomarkers. The choline requirement for the two abovementioned biomarkers were estimated to be at the level

where all signs of LMS were diminished; 2850 and 2310 mg/kg for the macroscopical and histological evaluation, respectively.

The results of the qPCR analyses showed a significantly decreased expression of both *chk* and *pcyt1a* with increasing choline levels. The genes *apoAI* and *apoAIV* showed a significant response with increasing choline level for the highest doses. The expression of both *mtp,* coding for transporters facilitating the transport of TAG by assisting in the assembly of the lipoprotein, and *fatp*, an actor in the transport of fatty acids from the enterocytes to the ER, was altered by increasing choline level. A down-regulation of *hmgcr*, coding for the rate-limiting enzyme in the biosynthesis of cholesterol, was observed with increasing levels of choline. The observed down-regulation of *plin2*, as a result of choline levels, confirms the findings presented in **Papers I** and **II**. Four of the studied genes: *apoAIV*, *apoAI*, *pcyt1a and plin2*, showed a choline dose-response making them suitable as biomarkers for the estimation of choline requirement. The analysed average choline requirement levels were 2593 ± 108 and 2610 ± 35 mg/kg for *apoAIV* and *apoAI* respectively and 3210 ± 404 and 3199 ± 360 mg/kg for *pcyt1a* and *plin2* respectively.

The results of the analysis of lipoproteins in blood plasma revealed a significant positive relationship between choline level and both LDL-CH and lathosterol, indicating increased cholesterol synthesis in the liver and an accelerated transport of cholesterol from the liver to peripheral tissues.

The average choline requirement for post-smolt Atlantic salmon was estimated to be 2936 mg/kg based on the average choline requirement for the selected biomarkers mentioned above. It should be a goal for an optimal diet to cover more than the needs of the average of the population. By adding 2 * SEM, the estimate will cover the requirement of 95% of the study groups. In the present study, the choline requirement was estimated to be 3350 mg/kg or 3.4 g/kg.

DISCUSSION OF ASPECTS REGARDING MATERIALS AND METHODS

Materials

Fish

The experimental trials in the present study were conducted at the Nofima's Research Station in Sunndalsøra, Norway. This research facility is approved by the Norwegian Animal Research Authority (NARA) and operates in accordance with the Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). Post-smolt Atlantic salmon (*Salmo salar*, Sunndalsøra breed) in seawater, were used in all three experiments. The fish were treated in accordance with the Aquaculture Operation Regulations during the trials. Previous nutrient requirement studies have highlighted that the type of culture system used for the experiment, stage of development of the fish, the response variables that are measured, environmental conditions, experimental design and statistical methods, could all have an impact on the estimated nutrient requirement level (Baker, 1986; Cowey, 1992; Mercer, 1992, 1989, 1982; Mercer et al., 1978; NRC, 2011; Robbins et al., 1979; Shearer, 2000), therefore all three experiments in this study were carried out in the same culture system with the same fish strain. The average start weight of the fish differed slightly between trials with fish starting at 362 g, 330 g and 456 g in **Papers I, II** and **III**, respectively. The average specific growth rate of the fish also differed between the experiments with values of 0.92 and 0.94 for the fish in **Papers I** and **III** respectively compared to 1.37 for the fish in **Paper II**. The differences in fish size at the end of the trial in **Papers I** and **III** were 944 g and 1340 g respectively and is a result of two additional weeks for the trial presented in **Paper III**. The differences in growth rate and potential differences in feed intake (and lipid intake) may have had an impact on the degree of the responses indicating lipid accumulation in the pyloric enterocytes and the accompanying molecular results.

Diets

The experimental diets varied due to the inclusion of the various plant raw materials, however the fishmeal level in the low fishmeal basal diets (LF) was 10% for all three experiments, meaning that the contribution of choline as phosphatidylcholine from the raw materials was quite similar. The analysed choline level for the LF diets were 944, 1190 and 1340 mg/kg for the work presented in **Papers I, II** and **III**, respectively.

Tables 1-5 show the diet formulations used in **Papers I, II** and **III**.

Table 1. Diet formulation and chemical composition for the 11-week trial presented in **Paper I**.

Diets	**LF***	**LFC****
Ingredients (g/kg)		
FM Super Prime	50	50
FM Nordic	50	50
Soy protein concentrate	190	194
Maize Gluten	150	150
Pea Protein 50	130	130
Dehulled Beans	140	130
Wheat Gluten	19.7	19.7
Fish oil	76.7	77.1
Rapeseed oil	176	177
Amino Acid mix	12.4	12.4
Mineral mix	3.0	3.0
Monocalcium phosphate	18.2	18.2
Lucantin Pink 10%	0.4	0.4
Yttrium	0.5	0.5
Choline chloride 70%	0	4.0
Analysed chemical composition (g/kg)		
Dry matter	975	972
Protein	417	418
Fat	286	297
Starch	107	102
Total choline (mg/kg)	944	4250
Total methionine	9.1	9.4
Total cysteine	5.1	5.8

*LF, low fishmeal basal diet (10%); **LFC, LF supplemented with choline chloride

Table 2. Diet formulation for the basal diets for the 12-week trial presented in **Paper II**.

Diets	**HF***	**LF****
Ingredients (g/kg)		
FM Westland	150	50
FM Super prime	150	50
Soy protein concentrate	83	200
Corn gluten	50	50
Pea protein	91	129
Wheat gluten	0	79
Beans, dehulled	128	129
Sunflower expeller	90.5	20
Fish oil	72	76
Rapeseed oil	167	178
Methionine	2.1	4.4
Lysine	0.1	6.6
Threonin	0.7	2.4
Histidine	2.7	3.9
Vit/Min mix	3.7	3.5
Mono calcium phosphate	12.7	24.1
Barox	0.2	0.2
Yttriuim	0.5	0.5
Analysed chemical composition (g/kg)		
Dry matter	929	943
Protein	398	412
Fat	290	260
Starch	53	69
Total choline (mg/kg)	1860	1190

*HF, high fishmeal diet (30%); **LF, low fishmeal basal diet (10%)

Table 3. Supplemented components to the LF basal diet for the trial presented in **Paper II**.

Diets	**TC1**	**TC2**	**CH**	**TA**	**PC**	**CL**	**CY**	**ME**
Ingredients (g/kg)								
Taurocholate; TC	6.9	3.5						
Cholesterol; CH			2.0					
Taurine; TA				0.8				
Phosphatidylcholine, 95%; PC					15.1			
Choline chloride, 70 %; CL						3.7		
Cysteine; CY							0.8	
Methionine; ME								1.0

Table 4. Diet formulation and chemical composition of the basal diet for the 13-week trial presented in **Paper III**.

Diets	**LF***
Ingredients (g/kg)	
FM Nordic	100
Soy protein concentrate	173
Maize Gluten	150
Pea Protein 50	130
Beans, dehulled	140
Wheat Gluten	27.5
Fish oil (Standard)	75.5
Rapeseed oil	176
Amino Acid mix	14.5
Mineral mix	3.5
Monocalcium phosphate	24.3
Lucantin Pink CWD 10%	0.4
Yttrium	0.5
Choline chloride 70%	0
Analysed chemical composition (g/kg)	
Dry matter	957
Protein	407
Fat	290
Starch	214

*LF, low fishmeal basal diet (10%)

Table 5. Supplemented and analysed choline (mg/kg) in experimental diets for the trial presented in **Paper III**.

	LF*	LF 1	LF 2	LF 3	LF 4	LF 5	LF 6	LF 7	LF 8
Supplemented		392	785	1177	1569	1962	2354	2746	3139
Analysed	1340	1540	1760	2310	2600	2850	3330	3830	4020

*LF, low fishmeal basal diet (10%)

Methods

Macro- and microscopic evaluation of lipid accumulation

For the work presented in **Papers I, II** and **III**, sampling and evaluation of the pyloric caeca and liver prepared for histological evaluation of lipid vacuolation was performed following well-established histomorphological methods. Both a macroscopic and a histological approach were used for evaluating the degree of lipid accumulation in the work presented in **Paper III**. The macroscopic method assessed the whiteness of the pyloric intestinal tissue. Fish showing no whiteness were given the score of zero, whereas fish showing whiteness were given a score of one (Figure 2), however the whiteness score did not take into consideration to what degree the whiteness occurred along the intestinal tissue (fish showing a slight white appearance within a small section of the anterior part of the pyloric intestine and fish with a whitish appearance within the whole pyloric intestine were both a score of one). The histological observations of vacuolation were assessed based on the appearance of lipid-like vacuoles, swelling and irregularly of the cells, and condensation of the nuclei. The vacuolation was assessed semi-quantitatively by the proportion of total histological picture affected: normal (≤ 10%), mild (10-25%), moderate (25-50%) or marked (≥ 50%; Figure 11).

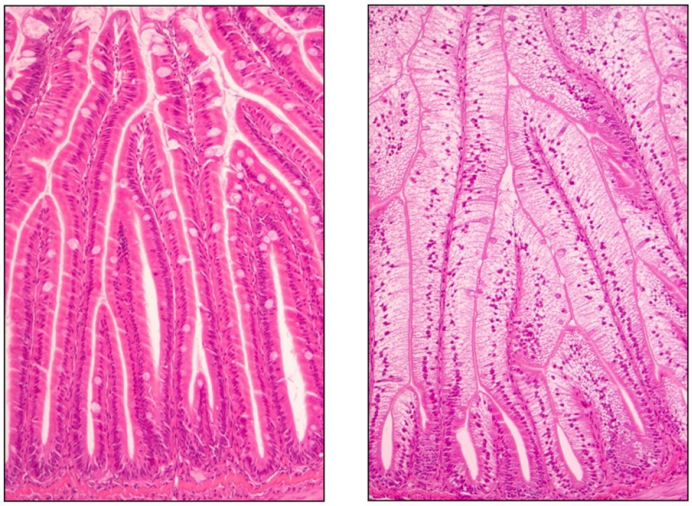

Figure 11: Histological severity of vacuolation of the pyloric caeca tissue. Pyloric caeca with enterocytes graded as normal (no hypervacuolation; left image) and marked (high degree of hypervacuolation; right image).

The level of choline where the fish showed no indication of lipid accumulation differed between the two methods (**Paper III**). The histological examination resulted in a lower estimate for choline requirement than the macroscopic evaluation. The difference may be related to the fact that macroscopic examination summarizes the characteristics of the whole pyloric intestinal tissue, whereas the histological examination observes a very limited area of a sample taken from a pyloric caecum located in the middle part of PI (Figure 5).

Lipid class analyses

The lipid classes TAG, DAG, PL and FFA of the tissues pyloric caeca tissues were analysed in fish fed the LF diets in the work presented in both **Papers I** and **III**. A choline supplemented diet was also analysed in both studies (i.e. LFC in the work presented in **Paper I** and LF5 for

Paper III; Table 1, 4 and 5). The pyloric intestine is a dynamic organ, with a primary role to absorb the dietary lipid and transport it further to the circulatory system and liver (Denstadli et al., 2004). Both studies observed a significant decrease of TAG (g/tissue) as a result of choline supplementation, but the analysed amount differed notably between the two studies. The analysed level of TAG in fish fed the LF diets were 12 mg TAG/g tissue in the experiment presented in **Paper I** compared to 178 mg TAG/g tissue (a 15-fold increase) in the work presented in **Paper III**. For the choline supplemented diets, the analysed value reported in **Paper III** was 9 times higher compared to the level reported in **Paper I,** 42 and 4.5 mg TAG/g tissue, respectively. It was observed throughout many of the previous experiments that fish appetite can vary from day to day. The observed difference of TAG in the tissue of pyloric caeca may be related to differences in feeding rate at the day of sampling between the two trials. Meaning that a higher feed intake would result in a higher intake of lipid and potentially affect the analysed values. Unfortunately. equipment for feed waste collection were not available for these two studies and therefore feed intake could not be estimated. It should be noted that there was a higher level of choline in the LFC diet, 4250 mg/kg (**Paper I**), compared to 2850 mg/kg for LF5 (**Paper III**). In **Paper III** a dose-response relationship between the dietary choline level and the degree of lipid vacuolation in the pyloric intestine, such as the transport of lipid out of the pyloric enterocytes, was observed. Both the effect of lipid and choline intake as a result of variation in feed intake or lipid content in the diets, on the degree level of TAG accumulated in the pyloric tissue and the development of LMS, warrants further investigations.

Gene expression profiling

Both microarray and quantitative real-time PCR (qPCR), are well established methods to study gene expression. Microarray is a global transcriptomic profiling measuring the expression of a large number of pre-defined genes (Krasnov et al., 2011). Microarray has been the preferred method for large scale discovery projects, such as whole genome or screening studies. The qPCR method is often considered to be more sensitive than the microarray and has traditionally been used to validate the microarray discoveries. It is also

the method of choice when analysing gene expression of a moderate number of genes (VanGuilder et al., 2008). QPCR is a more targeted method than microarray, meaning that the genes to be tested are defined by the person designing the experiment. Microarray was performed on liver samples in the first experiment (**Paper I**) and on pyloric caeca samples in the second experiment (**Paper II).** Although the overall picture was similar for the two independent profiling methods, microarray results did not reveal the same significant responses in the expression of selected genes related to lipid metabolism as qPCR. An exception was the down-regulation of *plin2* as a result of choline supplementation. Table 6 presents the overview of the genes tested by qPCR in the present thesis. Microarray and qPCR are independent technologies with differences in the preparation of the sample which could explain the observed divergences in the results. Another possibility is that the sequence used for identifying and measuring a specific gene transcript differed between the microarray chip and qPCR. Salmon have a large number of paralog genes where 10-20% of the salmon genome are maintaining tetraploid genetic characteristics (four copies of a gene) (Houston and Macqueen, 2019).

Although the qPCR method is an established method for profiling quantitative gene expression, bias could be introduced to the results if the normalization of the data from the reference genes is not performed correctly. There are several methods for normalizing gene expression but the use of reference genes is a commonly accepted method for relative normalization of qPCR assays (Kortner et al., 2011). There is a general agreement that a universal reference gene, displaying a constant expression regardless of species, tissue or experimental conditions, does not exist (Dheda et al., 2004; Schmittgen and Zakrajsek, 2000). Therefore, it is important to evaluate the stability of the selected reference genes for different experimental setups. False positive or negative results may be obtained by using unstable reference genes.

Table 6. Gene overview. Primer pair sequences, efficiency, amplicon size and annealing temperature for the genes used for quantitative real-time PCR.

Name	Gene symbols	Forward	Reverse	Amplicon size (bp)	Anealing temperature (°C)	Primer efficiency	GeneBank accession no.
		5'–3' primer sequence					
3-hydroxy-3-methylglutaryl-CoA reductase	*hmgcr*	ccttcagccatgaactggat	tcctgtccacaggcaatgta	224	60	1.9	NM_001173919
Acyl-coA cholesterol acyltransferase	*acat*	tgctggagtttgacctgttg	gctgcgatggtagagagtcc	139	60	2.0	GE793368
Adipophilin/perilipin 2	*plin2*	cccaggtctactccagcttc	cagcgactccttcatcttgc	104	60	2.0	XM_014155742
Apolipoprotein AI	*apo-AI*	ctggtcctcgcactaaccat	tggacctctgtgcagtcaac	144	60	2.0	NM_001123663
Apolipoprotein AIV	*apo-AIV*	caggaccagtctcagcaaca	gttgacttcctgtgccacct	131	60	1.9	BT048822
Apolipoprotein B100	*apo-B100*	ttgcagagaccctttaagttcattca	tgtgcagtggttgccttgac	120	60	1.9	X81856
Apolipoprotein B48	*apo-B48*	ccctgagatggtgtccgtat	gcgtcgacttccatagcttc	131	63	1.8	CB504205
ATP-binding cassette A1	*abca1*	acagtggagggaacatgagg	cccctccttgacgatactga	149	60	2.0	TC187143
ATP-binding cassette G5	*abcg5*	agactgcctcgtccaacact	ccattttcgtgaacgtgtacc	157	60	1.9	CU073172
Beta-actin	*actb**	caaagccaacagggagaagatga	accggagtccatgacgatac	133	60	1.9	AF012125
Choline kinase	*chk*	ctcaagtttgcccgtctgat	cacaggggaatgagtggagt	88	60	1.9	DY706802
Choline transporter-like protein 2	*slc44a2*	tcgtcatcattttgctgctc	aggcgatgacaatggatagg	152	60	2.0	NM_001140367
Choline-phosphate cytidyltransferase	*pcyt1a*	cgggtctatgcagatggaat	gctcgtcctcgttcatcact	166	60	2.1	BT045986
Cluster of differentiation 36	*cd36*	caagtcagcgacaaaccaga	aggagacatggcgatgtagg	91	60	1.9	AY606034
Cytochrome P450 51	*cyp51*	tgcattggggagaactttgc	atctgatgacggggttgtgt	148	60	1.9	XM_014177708
Elongation factor 1 alpha	*ef1a**	gtgctgtgcttatcgttgct	ggctctgtggagtccatctt	148	60	1.9	AF321836
Extracellular superoxide dismutase precursor	*sod3*	gggaagcctcaaagtcctct	ccgtactggtggatgtggat	87	60	1.9	NM_001140762
Farnesoid X receptor	*fxr*	ttcaacatctcaactcatca	tagcaggtcctcattgat	102	60	2.0	NM_001173830
Fatty acid binding protein 2a1	*fabp2a1*	ggtgctgaaaactaccagagcca	ggatttgaacgtagctcttcttgg	152	60	2.0	EU880417
Fatty acid binding protein 2a2	*fabp2a2*	cagctacgatggagtcgaagcca	ggttgtaaaatgttcagtgtcac	139	60	2.0	BT046863
Fatty acid binding protein 2B	*fabp2b*	tgccttcccctcattctcta	ggtgatacggtcttcatccaa	82	60	2.0	EU880419
Fatty acid transport protein	*fatp*	aggagagaacgtctccacca	cgcatcacagtcaaatgtcc	159	60	1.9	CA373015
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh**	aagtgaagcaggagggtgga	cagcctcaccccatttgatg	96	60	1.9	BT050045
Isopentenyl-diphosphate delta isomerase 1	*idi1*	tacctcccaaaatggcactc	cgtccctcatagcagctttc	132	60	1.9	XM_014157452
Liver X receptor	*lxr*	gccgccgctatctgaaatctg	caatccggcaaccaatctgtagg	210	60	1.9	FJ470290
Microsomal triglyceride transfer protein	*mtp*	aacgtgacagtggacatgga	ggaccgtggtgatgaagtct	89	60	2.0	CA042356
Monoacylglycerol acyltransferase 2-A	*mgat2a*	acgctacaggcttcaggaaa	ggaatcagacctgccatcat	116	60	2.0	NM_001140718
Niemann-Pick C1 like 1	*npc1l1*	ccaaagacctgatcctggaa	cgaagcacacatccttcaga	108	60	1.9	XM_014171081
Peroxisome proliferator activated receptor alpha	*pparα*	gcttcatcaccagggagttt	tcactgtcatccagctccag	113	60	2.0	NM_0011235960
Peroxisome proliferator activated receptor gamm	*pparγ*	tgctgcaggctgagtttatg	caggggaaagtgtctgtggt	107	58	2.0	NM_0011235946
Phosphatidylethanolamine N-methyltransferase	*pemt*	gttgctgtcatcgccatcat	gaggaggatgatgagggtgc	141	60	2.0	XM_014158251
Phosphomannomutase	*pmm*	cacacttgaggagcgaatcg	cttcccagcaaactcctcct	97	60	2.0	EG869621
Retinoid X receptor beta	*rxrb*	tccgagccatcatcctcttc	cgatgcgtacacttttctccc	89	60	1.9	XM_014177312
Ribosomal protein S20	*rps20**	agccgcaacgtcaagtct	gtcttggtgggcatacgg	98	60	2.0	NM_001140843
RNA polymerase II	*rnapolII**	ccaatacatgaccaaatatgaaagg	atgatgatggggatcttcctgc	157	60	1.8	BG936649
sterol element regulatory binding protein 1	*srebp1*	gccatgcgcaggttgtttcttca	tctggccaggacgcatctcacact	151	63	1.9	HM561860
Sterol element regulatory binding protein 2	*srebp2*	tcgcggcctcctgatgatt	agggctaggtgactgttctgg	147	60	1.9	HM561861
Taurine transporter	*slc6a6*	ggaggtggaaggacagatca	acatgccaccttttcgttacc	143	60	2.0	NM_00112363

* Reference genes

In accordance to the suggestions above, several reference genes were tested, to identify a stable reference gene for trials within this thesis. The reference genes were evaluated by ranking relative gene expression according to their overall coefficient of variation (CV) and their interspecific variation (Kortner et al., 2011). The chosen reference gene(s) in the assay(s) from the pyloric intestine of Atlantic salmon was *gapdh* for the experiments presented in **Papers I** and **II**, whereas *rnapolII* was the most stable in the last study, presented in **Paper III** (Table 6).

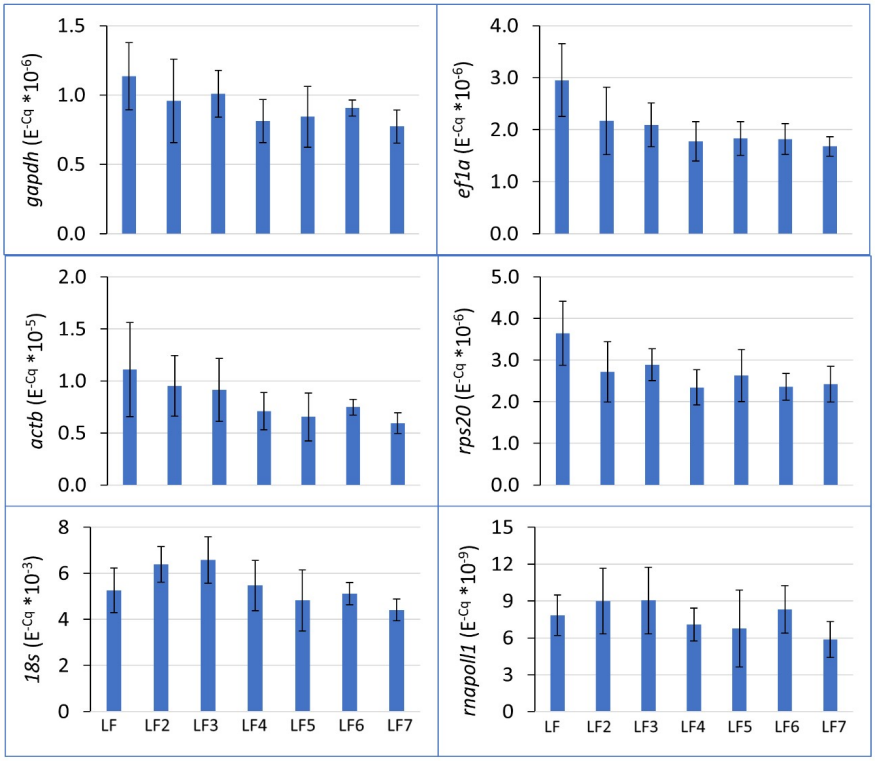

Figure 12: Pyloric caeca gene expression of candidate reference genes in the choline dose-response study (**Paper III**); glyceraldehyde-3-phosphate dehydrogenase (*gapdh*), elongation factor 1α (*ef1a*), β-actin (*actb*), ribosomal protein S20 (*rps20*), ribosomal protein 18S (*18S*) and RNA polymerase II (*rnapolII*). Values are means with standard deviations represented by vertical bars. E: Reaction efficiency, Cq: quantification cycle; LF, low fishmeal reference diet; LF2-7, choline chloride supplemented diets, See Tables 4 and 5.

In the experiment presented in **Paper I**, the expression of the first reference gene analysed, (*gapdh*), turned out to be stable so no additional analyses of alternative reference genes were needed. In the second experiment (**Paper II**), *actb*, *ef1a*, *gapdh* and *rps20* were evaluated as alternative reference genes. In the last choline dose-response study presented in **Paper III**, *gapdh*, *ef1a*, rnapolI, *actb*, *rps20* and *18S* were tested. Interestingly, a systematic variation was observed in the expression of *gapdh*, *ef1a*, *actb*, *18S* and to some degree of *rps20*, as a result of increasing inclusion levels of choline chloride (Figure 12), reiterating the importance to evaluate several reference genes for each experiment (Dheda et al., 2004; Kortner et al., 2011; Schmittgen and Zakrajsek, 2000).

Estimating choline requirement

Performing requirement studies with fish has additional challenges compared to studying requirements in terrestrial animals, since experiments with fish are carried out in water. Therefore, special considerations must be made in order to control the feed intake. There are important considerations which should be evaluated on methodology and data analysis for nutrient requirements studies, as already presented above.

The main finding in the two first experiments, presented in **Papers I** and **II**, was a clear diminishing effect of the hypervacuolation of the enterocytes, as a result of dietary choline supplementation. Overall, indicated by the observations in these two studies, PI was the most sensitive organ for studying the effects of variation in dietary choline. This formed the basis for the final choline dose-response study (**Paper III**), aiming to estimate the choline requirement for post-smolt Atlantic salmon. In previous investigations addressing choline function and requirement, indicators of lipid transport in gut mucosa have seldom been response criteria, including the NRC's (NRC, 2011) basis for estimates of choline requirement. Weight gain and liver lipid content have more often been ending points in studies addressing choline requirement (Craig and Gatlin, 1997; Griffin et al., 1994; Halver and Hardy, 2002b; Ketola, 1976; Rumsey, 1991; Shiau and Lo, 2000; Wilson and Poe, 1988). However, they are not necessarily the optimal biomarkers for estimating a choline

requirement, at least not in larger fish. Indicators of the efficiency of lipid transport in the mucosa of the pyloric caeca, supposedly the organ with the highest lipid turnover (Denstadli et al., 2004) (in particular in rapidly growing fish on high lipid diets) may be better biomarkers for choline requirement. The choline dose-response study **(Paper III)** confirmed the abovementioned hypothesis regarding biomarkers in choline requirement studies where intestinal responses and not liver responses fulfilled the criteria and turned out to be suitable as biomarkers for estimating the choline requirement.

The estimated average choline requirement indicated by the various biomarkers; whiteness of PI, hypervacuolation of pyloric enterocytes, OSIPI, OSIMI and the expression of *pcyt1a*, *apoAIV, apoAI* and *plin2* in pyloric caeca differed. Thinking of the key role of choline in lipid transport and metabolism observed in the present work, it is likely that choline requirement depends on dietary lipid level and feed intake, i.e., lipid load. The diets used in the experiment presented in **Paper III** contained 29% lipid and the average SGR was 0.94% (TGC: 2.7). Higher growth rates, higher feed intakes and/or higher dietary lipid levels are all factors which could potentially influence the choline requirement level of 3350 mg/kg (3.4 g/kg) for Atlantic salmon in seawater in the present study. Further studies are needed for the characterization of these relationships.

GENERAL DISCUSSION

The summaries of the results of this thesis led to the following topics for discussion:

1. Atlantic salmon in seawater requires dietary supply of choline.
2. The effects of choline chloride versus phosphatidylcholine.
3. Impact of choline on gene expression.
4. Consequences of LMS on performance.
5. Commercially available choline sources.

1. Atlantic salmon in seawater requires dietary supply of choline.

The results document, for the first time, that post-smolt Atlantic salmon requires dietary supply of choline. Choline requirement is defined for early life stages for many fish species (NRC, 2011) and the requirement appears to differ substantially between species. These requirement estimates are mostly based on the observation of effects on weight gain and liver lipid content (Craig and Gatlin, 1997; Griffin et al., 1994; Halver and Hardy, 2002b; Ketola, 1976; NRC, 2011; Rumsey, 1991; Sargent et al., 2003; Shiau and Lo, 2000; Wilson and Poe, 1988). Indicators of lipid transport in the gut mucosa have rarely been used as response criteria in studies addressing choline function and requirement in previous investigations for any species at any life stage (NRC, 2011). Weight gain and liver lipid indicators are, however, not necessarily optimal biomarkers when estimating choline requirement in larger fish. Indicators of efficiency of lipid transport in the mucosa of the pyloric intestine, particular in rapidly growing fish fed high lipid diets, may be more suitable biomarkers for choline requirement. In all the three independent studies in this PhD (**Papers I-III**), choline seemed to have a stronger impact on biomarkers related to the pyloric intestine and is therefore considered as a more relevant biomarker than both growth and liver lipid content.

The biomarkers which showed a clear dose-response relationship with dietary choline level in addition to having a close functional relationship with choline in the requirement study

presented in **Paper III**, are all involved in the lipid transport in the mucosa of the pyloric intestine. This raises the question whether choline requirement might be a function of both feed intake and dietary lipid level. The present estimate of choline requirement of 3350 mg/kg was obtained with a dietary lipid content of 29% and an average SGR of 2.7. Higher lipid levels and higher growth rates are common in salmon production. A higher lipid level might demand a higher choline level; higher than 3350 mg/kg. The impact of fish size is another potential factor which should be investigated in future studies.

2. The effects of choline chloride versus phosphatidylcholine.

The elimination of sign of LMS, the decreasing effects on organ indices, and the alteration of expression of genes involved in lipid transport observed with diets supplemented with phosphatidylcholine, were very similar to those caused by diets supplemented with choline chloride (**Paper II**). In line with the fact that choline is an integrated component of phosphatidylcholine, similar responses were expected and were in agreement with observations from a corresponding study on juvenile white sturgeon (Hung, 1989). Fish fed the high fishmeal diet showed very similar responses to those mentioned above for the two choline supplemented diets, compared to the low fishmeal diet. Interestingly, the high fishmeal group showed a significantly higher organ index of the pyloric intestine compared to the two choline supplemented groups, whereas no significant effects where seen for the histological vacuolation (LMS). This observation may be a consequence of the lower analysed dietary choline level in the high fishmeal diet (1860 mg/kg) compared to the two supplemented diets (2870 and 2980 mg/kg for the phosphatidylcholine and choline chloride group, respectively). Furthermore, the higher choline requirement for the organ index of the pyloric intestine, 3593 (± 226) mg/kg, found in the choline dose-response study presented in **Paper III**, might explain the observed significant difference for this biomarker and not for the histological vacuolation, where a lower requirement were revealed, 2310 mg/kg.

The expression of a few genes differed significantly between the choline and phosphatidylcholine fed fish. Expression of *srebp2*, the controlling transcription factor, and *mtp*, playing an important role in the formation of the intestinal lipoproteins, were significantly higher expression in the choline chloride fed fish. These lipoproteins are involved in export of soluble lipids such as TAG and cholesterol esters from the enterocytes. The impact of the abovementioned differences in gene expression between the two supplements remains unclear. Overall, the observed responses in the fish fed choline, supplemented either as phosphatidylcholine or choline chloride, were very similar.

An observation worth mentioning is the substantial increase in lipid digestibility observed in the phosphatidylcholine group (+2.4%) compared to the low fishmeal reference diet. Both the choline chloride supplemented group and the high fishmeal fed group (HF) showed higher lipid digestibility than the low fish meal group (LF), but the difference was smaller and not significant, +1.1% and +1.2%, respectively. The explanation for the positive effect of phosphatidylcholine, and not choline, on lipid digestibility, may be its important role in emulsification of lipid in the stomach and in the formation of micelles, whose role is to deliver soluble lipid components to the absorptive cells of the mucosa (Bauer et al., 2005) (Figure 6). The lower analysed level of choline in the high fishmeal diet, meaning a lower level of phosphatidylcholine, might explain the lower lipid digestibility for this diet compared to the phosphatidylcholine supplemented group.

3. Impact of choline on gene expression.

The changes in the degree of lipid accumulation, as a result of choline supplementation, were not reflected in major transcriptomic changes other than for genes involved in lipid metabolism. Choline supplementation, in general, resulted in moderate transcriptomic changes in the pyloric tissue performed both by qPCR in all three studies. The moderate transcriptomic changes were also confirmed by microarray, in the liver in the first experiment (**Paper I**) and in the pyloric caeca in the second experiment (**Paper II**). A previous study conducted on first feeding salmon fry documented relatively stable transcriptome profiles after dietary phospholipid supplementation (De Santis et al., 2015).

Of the 25 genes tested in all three of the present experiments, the expression of *pcyt1a*, *apoAIV* and *plin2* were significantly and systematically altered as a result of choline supplementation (Table 7). The direction of the modulations in expression were the same, however *pcyt1a* and *plin2* were reduced, whereas *apoAIV* was induced.

The *pcyt1a* is the rate limiting enzyme in the production of phosphatidylcholine (Gibellini and Smith, 2010; Li and Vance, 2008) whereas *apoAIV* codes for the apolipoprotein ApoAIV (Kamalam et al., 2013). One could question why choline affected *pcyt1a* and *apoAIV* in opposite directions when both play an important role in the formation of lipoproteins and are therefore essential in the transport of dietary lipid from the intestine. Insufficiency of phosphatidylcholine has been suggested to result in a disrupted assembly of lipoproteins and transport of lipids from the enterocytes (Daprà et al., 2011; De Santis et al., 2015; Fontagne et al., 1998; Gu et al., 2014; Olsen et al., 2003). Because of this, the expression of *pcyt1a* may become induced in periods with an insufficient supply of choline in order to secure that all available choline is fully utilized. The apolipoproteins, on the other hand, may be induced only when the supply of all the components needed for a successful lipoprotein assembly are present in sufficient amounts. Another possible explanation is that the synthesis of phosphatidylcholine is regulated by the availability of phosphatidylcholine.

The supplementation of choline increased the biosynthesis of phosphatidylcholine and the decreased expression of *pcyt1a* was the result of phosphatidylcholine inhibiting its own synthesis pathway through a negative feed-back mechanism. As the regulation of *pcyt1a* activity is very complicated, including several post translational steps (Cornell and Ridgway, 2015), further studies of this rate limiting enzyme are therefore needed to understand the mechanisms underlying the observed effect of choline on *pcyt1a.*

Table 7. Genes affected by choline and phosphatidylcholine (indicated by PC) supplementation in the work presented in **Papers I- III***.

Gene symbol	Paper I	Paper II[a]	Paper III
abca1			
abcg5		PC	
acat			
apoAI			*
apoAIV			*
apoB48		PC	
cd36			
chk			
fabp2b			
fatp			*
fxr			
hmgcr			*
lxr			
mgat2a			
mtp			*
npc1l1			
pcyt1a		PC	*
pemt			
plin2			*
pparα			
pparγ			
slc44a2			
slc6a6			
srebp1			
srebp2			

[a]Cells with no writing = the response is representative for both the choline chloride and phosphatidylcholine group (PC). PC = a significant response where observed only for the PC group. Grey colour = no significant differences, green colour = significantly down-regulated, red colour = significantly up-regulated. *genes significantly affected by increasing choline level.

In all the three studies, choline supplementation induced a marked reduction of *plin2,* a surface marker of intestinal lipid droplets (Schubert et al., 2003; Xiao et al., 2019). The roles and interaction of *plin2* and phosphatidylcholine in the formation and mobilization of lipid droplets, in intestinal lipoprotein formation and secretion, remains to be elucidated.

Molecular processes are of key importance in maintaining optimal functionality in any organ and show typical responses to various diet-related challenges such as in soybean meal induced inflammation (Gu et al., 2014; Kortner et al., 2014, 2012; Krogdahl et al., 2003). The lack of strong gene expression responses observed in the presented studies, despite the clear differences in gut mucosa structure and enterocyte hypervacuolation is interesting to investigate in further studies. Intestinal lipid absorption and transepithelial transport, including the temporary storage of lipid in the cytosolic lipid droplets, are through natural metabolic processes undertaken by all healthy animals upon ingestion of a high fat meal. A possibility is that the lipid load in the present study did not exceed the threshold requiring compensatory changes of the transcriptome. This may also explain the lack of responses of *pemt* in the liver (**Paper I**), encoding for the endogenous pathway for production of phosphatidylcholine from phosphatidylethanolamine (De Santis et al., 2015; Li and Vance, 2008).

4. Consequences of LMS on performance.

Symptoms of LMS may occur without a clear negative effect on performance. The significantly higher growth observed in fish fed the choline supplemented diet in the first study (**Paper I**), were not confirmed in the two following studies (**Papers II and III**). Previous studies report similar findings where no growth effects of choline were observed on adult Atlantic salmon (Espe et al., 2017), fingerling channel catfish (Wilson and Poe, 1988) nor juvenile giant grouper (Yeh et al., 2015). However, several other studies with juveniles of Atlantic salmon (Hung et al., 1997; Poston, 1990), carp (Duan et al., 2012; Wu et al., 2011) and blunt snout bream (Jiang et al., 2013; Li et al., 2016) report increased growth with choline supplementation. The increased growth in the choline fed group observed in the first experiment (**Paper I**) was not a result of more available energy since the diets

were isoproteic and isoenergetic and no significant differences in nutrient digestibilities were observed. A choline regression study with juvenile blunt snout bream reported an increase of crude lipid in the dressed carcass as a result of choline supplementation (Jiang et al., 2013). The impact of choline on lipid content of the muscle, as a result of the improved transport of lipid from the intestine to other tissues, warrants further investigations also for Atlantic salmon.

The present experiments showed that feed with high levels of plant ingredients and corresponding lower levels of fishmeal need to be supplemented with choline to prevent LMS in Atlantic salmon in seawater, which should not be accepted from a fish health perspective. The lipid level in the three trials varied from 27 to 29%, which is substantially lower than the highest levels, around 40%, used in commercial feeds for the largest sizes of farmed Atlantic salmon. Lipid intake of farmed fish increases with the increasing level in the feed throughout the lifecycle and over a longer time period than the fish experienced in the present studies. The question then arises whether fish fed very high lipid diets would develop a more severe LMS than observed in the present studies, with floating faeces as a result. These questions require further investigations to be answered.

5. Commercially available choline sources.

The results mentioned above indicate that fishmeal, phosphatidylcholine and choline chloride are good choline sources. On the other hand, from a sustainability and economical point of view, neither fishmeal nor phosphatidylcholine would be suitable for use in commercial feed for Atlantic salmon. An inclusion of around 60% of fishmeal would be needed to achieve the choline requirement level of 3350 mg/kg presented in **Paper III**. A level of 60% fishmeal is not an alternative for the industry from both a sustainable and economical point of view. Moreover, the phosphatidylcholine product used in the present thesis work, is too expensive to be considered as an alternative choline source for the commercial salmon feed industry. Soybean lecithin on the other hand, is a mixture of several phospholipids, and is a commonly used source for phospholipids in feed for aquaculture. In a previous report, we have presented results showing a small, 16%, but

significantly reduced weight of the proximal intestinal tissues by supplementing a low fishmeal basal diet (10%) with 15 g/kg soybean lecithin (Kortner et al., 2016). The histological examination (unpublished data) showed a corresponding trend for enterocyte hypervacuolation. The choline chloride supplemented group, reported in **Paper I**, showed a stronger significant reduction compared to the lecithin group by 38% for the relative weight of the proximal intestinal tissue and no observed hypervacuolation. The choline level in the abovementioned lecithin supplemented diet was 1153 mg/kg. The concentration of choline in the soybean lecithin product was therefore too low to be the only source of additional choline to achieve the requirement level of 3350 mg/kg. A supplement of 180 g/kg would then be needed which is not possible from a commercial point of view. The best option therefore seems to be choline chloride products which are available at a reasonable price and will make it possible to reach the required choline level at fairly low inclusion levels. There are no published reports claiming negative effects as a result of choline supplementation in feed for fish. In the choline dose-response study presented in **Paper III** doses between 400 – 3100 mg/kg choline (Table 5) were used and no negative effects on performance were observed.

MAIN CONCLUSIONS

- The present study indicates that a dietary choline level of 3350 mg/kg will cover the choline requirement in 95% of post-smolt Atlantic salmon fed a diet containing 29% lipid.

- Higher levels of choline may be required if higher dietary lipid levels are used and if higher growth rates are observed than in the present study.

- Choline, supplemented in the form of choline chloride or phosphatidylcholine, or as part of fishmeal, prevented LMS in post-smolt Atlantic salmon in seawater.

- Atlantic salmon in seawater fed a diet with 10% fishmeal and 28% lipid (70% rapeseed and 30% fish oil) develop LMS.

- LMS is a result of impaired lipid transport across the enterocytes due to impaired lipoprotein assembly, as a result of deficient availability of phosphatidylcholine.

- Supplementation of taurocholate (6.9 and 3.5 g/kg), cholesterol (2 g/kg), taurine (0.8 g/kg), cysteine (0.8 g/kg) or methionine (1.0 g/kg) did not affect the development of LMS.

FUTURE PERSPECTIVES

The present thesis has, for the first time, provided a requirement for choline in post-smolt Atlantic salmon. New knowledge of suitable biomarkers, indicating intestinal lipid transport capacity and development of LMS, when studying effects of choline in fish have been elucidated. However, there are still questions which deserve further investigation. A better understanding of the relationship between choline requirement, dietary lipid level and feed intake should be established. Knowledge of the functions, interactions and the impact of quantitative levels of choline, cholesterol and lipid should be further explored. The following questions still need to be elucidated:

- Would higher dietary lipid levels and higher growth rates increase the choline requirement?

- Would fish size, from smolt to harvest size of Atlantic salmon, have impact on the choline requirement?

- Does the improved transport of lipid from the intestine by choline supplementation result in increased lipid content in other peripheral tissues, i.e., heart, liver or muscle?

- How long does it take for an Atlantic salmon with LMS to revert to a normal state (no signs of LMS) after the dietary choline level is increased or dietary lipid level supply is reduced?

- What is the functional interaction of choline with other key components involved in lipid metabolism?

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RESEARCH ARTICLE Open Access

Choline supplementation prevents diet induced gut mucosa lipid accumulation in post-smolt Atlantic salmon (*Salmo salar* L.)

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Abstract

Background: Various intestinal morphological alterations have been reported in cultured fish fed diets with high contents of plant ingredients. Since 2000, salmon farmers have reported symptoms indicating an intestinal problem, which we suggest calling lipid malabsorption syndrome (LMS), characterized by pale and foamy appearance of the enterocytes of the pyloric caeca, the result of lipid accumulation. The objective of the present study was to investigate if insufficient dietary choline may be a key component in development of the LMS.

Results: The results showed that Atlantic salmon (*Salmo salar*), average weight 362 g, fed a plant based diet for 79 days developed signs of LMS. In fish fed a similar diet supplemented with 0.4% choline chloride no signs of LMS were seen. The relative weight of the pyloric caeca was 40% lower, reflecting 65% less triacylglycerol content and histologically normal gut mucosa. Choline supplementation further increased specific fish growth by 18%. The concomitant alterations in intestinal gene expression related to phosphatidylcholine synthesis (*chk* and *pcyt1a*), cholesterol transport (*abcg5* and *npc1l1*), lipid metabolism and transport (*mgat2a* and *fabp2*) and lipoprotein formation (*apoA1* and *apoAIV*) confirmed the importance of choline in lipid turnover in the intestine and its ability to prevent LMS. Another important observation was the apparent correlation between *plin2* expression and degree of enterocyte hyper-vacuolation observed in the current study, which suggests that *plin2* may serve as a marker for intestinal lipid accumulation and steatosis in fish. Future research should be conducted to strengthen the knowledge of choline's critical role in lipid transport, phospholipid synthesis and lipoprotein secretion to improve formulations of plant based diets for larger fish and to prevent LMS.

Conclusions: Choline prevents excessive lipid accumulation in the proximal intestine and is essential for Atlantic salmon in seawater.

Keywords: Choline, Lipid accumulation, Lipid transport, LMS, Lipid malabsorption, Gut health, Fish feed, Plant ingredients

Background

The main driver for replacement of marine raw materials with alternative plant ingredients in fish feed is the ambition to maintain growth of the aquaculture industry and to secure flexibility regarding raw materials in feed production. However, in parallel with the decrease in fishmeal and increase in plant meals in fish feed, the prevalence of various intestinal disturbances has increased. Therefore, it is likely that some of the observed intestinal challenges may be due to deficient supply of nutrients, which are present at lower levels in plant ingredients than in fishmeal, but not corrected for due to lack of information on their essentiality and/or required level. The requirements for many nutrients have been defined for many species but all nutrient requirements are far from defined [1].

The present work addresses symptoms of a well-known intestinal disorder for which we suggest the term lipid malabsorption syndrome (LMS) and which since 2000 have been reported by salmon farmers to affect

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young as well as more mature fish [2–4]. The typical sign is increased lipid accumulation in the enterocytes giving the pyloric caeca a pale and foamy appearance on the macroscopic level. Similar signs have been reported also in other fish species fed diets high in plant meal [5–8] or high in plant oil [7, 9–11]. The apparent disturbance in lipid transport is also observed on the molecular level. Plant based diets may influence the expression of genes involved in lipid metabolism in a manner reflecting reduced lipid export from the enterocytes [8, 12–20]. However, the mechanisms underlying the excessive lipid accumulation are not yet fully clarified. Some studies seem to indicate that phospholipid synthesis, and in particular phosphatidylcholine, might be the bottleneck in lipid export from the enterocytes in fish showing such lipid accumulation [9, 10, 21–27]. Phosphatidylcholine, however, is not established as an essential nutrient for Atlantic salmon nor for any other fish species (NRC, 2011). For choline, on the other hand, of which about 95% is found in phosphatidylcholine [28, 29], a requirement is established for several fish [1]. Due to insufficient information, the question of whether choline is essential, and if so, the required amount, cannot currently be determined for Atlantic salmon.

In animals, including fish, choline is necessary for synthesis of phosphatidylcholine for use in lipid digestion and absorption, as a component in lipoproteins for lipid transport, in production of the neurotransmitter acetylcholine, and as a methyl donor in a wide range of methylation processes. Poor growth and low feed efficiency, fatty liver, high mortality, and anorexia are all reported effects of choline deficiency in the species for which we have documentation [1, 30–32]. Lipid accumulation in the intestinal mucosa is, however, not a common endpoint in studies of choline deficiency and requirement and has only been observed in an early study of Japanese eel (*Anguilla japonica*) as "white-grey colored intestines" [33]. This gut observation appears similar to that observed in Atlantic salmon with LMS. No studies have been conducted to define whether choline is essential for Atlantic salmon, or how much can be synthesized. Accordingly, a requirement is not established, and the question whether high plant diets might be deficient in phosphatidylcholine or choline, cannot be answered. Rainbow trout have been found to require choline at earlier life stages due to an inability to produce sufficient choline even with a high supply of methyl donors such as betaine and methionine [31]. On the other hand, channel catfish were able to produce sufficient choline, if the supply of methionine was high [1].

The level of fishmeal in today's commercial salmon diets is in general low and decreases throughout the life cycle of the fish. A diet for salmon weighing 500 g or more typically contains between 5 and 10% fishmeal. Fishmeal would be the main contributor for choline in these diets, which means that supply of choline from the other main ingredients would be rather low. For example, the basal diet (LF) used in the present experiment was a commercially representative feed with 10% fishmeal which revealed a choline level of 944 mg/kg (Tables 1 and 2). With several recent reports from the salmon industry regarding LMS [4], investigation of the role of choline for LMS is needed. The present work therefore aimed to elucidate whether LMS is a result of insufficient choline supply and also addressed the role of

Table 1 Formulation and chemical composition of the experimental diets

Diets	LF [a]	LFC [b]
Ingredients (g/kg)		
Super Prime fra Peru[c]	50	50
Nordic LT 94 fishmeal [d]	50	50
Soya 60% (SPC) [e]	190	194
Maize Gluten [f]	150	150
Pea Protein 50 [g]	130	130
Dehulled Beans [h]	140	130
Wheat Gluten [i]	19.7	19.7
Fish oil (Standard) [j]	76.7	77.1
Rapeseed oil [k]	176	177
Amino Acid mix [l]	12.4	12.4
Mineral mix [l]	3.0	3.0
Monocalcium phosphate [l]	18.2	18.2
Lucantin Pink CWD 10% [l]	0.4	0.4
Yttrium [m]	0.5	0.5
Choline chloride 70%	0	4.0
Analyzed chemical composition (g/kg)		
DM	975	972
Protein	417	418
Fat	286	297
Starch	107	102
Total choline (mg/kg)	944	4250
Total methionine	9.1	9.4
Total cysteine	5.1	5.8

[a]Low fishmeal diet
[b]Choline supplemented low fishmeal diet
[c]Supplied by Kôster Marine Proteins GmbH
[d]Supplied by Norsildmel AS
[e]Supplied by Selecta S/A, Avenida Jamel Ceilio, 2496 – 12th region. SPC, soya protein concentrate
[f]Supplied by Cargill Nordic
[g]Supplied by DLG Food Grain
[h]Supplied by HC Handelscenter
[i]Supplied by Roquette
[j]Supplied by FF Skagen
[k]Supplied by Emmelev
[l]Supplemented to meet the requirements
[m]Inert marker for the evaluation of nutrient digestibility

Table 2 Growth performance (Mean values with their standard errors)

	LF[a]	LFC[b]	Pooled SEM	*P*-value[c]
IBW (g)[d]	364	354	7.3	0.501
Growth (g)	344	418	20.3	**< 0.001**
SGR (%d-1)[e]	0.84	0.99	0.04	**< 0.001**

[a]Control low fishmeal diet group ($n = 70$)
[b]Choline supplemented low fishmeal diet ($n = 68$)
[c]*P*-values obtained in t-test, values in bold indicate significant differences between the two treatments
[d]Initial body weight
[e]Specific growth rate

choline in enterocyte lipid transport in post-smolt Atlantic salmon.

Results

Growth performance and nutrient digestibilities

Growth performance was significantly higher for fish fed the choline supplemented feed (LFC) compared to those fed the unsupplemented basal diet (LF, Table 2). Choline inclusion did not affect apparent digestibility (AD) significantly for any of the nutrients. The average AD (± SEM) for the two test diets was 96.1 (± 0.24) for crude lipid, 90.1 (± 0.19) for crude protein and 75.8 (± 0.65) for starch.

Intestinal chyme dry matter and brush border leucine aminopeptidase

Choline supplementation tended to increase dry matter content of digesta along the intestine (Table 3). The increase was significant for the mid intestine (MI) and distal intestine (DI) sections of the intestine. The trend was clear also for proximal half of the pyloric intstine 1 (PI1) and PI2 ($p = 0.062$ and 0.086, respectively). Brush border membrane leucine aminopeptidase (LAP) activities for PI and DI are shown in Table 3. There were no significant differences in the enzyme activity between the two treatments either in PI or DI tissue.

Table 3 Intestinal dry matter and leucine aminopeptidase activity (LAP) (Mean values with their standard errors)

	LF[a]	LFC[b]	Pooled SEM	*P*-value[c]
Intestinal dry matter (%)				
PI1	9.3	10.3	0.65	*0.062*
PI2	11.0	12.2	0.71	*0.086*
MI	12.9	14.5	0.67	**0.005**
DI1	12.9	14.0	0.48	**0.011**
DI2	11.0	12.7	0.47	**<0.001**
LAP (mmol/h/kg BW)				
PI	244	235	16.1	0.729
DI	44	43	3.1	0.743
LAP (μmol/h/mg prot)				
PI	331	385	25.3	0.309
DI	228	244	17.3	0.237

[a]Control low fishmeal diet group ($n = 20$)
[b]Choline supplemented low fishmeal diet ($n = 20$)
[c]*P*-values in bold indicate significant differences between the two treatments; italicized values represent trends

Organosomatic indices, intestinal and liver lipid content and histology

Relative organ weights of the PI, MI, DI and liver (LI) are shown in Fig. 1. Choline supplementation reduced relative weights of PI, MI and LI significantly, but not of DI. Macroscopic observations revealed white and swollen pyloric caeca in most of the sampled individuals fed the LF diet, whereas this observation was not recorded for any of the fish fed the LFC diet (Fig. 2a). Accordingly, the histological examination showed a significantly higher degree of lipid droplet accumulation in the pyloric caeca in fish fed the LF diet compared to those fed the LFC diet (Fig. 2b and c, respectively, $p < 0.001$). The degree of vacuolation of the enterocytes was 0% in sampled fish fed the LFC diet compared to 100% in the LF fed group (Fig. 3). Choline supplementation significantly lowered triacylglycerol (TAG) concentration in the tissue of the PI (Fig. 4, $p = 0.024$). No significant differences due to supplementation were found for free fatty acids (FFA), monoacylglycerol (MAG), diacylglycerol (DAG) and phospholipid (PL). The histological examination of LI vacuolation did not indicate similar effects of choline supplementation as in the pyloric caeca. No significant differences in the degree of liver vacuolation was found between the two diets ($p = 0.867$). Likewise, calculation of absolute amount of liver lipid (g) did not reveal significant differences ($p = 0.867$) between LFC and LF fed fish, 0.33 (± 0.04) and 0.33 (± 0.03), respectively.

Intestinal gene expression

Figure 5 illustrates the molecular regulations of the studied genes involved in synthesis of phosphatidylcholine, cholesterol, and lipids, as well as intestinal lipid transport, lipoprotein assembly and secretion. Table 4 presents the results of the effect of choline supplementation on intestinal gene expression. Expression of genes encoding three enzymes involved in the pathway of phosphatidylcholine biosynthesis was analysed. The expression of the *pcyt1a* gene was significantly down-regulated whereas the effect for *chk* showed a trend towards down-regulation ($p = 0.068$). No significant effect was observed on expression of *pemt*. Genes involved in cholesterol (CH) transport were also significantly affected in fish fed the choline enriched diet. *Niemann-Pick C1 like 1* (*npc1l1)* and *abcg5* were up-regulated. Expression of *fabp2* homologs, encoding fatty acid transporters, and the transcription factors *pparα* and *pparγ* were significantly enhanced. Also *mgat2a*, responsible for TAG re-esterification, was significantly up-

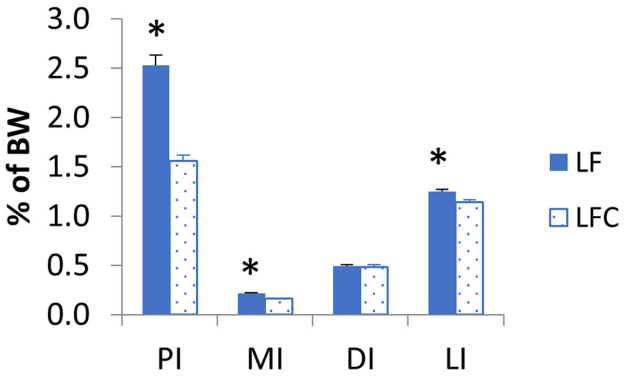

Fig. 1 Organ somatic indices of the intestinal sections, pyloric intestine (PI), mid-intestine (MI), distal intestine (DI) and liver (LI). Values are means (PI $n = 20$ and MI, DI and LI $n = 30$) with standard errors represented by vertical bars. Significant differences ($p < 0.05$) between the LF and LFC group are indicated with *. The inclusion of choline resulted in a significant lower organ somatic index for PI, MI and LI ($p < 0.05$)

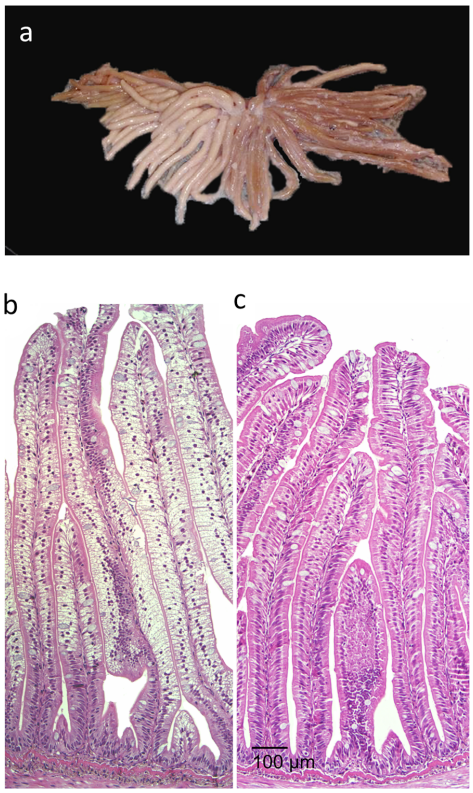

Fig. 2 a example of white pyloric caeca with grossly visible of lipid accumulation. Image credit: Vegard Denstadli. Histological appearance of pyloric caeca in fish fed (**b**) the low fishmeal diet, LF and (**c**) the choline supplemented diet, LFC. Scale bare = 100 µm

regulated. A similar up-regulation was seen for both *apoAI and apoAIV*, involved in lipoprotein assembly. The general marker for lipid load of non-adipogenic cells, *adipophilin/ perilipin 2* (*plin2)* was down-regulated. The taurine transporter *slc6a6* was up-regulated in the choline treated fish.

Hepatic gene expression

In the microarray analysis (fold change > 1.6, $p < 0.05$), 168 entities were found to be differentially expressed between the two diet groups (Additional file 2). The differentially expressed genes appeared to be distributed among many functional classes, and a search for enriched GO and KEGG terms provided little meaningful information (data not shown). Among the highest responding transcripts, two innate immunity-related lectins (*nattectin*, *c-type mbl-2 protein*) were markedly induced by the choline treatment. In contrast, *rxr*, *pmm* and *sod3* were down-regulated by choline supplementation. *Perilipin 2* (*plin2)* showed up-regulation in the liver in contrast to the down-regulation found in PI. To further verify the microarray data, *rxr*, *pmm*, *sod3* and *plin2* were quantified by qPCR (Table 5). In accordance with microarray data, *pmm* and *plin2* were down- and up-regulated, respectively, whereas no differences for *rxr* and *sod3* were observed with qPCR. The lipoprotein and sterol associated transcripts measured in pyloric caeca were also quantified in liver using qPCR (Table 5). In accordance with the microarray data, we observed no significant changes for any of these transcripts. Altogether, microarray and qPCR data were closely correlated (Person's correlation coefficient: 0.74, $p = 0.0003$).

Blood plasma endpoints

Most of both the TAG and cholesterol in plasma was present in the high-density lipoprotein (HDL) fraction

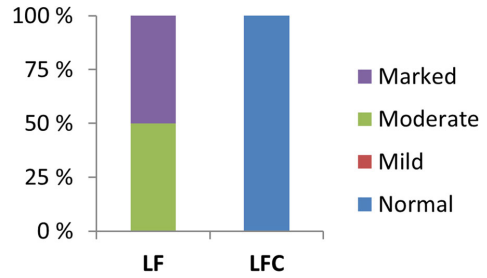

Fig. 3 Contingency charts of the pyloric intestine showing proportions of sampled individuals that scored vacuolation grade "normal", "moderate" and "marked" (none scored "mild"). Fish fed the low fishmeal diet displayed hyper-vacuolated enterocytes. Choline inclusion resulted in normal epithelium. The differences between the diets were significant ($p < 0.05$; Chi-squared test)

independent of treatment and the distribution of TAG and cholesterol among the lipoproteins were similar. Choline supplementation significantly decreased the plasma level of TAG reflecting reductions in HDL and low-densitylipoprotein (LDL) (Table 6). The opposite effect was seen on plasma cholesterol reflecting cholesterol increase in all the lipoprotein fractions. Plasma lathosterol, indicative of the rate of cholesterol synthesis, increased upon choline supplementation. The level of 7α-hydroxycholesterol, a metabolite in cholesterol catabolism and conversion to bile acids, also increased, whereas C4

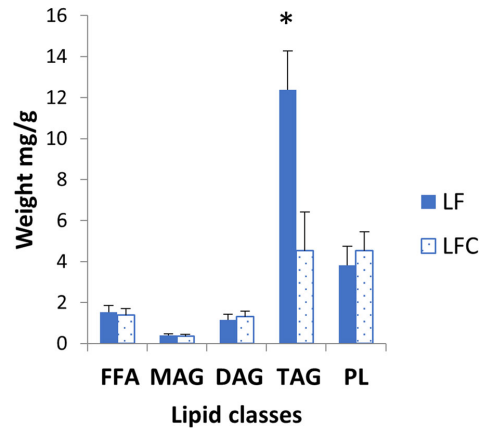

Fig. 4 Distribution of the lipid classes; free fatty acids (FFA), monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG) and phospholipid (PL) in pyloric caeca tissue. Values are means ($n = 10$) with standard errors represented by vertical bars. Significant differences ($p < 0.05$) between the LF and LFC group are indicated with *. The inclusion of choline resulted in a significant lower content of TAG ($p < 0.05$; T-test)

(7α-Hydroxy-4-cholesten-3-one), a later metabolite in the cholesterol catabolism, was not significantly affected. Plasma levels of other catabolic products of cholesterol, i.e. the oxysterols 7β- hydroxycholesterol, 7β-keto-hydroxycholesterol, 24-hydroxycholesterol and 27-hydroxycholesterol were increased by dietary choline supplementation.

Discussion

In brief, the present study revealed that choline supplementation to a plant based diet, 4.3 g/kg, improved growth by 18%, without effects on macronutrient digestibilities or other observed indicators of digestive function. The relative weight of the pyloric caeca decreased by 40% - reflecting a reduction in TAG and was shown histologically as elimination of enterocyte hypervacuolation. On the molecular level the supplementation caused down-regulation of genes involved in the CDP-choline pathway in which phosphatidylcholine is synthesized from free choline and a phosphorylated diglyceride (*chk* and *pcyt1a*), but had no significant effect on expression of *pemt* involved in synthesis of phosphatidylcholine from phosphatidylethanolamine via the PEMT pathway, the second pathway for phosphatidylcholine synthesis. Choline supplementation up-regulated two genes involved in cholesterol transport (*abcg5* and *npc1l1*), as well as genes involved in lipid metabolism and transport (*mgat2a* and *fabp2*), and lipoprotein formation (*apoA1* and *apoAIV*). The reduced intracellular lipid level was reflected in marked suppression of the lipid droplet marker *plin2*.

The aim of the present study was to elucidate if choline deficiency is a key contributor for LMS, and whether dietary supplementation with choline might prevent development of LMS. Our results clearly affirm these hypotheses. In this respect, our results are in line with the observations of lipid accumulation in Japanese eel fed choline deficient diets [33]. Our observations also highlight the importance of choline in lipid turnover in post-smolt Atlantic salmon, and supply information relevant for later developmental stages.

Choline effects on performance

Choline supplementation of the feed for Atlantic salmon of the size used in the present study, start weight 362 g and final weight 740 g, was found to have a great improving effect on SGR, by 18%. Similar improvements have been observed at juveniles stages in Atlantic salmon as well as in other species [32, 34–38]. Several studies have also confirmed the requirement for phospholipid in both freshwater and marine juveniles [31, 34, 39, 40]. The 18% increase in growth rate in fish fed the choline supplemented diet may give great expectations for improvement of efficiency in production of Atlantic salmon. It should, however, be kept in mind,

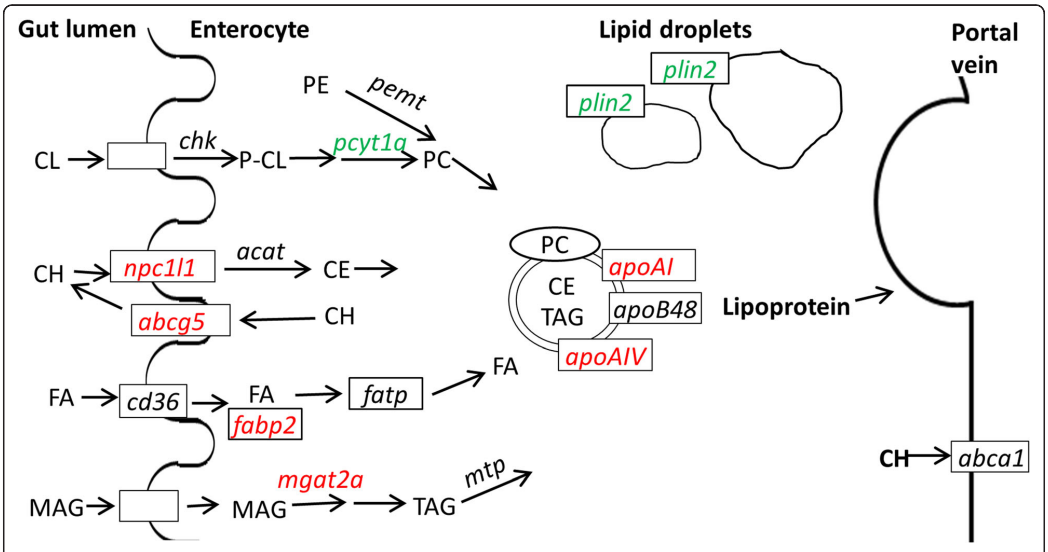

Fig. 5 Overview of genes involved in lipid digestion and absorption in the intestine of Atlantic salmon and studied in the present study. Arrows indicates steps in the pathways. Studied genes are italicized. Green color indicates genes which were significantly down-regulated and red color indicate up-regulated genes. No color represents genes not significantly affected. Dietary choline (CL) is synthesized by *choline kinase* (*chk*) to phosphocholine (P-CL) and after an intermediate step not studied here, *choline-phosphate cytidylyltransferase* (*pcyt1a*) to phosphatidylcholine (PC). PC could also be synthesized from endogenous phosphatidylethanolamine (PE) by *phosphatidylethanolamine N-methyltransferase* (*pemt*). PC is an important element in the membrane portion of lipoproteins preventing triacylglycerol (TAG) from leaking out. Cholesterol (CH) is transported from the lumen and over the membrane by *Niemann-Pick C1-Like1* (*npc1l1*). *Acyl-CoA cholesterol acyltransferase* (*acat*) located in ER, facilitates the esterification of CH to cholesterol esters (CE). *ATP-binding cassette G5* (*abcg5*) returns some of the free cholesterol back to the gut for reuse. Some of the free cholesterol is also shuttled to the basolateral membrane for biogenesis of high-density lipoprotein (HDL) mediated by *ATP-binding cassette A1* (*abca1*). Fatty acids (FA) are transported from the gut lumen over the brush border membrane and into the epithelial cell by *cd36* (*cluster of differentiation 36*). The *fatty acid-binding protein 2* (*fabp2*) shuttles the fatty acids within the epithelial cell and the *fatty acid transport protein (fatp)* further to the smooth endoplasmic reticulum (ER). Monoacylglycerol (MAG) is esterified by *monoacylglycerol acyltransferase* (*mgat2a*), located in ER, to diacylglycerol (DAG) which is further transformed into triacylglycerol (TAG), a step not studied here. *Microsomal triglyceride transfer protein* (*mtp*) further facilitates the transport of TAG by assisting in the assembly of the lipoprotein. The three apolipoproteins *apoB48*, *apoAI* and *apoAIV* are important elements for successful production and secretion of the lipoprotein. The formation of lipoproteins is again an essential step for export of lipid to the general circulation and to other organs such as the liver. Excess lipid is stored as lipid droplets in the enterocytes. The lipid droplet structure and formation are regulated by the amphiphilic structural protein, *adipophilin/perilipin 2* (*plin2*)

that the SEM indicates that the true difference might be much less, or much higher. Follow-up studies are therefore needed, to find whether similar improvements can be expected in the long run.

Effects of choline on lipid accumulation in the pyloric intestinal tissue

There is a general understanding that TAG is the primary lipid class in lipid stores [40] and an increased supply of fatty acids promotes TAG synthesis and storage in fat cells where lipid droplets increase in abundance and size [41]. The high TAG levels and the corresponding occurrence of large lipid vacuoles observed in the pyloric caeca of the control fish suffering from LMS in the present study are in line with this. The absence of lipid droplets in pyloric caeca in choline fed fish might also be explained by phosphatidylcholine playing an important role in lipoprotein formation, and therefore in the transport of lipids across cell membranes and an efficient transport of dietary lipids from the pyloric caeca [22, 42–44]. The relatively low TAG level observed in fish fed the choline supplemented feed could also be a result of phosphatidylcholine also acting as a surfactant stabilizing growing lipid droplets and further preventing lipid droplet coalescence [41]. The concomitant alterations in expression of genes involved in phosphatidylcholine synthesis, cholesterol synthesis, lipid droplet formation, lipid transport, and lipoprotein formation and metabolism tested in the present study confirmed the importance of choline in this respect.

The cytidine (CDP)-choline pathway is the main pathway for phosphatidylcholine synthesis from dietary choline [29]. Choline kinase (*chk*), catalyzing the initial and committing step, showed a tendency to be down-regulated by choline

Table 4 Gene expression profiling of pyloric caeca samples by qPCR

Gen category and function	Gen symbol	Fold change[a]	*P*-value[b]
Lipid uptake and transport			
Fatty acid transporter	*cd36*	1.13	0.175
Fatty acid transporter	*fabp2b*	1.37	**0.004**
Fatty acid transporter	*fabp2a1*	1.11	0.302
Fatty acid transporter	*fabp2a2*	1.01	0.962
Fatty acid transporter	*fatp*	0.89	0.120
Lipoprotein assembly	*mtp*	1.12	0.259
Lipoprotein component	*apoB48*	1.07	0.730
Lipoprotein component	*apoAIV*	1.58	**0.028**
Lipoprotein component	*apoAI*	1.42	**0.001**
Lipid droplet component	*plin2*	0.273	**< 0.001**
Nuclear receptor – regular of lipid metabolism	*ppara*	1.52	**0.037**
Nuclear receptor – regular of lipid metabolism	*pparγ*	1.38	**0.024**
Resynthesis of triacylglycerols	*mgat2a*	1.39	**0.023**
Phosphatidylcholine synthesis			
Choline transporter	*slc44a2*	1.09	0.329
Phosphatidylcholine biosynthesis	*pemt*	0.95	0.486
Phosphatidylcholine biosynthesis	*chk*	0.61	*0.068*
Phosphatidylcholine biosynthesis	*pcyt1a*	0.58	**0.004**
Cholesterol metabolism			
Bile acid nuclear receptor	*fxr*	1.15	0.415
Cholesterol biosynthesis	*hmgcr*	0.91	0.260
Cholesterol efflux transporter	*abca1*	0.95	0.650
Cholesterol efflux transporter	*abcg5*	1.61	**0.004**
Cholesterol esterification	*acat*	0.95	0.677
Cholesterol transporter	*npc1l1*	1.58	**< 0.001**
Nuclear receptor - regular of lipid and sterol metabolism	*lxr*	1.06	*0.580*
Nuclear receptor - regular of lipid and sterol metabolism	*srebp1*	0.55	0.159
Nuclear receptor - regular of lipid and sterol metabolism	*srebp2*	1.04	0.907
Taurine transporter - bile salt metabolism	*slc6a6*	1.39	**0.001**

[a]Values are mean fold change observed in the choline diet fed group in comparison with those in the control group
[b]*P*-values in bold indicate significant differences between the two treatments; italicized values represent trends

supplementation, whereas significant down-regulation was found for *pcyt1a*, regulating the second and rate-limiting step in the CDP-pathway [28, 29]. These results are in agreement with findings presented earlier [8] showing lower expression of *chk* and *pcyt1a* in the pyloric caeca of fish fed a high fishmeal diet, supposedly with a higher choline level, compared to the expression in hyper-vacuolated pyloric caeca of fish fed a plant meal based diet with a lower choline level. The down-regulation of *chk* and *pcyt1a*, as a result of choline supplementation in the present study, could be an indication that the fish received more than enough choline and that the phosphatidylcholine synthesis was regulated through a negative feed-back control. However, regulation of *pcyt1a* activity is very complicated with important post translational steps [45]. Further studies of this rate limiting enzyme are therefore needed to understand the impact of the observed effect on *pcyt1a*. Choline supplementation did not, however, alter the expression of *pemt* in the present study, which is in agreement with previous studies carried out with mammals which showed that *pemt* is expressed mainly in the liver [29].

Choline induced the expression of both *npc1l1*, involved in the absorption of cholesterol from the intestinal lumen into the enterocytes [46] and *abcg5*, catalyzing the transport of a proportion of the free cholesterol back to the gut for reuse [47]. As such, choline seemed to promote the circulation and reuse of free cholesterol, also indicated by the increased blood plasma CH levels in choline fed fish.

Table 5 Gene expression profiling of liver samples by qPCR

Gen category and function	Gen symbol	Fold change[a]	*P*-value[b]
Lipid uptake and transport			
Fatty acid transporter	*cd36*	0.948	0.711
Fatty acid transporter	*fatp*	0.992	0.923
Lipoprotein component	$apo(B100)_{liver}$	0.996	0.983
Lipid droplet component	*plin2*	1.626	**0.013**
Nuclear receptor – regular of lipid metabolism	*ppara*	0.660	0.182
Nuclear receptor – regular of lipid metabolism	*ppary*	0.973	0.834
Phosphatidylcholine synthesis			
Phosphatidylcholine biosynthesis	*pemt*	0.998	0.988
Phosphatidylcholine biosynthesis	*chk*	0.505	0.116
Phosphatidylcholine biosynthesis	*pcyt1a*	0.923	0.597
Cholesterol metabolism	*abc1a1*	0.851	0.310
Bile acid nuclear receptor	*fxr*	1.154	0.265
Cholesterol biosynthesis	*hmgcr*	0.936	0.479
Cholesterol biosynthesis	*cyp7a1*	0.925	0.580
Cholesterol efflux transporter	*abca1*	0.855	0.289
Cholesterol efflux transporter			
Cholesterol efflux transporter	*abcg5*	1.063	0.601
Cholesterol transporter	*npc1l1*	0.955	0.776
Nuclear receptor - regular of lipid and sterol metabolism	*lxr*	0.837	0.156
Nuclear receptor - regular of lipid and sterol metabolism	*srebp1*	0.944	0.851
Nuclear receptor - regular of lipid and sterol metabolism	*srebp2*	1.174	0.405
ROS metabolism / antioxidant			
Superoxide dismutation	*sod3*	0.739	0.374
Nuclear receptor – control of gene transcription			
Transcription factor	*rxr*	0.789	0.104
Mannose metabolism			
Glycosylation	*pmm*	0.759	**0.029**

[a]Values are mean fold change observed in the choline diet fed group in comparison with those in the control group
[b]*P*-values in bold indicate significant differences between the two treatments; italicized values represent trends

Choline supplementation seemed not to influence the transport of fatty acids across the brush border membrane from gut lumen to the enterocytes and further to ER as no significant alteration of *cd36* and *fatp* expressions were observed. On the other hand, choline seemed to influence the transport of fatty acids within the epithelial cell due to the induced expression of *fabp2* in the choline fed group [48, 49]. The up-regulation of *mgat2a* indicates that choline is also important in the synthesis of MAG to DAG, which is an important intermediate for the synthesis of both TAG and phosphatidylcholine [50]. The synthesized TAG is exported from the cells in lipoproteins. Both apoAI and apoAIV are major proteins in enterocyte lipoprotein assembly [51] and were up-regulated with choline supplementation. These results support our hypothesis regarding the importance and key roles of choline for efficient lipid supply and metabolism in salmon and strengthens the suggestion that choline is important for the synthesis and secretion of lipoproteins [10, 22, 27, 42, 52]. A study on rats [53] observed an increased intestinal lipid content and an impaired chylomicron secretion as a result of choline deficiency. These observations support our findings regarding the importance of choline for proper lipid metabolism.

Another important observation was the decreased expression of *plin2*, a general marker for the lipid load of non-adipogenic cells [54]. In humans, *plin2* has been suggested as a marker for detection of lipid droplets in tissues, which further are associated with various diseases such as hepatocyte steatosis [55]. Plin2 has also been reported to coat cytoplasmic lipid droplets in

Table 6 Blood plasma variables

	LF[a]	LFC[b]	Pooled SEM	*P*-value[c]
Glucose (mmol/L) [d]	5.3	5.9	0.22	**< 0.001**
Free Fatty Acids (mmol/L) [d]	0.27	0.25	0.02	0.35
Lipoptoteins				
Total CH (mmol/L) [d]	8.3	11.1	1.74	**< 0.001**
HDL-CH[e]	7.5	8.9		
LDL-CH[e]	1.3	1.5		
VLDL-CH[e]	0.1	0.3		
Total TAG (mmol/L) [d]	3.3	2.5	0.27	**0.01**
HDL-TAG[e]	3.2	2.4		
LDL-TAG[e]	1	0.7		
VLDL-TAG[e]	0.5	0.8		
Bile salts (μmol/l) [d]	20	19	8.47	0.822
Sitosterol (μg/ml) [f]	71	61	6.98	0.204
Campesterol (μg/ml) [f]	188	224	27.4	0.342
Lathosterol (μg/ml) [f]	3.8	9.2	0.48	**< 0.001**
C4 (μg/ml) [f]	0.01	0.01	0.02	0.921
Oxysterols (ng/ml) [e]				
7α-hydroxy-CH	130	295		
7β-hydroxy-CH	37	139		
7-keto-hydroxy-CH	101	538		
24-hydroxy-CH	2.2	4		
25-hydroxy-CH	5	5		
27-hydroxy-CH	21	33		

[a]Low fishmeal diet
[b]Choline supplemented low fishmeal diet
[c]*P*-values in bold indicate significant differences between the two treatments; italicized values represent trends
[d]Measured for n = 20 per diet
[e]Lipoprotein and oxysterol profiles were measured in pooled samples of *n* = 5 per diet
[f]Measured for n = 10 per diet. Mean values with their standard errors

enterocytes of chronic high-fat fed mice [54]. The apparent correlation between *plin2* expression and degree of enterocyte hyper-vacuolation observed in the current and previous studies [56], suggest that *plin2* may serve as a marker for intestinal lipid accumulation and steatosis in fish.

Effects of choline on liver

The choline fed fish had significantly lower hepatosomatic index than the control, but this was not reflected in lower content of lipid, nor in histological apparent vacuolation. Both diets resulted in relatively high degree of lipid accumulation. This is in accordance with previous observations in gibel carp [32] and red drum [57] showing that dietary choline deficiency did not cause an increased accumulation of liver lipid. On the other hand, studies on common carp [58], lake trout [30], rainbow trout [31] and blunt snout bream [59] reported fatty livers in fish fed choline deficient diets. In the present study, choline supplementation caused only minor effects on the hepatic transcriptome and no genes related to lipid metabolism showed altered expression. Collectively, the lack of response to choline supplementation in liver is in sharp contrast to the marked changes observed in the intestine and clearly points towards a focus on intestinal responses in future studies of lipid accumulation and choline requirements in salmon.

Choline effects on plasma indicators

Very low-density lipoprotein (VLDL) synthesis and assembly is regulated by the availability of triglycerides [60–63] and it seems from the tendency of the enhanced amount of both VLDL-TAG and VLDL-CH observed in the choline group that choline increased the VLDL synthesis and assembly. Even though an increase in VLDL-TAG was observed did the total level of TAG decrease in plasma in fish fed the choline enriched diet. The reduction was a result of reduced TAG in both HDL and LDL which could indicate that the lipids were successfully extracted from VLDL in the peripheral tissues [64]. A similar decrease in TAG level in plasma has been observed for juvenile lobsters [65] and cobia larvae [39] fed soy lecithin. Niu et al. [39] further suggested that this was a result of a positive effect of phospholipids on lipoprotein lipase and hepatic lipase activities for TAG uptake in liver and further distribution to other tissues. Choline also seemed to increase HDL's, in addition to VLDL and LDL's, capacity to bind and transport cholesterol due to the higher cholesterol amount. The present study further supports previous observations [66–68] showing that HDL is the most abundant lipoprotein carrying the main load of both cholesterol and TAG. The increase of cholesterol bound to HDL in the choline supplemented group could be a result of higher levels of phospholipids incorporated into the HDL, which in a study with rat, was shown to play a key role in modulating cholesterol efflux (transport and re-use of cholesterol) [69]. Phospholipid levels in the lipoproteins were not analysed in the present study, so this should be investigated in further studies.

Conclusion

Choline is an essential nutrient for Atlantic salmon, even after early developmental stages. Plant based diets must be supplemented with choline to ensure normal uptake, metabolism, and export of lipids across the intestinal mucosa.

Methods

Diets

A low fishmeal, high plant diet (LF) was used as a reference diet, containing 10% of a 50/50 mix of Nordic LT

fishmeal from the North Atlantic and Super Prime fishmeal from Peru. The total lipid content was 70% rape seed oil and 30% fish oil. The choline supplemented diet (LFC) was made by supplementing the LF diet with 4 g/kg of choline chloride. The diets contained approximately the same amount of methionine and cysteine. Table 1 shows diet formulation and analysed chemical composition. Both diets were supplemented with standard vitamin and mineral premixes in accordance with NRC guidelines (2011) and BioMar standards to meet requirements. Yttrium oxide (0.5 g/kg) was added as inert marker for estimation of nutrient apparent digestibility. The two experimental diets were produced by extrusion (feed pellet size 6 mm) at BioMar Feed Technology Centre (Brande, Denmark) using a BC 45 twin screw extruder (Clextral, France).

Experimental animals and conditions

Atlantic salmon (*Salmo salar* L., post smolt, Sunndalsøra breed) with mean initial weight of 362 g ± 95 (mean ± SD) were pit tagged, weighed individually, and randomly allocated into four fiberglass tanks with 270 l of saltwater, two replicate tanks per diet, 35 fish in each. Each tank was supplied with flow through seawater. Salinity ranged between 32 and 33 g/l. The water flow was increased accordingly to the increase in biomass and to maintain oxygen saturation at any time above 80%. The oxygen content of the outlet water was monitored once a week or more often in periods with larger temperature variations. Temperature varied between 7.0 and 14.5 °C during the experimental period (from July to September), with an average of 9.4 °C. A 24 h light regime was employed during the experimental period. The fish were fed continuously using disc feeders aiming at an excess feeding of 15% during the trial period. Equipment for recording feed waste and hence feed intake was not available for the present experiment.

Sampling

After 79 days, feeding was terminated. Weight and length were recorded for all fish. From each tank ten fish were anaesthetized with tricaine methane-sulfonate (MS-222). Blood was sampled from the caudal vein in vacutainers with lithium heparin. The vacutainers were stored on ice until plasma preparation. Plasma, 2 mL aliquots, was frozen in liquid nitrogen and stored at − 80 °C. Following blood sampling the fish were killed by a sharp blow to the head and opened ventrally. The gastro-intestinal tract was removed from the abdominal cavity, cleared of other organs and adipose tissue, and sectioned as follows. Pyloric intestine (PI): the section from the pyloric sphincter to the most distal pyloric caeca; mid intestine (MI): from the distal end of PI and proximal to the increase in intestinal diameter; distal intestine (DI): from the distal end of MI to the anus. The intestinal wall tissue of PI and DI was collected and weighed, whereas the digesta from these two sections were each split into two samples, i.e. the proximal half (PI1 and DI1, respectively) and distal half (PI2 and DI2, respectively). The intestinal samples were snap frozen in liquid nitrogen and stored at − 80 °C. The liver (LI) was also sampled and weighed. Another five fish per tank were euthanized and killed for sampling of LI and PI for histological and gene expression analyses. The 20 fish remaining in each tank were stripped for faeces as described by Austreng [70]. They were then fed for one more week for an additional stripping. The fecal samples were pooled for each tank, frozen immediately after stripping (N_2) and stored at − 80 °C until analysis. Tissues sampled for histological examination were fixed in 10% neutral buffered formalin (4% formaldehyde). Samples for gene expression analyses were rinsed in sterile saline water, submerged in RNAlater®, incubated at 4 °C for 24 h and subsequently stored at − 40 °C until analysis.

Histology

Pyloric caeca and liver samples were processed at the Norwegian University of Life Sciences (NMBU) using standard histological techniques: dehydration in ethanol, clearing in xylene, and embedding in paraffin before sectioning (5 μm). Hematoxylin and eosin were used for tissue staining. The samples were evaluated for enterocyte vacuolation blinded in a randomized order using a light microscope. Vacuolation was assessed based on appearance of lipid-like vacuoles, swelling and irregularity of the cells, and condensation of the nuclei. Vacuolation was assessed semi-quantitatively as the proportion of total tissue affected: normal (≤ 10%), mild (10–25%), moderate (25–50%) or marked (≥ 50%) and presented as percentage of vacuolated enterocytes (Fig. 6).

RNA extraction

Total RNA was extracted from pyloric caeca samples (~ 30 mg) using a Ultraturrax homogenizer, TRIzol® reagent (Invitrogen, ThermoFisher Scientific) and chloroform according to the manufacturer's protocol. Obtained RNA was DNase treated (TURBO™, Ambion, ThermoFisher Scientific) and purified with PureLink RNA mini kit (Invitrogen, ThermoFisher Scientific). Total RNA from liver samples (~ 30 mg) were also extracted using Trizol® /chloroform whereas the homogenization was carried out using a FastPrep-24 (MP Biomedicals) before the samples were purified with PureLink RNA mini kit including an on-column DNase treatment according to the manufacturer's protocol. The integrity of the RNA from pyloric caeca samples were assessed by gel electrophoresis, and in addition selected samples were verified with a 2100 Bioanalyzer using a RNA Nano Chip (Agilent Technologies). All liver samples were evaluated by

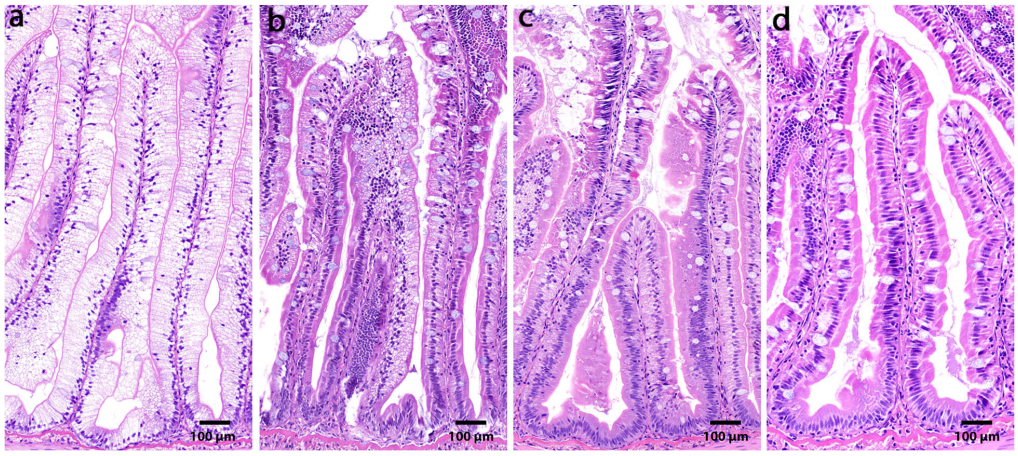

Fig. 6 Severity of vacuolation (steatosis) of the pyloric caeca tissue, representative for (**a**) marked (**b**) moderate (**c**) mild and (**d**) normal. Scale bar = 100 µm

Bioanalyzer. RIN values for both pyloric caeca and liver samples were all > 8. RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Total RNA was stored at − 80 °C until use.

Microarrays

A two-colour microarray design was used for liver transcriptome profiling. Samples from five fish per treatment were labeled with fluorescent Cy3 and hybridized against a common reference sample (pool of 10 individual fish fed a fishmeal-based diet) labeled with fluorescent Cy5. Nofima's Atlantic salmon 15 k oligonucleotide microarray SIQ-6 (GEO Omnibus GPL16555) was manufactured by Agilent Technologies (Santa Clara, CA USA). Reagents and equipment were from the same source unless indicated otherwise. RNA amplification and labelling were performed with a Two-Colour Quick Amp Labelling Kit and Gene Expression Hybridization kit was used for fragmentation of labelled RNA. A total of 200 ng RNA was used as input for each reaction. After hybridization in an oven over night (17 h, 65 °C, 10 rpm rotation speed), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with a GenePix 4100A (Molecular Devices, Sunnyvale, CA, USA). GenePix Pro 6.0 was used for spot to grid alignment, assessment of spot quality, feature extraction and quantification. STARS were used to carry out the subsequent bioinformatics data analysis [71]. Low quality spots were flagged by GenePix and filtrated away before Lowess normalization of $\log_2$-expression ratios (ER) was performed. Genes that passes quality control in at least four samples per group were included in subsequent analyses. The differentially expressed genes (DEG) were selected by the following criteria: fold difference > 1.6 and $p < 0.05$ (T-test). Enrichment of GO and KEGG terms in the list of DEG was assessed with Yates' corrected chi-square using all probes that passed quality control as reference. Enriched terms corresponding to at least five differentially expressed genes were selected.

Quantitative real-time PCR (qPCR)

Quantification of hepatic gene expression by qPCR was conducted to validate the microarray results and to examine particular genes of interest in detail. qPCR was also used for quantification of genes related to lipid and sterol metabolism and transport in pyloric caeca. Assays were carried out in accordance to the MIQE standards [72]. First strand cDNA synthesis was carried out using four fish from each tank giving a total of eight fish per treatment, and Superscript III in 20 µL reactions (Invitrogen) with total RNA (0.8 µg) and oligo $(dT)_{20}$ primers were used. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 before use and stored at − 20 °C. Quantitative PCR primers were obtained from literature or designed using Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/). Detailed information of the primers is shown in Additional file 1. PCR reaction efficiency (E) for each gene assay was determined separately for both pyloric caeca and liver using 2-fold serial dilutions of randomly pooled cDNA. A LightCycler 480 (Roche Diagnostics) was used for DNA amplification and analysis of the expression of individual gene targets. Each 10 µl DNA

amplification reaction contained 2 μl PCR-graded water, 2 μl of 1:10 diluted complementary DNA template, 5 μl of LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 μl of each forward and reverse primer. Each sample was assayed in duplicate in addition to a no template control. The three-step qPCR program included an enzyme activation step at 95 °C for 5 min followed by 40 or 45 cycles (depending on the individual gene tested) of 95 °C (10 s), 58, 60 or 63 °C (10 s depending on the individual gene tested) and 72 °C (15 s). Quantification cycle (Cq) values were calculated using the second derivative method. The PCR products were evaluated by analysis of melting curve and by agarose gel electrophoresis to confirm amplification specificity. All primer pairs gave a single band pattern on the gel for the expected amplicon of interest in all reactions. For target gene normalization, *actb*, *ef1a*, *gapdh*, *rnapolII* and *rps20* were evaluated for use as reference genes by ranking relative expression levels according to their stability, as described previously [73]. For liver samples, *rnapolII* was used as normalization factor, whereas *gapdh* was used for pyloric caeca. Relative expression of target genes was calculated using the $^{\Delta}{}^{\Delta}$CT method [74].

Chemical analyses

Diets and faecal samples were analysed for dry matter (after heating at 105 °C for 16–18 h), ash (combusted at 550 °C to constant weight), crude protein (by the semi-micro-Kjeldahl method, Kjeltec-Auto System, Tecator, Höganäs, Sweden), lipid (diethylether extraction in a Fosstec analyzer (Tecator) after HCL-hydrolysis), starch (measured as glucose after hydrolysis by alpha-amylase (Novo Nordisk A/S, Bagsvaerd, Denmark) and amyloglucosidase (Bohringer Mannheim GmbH, Mannheim, Germany), followed by glucose determination by the "Glut-Dh method" (Merck Darmstadt, Germany)), gross energy (using the Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA) and yttrium (by inductivity coupled plasma (ICP) mass-spectroscopy as described by Refstie et al. [75]. The plasma variables; free (non-esterified) fatty acids, cholesterol and total triacylglycerides were analysed according to standard procedures at the Central Laboratory of the Norwegian University of Life Sciences (NMBU). Lipoprotein profile analyses (HDL, LDL and VLDL) in plasma were carried out by size exclusion chromatography and measurements of cholesterol and triglycerides on-line using microliter sample volumes as described by Parini et al. [76]. Isotope dilution mass spectrometry as described by Lund et al. [77] was used for analyzing lathosterol. 7α-hydroxy-4-cholesten-3-one (C4) was analyzed by isotope dilution and combined HPLC-MS as described by Lövgren-Sandblom et al. [78]. Plasma levels of oxysterols, sitosterol and campesterol were analyzed by isotope dilution and combined GC-MS after hydrolysis as described by Dzeletovic et al. [79] for the first mentioned and by Acimovic et al. [80] for the last two mentioned. The lipid classes free fatty acids (FFA), monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG) and phospholipid (PL) in the pyloric caeca were extracted using the Folch procedure [81], then analysed using HPTLC Silica gel 60 F plates. DigiStore 2: documentation was used for visual documentation and the integration program WinCats was further used for calculating the amount of the lipid classes.

Enzyme analyses

Brush-border membrane enzyme activity were analysed by measuring the activity of the enzyme leucine aminopeptidase (LAP; EC 3.4.11.1) in intestinal tissue homogenates. The homogenates were prepared from tissue thawed on ice-cold tris-mannitol buffer (1:20 w/v) containing the serine proteinase inhibitor 4-[2-Aminoethyl] benzensulfonylfluoride HCL (Pefabloc® SC; Pentapharm Limited). LAP activity was then determined colorimetrically with a kit (Sigma procedure no. 251) using L-leucine-β-napthylamide as substrate.

Calculations

Growth of the fish was calculated as specific growth rate (percent growth per day): SGR = ((ln FBWg / ln IBWg) / D) X 100. IBW and FBW are the initial and final body weight (tank means) and D is number of feeding days. Organ somatic index was calculated as percentages of the weight of the organ in relation to body weight. Apparent digestibilities (AD) of main nutrients was estimated by using Y_2O_3 [82] as an inert marker and calculated as: $AD_n = 100 - (100 \text{ X } (M_{feed}/M_{faeces}) \text{ X } (N_{feed}/N_{faeces}))$, where M represents the percentage of the inert marker in feed and faeces and N represents the percentage of a nutrient in feed and faeces.

Statistical analysis

The diets in the present study were part of a larger trial. To obtain the best estimate of variance of tank means (SEM), results from all treatments were included. The other results of the experiment are published elsewhere [66, 67]. Tank was the experimental unit for all responses except for the histological observations for which the individual fish were the unit. Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC, USA). Data was analysed using the General Linear Model procedure with diets and tanks as class variables. Specific differences were evaluated by Duncan's test. The level of significance was set to $P < 0.05$, and P-values between 0.05 and 0.1 were considered as indications of effects and mentioned as trends. All data are means ± SEM. A Chi-squared test was used for analyzing histology data.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s12917-020-2252-7.

Additional file 1. Primer pair sequences, efficiency, amplicon size and annealing temperature for the genes used for real-time PCR.

Additional file 2. Differentially expressed genes between hepatic transcriptomes of fish fed the low fishmeal diet (LF) and fish fed the choline supplemented diet (LFC).

Abbreviations

AD: Apparent digestibility; DAG: Diacylglycerol; DI: Distal intestine; DI1: Proximal half of distal intestine; DI2: Distal half of distal intestine; FFA: Free fatty acids; HDL: High-density lipoproteins; IBW: Initital body weight; LAP: Leucine aminopeptidase activity; LDL: Low-density lipoproteins; LF: Low fishmeal; LFC: Choline supplemented low fishmeal; LI: Liver; LMS: Lipid malabsorption syndrome; MAG: Monoacylglycerol; MI: Mid intestine; OSI: Organosomatic indices; PI: Pyloric intestine; PI1: Proximal half of pyloric intestine; PI2: Distal half of pyloric intestine; PL: Phospholipid; qPCR: Quantitative real-time polymerase chain reaction; SGR: Specific growth rate; TAG: Triacylglycerol; VLDL: Very low-density lipoproteins

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Authors' contributions

The authors' contributions were as follows: A.K.G.H: experimental design, sampling, qPCR, data evaluation and interpretation and manuscript development, T.M.K: microarray, qPCR and manuscript review, A.K; microarray and manuscript review, I.B: biochemistry analyses, data interpretation and manuscript revision, M.P: experimental design, sampling and histology, Å.K: leadership, experimental design, data evaluation and interpretation and manuscript review. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The datasets generated and/or analysed during the current study are available in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus NCBI repository, with accession no. GSE51887.

Ethics approval and consent to participate

Rearing of the fish (Atlantic salmon, *Salmo salar*, Sunndalsøra breed) were conducted at Nofima's Research Station at Sunndalsøra, which is a research facility approved by Norwegian Animal Research Authority (NARA) and operates in accordance with Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). Trial fish were treated in accordance with the Aquaculture Operation Regulations during the trial. Fish were randomly sampled, anaesthetized and killed by a sharp blow to the head, in accordance with the Norwegian Animal Welfare act. No surgical manipulation of live fish was conducted, and tissue samples were only retrieved from euthanized fish. Ingredients commonly used in commercial diets were used in experimental diets and do not cause the fish any apparent distress. No NARA approval was required according to §2 of the Norwegian Regulation on Animal Experimentation.

Consent for publication

Not applicable.

Competing interests

The present study was partly funded by BioMar AS. Co-author Anne Kristine Hansen is employed by BioMar.

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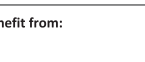

II

Choline and phosphatidylcholine, but not methionine, cysteine, taurine and taurocholate, eliminate excessive gut mucosal lipid accumulation in Atlantic salmon (*Salmo salar* L)

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Abstract

Excessive enterocyte lipid accumulation, with the suggested term lipid malabsorption syndrome (LMS), is frequently observed in Atlantic salmon (*Salmo salar* L), in small fish in fresh water as well as in large fish in seawater. The symptoms indicate insufficient supply of components involved in lipid assimilation. The questions addressed in the present work were whether dietary supply of components involved in phospholipid and sterol metabolism might prevent LMS. Atlantic salmon (35 fish, 330 g per 200 l tank) were fed a low fish meal diet (LF) as such or supplemented with taurocholate at two levels (3.5 and 6.9 g/kg), cholesterol (2.0 g/kg), taurine (0.8 g/kg), phosphatidylcholine (15.1 g/kg), choline (3.7 g/kg), cysteine (0.8 g/kg) or methionine (1.0 g/kg). A high fish meal diet (HF) was also included. The overall growth rate of the fish was high (TGC>4.2) with no significant effects of diet. Fish fed the LF diet showed increased relative weight of the pyloric and mid intestine and excessive lipid accumulation in the enterocytes, characteristics were nearly absent in fish fed the HF diet and the LF diet supplemented with choline and phosphatidylcholine. The phosphatidylcholine supplemented diet showed significantly higher lipid digestibility than the LF diet. None of the other supplements eliminated the signs of excessive enterocyte lipid accumulation. Phosphatidylcholine down-regulated *pcyt1a*, involved in the phosphatidylcholine synthesis and both choline and phosphatidylcholine induced *apoaIV,* important in lipoprotein assembly, and markedly suppressed the lipid droplet marker *plin2*. Methionine supplementation did not stimulate endogenous synthesis of choline. Cholesterol supplementation suppressed sterol uptake and de novo cholesterol synthesis, and induced sterol efflux from the intestinal mucosa. Taurocholate and taurine induced their respective metabolic pathways. All feed supplements, in particular cholesterol and cysteine, down-regulated immune genes with antiviral, chemokines, antigen presentation, immunoglobulins roles and extracellular proteases. The results of this study confirm the results

our previous study showing that in low fish meal diet choline or phosphatidylcholine is a necessary ingredient.

Keywords: High fishmeal, low fish meal, intestinal lipid accumulation, choline

1 Introduction

Various gut health challenges, such as excessive enterocyte lipid accumulation, inflammation, neoplasia and ulcers are observed, seemingly, with increasing frequency in cultivated salmon. This development may be related to the change in content of nutrients, non-nutrients and antinutrients in fish diets resulting from the shift in proportion of fish meal and plant ingredients which has taken place over the last decades (Ytrestøyl et al., 2015; Aas et al., 2019). Reduced cholesterol and bile salt levels in digesta and blood are common findings in fish fed diets with high levels of plant components (Kortner et al., 2013; Romarheim et al., 2008; Romarheim et al., 2006). Moreover, diets high in plant ingredients contain low levels of phospholipids. The symptoms of excessive lipid accumulation in the pyloric caeca of Atlantic salmon, which are commonly observed in salmon in both fresh water and seawater, and in severe cases results in floating faeces around the sea cages indicate impaired absorption of lipids. The suggested term for the condition is lipid malabsorption syndrome (LMS) (Penn, 2011). The condition raises questions whether disturbances and deficiencies in sterol and phospholipid metabolism may cause LMS.

The work presented herein was conducted to follow up the results of a previous feeding study which aimed to reveal mechanisms underlying effects of dietary supplementation with components involved in lipid and sterol metabolism on gut function and health (Kortner et al., 2016; Kortner et al., 2014). In the former study, a high plant diet was supplemented with either

taurocholate (1.8 %), a crude mix of bovine bile salts (1.8 %), taurine (0.4 %), lecithin (1.5 %) and cholesterol (1.5%), all key components in lipid and sterol metabolism. The results showed negative rather than positive effects of cholesterol and bile salt supplementation on gut inflammation (Kortner et al., 2016). The reason for this may have been that the levels chosen for bile salts and cholesterol were too high to be physiologically relevant for Atlantic salmon. The basis for the choices was levels used in former studies on rainbow trout by Japanese researchers giving results indicating beneficial effects of such levels (Iwashita et al., 2009; Iwashita et al., 2008). In our previous experiment we also included a crude preparation of phospholipids, i.e. soybean lecithin, observing no clear effect on enterocyte lipid accumulation. However, analyses of the lecithin showed that the content of phosphatidylcholine, the major phospholipid in the lipoproteins transporting lipids from the enterocytes, was very low (Kortner et al., 2016).

Our aim in the present work was therefore to gain more information on effects of various levels of pure taurocholate, the dominating bile salt in Atlantic salmon, and purified phosphatidylcholine, the dominating phospholipid involved in lipid transport across the intestinal mucosa, on gut inflammation and lipid transport. The role of free choline, one of the two essential nutrients of phosphatidylcholine besides essential fatty acids, was also studied. Moreover, we wanted to find if supplementation with methionine, a key substrate in synthesis of choline from ethanolamine, might promote lipid gut mucosal transport. If so, also the level of cysteine, produced from methionine, may play a role for production of choline. Cysteine is also a key substrate/metabolite in the production of taurocholate, and was also included in the study (Schubert, Blumenthal, Cheng, 2003). Figure 1 illustrates the main pathways and components in the supply and metabolism of compounds important in production of phosphatidylcholine and

indicates the position and role of the compounds studied in the present work (dopted from Harvey R.A., 2011).

2 Materials and methods

2.1 Experimental diets

Ten experimental diets were formulated: a high fish meal diet (HF), a low fish meal diet (LF), and eight diets based on the LF diet with supplementation of taurocholate at two levels (LF_TC1 and LF_TC2), cholesterol (LF_CH), taurine (LF_TA), phosphatidylcholine (LF_PC), choline (LF_Cl), cysteine (LF_CY) and methionine (LF_ME). The receipts are shown in Table 1A. The diets were supplemented with standard vitamin and mineral premixes in accordance with NRC guidelines (2011) and BioMar standards to meet the requirements. Yttrium oxide (0.50 g/kg) was added as inert marker for estimation of nutrient apparent digestibility. The experimental diets were produced by extrusion (feed pellet size 6 mm) at BioMar Feed Technology Centre (Brande, Denmark) using a BC 45 twin screw extruder (Clextral, France).

2.2 Experimental animals, feeding and rearing conditions

The feeding trial was performed at Nofima's research facility at Sunndalsøra, Norway, a research facility approved by Norwegian Animal Research Authority (NARA), operating in accordance with Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). Atlantic salmon (*Salmo salar* L., post smolt, Sunndalsøra breed) with mean initial weight of 330g ± 46 (mean ± SD) were pit tagged and randomly assigned to cylindrical fiberglass tanks ($1m^3$, 600L), 35 fish per tank. The fish were weighed individually when allocated to the experimental units, to assure similar

biomass in all tanks. The diets were allocated randomly to the tanks and two tanks were used per diet. The feeding period lasted 84 days. Each tank was supplied with flow through seawater at a rate of 6-7 l min^{-1} and constant light. During the feeding trial, water temperature decreased gradually from 11.5 to 8.0°C. Dissolved oxygen in the outlet water was measured daily and was maintained above 80% saturation throughout the experiment. The fish were fed continuously using disc feeders aiming at an excess feeding of 20% (Helland et al., 1996). Feed intake was recorded by collection of spilled feed pellets in the outlet water.

2.3 Trial termination - sampling

After 84 days, feeding was terminated. From each tank 18 fish, randomly selected, were anaesthetized with tricaine methane-sulfonate (MS-222), followed by a sharp blow to the head. Weight and length were recorded for all fish and blood was sampled from the caudal vein in vacutainers with lithium heparin. The vacutainers were stored on ice prior to plasma preparation. Plasma was sampled in 2 mL aliquots and snap frozen in liquid nitrogen and stored at -80°C. Following blood sampling the fish were opened ventrally. The gastro-intestinal tract was removed from the abdominal cavity, cleared of other organs and adipose tissue, and sectioned as follows: pyloric intestine (PI): the section from the pyloric sphincter to the most distal pyloric caeca; mid intestine (MI) from the distal end of PI and proximal to the increase in intestinal diameter; distal intestine (DI) section from the distal end of MI to the anus. The tissue of the PI and DI, cleared of external fat, was collected and weighed, and tissue from pyloric caeca and PI and DI tissues were sampled for enzyme analyses. Digesta from PI and DI was collected and split in two samples, i.e. the proximal half (PI1 and DI1, respectively) and distal half (PI2 and

DI2, respectively) for bile salt analyses. The intestinal samples were snap frozen in liquid nitrogen and stored at -80°C.

An additional eight fish per tank were euthanized prior to sampling of tissue from the pyloric caeca, mid and distal intestines, and liver for histological examination and gene expression analysis. Tissues for histology were fixed in 10% neutral buffered formalin (4% formaldehyde) for 24 hours and subsequently transferred to 70% EtOH for storage until processing. Samples for gene expression analyses were rinsed in sterile saline water, submerged in RNAlater®, incubated at 4°C for 24 hours and subsequently stored at -20°C until analysis. The remaining fish in each tank were stripped for faeces and fed for one more week for an additional stripping in order to collect enough sample for digestibility analysis. Faecal samples were pooled, frozen in liquid nitrogen and stored at -80°C until analysis.

2.4 Chemical analyses of feed and feaces

Diet and faecal samples were analyzed for dry matter (after heating at l05°C for 16-18 h), ash (combusted at 550°C to constant weight), nitrogen (crude protein) (by the semi-micro-Kjeldahl method, Kjeltec-Auto System, Tecator, Höganäs, Sweden), fat (diethyl ether extraction in a Fosstec analyzer (Tecator) after HCl-hydrolysis), starch (measured as glucose after hydrolysis by alpha-amylase (Novo Nordisk A/S, Bagsvaerd, Denmark) and amylo-glucosidase (Bohringer Mannheim GmbH, Mannheim, Germany), followed by glucose determination by the 'Glut-DH method' (Merck, Darmstadt, Germany)), gross energy (using the Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA), and yttrium (by inductivity coupled plasma (ICP) mass-spectroscopy as described by (Refstie et al., 1997).

2.5 Plasma analysis

Plasma was analysed for non-esterified (free) fatty acids (NEFA), total triglycerides, cholesterol and total bile acids following standard procedures at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo.

2.6 Intestinal histology

Evaluation of histological appearance of tissues from PI and DI was performed at the Norwegian University of Life Sciences (NMBU) using standard histological methods. Slides were randomized to ensure blinded examination and evaluated using a light microscope. Proximal intestine (pyloric caeca) tissue samples from four individuals per tank (i.e. eight per diet) were evaluated. Enterocyte hypervacuolation was assessed semi quantitatively, indicating the proportion of total mucosa affected: Score 1=no hypervacuolation (normal) (≤ 10%); Score 2= Mild to moderate hypervacuolation, some areas appear normal; (10 - 25 %); Score 3= Moderate hypervacuolation in almost all areas (25 – 50%) or Score 4= Moderate to severe hypervacuolation in almost all areas (clearly abnormal) (≥ 50%). Fig 2 shows representative pictures of pyloric caeca samples given scores 1 and 4.

Histological appearance of the DI, focusing on indications of processes corresponding to soybean induced enteritis, a scoring system with a scale of 0-10 was used where 0-2.5 represented normal, >2.5 to 4.5 mild changes, >4.5 to 6.5 moderate changes, >6.5-8 marked changes, and >8-10 severe changes. The scores were categorical variables and the differences between the diets were explored by contingency analysis using the chi-squared test. The following variables were observed: changes in mucosal fold length, width and cellularity of the submucosa and lamina propria, enterocyte supranuclear vacuolation, and frequency of goblet

cells, intra-epithelial lymphocytes, mitotic figures and apoptotic bodies within the epithelial layer.

2.7 RNA extraction

Based on the results of the histological examination, showing clear effects of diet in the pyloric caeca but hardly any in the distal intestine, we chose to focus the gene expression analyses on the pyloric caeca tissue.

Total RNA was extracted using a Precellys® homogenizer, Trizol® reagent and further purified with PureLink RNA mini kit (Invitrogen, Thermo Fisher Scientific, USA) including an on-column DNase treatment. The integrity of the RNA samples was verified by the 2100 Bioanalyzer in combination with an RNA Nano Chip (Agilent Technologies), and RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). RNA integrity number (RIN) was >8 for all samples and average RIN was 9.1, indicative of excellent RNA quality. Total RNA was stored at -80°C until use.

2.8 Microarrays

A two-color microarray design was used, where individual fish samples (five in each study group, two to three individuals from each tank duplicate) were labeled with fluorescent Cy3 and hybridized against a common reference sample labeled with fluorescent Cy5. The common reference sample consisted of a pool of equal amounts of RNA from all individual fish included in the analysis. Nofima's Atlantic salmon 15k oligonucleotide microarray SIQ-6 (GEO accession GPL16555) was manufactured by Agilent Technologies and unless indicated otherwise, the reagents and equipment were from the same source. RNA amplification and labelling were performed with a Two-Colour Quick Amp Labelling Kit and a Gene Expression Hybridization

kit was used for fragmentation of labelled RNA. The input of total RNA used in each reaction was 200ng. After overnight hybridization in an oven (17 hours, 65ºC, rotation speed 10 rpm), arrays were washed with Gene Expression Wash Buffers 1 and 2 and scanned with a GenePix 4100A (Molecular Devices, Sunnyvale, CA, USA). GenePix Pro 6.0 was used for spot to grid alignment, assessment of spot quality, feature extraction and quantification. Subsequent data analyses were performed with the bioinformatic system STARS (Krasnov et al., 2011). After filtration of low-quality spots flagged by GenePix, Lowess normalization of $\log_2$-expression ratios (ER) was performed. Genes that passed quality control in at least four samples per group were included in subsequent analyses. The HF diet and all LF supplemented diets were compared against the LF reference diet group. Differentially expressed genes (DEG) were selected by criteria: $\log_2$ fold difference > 0.8 and $p < 0.05$ (T-test). STARS annotated genes by GO, KEGG and custom vocabulary. Groups of functionally related genes were compared by mean $\log_2$-FC and difference from LF was assessed (T-test, $p < 0.05$). Complete data files were deposited in NCBI's Gene Expression Omnibus with accession no. xx. (will be published after article acceptance)

2.9 Quantitative real-time PCR

Quantification of pyloric caeca gene expression by quantitative real-time PCR (qPCR) was conducted to validate the microarray results, and to examine selected genes related to lipid and sterol metabolism. Totally, 24 genes involved in metabolism of lipids and bile acids were analyzed (Table S1). Assays were performed according to MIQE standards (Bustin et al., 2009) on eight animals from each diet group (four individuals from each tank duplicate). First strand cDNA synthesis was performed using 0.8µg total RNA from all samples using Superscript III

(Invitrogen, Thermo Fisher Scientific, USA) in 20µL reactions, and primed with a mixture of Oligo(dT)$_{20}$ and random hexamer primers according to the manufacturer's protocol. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 before use and stored at -20°C. PCR primers were obtained from the literature or designed using Primer3web software version 4.0.0 (http://primer3.ut.ee/). Detailed information of the primers is shown in Table S1. All primer pairs gave a single band pattern for the expected amplicon of interest in all reactions. PCR reaction efficiency (E) for each gene assay was determined using 2-fold serial dilutions of randomly pooled cDNA. Expression of individual gene targets was analyzed using the LightCycler 480 (Roche Diagnostics). Each 10µL DNA amplification reaction contained 2µL PCR-grade water, 2µL of 1:10 diluted cDNA template (corresponding to 8ng total RNA), 5µL of LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5µL (final concentration 500nM) of each forward and reverse primer. Each sample was assayed in duplicate, including a no template control (NTC). The three-step qPCR program included an enzyme activation step at 95°C (5 min) and 40 cycles of 95°C (10 s), 60°C (10 s) and 72°C (15 s). Quantification cycle (Cq) values were calculated using the second derivative method. The PCR products were evaluated by analysis of melting curve and by agarose gel electrophoresis to confirm amplification specificity. For target gene normalization, *actb, ef1a, gapdh* and *rps20* were evaluated for use as reference genes by ranking relative gene expression according to their overall coefficient of variation (CV) and their interspecific variance (Kortner et al., 2011). The graph showed a stable expression pattern and was therefore used as normalization factor. Relative expression of target genes was calculated using the $^{\Delta\Delta}C_q$ method (Livak and Schmittgen, 2001).

2.10 Calculations

Crude protein (CP) was calculated as N x 6.25. Thermal-unit growth coefficient (TGC) was calculated as: $TGC = (FBW^{1/3} - IBW^{1/3}) \times (\Sigma D°)^{-1}$, where IBW and FBW are the initial and final body weights (tank means) and ΣD° is the thermal sum (feeding days x average temperature in °C). Feed efficiency (FE) was calculated as: (FBW-IBW)/Feed eaten. Organosomatic indices were calculated as percentages of the weight of the organ in relation to body weight. Apparent digestibility (AD) was estimated by the indirect method using Y_2O_3 as an inert marker (Austreng et al., 2000) and calculated as: $AD_N = 100 - [100 \times (M_{feed} / M_{faeces}) \times (N_{faeces} / N_{feed})]$ where M_{feed} and M_{faeces} are percent concentration of the inert marker (Y_2O_3) in feed and faeces, respectively and N_{feed} and N_{faeces} represent percent concentration of a nutrient in feed and faeces, respectively.

2.11 Statistical analyses

Data was analyzed using one-way ANOVA followed by Duncan's multiple range test for post hoc comparison, unless otherwise noted. Tank means (i.e. the mean of all individuals per tank) were used as the statistical unit. For histological results the scores generated were categorical variables and the differences between the diets were explored by contingency analysis using the chi-squared test.

3 Results

The fish appeared healthy throughout the feeding period, and no mortality was recorded.

3.1 Diet composition, including cholesterol and bile salt level

The analysed content of nutrient in the diets showed results close to the intended composition (Table 1B). As expected, diet cholesterol level was higher in the HF (1.4 g/kg) and the LF_CH (4.4 g/kg) diet, than the LF basal diet (0.7 g/kg). The HF diet contained much more bile salts than the LF diet, and the T_CA supplemented diet clearly deviated from the others as intended. The diet differences reflected the level of cholesterol and bile salts of the ingredients and the supplementation.

3.2 Effects on feed growth and nutrient digestibility

Fish growth rates and feed efficiencies are shown in Table 2. Growth, as indicated by both TGC and SGR, was in general very high for all treatments. The differences between the treatments did not reach significance. The highest result was observed for fish fed the LF_PC diet.

Apparent digestibility of lipid is shown in Table 2. Lipid digestibility was in general high for all diets. Phosphatidylcholine supplementation to the LF diet (LF_PC) increased lipid digestibility significantly compared to the LF reference and showed a result similar to the HF diet. All diets, except the LF diet, showed significantly higher lipid digestibility than the diet supplemented with taurine (LF_TA). None of the other treatments differed significantly regarding lipid digestibility. Compared to the LF treatment, crude protein digestibility increased significantly by dietary supplementation with the lower level of taurocholate (LF_TC2), and with taurine (LF_TA)

(Table 2). None of the other supplementations caused significant differences compared to the LF. The lowest protein digestibility was observed for the HF diet and the result was significantly lower than for all other treatments. Among fish fed the LF based diets, significant reduction in starch digestibility was observed for LF_PC treated fish (Table 2). No other significant differences were observed between these treatments. For the HF fed fish, starch digestibility was significantly higher than for all the other treatments, supposedly due to the lower starch level in the HF diet.

3.3 Organosomatic indices

Relative organ weights (organosomatic indices, OSI) of the pyloric (PI), mid (MI) and distal (DI) intestines, as well as liver are shown in Fig 3. Somatic indices for PI and MI differed significantly between diet groups. The OSI for PI was significantly higher in fish fed LF in comparison with the HF diet. The tissues with high somatic indices also had a whitish and foamy appearance. Among the fish groups fed the supplemented LF diet, those fed choline and phosphatidylcholine showed significantly lower OSI for PI, as well as normal colouration and texture compared to the groups fed the other LF diets. Also compared to fish fed the HF diet, the LF_PC and LF_Cl groups showed lower OSI for PI. The OSI for MI showed a similar effect of diet as OSI for PI, whereas no dietary effect was observed for OSI of the DI. The greatest difference in liver OSI was observed between fish fed LF diet and those fed the LF_PC and LF_CL diets, and the difference was close to significant ($p=0.0530$).

3.4 Chyme bile salt concentration along the intestine

No difference was observed in chyme bile salt concentration along the intestine between the HF and LF fed fish (Table 3). Fish fed the LF diets supplemented with bile salt showed elevated chyme bile salt concentration. Compared to the LF diet, the LF_TC1 diet caused significantly elevated levels in PI2, whereas both these bile salt supplemented diets produced elevated bile salt levels in DI1 and DI2. In the two distal most intestinal sections, cholesterol supplementation, LF_CH, elevated chyme bile salt concentration significantly. Moreover, PC supplementation increased bile salt concentration in DI1, and the same trend was observed in DI2.

3.5 Histology

The degree of vacuolation of the pyloric caeca were significantly lower in fish fed the HF, LF_PC and LF_Cl diets compared to all the other treatments (Fig 4). No significant differences were observed between the HF, LF_PC and LF_Cl groups. The fish fed the other diets showed moderate to severe vacuolation, with no significant difference between the groups. The DI sections of all fish showed morphological appearance typical of healthy DI mucosa, except for one individual.

3.6 Blood plasma biochemistry

Blood plasma was analyzed for free fatty acids, triglycerides, cholesterol and total bile salts (Table 4). The plasma cholesterol was significantly higher in fish fed the LF_CH diet compared to all the other treatments except for LF_PC. No significant differences were observed for the other indicators analysed.

3.7 Pyloric caeca microarray

The LF diet was used as a reference for comparison with other diets. The numbers of DEG, which reflect the magnitude of transcriptome responses to additives ranged from 25 (LF_TC1) to 171 (CF_CH) (Table 5) (See Table S2 for the list of all DEGs). The difference between the HF and the LF diet control was small – only 42 DEG. Several functional groups of immune genes showed coordinated expression changes being down-regulated in fish given the supplemented diets (Fig 5). Of note is that in HF fed fish expression of these genes was also significantly lower than in LF fed fish. The largest group (50 DEG, Fig 5) was innate antiviral immunity related, which included a number of emblematic markers of viral infections, such as *mx, viperin, ifn-induced protein 44* and *very large inducible GTPases*. The metabolic responses were relatively small but some of them might have functional consequences. All LF diets except LF_TA caused down-regulation of a small set of extracellular proteases (*chymotrypsin b*, *carboxypeptidase a2*, *proproteinase e*, *duodenase* and *elastase*) and their expression further decreased for diets with additives (Fig 6A). Nine genes with the key roles in terpenoid and steroid biosynthesis were down-regulated by LF_CH (Fig 6B) suggesting suppression of the entire pathway.

3.8 Pyloric caeca qPCR

Gene expression profiles were further studied with PCR (qPCR) focusing on genes involved in biosynthesis and transport of fatty acids, cholesterol, bile acids and phospholipids. Results are presented in Fig 7A-C. In general, the qPCR results reflected alterations in lipid and sterol metabolic pathways. The strongest responses were seen in the LF_CH, LF_PC and LF_Cl groups. In accordance with the microarray data, a clear transcriptional suppression of cholesterol uptake and biosynthesis was observed for fish fed the LF_CH diet (Fig 7A). The cholesterol

influx transporter *npc1l1* was suppressed, whereas the apical efflux transporter *abcg5* was induced, indicative of possible reduction of cholesterol uptake from the gut. The marked down-regulation of cholesterol biosynthesis was confirmed by reduced levels of the enzymes IPP synthase (*idi1*) and *cyp51* as well as the controlling transcription factor *srebp2*. On the other hand, cholesterol supplementation produced increased expression levels of the fatty acid synthesis transcription factor *srebp1*.

Alterations of genes involved in lipid metabolism were also observed for the two choline supplemented groups (LF_PC and LF_Cl) (Fig7B and C). The rate-limiting enzyme in the phosphatidylcholine synthesis (*pcyt1a)* was down regulated by phosphatidylcholine inclusion, and a similar trend was observed for choline kinase (*chk*) and *pemt*. An interesting finding was the clear suppression of a proposed marker for lipid load of non-adipogenic cells, *adipophilin/perilipin 2* (*plin2*) in both choline supplemented groups and the HF group. Phosphatidylcholine inclusion also significantly up regulated both *apoAIV* and *apoB*, involved in lipoprotein assembly. A similar trend was seen for *mgat2a*, the fatty acid transporter *fabp2* and the connected transcription factors *pparα* and *pparγ*. Choline significantly induced the expression of *apoAIV*. Genes involved in cholesterol metabolism were also significantly affected in fish fed the choline enriched diets, indicative of increased cholesterol uptake and/or synthesis. Phosphatidylcholine significantly induced the cholesterol transporter *abcg5* and the master regulator *srebp2*. Choline induced the expression of the two cholesterol biosynthetic enzymes *idi1* and *cyp51* in addition to *srebp2* (Fig 7A).

For the other supplemented diets few significant changes in gene expression were seen. The two taurocholate groups, LF_TC1 and LF_TC2 showed stable transcript profiles for the selected genes, in accordance with microarray data. Induced expression of *idi1* and *cyp51* was seen for the LF_CY diet (Fig 7A), which could indicate an increased capacity for cholesterol biosynthesis. Some differences between the LF and the HF basal diets were seen, which could reflect the different degree of lipid accumulation between these two diet groups. As previously noted, the lipid load marker *plin2* was clearly induced in the LF diet as compared to the HF diet. In contrast, reduced levels of expression of apolipoproteins (*apoaI, apoaIV, apoB*) were observed (Fig 7B).

4 Discussion

The discussion below is organized as follows. Firstly, the results for the fish fed the LF diet are compared to the results for those fed the HF diet. Thereafter effects of the individual supplements are discussed.

4.1 LF versus HF diet

The 50% lower cholesterol and 30% lower bile salt content of the LF diet compared to the HF diet did not induce significant differences in plasma cholesterol, plasma bile salt level or sterol and bile-related gene expression, indicating that the body's endogenous cholesterol synthesis and further conversion to bile acids compensated for the lower supply to fish fed the LF diet. These findings are in line with the results of an earlier study comparing diets varying in plant ingredients (Kortner et al., 2016). The absence of effects on plasma free fatty acids, triglycerides and bile salts are in line with the results of the study by Kortner et al. (2016). However, in earlier

feeding experiments with salmon, major drops in plasma cholesterol and bile salt have been observed in fish fed diets with high inclusion of plant ingredients compared fish fed diets high in fish meal, in particular when soybean meal has been included (Kortner et al., 2013; Romarheim et al., 2008; Romarheim et al., 2006). Varying dietary levels of compounds with the ability to compete with cholesterol for absorption, such as phytosterols and saponins, may be the explanation for the difference between experiments (Krogdahl et al., 2015). Another point to keep in mind is that fish meal level in commercial salmon diets has dropped significantly the last 10-20 years. In earlier studies the fishmeal control diets contained typically around 50-60% of fish meal. Today diets with around 30% of fishmeal represents a high fishmeal control diets, based on today's practical salmon diets.

The macroscopically whitish and foamy appearance of the pyloric intestine and the histological observations of excessive lipid droplet accumulation in the fish fed the LF diet is in accordance with our previous study (Gu et al., 2014). The increased relative weight of the PI and MI observed in LF fed fish were most likely a result of an increase in lipid content due to increased lipid vacuolation [Hansen et al., 2019, manuscript submitted]. This lipid accumulation was most likely a result of reduced lipid transport from the intestinal mucosa to the circulatory system. The observed symptoms were similar to those described in detail for Arctic charr (200 – 250 g) by Olsen and co-workers (Olsen et al., 2000; Olsen et al., 1999). The charr were fed semi-purified diets with linseed oil as the only lipid source. The authors suggested deficiency of certain fatty acids as the plausible explanation for the lipid accumulation. Excessive lipid accumulation in the enterocytes due to deficiency of essential fatty acids has recently been documented also for Atlantic salmon (Bou et al., 2017). Enterocyte lipid accumulation has been observed in PI of

rainbow trout (500 g) fed a diet containing either fish oil, soybean oil or soybean lecithin as the only fat source (Olsen et al., 2003), whereas only minor accumulation was observed in fish fed diets with fish oil and soybean lecithin. Based on these results, the authors concluded as follows: fish may require exogenous phospholipids in order to sustain a sufficient rate of lipoprotein synthesis and phosphatidylcholine was suggested to be the key compound in this context. A study of development in fish of expression of genes involved in the pathways for the production of phospholipids have shown low values at the early stages (Carmona-Antonanzas et al., 2015; De Santis et al., 2015). However, a phosphatidylcholine requirement of Atlantic salmon after juvenile stage has not been established (NRC, 2011).

Despite clear differences in gut mucosa structure and enterocyte hypervacuolation, changes in gene expression were small or moderate by magnitude, as evaluated by a combination of microarray analyses and qPCR assays targeting the genes involved in lipid and sterol metabolism. This contrasts with the major transcriptional changes associated with other intestinal disturbances in salmon, such as dietary induced inflammation (Kortner et al., 2012). Slight but consistent down-regulation of immune genes from several functional groups and pathways might be interpreted as mild immune suppression with CH being the most potent. Down-regulation of several digestive proteolytic enzymes was observed, which, nonetheless did not affect growth and feed efficiency. Expression changes of genes involved in lipid metabolism were limited. Intestinal lipid absorption and transepithelial transport, including temporary storage of lipid in cytosolic lipid droplets, are natural metabolic processes undertaken by all healthy animals upon ingestion of a high fat meal. It is possible that the lipid load in the present study did not exceed the threshold that requires compensatory changes of transcriptome. However, the trend towards

lower lipid digestibility in the fish fed the LF diet may indicate that the fish' capacity for lipid absorption was surpassed, leading towards lipid malabsorption syndrome (LMS) (Hanche-Olsen, 2013; Penn, 2011). Lack of strong gene expression responses could also be related to the general understanding that Atlantic salmon most likely do not encounter such problems in their natural environment and mechanisms for adaptation to high-fat plant diets have not evolved.

4.2 Choline supplements to the LF diet

The most pronounced effects of the choline supplementation were the significant reduction in lipid vacuolation in pyloric intestine and the organo-somatic index for the pyloric intestine (PISI) and mid intestine (MISI) compared to all the other treatments. These observations corresponded to the histological findings showing normal, low degree of enterocyte vacuolation in the proximal intestine region in the LF_Cl fed fish, in contrast to the marked to severe vacuolation in all the other LF treatments, except the LF_PC (see discussion below). These results indicate that dietary choline is a key component for efficient transport of lipid across the intestinal mucosa and that the level in the LF basal diet, in the form of free and bound choline, was not sufficient. These results correspond to our previous study [Hansen et al., 2019, manuscript submitted] and are further in line with the key role choline plays in lipid transport as part of phosphatidylcholine, an essential, structural component of lipoproteins (Harvey, 2011). Choline metabolism in fish has not been studied in sufficient detail in fish. It is however, likely that choline is metabolized in salmonids as in monogastric mammals. A major difference may be the route from the enterocytes to the peripheral tissues as salmonids seems to lack a lymphatic system. Also birds lack lymphatic vessels in the mesentery (Whittow, 2000). Without lymphatic vessels, the major route for lipoproteins and their phosphatidylcholine would be via *vena porta*,

directly to the liver. Free dietary choline is absorbed by the enterocytes via choline transporters and immediately phosphorylated to phosphocholine and further bound to diacylglycerol to form phosphatidylcholine (Fig 1) (Li and Vance, 2008). Phosphatidylcholine entering the intestine, with food or in bile, is hydrolyzed by phospholipases to lysophosphatidylcholine before uptake into the enterocyte and re-esterification to phosphatidylcholine. Another supply of phosphatidylcholine is endogenous synthesis in the liver from serine, activated diglycerides, ethanolamine and methyl groups from methionine as described in Fig 1. Endogenous synthesis of phosphatidylcholine seems to be sufficient for many animals at most life stages. However, for some species, such as chickens, an absolute requirement for choline has been determined (NRC, 1994). A requirement is established also for several fish species, but only at the early life stages (NRC, 2011), and not yet for Atlantic salmon. An early study of effects of dietary supplementation of phospholipid and choline in Atlantic salmon weighing from 1.0 to 7.5 g indicated, based on growth rate, that the smallest fish required a dietary supply, but not the larger (Poston, 1990). Since these conclusions, research on phospholipid and choline metabolism in Atlantic salmon has been conducted only with very young salmon. The most recent studies, also investigating very young fish, have for the first time addressed lipoprotein metabolism in the intestine of the Atlantic salmon (Jalili et al., 2019; Jin et al., 2018a; Jin et al., 2018b). These studies seem to confirm that phospholipid metabolism is immature in the young Atlantic salmon and that an exogenous supply may be necessary.

The results of the molecular studies in the present study are in accordance with our previous work [Hansen et al., 2019, manuscript submitted] and confirm the role of choline in lipoprotein assembly and lipid transport. Choline supplementation, in both of our studies, seemed to cause a

down-regulation of phosphatidylcholine synthesis by decreasing *pcyt1a*. It also seemed to promote intracellular lipid transport by inducing *apoaIV* expression and reducing intracellular lipid storage as indicated by the reduced expression of *plin2*. However, the overall transcriptome response to the choline supplementation was low, as pointed out above. A previous study conducted on first feeding salmon fry has also documented relatively stable transcriptome profiles after dietary phospholipid supplementation (De Santis et al., 2015).

4.3 Phosphatidylcholine supplements to the LF diet

The effects of supplementation with PC, were very similar to those caused by supplementation with choline regarding organ indices, lipid vacuolation of the PI and intestinal gene expression. In line with the fact that choline is an integrated component of PC, this was as expected. PC supplementation significantly increased the lipid digestibility by 2.4%, compared to the unsupplemented diet. Choline supplementation elevated the lipid digestibility less than for PC with 1.1%. The explanation for greater effect of PC was most likely related to the role of PC, as one of several phospholipids, in emulsification of lipid in the stomach and intestine and its role in micelle formation following hydrolysis (Bauer et al., 2005). Based on these considerations, it appears likely that supplementation of a choline/PC deficient diet with PC would more efficiently rectify the effects of the deficiency on lipid digestibility. The changes in lipid digestibility by PC would be interesting to follow up in further investigations.

4.4 Methionine supplements to the LF diet

The lack of effects of methionine supplementation suggests that the methyl groups of methionine are not available for the synthesis of phosphatidylcholine from phosphatidylserine, via the

pathway known to function in the liver of some other animals, and as shown in Fig 1. This is in line with the results of Rumsey et al (Rumsey, 1991) who investigated choline requirement in the young rainbow trout. No replacement value for choline was observed for methionine supplementation. This implies that, Atlantic salmon in salt water, need a dietary supply of choline or phosphatidylcholine. According to nutritional principles, choline will be defined as the essential nutrient, as phosphatidylcholine can be synthesized if sufficient choline is present.

4.5 Taurocholate, taurine and cysteine supplements to the LF diet

Taurocholate, at both inclusion levels, affected only biomarkers related to bile salt metabolism, and the responses were as expected. The only exception was an elevation in protein digestibility observed for the low taurocholate level (LF_TC2), i.e. compared to LF. This might be due to the stabilizing effect of bile salts on intestinal proteases, such as trypsin and chymotrypsin making them more resistant to degradation. Higher stability may secure higher efficiency of protein hydrolysis (Maldonado-Valderrama et al., 2011; Gass et al., 2007). The highest level of taurocholate supplementation (LF_TC1) did not further increase protein digestibility. The explanation might be that the maximum effect was obtained with the lower dose. No indications of either beneficial or detrimental alterations associated with diet-induced enteritis were observed upon inclusion of taurocholate in the diet, in contrast to the detrimental effects of higher levels shown in our previous study (Kortner et al., 2014).

Supplementation with taurine, the conjugate, in taurocholate, did not affect any of the observed biomarkers. The exception was an elevated protein digestibility similar to the effects of the LF_TC2 diet. The plasma level of bile salts in fish fed the LF_TA diets were high, and similar to

the levels of the LF_TC fed fish. It may be suggested that the positive effect on protein digestibility, as for the LF_TC fed fish, was related to higher bile salt concentration in the chyme.

In line with the lack of responses of taurine supplementation, responses to supplementation with cysteine, a precursor of taurine, were also insignificant.

4.6 Cholesterol supplements to the LF diet

In general, the cholesterol supplementation produced expected effects on sterol and bile salt metabolism and confirms the findings in our previous study (Kortner et al., 2016; Kortner et al., 2014). Sterol uptake was suppressed as well as *de novo* cholesterol biosynthesis and, hence, induction of sterol efflux from the intestinal mucosa. The magnitude of response in gene expression was lower in the present study as compared to our earlier work. Lower cholesterol dose used in the present study (0.2% vs. 1.5%) is the likely explanation for this difference.

5 Conclusions

Choline is an essential nutrient for Atlantic salmon in seawater, particularly important in lipid transport across the intestinal mucosa. Phosphatidylcholine is a good source of choline. Neither supplementation with methionine, cysteine, taurine nor taurocholate diminished the symptoms of choline deficiency. High plant diets for Atlantic salmon must include a choline or phosphatidylcholine source.

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Competing interests

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Supplementary data

S1 Table. Primer pair sequences, efficiency, amplicon size and annealing temperature for the genes used for real-time PCR.

S2 Table. Normalized log2-FC to the means of all samples. Significant differentially expressed genes (DEG) as compared to the LF reference are underlined.

Figure captions

Fig 1. Main pathways of compounds important for supply of phosphatidylcholine. Phosphatidylcholine supplied by the diet is hydrolyzed to lysophosphatidylcholine in the intestine, absorbed and reesterified to phosphatidylcholine in the enterocyte. Dietary free choline is transported as such through the brush border into the enterocytes, followed by activation via the Kennedy pathway, i.e. by use of both ATP and cytidine triphosphate (CTP) and fusion with diacylglycerol (DAG), producing phosphatidylcholine. Phosphatidylcholine circulating in the blood and bile is also part of the phosphatidylcholine pool. Endogenous synthesis is possible if the necessary substrates are available, i.e. phosphatidylethanolamine which can be produced from serine by incorporation of serine into phosphatidylserine, through the Kennedy pathway, and subsequent decarboxylation to generate phosphatidylethanolamine. However, the Kennedy pathway from serine appears to be insufficiently developed in many animal species, in particular in young individuals. Ethanolamine is converted to phosphatidylcholine after three methylation steps catalyzed by phosphatidylethanolamine methyl transferase (PEMT). The condition for the methylations, is sufficient supply of methionine as methyl donor as well as of the B-vitamins folic acid, cobalamine (B12), pyridoxine (B6) and niacin, necessary for remethylation of the donor molecules. Dietary supply of taurine may also be of importance for the size of the

740 phosphatidylcholine pool, as taurine is needed for conjugation of bile acids, and is produced
741 from methionine, via cysteine. Low supply may reduce availability of methyl groups for
742 formation of choline. Phosphatidylethanolamine can also be supplied from the diet either as
743 such, or as free ethanolamine, and follows the same pathways of absorption as indicated for
744 dietary phosphatidylcholine and choline. In the figure, blue colored compound indicate those
745 investigated in the present study regarding importance for phosphatidylcholine availability in an
746 animal. (Adopted from Harvey R.A., 2011).
747 Fig 2. Histological severity of vacuolation of the pyloric caeca tissue. Pyloric caeca with enterocyte
748 hypervacuolation graded as 1=no vacuolation/normal (left image) and 4=moderate to severe
749 hypervacuolation (right image).

750 Fig 3. Gut and liver somatic indices (OSI). PI=pyloric intestine; MI=mid intestine, DI=distal
751 intestine. Different letters denote significant differences between diet groups. Error bars indicate
752 SEM.

753 Fig 4. Degree of vacuolation of PI tissue. The columns indicate average score for fish fed the
754 different diets. Score 1=Normal, Score 4=Moderate to high vacuolation. Different letters denote
755 significant differences between diet groups. Error bars indicate SEM.
756 Fig 5. Groups of immune genes with correlated expression profiles (microarray analyses). The
757 numbers of genes are indicated in parentheses. Data are mean log^2-Expression Ratios ± SE,
758 significant differences from LF are indicated with asterisks.

759 Fig 6. Differentially expressed genes with metabolic functions (microarray analyses). A:
760 extracellular proteases. Data are mean log2-Expression Ratios ± SE, significant differences from
761 LF are indicated with asterisks. B: genes of steroid biosynthesis pathways, data are LF_CF to LF
762 ratio (folds), all differences are significant.

763 Fig 7A-C. Gene expression profiling of pyloric caeca samples by qPCR. Values are expressed as
764 mean normalized expression (MNE), with their standard errors represented by bars (n=8 fish per
765 group). Different levels denote significant differences between diet groups ($p<0.05$). For full
766 genes names see S1 Table.

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768 ## Tables

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777 **Table 1A. Feed ingredient composition*.**

Ingredients	HF	LF	LF_TC1	LF_TC2	LF_CH	LF_TA	LF_PC	LF_CI	LF_CY	LF_ME
FM Westland, %	14.78	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95
FM Super prime, %	14.78	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95
Soy protein concentrate, %	8.31	19.80	19.80	19.80	19.80	19.80	19.80	19.80	19.80	19.80
Corn gluten, %	4.93	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95	4.95
Pea protein, %	9.14	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87
Wheat gluten, %	0.00	7.84	7.84	7.84	7.84	7.84	7.84	7.84	7.84	7.84
Beans dehulled, %	12.81	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87	12.87
Sunflower exp, %	9.05	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76	1.76
Fish oil (std), %	7.17	7.63	7.63	7.63	7.63	7.63	7.63	7.63	7.63	7.63
Rapeseed oil, %	16.72	17.80	17.11	17.45	17.60	17.72	16.29	17.43	17.72	17.70
Methionine, %	0.21	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44	0.44
Lysine, %	0.01	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66
Threonin, %	0.07	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Histidine, %	0.27	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39	0.39
Vit/Min mix, %	0.37	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Mono calcium phosphate, %	1.27	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41	2.41
Barox %	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Yttriuim, %	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Taurocholate, g/kg			6.9	3.5						
Cholesterol, g/kg					2.0					
Taurine, g/kg						0.8				
Phosphatidylcholine (95%), g/kg							15.1			
Choline chloride (70 %), g/kg								3.7		
Cysteine, g/kg									0.8	
Methionine, g/kg										1.0

778

779 *HF, high fishmeal diet; LF, low fishmeal diet; LF_TC1 and TC2, supplemented with

780 taurocholate; LF_CH, supplemented with cholesterol, LF_TA, supplemented with taurine;

781 LF_PC, supplemented with phosphatidylcholine; LF_Cl, supplemented with choline chloride,

782 LF_CY, supplemented with cysteine; LF_ME, supplemented with methionine.

783

784 Table 1B. Macronutrient (%), cholesterol, choline and bile salt content of the diets (mg/kg) as
785 analyzed

Diet	HF	LF	LF_TC1	LF_TC2	LF_CH	LF_TA	LF_PC	LF_Cl	LF_CY	LF_ME
Lipid	29.0	26.0	26.9	26.1	26.6	27.3	28.3	26.4	27.0	27.7
Protein	39.8	41.2	40.0	41.1	40.6	40.7	41.3	40.9	39.2	40.5
Starch	5.3	6.9	6.8	7.0	6.8	6.8	6.5	7.0	7.1	7.0
Choline, free	410	411	403	407	419	408	401	2180	423	393
Choline, total	1860	1190	1180	1170	1190	1180	2870	2980	1200	1160
Cholesterol	1.42	0.70	0.75	0.65	4.45	0.66	0.74	0.63	0.61	0.67
Tot bile salt	274	86	4840	2534	86	86	86	86	86	86

786

787

788 Table 2. Growth (SGR/TGC), feed efficiency (FE) and apparent digestibilities.

				Apparent digestibility		
Diet	SGR	TGC	FE	Crude lipid	Crude protein	Starch
HF	1.41	4.39	1.20	96.9^{abc}	85.9^{d}	73.8^{a}
LF	1.37	4.22	1.27	95.7^{bcd}	89.4^{bc}	63.2^{bcde}
LF_TC1	1.36	4.18	1.24	97.4^{ab}	89.9^{abc}	68.2^{b}
LF_TC2	1.34	4.15	1.25	97.7^{ab}	90.4^{a}	65.7^{bcd}
LF_CH	1.37	4.31	1.27	96.8^{abc}	89.2^{c}	59.6^{ef}
LF_TA	1.37	4.25	1.24	94.3^{d}	90.2^{a}	67.5^{bc}
LF_PC	1.45	4.53	1.24	98.1^{a}	90.1^{ab}	57.0^{f}
LF_CI	1.36	4.21	1.21	96.8^{abc}	90.3^{abc}	60.9^{def}
LF_CY	1.35	4.15	1.31	97.3^{abc}	89.8^{abc}	67.5^{bc}
LF_ME	1.32	4.08	1.30	96.6^{abc}	89.9^{abc}	62.4^{cde}
P(model)	0.0642	0.0768	0.1590	0.0412	<0.0001	0.0005
Pooled SEM	0.022	0.081	0.025	0.64	0.23	1.56

789 SGR=specific growth rate; TGC=thermal growth coefficient; FE=feed efficiency. For
790 explanation of diet codes see Table 1A and B.

791

792 Table 3. Bile salt concentration in chyme along the intestinal tract, mg/g dry matter.

Diet	PI1	PI2	MI	DI1	DI2
HF	228	170^{b}	127	52de	11^{b}
LF	212	149^{b}	121	39^{e}	5^{b}
LF_TC1	259	234^{a}	154	77^{a}	19^{a}
LF_TC2	249	190^{b}	154	74^{ab}	18^{a}
LF_CH	219	165^{b}	132	71^{abc}	18^{a}
LF_TA	219	187^{b}	132	53de	8^{b}
LF_PC	212	182^{b}	155	58^{bcd}	11^{b}
LF_CI	208	190^{b}	156	56^{cde}	10^{b}
LF_CY	188	160^{b}	143	44de	6^{b}
LF_ME	251	177^{b}	129	56^{cde}	12^{ab}
P (model)	0.1923	0.0313	0.7225	0.0039	0.0041
Pooled SEM	12.5	9.4	13.0	4.7	1.6

793 PI=pyloric intestine; MI=mid intestine, DI=distal intestine. For explanation of diet codes see
794 Table 1A and B.

795

796 Table 4. Blood indicators of lipid and sterol metabolism.

Diet	FFA, mM	Cholesterol mM	TG mM	Bile salts, uM
HF	0.30	10.3[b]	3.5	14
LF	0.27	8.8[b]	2.9	20
LF_TC1	0.42	9.8[b]	2.9	35
LF_TC2	0.36	9.2[b]	3.2	34
LF_CH	0.41	13.6[a]	3.2	30
LF_TA	0.41	9.0 [b]	3.2	34
LF_PC	0.26	11.7[ab]	4.1	20
LF_CI	0.36	10.6[b]	3.5	26
LF_CY	0.31	8.8[b]	3.1	20
LF_ME	0.38	8.8[b]	3.2	25
P(model)	0.8764	0.0255	0.7433	0.4438
Pooled SEM	0.08	0.86	0.40	6.54

797 FFA=free fatty acids. TG=triglycerides. For explanation of diet codes see Table 1A and B.

798

799 Table 5. The number of differentially expressed genes (DEG).

Supplemented diets	HF	LF_TC1	LF_TC2	LF_CH	LF_TA	LF_PC	LH_Cl	LF_CY	LF_ME
Number of DEG	42	25	40	171	75	65	38	57	78

800 Supplement diet groups compared to the low fish meal control group (LF).

801

Figure 1:

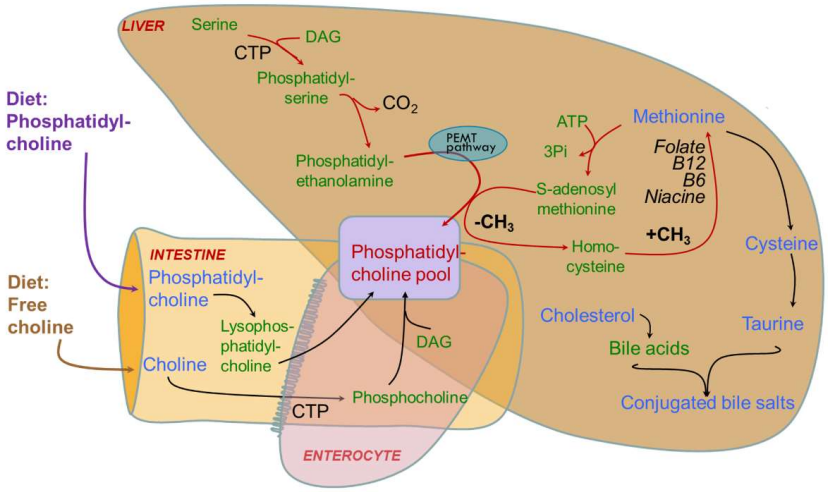


Figure 2:

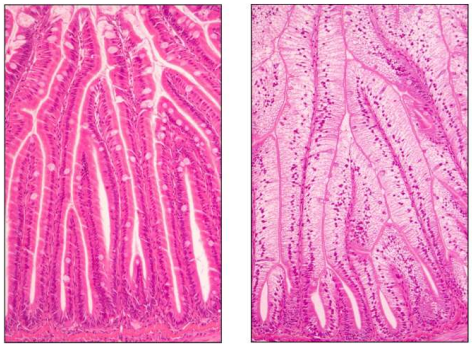


Figure 3:

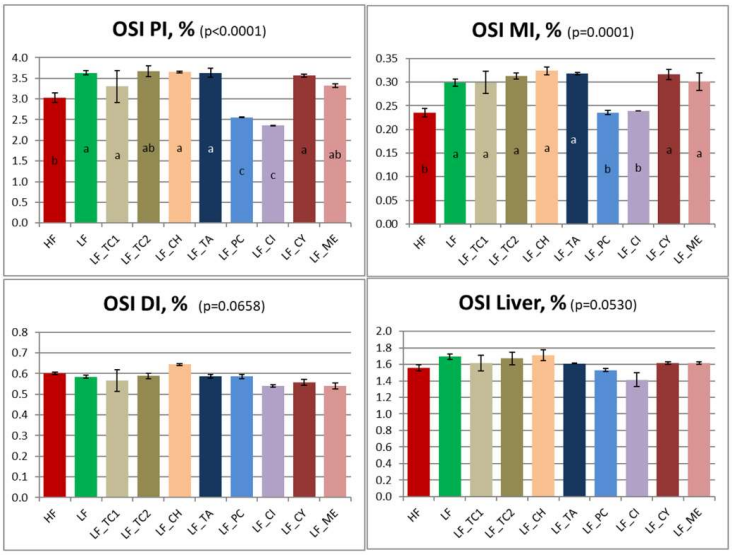


Figure 4:

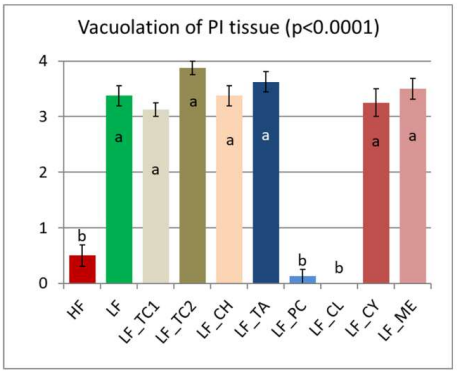


Figure 5:

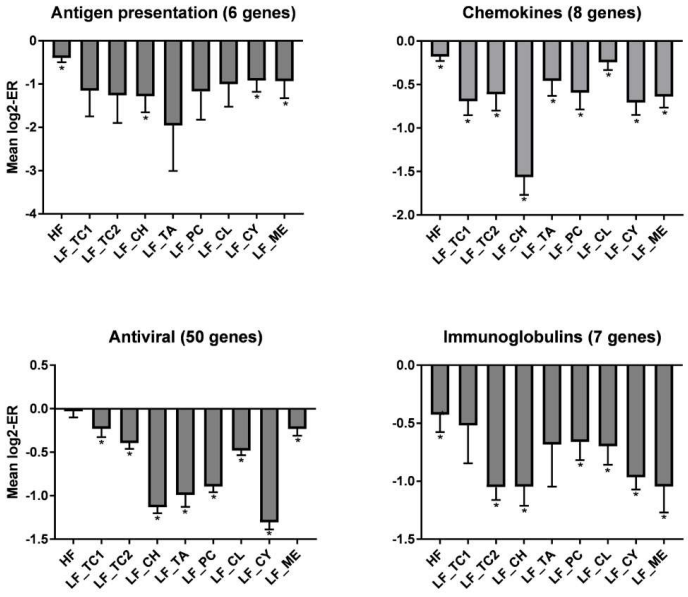
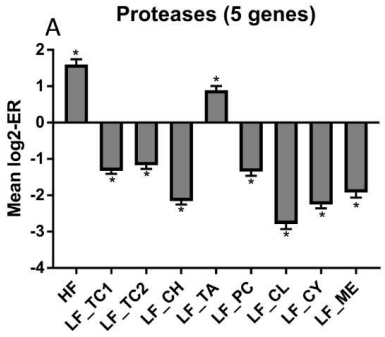


Figure 6:



B

Genes	CH/LF Fold
3-keto-steroid reductase	-2.26
7-dehydrocholesterol reductase	-2.43
Diphosphomevalonate decarboxylase	-4.05
Farnesyl pyrophosphate synthetase	-3.10
Hydroxymethylglutaryl-CoA synthase	-2.47
Isopentenyl-diphosphate Delta-isomerase	-2.88
Lanosterol 14-alpha demethylase	-4.18
Squalene monooxygenase	-4.49
Squalene synthase	-2.87

Figure 7A:

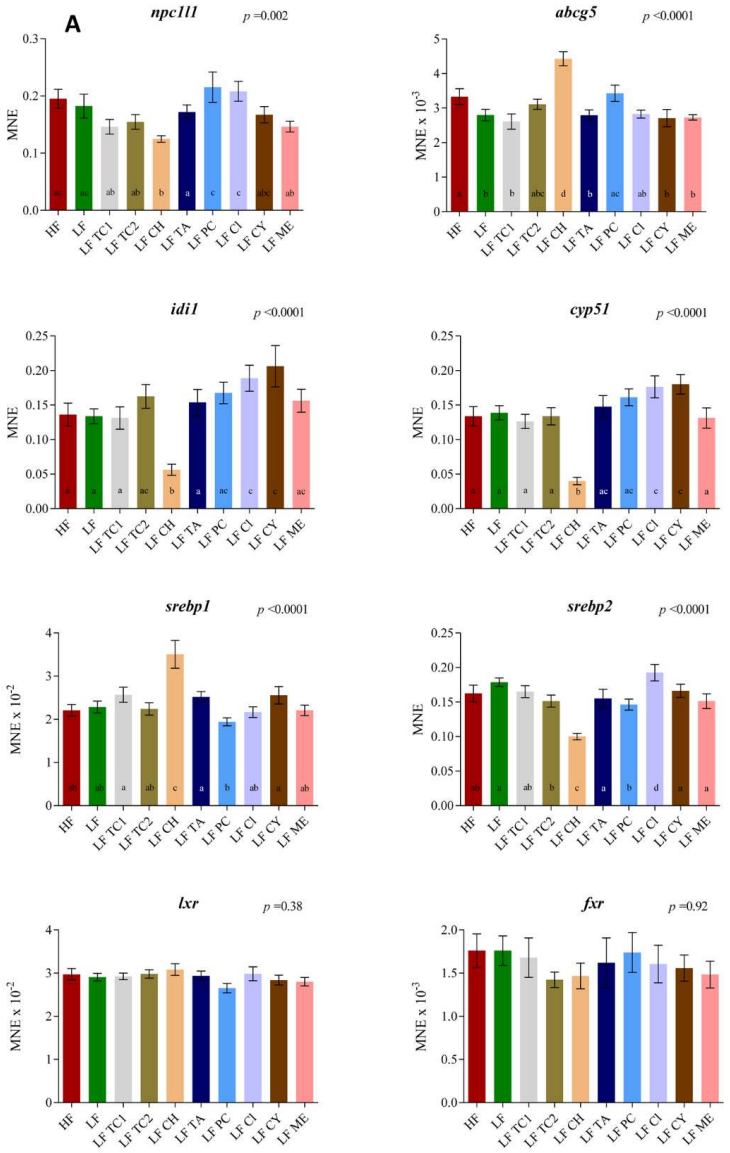

A
npc1l1
p =0.002
abcg5
p <0.0001
idi1
p <0.0001
cyp51
p <0.0001
srebp1
p <0.0001
srebp2
p <0.0001
lxr
p =0.38
fxr
p =0.92
MNE
MNE x 10-2
MNE x 10-3
HF
LF
LF TC1
LF TC2
LF CH
LF TA
LF PC
LF CI
LF CY
LF ME

Figure 7B:

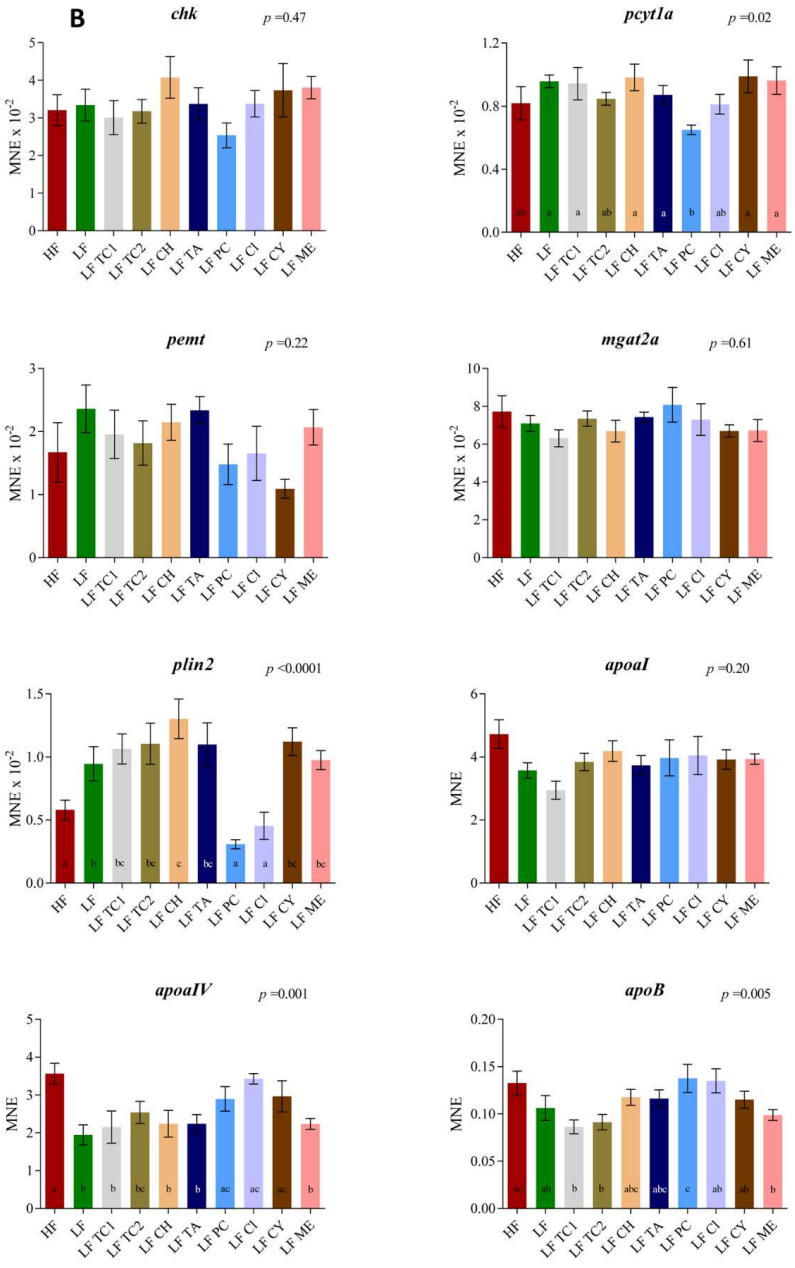
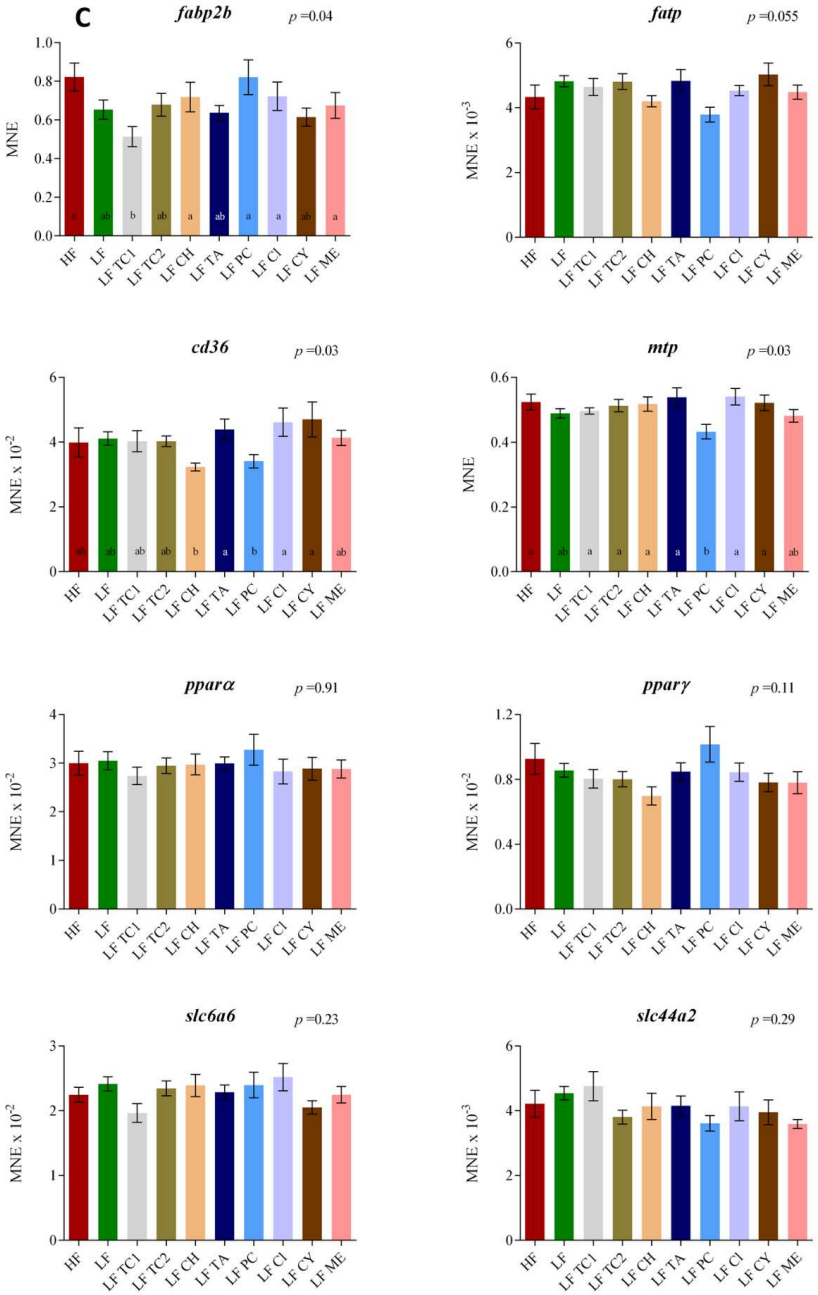

B
chk
p =0.47
MNE x 10-2
pcyt1a
p =0.02
MNE x 10-2
pemt
p =0.22
MNE x 10-2
mgat2a
p =0.61
MNE x 10-2
plin2
p <0.0001
MNE x 10-2
apoaI
p =0.20
MNE
apoaIV
p =0.001
MNE
apoB
p =0.005
MNE
HF
LF
LF TC1
LF TC2
LF CH
LF TA
LF PC
LF CI
LF CY
LF ME

Figure 7C:


C
fabp2b
p =0.04
MNE
fatp
p =0.055
MNE x 10-3
cd36
p =0.03
MNE x 10-2
mtp
p =0.03
MNE
pparα
p =0.91
MNE x 10-2
pparγ
p =0.11
MNE x 10-2
slc6a6
p =0.23
MNE x 10-2
slc44a2
p =0.29
MNE x 10-3
HF
LF
LF TC1
LF TC2
LF CH
LF TA
LF PC
LF CI
LF CY
LF ME

S1 Table. Primer pair sequences, efficiency, amplicon size and annealing temperature for the genes used for real-time PCR

		5'–3' primer sequence						
Name	Gene symbols	Forward	Reverse	Amplicon size (bp)	Anealing temperature (°C)	Primer efficiency	GeneBank accession no.	Reference
Adipophilin/perilipin 2	*plin2*	cccaggtctactccagcttc	cagcgactccttcatcttgc	104	60	2.0	XM_014155742	This study
Apolipoprotein AI	*apo-AI*	ctggtcctcgcactaaccat	tggacctctgtgcagtcaac	144	60	2.0	NM_001123663	7
Apolipoprotein AIV	*apo-AIV*	caggaccagtctcagcaaca	gttgacttcctgtgccacct	131	60	1.9	BT048822	7
Apolipoprotein B48	*apo-B48*	ccctgagatggtgtccgtat	gcgtcgacttccatagcttc	131	63	1.8	CB504205	7
ATP-binding cassette G5	*abcg5*	agactgcctcgtccaacact	ccattttcgtgaacgtgtacc	157	60	1.9	CU073172	5
Beta-actin	*actb**	caaagccaacagggagaagatga	accggagtccatgacgatac	133	60	1.9	AF012125	1
Choline kinase	*chk*	ctcaagtttgcccgtctgat	cacaggggaatgagtggagt	88	60	1.9	DY706802	7
Choline transporter-like protein 2	*slc44a2*	tcgtcatcattttgctgctc	aggcgatgacaatggatagg	152	60	2.0	NM_001140367	This study
Choline-phosphate cytidyltransferase	*pcyt1a*	cgggtctatgcagatggaat	gctcgtcctcgttcatcact	166	60	2.1	BT045986	7
Cluster of differentiation 36	*cd36*	caagtcagcgacaaaccaga	aggagacatggcgatgtagg	91	60	1.9	AY606034	7
Cytochrome P450 51	*cyp51*	tgcattggggagaacttgc	atctgatgacggggttgtgt	148	60	1.9	XM_014177708	2
Elongation factor 1 alpha	*ef1a**	gtgctgtgcttatcgttgct	ggctctgtggagtccatctt	148	60	1.9	AF321836	1
Farnesoid X receptor	*fxr*	ttcaacatctcaactcatca	tagcaggtcctcattgat	102	60	2.0	NM_001173830	5
Fatty acid binding protein 2B	*fabp2b*	tgccttcccctcattctcta	ggtgatacggtcttcatccaa	82	60	2.0	EU880419	4
Fatty acid transport protein	*fatp*	aggagagaacgtctccacca	cgcatcacagtcaaatgtcc	159	60	1.9	CA373015	7
Glyceraldehyde-3-phosphate dehydrogenase	*gapdh**	aagtgaagcaggagggtgga	cagcctcacccatttgatg	96	60	1.9	BT050045	1
Isopentenyl-diphosphate delta isomerase 1	*idi1*	tacctcccaaaatggcactc	cgtccctcatagcagcttc	132	60	1.9	XM_014157452	2
Liver X receptor	*lxr*	gccgccgctatctgaaatctg	caatccggcaaccaatctgtagg	210	60	1.9	FJ470290	3
Microsomal triglyceride transfer protein	*mtp*	aacgtgacagtggacatgga	ggaccgtggtgatgaagtct	89	60	2.0	CA042356	7
Monoacylglycerol acyltransferase 2-A	*mgat2a*	acgctacaggcttcaggaaa	ggaatcagacctgccatcat	116	60	2.0	NM_001140718	7
Niemann-Pick C1 like 1	*npc1l1*	ccaaagacctgatcctggaa	cgaagcacacatccttcaga	108	60	1.9	XM_014171081	2
Peroxisome proliferator activated receptor alpha	*ppara*	gcttcatcaccagggagttt	tcactgtcatccagctccag	113	60	2.0	NM_001235960	4
Peroxisome proliferator activated receptor gamma	*pparγ*	tgctgcaggctgagtttatg	caggggaaagtgtctgtggt	107	58	2.0	NM_001235946	4
Phosphatidylethanolamine N-methyltransferase	*pemt*	gttgctgtcatcgccatcat	gaggaggatgatgagggtgc	141	60	2.0	XM_014158251	This study
Ribosomal protein S20	*rps20**	agccgcaacgtcaagtct	gtcttggtgggcatacgg	98	60	2.0	NM_001140843	1
sterol element regulatory binding protein 1	*srebp1*	gccatgcgcaggttgtttcttca	tctggccaggacgcatctcacact	151	63	1.9	HM561860	3
Sterol element regulatory binding protein 2	*srebp2*	tcgcggcctcctgatgatt	agggctaggtgactgttctgg	147	60	1.9	HM561861	3
Taurine transporter	*slc6a6*	ggaggtggaaggacagatca	acatgccacctttcgttacc	143	60	2.0	NM_00112363	6

* reference genes

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S2 Table. Normalized log2-FC to the means of all samples

Significant differentially expressed genes (DEG) as compared to the LF reference are underlined.

Accession	Gene	HF	LF-CH	LF-CI	LF-CY	LF-ME	LF-PC	LF-TA	LF-TC1	LF-TC2	LF
EG835598	11-beta-hydroxysteroid dehydrogenase	0.79	-0.15	-0.14	0.14	-0.33	0.22	-0.27	0.03	-0.09	-0.19
89865375	40S ribosomal protein S2 [Salmo salar]	0.09	0.19	-0.20	0.26	0.40	-0.21	-0.32	-0.11	-0.07	-0.03
89865458	Tyrosine-protein kinase yes - Ident 43	0.23	0.61	-0.12	-0.04	0.45	-0.34	-0.05	-0.37	-0.15	-0.21
89866541	Metastasis associated family_ member 3	0.22	0.79	-0.16	0.01	0.75	-0.82	-0.12	-0.33	-0.04	-0.31
89869070	insulin receptor 2 [Oncorhynchus mykiss]	-0.50	-0.02	0.18	-0.22	0.30	0.15	0.00	-0.08	-0.17	0.37
89870321	Unknown	0.34	-0.54	-0.23	-1.04	0.58	-0.56	0.70	-0.12	0.42	0.45
89870381	actin related protein 2/3 complex subunit 2 [Salmo salar]	0.22	0.62	-0.04	-0.10	0.50	-0.47	-0.13	-0.17	-0.23	-0.19
89873507	Transcription factor RelB [Salmo salar]	-0.31	0.60	-0.57	-0.25	0.37	-0.25	0.00	-0.05	0.05	0.42
89874626	Gelsolin precursor [Salmo salar]	0.15	-0.45	0.75	0.52	-0.47	0.71	-0.12	0.15	-0.79	-0.44
89874750	AF133701_10 NADH dehydrogenase subunit 4 [Salmo salar]	0.38	0.66	-0.16	0.00	0.39	-0.49	-0.47	0.10	-0.16	-0.26
89880186	Proteasome assembly chaperone 2	0.25	0.03	-0.21	0.07	-0.12	-0.42	-0.04	0.33	-0.21	0.32
89880639	Acid phosphatase-like protein 2 - Ident 23	-0.58	-0.06	-0.23	0.20	-0.98	0.27	0.14	0.40	0.43	0.42
89882046	Eukaryotic initiation factor 4A-III	0.05	0.68	0.00	0.26	0.35	-0.49	-0.35	0.29	-0.30	-0.50
89884753	Cytoplasmic polyadenylation element-binding protein 4 [CPE-BP4]	0.14	0.60	-0.05	0.02	0.28	-0.35	-0.07	-0.38	0.29	-0.48
117428815	Novel protein similar to vertebrate spectrin_ beta_ non-erythrocytic 1 (SPTBN1) - Ident 24	0.83	-0.14	0.20	0.06	-1.10	0.21	-0.73	0.00	0.18	0.50
117429126	Novel protein with a Zinc finger_ C3HC4 type (RING finger) domain - Ident 39	0.12	-0.62	0.59	-0.21	-0.34	0.03	-0.07	0.30	-0.29	0.48
117430714	G protein-coupled receptor kinase 4	0.34	-0.11	-0.15	-0.11	-0.10	0.22	0.50	-0.23	0.15	-0.52
117436038	Polyprotein - Ident 24	-0.02	-0.64	0.51	0.23	-0.83	0.46	-0.14	-0.31	0.05	0.69
117441735	T cell receptor alpha [Salmo salar]	0.33	0.24	0.08	-0.18	0.17	-0.11	0.09	-0.05	-0.71	0.15
117456696	PDZ and LIM domain protein 7 - Ident 33	-0.05	0.06	0.17	-0.25	-0.05	-0.06	-0.12	-0.03	0.06	0.27
117462036	Coatomer subunit epsilon [Oncorhynchus mykiss]	-0.26	-0.20	0.13	0.09	-0.60	-0.04	0.02	0.12	0.44	0.29
117469011	Ubiquitin carboxyl-terminal hydrolase 12	0.13	0.57	-0.26	0.04	0.57	-0.15	-0.37	-0.32	-0.16	-0.06
117474887	Bloodthirsty - Ident 38	0.04	-0.28	0.64	0.05	-0.30	0.44	-0.19	-0.32	0.26	-0.35
117475077	Drebrin-like protein [Salmo salar]	-0.29	-0.79	0.07	-0.16	-0.22	0.68	-0.02	0.23	-0.05	0.54
117491424	Optineurin [Salmo salar]	-0.07	-0.46	0.17	-0.12	-0.65	0.81	-0.25	0.13	-0.32	0.76
117492434	ATPase_ Na+/K+ transporting_ beta 3a polypeptide	0.44	-0.65	0.37	0.13	-0.83	0.28	0.08	-0.34	0.07	0.45
117493207	Dspa protein - Ident 53	-0.27	0.11	-0.11	-0.17	0.14	0.32	0.12	-0.07	-0.02	-0.05
117493558	Receptor transporting protein 3	0.73	-0.60	0.02	-1.64	0.63	-0.36	0.80	-0.21	0.25	0.38
117495885	Gig2	-0.22	-0.46	0.35	-1.06	0.45	-0.75	1.06	-0.07	0.29	0.40
117496465	Cytochrome P450 3A27 [Salmo salar]	0.25	0.56	0.06	0.36	0.21	-0.18	0.30	-0.85	-0.05	-0.66
117498019	Isopentenyl-diphosphate delta-isomerase 1 [Oncorhynchus mykiss]	0.23	-1.37	0.37	0.62	-0.27	0.58	0.02	-0.38	0.15	0.04
117500470	enolase 3-2 [Salmo salar]	0.35	0.46	-0.08	-0.12	0.55	-0.20	-0.02	-0.51	-0.15	-0.28
117501468	Actopaxin - Ident 89	0.34	0.08	0.49	0.02	-0.77	0.28	-0.13	-0.49	-0.05	0.24
117501945	cytochrome b558 alpha-subunit [Oncorhynchus mykiss]	-0.35	0.17	-0.54	0.71	-0.17	0.52	0.11	0.15	-0.46	-0.13
117503807	Interleukin-17D precursor [Salmo salar]	0.44	-0.31	0.57	-0.42	-0.51	0.33	-0.21	-0.56	0.10	0.57
117509697	Very large inducible GTPase 1-2	0.01	-1.21	0.22	-0.56	0.83	-0.40	0.10	-0.07	0.23	0.86
117510490	Interferon-induced guanylate-binding protein 1 [Salmo salar]	0.36	-0.64	0.00	-0.42	-0.20	-0.11	-0.26	0.99	-0.27	0.55
117511857	CTP synthase 1 [Salmo salar]	-0.26	0.12	0.04	-0.60	0.17	-0.12	-0.14	0.60	-0.08	0.26
117517242	Hydroxysteroid (11-beta) dehydrogenase 3	0.17	0.43	0.21	0.24	0.35	-0.42	-0.20	-0.30	-0.05	-0.44
117537592	VHSV-induced protein [Salmo salar]	0.00	-0.25	0.16	-0.68	0.25	-0.53	0.41	-0.02	0.31	0.36
117537786	C1q-like adipose specific protein	0.07	-0.72	0.52	-0.42	-0.40	0.13	-0.20	0.67	-0.33	0.67
117541850	Tissue factor pathway inhibitor 2 precursor [Salmo salar]	0.23	0.70	-0.10	-0.13	0.39	-0.66	-0.04	-0.17	-0.03	-0.20
117543911	Methylosome protein 50 [Salmo salar]	-0.01	0.30	-0.02	0.06	0.08	-0.84	0.10	0.13	0.14	0.06
117821026	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4 [Salmo salar]	-0.23	0.02	-0.16	0.06	-0.65	0.52	-0.11	0.23	-0.15	0.46
117823520	CD209 antigen-like protein A [Salmo salar]	-0.19	-0.04	-0.04	-0.14	0.12	-0.50	0.22	0.85	-0.32	0.04
117836117	Armadillo repeat-containing protein 8 - Ident 38	-0.54	-0.27	0.14	0.32	-0.28	0.59	-0.32	0.12	0.61	-0.38
117843187	properdin P factor 2 [Oncorhynchus mykiss]	-0.51	0.12	0.63	0.13	-0.61	0.69	0.44	0.16	-0.49	-0.55
117851935	Cysteine and glycine-rich protein 1 [Salmo salar]	0.26	-0.29	0.21	-0.06	-0.51	0.24	0.04	-0.02	-0.16	0.30
209153085	Interferon-induced GTP-binding protein Mx [Salmo salar]	0.04	-0.59	-0.03	-0.99	0.38	-0.43	0.64	0.21	0.33	0.42
209153949	Acetyl-CoA acetyltransferase, cytosolic [Salmo salar]	-0.19	-0.78	-0.02	0.52	0.17	0.10	0.08	-0.02	0.00	0.13

209154495	C-ets-2 [Salmo salar]	0.18	-0.88	-0.10	-0.29	0.17	0.12	0.31	0.34	-0.29	0.45
209154507	AP1-1	-0.29	0.19	-0.21	0.32	0.08	-0.26	0.16	-0.13	0.33	-0.19
209154567	Kelch-like protein 13 [Salmo salar]	-0.16	-0.63	0.47	-0.06	-0.20	0.34	-0.28	0.39	-0.09	0.21
209154585	Ornithine decarboxylase 1 [Salmo salar]	-0.41	-0.23	-0.05	0.59	0.16	-0.18	0.02	-0.05	0.48	-0.33
209154595	Lathosterol oxidase [Salmo salar]	0.04	-0.78	0.10	0.31	-0.16	0.49	-0.14	-0.03	0.12	0.04
209154675	AP1-2	-0.33	0.29	-0.49	1.02	0.15	-0.50	0.19	-0.44	0.19	-0.09
209154815	Probable ATP-dependent RNA helicase DHX58 [Salmo salar]	0.13	-0.48	-0.08	-0.63	0.61	-0.46	0.67	-0.26	0.11	0.40
209155005	Forkhead box protein C1 [Salmo salar]	-0.20	0.23	0.35	0.06	-0.51	-0.50	0.02	-0.14	0.18	0.51
209155213	Gap junction beta-6 protein [Salmo salar]	-0.14	-0.59	0.37	0.07	-0.26	-0.25	0.04	0.61	-0.13	0.27
209155565	Farnesyl pyrophosphate synthetase [Salmo salar]	0.07	-1.49	0.67	0.43	-0.02	0.28	-0.11	-0.29	0.31	0.15
209155627	Tripartite motif-containing protein 25 [Salmo salar]	0.98	0.37	0.21	-0.49	-0.33	0.08	-0.09	1.25	-1.38	-0.60
209155649	Interferon-induced protein 44 [Salmo salar]	0.27	-0.93	0.06	-0.44	0.15	0.00	0.14	0.42	-0.23	0.57
209155657	CD83 antigen precursor [Salmo salar]	0.04	-0.52	0.11	-0.07	-0.04	0.15	0.06	0.12	-0.14	0.29
209155743	Synaptophysin-like protein 1 [Salmo salar]	-0.39	-0.26	-0.18	0.00	-0.02	0.33	0.05	-0.25	0.26	0.47
209155867	Peroxisomal biogenesis factor 7	-0.13	0.16	0.00	0.06	0.03	-0.60	0.03	0.08	0.15	0.23
209156003	Diphosphomevalonate decarboxylase	-0.15	-1.89	0.53	0.79	-0.18	0.63	-0.15	-0.10	0.38	0.13
209156053	Tapasin-related protein [Salmo salar]	0.18	-0.45	0.21	-0.16	0.06	-0.05	-0.14	-0.06	0.04	0.36
209156101	Interferon-induced protein with tetratricopeptide repeats 5-2	0.27	-0.40	-0.28	-1.04	0.66	-0.63	0.84	0.00	0.27	0.31
209730791	Pleckstrin [Salmo salar]	-0.29	-0.10	-0.35	-0.16	-0.16	-0.11	0.03	0.19	0.41	0.54
209730903	Tripartite motif-containing protein 55 [Salmo salar]	-0.03	-0.77	0.18	-0.31	-0.28	0.77	-0.11	0.14	-0.27	0.69
209732275	Branched-chain-amino-acid aminotransferase, cytosolic [Salmo salar]	-0.09	0.04	0.05	-0.15	-0.08	0.10	-0.16	0.27	-0.31	0.31
209732385	17-beta-hydroxysteroid dehydrogenase 14 [Salmo salar]	-0.16	0.02	0.09	0.22	-0.26	0.52	0.01	0.03	-0.19	-0.28
209732475	Cholesterol 25-hydroxylase-like protein A [Salmo salar]	-0.04	-0.43	-0.09	-0.60	0.34	-0.83	0.90	0.05	0.45	0.25
209732509	Transcription factor Spi-C [Salmo salar]	-0.13	-0.19	0.20	0.05	-0.59	0.88	0.13	0.30	-0.30	-0.34
209732591	Inositol 1,4,5-trisphosphate receptor type 2 [Salmo salar]	0.22	-0.09	0.23	-0.28	-0.93	0.18	-0.41	0.33	0.36	0.39
209732661	Carboxypeptidase A1 precursor [Salmo salar]	0.34	-0.39	-1.34	-0.45	-0.18	0.47	0.30	-1.31	0.76	1.79
209732765	Macrophage migration inhibitory factor [Salmo salar]	-0.12	-0.01	-0.20	0.12	-0.05	-0.10	0.18	0.13	0.32	-0.27
209732957	Beta-neoendorphin-dynorphin precursor [Salmo salar]	0.30	-0.63	0.16	-0.21	-0.04	0.42	-0.25	0.01	-0.07	0.31
209733083	Radical S-adenosyl methionine domain-containing protein 2 [Salmo salar]	0.51	-0.85	-0.56	-1.62	0.55	-0.94	1.03	0.37	0.88	0.63
209733651	Troponin C, skeletal muscle	-0.40	-0.57	-0.41	0.30	0.26	-0.67	0.68	0.15	0.36	0.29
209733949	CD209 antigen-like protein D [Salmo salar]	-0.37	-0.05	0.23	-0.12	-0.12	-0.32	0.08	0.89	-0.01	-0.20
209734305	Chymotrypsin B [Salmo salar]	0.66	-0.46	-1.15	-0.68	-0.29	0.20	0.50	-1.22	0.49	1.96
209734511	Acyl-CoA-binding domain-containing protein 7 [Salmo salar]	-0.13	-0.37	0.13	-0.50	0.12	0.25	-0.06	0.79	-0.55	0.33
209734583	Caspase-14 precursor [Salmo salar]	0.15	-0.88	1.49	-0.76	-0.53	-0.41	-0.43	0.20	-0.25	1.42
209734939	Interferon regulatory factor 1 [Salmo salar]	0.27	-0.85	0.28	-0.37	-0.12	-0.31	0.26	0.39	-0.10	0.54
209735205	Cell death activator CIDE-3 [Salmo salar]	-0.53	0.23	0.31	0.64	-0.17	-0.50	0.06	-0.26	0.57	-0.34
209735921	Phosphatidylethanolamine N-methyltransferase [Salmo salar]	-0.60	0.34	-0.42	0.46	0.08	0.09	0.38	0.06	-0.17	-0.23
209736049	Tumor necrosis factor ligand superfamily member 6, TNFL6	-0.06	-0.41	0.56	-0.15	-0.37	0.13	-0.08	0.02	-0.06	0.41
209736251	T-cell receptor beta chain T17T-22 precursor [Salmo salar]	-0.35	-0.09	-0.25	-0.16	-0.72	-0.35	-0.07	1.26	0.41	0.31
209736373	Cytosolic sulfotransferase 3 [Salmo salar]	0.65	-1.03	0.75	-0.26	0.15	0.04	-0.70	-0.07	-0.34	0.80
209736439	Proproteinase E precursor [Salmo salar]	0.37	-0.46	-0.86	-0.45	-0.25	0.40	0.47	-1.35	0.43	1.70
209736579	TNF receptor member 11B	0.29	-0.78	0.69	-0.30	-0.17	0.18	-0.28	0.29	-0.26	0.33
209737177	Epoxide hydrolase 1 [Salmo salar]	0.38	0.31	-0.09	0.57	-0.20	0.18	-0.03	-0.37	-0.06	-0.69
209738229	Mitochondrial 2-oxoglutarate/malate carrier protein [Salmo salar]	0.49	0.48	-0.72	0.43	0.68	-0.24	0.29	-0.44	-0.19	-0.78
209738473	Flavin reductase [Oncorhynchus mykiss]	0.22	-0.08	0.13	-0.02	0.18	0.43	0.05	-0.54	0.00	-0.38
209738479	Duodenase-1 precursor [Salmo salar]	-0.14	-0.62	0.77	-0.42	0.02	0.04	0.02	-0.17	-0.12	0.63
213061166	interferon-gamma receptor 1 [Oncorhynchus mykiss]	0.79	-0.04	0.14	-0.07	-0.22	0.13	0.05	-0.52	-0.03	-0.24
213066809	Ryanodine receptor 2b protein - Ident 36	-0.19	0.84	0.06	-0.03	-0.13	-0.08	-0.11	-0.30	0.06	-0.11
213069558	Myeloperoxidase precursor [Salmo salar]	-0.35	-0.38	0.65	-0.14	-0.35	1.50	-0.25	-0.05	-0.42	-0.21
213070818	Novel protein similar to human and mouse poliovirus receptor-related 3 (PVRL3) - Ident 24	0.28	-0.78	-0.09	-0.26	0.16	0.06	0.13	0.20	-0.12	0.42
213075604	L-asparaginase	0.22	0.16	-0.75	0.44	0.26	-0.32	0.29	0.22	-0.17	-0.36
213079353	Suppressor of fused homolog (Drosophila)	-0.23	-0.17	-0.32	0.10	-0.11	0.07	0.29	-0.39	0.29	0.48
221219675	GTPase IMAP family member 7 [Salmo salar]	-0.45	-0.16	0.38	-0.05	-0.18	0.30	0.35	0.01	-0.04	-0.16
221220195	Tubulin polymerization-promoting protein family member 3 [Salmo salar]	0.01	0.00	-0.16	-0.55	0.11	0.17	0.60	0.10	-0.07	-0.21

221220363	Growth arrest and DNA-damage-inducible protein GADD45 gamma [Salmo salar]	-0.10	-0.49	0.81	-0.26	-0.61	0.74	-0.08	0.27	-0.50	0.22
221220567	Arachidonate 5-lipoxygenase-activating protein [Salmo salar]	-0.29	-0.02	0.08	-0.20	-0.43	0.62	0.17	0.34	0.00	-0.28
221220811	SH3 domain-binding glutamic acid-rich-like protein [Salmo salar]	-0.06	-0.31	0.06	0.15	-0.64	0.27	0.14	-0.19	0.31	0.27
221221677	cholecystokinin-L [Salmo salar]	-0.06	0.23	-0.60	0.26	0.35	-0.46	0.05	-0.04	-0.16	0.44
221222221	BTB/POZ domain-containing protein KCTD17 [Salmo salar]	-0.32	-0.04	-0.03	-0.03	-0.54	0.12	-0.10	0.49	0.06	0.40
221222227	Ubiquitin thioesterase OTUB1 [Salmo salar]	-0.07	0.48	0.00	-0.12	0.58	-0.26	-0.22	-0.18	0.04	-0.23
223567435	Pseudogene with tubulin tyrosine ligase-like family member 6a TC1 transposase-like sequences	0.25	-0.97	0.42	-0.41	0.35	-0.10	-0.12	-0.32	-0.11	1.02
223576393	Unknown	-0.07	-0.45	0.02	-0.05	-0.31	0.37	-0.08	0.76	-0.08	-0.10
223577024	Probable E3 ubiquitin-protein ligase RNF144A-A	0.28	-0.65	0.02	-0.43	-0.16	0.65	0.01	0.23	-0.32	0.36
223577238	Sulfide quinone reductase-like (Yeast)	-0.16	0.22	-0.11	0.07	-0.10	0.68	0.07	-0.21	-0.09	-0.36
223578554	Glutaredoxin 3	0.27	0.78	-0.35	0.11	0.89	-0.78	-0.18	-0.12	-0.41	-0.22
223583864	PLAC8-like	-0.21	0.02	0.07	-0.44	0.26	-0.65	0.46	0.18	0.07	0.23
223647433	P2Y purinoceptor 8 [Salmo salar]	0.00	-0.27	0.18	-0.84	0.24	-0.39	0.47	0.09	0.23	0.30
223647491	Tumor protein D52 [Salmo salar]	-0.08	1.17	-0.05	-0.07	1.00	-1.15	-0.03	-0.42	-0.09	-0.29
223647567	neutrophil cytosolic factor 1 [Salmo salar]	-0.51	-0.03	-0.82	0.52	0.19	-0.51	0.59	0.02	0.29	0.26
223647645	Centromere protein S [Salmo salar]	-0.05	-0.38	-0.13	0.17	-0.02	-0.23	-0.10	0.07	0.23	0.44
223647743	5-aminolevulinate synthase, erythroid-specific, mitochondrial precursor [Salmo salar]	-0.03	0.23	0.25	-0.03	-0.33	0.43	-0.50	-0.45	-0.22	0.66
223647899	Chloride channel protein 3 [Salmo salar]	0.27	0.23	0.05	-0.26	0.33	-0.25	-0.21	-0.81	0.55	0.11
223648101	DNA replication licensing factor mcm2 [Salmo salar]	-0.20	-0.62	0.28	0.02	-0.21	0.05	-0.09	0.27	0.14	0.36
223648205	Cell division cycle 5-like protein [Salmo salar]	-0.27	-0.67	-0.06	0.04	-0.03	0.57	0.24	0.87	0.14	-0.83
223648283	14-3-3 protein gamma-1 [Salmo salar]	0.00	0.70	-0.17	0.09	0.08	0.23	-0.15	-0.37	-0.01	-0.41
223648415	interleukin-1 receptor-like protein [Salmo salar]	-0.80	0.39	-0.37	0.91	-0.59	-0.12	0.07	0.67	0.24	-0.41
223648565	Acyl-CoA desaturase [Salmo salar]	-0.22	-0.10	-0.91	0.89	-0.54	-0.58	-0.44	0.21	1.28	0.41
223648621	RNA-binding protein with multiple splicing [Salmo salar]	0.27	-0.44	0.24	-0.09	-0.64	0.39	0.17	-0.34	0.04	0.40
223648651	Arylacetamide deacetylase [Salmo salar]	0.12	-0.35	0.50	0.25	-0.66	0.85	-0.26	0.03	-0.25	-0.23
223648747	Zygotic DNA replication licensing factor mcm6-B [Salmo salar]	0.02	-0.52	0.10	0.28	-0.49	0.00	-0.01	0.15	0.14	0.31
223648991	Ganglioside GM2 activator precursor [Salmo salar]	0.00	-0.27	-0.12	0.16	-0.29	0.69	-0.21	0.22	-0.01	-0.17
223649065	Programmed cell death protein 4 [Salmo salar]	0.19	-0.30	0.13	-0.39	-0.02	0.74	-0.09	0.15	-0.17	-0.24
223649067	B0,+-type amino acid transporter 1 [Salmo salar]	0.39	0.39	0.51	0.51	-0.06	0.08	-0.27	-0.56	-0.53	-0.45
223649079	Rho-related GTP-binding protein RhoE precursor [Salmo salar]	-0.25	-0.16	0.29	-0.01	-0.43	0.40	-0.30	-0.09	0.11	0.46
223649107	Complement C1qC	0.46	0.57	-0.04	0.01	0.57	-1.16	-0.12	0.28	-0.44	-0.14
223649173	Cyclic AMP-dependent transcription factor ATF-5 [Salmo salar]	-0.21	0.15	-1.17	1.03	0.11	-0.86	0.18	-0.19	0.72	0.25
223649223	60 kDa heat shock protein, mitochondrial precursor [Salmo salar]	-0.10	0.34	-0.06	0.25	-0.09	-0.62	-0.15	-0.05	0.27	0.22
223649253	Ras-related and estrogen-regulated growth inhibitor [Salmo salar]	0.03	-0.86	1.33	0.18	-0.82	1.52	-0.06	-0.65	-0.06	-0.60
223649367	JunD-2	-0.24	0.22	-0.31	0.33	0.10	-0.04	0.22	-0.65	0.12	0.25
223649429	Casein kinase II subunit beta [Salmo salar]	-0.09	-0.58	0.62	0.54	-0.59	-0.40	0.09	0.40	0.24	-0.23
223649437	Plasticin [Salmo salar]	0.48	-0.28	0.49	-0.19	-0.70	0.08	0.24	-0.36	-0.12	0.36
223672078	C-C motif chemokine 19 precursor-1	0.06	-1.30	0.52	0.07	-0.15	0.08	0.16	0.14	-0.25	0.67
223672662	Proteasome subunit beta type-8 precursor [Salmo salar]	-0.83	0.38	0.28	-1.54	0.02	-0.09	-1.28	1.73	-0.16	1.48
223673088	Proline-serine-threonine phosphatase-interacting protein 2 [Salmo salar]	0.23	-0.65	0.08	0.31	-0.42	0.85	-0.17	-0.06	-0.06	-0.11
377782457	Vesicle amine transport protein 1 homolog (T californica)	-0.04	-0.29	0.21	0.12	-0.58	0.56	-0.46	0.29	-0.28	0.45
377792223	Secretory carrier-associated membrane protein 4 [Salmo salar]	0.38	0.18	-0.13	0.09	0.46	-0.66	-0.56	-0.38	0.22	0.39
AF076620	Radical S-adenosyl methionine domain-containing protein 2; AltName: Full=Virus inhibitory protein, endoplasmic reticulum	0.35	-0.61	-0.77	-1.64	0.52	-1.07	1.27	0.46	0.94	0.55
AF141606	Immunoglobulin heavy chain [Salmo salar] AF141606_1	-0.13	-0.54	-0.10	-0.07	-0.73	0.24	-0.86	0.96	-0.05	1.27
AF273410	Ig heavy chain	-0.13	-0.35	-0.23	-0.03	-0.30	0.23	0.60	0.15	-0.50	0.56
AF273418	Ig heavy chain	-0.92	0.10	0.56	-0.27	0.07	0.34	0.16	0.71	-0.39	-0.34
AJ425734	Neuroligin 1 - Ident 50	0.27	-0.81	-1.07	-0.61	-0.53	0.72	0.58	-1.01	0.84	1.61
AM402497	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (Soluble)	0.05	-1.30	0.59	0.49	0.08	0.47	-0.09	-0.27	-0.02	0.00
AM402501	Phosphate carrier protein, mitochondrial precursor [Salmo salar]	0.19	0.62	-0.09	0.00	0.61	-0.63	-0.18	-0.07	-0.26	-0.19
AM402527	UDP glucuronosyltransferase 1 family polypeptide b7 short isoform	0.50	0.07	-0.19	-0.57	-0.07	-0.22	-0.01	0.45	0.41	-0.38
AM412213	AF392054_3 cytochrome c oxidase subunit I [Oncorhynchus tshawytscha]	0.25	0.39	-0.20	-0.11	0.77	-0.32	-0.18	-0.12	-0.30	-0.19
AY362187	liver-expressed antimicrobial peptide 2B [Oncorhynchus mykiss]	-0.12	0.02	-0.06	-0.02	-0.22	0.26	-0.12	-0.44	0.11	0.59
AY572834	C type lectin receptor C [Salmo salar]	-0.06	-0.45	-0.33	0.08	-0.07	0.14	0.00	0.28	-0.07	0.50
BG935573	haptoglobin [Oncorhynchus mykiss]	0.17	0.26	0.53	0.02	0.05	-0.12	-0.49	0.49	-0.02	-0.86

BM414052	Serine/threonine-protein kinase SIK3 homolog - Ident 45	0.23	0.67	-0.01	-0.02	0.23	-0.35	-0.13	-0.20	-0.28	-0.13
BM414372	Y-box-binding protein 2-A [Salmo salar]	0.28	0.55	-0.21	-0.34	0.66	-0.53	0.00	-0.16	-0.04	-0.23
BQ035726	Very large inducible GTPase 1-3	0.11	-0.89	0.03	-0.67	0.52	-0.38	0.11	0.20	0.07	0.91
BQ036296	Cytochrome P450_ family 4_ subfamily V_ polypeptide 2 - Ident 68	0.33	-0.21	-0.14	0.07	0.45	-0.03	0.12	-0.38	0.17	-0.38
BT043592	VHSV-induced protein [Salmo salar]	-0.03	-0.35	0.23	-1.43	0.44	-0.67	0.67	0.47	0.28	0.40
BT043723.1	myxovirus resistance 1 [Salmo salar]	-0.07	-0.58	-0.51	-1.10	1.80	-0.45	0.36	0.27	0.28	0.00
BT045308	Jun C	-0.62	0.41	-0.22	0.77	0.00	-0.69	0.13	-0.19	0.46	-0.06
BT045626	Rhomboid domain-containing protein 1 [Salmo salar]	0.07	-0.46	-0.17	0.41	-0.54	0.54	0.04	-0.04	0.43	-0.28
BT047975	Proteasome subunit beta type-7 precursor [Salmo salar]	-0.82	-0.27	0.79	0.38	0.27	0.47	0.67	-1.68	0.65	-0.47
BT048158	Ig kappa chain V-IV region B17 precursor [Salmo salar]	0.21	-0.44	0.37	-0.39	-0.30	-0.19	0.32	0.01	-0.23	0.64
BT056389	Ig kappa chain V-IV region B17 precursor [Salmo salar]	0.29	-0.14	-0.34	-0.53	-0.86	0.43	0.21	0.62	-0.15	0.48
BT072557	VHSV-induced protein-1	-0.08	-0.54	0.20	-1.01	0.31	-0.57	0.57	0.44	0.30	0.38
BT072598	Adipophilin [Salmo salar]	-0.69	0.79	-0.53	0.62	0.29	-1.27	0.21	0.04	0.53	0.02
BU694011	Keratin 14	-0.15	0.28	-0.18	-0.01	0.63	-0.08	-0.07	-0.26	0.01	-0.18
BX081897	Cholesterol 25-hydroxylase-like protein A [Salmo salar]	-0.45	0.04	0.32	1.00	-0.06	1.06	-0.71	-1.08	0.61	-0.71
BX302486	EGF-like domain-containing protein 7 precursor [Salmo salar]	0.01	-0.63	0.23	-0.29	-0.72	0.85	-0.11	0.29	-0.48	0.86
BX307538	AT-rich interactive domain-containing protein 3A [ARID domain-containing protein 3A] - Ident 37	0.23	0.64	-0.03	-0.18	0.34	-0.18	-0.19	-0.12	-0.33	-0.19
BX309109	Novel zinc finger protein - Ident 39	0.22	-1.63	0.89	0.36	0.06	0.41	0.01	-0.46	0.13	0.00
BX860471	Kunitz-type protease inhibitor 1 precursor [Salmo salar]	-0.02	0.28	0.02	0.10	-0.21	-0.15	0.41	-0.15	0.05	-0.32
BX875508	mitogen activated protein kinase p38b [Salmo salar]	0.11	-0.09	-0.20	0.05	0.31	0.24	-0.70	0.10	-0.08	0.25
BX879672	Novel protein similar to vertebrate lactase (LCT) - Ident 98	0.90	0.12	-0.22	-0.37	0.36	0.31	-0.12	-0.91	0.08	-0.14
BX879710	Regeneration associated muscle protease isoform a [Homo sapiens]	0.31	-0.78	0.27	-0.32	-0.79	0.79	-0.09	0.23	-0.21	0.58
BX881541	Myb-binding protein 1A-like protein	0.16	0.12	0.20	0.08	-0.34	-0.24	-0.33	-0.14	0.14	0.35
BX881802	T-cell surface antigen CD2 precursor [Salmo salar]	-0.75	-0.22	0.21	0.23	-0.53	0.38	0.29	0.64	0.12	-0.38
BX883335	DEAH (Asp-Glu-Ala-His) box polypeptide 16 - Ident 36	0.07	0.35	0.08	-0.10	0.22	0.12	-0.13	-0.05	0.07	-0.62
BX884595	Neutral ceramidase [N-CDase]	0.69	-0.33	-0.02	-0.19	0.49	0.24	-0.03	-0.66	0.01	-0.19
BX889811	Small inducible cytokine A13 [Oncorhynchus mykiss]	-0.47	-0.17	-0.08	0.65	-0.60	-0.24	0.13	1.30	-0.12	-0.41
BX909624	PERQ amino acid-rich with GYF domain-containing protein 2 - Ident 44	0.37	-1.83	0.88	0.02	0.19	0.47	-0.04	-0.34	0.13	0.15
CA037592	Unconventional myosin-Ic - Ident 42	-0.29	-0.09	0.80	0.03	-1.24	1.45	0.19	0.45	-0.48	-0.82
CA037937	Trypsin-2 TRY2_SALSA RecName: Full=; AltName: Full=Trypsin II; Flags: Precursor	0.24	-0.53	-0.20	-0.36	-0.12	0.97	-0.13	-0.63	0.24	0.54
CA042221	Rho guanine nucleotide exchange factor 18	0.31	-0.07	0.20	-0.49	-0.65	0.21	0.10	0.23	-0.37	0.53
CA043269	Meprin A subunit alpha	0.60	0.15	0.02	-0.21	0.32	0.09	-0.21	-0.60	0.04	-0.21
CA050533	LDL receptor [Oncorhynchus mykiss]	-0.12	-1.04	0.26	0.22	-0.06	0.07	0.13	0.25	0.17	0.13
CA054354	Solute carrier family 22 member 6 [Salmo salar]	0.05	0.68	-0.25	-0.12	-0.30	0.20	0.03	-0.32	0.22	-0.19
CA058382	Sodium- and chloride-dependent GABA transporter 2 [Salmo salar]	-0.07	-0.35	0.02	0.23	0.53	-0.14	0.37	-0.20	-0.29	-0.11
CA060324	Annexin A1 [Salmo salar]	-0.07	0.14	-0.37	0.04	0.09	0.13	0.22	0.73	-0.31	-0.60
CA061541	PRKCA-binding protein [Salmo salar]	-0.53	0.09	-0.46	-0.60	0.86	-0.23	0.07	0.35	0.03	0.42
CA357037	Exosome complex exonuclease RRP4 [Salmo salar]	-0.06	-0.54	0.18	-0.15	-0.65	0.82	-0.11	0.11	-0.10	0.50
CA388053	Tyrosine-protein phosphatase non-receptor type 6 [Salmo salar]	-0.16	-0.25	-0.13	0.18	0.13	0.38	0.00	0.02	-0.80	0.63
CB498981	Intersectin 1 (SH3 domain protein) - Ident 31	0.09	0.10	0.27	0.06	0.02	0.20	0.06	-0.22	-0.06	-0.53
CB500831	C-C motif chemokine 19-2	0.28	-1.46	0.62	-0.22	0.21	-0.12	-0.02	0.06	-0.48	1.13
CB503941	Euchromatic histone lysine N-mthyltransferase EHMT2/G9a - Ident 36	0.01	-0.74	0.40	-0.25	-0.75	0.75	-0.02	0.18	-0.31	0.72
CB507021	Cell division protein kinase 2 [Salmo salar]	-0.21	-0.36	0.29	0.22	-0.48	0.11	-0.16	-0.21	0.43	0.37
CB507035	Transmembrane 4 L6 family member 1 [Salmo salar]	-0.55	-0.06	0.00	0.03	-0.05	-0.50	0.22	0.47	0.11	0.33
CB511089	complement C4 [Oncorhynchus mykiss]	0.01	0.00	0.36	-0.74	-0.18	0.15	-0.01	0.51	-0.26	0.17
CB511435	Complement factor Bf-1	-0.08	-0.31	0.12	-0.13	0.10	0.73	-0.17	0.39	-0.39	-0.26
CB511660	Immune-related lectin-like receptor-like	-0.43	-0.63	-0.25	0.02	0.05	-0.28	0.19	0.93	0.00	0.39
CB513054	Signal transducer and activator of transcription 1-alpha/beta [Salmo salar]	0.26	-0.49	-0.20	-0.61	0.33	-0.65	0.42	0.18	0.33	0.41
CB515442	glyceraldehyde 3-phosphate dehydrogenase [Oncorhynchus mykiss]	0.10	0.79	-0.28	-0.06	0.58	-0.47	-0.25	-0.15	-0.19	-0.06
CB517025	U4/U6 small nuclear ribonucleoprotein Prp4 [Salmo salar]	-0.33	-0.08	0.27	0.05	0.06	0.27	-0.17	0.41	0.06	-0.55
CB517736	GLUT6, facilitated glucose transporter member 3 [Salmo salar]	-0.21	-0.69	0.22	0.14	-0.25	-0.09	-0.15	0.53	0.08	0.41
CK873801	Dynamin-1-like protein [Salmo salar]	-0.10	0.23	0.05	-0.21	0.42	-0.52	-0.08	-0.04	0.13	0.12
CK884746	Tropomyosin-1 alpha chain [Salmo salar]	0.19	-0.38	0.31	-0.13	-0.69	0.49	-0.09	-0.14	0.05	0.39
CK885007	Procollagen C-endopeptidase enhancer 1 precursor [Salmo salar]	0.40	-0.29	0.43	-0.37	0.06	0.50	-0.10	-0.10	-0.15	-0.38

CK886108	Olfactomedin 2 - Ident 25	0.94	-0.43	0.23	-0.74	0.11	-0.07	-0.65	0.30	0.00	0.30
CK886159	Profilin-2 [Salmo salar]	0.25	0.50	-0.07	-0.03	0.63	-0.60	-0.02	-0.27	-0.08	-0.31
CK889727	Novel zona pellucida-like domain containg protein - Ident 23	0.85	-1.59	0.78	-0.80	0.09	1.03	-0.39	-0.84	-0.07	0.95
CK891531	AF231708_1 vitelline envelope protein gamma [Oncorhynchus mykiss]	-0.72	0.20	-0.22	-0.03	-0.15	-0.07	0.45	0.45	-0.09	0.18
CK897012	Lectin precursor [Salmo salar]	-0.08	-0.08	0.41	-0.09	-0.48	-0.17	0.26	-0.54	0.02	0.76
CK898270	Peroxisomal biogenesis factor 7	-0.14	0.01	0.21	0.06	0.06	-0.04	0.26	0.35	-0.54	-0.23
CK990896	NADH dehydrogenase subunit 3 [Salmo trutta]	0.16	0.65	-0.16	0.05	0.42	-0.65	-0.25	-0.05	-0.01	-0.19
CR943778	Novel protein with transmembrane receptor (Rhodopsin family) domain - Ident 24	-0.07	-0.61	0.26	-0.01	-0.15	-0.05	0.32	0.16	-0.22	0.38
CT962732	UDP-GlcNAc:betaGal beta-1_3-N-acetylglucosaminyltransferase 7 - Ident 99	0.07	0.07	-0.16	0.87	-0.24	-0.03	0.30	-0.45	-0.07	-0.36
CU070695	Short stature homeobox - Ident 86	0.32	-0.32	0.40	-0.19	-0.54	1.17	-0.15	-0.04	-0.66	0.02
CU071711	Novel protein similar to vertebrate NGFI-AB0S788 Novel protein similar to vertebrate NGFI-A	0.09	0.12	0.02	0.01	0.11	0.06	0.12	-0.57	-0.26	0.29
CU072347	E3 ubiquitin-protein ligase MARCH4	0.10	-0.39	0.08	-0.08	-0.69	0.61	-0.07	0.50	-0.46	0.42
CU073629	Procollagen lysine 2-oxoglutarate 5-dioxygenase 2b isoform - Ident 27	0.15	-0.93	0.35	0.41	-0.67	1.06	0.00	0.00	-0.20	-0.17
CV428775	NADH dehydrogenase subunit 2 [Salmo salar]	0.33	0.90	-0.20	0.25	0.62	-0.81	-0.25	-0.09	-0.35	-0.40
CX030163	Chromodomain-helicase-DNA-binding protein 8 - Ident 61	0.29	-0.13	0.07	-0.03	0.08	0.07	-0.43	0.01	-0.23	0.29
CX353208	Protein kinase_ cGMP-dependent_ type I	0.00	-0.28	0.45	-0.07	-0.47	0.85	-0.04	-0.15	-0.24	-0.05
CX353894	fish virus induced TRIM protein [Oncorhynchus mykiss]	0.24	-0.53	0.15	-0.12	-0.77	0.73	-0.20	0.25	-0.37	0.61
CX721196	Acyl-CoA synthetase family member 4	0.02	0.46	0.01	-0.06	0.51	-0.54	-0.07	0.10	-0.02	-0.41
CX721876	FLJ25371 [Salmo salar]	0.09	-0.38	0.57	-0.19	0.12	0.04	-0.40	-0.56	0.24	0.47
DN047839	RNase 2 [Oncorhynchus masou formosanus]	-0.67	0.34	-0.56	-0.68	0.34	0.30	-0.17	0.38	0.28	0.44
DN163049	Striated muscle preferentially expressed protein kinase - Ident 62	0.19	0.35	0.28	0.18	0.02	0.03	-0.16	-0.25	-0.10	-0.54
DQ459470	Toll-like receptor 3-like protein [Oncorhynchus mykiss]	-0.14	-0.43	-0.05	-0.69	-0.08	0.23	0.46	0.46	0.12	0.12
DR696497	Serpin peptidase inhibitor_ clade A (Alpha-1 antiproteinase_ antitrypsin)_ member 7 - Ident 90	-0.36	0.05	-0.15	0.38	0.01	0.27	0.01	-0.63	0.18	0.25
DT317734	enolase 3-2 [Salmo salar]	0.41	0.44	-0.04	-0.16	0.58	-0.21	-0.05	-0.46	-0.19	-0.32
DV106832	Tax1-binding protein 1 homolog B	0.18	0.34	-0.02	0.16	0.47	-0.56	-0.30	0.11	0.03	-0.41
DV107337	glutathione peroxidase 4b [Salmo salar]	0.05	0.66	0.44	0.33	-0.16	-0.44	0.07	-1.00	0.22	-0.16
DW005432	Acta1 protein - Ident 25	-0.04	0.47	0.00	-0.17	0.59	-0.22	-0.12	-0.19	-0.09	-0.24
DW178237	Myelin protein zero-like 3 - Ident 87	0.27	-0.14	-0.15	0.21	-0.51	0.05	-0.07	-0.16	0.17	0.33
DW179498	Occludin - Ident 77	-0.31	-0.33	0.16	-0.18	-0.54	0.59	-0.02	-0.06	0.22	0.47
DW471462	Ig kappa chain	0.22	-0.39	0.17	-0.20	0.00	-0.29	0.42	-0.05	-0.48	0.59
DW531881	polyunsaturated fatty acid elongase elov12 [Salmo salar]	0.46	-0.84	0.03	0.17	0.16	0.32	-0.09	-0.29	0.11	-0.03
DW532027	High choriolytic enzyme 1 precursor [Salmo salar]	0.63	-0.61	-1.10	-0.58	-0.32	0.81	0.39	-1.18	0.51	1.45
DW533708	Lectin precursor [Salmo salar]	0.17	-0.05	0.18	-0.24	-0.20	0.66	-0.10	0.09	-0.33	-0.16
DW534482	A Chain A, Structure Of Native Pancreatic Elastase From North Atlantic Salmon At 1.61 Angstroms Resolution	0.45	-0.38	-1.06	-0.59	0.04	0.36	0.38	-1.19	0.57	1.43
DW535351	cytochrome P450 2K5 [Oncorhynchus mykiss]	-0.14	0.24	-1.00	0.22	-0.02	0.03	0.27	0.24	0.16	0.00
DW536955	TBC1 domain family_ member 5 - Ident 96	0.30	-0.20	-0.07	0.11	0.27	0.32	-0.51	-0.14	-0.26	0.20
DW537096	AF504013_1 MHC class I [Salmo salar] MHC class I [Salmo salar]	1.44	-0.50	-0.73	1.27	-0.16	-1.61	-0.95	0.40	-1.56	2.41
DW537917	EH domain-binding protein 1-like protein 1 [Salmo salar]	0.28	0.13	-0.44	-0.17	-0.16	0.12	0.14	0.32	-0.60	0.37
DW537935	Cmn protein - Ident 31	0.06	0.00	-0.20	0.00	0.10	0.52	-0.09	-0.17	0.07	-0.28
DW538720	actin related protein 2/3 complex subunit 2 [Salmo salar]	0.22	0.59	-0.04	-0.13	0.53	-0.47	-0.21	-0.06	-0.08	-0.36
DW539357	C-C motif chemokine 19-3	-0.08	-1.19	0.18	0.03	-0.09	-0.03	-0.09	0.46	0.43	0.37
DW541953	Interferon-induced protein 44 [Salmo salar]	0.42	-1.02	0.53	-0.84	0.25	-0.38	-0.33	0.15	-0.09	1.33
DW549788	Serine/threonine-protein kinase 6 [Salmo salar]	0.00	-0.17	-0.07	-0.13	0.15	0.05	-0.13	-0.38	0.28	0.40
DW551663	SAPS domain family member 3 [Salmo salar]	0.01	-0.70	0.24	-0.31	-0.57	0.94	0.07	0.10	-0.29	0.52
DW553532	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 2 [Salmo salar]	0.18	0.62	0.05	-0.13	0.35	-0.19	-0.26	-0.02	-0.34	-0.26
DW553824	STAM-binding protein-like A - Ident 50	-0.27	0.51	-0.04	0.01	0.43	-0.38	-0.14	0.06	0.18	-0.37
DW556533	MHC class I antigene [Salmo salar]	0.10	-0.66	0.24	-0.09	0.00	-0.01	0.13	0.33	-0.20	0.16
DW556649	Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1 [Salmo salar]	0.95	1.94	-2.94	1.97	1.02	-0.22	0.15	-0.88	-2.00	0.01
DW556796	Interferon-induced protein 44-1	0.01	-0.16	-0.14	-1.05	0.57	-0.81	0.48	0.32	0.45	0.34
DW557248	Keratin_ type I cytoskeletal 18 - Ident 25	0.07	-0.46	0.21	-0.34	-0.38	0.34	0.19	0.13	-0.37	0.60
DW558834	Glutamate-rich WD repeat containing 1	-0.10	-0.01	0.01	-0.58	0.13	-0.40	0.03	0.19	0.42	0.32
DW560686	Prpf8 protein - Ident 87	0.20	0.13	0.00	0.30	0.67	-0.41	-0.22	-0.42	0.10	-0.35
DW560876	Syntaxin binding protein 6 (Amisyn)	0.09	0.08	0.35	-0.40	0.35	-0.25	0.30	0.07	-0.05	-0.53
DW563628	sub-family B ATP-binding cassette transporter 2 [Oncorhynchus mykiss]	-0.22	-0.05	-0.14	-0.89	0.04	0.31	-0.86	1.13	-0.31	1.00

DW563656	AF321816_1 hepatic glucose transporter GLUT2 [Oncorhynchus mykiss]	0.20	0.47	-0.26	0.10	0.28	-0.25	-0.11	-0.19	0.11	-0.35
DW566599	Hypocretin receptor	1.21	2.03	-0.23	-0.28	-0.92	0.13	-1.00	-0.29	-0.38	-0.65
DW567934	Methylcrotonoyl-CoA carboxylase beta chain, mitochondrial precursor [Salmo salar]	-0.11	0.58	-0.14	-0.23	0.24	-0.05	0.04	-0.13	0.10	-0.29
DW568290	Protein MCM10 homolog	-0.42	0.14	0.43	0.38	0.24	-0.07	-0.16	0.14	0.14	-0.81
DW569404	Solute carrier family 5 (Sodium/glucose cotransporter)_ member 11 - Ident 24	0.76	-0.38	0.28	-0.38	-0.10	0.37	0.03	-0.08	-0.04	-0.47
DW569481	Novel protein similar to H.sapiens ERCC6_ excision repair cross-complementing rodent repair deficiency_ complementation	0.29	0.25	0.03	0.15	0.27	-0.61	0.09	0.21	-0.09	-0.59
DW569920	52 kDa Ro protein [Salmo salar]	0.35	-0.82	0.33	-0.65	0.26	-0.09	0.06	0.16	-0.26	0.65
DW570698	Putative spalt protein - Ident 68	-0.78	-0.09	-0.04	0.32	-0.08	0.13	0.10	0.28	-0.01	0.17
DW577519	E3 ubiquitin-protein ligase LINCR [Salmo salar]	0.60	-1.40	0.73	-0.89	0.98	-1.97	-0.36	0.83	-0.29	1.76
DW577631	PRP4 pre-mRNA processing factor 4 homolog B	0.45	0.17	-0.02	0.03	0.23	0.06	-0.22	-0.13	-0.19	-0.39
DW578263	Transforming growth factor beta-1-induced transcript 1 protein [Salmo salar]	0.21	0.47	-0.32	-0.03	0.37	0.11	-0.20	-0.32	0.05	-0.33
DW579517	CD36 antigen [Oncorhynchus mykiss]	-0.02	0.22	-0.04	-0.08	0.56	-0.08	0.15	-0.19	-0.28	-0.23
DW580583	Oxidation resistance protein 1 [Salmo salar]	0.01	0.25	0.14	0.07	0.11	-0.47	0.23	-0.02	0.03	-0.36
DW580609	ADP-dependent glucokinase	0.40	-0.19	0.11	0.25	-0.05	-0.02	-0.08	-0.01	0.03	-0.45
DW581391	Hemicentin1 protein - Ident 31	0.20	0.26	-0.23	-0.10	0.55	-0.02	-0.08	-0.15	-0.06	-0.38
DW582013	Solute carrier family 25_ member 16	0.17	0.43	0.03	0.06	0.47	-0.41	-0.14	-0.02	-0.21	-0.37
DW582174	Nucleolar and spindle associated protein 1	0.12	-0.19	0.16	-0.07	0.07	-0.16	-0.21	-0.06	-0.04	0.38
DW582459	Ubiquitin carboxyl-terminal hydrolase 4 [Salmo salar]	0.03	0.02	0.00	0.18	0.54	-0.21	-0.02	-0.58	0.38	-0.34
DW582692	Novel protein similar to vertebrate calcium channel_ voltage-dependent_ alpha 2/delta 3 subunit (CACNA2D3) - Ident 30	0.31	0.02	-0.13	0.36	0.02	-0.12	-0.54	-0.09	-0.01	0.18
DW590610	desmin [Oncorhynchus mykiss]	0.55	-0.19	0.58	-0.17	-0.91	0.41	-0.05	-0.98	0.22	0.54
DY691527	Nuclease EXOG_ mitochondrial - Ident 28	0.29	0.26	0.27	-0.77	0.44	0.89	-1.01	0.21	-0.41	-0.18
DY691889	Lamin B receptor	0.07	-0.96	0.10	0.48	-0.19	0.38	0.01	-0.05	0.20	-0.03
DY692070	Coronin-1C [Salmo salar]	0.37	0.62	-0.18	-0.09	0.67	-0.53	-0.12	-0.20	-0.29	-0.25
DY692087	Acyl-CoA thioesterase 11 - Ident 95	0.50	-0.24	-0.07	0.36	-0.30	0.21	0.22	-0.57	0.34	-0.46
DY692126	Elastase-1	0.61	-0.50	-0.94	-0.52	-0.34	0.41	0.29	-1.20	0.48	1.70
DY692281	Peroxisomal proliferator-activated receptor A-interacting complex 285 kDa-2	0.47	-0.62	-0.20	-1.00	0.42	-0.18	0.44	0.06	0.30	0.32
DY692550	Glycoprotein_ synaptic 2	0.42	0.90	-0.38	0.06	1.02	-0.85	-0.06	-0.38	-0.40	-0.33
DY692568	Keratin_ type I cytoskeletal 17	-0.09	0.21	-0.59	0.10	-0.06	-0.55	0.28	0.43	0.05	0.21
DY692675	Solute carrier family 13 member 3 [Salmo salar]	-0.36	-1.19	0.73	0.53	-0.55	1.34	0.13	-0.77	0.36	-0.21
DY692879	CD36 antigen	0.60	-0.46	0.41	-0.51	-0.07	1.11	-0.21	-0.34	-0.32	-0.20
DY692918	Programmed cell death 8	0.21	0.40	0.05	0.44	-0.21	-0.16	-0.03	-0.30	0.11	-0.51
DY693133	Succinyl-CoA ligase subunit alpha, mitochondrial precursor [Salmo salar]	0.41	0.53	-0.17	0.13	0.69	-0.68	-0.16	-0.54	0.01	-0.23
DY694469	Lamin B receptor	0.22	-1.26	0.29	0.60	-0.18	0.45	0.09	-0.35	0.13	0.02
DY699233	sex hormone-binding globulin beta [Oncorhynchus tshawytscha]	-0.22	0.00	0.26	-0.72	0.03	0.25	-0.18	0.90	-0.19	-0.14
DY701037	NtA agrin	0.08	-0.12	0.05	0.31	-0.14	-0.32	0.16	-0.18	-0.54	0.69
DY706235	Proprotein convertase subtilisin/kexin type 5b	0.31	0.67	-0.05	-0.01	0.41	-0.41	-0.10	-0.25	-0.26	-0.32
DY706495	Novel protein similar to vertebrate squalene epoxidase (SQLE) - Ident 61	0.47	-2.13	0.69	0.57	0.20	0.35	0.01	-0.31	0.10	0.03
DY706723	Glucocorticoid-induced transcript 1 protein [Salmo salar]	-0.04	-0.08	-0.23	-0.05	0.27	0.42	-0.36	0.03	0.00	0.04
DY707492	Interferon-induced protein 44 [Salmo salar]	0.15	-0.03	-0.26	-0.97	0.57	-0.71	0.47	0.20	0.21	0.37
DY708412	Novel protein similar to vertebrateA2RUY5 Novel protein similar to vertebrate	-0.01	0.58	0.11	-0.15	0.37	-0.45	-0.12	-0.15	0.08	-0.27
DY709730	FAT tumor suppressor homolog 1 - Ident 27	0.82	-0.38	0.06	0.01	-0.18	0.14	-0.16	-0.05	-0.08	-0.18
DY710461	RAB28_ member RAS oncogene family	0.52	0.60	-0.12	-0.10	0.81	-0.49	-0.15	-0.66	-0.14	-0.27
DY710578	DDB1- and CUL4-associated factor 10 - Ident 90	0.26	0.46	-0.13	0.13	0.28	-0.20	-0.15	-0.20	-0.11	-0.34
DY710736	Transgelin [Salmo salar]	0.17	-0.25	0.24	-0.11	-0.74	0.31	0.12	-0.07	0.00	0.33
DY712593	Novel proteinA2BG56 Novel protein	-0.39	0.63	-0.06	0.11	0.22	-0.23	-0.02	0.11	-0.03	-0.34
DY712854	Hydroxyacylglutathione hydrolase [Salmo salar]	-0.50	-0.13	0.40	-0.04	0.26	-0.20	0.05	0.05	-0.21	0.37
DY713827	Transcription factor Sox-2 [Salmo salar]	-0.37	0.38	-0.08	0.00	0.23	0.19	0.29	-0.03	-0.31	-0.31
DY715894	GTP-binding protein GEM [Salmo salar]	0.10	0.09	0.11	-0.05	-0.70	0.34	0.07	0.38	0.16	-0.49
DY716039	IFN-induced 44	0.21	-0.03	-0.12	-0.35	0.46	-0.46	0.32	-0.22	0.08	0.11
DY717161	Squalene synthetase [Salmo salar]	0.17	-1.49	0.41	0.54	0.33	-0.04	0.12	-0.30	0.23	0.03
DY717556	Integrin_ alpha 5 (Fibronectin receptor_ alpha polypeptide)	0.23	-0.61	0.04	-0.17	-0.47	0.17	0.31	0.02	0.09	0.38
DY718635	Interferon-induced guanylate-binding protein 1 [Salmo salar]	0.22	-0.51	-0.07	-0.21	-0.13	0.11	-0.45	0.32	-0.10	0.81
DY721106	Protein transport protein Sec24D	0.14	0.57	0.28	-0.15	0.19	-0.26	0.21	-0.52	-0.18	-0.27
DY721474	Protein phosphatase 1_ regulatory subunit 10	0.67	0.17	0.17	-0.45	-0.11	-0.34	-0.17	-0.08	0.30	-0.27

DY722579	Acid phosphatase 5a_ tartrate resistant - Ident 39	0.24	0.41	-0.08	-0.16	0.86	-0.40	-0.11	-0.20	-0.28	-0.28
DY723052	Disabled homolog 2 [Salmo salar]	0.50	0.06	0.25	0.18	0.32	-0.44	-0.30	-0.19	-0.09	-0.30
DY725214	Sorbin and SH3 domain containing 3	0.08	0.95	-0.23	0.27	0.28	-0.21	-0.36	-0.01	-0.70	-0.08
DY725243	Ubiquitin [Salmo salar]	0.31	-0.06	-0.16	-0.12	0.19	-0.05	0.01	-0.11	-0.12	0.10
DY726242	Spectrin_ beta_ erythrocytic - Ident 39	0.19	-0.57	0.33	-0.30	-0.71	0.82	-0.16	0.10	-0.32	0.62
DY727861	Collectin-12	-0.14	-0.07	0.02	-0.18	0.18	0.28	0.17	0.34	-0.33	-0.27
DY728981	Nxf1 protein - Ident 92	-0.12	-0.07	-0.08	-0.03	0.59	0.04	-0.02	0.11	-0.02	-0.39
DY729349	Retrovirus-related Pol polyprotein from transposon 17.6 [Salmo salar]	-0.54	0.05	-0.18	-0.99	1.16	-0.26	0.57	0.25	0.48	-0.54
DY729659	Phosphatidylinositol-binding clathrin assembly protein [Salmo salar]	-0.19	-0.09	-0.06	-0.08	0.17	0.10	0.08	-0.26	-0.14	0.48
DY729902	52 kDa Ro protein [Salmo salar]	-0.14	-0.54	0.57	-0.05	-0.17	0.03	-0.43	0.19	-0.07	0.60
DY730951	phosphatase 1H [Salmo salar]	-0.66	0.06	-0.24	0.18	0.06	-0.02	-0.15	0.31	0.08	0.37
DY731118	Cytochrome P450_ family 51	0.20	-1.93	0.49	0.59	0.03	0.51	0.04	-0.38	0.30	0.14
DY733157	Ribose 5-phosphate isomerase A (Ribose 5-phosphate epimerase)	0.09	0.35	-0.03	0.14	0.22	-0.65	-0.37	-0.14	0.30	0.10
DY735727	R-spondin-1	0.03	0.03	0.20	-0.14	-0.29	-0.32	0.16	0.35	0.42	-0.45
DY736395	Novel protein similar to H.sapiens CNNM2_ cyclin M2 (CNNM2) - Ident 77	-0.13	0.22	0.11	-0.04	0.55	-0.33	-0.04	-0.15	0.10	-0.28
DY737052	Myeloid-restricted CCAAT/enhancer-binding protein 1 - Ident 32	-0.28	0.52	-0.96	0.57	0.38	-0.87	0.50	-0.02	0.21	-0.05
DY737280	vacuolar protein sorting 52 [Salmo salar]	0.43	-0.27	-0.03	0.23	0.02	-0.16	0.02	0.38	-0.17	-0.45
DY737540	Novel NACHT domanin containing protein - Ident 27	0.03	-0.36	-0.33	0.35	-0.56	0.78	0.29	-0.10	0.12	-0.22
DY737846	P2Y purinoceptor 2 [Oncorhynchus mykiss]	-0.13	0.14	-0.05	0.20	0.03	-0.55	0.02	-0.12	0.10	0.37
DY739459	Angiopoeitin like 2	0.54	-0.34	0.15	-0.15	-0.70	0.48	-0.25	-0.22	0.13	0.36
DY739699	Novel tub family member protein - Ident 43	-0.05	-0.41	-0.22	0.01	0.41	0.12	0.18	-0.14	0.63	-0.53
DY740624	CPI-17 - Ident 29	-0.08	0.77	-0.27	-0.11	0.56	-0.46	-0.05	-0.14	-0.10	-0.11
DY741343	Cytochrome P450_ family 51	0.15	-1.44	0.47	0.45	0.13	0.38	0.14	-0.32	-0.05	0.09
EF410001	thymidylate kinase [Salmo salar]	0.07	-0.07	-0.19	-0.28	0.38	-0.66	0.25	-0.05	0.36	0.19
EF466870	T cell receptor alpha [Salmo salar]	-0.14	-0.44	0.25	-0.33	-0.45	0.60	0.01	0.01	-0.22	0.70
EF695272	homeobox protein HoxC8ba [Salmo salar]	-0.10	-0.92	0.21	-0.16	-0.52	0.85	0.21	0.19	-0.35	0.59
EG647756	Protein (Peptidyl-prolyl cis/trans isomerase) NIMA-interacting 1	0.21	0.50	0.03	0.17	0.46	-0.71	-0.21	0.06	-0.18	-0.32
EG648470	Lactosylceramide 1_3-N-acetyl-beta-D-glucosaminyltransferase A	-0.10	0.31	0.49	-0.18	0.05	-0.01	0.28	-0.32	-0.25	-0.29
EG756571	Titin a - Ident 21	0.13	0.56	-0.13	0.11	0.38	-0.23	-0.24	-0.12	-0.21	-0.26
EG758412	Cell division protein kinase 2 [Salmo salar]	-0.04	-0.40	0.30	0.25	-0.34	0.00	-0.20	-0.32	0.35	0.41
EG760122	CC chemokine with stalk CK2 [Oncorhynchus mykiss]	-0.61	0.42	0.18	-0.21	-0.09	-0.30	-0.13	0.31	0.15	0.27
EG761046	tRNA (guanine-N(7)-)-methyltransferase	-0.15	0.79	-0.23	0.03	0.47	-0.44	-0.18	-0.28	0.21	-0.20
EG763652	nel-like 1 [Oncorhynchus mykiss]	-0.12	0.01	-0.79	-0.05	0.24	-0.10	-0.36	0.60	0.15	0.42
EG769368	GMP Giant mucus protein	0.13	-0.46	0.32	-0.54	-0.39	0.49	-0.11	0.58	-0.50	0.48
EG776091	Mx2 protein [Oncorhynchus mykiss]	0.14	-0.91	0.50	-0.48	0.15	-0.73	-0.18	0.37	0.17	0.97
EG782702	Thrombospondin 4b	0.37	-0.87	0.66	-0.13	-0.61	0.56	0.20	-0.36	-0.16	0.35
EG786166	PaTched Related family member	0.08	-1.14	0.72	-0.96	-0.13	-0.20	0.48	0.73	-0.11	0.53
EG786828	H-2 class II histocompatibility antigen gamma chain [Salmo salar]	-0.31	-0.20	0.30	0.06	-0.02	-0.17	0.21	0.13	0.05	-0.07
EG787057	C-C motif chemokine 19 precursor [Salmo salar]	0.26	-0.72	0.61	-0.47	-0.13	-0.53	-0.80	0.77	0.23	0.79
EG787743	Prolylcarboxypeptidase (Angiotensinase C)	-0.04	-0.08	-0.07	-0.13	-0.15	0.56	-0.07	0.14	0.07	-0.24
EG788483	C-C motif chemokine 4 precursor [Salmo salar]	0.06	-0.80	0.39	-0.07	-0.22	0.42	-0.14	0.17	-0.08	0.27
EG788755	Dspa protein - Ident 90	-0.54	-0.62	0.45	0.42	-0.55	0.35	0.35	-0.21	0.65	-0.29
EG792923	interleukin-15 [Salmo salar]	0.27	-0.85	0.10	-0.14	0.19	0.24	-0.15	0.14	-0.17	0.38
EG804767	valosin containing protein [Oncorhynchus mykiss]	0.25	-0.28	0.20	-0.11	-0.53	0.17	-0.11	-0.14	0.20	0.36
EG805683	Gig2-6	-0.05	-0.45	0.03	-0.20	0.11	-0.23	0.16	0.24	0.03	0.36
EG806118	Uncharacterized proteinF1R6C4 Uncharacterized protein	-0.26	0.23	0.02	-0.54	-0.11	0.06	-0.18	0.10	0.43	0.26
EG806570	Small muscle protein_ X-linked	-0.04	0.34	0.11	-0.07	0.04	-0.05	-0.15	-0.09	-0.15	0.07
EG810100	Kelch-like protein 13 [Salmo salar]	0.31	0.46	0.08	0.17	0.21	-0.66	-0.50	-0.08	0.08	-0.06
EG818391	adenylosuccinate synthase like 2 [Salmo salar]	-0.03	0.19	0.03	0.23	0.46	-0.08	0.06	-0.25	-0.11	-0.50
EG819885	Cytotoxic T-lymphocyte protein 4 - Ident 49	-0.95	-0.59	0.00	0.65	0.35	0.52	0.22	0.44	0.10	-0.74
EG821178	Protein-glutamine gamma-glutamyltransferase 2 [Salmo salar]	-0.31	-0.51	0.25	-0.30	0.07	-0.25	-0.11	0.62	0.05	0.49
EG823993	C-C motif chemokine 19 precursor [Salmo salar]	0.11	-1.19	0.42	-0.13	-0.44	-0.25	-0.07	1.09	-0.23	0.68
EG824979	C-X-C motif chemokine 10 precursor [Salmo salar]	-0.11	-0.44	-0.27	-0.18	0.22	0.00	0.03	0.35	-0.02	0.42
EG828714	actin [Oncorhynchus keta]	0.46	-0.48	0.55	0.26	-1.00	0.65	-0.07	-0.96	0.33	0.26

EG829823	Cartilage associated protein - Ident 48	0.53	-0.07	-0.18	0.10	0.03	0.22	-0.27	0.09	-0.04	-0.41
EG834184	Lymphocyte protein tyrosine kinase - Ident 96	0.11	-0.03	0.22	0.23	-0.22	0.58	0.01	-0.30	-0.32	-0.27
EG836160	Ubiquitin-like protein-1	0.57	-0.72	-0.54	-1.58	0.85	-1.00	0.82	0.07	0.78	0.76
EG836953	Novel protein similar to vertebrate patched domain containing 3 (PTCHD3)	0.11	-1.05	0.57	-1.25	0.08	-0.36	0.52	0.91	-0.12	0.58
EG840055	Isopentenyl-diphosphate delta-isomerase 1 [Oncorhynchus mykiss]	-0.02	-1.51	0.37	0.69	-0.15	0.47	0.02	-0.21	0.32	0.02
EG841455	Very large inducible GTPase 1-1	0.06	-1.22	0.09	-0.69	0.65	-0.39	0.19	0.19	0.08	1.03
EG841846	similar to hect domain and RLD3	-0.01	-0.32	-0.13	-1.75	0.80	-0.96	0.87	0.44	0.49	0.57
EG842682	Novel protein similar to vertebrate spectrin_ beta_ non-erythrocytic 1 (SPTBN1) - Ident 24	-0.47	-0.22	-0.19	-0.21	0.22	-0.03	0.38	0.35	0.58	-0.42
EG844312	Ubiquitin-like modifier activating enzyme 1	0.10	-0.57	0.22	-0.30	0.05	0.10	-0.02	0.02	0.09	0.30
EG845528	Electron transfer flavoprotein subunit alpha, mitochondrial precursor [Salmo salar]	0.40	-0.13	0.58	-0.46	0.32	0.63	-0.01	-0.86	0.13	-0.60
EG845583	Protein kinase C and casein kinase substrate in neurons 1 - Ident 36	0.50	-0.03	-0.04	0.05	-0.61	-0.02	0.24	-0.61	0.23	0.29
EG847712	3-oxoacyl-[acyl-carrier-protein] synthase - Ident 27	0.27	-0.14	0.09	-0.63	-0.16	0.72	0.14	0.46	-0.79	0.04
EG852935	Cathepsin K precursor [Salmo salar]	0.05	-0.77	0.63	-0.38	-0.27	0.40	0.03	0.43	-0.19	0.07
EG861089	Retinoid-binding protein 7 [Salmo salar]	-0.24	0.09	-0.13	0.15	-0.63	0.31	0.45	-0.16	-0.07	0.23
EG868420	Secreted frizzled-related protein 1 [Salmo salar]	-0.29	0.00	0.16	-0.34	-0.69	-0.54	-0.22	-0.21	-0.35	0.47
EG868838	Glutamate decarboxylase-like protein 1 [Salmo salar]	-0.22	0.09	0.47	0.03	0.10	0.12	-0.22	-0.04	0.08	-0.42
EG868979	C-C motif chemokine 13 precursor [Salmo salar]	-0.74	-0.36	-0.56	0.91	0.11	-0.38	0.26	0.79	0.60	-0.61
EG869207	Ubiquitin-conjugating enzyme E2 G1 [Salmo salar]	0.04	0.48	-0.12	0.24	0.47	-0.66	0.14	-0.04	-0.07	-0.47
EG869777	TsetseEP precursor [Salmo salar]	0.30	-1.06	0.24	-0.48	0.58	-0.01	0.24	-0.12	0.07	0.24
EG869820	Tripartite motif-containing protein 65 [Salmo salar]	-0.02	-0.52	-0.02	0.00	-0.21	-0.15	0.11	0.40	-0.13	0.55
EG871889	Caspase-1 precursor [Salmo salar]	0.16	-0.59	0.33	-0.03	0.27	0.62	-0.29	0.42	-0.04	-0.86
EG871928	Gap junction beta-6 protein [Salmo salar]	0.12	0.68	-0.13	0.10	0.72	-0.56	-0.24	-0.23	-0.29	-0.18
EG872078	Annexin A1 [Salmo salar]	-0.20	-0.03	-0.25	0.36	-0.09	0.27	0.19	0.68	-0.34	-0.58
EG874981	Growth arrest-specific 1b	0.45	-0.10	0.75	-0.18	-0.27	0.09	-0.36	-0.11	-0.08	-0.19
EG876930	Tropomyosin-1 alpha chain [Salmo salar]	0.36	-0.25	0.25	-0.13	-0.64	0.41	-0.02	-0.35	-0.12	0.49
EG877386	Interferon-induced protein 44 [Salmo salar]	0.08	-0.53	0.23	-0.44	0.37	0.01	0.11	-0.05	-0.12	0.34
EG878935	Complement factor D precursor [Salmo salar]	0.39	-0.54	0.47	-0.40	0.50	-0.04	-0.35	-0.10	-0.39	0.46
EG878939	Synaptotagmin V - Ident 25	0.56	-0.32	0.48	0.03	0.07	-0.25	-0.21	-0.33	-0.15	0.12
EG879077	Angiopoietin-like 7	-0.15	-0.02	0.05	0.76	0.01	-0.16	-0.20	-0.57	0.10	0.15
EG879132	Phytanoyl-CoA dioxygenase, peroxisomal [Salmo salar]	0.13	0.39	0.04	-0.18	0.54	-0.10	-0.11	-0.21	-0.04	-0.47
EG881366	Synaptosomal-associated protein 25-A [Salmo salar]	0.02	-0.52	0.35	-0.24	-0.80	0.94	-0.11	0.27	-0.48	0.59
EG884962	Novel protein similar to vertebrate containing RUN and TBC1 domain - Ident 80	-0.17	0.44	-0.24	-0.04	0.23	0.00	-0.10	0.19	0.05	-0.37
EG891995	SET protein [Salmo salar]	0.10	0.02	0.30	0.09	-0.37	0.11	-0.02	-0.86	0.09	0.54
EG893818	Claudin-like protein ZF4A22 [Salmo salar]	0.08	-0.84	0.15	0.15	-0.34	0.49	-0.01	0.23	-0.39	0.48
EG896159	Fructose-1,6-bisphosphatase 1 [Salmo salar]	0.16	0.21	-0.02	0.06	0.09	0.03	0.00	0.02	-0.12	-0.41
EG896619	Complement factor D precursor [Salmo salar]	0.05	-0.23	0.26	-0.59	-0.17	0.18	-0.02	0.07	0.18	0.26
EG898541	Complement C1q-like protein 2 precursor [Salmo salar]	-0.17	-0.38	0.19	-0.36	0.46	-0.64	0.06	0.78	0.17	-0.11
EG898949	PREDICTED: similar to SI:zC220F6.1 (novel protein similar to human dynein heavy chain (DHC)) [Danio rerio]	0.32	-0.75	0.36	-0.66	0.19	-0.24	-0.15	0.26	-0.14	0.81
EG904504	Poly polymerase 12-2	0.10	-0.44	-0.52	-0.80	0.49	-0.46	0.72	0.39	0.18	0.35
EG906096	Sacsin	0.20	-0.72	-0.32	-1.05	0.41	-0.38	0.70	0.31	0.69	0.15
EG910288	Quattro	0.17	0.28	0.03	-0.01	-0.02	0.42	-0.13	0.12	-0.45	-0.42
EG912551	Forkhead box protein A2	-0.36	-1.20	0.43	-1.08	-0.31	0.20	0.11	0.56	0.50	1.15
EG915565	interleukin-1 receptor antagonist [Oncorhynchus mykiss]	-0.01	-0.19	0.03	-0.05	-0.10	0.20	0.38	0.11	-0.13	-0.24
EG916718	Dynein cytoplasmic 1 heavy chain 1 - Ident 25	0.36	0.06	0.13	-0.10	0.07	-0.36	-0.46	0.08	0.07	0.15
EG922774	UDP-GlcNAc:betaGal beta-1_3-N-acetylglucosaminyltransferase-like 1 - Ident 99	0.28	-0.60	-0.02	-0.46	-0.40	0.65	0.24	0.04	-0.18	0.45
EG924531	C-C motif chemokine 8 precursor [Salmo salar]	-0.79	-0.40	0.09	0.39	-0.50	0.07	0.02	0.83	0.24	0.05
EG928302	Metalloproteinase inhibitor 2 precursor [Salmo salar]	-0.21	0.09	-0.16	-0.36	-0.22	0.29	0.16	0.67	-0.06	-0.21
EG930210	Cytochrome P450 4F3 [Salmo salar]	0.18	0.21	-0.15	0.17	-0.44	0.45	0.16	-0.04	-0.08	-0.45
EG934966	Cytochrome P450 24A1, mitochondrial precursor [Salmo salar]	0.63	0.14	0.00	-0.07	0.32	0.13	-0.31	-0.56	0.00	-0.28
EG938049	Complement C1q-like protein 2 precursor [Salmo salar]	0.28	0.29	-1.08	0.88	0.03	0.08	-0.42	-0.67	0.31	0.30
EG939031	VHSV-induced protein-10 [Oncorhynchus mykiss] VHSV-induced protein-10 [Oncorhynchus mykiss]	-0.14	-0.83	0.82	-0.94	0.41	-0.28	-0.11	0.75	-0.12	0.44
EG942280	Retinol dehydrogenase 12_ like - Ident 27	-0.11	-1.19	0.24	0.33	0.02	0.42	0.01	0.02	0.27	-0.01
EH033578	Titin b - Ident 97	-0.06	-0.04	0.00	0.09	0.14	0.26	0.29	-0.31	-0.08	-0.33
EST1-3A_B03	Natterin-like protein [Salmo salar]	-0.02	-0.07	0.09	-0.25	-0.20	-0.09	-0.37	0.46	0.21	0.23

FC072812	fast myotomal muscle actin 2 [Salmo salar]	-0.08	0.53	0.12	0.26	0.01	0.01	-0.42	-0.10	-0.13	-0.19
FJ184374	interferon gamma 1 [Oncorhynchus mykiss]	0.16	-0.69	0.29	0.04	0.02	0.02	-0.16	0.41	-0.30	0.21
FJ184375	interferon gamma 2 [Oncorhynchus mykiss]	0.00	-0.46	0.09	-0.02	-0.08	-0.37	0.23	0.13	-0.13	0.61
FP319330	Diaphanous 2	0.25	-0.74	0.22	-0.41	-0.77	0.80	-0.02	0.11	0.00	0.57
GE768559	Ubiquitin [Salmo salar]	0.34	0.78	-0.11	-0.14	0.76	-0.55	-0.24	-0.10	-0.52	-0.20
GE774984	fish virus induced TRIM protein [Oncorhynchus mykiss]	0.08	-0.81	0.38	-0.13	-0.63	0.90	-0.15	0.11	-0.37	0.62
GE779849	Ganglioside GM2 activator precursor [Salmo salar]	0.16	-0.11	-0.12	0.06	-0.02	0.46	-0.07	-0.02	0.03	-0.36
GE790164	Myocyte enhancer factor 2cb	0.20	0.05	-0.53	0.21	-0.04	-0.21	0.42	-0.12	0.27	-0.26
GE828738	C-Myc-binding protein [Oncorhynchus mykiss]	-0.10	0.32	-0.03	0.11	0.73	-0.66	-0.05	-0.12	-0.03	-0.17
GE834131	C-C motif chemokine 4 precursor [Salmo salar]	0.04	-0.88	0.14	-0.13	0.04	0.23	-0.04	0.32	0.06	0.21
GO049421	NBP - Ident 90	0.32	0.29	-0.07	0.15	0.64	-0.58	-0.12	-0.31	-0.10	-0.21
GO056376	Galectin-4 [Salmo salar]	-0.76	-0.10	-0.39	0.35	0.18	-0.20	0.12	0.10	0.28	0.41
GO057285	Rho guanine nucleotide exchange factor (GEF) 7b	0.01	0.68	-0.20	0.21	0.18	-0.40	-0.10	-0.16	-0.02	-0.19
GO057450	Minichromosome maintenance complex component 10	-0.36	-0.06	0.58	0.28	0.29	-0.28	0.00	0.28	-0.18	-0.55
GO057850	Transmembrane protein 179B [Salmo salar]	0.44	-0.18	0.04	0.17	-0.40	0.04	0.12	0.35	-0.21	-0.37
S25807320	matrix metalloproteinase [Oncorhynchus mykiss]	0.12	0.26	0.00	0.00	-0.62	0.04	-0.07	0.00	-0.10	0.36
S30293225	Galectin-9 [Salmo salar]	-0.02	-0.16	-0.27	-0.65	0.56	-0.59	0.62	-0.01	0.72	-0.19
S48394090	UDP-N-acetylglucosamine--peptide N-acetylglucosaminyltransferase 110 kDa subunit [Salmo salar]	0.09	0.38	0.10	-0.04	0.22	-0.11	0.15	-0.16	-0.19	-0.43
S48416864	Tpr protein - Ident 89	0.05	-0.51	0.07	-0.19	-0.33	0.42	-0.14	0.30	-0.05	0.38
S48425785	NipSnap2 [Oncorhynchus mykiss]	0.16	-0.76	0.57	-0.13	0.17	0.21	0.04	-0.33	-0.01	0.09
S48436177	Synaptobrevin homolog ykt6 [Oncorhynchus mykiss]	-0.07	-0.46	0.16	-0.34	-0.28	0.32	-0.03	-0.03	0.19	0.54
S48437928	Gap junction beta-6 protein [Salmo salar]	-0.11	-1.25	0.13	0.58	-0.41	0.77	0.13	-0.19	0.29	0.07
TC103532	GrpE protein homolog 1, mitochondrial precursor [Salmo salar]	0.34	-0.24	0.34	-0.24	-0.56	1.25	-0.20	-0.09	-0.54	-0.06
TC104008	vomeronasal receptor-like protein [Salmo salar]	0.01	0.61	0.08	0.09	-0.04	-0.60	0.20	0.07	-0.19	-0.23
TC104277	Ectonucleoside triphosphate diphosphohydrolase 2 [Salmo salar]	-0.17	-0.15	0.14	-0.17	-0.52	0.32	-0.02	0.48	-0.46	0.56
TC105613	fish virus induced TRIM protein [Oncorhynchus mykiss]	0.34	-0.13	0.03	0.00	0.02	0.06	0.12	0.19	-0.77	0.13
TC106062	interleukin 22 [Oncorhynchus mykiss]	0.00	-0.49	0.53	-0.09	-0.90	1.12	-0.40	0.11	-0.11	0.24
TC106398	52 kDa Ro protein-1	-0.10	-0.28	-0.39	-0.81	0.14	-0.59	0.80	0.55	0.45	0.22
TC111220	CD86 molecule [Oncorhynchus mykiss]	-0.17	-0.30	-0.15	-0.29	-0.18	-0.06	0.49	-0.02	0.17	0.51
TC166906	Rho GTPase activating protein 12 - Ident 31	-0.37	-0.48	0.22	-0.35	-0.35	0.48	0.13	0.23	-0.09	0.59
TC88700	Dimethylaniline monooxygenase 5 [Salmo salar]	0.58	0.14	-0.16	-0.33	0.52	-0.26	-0.04	-0.12	-0.07	-0.25
TC90018	Fads2 protein - Ident 21	0.39	0.27	0.08	-0.26	0.55	-0.27	-0.11	-0.28	-0.10	-0.28
Y12456	Ig mu chain C region membrane-bound form [Salmo salar]	0.22	-0.18	0.18	-0.33	0.15	-0.16	0.43	-0.34	-0.66	0.70
KSS5151	B-cell lymphoma 6 protein homolog [Salmo salar]	0.08	0.59	0.05	0.00	0.40	-0.60	-0.11	-0.13	0.02	-0.31
STIR35406	CXC chemokine d1 [Oncorhynchus mykiss]	-0.41	-0.25	0.02	0.20	-0.33	0.01	0.36	0.13	-0.13	0.40
KSS3392	GMP Giant mucus protein	1.51	-1.20	-0.43	-0.02	0.16	0.03	-2.14	-0.34	0.96	1.48
KSS976	TBC1 domain family_ member 5 - Ident 38	0.08	0.75	-0.14	-0.22	0.42	-0.17	-0.25	0.00	-0.31	-0.16
KSS2413	Unknown	-0.06	0.36	0.18	-0.05	0.22	0.07	-0.05	-0.36	0.13	-0.44

III

Dose-response relationship between dietary choline and lipid accumulation in pyloric enterocytes of Atlantic salmon (*Salmo salar* L.) in seawater

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Short title: Choline prevents gut lipid accumulation

Key words: Choline requirement, Lipid accumulation, Gut health, Fish feed, Plant ingredients

Abstract

Foamy, whitish appearance of the pyloric caeca, reflecting elevated lipid content, histologically visible as hypervacuolation, is frequently observed in Atlantic salmon fed high plant diets. Lipid malabsorption syndrome (LMS) is suggested as term for the phenomenon. Earlier studies have shown that insufficient supply of phospholipids may cause similar symptoms. The objective of the present study was to strengthen knowledge on the role of choline, the key component of phosphatidylcholine, in development of LMS as well as finding the dietary required choline level in Atlantic salmon. A regression design was chosen to be able to estimate the dietary requirement level of choline, if found essential for the prevention of LMS. Atlantic salmon (456 g) were fed diets supplemented with 0, 392, 785, 1177, 1569, 1962, 2354, 2746 and 3139 mg/kg choline chloride. Fish fed the lowest choline diet had pyloric caeca with whitish foamy surface, elevated relative weight, and the enterocytes were hypervacuolated. These characteristics diminished with increasing choline level and levelled off at levels of 2850, 3593 and 2310 mg/kg, respectively.

The concomitant alterations in expression of genes related to phosphatidylcholine synthesis, cholesterol biosynthesis, lipid transport and storage, confirmed the importance of choline in lipid turnover in the intestine and ability to prevent LMS. Based on the observations of the present study the lowest level of choline which prevents LMS and intestinal lipid hypervacuolation in post smolt Atlantic salmon is 3.4 g/kg. However, the optimal level most likely depends on the feed intake and dietary lipid level.

Introduction

Lipid malabsorption syndrome (LMS) has been observed for more than a decade in farmed Atlantic salmon in Norway [(1)]. An ongoing screening of gut health in Norwegian cultivated salmon shows that LMS still is a frequent occurrence at all developmental stages [(2)]. The typical macroscopic characteristics of fish with LMS is a pale and foamy surface of the pyloric intestine and pyloric caeca, in some cases extending to the mid intestine, due to excessive lipid accumulation in the enterocytes. The likely explanation is limited lipid transport capacity. Our previous study [(3)] indicates that choline synthesis in post smolt Atlantic salmon in seawater is insufficient to cover the requirement. One consequence of the shift from marine to plant-based diets is a lower content of choline, in the form of phosphatidylcholine in the raw materials [(4)]. An exogenous supply of choline may therefore be essential for normal metabolism, transport and export of lipids across the mucosa of the pyloric caeca and for prevention of LMS in salmon, in the seawater phase [(3)].

Choline is defined as an essential nutrient for mammals [(5)], whereas phosphatidylcholine is not, as it can be synthesized if choline is present. Regarding fish, essentiality has been established, as reported in the NRC [(6)], but only for early stages, and the requirement differs between the species [(6–12)]. For salmonids, the NRC suggests a requirement of 800 mg/kg for both rainbow trout and some species of Pacific salmon [(6)]. However, also higher requirements around 3000 mg/kg have been indicated for Pacific salmonids [(13)]. Studies on juvenile rainbow trout suggest an inverse relationship between initial body weight and choline requirement. Estimated choline requirement for 0.12, 1.4, 3.2 and 3.5 g fish is 3000, 813, 714 and between 50-100 mg/kg diet, respectively

[6,14,15]. At present no requirement estimates are established in the scientific literature for any life stages of Atlantic salmon [6]. The available studies addressing choline requirement for early stages of fish development shows that deficient supply causes symptoms as poor growth and low feed efficiency, fatty liver, high mortality and anorexia [11,15,16]. Lipid accumulation in the intestinal mucosa is seldom an observed endpoint in studies of choline deficiency and requirement but was observed in an early study of Japanese eel (*Anguilla japonica*) as "white-grey colored intestines" [17]. The symptoms appear similar to those observed in Atlantic salmon with LMS. Other studies have shown that phosphatidylcholine may prevent lipid accumulation in the intestinal mucosa and might be the limiting factor for lipid cell transport [18–24]. In these, dietary supplementation with phosphatidylcholine was observed to reduce the formation of enterocytic lipid droplets and was an important factor in lipoprotein formation [22,25–27] necessary for exporting dietary lipid from the gut [20,28,29]. The apparent disturbance in lipid metabolism of fish fed diets with high level of plant ingredients is also observed on the molecular level. Expression of genes involved in lipid metabolism seems to reflect reduced lipid export from the enterocytes [10,20–38].

However, the key components and mechanisms involved and the mechanisms underlying the excessive lipid accumulation are not yet understood. The present study is part of a PhD-program aiming to gain knowledge on mechanisms underlying development of LMS in Atlantic salmon and to find optimum levels of choline in diets for Atlantic salmon. As far as our literature review has shown, no dose-response studies with choline in diets for Atlantic salmon in seawater has been reported so far. The aim of the present study was, therefore, to estimate choline requirement of Atlantic salmon under saltwater conditions, as indicated by excessive lipid accumulation in the intestine.

Materials and methods

Diets

A low fishmeal, high plant diet (LF1) was used as a reference diet, containing 10% Nordic LT fishmeal. The total lipid content was 70% rapeseed oil and 30% fish oil. The diet formulation and analysed chemical composition can be found in Table 1. The choline supplemented diets (LF2-

LF9) were made by supplementing the LF1 diet with eight levels of choline chloride 70%. Table 2 shows supplemented and analysed choline content in the experimental diets. The choline analyses indicate a higher than expected variation in the results. However, there was no systematic deviation. Therefore, the high variation should not affect the estimates of choline requirement. The diets were supplemented with standard vitamin and mineral premixes in accordance with NRC guidelines (2011) and BioMar standards to meet the requirements. Yttrium oxide (0.50 g/kg) was added as inert marker for estimation of nutrient apparent digestibility. The experimental diets were produced by extrusion (feed pellet size 6 mm) at BioMar Feed Technology Centre (Brande, Denmark) using a BC 45 twin screw extruder (Clextral, France).

Experimental animals and conditions

The experiment was conducted at Nofima's Research Station at Sunndalsøra, which is a research facility approved by Norwegian Animal Research Authority (NARA) and operates in accordance with Norwegian Regulations of 17th of June 2008 No. 822: Regulations relating to Operation of Aquaculture Establishments (Aquaculture Operation Regulations). Trial fish were treated in accordance with the Aquaculture Operation Regulations during the trial. Fish were randomly sampled, anaesthetized and killed by a sharp blow to the head, in accordance with the Norwegian Animal Welfare act. No surgical manipulation of live fish was conducted, and tissue samples were only retrieved from euthanized fish. Ingredients commonly used in commercial diets were used in the experimental diets and did not cause the fish any apparent distress. No NARA approval was required according to §2 of the Norwegian Regulation on Animal Experimentation.

Atlantic salmon (*Salmo salar* L., post smolt, Sunndalsøra strain) with mean initial weight of 456 g ± 65 (mean ± SD) were pit tagged, individually weighed, and randomly allocated into nine fiberglass tanks. Each tank contained 300 l of saltwater, 35 fish and an initial and final fish density of 53 and 127 kg/m^3, respectively. The density was high, but within limits found compatible with good growth, health and welfare of fish under the condition that oxygen supply is sufficient [39]. The tanks were supplied with flow through seawater. Salinity ranged between 32 and 33 g/l. The water flow was increased accordingly to the increase in biomass over time and to maintain oxygen saturation above 80%. The oxygen content of the outlet water was monitored once a week or more often in periods with larger temperature variations. The water temperature

varied between 7.5 and 14.0°C during the experimental period (from July to September 2012), with an average of 10.5°C and a constant 24 h light regime was employed during the experimental period. Each tank was fed one experimental diet. Feed waste could not be collected and therefore feed intake was not measured. The daily amount of feed given were calculated from the expected biomass and daily growth rate and added with 15% to secure feeding to ad-libitum. The fish were fed using disc feeders.

Sampling

After 92 days, feeding was terminated. From each tank ten fish were anaesthetized with tricaine methane-sulfonate (MS-222). Weight and length were recorded for all fish and blood was sampled from the caudal vein in vacutainers with lithium heparin. The vacutainers were stored on ice prior to plasma preparation. Plasma was sampled in 2 mL aliquots and snap frozen in liquid nitrogen and stored at -80°C. Following blood sampling the fish were killed by a sharp blow to the head and opened ventrally. The gastro-intestinal tract was removed from the abdominal cavity, cleared of other organs and adipose tissue, and sectioned as follows: pyloric intestine (PI): the section from the sphincter to the most distal pyloric caeca; mid intestine (MI) from the distal end of PI and proximal to the increase in intestinal diameter; distal intestine (DI) section from the distal end of MI to the anus. The tissue of the PI and DI were collected and weighed, whereas the digesta from these two sections were split in two samples, i.e. the proximal half (PI1 and DI1, respectively) and distal half (PI2 and DI2, respectively). The intestinal samples were snap frozen in liquid nitrogen and stored at -80°C. The liver (LI) was also weighed. An additional eight fish per tank were euthanized prior to sampling of the pyloric caeca for histological and gene expression analyses. The remaining fish in each tank were stripped for faeces as described by Austreng [40]. The remaining fish were then fed for one more week for an additional stripping in order to collect enough sample for analysis. The fecal samples were pooled for each tank, frozen immediately in liquid nitrogen after stripping and stored at -80°C until analysis. Tissues sampled for histological examination were fixed in 10% neutral buffered formalin (4% formaldehyde). Samples for gene expression analyses were rinsed in sterile saline water, submerged in RNAlater® and kept at 4°C for 24 hours and subsequently kept at -40°C until analysis.

Histology

Pyloric caeca samples were processed at the Norwegian University of Life Sciences (NMBU) using standard histological techniques and stained with haematoxyling and eosin. The samples were evaluated for enterocyte vacuolation blinded in a randomized order using a light microscope. The appearance of lipid-like vacuoles was assessed semi quantitatively by the proportion of total tissue affected: marked (≥ 50%), moderate (25-50%), mild (10-25%) and normal (≤ 10%) and presented as percentage of vacuolated enterocytes (Figure 1).

Gene expression

Quantification of gene expression was conducted on pyloric caeca samples from fish fed the LF1 and LF3-8, in accordance to MIQE standards [41]. Total RNA from pyloric caeca samples (~30 mg) were extracted using a Ultraturrax homogenizer, Trizol® reagent (Invitrogen) and chloroform according to the manufacturer's protocol. Obtained RNA was DNase treated (TURBO™, Ambion, ThermoFisher Scientific) and purified using a Direct-zol RNA purification kit (Zymo Research). RNA integrity of all samples was assessed by gel electrophoresis, and in addition selected samples were verified with a 2100 Bioanalyzer using a RNA Nano Chip (Agilent Technologies). The RIN values were all >8. RNA purity and concentrations were measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Total RNA was stored at -80°C for future use.

First strand cDNA synthesis was carried out using five fish from each tank and Superscript III in 20 µL reactions (Invitrogen, ThermoFisher Scientific) using 0.8 µg total RNA and oligo $(dT)_{20}$ primers. Negative controls were performed in parallel by omitting RNA or enzyme. Obtained cDNA was diluted 1:10 before use and stored at -20°C. A total of 26 target genes with key functions in lipid and sterol metabolism were profiled by quantitative PCR. Information of gene categories and functions is provided in Supplementary table 1. PCR primers were obtained from the literature or designed using Primer3web version 4.0.0 (http://bioinfo.ut.ee/primer3/). Detailed information of the primers is shown in Supplementary Table 1. PCR reaction efficiency (E) for each gene assay was determined separately using 2-fold serial dilutions of randomly pooled cDNA. A LightCycler 480 (Roche Diagnostics) was used for DNA amplification and analysis of

the expression of individual gene targets. Each 10 µl DNA amplification reaction contained 2 µl PCR-graded water, 2 µl of 1:10 diluted cDNA template, 5 µl of LightCycler 480 SYBR Green I Master (Roche Diagnostics) and 0.5 µl of each forward and reverse primer. Each sample was assayed in duplicate in addition to a no template control. The three-step qPCR program included an enzyme activation step at 95°C for 5 minutes followed by forty or forty-five cycles (depending on the individual gene tested) of 95°C (10 s), 58, 60 or 63°C (10 s depending on the individual gene tested) and 72°C (15 s). Quantification cycle (Cq) values were calculated using the second derivative method. The PCR products were evaluated by analysis of melting curve and by agarose gel electrophoresis to confirm amplification specificity. All primer pairs gave a single band pattern on the gel for the expected amplicon of interest in all reactions. For target gene normalization, *actb*, *ef1a*, *gapdh*, *rnapolII* and *rps20* were evaluated for use as reference genes by ranking relative expression levels according to their stability, as described previously [(42)]. The *rnapolII* showed a stable expression pattern and was therefore used as normalization factor. Mean normalized expression of the target genes was calculated from raw Cq values by relative quantification [(43)].

Chemical analyses

Diets and faecal samples were analysed for dry matter (after heating at 105°C for 16-18 hours), ash (combusted at 550°C to constant weight), crude protein (by the semi-micro-Kjeldahl method, Kjeltec-Auto System, Tecator, Höganäs, Sweden), lipid (diethylether extraction in a Fosstec analyzer (Tecator) after HCL-hydrolysis), starch (measured as glucose after hydrolysis by alpha-amylase (Novo Nordisk A/S, Bagsvaerd, Denmark) and amylo-glucosidase (Bohringer Mannheim GmbH, Mannheim, Germany), followed by glucose determination by the "Glut-Dh method" (Merck Darmstadt, Germany)), gross energy (using the Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA) and yttrium (by inductivity coupled plasma (ICP) mass-spectroscopy as described by Refstie et al. [(44)]. The plasma variables; free (non-esterified) fatty acids, cholesterol and total triacylglycerides were analysed according to standard procedures at the Central Laboratory of the Norwegian University of Life Sciences (NMBU). Lipoprotein profile analyses (HDL, LDL and VLDL) in plasma were carried out by size exclusion chromatography and measurements of cholesterol and triglycerides on-line using microliter sample volumes as

described by Parini et al. [45]. Isotop dilution mass spectrometry as described by Lund et al. [46] was used for analyzing lathosterol. 7α-hydroxy-4-cholesten-3-one (C4) was analyzed by isotope dilution and combined HPLC-MS as described by Lövgren-Sandblom et al. [47]. Plasma levels of oxysterols, sitosterol and camposterol were analyzed by isotope dilution and combined GC-MS after hydrolysis as described by Dzeletovic et al. [48] for the first mentioned and by Acimovic et al. [49] for the last two mentioned. The pyloric caeca tissue from four fish from each of the LF1 and LF6 diets were analysed for the lipid classes free fatty acids (FFA), monoacylglycerol (MAG), diacylglycerol (DAG), triacylglycerol (TAG) and phospholipid (PL). Four fish was considered sufficient to reveal differences in lipid characteristics of fish from these treatments. Lipid were extracted according to a modified Folch procedure [50,51]. Duplicate samples were individually applied to thin layer TLC plates to separate the lipid classes; FFA, PL, TAG, and DAG. The various lipid classes were identified by comparison with standards [52]. The respective spots were scraped into reaction vials and the results of the further chemical [53, 54] where analysed using the Hewlett-Packard Chem Station Software. In the analysis the phospholipid composition was related to the C23:0 peak, the free fatty acid composition to the C12:0 peak, and the tri- and diacylglyceride compositions to the C13:0 peak. The combined weights of each lipid class sample thus calculated were corrected for contents of non-fatty acid material (e.g glycerol phosphate) by multiplying each weight with appropriate factors as given by Christie [55].

Calculations

Growth of the fish was calculated as specific growth rate (percent growth per day): SGR = ((ln FBWg / ln IBWg) / D) X 100. IBW and FBW are the initial and final body weight (tank means) and D is number of feeding days. The condition factor (CF) as: CF = FBW * 100) / Fork length cm^3). Organosomatic Indices (OSI) were calculated as: (organ weight g / body weight g) X 100). Apparent digestibilities (AD) of main nutrients was estimated by using Y_2O_3 [56] as an inert marker and calculated as follows: $AD_n = 100 - (100\ X\ (M_{feed} / M_{faeces})\ X\ (N_{feed} / N_{faeces}))$, where M represents the percentage of the inert marker in feed and faeces and N represents the percentage of a nutrient in feed and faeces. The 95% confidence range for choline requirement for the selected biomarkers = choline requirement level ± * 2SEM.

Statistical analysis

Data were tested for normality and homogeneity of variance using Shapiro-Wilk and Brown-Forsythe test respectively. When necessary, data were log transformed to obtain homogenous variance (indicated by "^{X}" in Supplementary Table 4). Responses of the supplemented levels of choline were evaluated using polynomial regression analyses. Visual examination of the results indicated that, for the data showing a clear relationship with dietary choline level, a second-degree function would fit the biomarkers well and be suitable for the main aim of the present study; to estimate a minimum required level of choline in salmonid diets for fish raised under conditions similar to the present. For the OSI of PI and DI, and the genes which showed significant correlation with choline level, the quadratic broken line model was applied for estimation of required level of choline [57]. Tank mean was used as the statistical unit. Effects on lipid classes and fatty acids in lipid classes were tested for significance by t-test with individual fish as the statistical unit. All data are presented as mean values with standard error of mean (SEM). The level of significance for all analyses was set at $P < 0.05$, and p-values between 0.05 and 0.1 were considered as indications of effects and mentioned as trends.

Results

Apparent nutrient digestibility and growth performance

Increasing choline inclusion did not affect apparent nutrient digestibility (AD) significantly for either protein, lipid or starch. The average AD (± SEM) was 89.8 (± 0.74) for protein, 98.0 (± 0.14) for lipid and 69.2 (± 0.95) for starch. No important relationships were observed between choline level and fatty acid digestibility (Supplementary Table 2). Regression analysis did not reveal any significant relationship between choline inclusion level and growth (SGR). However, a significant inverse relationship between CF and choline level was observed, but the R^2 for the model was very low ($R^2 = 0.059$, $p = 0.008$; Supplementary Table 2), and therefore the results were not considered beneficial for the estimation of choline requirement. The mean values are presented in Table 3.

Organosomatic indices, pyloric tissue lipid content and histology

Among the organosomatic indices (OSI), those for PI and MI (OSIPI and OSIMI) decreased significantly with increasing choline levels. For the other OSIs, i.e. DI and LI, no significant relationship with increasing choline levels was observed (Supplementary Table 2). Mean values are presented in Table 3. The choline (mg/kg) requirement level, (± SEM), estimated by a linear broken line model was lower for OSIPI (3090 ± 212) and OSIMI (2496 ± 538) than estimated by the quadratic model which indicated an average requirement of 3593 (± 226) and 3031 (±195) mg/kg diet respectively (Figure 2).

Regression analysis revealed a significant inverse relationship also between choline level and the macroscopically observed degree of whiteness and histologically observed lipid vacuolation (Supplementary Table 2). Results of the macroscopically and histologically examinations of the pyloric caeca showed an absence of symptoms at a choline level of, 2850 and 2310 mg/kg respectively (Figure 3-5).

Concentration of TAG ($p = 0.026$) and DAG ($p = 0.039$) in the pyloric tissue, analyzed only for fish fed the LF1 (1340 mg choline/kg) and LF6 (2850 mg choline/kg) diets, showed great and significant effects of choline supplementation (Figure 6). Compared to the results for the LF1 diet, the concentrations of TAG and DAG decreased by 76% and 63% respectively, in fish fed the LF6 diet. No significant difference due to choline supplementation was found for FFA or PL. Fatty acid profiles within the lipid classes TAG, DAG, FFA and PL of the pyloric caeca tissue are presented in Supplementary Table 3. Significant effects of choline supplementation are highlighted in Figure 7. Choline affected a higher number of fatty acids in PL than in the other lipid classes.

Intestinal chyme dry matter and bile salt concentration

Increasing choline inclusion did not significantly affect the dry matter content of digesta nor the bile salt concentration along the intestine (Table 4 and Supplementary Table 2).

Intestinal gene expression

The regression analysis results and the mean values of the results from the qPCR analyses of the pyloric tissue are presented in Supplementary Tables 2 and 4 respectively. Figure 8 illustrates the expression of the genes showing a significant regression or a trend ($0.05 < p > 0.1$) and an estimated choline requirement are shown for *pcyt1a* which is involved in the phosphatidylcholine synthesis, for *apoAIV* and *apoAI,* involved in the lipoprotein assembly and *plin2*, the general marker for lipid load of non-adipogenic cells. Among three enzymes *chk*, *pcyt1a* and *pemt*, all involved in the pathway of phosphatidylcholine biosynthesis, *pcyt1a* decreased significantly and *chk* showed the same trend with increasing levels of choline, whereas *pemt* was not significantly affected. A significant reduction in expression by an increased level of choline was also observed for *mtp* and *fatp*, involved in lipid transport. Moreover, both *apoAIV* and *apoAI*, showed a dose-response curve, increasing with increasing choline doses, levelling off at the highest inclusion level. A decreasing response was observed for *hmgcr*, an important enzyme in the regulation of the cholesterol biosynthesis. The expression of *plin2,* also decreased for then to plateauing for the highest choline inclusion levels. The *apoAIV* and *apoAI* revealed choline requirement levels not far from each other, 2593 ± 108 and 2610 ± 35 mg/kg, respectively. Similarities in choline requirement levels (± SEM) were also observed for *pcyt1a* and *plin2*, 3210 ± 404 and 3199 ± 360 mg/kg, respectively (Figure 8).

Blood plasma endpoints

The regression analysis revealed a significant positive relationship between choline level and LDL-CH, and a similar trend was observed for total plasma cholesterol levels (Supplementary Table 2). Blood plasma mean values are presented in Table 5. The HDL contained most of the plasma cholesterol and there was a trend towards increasing levels with increased dietary choline levels. Lathosterol is a steroid intermediate in the cholesterol synthesis and the circulating level reflects cholesterol synthesis in the liver. The circulating lathosterol levels were significantly and positively correlated with dietary choline level (Supplementary Table 2). For the other blood plasma values, presented in Supplementary Table 2, no significant relationship with increasing choline levels were observed.

Estimates of choline requirement level

Among all biomarkers observed in the present investigation, those showing significant effects of dietary choline level, and which may indicate choline requirement level: macroscopically observed whiteness, histologically observed vacuolation, OSIPI, OSIMI, *pcyt1a*, *apoAIV, apoAI* and *plin2*, are presented in Figure 9. The statistical analyses of OSIPI and OSIMI revealed SEM values of 6% of the estimated requirement of choline for both biomarkers. Assuming that the SEMs for the biomarkers macroscopic whiteness and histological vacuolation were of similar magnitude, the means and ranges indicating 95% confidence limits are illustrated in the Figure 9. The means ranged between 2310 and 3593 mg/kg of choline, with an average of 2936 mg/kg.

Discussion

The main finding of the present study was a clear inverse relationship between dietary choline level and the degree of vacuolation in the mucosa of the pyloric intestine of Atlantic salmon reared in seawater. Characterization of the lipid content of the mucosa showed a marked decrease also in lipid content, mainly due to a reduction in TAG and DAG in the pyloric caeca. The concomitant alterations in expression of genes related to phosphatidylcholine synthesis, cholesterol (CH) biosynthesis, lipid transport and the general marker for lipid load, confirmed the essentiality of choline in lipid turnover in the intestinal mucosa and ability to prevent lipid vacuolation and LMS. These findings form a basis for estimation of choline requirement as well as for understanding the mechanisms underlying lipid transport across the intestinal mucosa to the peripheral circulation.

Estimation of choline requirement

Indicators of gut mucosa lipid transport have rarely been used as response criteria in studies addressing choline function and requirement in previous investigations. Accordingly, the same is the situation regarding the literature reviewed as the basis for the estimates of choline requirement such as in the NRC's Nutrient requirement of fish and shrimp [6]. Weight gain and

liver lipid content have more often been end-points in studies addressing choline requirement [7,9,13,15,16,58,59]. Those indicators, however, are not necessarily the optimal biomarkers for the estimation of choline requirement in larger fish. Indicators of efficiency of lipid transport in the mucosa of the PI, supposedly the organ with the highest lipid turnover [60], particular in rapidly growing fish fed high lipid diets, may be more suitable biomarkers for choline requirement. In our previous study [3], we observed that the PI was the most sensitive tissue for studying the effects of variation in dietary choline. The present study confirms this observation, showing clear effects of choline level on the PI somatic index, macroscopically observed degree of whiteness and histologically observed lipid vacuolation in PI, whereas no significant effect on liver index was observed. The biomarkers presented in Figure 9 are those showing significant correlation to dietary choline levels in our study, i.e. with a response curve which make them potential indicators for choline requirement.

The mean requirements indicated by the biomarkers differed. Running requirements studies with fish, differs from terrestrial animal nutrition since the experiments are carried out in water and this requires special considerations related to controlling the actual feed intake. The book, Nutrient requirement of fish and shrimp [6], provides guidance on methodology and data analysis for nutrient requirement studies. There is no general agreement regarding which biomarkers gives the most relevant estimates for requirements, but it is generally agreed upon that the biomarker should functionally have a close relationship with the nutrient in question and show a clear dose-response relationship with intake at levels below requirement. Although the OSIPI indicated the highest choline requirement, it may not be appropriate as a biomarker to be used for the requirement estimation. The PI, as with the other parts of the intestine, has the ability to enlarge and diminish according to needs as a natural adaptation to variation in nutritional quality of the diet [61]. The histologically observed degree of vacuolation and the macroscopically observed appearance of whiteness, may be biomarkers more closely related to choline requirement and may be more relevant biomarkers, indicating overall lipid transport processes, and may therefore be the biomarkers best suited for the estimation of choline requirement. The PI whiteness gave the highest estimate of choline requirement of the two, i.e. 2850 mg/kg for whiteness and 2310 mg/kg for hypervacuolation. The difference between the two may be related to the fact that macroscopically examination summarizes characteristics of the whole pyloric intestinal tissue

whereas the histological examination observes a very limited area of a small sample of the pyloric caeca.

Regarding the four gene expression biomarkers suitable for requirement estimation, *apoAIV* (2592 mg/kg) and *apoAI* (2610 mg/kg) suggest average choline requirements between those of whiteness (2850 mg/kg) and hypervacuolation (2310 mg/kg), whereas *pcyt1a* (3210 mg/kg), as well as *plin2* (3199 mg/kg), suggest higher requirements. The 95% confidence range for the biomarker macroscopic whiteness overlap with all the ranges for the other selected biomarkers for choline requirement, making it reasonable to suggest the average of the choline requirements for the biomarkers as the estimate for this study, i.e. 2936 mg/kg. It should be a goal for an optimal diet to cover the needs of 95% of the fish, i.e. 2 x SEM should be added to the mean. In the present experiment the recommended choline level, for prevention of LMS is estimated to be 3350 mg/kg or 3.4 g/kg (Figure 9).

With a key role in lipid transport and metabolism, it is likely that choline requirement depends on dietary lipid level and feed intake, which we may call lipid load. In the present study, the diets contained 29% lipid and the average SGR was 0.94% (TGC: 2.7). At higher growth rates, higher feed intakes and/or higher dietary lipid levels, higher choline level may be required. Further studies are needed for the characterization of these relationships. The natural prey of wild Atlantic salmon can have a very high lipid content. Depending on the season, the lipid content in a herring from the North Sea can be as high as 50% on a dry matter basis (25% as is). There is a general lack of knowledge regarding the degree of lipid vacuolation in the intestines in a wild salmon during optimal periods where access to different foodstuff is high.

Dietary choline level effects on intestinal lipid transport

The present study showed a clear decreasing effect of choline level on the somatic index of PI, macroscopically observed degree of whiteness and histologically observed lipid vacuolation in PI. In the most serious cases of vacuolation, observed for the lowest levels of choline, the macroscopic whiteness continued from the PI and further down into the MI. Previous studies have shown that Atlantic salmon can use both PI and MI for absorption of long chain fatty acids, e.g. C18:1 [60]. It seems possible that our observation of lipid vacuolation in the MI could be

related to periods when the fish experience a lipid load higher than the capacity of the PI. These results substantiate that dietary choline increase the capacity of the PI to absorb and transport the lipid from the enterocytes [20–22,28,29]. The observed lower level of TAG in the fish given the diet with 2850 mg/kg choline compared to the un-supplemented diet with 1340 mg/kg, in combination with the absence of lipid accumulation and no significant differences in lipid digestibility, indicated as an increased flux of TAG across the tissue from the gut lumen to the portal vein, as a result of choline supplementation [62]. This conclusion is consistent with other studies observing that dietary phosphatidylcholine prevents accumulation of TAG in the intestinal mucosa [21,23]. Our results also support the general understanding that TAG is the primary lipid class in lipid stores [29], also in the intestinal mucosa, in the form of lipid droplets in the enterocytes [21,28,63,64].

The lower content of DAG in fish fed the choline supplemented diet, may indicate increased use for incorporation into phosphatidylcholine [24]. Since all the diets had the same fatty acid compositions, the fatty acid profile in the lipid fractions of the pyloric caeca was not expected to vary. Yet, some compositional differences were observed within all lipid fractions. Choline supplementation seemed to have the greatest impact on the fatty acid profile in PL. A significant impact was observed for several fatty acids ranging from C16 to C22, with the largest effects on the levels of 20:5n-3 (EPA) and 18:3n-3. A modulation of the fatty acids in the PL fraction of the tissue was also observed in a previous study on gilthead seabream larvae showing that lecithin and phosphatidylcholine supplementation increased the incorporation of 18:1n-9 [25,26]. Our observation of a lower content of EPA in the PL fraction with increasing choline level is suggested to be a result of increased lipid transport from the gut and might, if analyzed, have been seen as an increase of EPA in other body compartments of the fish such as heart, liver, brain or muscle. The magnitude of the changes in the fatty acid profile of the PL, as a result of choline supplementation, warrants further investigations.

For further transport to the systemic circulation and to the peripheral tissues from the epithelial cells, the TAG must be incorporated into lipoproteins [63]. This lipoprotein production involves a series of biosynthetic processes whereby the large hydrophobic core of the lipoprotein, containing TAG, cholesterol esters, lipid soluble vitamins and other highly lipophilic compounds are covered by a thin coat in which phosphatidylcholine plays an important role, in addition to

apolipoproteins and free cholesterol [65,66]. Our observations of dietary choline increasing the flux of TAG across the epithelial cells and preventing accumulation of TAG in the epithelial mucosa were further supported by concomitant expression changes in genes involved in several processes of the lipid turnover. The decreased expression of *mtp,* coding for transporters facilitating the transport of TAG by assisting in the assembly of the lipoprotein, with increasing choline level, is in agreement with findings presented earlier. These findings show lower expression of *mtp* in fish fed a fishmeal diet, supposedly with higher choline level, compared to the plant-based diet [36]. The same decreasing expression, induced by choline, was observed for the fatty acid transporter *fatp*. Dietary choline is rapidly transformed to phosphatidylcholine by the cytidine (CDP)-choline pathway when entering the pyloric intestine [4,5]. The present study confirmed the results from our previous study [3], showing suppressed levels of *chk* and *pcyt1a*, which proteins catalyzes the initial and the second rate-limiting step in the cytidine (CDP)-choline pathway for phosphatidylcholine synthesis, respectively. The concomitant decrease of both *chk* and *pcyt1a* by increasing choline levels could be a result of an increased production of phosphatidylcholine in the fish by increasing dietary choline doses and that phosphatidylcholine inhibits its own synthesis pathway through a negative feed-back control. The genes coding for apolipoproteins apoAI and apoAIV, both important components for a successful production and secretion of the lipoproteins, showed a significant response with increasing choline level. In studies with various fish species at larval stage [19–21,27] and in one of our previous studies with Atlantic salmon in seawater [36], insufficiency of phosphatidylcholine has been suggested to result in a disturbed assembly of lipoproteins and transport of lipids across the intestinal mucosa. Another important and confirming result from our previous study, was the marked suppression of *plin2*, whose role is to stabilize the lipid droplets [67] and is further suggested to be a surface marker of lipid droplets [68].

Dietary choline level effects on plasma indicators

For the plasma indicators the observed positive relationship between lathosterol and LDL-CH and choline level indicates that choline increased the level of cholesterol synthesis in the liver [69] and accelerated the transport of cholesterol from the liver to peripheral tissue, seen as an increase in the LDL-CH [70,71]. Similar responses to choline supplementation was also observed in our

previous study [3]. Most likely, the increased plasma cholesterol levels were also a result of the increasing capacity of the PI to assemble and transport lipoproteins, which also are responsible for the transport of cholesterol from the intestine to the liver [66].

Dietary choline level effects on performance

The overall growth rate in the present choline dose-response study was within the range observed in fish both under experimental and commercial conditions. However, there were no apparent indications of reduced performance as a result of choline dose nor LMS, for which no significant differences on growth nor macronutrient digestibilities were observed. The present observation of choline not affecting growth agree with some previous studies on adult Atlantic salmon [72], channel catfish [7] and giant grouper [73]. However, in our previous study with large Atlantic salmon [3] and in several other studies with juveniles from different species [5,10,13,73–76], increased growth with choline supplementation has been observed. The relationship between feed intake, growth and choline requirement should be addressed in future studies.

Conclusion

The results of the present study indicate that a dietary choline level of 3.4 g/kg is required for prevention of LMS and intestinal lipid hypervacuolation in 95% of the post smolt Atlantic salmon kept under similar conditions as in the present study. Higher levels may be required at higher feed intakes and/or higher lipid levels in the diet.

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Conflict of Interest

The present study was partly funded by BioMar AS. Co-author Anne Kristine Hansen is employed by BioMar.

Authorship

The authors' contributions were as follows: A.K.G.H: experimental design, performed the molecular analyses, data evaluation and interpretation and manuscript development; T.M.K: data evaluation and manuscript review, VD: sampling, data evaluation and manuscript revision, K.M: experimental design and manuscript revision, I.B: Biochemistry analyses and data interpretation and manuscript revision, H.J.G: lipid class analyses and data interpretation and manuscript revision, Å.K: leadership, experimental design, data evaluation and interpretation and manuscript development. All authors read and approved the final manuscript.

509 Figure 1. Histological Severity of vacuolation of the pyloric caeca tissue, representative for a)
510 marked, b) moderate, c) mild and d) normal.

511 Figure 2. Illustration of choline requirement (mg/kg) by broken line models with linear (A and C)
512 and quadratic (B and D) portion of OSIPI (% of body weight) (A and B) and OSIMI (% of body
513 weight) (C and D).

514 Figure 3. Results of visual examination of the macroscopic appearance of whiteness of the
515 pyloric intestine (n = 8).

516 Figure 4. Results of histological examination and evaluation of degree of vacuolation in the
517 pyloric caeca (n = 8).

518 Figure 5. Representative images of histological appearance of lipid in pyloric caeca in fish fed
519 diets with various levels (mg/kg) of choline (A) 1340, (B) 1540, (C) 1760 and (D) 2310.

520 Figure 6. Concentration (mg/g tissue) of triacylglycerol (TAG), diacylglycerol (DAG), free fatty
521 acids (FFA) and phospholipids (PL) in pyloric tissue from fish fed diets with 1340 mg/kg choline
522 (LF1, n = 4) and 2850 mg/kg choline chloride (LF6, n = 4). Monoacylglycerol did not show
523 measurable concentrations in the tissue. Concentration of TAG and DAG differed significantly,
524 indicated by * and were $p = 0.026$ and $p = 0.039$, respectively. For FFA and PL the differences
525 were not significant, $p = 0.328$ and $p = 0.253$, respectively (data log transformed).

526 Figure 7. Fatty acids (% of lipid) in triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids
527 (FFA) and phospholipids (PL) of pyloric caeca tissue showing significant ($p < 0.05$, data log
528 transformed) effects of choline supplementation, i.e. comparing samples from fish fed the diets
529 LF1 (n = 4) and LF 6 (n = 4). Monoacylglycerol did not show measurable levels.

530 Figure 8. Illustration of relationships between dietary choline concentration levels (mg/kg) and
531 mean normalized expression (MNE) of *chk*, *pcyt1a*, *fatp*, *mtp*, *apoAIV*, *apoAI*, *hmgcr* and *plin2* in
532 pyloric caeca tissue from fish fed the diets LF1 and LF3-8 (n = 5). The curves illustrate the
533 regression that fits the results best. The dotted vertical lines indicate estimated choline
534 requirement level.

535 Figure 9. Choline requirement level as indicated by biomarkers of various indicators of lipid
536 assembly, storage and transport. Horizontal lines (—) indicate estimated 95% confidence range
537 (estimated mean ± 2D) for optimum choline level for each biomarker.

Table 1: Formulation and chemical composition of the basal diet

Diets	**LF 1**[*]
Ingredients (g/kg)	
Nordic LT 94 fishmeal[†]	100
Soya 60% (SPC)[‡]	173
Maize Gluten[§]	150
Pea Protein 50[ǀ]	130
Dehulled Beans[¶]	140
Wheat Gluten[**]	27.5
Fish oil (Standard)[††]	75.5
Rapeseed oil[‡‡]	176
Amino Acid mix[§§]	14.5
Mineral mix[§§]	3.5
Monocalcium phosphate[§§]	24.3
Lucantin Pink CWD 10%[§§]	0.4
Yttrium[ǁ]	0.5
Choline chloride 70%[¶¶]	0
Analysed chemical composition (g/kg)	
Dry Matter	957
Protein	407
Fat	290
Starch	214

[*]Low fishmeal basal diet, [†]Supplied by Norsildmel AS, [‡]Supplied by Selecta S/A, Avenida Jamel Ceilio, 2496 – 12th region, [§]Supplied by Cargill Nordic, [ǀ]Supplied by DLG Food Grain, [¶]Supplied by HC Handelscenter, [**]Supplied by Roquette, [††]Supplied by FF Skagen, [‡‡]Supplied by Emmelev, [§§]Supplemented to meet the requirements, [ǁ]Inert marker for the evaluation of nutrient digestibility, [¶¶]Supplied by Balchem.

547 Table 2: Supplemented and analysed choline (mg/kg) in experimental diets

	LF1	LF 2	LF 3	LF 4	LF 5	LF 6	LF 7	LF 8	LF 9
Supplemented		392	785	1177	1569	1962	2354	2746	3139
Analysed	1340	1540	1760	2310	2600	2850	3330	3830	4020

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550 Table 3: Choline effects on specific growth rate (SGR), condition factor (CF) and organosomatic
551 indices (OSI)

	LF1	LF2	LF3	LF4	LF5	LF6	LF7	LF8	LF9	Pooled SEM
SGR (%d-1)	0.97	0.91	0.93	0.99	1.01	0.84	0.96	0.94	0.95	0.03
CF	1.58	1.56	1.60	1.62	1.60	1.49	1.55	1.49	1.54	0.03
OSI PI	2.31	2.15	2.09	1.85	1.75	1.82	1.57	1.68	1.68	0.090
OSI MI	0.19	0.17	0.17	0.17	0.16	0.14	0.17	0.16	0.17	0.007
OSI DI	0.42	0.46	0.49	0.43	0.49	0.45	0.47	0.52	0.44	0.021
OSI LI	1.15	1.28	1.20	1.18	1.23	1.24	1.23	1.31	1.11	0.057

552 Table 4: Intestinal dry matter and bile salt levels in digesta of Atlantic salmon

		LF1	LF 2	LF 3	LF 4	LF 5	LF 6	LF 7	LF 8	LF 9	Pooled SEM
Dry matter (%)											
	PI1	13	13	11	14	14	16	13	12	15	0.92
	PI2	14	15	15	16	17	16	16	14	17	0.82
	MI	15	16	16	17	16	16	16	15	17	0.48
	DI1	15	14	14	15	14	15	14	14	15	0.54
	DI2	14	12	13	14	13	13	13	13	14	0.56
Bile salt (mg/g DM)											
	PI1	110	110	110	93	114	109	105	137	123	4.0
	PI2	89	88	88	85	111	104	97	93	93	2.8
	MI	125	101	107	110	115	121	136	132	117	3.8
	DI1	95	105	90	80	105	101	95	121	93	3.8
	DI2	27	15	13	42	20	22	37	18	17	3.3

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559 Table 5: Blood plasma variables (n = eight fish per diet)

	LF1	LF 2	LF 3	LF 4	LF 5	LF 6	LF 7	LF 8	LF 9	Pooled SEM
Free Fatty Acids (mmol/L)	0.24	0.22	0.20	0.26	0.22	0.20	0.20	0.22	0.26	0.01
Total CH (mmol/L) *	8.89	9.97	11.1	10.7	10.6	10.7	10.0	12.3	12.1	0.35
HDL-CH†	7.88	8.29	9.73	9.25	8.92	8.66	8.20	10.55	10.17	0.31
LDL-CH†	0.72	1.19	1.08	1.07	1.29	1.39	1.36	1.16	1.53	0.08
VLDL-CH†	0.28	0.49	0.29	0.40	0.42	0.64	0.48	0.60	0.40	0.04
Total TAG (mmol/L)‡	2.04	2.41	1.99	1.78	2.13	1.92	1.51	2.12	2.07	0.08
HDL-TAG†	1.21	1.68	1.51	1.34	1.15	1.32	1.09	1.53	1.13	0.07
LDL-TAG†	0.35	0.34	0.28	0.22	0.37	0.34	0.27	0.20	0.46	0.03
VLDL-TAG†	0.48	0.38	0.20	0.22	0.61	0.27	0.15	0.38	0.49	0.05
C4 (nmol/L)	16.9				38.6				37,3	
Sitosterol (µg/ml)	69.5	58.4	71.4	69.1	65.8	69.4	55.0	82.8	69.9	2.65
Camposterol (µg/ml)	129	116	149	143	147	140	104	171	151	6.71
Lathosterol (µg/ml)	0.80	0.86	0.94	1.47	1.54	1.69	1.75	1.13	1.78	0.13

560 *Total CH: total cholesterol

561 †HDL: high-density lipoproteins; LDL: low-density lipoproteins; VLDL: very low-density lipoproteins

562 ‡Total TAG: total triacylglycerol

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Figure 1

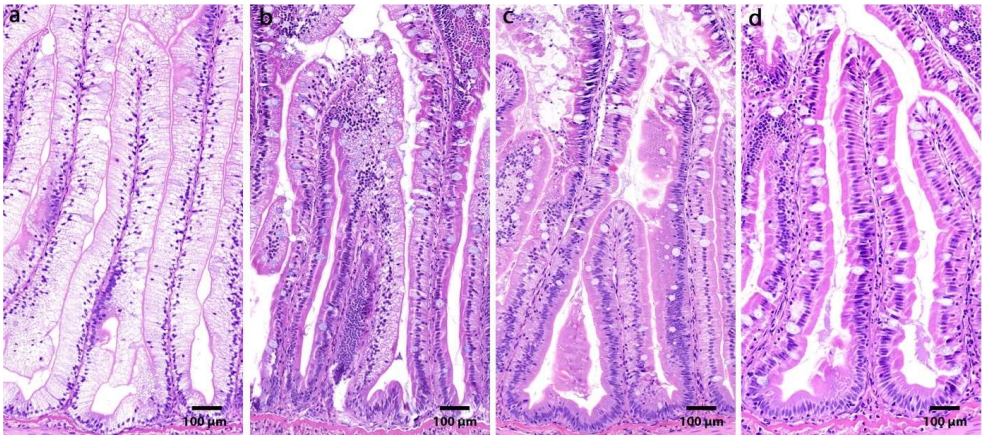


Figure 2

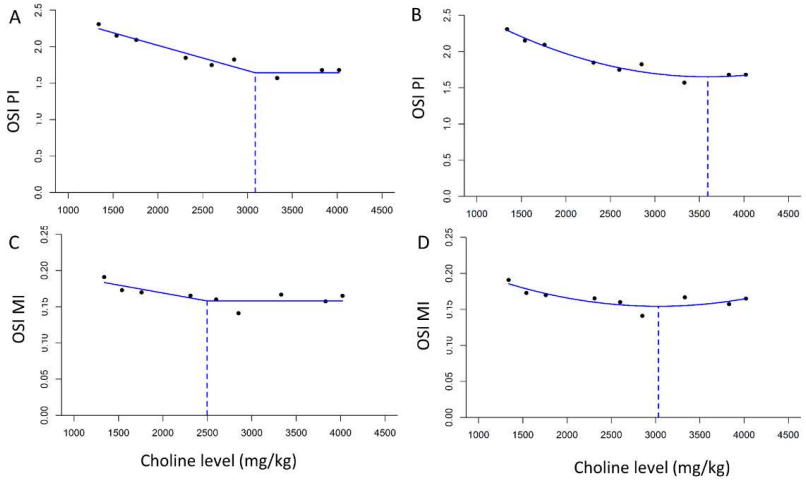


Figure 3

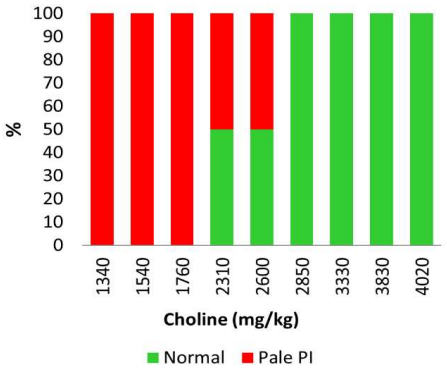


Figure 4

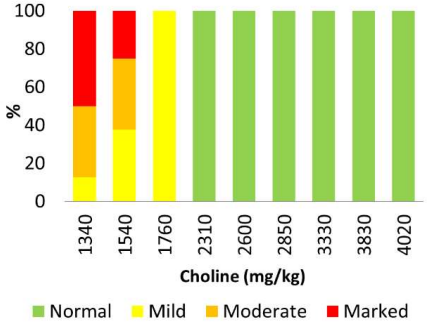
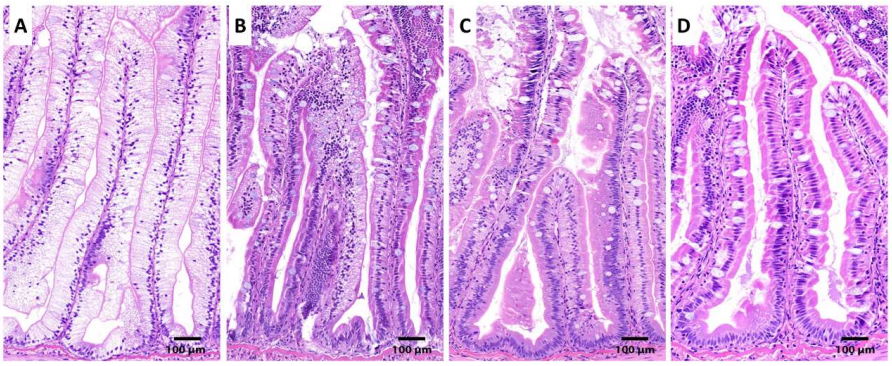


Figure 5



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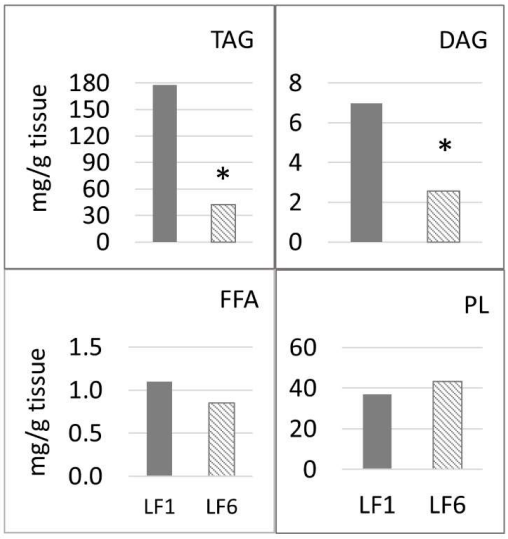

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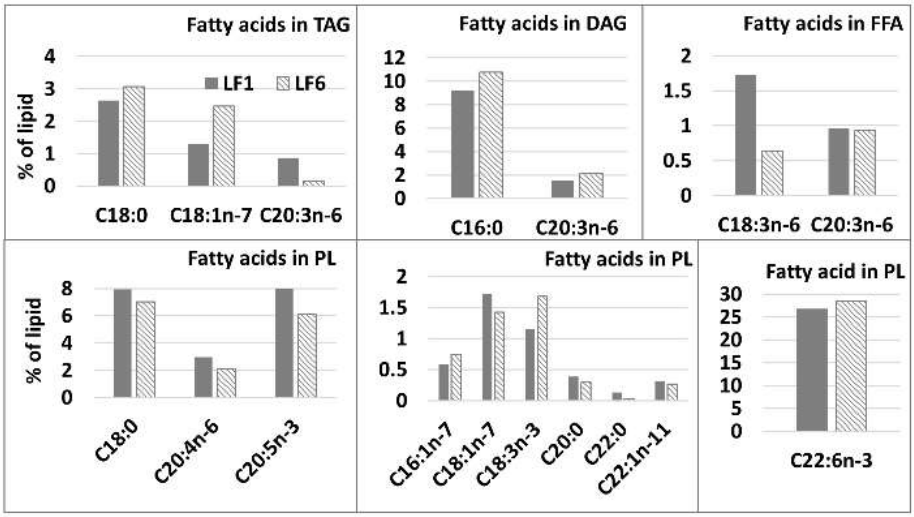

Fatty acids in TAG
% of lipid
LF1
LF6
C18:0
C18:1n-7
C20:3n-6
Fatty acids in DAG
C16:0
C20:3n-6
Fatty acids in FFA
C18:3n-6
C20:3n-6
Fatty acids in PL
% of lipid
C18:0
C20:4n-6
C20:5n-3
Fatty acids in PL
C16:1n-7
C18:1n-7
C18:3n-3
C20:0
C22:0
C22:1n-11
Fatty acid in PL
C22:6n-3

Figure 8

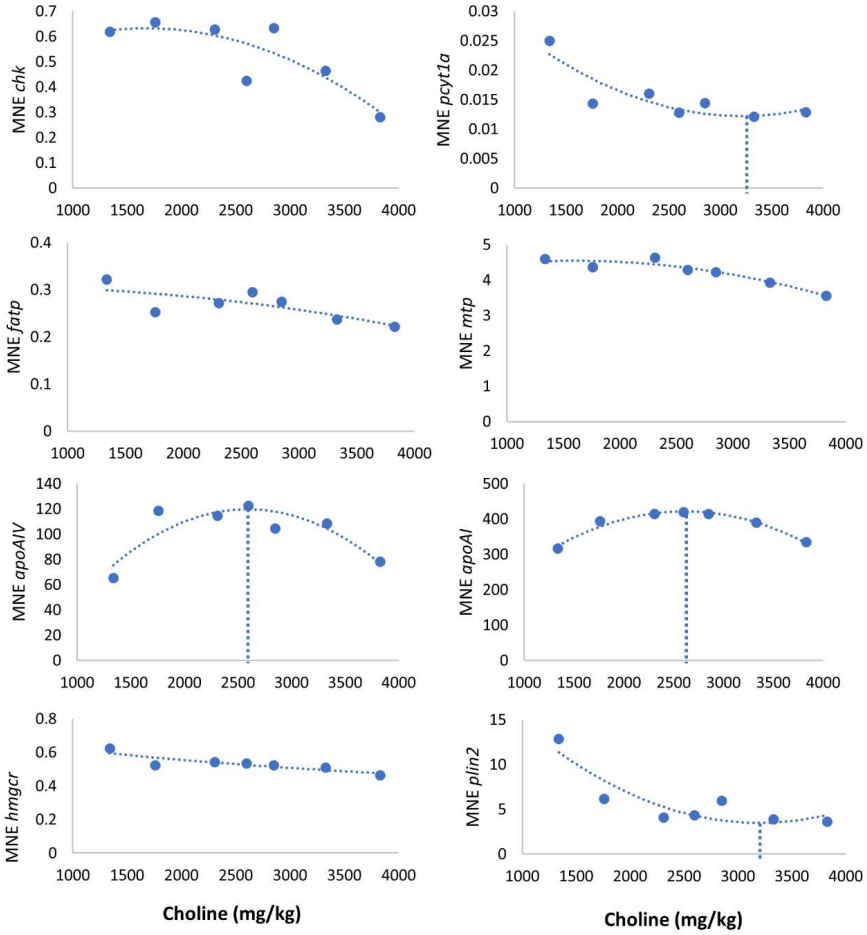
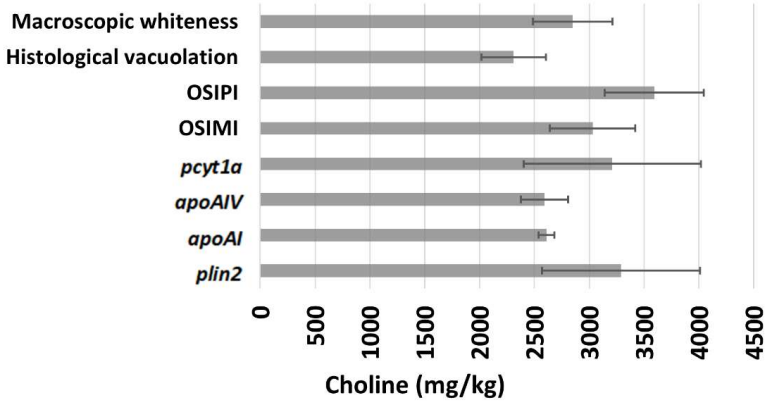

MNE chk
MNE pcyt1a
MNE fatp
MNE mtp
MNE apoAIV
MNE apoAI
MNE hmgcr
MNE plin2
Choline (mg/kg)
Choline (mg/kg)

Figure 9


Macroscopic whiteness
Histological vacuolation
OSIPI
OSIMI
pcyt1a
apoAIV
apoAI
plin2
0
500
1000
1500
2000
2500
3000
3500
4000
4500
Choline (mg/kg)

Supplementary Table 1. Primer pair sequences, efficiency, amplicon size and annealing temperature for the genes used for real-time PCR

			5'–3' primer sequence						
Gene name and category	**Gene function**	**Gene symbols**	**Forward**	**Reverse**	**Amplicon size (bp)**	**Anealing temperature (°C)**	**Primer efficiency**	**GeneBank accession no.**	**Reference**
Lipid uptake and transport									
Adipophilin/perilipin 2	Lipid droplet component	*plin2*	cccaggtctactccagcttc	cagcgactccttcatcttgc	104	60	2.0	XM_014155742	This study
Apolipoprotein AI	Lipoprotein component	*apoAI*	ctggtcctcgcactaaccat	tggacctctgtgcagtcaac	144	60	2.0	NM_001123663	7
Apolipoprotein AIV	Lipoprotein component	*apoAIV*	caggaccagtctcagcaaca	gttgacttcctgtgccacct	131	60	1.9	BT048822	7
Apolipoprotein B48	Lipoprotein component	*apoB48*	ccctgagatggtgtccgtat	gcgtcgacttccatagcttc	131	63	1.8	CB504205	7
Cluster of differentiation 36	Fatty acid transporter	*cd36*	caagtcagcgacaaaccaga	aggagacatggcgatgtagg	91	60	1.9	AY606034	7
Fatty acid binding protein 2B	Fatty acid transporter	*fabp2b*	tgccttcccctcattctcta	ggtgatacggtcttcatccaa	82	60	2.0	EU880419	4
Fatty acid transport protein	Fatty acid transporter	*fatp*	aggagagaacgtctccacca	cgcatcacagtcaaatgtcc	159	60	1.9	CA373015	7
Microsomal triglyceride transfer protein	Lipoprotein assembly	*mtp*	aacgtgacagtggacatgga	ggaccgtggtgatgaagtct	89	60	2.0	CA042356	7
Monoacylglycerol acyltransferase 2-A	Resynthesis of triacylglycerols	*mgat2a*	acgctacaggcttcaggaaa	ggaatcagacctgccatcat	116	60	2.0	NM_001140718	7
Peroxisome proliferator activated receptor alpha	Nuclear receptor - regular of lipid metabolism	*pparα*	gcttcatcaccaggagattt	tcactgtcatccagctccag	113	60	2.0	NM_0011235960	4
Peroxisome proliferator activated receptor gamma	Nuclear receptor - regular of lipid metabolism	*pparγ*	tgctgcaggctgagtttatg	caggggaaagtgtctgtggt	107	58	2.0	NM_0011235946	4
Phosphatidylcholine synthesis									
Choline kinase	Phosphatidylcholine biosynthesis	*chk*	ctcaagtttgcccgtctgat	cacagggaatgagtggagt	88	60	1.9	DY706802	7
Choline transporter-like protein 2	Choline transporter	*slc44a2*	tcgtcatcattttgctgctc	aggcgatgacaatggatagg	152	60	2.0	NM_001140367	This study
Choline-phosphate cytidyltransferase	Phosphatidylcholine biosynthesis	*pcyt1a*	cgggtctatgcagatggaat	gctcgtcctcgttcatcact	166	60	2.1	BT045986	7
Phosphatidylethanolamine N-methyltransferase	Phosphatidylcholine biosynthesis	*pemt*	gttgctgtcatcgccatcat	gaggaggatgatgagggtgc	141	60	2.0	XM_014158251	This study
Cholesterol metabolism									
3-hydroxy-3-methylglutaryl-CoA reductase	Cholesterol biosynthesis	*hmgcr*	ccttcagccatgaactggat	tcctgtccacaggcaatgta	224	60	1.9	NM_001173919	3
Acyl-coA:cholesterol acyltransferase	Cholesterol esterification	*acat*	tgctggagtttgacctgttg	gctgcgatggtagagagtcc	139	60	2.0	GE793368	7
ATP-binding cassette A1	Cholesterol efflux transporter	*abca1*	acagtggaggaacatgagg	cccctccttgacgatactga	149	60	2.0	TC187143	2
ATP-binding cassette G5	Cholesterol efflux transporter	*abcg5*	agactgcctcgtccaacact	ccattttcgtgaacgtgtacc	157	60	1.9	CU073172	5
Farnesoid X receptor	Bile acid nuclear receptor	*fxr*	ttcaacatctcaactcatca	tagcaggtcctcattgat	102	60	2.0	NM_001173830	5
Liver X receptor	Nuclear receptor - regular of lipid and sterol metabolism	*lxr*	gccgccgctatctgaaatctg	caatccggcaaccaatctgtagg	210	60	1.9	FJ470290	3
Niemann-Pick C1 like 1	Cholesterol transporter	*npc1l1*	ccaaagacctgatcctggaa	cgaagcacacatccttcaga	108	60	1.9	XM_014171081	2
sterol element regulatory binding protein 1	Nuclear receptor - regular of lipid and sterol metabolism	*srebp1*	gccatgcgcaggttgtttcttca	tctggccaggacgcatctcacact	151	63	1.9	HM561860	3
Sterol element regulatory binding protein 2	Nuclear receptor - regular of lipid and sterol metabolism	*srebp2*	tcgcggcctcctgatgatt	agggctaggtgactgttctgg	147	60	1.9	HM561861	3
Taurine transporter	Taurine transporter - bile salt metabolism	*slc6a6*	ggaggtggaaggacagatca	acatgccacctttcgttacc	143	60	2.0	NM_00112363	6
Reference genes									
Beta-actin	Cytoskeleton protein	*actb*	caaagccaacaggagaagatga	acggagtccatgacgatac	133	60	1.9	AF012125	1
Elongation factor 1 alpha	Translation	*ef1a*	gtgctgtgcttatcgttgct	ggctctgtggagtccatctt	148	60	1.9	AF321836	1
Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	*gapdh*	aagtgaagcaggagggtgga	cagcctcacccatttgatg	96	60	1.9	BT050045	1
Ribosomal protein S20	Ribosomal subunit	*rps20*	agcgcaacgtcaagtct	gtcttggtgggcatacgg	98	60	2.0	NM_001140843	1
RNA polymerase II	Transcription	*mapolli*	ccaatacatgaccaaatatgaaagg	atgatgatggggatcttcctgc	157	60	1.8	BG936649	1

References:

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Supplementary Table 2. Results of regression analyses, employing a second degre polynome as model, regarding results in Atlantic salmon fed increasing doses of choline (model: $Y=a+bX+cX^2$)

	a	b	c	R^2	p(model)
Apparent Nutrient Digestibility					
Protein AD	91	-0.07	0.002	0.094	0.741
Lipid AD	98	-0.01	0.008	0.19	0.574
Starch AD	73	-1.94	0.19	0.174	0.563
C14:0	97	-0.69	0.07	0.24	0.431
C16:0	94	-0.06	0.03	0.19	0.526
C16:1n7	100	0.05	0.00	0.40	0.218
C18:0	96	-3.36	0.37	0.60	0.063
C18:1n9	99	0.09	-0.004	0.36	0.264
C18:2n6	99	-0.07	0.01	0.13	0.661
C18:3n3	99	-0.01	0.003	0.09	0.761
C20:0	96	-0.002	0.02	0.29	0.351
C20:1n9	97	0.07	0.003	0.20	0.513
C20:5n3	99	0.25	-0.02	0.09	0.747
C22:6n3	99	-0.45	0.05	0.63	0.049
Performance					
SGR	0.95	-4.02E-06	5.96E-10	5.89E-05	0.995
CF	1.56	3.19E-05	-1.09E-08	0.059	0.008
Organosomtatic indices					
OSI PI	3.29	-9.11E-04	1.27E-07	0.41	<0.0001
OSI MI	0.25	-6.63E-05	1,09E-08	0.18	0.0002
OSI DI	0.41	3.53E-05	-4.58E-09	0.022	0.373
OSI LI	1.06	1.30E-04	-2.37E-08	0.0083	0.695

Pyloric caeca vacuolization					
Macroscopic	2.40	-0.25	0.009	0.69	<0.0001
Histological	4.43	-1.06	0.078	0.82	<0.0001
Dry matter (%)					
PI1	11.7	0.53	-0.032	0.036	0.200
PI2	14.1	0.64	-0.054	0.025	0.339
MI1	15.2	0.31	-0.024	0.022	0.386
DI1	14.3	-0.28	0.004	0.0008	0.967
DI2	13.3	-0.14	0.020	0.011	0.614
Bile salt (mg/g DM)					
PI1	117	-6.55	0.89	0.48	0.142
PI2	77.6	7.09	-0.60	0.32	0.311
MI1	112	-0.33	0.255	0.284	0.367
DI1	96.2	-0.86	0.20	0.091	0.751
DI2	15.3	4.55	-0.46	0.081	0.776
Gene expression					
chk	0.43	2.42E-04	-7.19E-08	0.16	0.063
pcyt1a	0.043	-1,92E-05	2.99E-09	0.47	<0.0001
pemt	0.58	-6.71E-05	-4.81E-10	0.11	0.14
mgat2	0.76	-2.33E-04	3.90E-08	0.074	0.29
cd36	0.95	-1.63E-05	-7.41E-09	0.087	0.23
fabp2b	2.7	0.003	-7.04E-07	0.087	0.23
fatp	0.31	3.029E-06	-6.42E-09	0.20	0.028
mtp	4.0	6.41E-04	-2.00E-07	0.26	0.008
acat	1.0	-3.71E-04	6.45E-08	0.14	0.090
apoB	26	0.011	-2.63E-06	0.061	0.36
apoAIV	-69	0.15	-2.81E-05	0.41	0.0002
apoAI	12	0.31	-6.01E-05	0.32	0.002
npc1l1	7.5	0.001	2.10E-07	0.024	0.68

abcg5	0.003	1.69E-06	-2.70E-10	0.04	0.50
abca1	0.16	-4.31E-05	7.42E-09	0.056	0.40
pparα	0.41	6.38E-06	-7.49E-09	0.097	0.20
pparγ	0.13	2.88E-05	-5.11E-09	0.006	0.90
srebp1	1.4	-3.75E-04	5.36E-08	0.10	0.19
srebp2	3.3	9.20E-04	-2.15E-07	0.055	0.40
hmgcr	0.69	-7.69E-05	5.69E-09	0.25	0.010
lxr	1.2	-8.41E-05	9.13E-09	0.042	0.51
fxr	0.021	-7.55E-07	3.56E-10	0.027	0.64
slc6A6	0.49	-9.83E-05	1.80E-08	0.037	0.55
scl44a2	0.068	-2.32E-05	4.11E-09	0.13	0.12
plin2	27	-0.015	2.28E-06	0.40	0.0003
Blood plasma values b					
Free Fatty Acids (mmol/L)	0.26	-0.018	0.002	0.22	0.481
Total CH (mmol/L)	9.20	0.32	-0.002	0.59	0.071
HDL-CH	8.27	0.086	0.012	0.37	0.246
LDL-CH	0.72	0.15	-0.008	0.66	0.041
VLDL-CH	0.22	0.083	-0.006	0.38	0.241
Total TAG (mmol/L)	2.39	-0.17	0.014	0.21	0.499
HDL-TAG	1.45	-0.017	-0.001	0.13	0.650
LDL-TAG	0.41	-0.054	0.006	0.19	0.526
VLDL-TAG	0.53	-0.099	0.010	0.16	0.600
Sitosterol (µg/ml)	68.6	-1.6	0.23	0.090	0.754
Camposterol (µg/ml)	127	2.1	0.057	0.13	0.659
Lathosterol (µg/ml)	0.39	0.32	-0.022	0.68	0.031

b Pooled samples per diet n=8

c Pooled samples per diet n=8 from LF1, LF5 and LF9

Supplementary Table 3. Fatty acid profile of triacylglycerol (TAG), diacylglycerol (DAG), free fatty acids (FFA) and phospholipids (PL) isolated from pyloric caeca tissue (n = 4). Results within the lipid classes showing significant ($p<0.05$, data log transformed) difference between diet LF1 and LF6 are coloured.

Lipid fractions (% of lipid)	TAG	TAG	DAG	DAG	FFA	FFA	PL	PL
Diet	LF1	LF6	LF1	LF6	LF1	LF6	LF1	LF6
C14:0	1.6	1.9	0.8	0.9	1.8	1.8	0.6	0.5
C16:0	8.4	9.6	9.2	10.8	11.8	12.1	16.0	14.7
C16:1n-7	1.8	2.1	1.2	1.4	1.6	1.9	0.6	0.8
C18:0	2.6	3.1	5.5	6.7	6.0	6.8	7.9	7.0
C18:1n-9	37.3	33.9	30.3	26.2	29.2	27.0	13.1	14.1
C18:1n-7	1.3	2.5	2.2	1.9	1.9	1.9	1.7	1.4
C18:2n-6	25.5	23.8	19.0	16.7	22.1	21.0	9.2	11.4
C18:3n-6	0.3	0.5	0.6	0.3	1.7	0.6	0.3	0.2
C18:3n-3	3.9	4.5	3.0	3.2	3.7	3.8	1.2	1.7
C20:0	0.0	0.0	0.5	0.4	0.5	0.4	0.4	0.3
C20:1n-11	0.4	0.4	0.9	0.7	1.5	1.0	0.3	0.2
C20:4n-3	1.4	1.0	0.2	0.1	0.2	0.5	0.1	0.1
C20:1n-9	3.2	3.4	2.7	2.2	2.2	1.6	1.3	1.2
C20:1n-7	0.1	0.1	0.0	0.2	0.2	0.5	0.1	0.1
C20:2n-6	1.3	1.2	2.0	1.6	1.2	1.1	0.0	0.0
C20:3n-6	0.9	0.1	1.5	2.2	1.0	0.9	2.7	2.8
C20:4n-6	0.1	0.1	1.5	2.0	0.6	0.8	3.0	2.1
C22:0	0.4	0.3	0.3	0.1	0.6	0.8	0.1	0.0
C22:1n-11	2.4	2.6	1.9	1.5	1.9	1.0	0.3	0.3
C20:5n-3	0.7	1.3	2.4	2.6	2.1	3.1	8.6	6.1
C22:5n-3	0.2	0.5	0.6	0.9	0.5	0.6	1.3	1.7
C22:6n-3	1.0	2.4	9.3	11.5	3.3	4.6	26.8	28.5
SLCFA	1.8	3.7	11.8	14.1	5.4	7.8	35.4	34.6
Sum n-3	3.6	5.4	12.7	15.2	6.2	8.8	37.0	36.7
Sum n-6	28.3	25.9	24.9	22.7	26.8	24.9	15.5	16.9
Sum Sat	2.1	2.2	1.8	1.5	2.9	3.0	1.6	1.2

Supplementary Table 4. The results of increasing doses of choline on gene expression profiling of the pyloric caeca samples by qPCR (n = 5). Values are mean normalized expression (MNE)

Gene symbols	LF1	LF 3	LF 4	LF 5	LF 6	LF 7	LF 8	Pooled SEM
chk	0.62	0.66	0.63	0.42	0.63	0.46	0.28	0.045
pcyt1a	0.025	0.014	0.016	0.013	0.014	0.012	0.013	0.001
pemt	0.53	0.38	0.39	0.43	0.44	0.32	0.31	0.026
cd36	0.86	0.97	0.86	0.9	0.81	0.73	0.83	0.024
fabp2b	5.4	8	6	6.7	6.3	7	5.3	0.253
fatp	0.32	0.25	0.27	0.29	0.27	0.24	0.22	0.009
mtp	4.6	4.4	4.6	4.3	4.2	3.9	3.6	0.106
acat	0.65	0.54	0.54	0.53	0.44	0.5	0.56	0.022
apoB	35	39	42	38	31	37	32	1.51
apoAIV	65	119	115	123	104	108	78	4.4
apoAI	317	393	414	420	414	390	334	10.7
npc1l1	5.8	6.4	6	5.4	5.5	5.3	6.0	0.201
abcg5	0.004	0.006	0.005	0.005	0.006	0.004	0.005	0.000
abca1	0.11	0.1	0.09	0.11	0.09	0.1	0.1	0.004
pparα	0.42	0.4	0.37	0.37	0.41	0.34	0.33	0.014
pparγ	0.15	0.18	0.17	0.18	0.16	0.16	0.17	0.008
srebp1	1.09	0.81	0.75	0.82	0.94	0.73	0.74	0.044
srebp2[x]	4.3	3.9	4.5	4.3	4.1	4	3.6	0.138
hmgcr	0.62	0.52	0.54	0.53	0.52	0.51	0.46	0.012
lxr	1.1	1.1	1.1	1.0	1.0	1.0	1.0	0.024
fxr	0.012	0.02	0.02	0.02	0.02	0.02	0.02	0.001
slc6a6	0.38	0.39	0.36	0.35	0.34	0.38	0.37	0.01
mgat2	0.56	0.41	0.42	0.41	0.5	0.4	0.44	0.021
scl44a2	0.045	0.04	0.034	0.04	0.03	0.034	0.034	0.001
plin2	12.9	6.2	4.1	4.3	6.0	3.9	3.6	0.685

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